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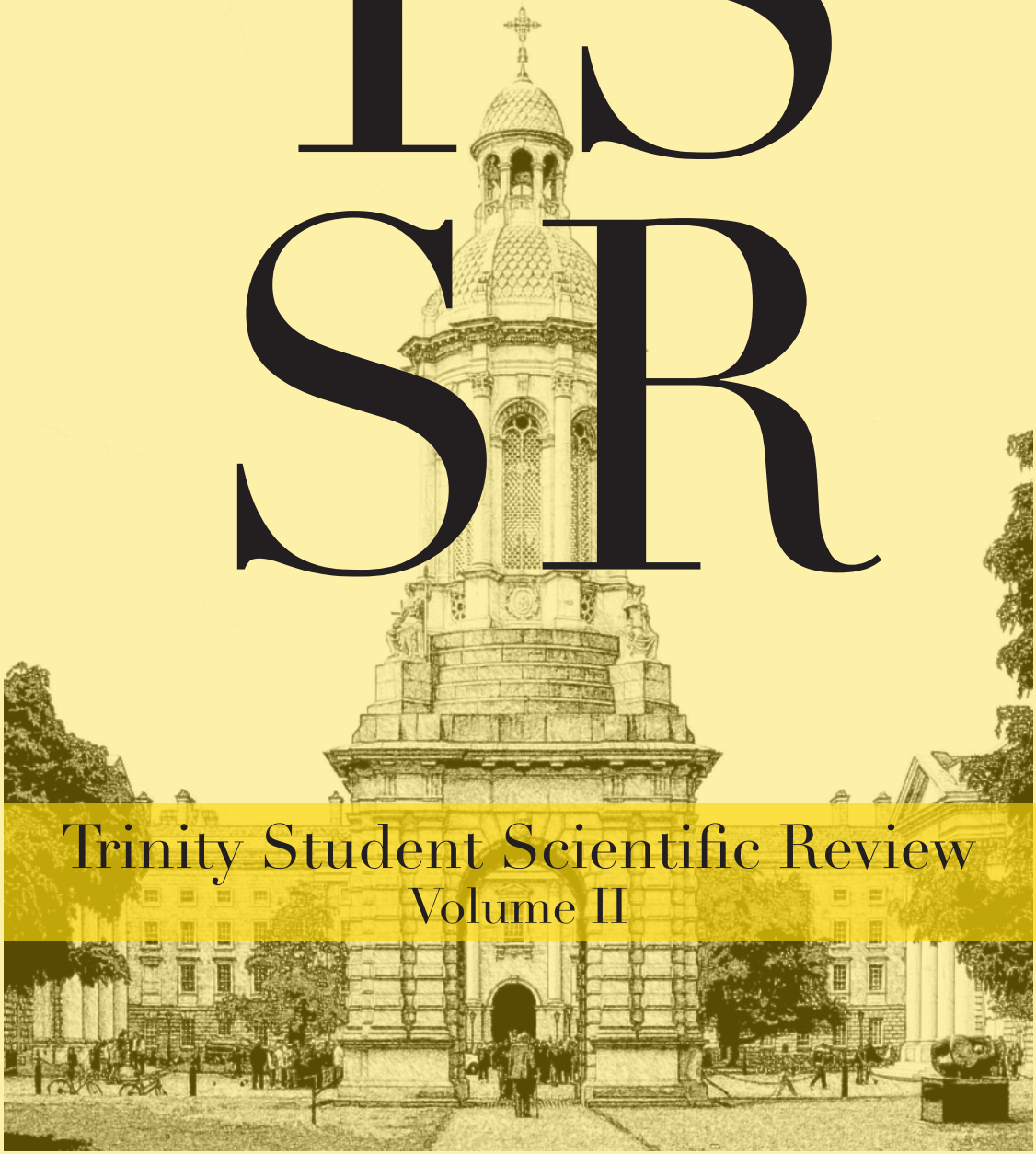

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Volume II

TSSR

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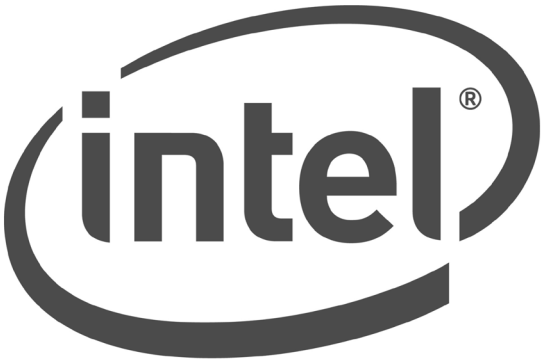

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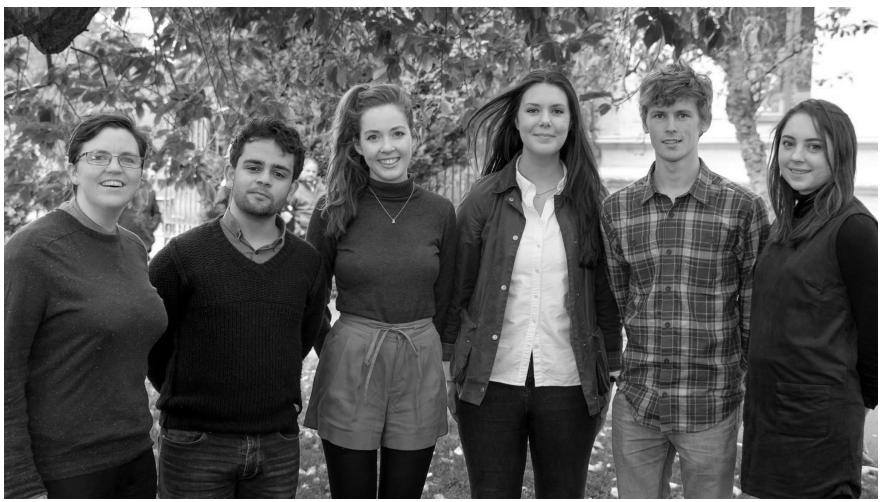

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Best Overall Essay:
Elongation, Termination and Antitermination: the Final Stages of Transcription in *Escherichia coli*
Kevin Lyons

Best Life Sciences Essay:
Inhibitor of Apoptosis Proteins and Their Potential in Cancer Therapy
Karen Slattery

Best Animal, Plant and Earth Sciences Essay:
Deafenaing Silence: The Impact of Naval Sonar Activity on Cetaceans
Andrew Mooney

Best Chemistry Essay:
Climate Change Mitigation using Metal-Organic Frameworks for Direct Air Capture of CO_2
Dónal Ring

Best Physics Essay:
The Nuclear Option: Advanced Radiotherapy Techniques for Cancer Treatment
Oskar Ronan

Best Freshman Essay:
Prebiotic Chemistry: Common Origins of Glycerol, Amino Acids, and Pyrimidines, and Cosmic Origin of Nature's Enantiomeric Excess of Amino Acids
Stephen Byrne

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Life Sciences

Animal, Plant and Earth Sciences

Chemistry

Physics

Welcome

Dear Reader,

2016 has seen one of the largest scientific breakthroughs of our generation. Decades in the making, the detection of gravitational waves is a discovery of paramount importance. Einstein first proposed their existence in his 1916 Theory of General Relativity. Their detection, coming 100 years later, is the embodiment of perseverance, ingenuity, and dedication of scientists worldwide to an idea. This is but one example of how scientific research in the last century has allowed us to push the boundaries of our knowledge and question the nature of the entire world around us.

Here at the TSSR we endeavour to provide undergraduates with a platform where they can begin to pose their own questions and perform their own investigations.

Undergraduate research has a long history in Trinity College Dublin. Dr. William Campbell, Nobel laureate and graduate of the college, first became interested in his field of research, parasitic worms, as an undergraduate in Trinity in the early 1950's. It was in the late 1970's that his team made the discovery of the drug avermectin and, almost 65 years after he first began his research, he was awarded the Nobel Prize in Physiology or Medicine in 2015. Dr. Campbell is a shining example of perseverance and should be remembered as an inspiration to all students as they begin their scientific careers. His undergraduate research was an important stepping stone in the future direction of his career and we hope to set that example and engage current Trinity students to see that their work now has the potential to lead to greater successes in the future.

Founded in 2015 by a group of pioneering undergraduates, the TSSR became the first peer-reviewed undergraduate scientific review journal of its kind in Ireland. It was created with the aim of showcasing student research, increasing awareness of scientific publication, and demonstrating the level of work done by students, to readers both inside and outside the college community. To the founding team I must extend a sincere gratitude. Without their initiative and passion the TSSR would not exist today.

The multidisciplinary nature of the TSSR is a key facet of the journal and one of its greatest strengths. This year we were inspired by the exceptional scientific writing put forward by undergraduates, with submissions spanning a wide range of scientific disciplines, from climate change, to quantum encryption, to name but a few. We hope this diversity will allow you, the reader, to sample the burgeoning talent within Trinity College Dublin,to kindle your curiosity, and inspire you to delve deep into the boundless world of scientific research.

Volume 2 would not exist without the help, advice, and hard work of many individuals and to them all I am incredibly grateful.

To every student who submitted to the journal, we are sincerely grateful for all the hard work you put into your pieces. We were blown away by your exceptional talent and, regardless of whether you made the final publication or not, we hope you benefited and learned from this experience.

To all our Academic Advisors, PhDs, Assistant Editors and anyone who has lent an ear to my questions throughout the year, your faithful support and advice has been thoroughly appreciated. To our sponsors who have gratefully supported us this year: Intel, Identigen, NSilico, TCD Association and Trust, and The Science Gallery, we are sincerely grateful.

To Prof. Vinny Cahill, Dean of the Faculty of Engineering, Mathematics and Science, and everyone in the Faculty office, we wholeheartedly thank you for all your support and advice throughout the year, without which we would not be here today.

My final thank you must be to my wonderful Editorial Board and Committee. Amy Worrall, Sarah Deegan, Kate Reidy, Blaise Delaney and James Orr, you have done amazing work these past few months and it has been an absolute pleasure to be able to call you both esteemed colleagues and friends.

With that, on behalf of myself, the Editorial Board and Committee, I welcome you to Volume 2.

Alison Hennessy
General Manager
Trinity Student Scientific Review 2015/16

Life Sciences

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Letter from the Editor

As the Trinity Student Scientific Review journal enters its second year of publication the notable splitting of the "Biology" category means that there is now a distinct "Life Sciences" category. Life Sciences is a suitable catch-all term for scientific work investigating living organisms, without ignoring technological, pharmaceutical, and even ethical considerations pertaining to that research. It is broad enough to provide scope for a vast array of research fields and indeed all of the submissions and the successful publications this year reflect that diversity.

This last year has seen amazing scientific advances in life sciences and many of the publications this year reveal that those novel developments are being taught in University and inspiring undergraduates to do their own research into those fields. This years TSSR addresses the novel, and somewhat controversial genome editing technology CRISPR/Cas9, which uses guideRNA to target specific genes. CRISPR technology has been in the news since the National Institute of Health, the major scientific funding body in the USA, announced that they would not be funding CRISPR technology research involving human development until further ethical discussions and concerns were addressed. Advancements in genetics, epigenetics, transcription and the broad spectrum of RNAs, including long non-coding RNA and competitive endogenous RNA, are other prominent areas of research that made the short list for publication. Research into biomolecular targets in oncology continues to be in demand, with the hunt for specific targets, and small molecules and biologicals to inhibit them. The immune system and immune-modulatory effects on organ systems remains a popular and ever growing area of research. The fact that authors captured some of hottest areas currently in the life science field is a testament to their understanding and engagement with scientific academia, industry and the world of research.

I would like to extend sincere thanks to Prof. Rachel McLoughlin who is an unwavering support to the TSSR and makes sure always to consider the long term goals and vision for the journal. The editing and reviewing of the submissions were done by a dedicated team including David O'Driscoll, Ciaran Doyle, Proinnsias Fox, Mimmi Lundahl, Alexandros Rammos, Peter O'Byrne and Daniel Johnston. I am indebted to them for their support throughout the editing process.

Finally, all authors that submitted to the TSSR must be thanked. It is daunting submitting your own work for review and critique, and we do not underestimate the work and time that we are sure went into producing their submission, irrespective of whether they were published. We are proud to establish a constructive forum for feedback to authors that were not published and want to heavily encourage them to re-submit in the future.

Amy Worrall
Life Sciences Editor
Trinity Student Scientific Review 2016

TS
SR

Elongation, Termination and Antitermination: The Final Stages of Transcription in Escherichia coli

Kevin Lyons
Junior Sophister
Microbiology

Termination signals in bacteria such as Escherichia coli trigger the end of transcript elongation by causing dissociation of RNA polymerase (RNAP) from both the RNA transcript and template DNA, thus preventing RNAP from carrying out further processive nucleotide addition to the 3'-end of the transcript. Two principal classes of transcription terminators are known to regulate gene expression in E. coli: intrinsic (Rho-independent) terminators – whereby termination is induced by the structure and nucleotide composition of the transcript at a specific template sequence, without the need for auxiliary factors; and Rho-dependent terminators – whereby termination is induced by the actions of Rho factor (a homohexameric ring-shaped RNA-dependent ATPase with translocase and RNA:DNA helicase activities). Although some finer molecular-level details remain elusive, many of the general mechanisms and consequences of transcript elongation, termination and antitermination are by now well-characterised and supported by a substantial body of evidence. A notable caveat, however, regarding termination, is that much of our current understanding stems from investigations involving only a few model terminators – for example, λ tR2 (intrinsic), and λ tR1 (Rho-dependent) – and despite recent advances in bioinformatics, computational methods of terminator identification in the E. coli genome are typically constrained by our limited understanding of the exact sequences and structural elements involved.

Introduction

The Gram-negative, rod-shaped bacterium *Escherichia coli* is by now one of the most intensively studied and best understood organisms on Earth (Blount, 2015; Keseler *et al*., 2013). A hardy, versatile, easily manipulable model organism, *E. coli* played a key role in elucidating the fundamental principles of life in the early days of molecular biology: including the nature of the genetic code (Crick *et al*., 1961), as well as the processes of DNA replication (Lehman *et al*., 1958), gene regulation (Jacob and Monod, 1961), translation (Nirenberg *et al*., 1965) and transcription (Stevens, 1960). Fifty to sixty years later, the study of transcription in *E. coli* continues to have a significant impact on the fields of prokaryotic and eukaryotic genetics, due, in part, to the fact that the principal structural determinants of multisubunit RNAPs have been highly conserved throughout evolution (Werner and Grohmann, 2011). In addition, our enhanced understanding of the structure and function of both the bacterial RNAP and Rho factor has contributed towards the development of clinically-relevant antibiotics used in the treatment of diseases such as tuberculosis and leprosy – namely, rifampicin and bicyclomycin, which inhibit RNAP and Rho respectively (Campbell *et al*., 2001; Vincent *et al*., 2000).

Transcription in *E. coli* can be divided into five major stages: (i) promoter engagement, (ii) initiation, (iii) promoter clearance, (iv) transcript elongation, and (v) termination (Mooney *et al*., 1998). Intrinsic and Rho-dependent terminators situated at the ends of operons; as well as between, within and upstream from genes, are involved in the regulation of gene expression (Peters *et al*., 2011), and can be overridden or inhibited by the actions of various opposing 'antitermination' mechanisms (Santangelo and Artsimovitch, 2011). The interconnected processes of transcript elongation, termination and antitermination will be discussed, with an emphasis on the two principal types of signal which give rise to termination in *E. coli* – intrinsic terminators, and Rho-dependent terminators. While there is currently little doubt about the broad, fundamental mechanisms and consequences of both intrinsic and Rho-dependent termination, many molecular-level ambiguities remain, and this has led to the development of three major competing models of termination: (i) the 'hybrid shearing' model, (ii) the 'hypertranslocation' model, and (iii) the 'invasion' (allosteric) model. All three models are supported to differing extents by biochemical and single-molecule analyses (Komissarova *et al*., 2002, Santangelo and Roberts, 2004, Epshtein *et al*., 2007, Datta and von Hippel, 2008, Larson *et al*., 2008, Epshtein *et al*., 2010), and are repeatedly posited in the literature without any clear consensus. The basis for each model will be addressed, along with a number of unanswered questions and recent developments (e.g. regarding the mechanism of Rho translocation). However, transcript elongation should be considered first, as it is the breakdown of this process, through the pausing of the transcription elongation complex (TEC), which ultimately allows termination to occur.

Reducing TEC Stability

The process of transcript elongation (Figure 1) is carried out by a single, multisubunit, DNA-dependent RNA polymerase (RNAP) and involves the synthesis, via nucleotide addition, of a single-stranded RNA molecule with a nucleotide sequence complementary to that of an antisense, template strand of DNA (Mooney *et al*., 1998, Nudler, 1999). The *E. coli* TEC consists of a characteristic and typically dynamic arrangement of RNAP, DNA and RNA, containing a short (~8–9 bp), stable RNA:DNA hybrid within a slightly longer (12–14 bp) transcription bubble of unwound (melted) DNA (Nudler *et al*., 1997, Korzheva *et al*., 2000). Inducing a reduction in *E. coli* TEC stability or in the rate of transcript elongation, in order to facilitate termination, is not easy. During a typical phase of uninterrupted elongation, the TEC is highly stable, continuing to successfully mediate nucleotide addition at temperatures as high as 70°C (Wilson and von Hippel, 1994) and against applied forces of up to 14 pN (Yin *et al*., 1995). Although the stability of the RNA:DNA hybrid, and its contacts with the RNAP, account for the larger part of overall TEC stability (Sidorenkov *et al*., 1998), RNAP also maintains contacts with ~7 nt of the single-stranded RNA transcript as it exits via the RNA exit channel; as well as with ~14 bp of unmelted, double-stranded DNA downstream of the transcription bubble (Vassylyev *et al*., 2007). These protein-nucleic acid interactions are now also thought to contribute to TEC stability: for example, the rudder loop of RNAP forms stabilizing bridging contacts with the RNA:DNA hybrid and the downstream DNA (Vassylyev *et al*., 2007). The rate of transcript elongation can be reduced by sequences in the template DNA (Bochkareva *et al*., 2012), as well as by the actions of many different accessory protein transcription factors (e.g. NusA and NusG) (Schmidt and Chamberlin, 1987, Sullivan and Gottesman, 1992). This can bring the TEC to a temporary halt (pausing) or a complete halt (arrest); or induce backwards movement of the TEC towards the promoter by 1 bp, known as 'backstepping', or by >1 bp, known as 'backtracking' (Washburn and Gottesman, 2015, Belogurov and Artsimovitch, 2015, Landick, 2006, Nudler, 2012). Low elongation rates can induce or enhance termination mechanisms via paused RNAP intermediates (Schmidt and Chamberlin, 1987, Sullivan and Gottesman, 1992). Hence, antiterminators often employ strategies to prevent pausing and/or increase the elongation rate (Santangelo and Artsimovitch, 2011).

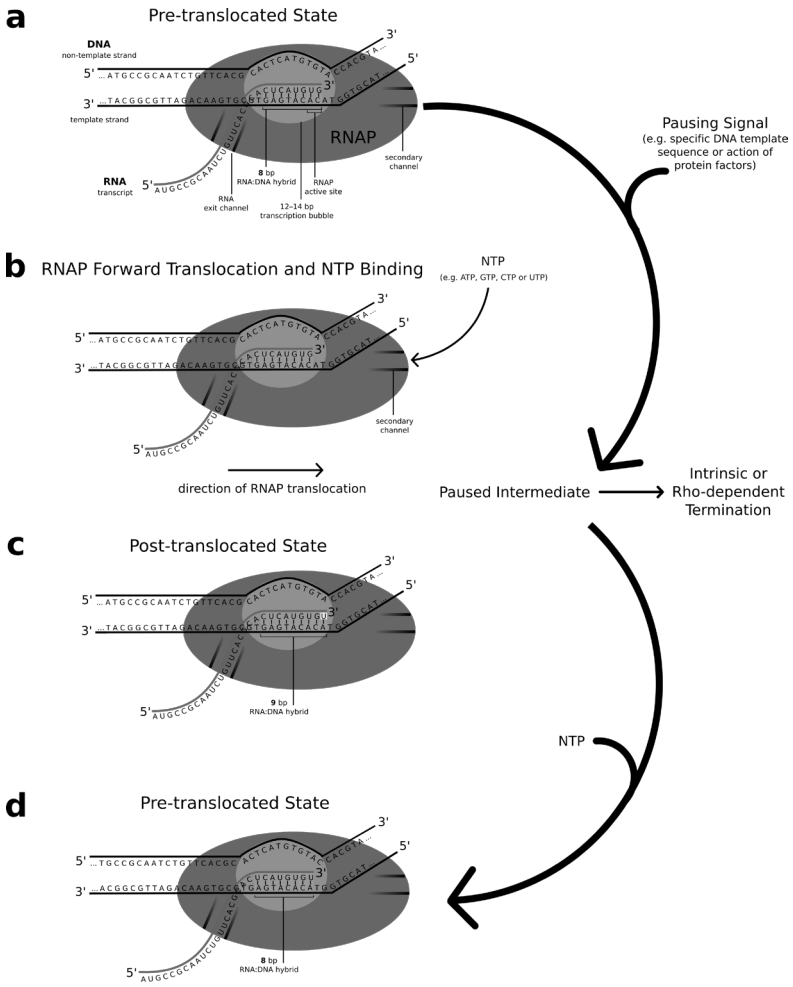

Figure 1. *Summary of transcript elongation, and the relationship between elongation, pausing and termination: (a) the most-recently-added nucleoside triphosphate (NTP) at the 3'-end of the RNA transcript (GTP in this example) is blocking the 3'-most part of the RNAP active site, and hence, binding of another NTP is not possible; (b) the incoming NTP (UTP in this example) is thought to enter via the RNAP secondary channel; (c) RNAP translocation allows addition of the incoming NTP to the 3'-end of the RNA transcript; (d) displacement of 1 nt of RNA from the upstream edge of the RNA:DNA hybrid re-establishes the 8 bp RNA:DNA hybrid (Santangelo and Artsimovitch, 2011). Adapted from Nudler et al., 1997; Korzheva et al., 2000; Santangelo and Artsimovitch, 2011.*

Overview of Intrinsic Termination

Intrinsic (Rho-independent) terminators in *E. coli* typically involve a relatively short (~20 nt), guanine-and-cytosine-rich (G+C-rich) region of template DNA with dyad symmetry (i.e. consisting of inverted repeats joined by a short, unrepeated intervening sequence) followed by a sequence typically consisting entirely of deoxyadenosine (dA) residues – i.e. an oligo(dA) region (Wilson and von Hippel, 1995, Lesnik *et al*., 2001, Gusarov and Nudler, 1999). Transcription of this template DNA gives rise to a correspondingly G+C-rich region of RNA with dyad symmetry, followed by a sequence, at the RNA 3'-end, typically consisting entirely of uridine (rU) residues – i.e. an oligo(rU) region. Base-pairing of the inverted repeats in the RNA induces the formation of a stable G+C-rich RNA hairpin (stem-loop structure), which, when combined with the destabilizing dA:rU duplex of the RNA:DNA hybrid, leads to transcription termination via breakdown of the TEC and dissociation of RNAP from both DNA and RNA (Tomizawa and Masukata, 1987, Wilson and von Hippel, 1995). The process of intrinsic termination can be divided into four major stages: (i) pausing, (ii) hairpin nucleation, (iii) hairpin completion (which causes disruption of the TEC), and (iv) TEC dissociation (Peters *et al*., 2011). Approximately 80% of the known protein-coding (mRNA) transcripts in *E. coli* are terminated by intrinsic terminators (Peters *et al*., 2009). One example of a model intrinsic terminator is the phage λ tR2 terminator (Wilson and von Hippel, 1995).

Overview of Rho-dependent Termination

Unlike intrinsic termination, which relies principally on the structure and nucleotide composition of the nascent RNA transcript at a specific template sequence, Rho-dependent termination relies on both cis-acting RNA elements and trans-acting factors, such as Rho, NusA and NusG (Richardson, 2002, Banerjee *et al*., 2006, Ciampi, 2006). Rho-dependent terminators in *E. coli* are bipartite elements, consisting of a Rho-utilization (rut) site and termination sites called transcription stop points (tsp) (Ciampi, 2006). The rut site – an 83–87 nt Rho-binding site on an untranslated RNA transcript (Koslover *et al*., 2012) – has a high affinity for Rho due to the fact that it has a high proportion of cytosine residues relative to guanine, and little secondary structure (Hart and Roberts, 1991, Platt, 1994, Richardson and Richardson, 1996). Rho-dependent termination requires an untranslated (i.e. ribosome-free) RNA transcript with a minimum length of 85–90 nt (Hart and Roberts, 1994). The tsp release sites, are the RNA sites situated at the TEC, at which Rho-dependent termination occurs – these can be separated from the rut site by hundreds of nucleotides (Richardson and Richardson, 1996). The protein which mediates this termination mechanism – Rho factor – is a homohexameric ring-shaped (Skordalakes and Berger, 2003) RNA-dependent ATPase (Lowery-Goldhammer and Richardson, 1974) with translocase and RNA:DNA helicase

activities (Brennan *et al*., 1987). Prior to termination, Rho binds to the rut site and begins to actively thread the RNA transcript through its central cavity using energy from ATP hydrolysis (Figure 2) (Gocheva *et al*., 2015). In this way, Rho translocates along the transcript in a 5′->3′ direction, while maintaining contacts with the rut site – a mechanism called 'tethered tracking' (Gocheva *et al*., 2015) – before inducing termination at a transcript release site by an unknown mechanism (Ciampi, 2006). Approximately 20% of the known protein-coding (mRNA) transcripts in *E. coli* are terminated by Rho-dependent terminators (Peters *et al*., 2009). One example of a model Rho-dependent terminator is the phage λ tR1 terminator, which terminates the rightward phage λ operon (Lau *et al*., 1982).

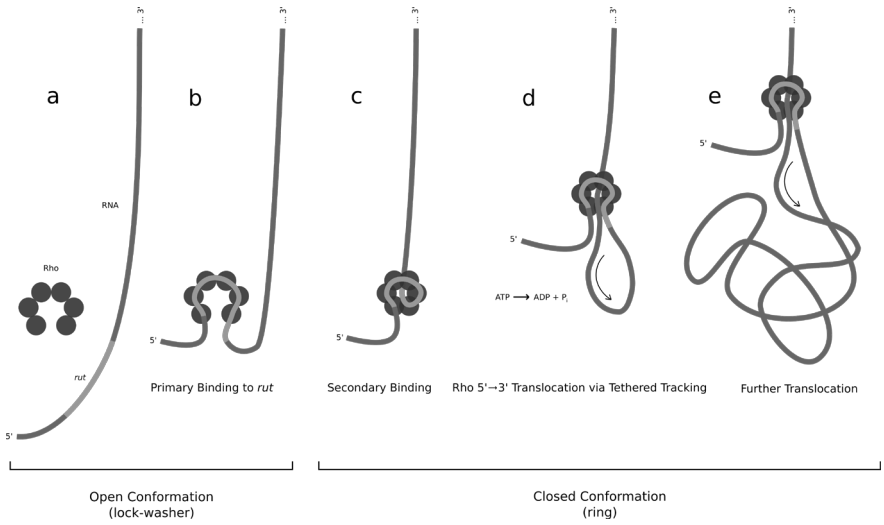

Figure 2. *Mechanism of Rho-rut binding and Rho 5′->3′ translocation via tethered tracking: (a) unbound Rho in the open (lock-washer) conformation; (b) primary binding of Rho to rut (Rho is in contact with 55–59 nt of RNA); (c) during secondary binding the transcript is passed through the central cavity of Rho, and Rho undergoes a conformation change to the closed (ring) conformation (Rho is in contact with 83–87 nt of RNA); (d) Rho 5′->3′ translocation occurs via tethered tracking, the RNA is actively threaded through the central cavity of Rho using energy from ATP hydrolysis; (e) translocation continues until Rho reaches a release site at the TEC, where it terminates transcription by an unknown mechanism. Adapted from Koslover et al. (2012).*

Overview of Antitermination

A wide variety of host-generated (Table 1) and phage-generated (Table 2) mechanisms are known to prevent termination in *E. coli*. The modes and sites of action of these bacterial and phage antiterminators are highly varied (Santangelo and Artsimovitch, 2011): some require specific DNA and/or RNA sequences or auxiliary co-factors in order to carry out their function; others do not; some act as general antiterminators, preventing termination at many different sites; others may be specific to only one or a small number of sites, or may be specific to preventing either intrinsic or Rho-dependent termination. Antiterminators in *E. coli* typically act to prevent termination by one or more of the following strategies: (i) direct disruption of the termination signal – that is, in the case of intrinsic termination, by destabilizing RNA secondary structure and preventing formation of the terminator hairpin (e.g. *E. coli* cold-shock proteins or BglG) (Phadtare and Severinov, 2010, Nussbaum-Shochat and Amster-Choder, 1999); or in the case of Rho-dependent termination, by disrupting the actions of Rho either by preventing binding of Rho to the RNA rut site (e.g. *E. coli* protein YaeO) (Gutierrez *et al*., 2007), or preventing Rho translocation along the transcript (e.g. *E. coli* protein Hfq or phage P4 protein Psu) (Ranjan *et al*., 2013, Rabhi *et al*., 2011), (ii) converting RNAP into a pause-resistant or termination-resistant form (e.g. *E. coli* proteins RfaH and S4; phage λ proteins N and Q; HK022 polymerase utilization (put) RNAs; put/put-like RNAs of phage HK639, HK75 and prophages) (Artsimovitch and Landick, 2002, Torres *et al*., 2001, Mason *et al*., 1992, Shankar *et al*., 2007, King *et al*., 2011, Komissarova *et al*., 2008), or (iii) inhibiting RNAP translocation (e.g. phage HK022 protein Nun) (Vitiello *et al*., 2014).

Antiterminators which employ the first strategy are known as passive antiterminators, and typically enable RNAP to bypass a single terminator. Those which employ the second strategy are known as active (or processive) antiterminators, and typically allow RNAP to read through multiple, consecutive terminators. Although most known antiterminators in *E. coli* fall into one of the two aforementioned categories, the Nun protein of phage HK022 is a notable exception, as it instead acts to inhibit RNAP translocation entirely (Vitiello *et al*., 2014), as well as to strongly prevent both RNAP and transcript dissociation (Chung Hung and Gottesman, 1995). Phage HK022 protein Nun therefore represents a third class of antiterminator. Passive antitermination mechanisms not involving host or phage proteins are also known. These typically involve stalled or translating ribosomes bound to the transcript, which can either prevent terminator hairpin formation through attenuation (Henkin and Yanofsky, 2002), or obstruct and prevent Rho binding to the rut site (Konan and Yanofsky, 2000). Active antitermination can also occur in the regions of the *E. coli* genome which encode non-protein-coding RNA transcripts (e.g. the rRNA (rrn) operons). Rho-dependent termination of rRNA transcripts is rare, as they typically have considerable secondary structure and interact heavily with ribosomal proteins during transcription (Kaczanowska and Ryden-Aulin,

2007). Both features contribute to preventing Rho binding to rut sites, and in addition to this, an rrn antitermination complex can convert RNAP to a Rho-resistant form (Condon *et al*., 1995).

Table 1. *Simplified summary of bacterial antiterminators in E. coli (Santangelo and Artsimovitch, 2011, with additional data derived from Artsimovitch and Landick, 2002; Condon et al., 1995; Gutierrez et al., 2007; Henkin and Yanofsky, 2002; Konan and Yanofsky, 2000; Nussbaum-Shochat and Amster-Choder, 1999; Phadtare and Severinov, 2010; Rabhi et al., 2011; Torres et al., 2001). YaeO, antitermination protein (Rho-specific inhibitor); BglG, β-glucoside (bgl) operon antiterminator protein; Csp, cold-shock proteins; Hfq, RNA-binding protein (Rho-specific inhibitor); RfaH, antiterminator protein; S4, ribosomal protein (Rho-dependent antiterminator); rrn complex, rRNA (rrn) operon antiterminator protein complex.*

Name	Passive/ Active	Mechanism	Reference
YaeO	Passive	YaeO-Rho binding inhibits Rho-RNA binding	(Gutierrez *et al*., 2007)
BglG	Passive	BglG-RNA binding prevents hairpin formation	(Nussbaum-Shochat and Amster-Choder, 1999)
Csp	Passive	Csp-RNA binding destabilizes RNA secondary structures and prevents hairpin formation	(Phadtare and Severinov, 2010
Hfq	Passive	Hfq-Rho binding prevents Rho translocation	(Rabhi *et al*., 2011)
RfaH	Active	RfaH-RNAP binding converts RNAP to termination-resistant form	(Artsimovitch and Landick, 2002)
S4	Active	S4-RNAP binding converts RNAP to Rho-resistant form	(Torres *et al*., 2001)
Ribosome (i)	Passive	Ribosome-RNA interaction prevents hairpin formation	(Henkin and Yanofsky, 2002)
(ii)	Passive	Ribosome-RNA interaction prevents Rho-RNA binding	(Konan and Yanofsky, 2000)
rrn complex	Active	Converts RNAP into a Rho-resistant form	(Condon *et al*., 1995)

Table 2. *Simplified summary of phage antiterminators in E. coli (Santangelo and Artsimovitch, 2011, with additional data derived from King et al., 2011; Komissarova et al., 2008; Mason et al., 1992; Ranjan et al., 2013; Shankar et al., 2007 and Vitiello et al., 2014). Psu, polarity suppression protein (Rho-specific inhibitor); N, phage λ protein N; Q, phage λ protein Q; Nun, phage HK022 protein Nun (E. coli RNAP translocation inhibitor); put, polymerase utilization.*

Name	Phage	Passive/ Active	Mechanism	Reference
Psu	P4	Passive	Psu-Rho binding prevents Rho translocation	(Ranjan *et al.*, 2013)
N	λ	Active	Converts RNAP to termination-resistant form (aided by several host Nus proteins)	(Mason *et al.*, 1992)
Q	λ	Active	Converts RNAP to termination-resistant form (aided by NusA)	(Shankar *et al.*, 2007)
Nun	HK022	–	Prevents RNAP translocation	(Vitiello *et al.*, 2014)
put RNAs	HK022	Active	Convert RNAP to termination-resistant form, via *put* RNA-RNAP interaction	(Komissarova *et al.*, 2008)
put/*put*-like RNAs	HK639, HK75 and prophages	Active	Convert RNAP to termination-resistant form, via *put*/*put*-like RNA-RNAP interaction	(King *et al.*, 2011)

Comparing and Contrasting Intrinsic Termination and Rho-dependent Termination

Intrinsic Termination

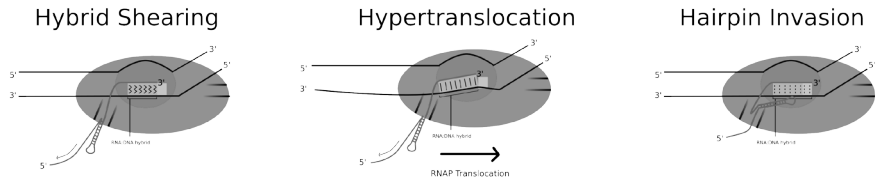

Figure 3. *Current competing models of intrinsic termination: Hybrid Shearing: the 3'-end of the transcript is pulled from the RNAP active site by the extending, upstream RNA hairpin, causing RNA:DNA hybrid shearing (i.e. disruption of the complementary base-pairing between template and transcript). Hypertranslocation: the extending, upstream RNA hairpin promotes forward translocation of RNAP (without nucleotide addition) while the RNA:DNA hybrid is maintained by translocation of RNA and DNA in the opposite direction – resulting in removal of the 3'-end of the transcript from the RNAP active site, as in the hybrid shearing model. Hairpin Invasion: the 3'-end of the transcript is not pulled from the active site, rather remains in place until movement of the hairpin into the RNAP main channel promotes hybrid melting via steric interactions (Peters et al., 2011).*

Rho-dependent Termination

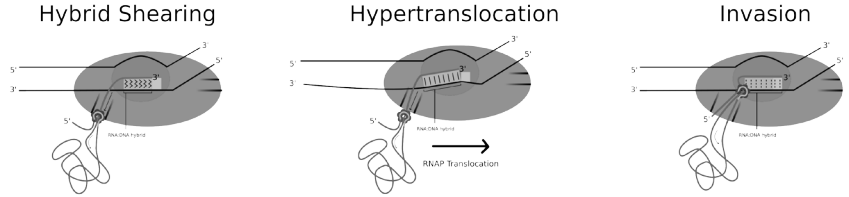

Figure. 4. *Current competing models of Rho-dependent termination: Hybrid Shearing: the 3'-end of the transcript is pulled from the RNAP active site by the pulling force of the increasingly taut RNA transcript being threaded through the central cavity of Rho. This causes RNA:DNA hybrid shearing (i.e. disruption of the complementary base-pairing between template and transcript). Hypertranslocation: Rho exerts a pushing force, promoting forward translocation of RNAP (without nucleotide addition) while the RNA:DNA hybrid is maintained by translocation of RNA and DNA in the opposite direction – resulting in removal of the 3'-end of the transcript from the RNAP active site as in the hybrid shearing model. Invasion: the 3'-end of the transcript is not pulled from the active site, rather remains in place until Rho directly unwinds the RNA:DNA hybrid using its RNA:DNA helicase activity (Peters et al., 2011).*

Rho Translocation

An example of a recent major advance in our understanding of Rho-dependent termination, involves the mechanism of Rho translocation (Figure 2), and the nature of the Rho-RNAP interaction. Recent chromatin immunoprecipitation and DNA microarray (ChIP-chip) analysis revealed that Rho associates directly with RNAP throughout the entire process of transcript elongation (Mooney *et al*., 2009). This was confirmed by an in vitro study (Epshtein et al., 2010), which appeared to show direct Rho-RNAP binding independent of proteins, DNA and RNA. It was also suggested that Rho might play a role in altering the properties of RNAP during elongation (Epshtein *et al*., 2010). If these findings were to be confirmed, rut site binding (or Rho-RNA interactions of any kind), would be unnecessary for Rho-RNAP association; and, assuming a Rho-RNAP association during elongation, Rho binding to rut would result in an RNA loop which would become shorter and more taut as the transcript was actively threaded through the central cavity of Rho. In fact, the opposite is true, and hence these radical findings have been challenged by other investigations, which maintain that Rho does not bind to RNAP in the absence of a rut site, nor does Rho directly associate with RNAP except during termination (Kalyani *et al*., 2011).

Single-molecule studies also show no evidence of direct Rho-RNAP binding (Koslover *et al*., 2012). As such, alternative models of Rho translocation (e.g. pure/simple translocation and rut-free translocation) have become increasingly unlikely and the previously described 'tethered tracking' model has gradually emerged as the most probable (Koslover *et al*., 2012). Support for the tethered tracking model has come, in part, from the fact that Rho can generate >200 pN of force (Schwartz *et al*., 2007): more than enough to overcome the hindrance of maintaining rut contacts during translocation, and surely sufficient to provide the >30 pN of force required to displace RNA from RNAP at one of several tsp release sites (Dalal *et al*., 2006). Recent single-molecule manipulations and fluorescence methods seem to have confirmed tethered tracking as the principal mechanism of Rho translocation in bacteria such as *E. coli* (Gocheva *et al*., 2015), and further investigation has also led to the observation that Rho can translocate against a relatively large applied force (7 pN), and can translocate approximately 2-5 times faster than RNAP (Gocheva *et al*., 2015).

Discussion and Conclusions

Unanswered Questions and Future Prospects

Our knowledge of transcription termination has greatly accelerated in recent years, thanks to structural and biochemical advances in our understanding of the multisubunit bacterial RNAP and TEC, as well as genetic analyses of terminators and single-molecule analyses of their associated protein factors (e.g. Rho, NusA,

NusG). However, certain fundamental elements of the termination mechanism remain elusive: for example, how Rho binding to the RNA rut site activates its ATPase activity, and if the intrinsic-terminator-associated pause involves backtracking. Further study of the interconnected mechanisms of elongation, termination and antitermination, as well as the continual and rapid development of more sophisticated investigative techniques – such as monitoring methods which allow the real-time observation of transcription (Greive *et al*., 2008) and backtracking (Lass-Napiorkowska and Heyduk, 2016) by *E. coli* RNAP using surface plasmon resonance (SPR) and various biochemical approaches – will likely lead to answers for these questions and others sometime in the near future. In addition, a greater understanding of elongation, termination and antitermination could have many practical and economical implications, due to the widespread use of *E. coli* in both the biotechnological and pharmaceutical industries (Blount, 2015). Further elucidation of these processes could potentially act to improve the efficiencies or yields of many commercial processes involving *E. coli*, including the production of biofuels (Liu and Khosla, 2010) and the production of recombinant therapeutic proteins such as insulin (Goeddel *et al*., 1979).

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Inhibitor of Apoptosis Proteins and Their Potential in Cancer Therapy

Karen Slattery
Senior Sophister
Molecular Medicine

Inhibitor of apoptosis proteins (IAPs) are a family of endogenous, pro-survival molecules found within the cell. They prevent apoptosis, a form of programmed cell death, by interfering with apoptotic pathways and inhibiting caspase cascades. IAPs are over expressed in many forms of cancer and thus contribute to one of the key hallmarks of cancer- the evasion of apoptosis. As a result, IAPs represent a novel target for the specific treatment of a range of cancers, especially those resistant to standard treatments such as chemotherapy and radiotherapy. The use of IAP inhibitors, antagonists and antisense oligonucleotides to downregulate IAPs has been shown to produce potent anti-tumour activity. Furthermore, targeting IAPs has proven markedly effective when used as part of a combination treatment, such as with the pro-apoptotic cytokine TRAIL.

Introduction

Apoptosis is an energy dependent form of programmed cell death. It is critical during development, in the maintenance of homeostasis and as an immunological and anti-tumor defence mechanism. Dysregulation of apoptosis contributes to a wide variety of human conditions such as cancer, neurodegenerative diseases and autoimmune disorders (Favaloro *et al.*, 2012). Distinct morphological changes characterise apoptosis. These include blebbing, cell shrinkage, chromatin condensation and DNA fagmentation (Elmore, 2007). Initiation of cell death pathways triggers sophisticated caspase cascades leading to cell death. Caspases are a family of aspartic acid specific proteases that are endogenous in

the cytoplasm as single chain zymogens (Nicholson, 1999). Upon apoptotic signal initiation upstream initiator caspases, such as caspase 8, cleave effector caspases at internal aspartic acid residues, transforming them into their tetrameric active form (Thornberry and Lazebnik, 1998). These effector caspases, such as caspase 3, activate targeted cellular proteins to trigger apoptosis. Apoptosis can be initiated intracellularly or extracellularly resulting in two distinct pathways: the intrinsic and the extrinsic pathway (Figure 1).

The intrinsic or mitochondrial pathway involves diverse, non-receptor mediated stimuli such as genotoxic agents, cell stress or loss of cell survival factors. These apoptotic signals result in the formation of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential and release of pro-apoptotic proteins into the cytosol (Saelens *et al.*, 2004). Smac (Second Mitochondrial Activator of Caspases) binds to and neutralises X-linked IAP (XIAP) which releases and facilitates the activation of caspase 3, 7 and 9 (Schimmer, 2004). Similarly, the serine protease Omi/HtrA2 binds to and inhibits XIAP (Suzuki *et al.*, 2001a). Cytochrome c binds to and activates apoptotic protease factor 1 (Apaf-1) along with ATP and pro-caspase 9 forming the apoptosome (Chinnaiyan, 1999). Caspase 9 is activated, initiating the caspase cascade. It induces the cleavage of pro-caspase 3 releasing the active effector caspase 3, and ultimately resulting in programmed cell death. Many conventional cancer treatments, such as chemotherapy and radiotherapy, induce DNA damage thus targeting the intrinsic apoptotic pathway in a p53 dependent manner (Fulda and Debatin, 2006).

The extrinsic pathway is initiated when a transmembrane death receptor (DR) is bound by its corresponding death receptor ligand (DRL). A number of these DR and DRL pairs have been described including members of the TNF superfamily, such as TNF-α with TNFR1, FasL with FasR, and TRAIL with DR4 or DR5 (Guicciardi and Gores, 2009). Upon ligation, adaptor proteins with corresponding death domains are recruited, such as Fas-Associated protein with Death Domain (FADD) and TNF receptor associated factor (TRAF), which then associate with pro-caspase 8 forming the death inducible signalling complex (DISC) (Kischkel *et al.*, 1995). Pro-caspase 8 is auto cleaved to caspase 8 which goes on to activate caspase 3, triggering the proteolytic cascade and apoptosis. Caspase 8 may also feedback into the intrinsic pathway by processing and activating BH3 interacting domain death agonist (Bid). Bid provokes the release of cytochrome c, fuelling apoptosome mediated cell death (Roy and Nicholson, 2000).

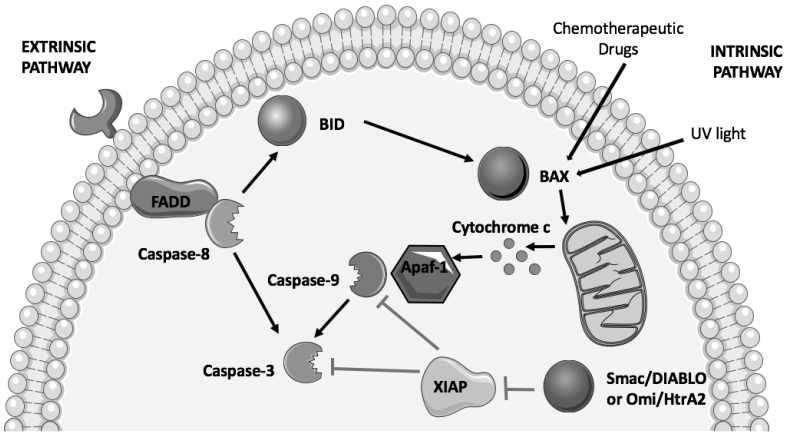

Figure 1. *The intrinsic and extrinsic cell death pathways Intrinsic signals trigger the release of pro-apoptotic proteins from the mitochondria. Smac and Omi/HtrA2 inhibit XIAP while cytochrome c, Apaf-1 and pro-caspase 9 form the apoptosome. Activated caspase 9 further activates caspase 3 thus triggering the caspase cascade resulting in apoptosis. Extracellular DRLs bind their DRs, adaptor proteins are recruited and caspase 8 is activated. This either goes on to activate caspase 3 and initiate the cascade or else activates Bid which promotes release of cytochrome c from the mitochondria. Adapted from Salvesen and Duckett (2002).*

Evasion of apoptosis is one of the key hallmarks of cancer and allows cells to divide and grow uncontrollably (Hanahan and Weinberg, 2011). Transformed cells employ a plethora of mechanisms in order to escape death. These include loss of p53, downregulation of Bid and importantly, upregulation of IAPs (Fernald and Kurokawa, 2013). Indeed, targeting IAPs represents a novel strategy for treating tumours. Because of this, many efforts have been made to develop antagonists, inhibitors and antisense oligonucleotides that downregulate IAPs in tumour cells, particularly in those that are already multi drug resistant. This review will discuss the molecular and cellular characteristics of the IAP family, their potential role in cancer therapy and the emerging importance of combination therapies.

Inhibitor of Apoptosis Proteins

IAPs are a family of endogenously expressed anti-apoptotic proteins and hence are promising targets in cancer drug discovery. There are eight members of the

IAP family in humans: cellular IAP1 (cIAP1), cIAP2, XIAP, melanoma IAP (ML-IAP), survivin, IAP-like protein 2 (ILP-2), neuronal apoptosis inhibiting protein (NAIP) and Apollon (de Almagro and Vucic, 2012). The activity of IAPs is regulated by endogenous antagonists such as Smac. Overexpression of IAPs has been found in many cancers and facilitate cancers in the evasion of apoptosis and ultimately contribute to tumorigenesis and metastasis (Sung *et al.*, 2009).

The Structure of IAPs

IAPs are characterised by protein domains in their tertiary structure (Figure 2). All IAPs have at least one Bacculoviral IAP Repeat (BIR) domain located at their N terminal (Fulda and Vucic, 2012). cIAP1, cIAP2, NAIP and XIAP each have three BIR domains, while ML-IAP, survivin, Apollon and ILP-2 have one. BIR domains consist of a highly conserved sequence of eighty amino acids with a characteristic arrangement of $CX_2CX_{16}HX_6C$, where C is cysteine, H is histidine and X can be any amino acid. Within the BIR fold, a zinc atom is tetrahedral coordinated by the histidine and the three cysteine residues to establish a hydrophobic environment (Hinds *et al.*, 1999). BIR domains mediate protein-protein interactions between IAPs and their targets. For example, XIAP binds caspases via its BIR2 or BIR3 domain, while cIAP1 and cIAP2 bind TRAFF via their BIR1 domain (Zheng *et al.*, 2010, Huang *et al.*, 2001).

Other common domains include the Really Interesting New Gene (RING) domain which provides E3 ubiquitin ligase activity, the Ubiquitin-Associated (UBA) domain which allows IAPs to bind ubiquitin chains and the Caspase-Activating And Recruitment Domain (CARD domain) which functions as a protein-protein interacting domain and mediates protein oligomerization (Vaux and Silke, 2005) (Gyrd-Hansen *et al.*, 2008).

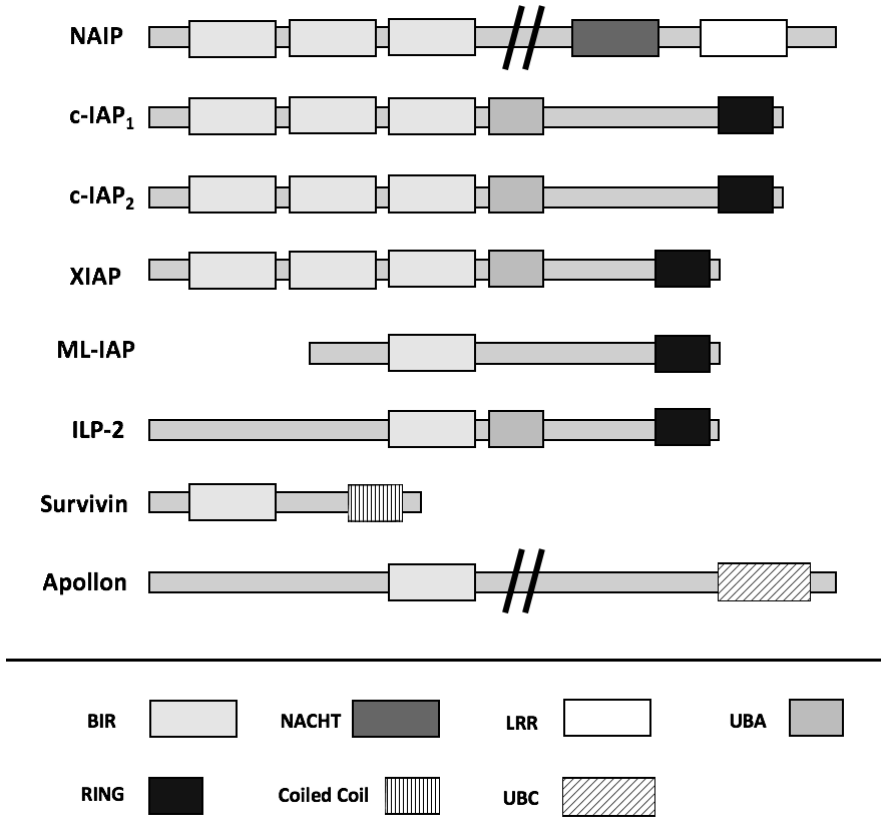

Figure 2. *The protein domains of IAPs. BIR (Bacculoviral IAP Repeat): protein-protein interactions. RING (really interesting new gene): E3 ubiquitin ligase activity. Coiled coil: super-secondary protein structure of coiled alpha helices. NAHCT (Neuronal Apoptosis Inhibitor Protein): NTPase activity. CARD (Caspase-Activating and Recruitment Domain): protein oligomerization. UBA (Ubiquitin-Associated Domain): polyubiquitin binding. UBC (Ubiquitin-Conjugating Enzyme): addition of ubiquitin. LRR (Leucine-Rich Repeat): secondary protein structure of an α/β horseshoe fold (Nishikawa and Scheraga, 1976, Koonin and Aravind, 2000, Gyrd-Hansen et al., 2008, Sekine et al., 2005, Enkhbayar et al., 2004). Adapted from Fulda and Vucic (2012).*

The Functions of IAPs

The members of the IAP family each have different functions within the cell. cIAP1/ cIAP2 serve to promote cell survival signals induced by members of the TNF superfamily. Upon ligation, they are recruited to the TNF receptor via TRAFF1/2

(Benetatos *et al*., 2014). Here they function as ubiquitin ligases by catalysing the addition of ubiquitin chains using their RING domain, for example to NFκB-inducing kinase. These chains can then serve as a scaffold, as signal transducers or as signals to target specific proteins for degradation (Walczak, 2011).

ML-IAP, also known as livin, has pro- and anti-apoptotic activity. Primarily, it binds Smac and promotes XIAP activity and caspase 9 degradation. It also promotes degradation of caspases via its E_3 ligase activity (Vucic *et al*., 2005). In contrast, ML-IAP can be cleaved at aspartate-52 by caspases forming a truncated form with pro-apoptotic activity (Nachmias *et al*., 2003). Thus, ML-IAP overexpression has been associated with drug resistance in melanoma, as well as leukaemia, bladder, cervical, colon, lung, breast and colorectal cancer (Vucic *et al*., 2000, Li *et al*., 2013, Tamm *et al*., 2000).

Survivin is structurally unique in that it is the smallest IAP in the family and only contains one protein domain, a BIR domain (Ambrosini *et al*., 1997). It has roles in the cellular stress response, in development and in mitosis (Altieri, 2010). Survivin has been shown to interact with heat shock proteins *in vivo*, which may help in its subcellular localisation or in the preservation of survivin stability (Fortugno *et al*., 2003, Yano *et al*., 2003). Within the cell survivin binds to and enhances the stability and activity of XIAP via their BIR domains (Dohi *et al*., 2004). During mitosis it has been reported to regulate chromosomal alignment, spindle assembly and stabilisation and cytokinesis (Lens *et al*., 2006).

XIAP is the best characterised member of the family and has the greatest anti-apoptotic activity. Two domains in XIAP are capable of binding and inhibiting nascent active caspases. The BIR3 domain binds directly to the C terminal of caspase 9. The BIR2 domain does not actually bind substrate but serves as a regulatory element for caspase binding and Smac neutralisation. Instead, a small segment directly at the amino terminal side of BIR2 is required for binding of caspase 3 and 7 (Stennicke *et al*., 2002). The sequence of this segment, known as 'the linker', binds the caspases with high affinity in the opposite orientation to their substrates (Huang *et al*., 2001). The BIR1 domain of XIAP induces NFκB and MAP kinase activation via TGF-beta activated kinase 1 (TAB1), an interaction that is subject to inhibition by Smac (Lu *et al*., 2007). Other functions of XIAP include regulation of the cell cycle (Levkau *et al*., 2001) and ubiquitination via its E3 ligase activity (Suzuki *et al*., 2001b)

IAPs in Cancer

The evasion of apoptosis allows cells to divide and grow uncontrollably thus contributing to tumorigenesis and metastasis. Upregulation of IAPs is believed to be a crucial mechanism by which tumours achieve this (de Almagro and Vucic, 2012). IAPs not only prevent cell death but also promote cell survival via NFκB and MAP kinase activation. Tamm *et al.* (2000) conducted an analysis of the expression of all of the IAPs in 60 human tumour cell lines and the expression

of XIAP in 78 untreated patients. Unsurprisingly, XIAP and $cIAP_1$ were overexpressed in most of the cell lines and $cIAP_2$ was overexpressed in more than half.

Each of the IAPs have different associations with particular cancers. For example, cIAP is associated with cervical cancer (Imoto *et al.*, 2002), survin with and livin with neuroblastoma (Islam *et al.*, 2000) and XIAP with pediatric leukemia (Sung *et al.*, 2009). The overexpression of IAPs in cancer is frequently an unfavourable prognostic parameter associated with poor treatment response and reduced relapse free survival. Notably, it seems that mRNA levels are often higher than protein levels, hinting at post-translational or post-transcriptional regulatory mechanisms (Tamm *et al.*, 2000, Hundsdoerfer *et al.*, 2010).

IAP Inhibition and Antagonism

Over the past decade, various strategies for targeting and inhibiting IAPs have been designed. These include IAP inhibitors and antagonists and antisense oligonucleotides (Fulda and Vucic, 2012). Antisense oligonucleotides are single stranded short pieces of synthetic DNA normally containing 12-30 oligonucleotides. These are designed to downregulate target protein expression by binding to complimentary stretches of mRNA and initiating its degradation (Jansen and Zangemeister-Wittke, 2002). AEG35156 is an example of an antisense oligonucleotide that targets XIAP expression (LaCasse *et al.*, 2006). It has displayed potent anti-tumour activity in mouse models of prostate, colon, ovarian and lung cancer (Fulda and Vucic, 2012). It has also been shown to sensitize cancer patients to cytotoxic agents such as chemotherapy and thus has completed Phase I/II of clinical trials (Holt *et al.*, 2011).

Smac mimetics are IAP inhibitors that mimic the N terminal portion of the endogenous protein Smac. This portion comprises just four hydrophobic amino acids in a conserved sequence: Ala-Val-Pro-Ille. The chain is essential for interacting with the hydrophobic environment of the BIR2 and BIR3 domains of the IAP proteins (Liu *et al.*, 2000). Monovalent as well as bivalent Smac mimetics have been designed (Figure 3). Dispute remains as to which is more favourable – bivalent mimetics seem to work more efficiently yet require intravenous administration while monovalent mimetics have less severe side effects and can be taken orally (Fulda, 2015).

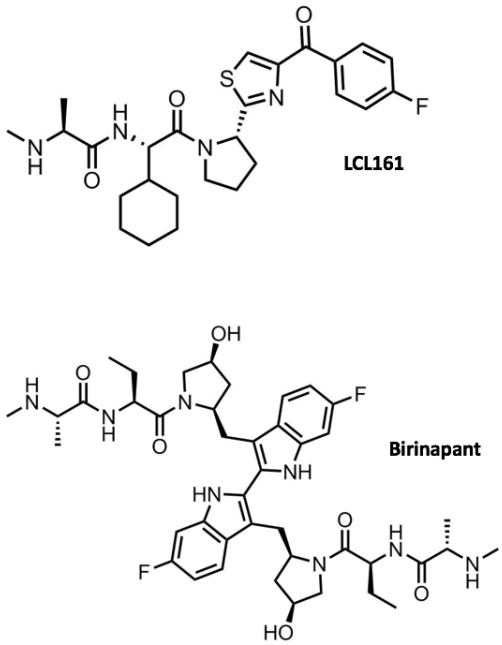

Figure 3. *The chemical structure of Smac mimetics. Examples of a monovalent (LCL161) and bivalent (Birinapant) Smac mimetic that are in the early stages of clinical trials. Adapted from Fulda (2015).*

Smac mimetics bind to several IAPs such as XIAP and cIAP to release XIAP from its inhibitory function and promote activation of activator caspases 3, 7 and 9 (Fulda and Vucic, 2012). They also induce cIAP1/2 proteasomal degradation by stimulating the auto-ubiquitination of cIAP1/2 via its E3 ubiquitin ligase activity (Guicciardi *et al.*, 2011). Furthermore, the reduction in cIAP1/2 results in the activation of the NFκB signalling pathway, resulting in the production of pro-inflammatory cytokines such as TNFα and interferon-γ, both of which have anti-tumour properties (Zarnegar *et al.*, 2008).

Embelin is a unique Smac mimetic in that it is a naturally occurring, non-peptidic compound. It effectively neutralises XIAP by binding to its BIR3 domain (Nikolovska-Coleska *et al.*, 2004). It was discovered via computational structure-based screening of a library of Japanese traditional herbal medicines. Nikolovska-Coleska *et al.* showed that it binds XIAP with an affinity similar to that of endogenous Smac and to crucial residues within the BIR3 domain that Smac and caspase 9 bind to. Embelin promotes apoptosis by relieving caspase 9 inhibition and inhibiting NFκB signalling (Park *et al.*, 2013). Indeed, it has been shown to be effective in the treatment of many cancers such as glioma, where it blocks cell proliferation and induces apoptosis via inhibition of NFκB, and also prostate

cancer, where it was shown to increase mitochondrial apoptosis and reduce Akt and β-catenin signalling (Park *et al*., 2013, Park *et al*., 2015).

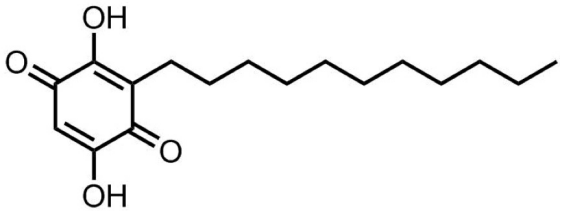

Figure 4. *The chemical structure of Embelin. Embelin is an alkyl substituted hydroxyl benzoquinone. The alkyl chain is crucial for XIAP binding; truncation to an ethyl group abolishes affinity. Taken from Nikolovska-Coleska et al. (2004).*

Combination Therapies

Due to the heterogeneous nature of tumours and their ability to rapidly develop resistance to treatment, IAP inhibitors and antagonists often have low efficacy when used as single agents and therefore research is now focused on combination therapies. These combination therapies make use of a wide range of cytotoxic stimuli, such as chemotherapy, radiation, DR agonists, signal transduction modulators and immune stimuli (Fulda, 2015). These have been shown to treat a wide variety of solid and haematological cancers (Table 1).

Of these stimuli, TRAIL has shown exceptional potential as a combination treatment for IAP inhibitors. TRAIL is a pro-apoptotic cytokine expressed by many tissues and immune cells (Wiley *et al*., 1995). Unlike many of the other stimuli, TRAIL exhibits cytotoxic selectivity towards cancer cells whilst sparing healthy cells (Walczak *et al*., 1999).

Table 1. *Combination Smac mimetic therapy in the treatment of cancer. Various Smac mimetics have shown potent anti-tumour activity when used in combination with treatments such as chemotherapy and radiotherapy. This has been shown in vitro and in vivo, in solid as well as haematological malignancies. CLL; Chronic lymphocytic leukemia, ALL; acute lymphoblastic leukemia, AML; acute myeloid leukemia.*

Combination Treatment	Smac Mimetic	Stimulus	Cancer Type	Reference
Chemotherapy	JP1400	Cisplatin	Lung	(Probst *et al.*, 2010)
	BV6	Glucocorticoids/ Cytarabine	ALL	(Belz *et al.*, 2014, Chromik *et al.*, 2014)
Death Receptor Agonists	Compound 3	TNF α	Solid tumours	(Wang *et al.*, 2008)
	IDN	TRAIL	Pancreatic carcinoma/ CLL/ ALL	(Vogler *et al.*, 2009, Stadel *et al.*, 2010, Loeder *et al.*, 2009, Fakler *et al.*, 2009)
Radiation	IDN	γ irradiation	Pancreatic carcinoma	(Giagkousiklidis *et al.*, 2007)
	BV6/ LBW242/ IDN	γ irradiation	Glioblastoma	(Berger *et al.*, 2011, Ziegler *et al.*, 2011, Vellanki *et al.*, 2009)
Signal transduction inhibitor	LBW242	Imatinib	Glioblastoma	(Ziegler *et al.*, 2008)
	BV6/ Birinapant	5-Aza, DAC	AML	(Steinhart *et al.*, 2013, Carter *et al.*, 2014)
Immune Stimuli	LCL161	Oncolytic virus/ poly(I:C)/ CpG oligonucleotides	Solid tumours	(Beug *et al.*)
	BV6	IFN α	AML	(Bake *et al.*, 2014)

The key feature of TRAIL cell signalling is the involvement of four diverse transmembrane receptors that each bind the cytokine. Two of them, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/ Apo2L), transmit pro-apoptotic signals into the cell. Binding of TRAIL, which naturally occurs as a trimer, to one of these induces receptor trimerisation, recruitment of FADD, formation of the DISC and ultimately apoptosis (Kischkel *et al*., 2000). The two other receptors, TRAIL-R3 (decoy receptor 1 (DcR1)) and TRAIL-R4 (DcR2), lack functional intracellular death domains and therefore do not transmit pro-apoptotic signals (Sheridan *et al*., 1997). DcR1 is a glycosyl-phosphatidyl-inositol-anchored receptor lacking any intracellular domain. DcR2 contains a truncated, non-functional death domain which has been reported to form inactive hetero-complexes with DR5, and trigger cell survival signalling pathways such as NFκB and PKB/Akt (Degli-Esposti *et al*., 1997, Lalaoui *et al*., 2011).

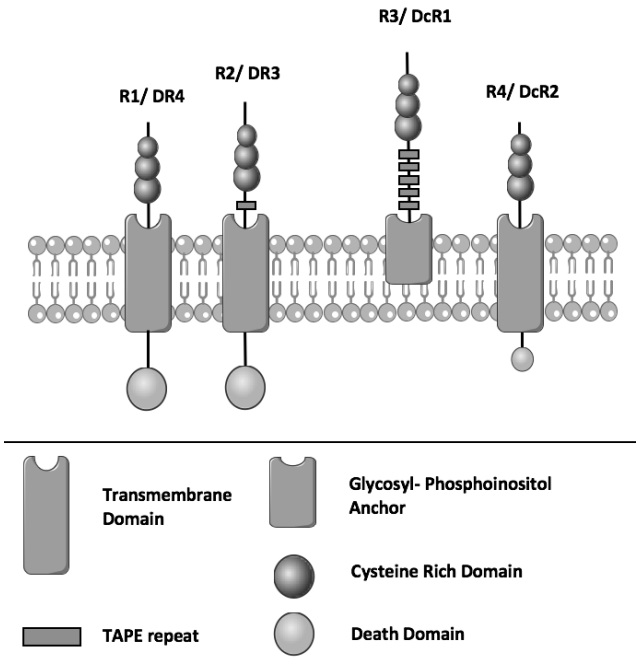

Figure 5. *The TRAIL death and decoy receptors. TRAIL has two death receptors, DR4 and DR5, which are capable of transmitting pro-apoptotic signals. Adapted from Lemke et al. (2014).*

Indeed, it is the preferential expression of decoy receptors in healthy cells and pro-apoptotic receptors in cancer cells that makes TRAIL signalling selective. This unique feature results in less toxic side effects in patients (Kruyt, 2008). Since this profound discovery, TRAIL receptor agonists and human recombinant TRAIL have been developed. Numerous Smac mimetics have exhibited promising broad clinical activity when combined with TRAIL *in vitro* and *in vivo* (Fulda *et al*., 2002, Li *et al*., 2004). The Smac mimetic Birinapant in combination with the TRAIL DR5 agonist Conatumumab has recently successfully completed its Phase 1 trial (clinicaltrials.gov).

Conclusion

IAPs, which are recurrently found upregulated in cancer, not only control cell death but also influence signal transduction pathways and progression of the cell cycle. With the recognition of evasion of apoptosis as a fundamental hallmark of cancer, the targeting of IAP proteins is now acknowledged as an incredibly promising and novel treatment strategy. Indeed, many aspect of IAP molecular biology and their contribution to cancer remains to be discovered and so they are being heavily researched. Future work must focus on implementing protocols to determine what patients will benefit the most from this novel modality of cancer therapy, in particular patients with tumour profiles specifically overexpressing IAPs. In addition, further trials need to be conducted in order to elucidate the combinations of therapies that maximise efficacy for specific patient cohorts.

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A Thief's Toolbox: Bacterial Strategies to Acquire Iron From The Human Host

Kelly Murray
Senior Sophister
Microbiology

Iron acquisition is a critical determinant in the success of bacterial infection. Bacteria are faced with a low availability of iron in the host, as it is sequestered by host proteins. In order to combat this reality, bacteria have devised multiple mechanisms to exploit iron sources by producing haemophores and siderophores themselves, or stealing them from other bacteria. A variety of different types of haemophores and siderophores are produced by both Gram-positive and Gram-negative pathogenic bacteria. The regulation of these iron uptake systems is crucial in order to maintain iron homeostasis. Knowledge of these systems can aid in the development of new therapeutic strategies such as conjugating antibiotics to the siderophores, enabling direct insertion of antibiotics into bacteria.

Introduction

Iron is an essential element for all organisms, including pathogenic bacteria. Iron can be found in two different positively charged ionic forms, Fe^{2+} and Fe^{3+} , useful in biological processes such as, oxidative phosphorylation, and DNA replication and repair. In the human host, free Fe^{3+} can react with oxygen via the Fenton and Haber-Weiss reaction, to generate reactive oxygen species that damages DNA, lipid and membrane haem proteins. To protect against the potential toxicity of Fe^{3+}, iron is sequestered by proteins such as, lactoferrin, transferrin, and ferritins. Iron may also be stored as haem, incorporated into a protoporphyrin ring in haemoproteins(Hammer and Skaar, 2011). This also reduces the availability of free iron for use by pathogenic bacteria, known as nutritional immunity. To combat this nutritional immunity, pathogenic bacteria have evolved many ways to extract

iron that is complexed to a variety of proteins. In order to acquire haem, bacteria secrete proteins known as haemophores that bind to haemoproteins to acquire haem. In order to obtain iron sequestered by lactoferrin or transferrin, bacteria can use secreted molecules known as siderophores (Caza and Kronstad, 2013). Haemophores are proteins while siderophores are not. Most haem iron uptake systems are negatively regulated by the ferric uptake regulator, Fur (Figure 1A)(Carpenter *et al*., 2009). However, this regulatory function is carried out by diphtheria toxin repressor, DtxR, in *Corynebacterium diphtheria*. *Streptococcus pyogenes* iron regulation is mediated by the metal transporter of streptococci regulator, MtsR, a DtxR divergent homolog. Analysis shows that despite their low sequence homology, MtsR, DtxR and Fur, share structural and functional similarity as transcriptional repressors of iron-responsive genes (Bates *et al*., 2005, Sheldon and Heinrichs, 2015). Some bacteria regulate iron acquisition using extracytoplasmic function (ECF) sigma factors (Figure 1B). This mechanism is employed by the has operon in *Serratia marcescens* (*S. marcescens*).

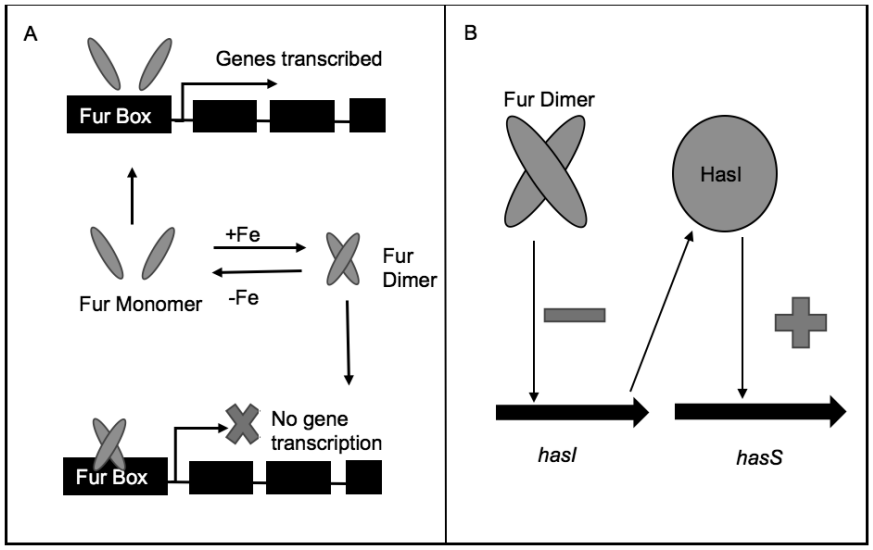

Figure 1. A) *The transcriptional repression of iron acquisition genes upon the binding of the Fur dimer to the Fur box, when iron levels are sufficient. Upon sensing of iron limiting conditions, Fur binding is diminished, resulting in the transcription of iron acquisition genes. Adapted from Nobles & Maresso (Nobles and Maresso, 2011).* B) *the regulation of iron acquisition genes by using ECF sigma factors. Fur negatively represses sigma factor gene, hasI, which results in the expression of the hasS gene which represses the hasR promoter involved in iron acquisition in S. marcescens. Adapted from Cescau et al. (Cescau et al., 2007).*

Haem-Iron acquisition strategies in bacteria

Haem accounts for 80% of the total iron available in the human host, and is found associated within haemoglobin, primarily in erythrocytes (Skaar *et al*., 2004). Many gram-positive pathogenic bacteria lyse these erythrocytes by releasing haemolysins. Upon lysis, free haem and hemoglobin are sequestered by host proteins, haptoglobin or haemopexin. Bacteria produce haemophores in order to gain access to this sequestered iron (Ma *et al*., 2015).

The NEAT-type haemophores

Haem is acquired through a relay system of proteins, known as iron-regulated surface-determinant (Isd) proteins in *Staphylococcus aureus*. The sortase B operon was found to be important for *S. aureus* pathogenesis, particularly iron acquisition. This operon, subsequently named Isd locus, encodes three of the surface-bound Isd proteins, IsdA, IsdB, and IsdC which are anchored to the cell wall by either Sortase A or Sortase B (Nobles and Maresso, 2011). This led to the proposed relay protein system, that IsdA, IsdB and IsdH are hemoprotein receptors on the cell surface that pass haem to IsdC or IsdE (Mazmanian et al., 2003). Haem is then transported through the cell wall via, ABC transport system IsdDEF or HtsBC, using energy from an ATPase, IdsF. Once inside the cytoplasm, haem oxygenases, IsdG and IsdI, break down the porphyrin ring, releasing iron for use by the bacterium (Figure 2A). The surface-bound Isd proteins, IsdB and IsdH, contain one or more near iron transporter (NEAT) motifs, which binds haem from either haemoglobin or haptoglobin. IsdH contains three NEAT domains and IsdB contains two domains, while IsdA and IsdC contain one each. IsdH (NEAT1 and NEAT2) and IsdB (NEAT1) can bind haemoglobin and haptoglobin but not haem. The haem that is stripped from the haemoglobin during the Isd protein relay, is captured by the IsdH (NEAT3) and IsdB (NEAT2) and then passed IsdA and IsdC, then passed to the ABC transporter IsdDEF (Sheldon and Heinrichs, 2015). Recent research using QM/MM and MD stimulations lead to the proposal of a reaction scheme for haem transfer between NEAT domains. The results from these experiments indicated that deprotonation of two tyrosine residues, Tyr166 and Tyr170 in IsdA , were crucial in the transfer of haem from IsdH by IsdA, to IsdC (Moriwaki *et al*., 2015).

Bacillus anthracis was found to contain homologs of the *S. aureus* Isd system, IsdC and SrtB while possessing some distinct features, such as IsdX1 and IsdX2 (Maresso *et al*., 2006, Maresso *et al*., 2008). IsdX1 and IsdX2 are secreted proteins containing NEAT domains. These were the first haemophores identified in Gram-positive bacteria. These were found to acquire haem from hemoglobin and transfer it on to IsdC. Interestingly, IsdX1 can transfer haem directly to IsdC or, pass haem to IsdX2, which then passes it to IsdC. It may seem like IsdX2 is functionally redundant, but it has been suggested that IsdX2 can act as a 'haem sponge' to control the rate of iron uptake (Honsa *et al*., 2011). The common property

of iron uptake systems in most Gram-positive bacteria, is the presence of NEAT domains. These 128 kDa NEAT domains show functional diversity, although their structural similarities are high (Honsa *et al*., 2014). Analysis of the NEAT1 (binds hemoglobin/haptoglobin) and NEAT2 (binds only haem) domains of IsdB in *S. aureus*, show 41% sequence homology. This is also the case for IsdH NEAT domains, where haem binding NEAT3 shows 38%-41% sequence homology to the first two domains (Dickson *et al*., 2014).

HasA-type haemophores

Haem acquisition system, HasA is a haemophore first identified in *Serratia marcescens* (*S. marcescens*), known as HasASM. Homologues of this system have only been identified in other Gram-negative bacteria such as, *Pseudomonas Aeruginosa* (*P. aeruginosa*), Pseudomonas fluorescens, Yersinia pestis, and Yersinia enterocolitica (Rossi *et al*., 2001, Ochsner *et al*., 2000). The HasA system allows bacteria to extract haem from haemoglobin, haemopexin and myoglobin. The crystal structure of holo-HasA has been determined, showing it to be a globular protein with two faces, one with four α-helices, and the other with seven β-strands. Between the two faces, there are two loops containing two iron axial ligands, His-32 (Loop1) and Tyr-75 (Loop 2), where haem is tightly bound (Arnoux *et al*., 1999). The Tyr-75 bond is stabilized by hydrogen bonds formed with His-83. Multiple sequence alignments of HasA from the bacteria mentioned above showed 30-50% homology, while His-32 and Tyr-75 are conserved among all species (Tong and Guo, 2009). Kinetic and biochemical studies on haem binding show that haem initially binds to Tyr-75, triggering Loop 1 to close over it, thus facilitating His-32 binding. Mutational analysis of these binding sites demonstrated that HasASM retains the ability to bind haem when one of the iron ligands is replaced by alanine, it is thought that water may help compensate the axial coordination (Caillet-Saguy *et al*., 2012). It was also suggested that His-83 may act as substitute iron ligand in the absence of Tyr-75, although this needs more examination (Kumar *et al*., 2014).

HasA then binds with high affinity to its receptor, HasR. Both holo-HasA and apo-HasA can bind to the receptor. The HasR receptor is composed of a transmembrane β-barrel, 22 antiparallel β-strands and an N-terminal plug that closes the pore of the β-barrel. Upon receptor binding, only haem is internalized while the HasA remains outside (Figure 2B) (Caillet-Saguy *et al*., 2009). Biochemical studies of this interaction revealed that haem transfer from HasA to HasR is Ton-B independent, and is actually caused by protein-protein interactions alone (Izadi-Pruneyre *et al*., 2006). It is thought that receptor binding disrupts the hydrogen bond between Tyr-75 and His-83 thus, weakening the iron binding sites affinity for haem in HasA. There are two conserved haemophore binding regions, β-strands 51-60 and 95-105. Interaction of these sites with loops 6, 8 and 9 of HasR has been indicated to cause a decrease in HasA affinity for haem, lower than HasR-haem affinity, resulting in the transfer of haem from HasA to HasR (Smith *et al*., 2015, Caillet-Saguy *et al*., 2012).

The haem that is released into the periplasm is bound by periplasmic binding haem proteins and transported across an ABC transporter into the cytoplasm, where it is degraded to biliverdin (Marvig *et al.,* 2014).

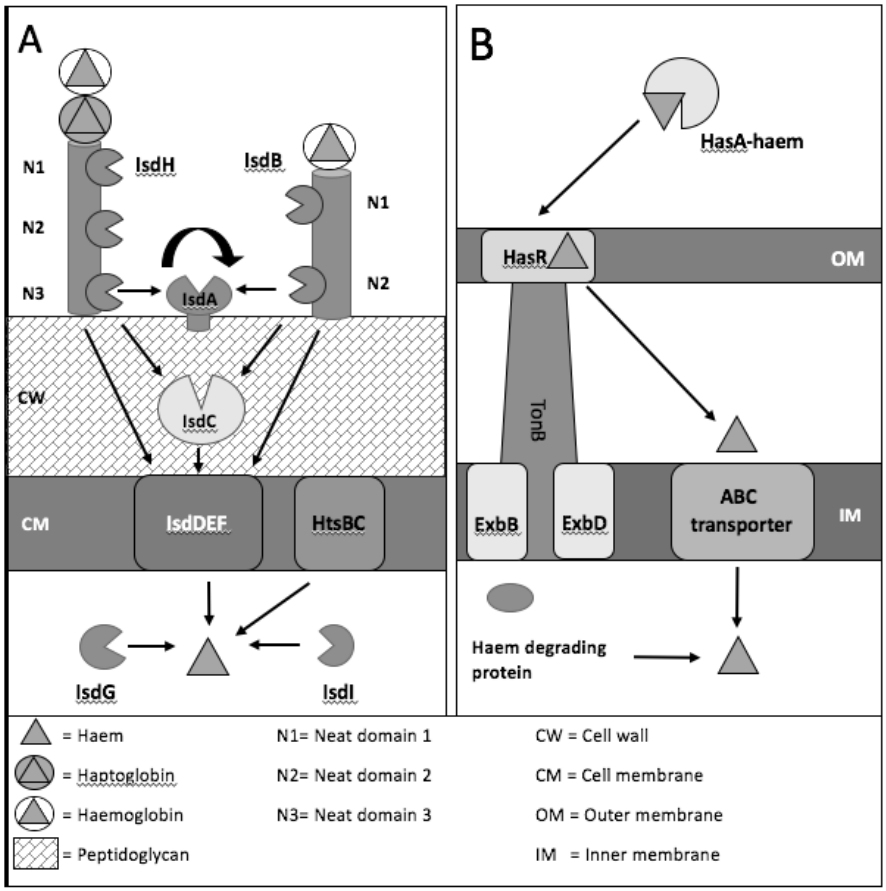

Figure 2. A) *The transport of haem along the NEAT domains (N1-N3) from IsdH, IsdB and IsdA, to IsdC or directly to IsdDEF. The black arrows represent direction of haem transport between the Isd proteins. Haem is transported across the membrane through IsdDEF or HtsBC transporters. The haem is then degraded by enzymes IsdG and IsdI. Adapted from Hammer & Skaar (Hammer and Skaar, 2011).* B) *The HasA-haem complex binding to the receptor HasR. The haem passes though the outer membrane with energy generated by the TonB analogue, HasB with proteins ExbB and ExbD. Haem transport into the cytoplasm occurs via an ABC transporter. Adapted from Contreras et al. (Contreras et al., 2014).*

Iron acquisition from transferrin, lactoferrin and ferritin

Iron complexed to lactoferrin, transferrin or ferritin can be exploited using Siderophores. Siderophores are small, secreted, high-affinity iron chelating molecules and unlike haemophores, are not proteins. Many bacterial pathogens use siderophores, but only one system from both Gram-positive and Gram-negative bacteria will be discussed in this review.

Siderophores in Gram-positive bacteria

S. aureus produces two siderophores, staphyloferrin A and staphyloferrin B, which are part of the carboxylate family of siderophores. The genes for expression of both siderophores are regulated by Fur, as previously discussed above (Hammer and Skaar, 2011).

Staphyloferrin A is encoded by the sfnABCD operon. The structure of staphyloferrin A has been determined to be a D-ornithine molecule that links two molecules of citrate through amide bonds. HtsABC is the ABC transporter involved in transporting Staphyloferrin A into the cytoplasm, as genes encoding it are found adjacent to the sfn operon (Beasley *et al*., 2009). Analysis of the crystal structure of the siderophore receptor HtsA, demonstrates that it undergoes a conformational change upon staphyloferrin A binding, trapping it. Transport across the membrane requires energy, however the snf operon does not encode an ATPase. The ferric hydroxymate uptake operon (fhuCBG) was found to encode an ATPase. FhuC was shown to be the ATPase required for both staphyloferrin A and staphyloferrin B transport, as transport was inhibited when fhuCBG operon encoding fhuC, was knocked out (Dale *et al*., 2004).

Staphyloferrin B is composed of L-2,3diaminopropionic acid, 1,2-diaminoethane and α-ketoglutaric acid. Staphyloferrin B is encoded by the sbnABCDEFGHI. The staphylococcal iron regulated transporter (sirABC) is thought to transport staphyloferrin B across the membrane in a similar manner to HtsABC with staphyloferrin A (Figure 3A). SirA, the receptor undergoes conformational change upon staphyloferrin B binding, trapping it just as HtsA does with staphyloferrin A. As with HtsABC, FhuC is the ATPase required for staphyloferrin B transport across the membrane (Madsen *et al*., 2015).

S. aureus also possesses the ability to steal siderophores produced by other bacteria, by producing xenosiderophores. These xenosiderophores are transported across the membrane using the FhuCBG system, which provides its ATPase for both staphyloferrin A and staphyloferrin B transport. FhuD1 and FhuD2 are the lipoprotein receptors and only undergo a minimal conformational change upon xenosiderophore binding, unlike HtsA and SirA. This is thought to enable broad-spectrum binding to many different types of xenosiderophores such as, erobactin, ferrichrome, ferrioxamine B and coprogen. The use of the Fhu ATPase is critical in *S. aureus* iron acquisition, making it a potential target for future therapeutic treatments.

Siderophores in Gram-negative bacteria

Similar to siderophore transport in gram-positive bacteria, ABC transporters are also required for siderophore transport in gram-negative bacteria, such as *P. aeruginosa*. *P. aeruginosa* is unable to take up iron from transferrin directly, to combat this it produces two siderophores, pyoverdine and pyochelin (Cezard *et al*., 2015). Pyoverdine has been well studied due to its fluorescent nature, however this fluorescence is diminished upon iron binding. Pyoverdine consists of a chromophore with a peptide chain bound to it. The length and composition of the peptide chain are different for each strain. *P. aeruginosa* can produce one of three structurally different types of pyoverdines. The pyoverdine-iron complex binds to the TonB-dependent membrane receptor, FpvA (Schalk, 2008). As previously discussed, this binding driven by cytoplasmic membrane potential results in a conformational change in the plug of the receptor, allowing the siderophore-iron complex passage into the periplasm. The iron is then taken by a periplasmic binding protein to the cytoplasm, via an ABC transporter. Pyoverdine is then recycled back out of the cell by an ABC efflux transporter, PvdRT-OmpQ (Yeterian *et al*., 2010). This efflux system has also been shown to be involved in the export of newly synthesized pyoverdine out of the cell, this had been disputed in the past (Imperi *et al*., 2009). Ferribactin, a pyoverdine precursor is exported into the periplasm by PvdE, where it undergoes maturation aided by PvdN, PvdO and PvdP. The newly synthesized pyoverdine is then exported out of the cell by PvdRT-OmpQ (Figure 3B).

Unlike Pyoverdine, Pyochelin does not undergo any periplasmic maturation. It is thought that Pyochelin is directly exported out of the cytoplasm through an ABC transporter composed of PchH and PchI, however little is known about the mechanisms involved. Pyochelin has a reasonably low affinity for iron and has higher affinities for both copper and zinc. However, studies indicate that pyochelin transport of copper and zinc is not as efficient as iron transport (Brandel *et al*., 2012). There is increasing evidence that both pyoverdine and pyochelin biosynthesis occur in siderosomes, in order to compartmentalize the siderophore precursors. This may be a protective mechanism for bacteria against the potentially toxic build-up of harmful molecules, such as siderophores or their precursors (Gasser *et al*., 2015). Recently, a novel iron acquisition system has been reported that is essential for the growth of *P. aeruginosa* in the airway of CF patients. *P. aeruginosa* strains defective in pyoverdine and pyochelin have been detected, suggesting an alternate iron acquisition strategy. Deletion experiments indicated that a genetic element encoding PA4834 gene was responsible for the iron acquisition of *P. aeruginosa* in airway mucosa cells. The mechanism of action of this novel iron acquisition system has yet to be determined, but suppression of this PA4834 gene may be a novel therapeutic approach against *P. aeruginosa* infection (Gi *et al*., 2015).

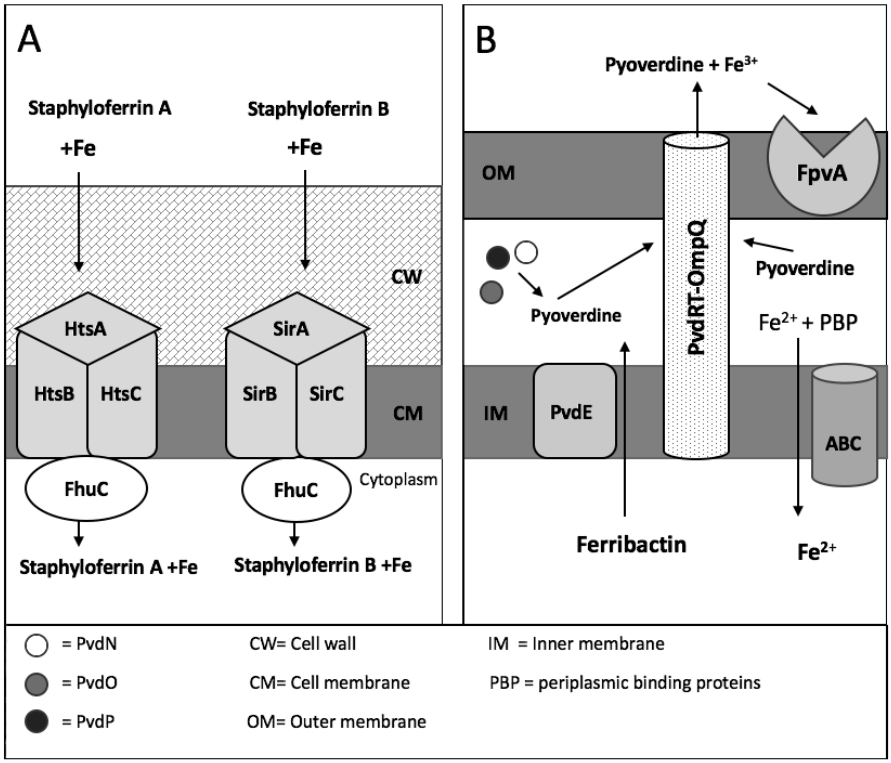

Figure 3. A) *The transport of haem complexed to siderophores, Staphyloferrin A and Staphyloferrin B produced by S. aureus. Staphyloferrin A transports iron into the cytoplasm via the HtsABC transporter, powered by ATPase, FhuC. Staphyloferrin B transports iron into the cytoplasm, via the SirABC transporter, also powered by the ATPase, FhuC. Adapted from Hammer and Skaar (2011).* B) *The generation of pyoverdine, one of the siderophores produced by P. aeruginosa. Ferribactin, a pyoverdine precursor is exported into the periplasm by PvdE, where it matures aided by proteins PvdN, PvdO and PvdP. The mature pyoverdine is then exported out of the cell by PvdRT-OmpQ. Pyoverdine extracts haem from host haemoproteins, then enters the periplasm through the receptor, FpvA. The pyoverdine molecule is recycled back out of the cell, via the PvdRT-OmpQ. The haem is transported into the cytoplasm, through an ABC transporter, aided by Periplasmic binding proteins. Adapted from Schalk (2008).*

Trojan horse strategy and medical applications

The rapid rate of antibiotic resistance is posing a great difficulty in the treatment of bacterial infections. New therapies are being developed in order to counteract this growing resistance such as, siderophore-drug complexes (SDC) known as the Trojan horse strategy. It is a way of increasing the efficacy of antibiotics by transporting it directly into the bacterium via their iron uptake systems (Page, 2012). The SDC are typically composed of three parts; siderophore, a linker and a drug. Upon uptake of the SDC, the linker region is cleaved, releasing the antibiotic, which is then functional. In the case of gram-negative bacteria, the antibiotic is transported through an ABC transporter into the cytoplasm. Naturally occurring siderophores covalently linked to an antibiotic moiety, known as sideromycins, has led to increasing interest in synthetic SDC derivatives.

The potential use of SDC against *P. aeruginosa* infections is of interest, as the bacterium is resistant to many antibiotics due to its chromosome-encoded antibiotic resistant genes. Recent studies have found that use of triscatecholate siderophores conjugated with either ampicillin, or amoxicillin, can inhibit the growth of gram-negative bacteria, such as *P. aeruginosa* (Ji *et al.*, 2012). As *P. aeruginosa* produces one of three different types of its pyoverdine siderophore, a pyoverdine-antibiotic conjugate would not be effective against all strains of *P. aeruginosa*. A pyochelin-antibiotic conjugate may be a more promising treatment, active against all strains of *P. aeruginosa* (Mislin and Schalk, 2014). Although recent research suggests that resistance to potential SDC against *P. aeruginosa* may still develop (Kim *et al.*, 2015). Research into the use of SDC against gram-positive bacteria, such as *S. aureus*, have shown some antimicrobial activity. A staphyloferrin A-fluoroquinolone conjugate has been designed to potentially treat staphylococcal skin infections (Milner *et al.*, 2013).

Conclusions

It is clear to see that iron is a critical element for bacterial survival, thus the vast amount of different iron acquisition systems that they possess in order to obtain it. While many of these systems have been determined, many are yet to be elucidated. Some systems are homologous across a wide range of bacteria, such as the presence of a TonB-ExbB-ExbD complex that provides the energy for transport of haem across the outer membrane, present in many Gram-negative bacterial iron acquisition systems. It is these common elements that provide the most hope as broad targets for future therapies against pathogenic bacteria. The Trojan horse strategy using siderophore-drug complexes, is already being used to treat certain iron related conditions, however there is increasing interest in their use against multidrug resistant bacterial infections, particularly caused by *S. aureus* and *P. aeruginosa*. Although some synthetic SDC have shown some antimicrobial efficacy, in order for these to be fully effective, the many iron acquisition pathways of bacterial pathogens need to be fully elucidated.

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Advances in Genome Engineering: The CRISPR/Cas9 Revolution

Aaron Fleming
Senior Sophister
Immunology

The ability to specifically alter and regulate gene in living organisms has long been a tantalising prospect in the field of cellular biology ever since the initial descriptions of how DNA encoded genetic information. The 2012 discovery of the RNA-guided DNA endonuclease activity of the bacterial Cas9 protein has spurred the beginning of a genome editing revolution which has attracted considerable attention outside the field of genetics alone. The CRISPR/Cas9 technology has usurped previous gene editing techniques and allowed researchers to explore new areas of genome engineering. The earliest discoveries concerning the clustered, regularly interspaced, short palindromic repeat (CRISPR) loci in prokaryotes led to the subsequent birth of the CRISPR/Cas9 genome editing tool and with it the most profound gene editing technology discovered to date. Novel attempts to improve the precision and efficiency of CRISPR/Cas9 are detailed and recent breakthroughs in the discovery of other CRISPR proteins are highlighted. The ease-of-use and efficiency of CRISPR/Cas9 will more than likely continue to expand our knowledge of genome function and potentially lead to its application in gene therapy for human disease.

Introduction

Genomic DNA sequences encode chemical databases for directing a vast array of biochemical processes. The human genome alone contains a staggering $3x10^9$ base pairs. Elucidating how this genome is regulated and expressed is of utmost importance for comprehending, predicting, and preventing disease. Central to our ability to investigate genome function is the power to test hypotheses by disrupting sequences implicated in a given process. The ability to alter or excise sequences complicit in disease states offers a powerful tool for potentially curing devastating genetic disorders. Controlled and targeted manipulation of DNA in this manner is being ushered in by widespread use of a new gene editing technology, CRISPR/Cas9, which is poised to transform our understanding of the complex interplay between genetics and disease states.

Targeted genome editing necessitates the creation of a double-stranded break (DSB) at a specified DNA sequence. Two different repair pathways predominate in cellular repair of DSBs. Non homologous end-joining (NHEJ) causes insertion/deletion mutations (indels) which can perturb reading frames and DNA regulatory elements, whilst homology-directed repair (HDR) employs the use of "donor" DNA molecules for repairing genetic sequences (Sancar *et al.*, 2004). Prior to CRISPR, previous methods for inducing DSBs at target loci relied on either zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), both of which comprise a customisable DNA binding protein domain fused to a nuclease domain.

Despite their potential, widespread adoption of ZFNs and TALENs was prevented by the expense and complexity of protein engineering (Sander and Joung, 2014). The 2012 discovery that *Streptococcus pyogenes*-derived CRISPR-associated protein 9 (Cas9) could induce targeted DSBs based on simple complementary base pairing between an RNA molecule and a target DNA sequence has led to a sustained uptake by research groups in the field of genome editing and engineering. Combining the relative ease of array-based oligonucleotide synthesis with the RNA-guided DNA endonuclease activity of Cas9 has allowed CRISPR technology to dwarf previous genome editing technologies. Twenty years of research assessing a specific prokaryotic loci has resulted in a genome engineering revolution which has seen the applications of CRISPR technology in research increase significantly in a relatively short period of time (Figure 1).

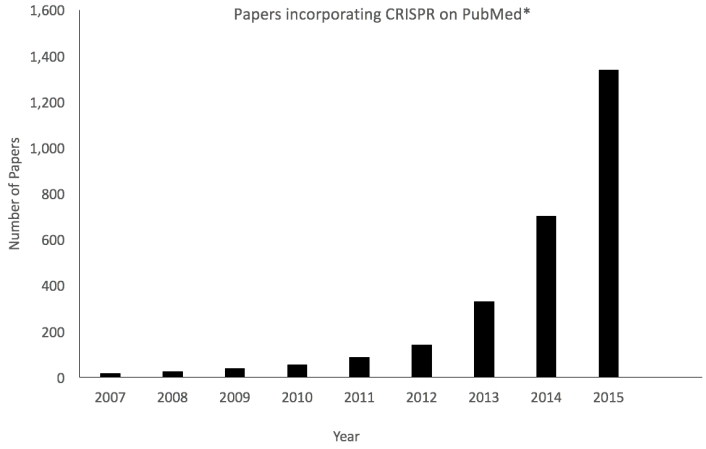

Figure 1. *Significant increase in the study and application of CRISPR technology. CRISPR research has seen an exponential growth since it was first described for genome editing in 2012. A PubMed search was conducted for the term "CRISPR", all primary papers and reviews returned were filtered by year and included.*

From peculiar prokaryotic sequence to robust genome-engineering tool: The CRIPSR story

Sequencing the *iap* gene from *Escherichia coli*, Ishino *et al.* (1987) noticed an unusual genetic element consisting of a repeated pattern of 29 conserved base pairs with a weakly palindromic nature separated by 32 nucleotides of a variable sequence. Genome sequencing advances in the 1990s led to the discovery of similar such sequences in a range of bacterial and archaeal organisms including *Mycobacterium tuberculosis* (Kamerbeek *et al.*, 1997), *Haloferax mediterranei* (Mojica *et al.*, 1995), and *Methanococcus jannaschii* (Bult *et al.*, 1996). Mojica *et al.* (2000) proposed grouping them together into a family of similar sequences defined by the presence of short, often palindromic nucleotide stretches separated by highly diverse "spacer" sequences. The CRISPR acronym (clustered, regularly interspaced, short palindromic repeats) was born two years later, in tandem with the designation *cas* (CRISPR-associated genes) to delineate the operon of genes found in close proximity to CRISPR arrays (Jansen *et al.*, 2002). Bolotin *et al.* (2005) revealed that spacer sequences of *Streptococcus thermophilus* CRISPR arrays are homologous to those found in *S. thermophilus*-specific phages and plasmids whilst Barrangou *et al.* (2007) showed that *S. thermophilus* is capable of directly incorporating short sequences of a phage genome (the protospacer) into the spacer region of its CRISPR array. Such acquisition confers protection against phages containing sequences

identical to the acquired one, revealing that CRISPR is the prokaryotic analogue of an adaptive immune system capable of targeting extra-chromosomal genetic elements (Figure 2).

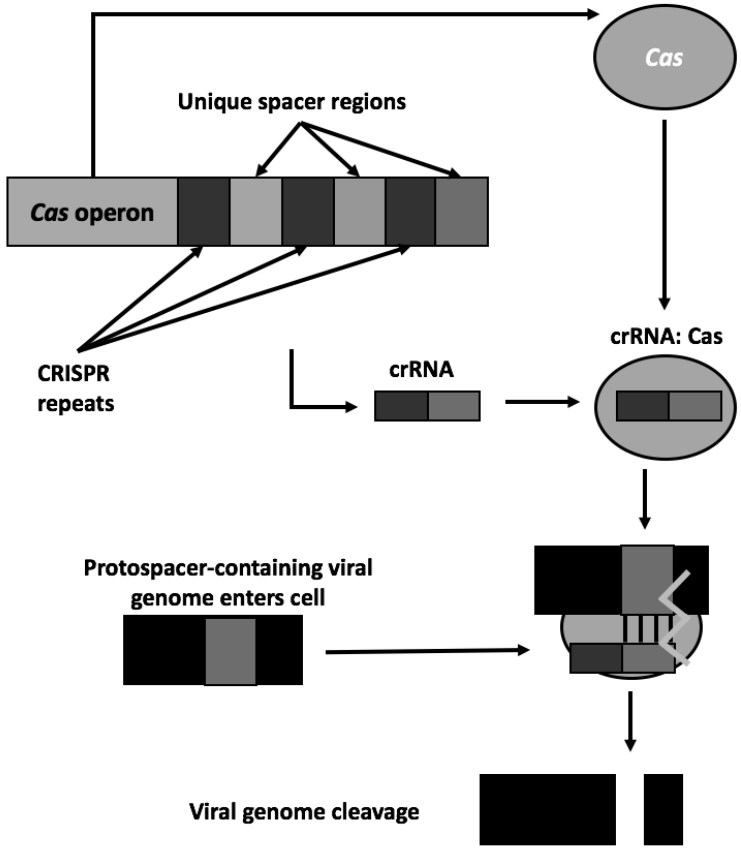

Figure 2. *CRISPR is a prokaryotic adaptive immune system which can be adapted for targeted and precise induction of double-stranded DNA breaks. In prokaryotes, CRISPR/Cas functions as an anti-viral defence mechanism whereby DNA-encoded portions of previous viral encounters (spacers) are used to target and cut viral genomes at precise sequences (protospacer) upon re-exposure.*

Brouns *et al.* (2008) showed that in *E. coli* the CRISPR array was first transcribed into a large pre-crRNA, which was then cleaved into smaller crRNAs containing both a spacer and repeat regions. In addition, Mojica *et al.,* (2009) revealed that small tri-

or di-nucleotide species-specific protospacer-adjacent motifs (PAMs) needed to be located upstream of the target sequence for effective phage defence, while Garneau *et al.* (2010) demonstrated that CRISPR/Cas targets foreign DNA by complementary base-pairing, resulting in the creation of DSBs with blunt ends. Differential RNA sequencing in *S. pyogenes* exposed the existence of small *trans*-encoded RNAs (tracrRNAs) in Type II CRISPR systems which hybridize to the repeat region of pre-crRNA at a 25 nucleotide sequence to form an RNA duplex held together by the Cas9 protein (Deltcheva *et al.*, 2011). Subsequent cleavage of the pre-crRNA produces an active crRNA:tracrRNA complex whereby the 20 nucleotide spacer sequence in the 5′ end of the crRNA portion can target complementary DNA sequences for Cas9-mediated cleavage (Figure 3). This two RNA/one cas protein mechanism contrasts with that of Type I and Type III CRISPR systems where a large multi-protein complex is required to process pre-crRNA (Charpentier *et al.*, 2015). Jinek *et al.* (2012) promptly capitalised upon this unique feature of Type II systems by capturing the *S. pyogenes* crRNA:tracrRNA interaction in a single chimeric "guide RNA" molecule (gRNA). Changes to the 20 nucleotide spacer region of the gRNA were sufficient to target the Cas9 endonuclease to multiple sites within a bacterial plasmid, provided they were downstream of the *S. pyogenes* PAM. This pivotal research provided proof-of-principle that a programmable Cas9-guided gRNA could theoretically be targeted to any PAM-adjacent portion of the genome and served as the impetus for the current CRISRP/Cas9 genome engineering revolution (Figure 4).

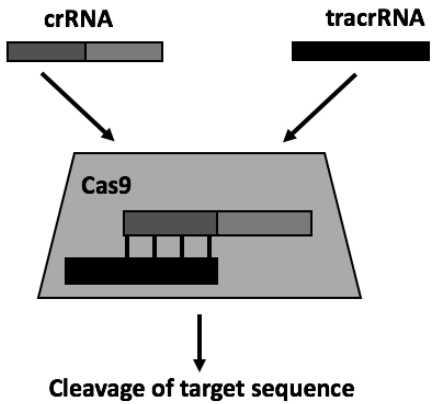

Figure 3. *Type II CRISPR systems harness Cas9 endonucleases to cleave dsDNA breaks. In Type II CRISPR systems, a crRNA:tracrRNA complex guides the Cas9 endonuclease to cleave target sequences. Adapted from Jinek et. al (2012).*

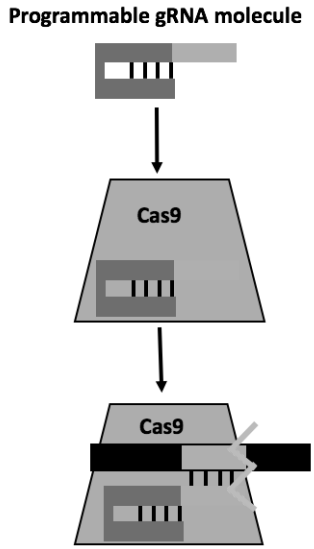

Figure 4. *The Type II CRISPR mechanism uses a guide RNA to direct the Cas9 endonuclease. The basic components of Type II CRISPR systems can be captured in a single guide RNA molecule (gRNA), whereby changes to the 20 nucleotide protospacer region can direct the Cas9 endonuclease to any PAM-adjacent portion of a target genome. Adapted from Jinek et. al (2012).*

Expanding the potential of CRISPR

Studies published in early 2013 demonstrated the versatility of CRISPR/Cas9 in a wide array of cell types. Jiang *et al.* (2013) showed that a Type II CRISPR system could be used to edit the endogenous genes of *E. coli* while Mali *et al.* (2013) engineered a human codon-optimized version of Cas9 encompassing a nuclear localisation signal (NLS) for eukaryotic gene editing. The capabilities of the CRISPR/Cas9 technology continued to be explored as numerous research teams employed it to edit zygotic genomes and create precise gene-edited analogues of key model organisms including *Arabidopsis thaliana* (Li *et al.*, 2013), *Caenorhabditis elegans* (Friedland *et al.*, 2013) , *Mus musculus* (Shen *et al.*, 2013), and *Macaca fascicularis* (Niu *et al.*, 2014). Introduction of stable and heritable changes in embryonic genomes using CRISPR/Cas9 has expedited the time and cost of creating transgenic animals whose study has traditionally been central to exploring the intertwined relationship between genetics and processes such as development and disease progression.

Several of the earliest studies demonstrating the genome-editing power of CRISPR/Cas9 anticipated its eventual use to correct causative mutations in heritable genetic disorders. Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder resulting in the absence of a functional dystrophin, a muscle protein, and causes decreased muscle cell integrity and usually death by the age of thirty from respiratory and cardiac failure (van Deutekom and van Ommen, 2003). Due to the considerable size of the dystrophin gene, it is refractory to traditional viral-mediated gene therapy. Long *et al.* (2014) employed a CRISPR-mediated gene editing therapy to correct the causative genetic lesion in embryos of the DMD mouse model. Subsequent to this, Ousterout *et al.* (2015) used a multiplexing approach comprising several gRNAs to delete ten deleterious exons in a "mutation hotspot" region of the dystrophin gene in cultured myoblast cells derived from human DMD patients. Exon deletion maintained the reading frame of the gene and resulted in the expression of a truncated yet functional dystrophin protein capable of maintaining correct localisation *in vivo* when engrafted onto immunodeficient mice. Furthermore, three research teams independently reported that adeno-virus associated (AAV) vectors could deliver the components of a CRISPR/Cas system for correction of a mutated dystrophin gene in adult mice (Nelson *et al*., 2015, Tabebordbar *et al*., 2015, Long *et al*., 2015). The novelty of these three studies was their systemic delivery of CRISPR/Cas9 gene editing components to treat fully grown animals with a genetic disease, potentially paving the way for its eventual use in DMD-afflicted patients.

Beyond gene editing: Repurposing CRISPR

The genome targeting function of Cas9 has been repurposed beyond its endogenous DNA endonuclease role to equip researchers with new tools to alter gene transcription and map genetic networks. Point mutations in the endonuclease domains of Cas9 create a catalytically inactive version (dCas9) which can remain bound to target sequences and serves as a convenient RNA-guided DNA binding platform. A technique christened CRISPR interference (CRISPRi) can target dCas9 to promoter and coding regions in *E. coli* to downregulate gene expression by physically hindering transcriptional factor recruitment (Qi *et al*., 2013). Eukaryotic transcriptional regulation however is more complex and involves distal and proximal regulatory elements including silencers, enhancers, and histone modifications. Gilbert *et al.* (2013) engineered a version of dCas9 fused to a transcriptional repressive domain to knock down protein expression in human cell lines, obtaining repression levels similar to that obtained using RNA interference (RNAi). Doudna and Charpentier (2014) have suggested that CRISPRi may be particularly potent in eukaryotic cells because it does not compete with endogenous gene knockdown pathways as in RNAi. Fusion of the catalytic core of a p300 acetyltransferase to dCas9 can also induce targeted histone acetylation at enhancer elements to activate nearby gene expression (Hilton *et al*., 2015).

The determination of the crystal structure of Cas9/gRNA with target sequences has also facilitated a more rational and structural approach to improving dCas9 by optimizing the location of associated effector domains (Nishimasu *et al.*, 2014). Konermann *et al.* (2015) built upon this crystal structure by fusing three activation domains to dCas9 to develop a potent transcription activation system termed synergistic activation mediator (SAM), creating a genome-wide gRNA library to identify novel gain-of-function mutations involved in resistance to a melanoma inhibitor. In a manner analogous to genome-wide association studies (GWAS), genome-wide CRISPR genetic perturbation studies will enable researchers to perform unbiased screenings to identify key components of cellular processes.

Improving reliability and efficiency: Next generation CRISPR techniques

The wide range of uses for CRISPR technology is an expanding area of research (Figure 5), but requires improvement in efficacy and reliability. One of the most important issues relating to the application of CRISPR is to what extent the gRNA can tolerate mismatches with the complementary sequence and thus to what extent Cas9 is able to induce unintended off-target DNA cleavage events. Hsu *et al.* (2013) demonstrated that PAM-proximal mismatches were better tolerated than mismatches distal to the PAM, in agreement with work done by Sternberg *et al.* (2014) showing that both gRNA-DNA duplex formation and DNA strand separation are initiated at the PAM. Hsu *et al.* (2013) proposed that a "seed sequence" of 8-14 nucleotides proximal to the PAM was critical for precise DNA targeting and that whether mismatches were adjacent to one another or interspaced affected the promiscuity of the gRNA. Furthermore, Fu *et al.* (2013) showed that off-target sites with up to five mismatches could be cleaved in a human cell-based reporter assay and most noticeably that there were often higher editing levels at predicted off-target sites than the intended on-target sites. Refuting this, Smith *et al.* (2014) found a low frequency of off-target effects in human stem cells using an unbiased and non-predictive whole genome sequencing approach, although it is possible that cell type architecture may affect the on/off-target ratios. Since there could potentially be thousands of off-target sites in the human genome if up to five mismatches were tolerated in a 20 base pair sequence, a clearer picture for how to both account for and avoid off-target effects will need to emerge before any CRISPR therapies can enter human clinical trials.

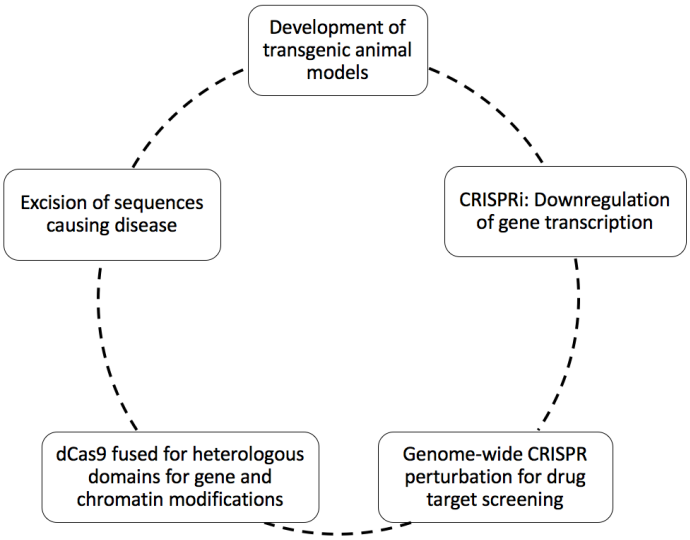

Figure 5. *Multitude of Uses for CRISPR/Cas9 Technology. CRISPR has been used for various applications in a wide range of cell types, animal models, scientific applications and has significant potential to revolutionise each of the areas detailed.*

Ran *et al.* (2013) adopted a "paired nickase" approach for reducing off-target mutagenesis whereby only one of the endonuclease domains of Cas9 is mutated (Cas9n) to produce a nickase capable of cutting only one strand of the DNA. Pairing nickases with offset guide RNAs effectively doubles the number of target bases and thus reduces the number of potential off-target. When a Cas9n/gRNA does nick an off-target site, these single nicks can be repaired by highly efficient base excision repair (BER) with concomitant low levels of unwarranted indel mutations (Mali *et al.*, 2013a). Adaptation of other CRISPR endonucleases for genome engineering purposes may also help to improve editing efficiencies. Ran *et al.* (2015) isolated a Cas9 orthologue from *S. aureus* (SaCas9) which is smaller than the more widely used S. pyogenes Cas9 and thus may be more compliant for cloning into size-restricted viral vectors. Zetsche *et al.* (2015) also isolated a novel CRISPR endonuclease called Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) which does not require a tracrRNA and induces a DSB with a 4 or 5 base pair 5′ overhang. Use of Cpf1 may simplify the design of guide RNAs due to the lack of tracrRNA requirement.

Conclusions

The initial discovery of CRISPR underscores the prime importance of research into basic cellular mechanisms and how we might utilise such research tools for eventual biomedical advances. However for all the excitement that CRISPR/Cas9 has generated, it has garnered a host of concomitant ethical questions concerning its application. The announcement in April 2015 that Chinese researchers had used CRISPR/Cas9 to edit human embryos with considerable off-target effects compelled the National Institutes of Health (NIH) to declare that they would not fund any CRISPR applications involving human embryos (Liang *et al*., 2015). Prof. Doudna, head of one of the research teams which initially described the adaptation of CRISPR systems for genome editing, has referred to the amount of CRISPR/Cas9 papers as a "tsunami" and called for increased education and reflection among scientists as to the sociological and ethical implications of quick and efficient genome editing (Doudna, 2015).

As the use of CRISPR continues to push gene editing boundaries, a measured approach will be required to balance the numerous advantages of such a powerful genetic tool against growing ethical concerns over the fast-paced application of the technology, particularly in the absence of universally agreed upon limits to gene editing. Despite this, it is becoming increasingly apparent that continued improvement of CRISPR systems will enable researchers to interrogate genetic networks on a scale previously unattainable with other technologies and to potentially further our knowledge and treatment of a wide range of complex human diseases.

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The Genesis of Malignant Rhabdoid Tumours

Cillian Brophy
Junior Sophister
Biochemistry

Malignant rhabdoid tumours (MRTs) are rare tumours found mainly in the kidneys and central nervous system, most commonly present in young infants and children and are typically associated with a very poor prognosis. Almost all rhabdoid tumours harbour a biallelic mutation of SMARCB1, a core subunit of the SWI/SNF chromatin remodelling complex. It has become apparent that epigenetic influences are a major driver of rhabdoid tumour growth. Current treatment regimens consist of aggressive multimodal therapies which can result in high treatment toxicity to patients. Only recently have specifically-designed treatment strategies been laid out for rhabdoid tumours in an official framework, and prognosis has consequently improved significantly. Most of these trials use molecular inhibitors to block tumour growth. Rhabdoid tumours and their oncogenic processes that drive their growth are the key targets for novel therapies and future treatment potential for patietns with MRTs.

Introduction

Malignant rhabdoid tumours (MRTs) are very rare but aggressive childhood cancers. MRTs are distinct from rhabdomyosarcomas (a cancer most commonly associated with rhabdoid cells). The term rhabdoid originally derived from cells that were 'rhabdoid' in nature when histological analysis of specimens was conducted by light microscopy. Rhabdoid cells are characterised by prominent nuclei, a single large nucleolus and a cytoplasm with globular eosinophilic inclusions (Barresi *et al.*, 2015).

MRTs are most commonly found in the kidneys (rhabdoid tumour of the kidney, RTK) and the central nervous system, in particular in the brain (atypical teratoid rhabdoid tumour, AT/RT). Rhabdoid tumours can also, more rarely, occur in extrarenal and extracranial soft tissues (Sredni and Tomita, 2015, Kerl *et al*., 2013). The presence of these rhabdoid cells and crucially the reduced expression of the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin B1 (SMARCB1) tumour suppressor gene are the key diagnostic factors of MRTs (Kieran *et al*., 2012). Analyses of factors such as expression of smooth muscle actin and epithelial membrane antigen is also often used to differentially diagnose the tumours in histopathology (Margol and Judkins, 2014).

RTK was the first rhabdoid tumour to be discovered and was initially thought to be a variant of Wilms' tumour, which is itself a renal tumour (Beckwith and Palmer, 1978), but was soon found to be morphologically and biologically distinct (Weeks *et al*., 1989). AT/RT, on the other hand, had originally often been thought to be primitive neuroectodermal tumour until it was ultimately identified as a separate entity (Rorke *et al*., 1996). Although there has been debate on the distinctiveness of AT/RT and RTK (Parham *et al*., 1994), these two major forms have striking similarities and are seen as counterparts, and MRTs from all anatomic sites are considered to be the same tumour type (Grupenmacher *et al*., 2013).

The majority of rhabdoid tumours share a biallelic inactivation of the SMARCB1 gene (Versteege *et al*., 1998, Jackson *et al*., 2009). These genes encode proteins that are subunits of a larger chromatin remodelling complex (Roberts and Biegel, 2009). The exact mechanisms by which SMARCB1 deficiency causes tumour growth is not fully understood, but it is known that the cell cycle and sonic hedgehog pathways are affected (Mora-Blanco *et al*., 2014, Tsikitis *et al*., 2005). Ongoing research is being done to investigate intermediaries involved in these pathways with the aim of developing and testing the efficacy of drugs and novel therapeutic strategies to combat these devastating tumours. Some of these drugs include small molecule inhibitors such as EZH2 inhibitors (Knutson *et al*., 2014).

MRT usually presents at 3 years or younger (Ahmed *et al*., 2007, Woehrer *et al*., 2010). Prognosis is poor compared with other early paediatric cancers and survival rates are low. Currently, the European Rhabdoid Registry recommends multimodal treatment consisting of surgery, radiotherapy and chemotherapy (Frühwald and Krefeld, 2010), which is aggressive and can result in toxic deaths (Chi *et al*., 2009).

A review of the Irish cohort of MRT documented a total of 25 patients that were diagnosed with rhabdoid tumours from 1986-2013 in the Republic of Ireland, all under the age of 17 (Uwineza *et al*., 2014). According to Uwineza *et al.* (2014) there was an equal sex incidence with a mean age at diagnosis of 38.8 months. These patients were treated based on the anatomic site of the tumours, because there was no standardised treatment for rhabdoid tumours in place (such as the European Rhabdoid Registry, which was only founded in 2010). Ten patients (43.5%) relapsed and the mean time to death was just less than 9 months (Uwineza *et al*., 2014).

SMARCB1/SMARCA4 as Tumour Suppressors

Chromatin remodelling complexes modify the condensed DNA (chromatin) in a cell to allow access for transcriptional machinery. This opening and closing of chromatin can effectively switch genes on or off, thus altering the expression of genes through what is called epigenetics, rather than by changes in the DNA sequence (Figure 1).

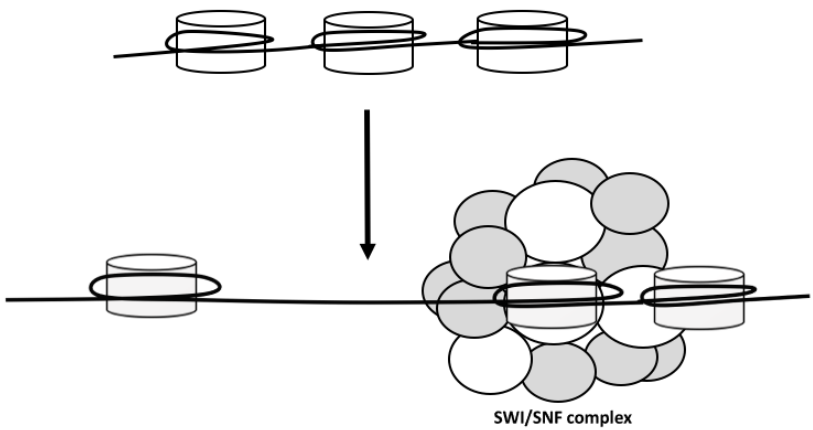

Figure 1. *Function of the SWI/SNF chromatin remodelling complex. The tightly wound DNA is opened and closed by the SWI/SNF chromatin remodelling complex, of which SMARCB1 and SMARCA4 are components. Chromatin modification in this way allows or denies access to the DNA for transcription factors, which are proteins involved in the regulation of gene expression. Mutation in SMARCB1 or SMARCA4 affects the ability of the complex to perform its functions, thereby altering the levels of expression of certain genes which in some cases leads to tumour development. Adapted from Wilson and Roberts (2011).*

SMARCB1 (also known as INI1, BAF47 or SNF5) is a gene that encodes the SMARCB1 protein, which is a subunit of the larger SWI/SNF chromatin remodelling complex (Roberts and Orkin, 2004). The genes encoding subunits of SWI/SNF complexes are particularly oncogenic, with mutations occurring in ~20% of all human cancers (Helming *et al.*, 2014). *SMARCB1* mutation can lead to loss of production of the SMARCB1 protein, which can result in interference of many important cell pathways. SMARCB1 mutation is a hallmark of rhabdoid tumours and is how they are genetically characterised, regardless of where they present in the body.

Many studies have established that SMARCB1 significantly influences MRT growth. In one study, 5 of 29 rhabdoid tumours from all recognised anatomic sites for MRTs had a biallelic deletion of the entire coding sequence for SMARCB1, a further 10 had a homozygous deletion of exon 1, and the remaining 14 were frameshift or nonsense mutations (Biegel *et al.*, 1999). In a similar study, biallelic inactivating events for SMARCB1 were found in 50 of 51 rhabdoid tumours (Jackson *et al.*, 2009). Thus, the notion that SMARCB1 is a tumour suppressor gene is strongly supported as its inactivation seems to be the single most important driver of rhabdoid tumour growth.

Lee *et al.* (2012) studied 32 frozen rhabdoid tumour samples and found that the genomes were incredibly simple, genomically stable and diploid and that loss of SMARCB1 was essentially the only recurrent event. In the case of 2 tumours, it was the only identified mutation. Another result of note from this study is that the mean mutation rate was just 0.19 mutations per DNA Mega base pair (Mbp). The research concluded that this is possibly the lowest rate of all currently-sequenced cancers, and particularly stands out because of the extremely aggressive and lethal nature of the cancer (Lee *et al.*, 2012).

Epigenetic changes are increasingly implicated in oncogenesis (McKenna and Roberts, 2009, Sparmann and van Lohuizen, 2006), however because most cancers are genomically unstable and have many mutations, the oncogenic effects of aberrant epigenetic influences is difficult to study and determine which (if any) epigenetic modifications are mainly responsible. In contrast, the simplicity of rhabdoid tumour genomes, along with most other SMARCB1-deficient cancers, allow rhabdoid cancers to be a useful model for further elucidation of the role of epigenetics in oncogenesis. It is highly likely that the role of SMARCB1 loss, leading to tumorigenesis is influenced by its role in epigenetic modification as a subunit of the SWI/SNF complex, however this requires further confirmation (Kim and Roberts, 2014).

In three separate studies the role of SMARCB1 in tumour growth was investigated using genetically engineered SMARCB1 knockout mice (Roberts *et al.*, 2000, Klochendler-Yeivin *et al.*, 2000, Guidi *et al.*, 2001). Homozygous inactivation of SMARCB1 leads to embryonic lethality, whereas heterozygous mice are born normally but are predisposed to cancer. Of the heterozygous mice, approximately 20% developed sarcomas with a median age of 12 months. While the location of these tumours are different to the sites of rhabdoid tumours in humans (all were extra-renal), there are clear comparisons to be made. The murine tumours closely resemble human rhabdoid tumours and include rhabdoid cells with standard human rhabdoid morphology. They were highly aggressive - as in humans , locally invasive, and often metastatic (Roberts and Orkin, 2004).

In other experiments, conditional biallelic SMARCB1 inactivation in mice results in a cancer predisposition and rapid aggressive tumorigenesis (Roberts *et al.*, 2002). All mice in this experiment developed tumours at a median age of

11 weeks. This pace of cancer development is incredibly rapid. For example, p53 deficiency results in cancer development at 20 weeks and p19ARF inactivation leads to cancer at 38 weeks. It is also interesting to note that reduced function or inactivation of SMARCB1 has been implicated in other cancers such as in familial schwannomatosis, a rare genetic disorder that can result in central nervous system tumours (Hulsebos *et al*., 2007), and epithelioid sarcoma, a rare sarcoma of soft tissue that frequently presents in the limbs of young adults (Hornick *et al*., 2009).

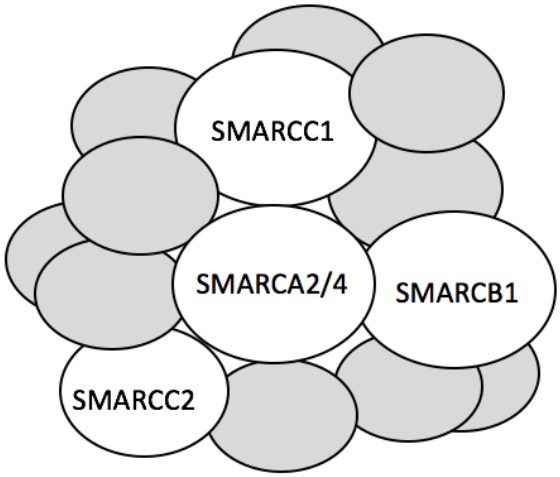

Figure 2. *An example of an SWI/SNF chromatin remodelling complex. Each SWI/SNF complex consists of an ATPase subunit (SMARCA4/SMARCA2), core subunits (SMARCB1, SMARCC1 and SMARCC2) and between 10-15 other accessory subunits (grey), enabling a vast number of possible combinations. SMARCB1 and SMARCA4 mutations have been directly linked to the proliferation of rhabdoid tumours. Adapted from Schneppenheim et al. (2010).*

SMARCA4 (also known as BRG1), the gene responsible for the expression of one of the two ATPase subunits in the SWI/SNF complex, has also been implicated in rhabdoid tumours (Figure 2). In a German study, it was found that two patients lacked SMARCA4 expression which suggests that SMARCA4 is also a tumour suppressor, and its inactivation can lead to rhabdoid tumours (Schneppenheim *et al*., 2010). This result is of note, because while another gene locus had been implicated in rhabdoid tumours it had not been identified in the literature previously (Frühwald *et al*., 2006). SMARCA4 inactivation has also been seen in small cell carcinoma of the ovary (SCCO), and it is thought that SCCO is consequently closely related to rhabdoid tumours (Ramos *et al*., 2014) or is a rhabdoid tumour itself (Fahiminiya *et al*., 2015). Rhabdoid tumour growth as a result of SMARCA4 mutations have been

linked to a higher rate of germline alterations than what is associated with SMARCB1 mutations, and affected individuals should seek genetic counselling (Hasselblatt *et al.*, 2014). Due to the potency of SMARCB1 as a tumour suppressor, significant efforts have been made to identify the cellular pathways affected by it and the mechanism through which it operates, although much is still unclear.

Pathways and Novel Therapies

Cyclin D1 and the Cell Cycle Pathway

Cyclin D1 is a critical sensor of extracellular stimuli and functions as a regulator of the cyclin-dependent kinases (CDKs). It was found in 2002 that re-introducing SMARCB1 to rhabdoid tumour cell lines induces cell cycle arrest by repressing cyclin D1 transcription via facilitation of histone deacetylase activity to the cyclin D1 promoter (Zhang *et al.*, 2002). Non-specific CDK inhibitors have been shown to inhibit rhabdoid tumour growth (Smith *et al.*, 2008). Mice which are SMARCB1 heterozygous and are lacking cyclin D1 do not produce rhabdoid tumours. This result provides clear *in vivo* evidence of cyclin D1 being at least one important factor for rhabdoid tumour growth, and that deletion of cyclin D1 is enough to block tumour development (Tsikitis *et al.*, 2005).

Pharmacological disruption of the cyclin D1 pathway is seen as a key potential target for future treatment. LEE011 is a small molecule inhibitor of CDK4/6 and is currently being used in a phase I clinical trial to investigate the effect inhibiting CDK4/6 has on patients (NCT02187783, 2016).

Sonic Hedgehog Pathway (SHH)

The SHH pathway plays an important role in differentiation during development. It is known to have a catalytic effect in oncogenesis. Loss-of-function mutations in the SHH pathway have been identified in medulloblastomas (a common paediatric brain cancer) and in familial and sporadic basal cell carcinomas (a common skin cancer) (Gailani *et al.*, 1996, Raffel *et al.*, 1997). GLI1 (glioma-associated oncogene homologue) is a transcription factor, making it involved in controlling the rate at which genes are read, and is a key mediator of the SHH pathway. The oncogenic potential of GLI1 has been shown in transgenic mice (Ruiz i Altaba *et al.*, 2007).

A link between SMARCB1 and GLI1 was investigated (Jagani *et al.*, 2010) and SMARCB1 and GLI1 were found to closely interact. This study also showed that when SMARCB1 is inactivated, GLI1 expression is increased eight- to tenfold. This result indicates that SMARCB1 plays a key role in repressing GLI signalling. Similarly SMARCA4 inactivation, and simultaneous inactivation of both SMARCA4 and SMARCB1 led to increased GLI1 expression, perhaps suggesting that GLI1 is a shared target for the SWI/SNF complex. GLI1 was upregulated in AT/RTs compared with control samples.

Arsenic trioxide (As_2O_3) targets GLI expression (Beauchamp *et al*., 2011), and it is an inhibitor of tumour cell growth of SHH-activated medulloblastoma (Kim *et al*., 2013). It is unknown how arsenic trioxide inhibits GLI expression, but it has been shown to be effective at inhibiting rhabdoid tumour cell growth both *in vitro* and *in vivo*. However, one concern with development of arsenic trioxide as a treatment for patients with rhabdoid tumours is the potential movement of the compound through the blood-brain barrier (Kerl *et al*., 2014).

Epigenetic antagonism between SWI/SNF and Polycomb complexes

Polycomb-group proteins (PcG) open or close chromatin and have well-known gene silencing abilities due to their epigenetic capability. Enhancer of Zeste Homolog 2 (EZH2) is the enzymatic component of the polycomb repressive complex 2 (PRC2), and it is overexpressed in many cancers with it being implicated in tumour progression (Chang and Hung, 2012). The apparent importance of EZH2 in oncogenesis has recently gained attention in and recent studies its role in cancer has been investigated (Suvà *et al*., 2009). It has been shown *in vitro* that polycomb proteins can directly repress the activity of the SWI/SNF complex (Shao *et al*., 1999). Inactivation of SMARCB1 leads to increased expression of EZH2 and its recruitment to polycomb targets. Thus, there appears to be epigenetic antagonistic properties between the SWI/SNF complex and PRC2. This observation was applied to an experiment wherein a mouse model's EZH2 gene was inactivated which thereby resulted in completely blocked tumour growth from SMARCB1 loss. This effect seems to be highly specific, as while the EZH2 loss resulted in tumour growth inhibition in the case of SMARCB1-deficient cancers, it had no effect on osteosarcomas resulting from p53/Rb loss (Wilson *et al*., 2010). In related studies, the efficacy of a small molecule EZH2 inhibitor called EPZ-6438 has been tested. EZH2-mutant xenograft-carrying mice models were given EPZ-6438 which was found to inhibit tumour growth and in some cases included complete tumour regression (Knutson *et al*., 2014). It was found that in the case of AT/RT, inhibiting EZH2 effectively sensitised rhabdoid tumour cells to radiation, identifying EZH2 as a potential therapeutic target (Alimova *et al*., 2013).

Aurora A

Aurora A (also referred to as Aurora Kinase A, STK6) is a mitotic gene and is often overexpressed in cancers, such as pancreatic and ovarian carcinomas (Wang *et al*., 2009), and this overexpression is also seen with SMARCB1-deficient rhabdoid tumours. Importantly, overexpression of Aurora A can transform normal cells (Katayama *et al*., 2003), which shows that it has strong evidence of being oncogenic. In another study (Lee *et al*., 2011), downmodulation of Aurora A resulted in a significant decrease in cell number and inhibition of cell proliferation, ultimately demonstrating that downmodulation of Aurora A is deleterious to rhabdoid

tumour cell growth. Alisertib (MLN8237) is a small, selective molecule inhibitor of Aurora A and due to strong results indicating anti-tumour activity preclinically (Maris *et al.*, 2010), a study was carried out to investigate its effect on rhabdoid tumours. Four patients with recurrent AT/RT were treated with alisertib and all four patients experienced disease stabilisation and/or decrease in tumour size after approximately 3-6 weeks. Two of the patients exhibited stable regression for 1 and 2 years. Alisertib had moderate toxicities including a decreased total WBC, neutropenia and thrombocytopenia (Wetmore *et al.*, 2015). Alisertib shows promise and is currently in phase II clinical trials (NCT02114229, 2015) .

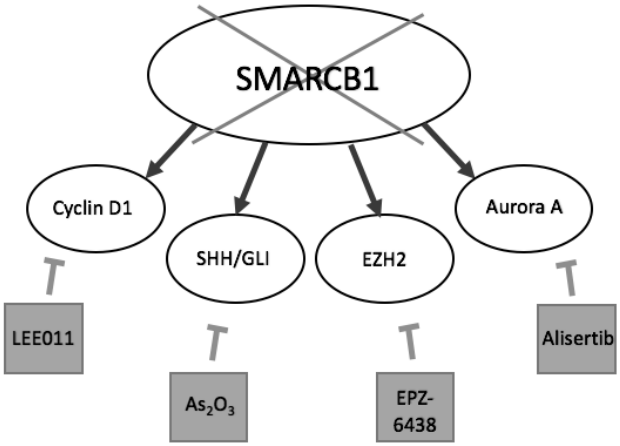

Figure 3. *Consequences of SMARCB1 mutations and therapeutic potential using inhibitors of affected pathways. Inactivation or loss-of-function of SMARCB1 upregulates the expression of many important pathway intermediary molecules such as cyclin D1, GLI1, EZH2 and Aurora A. Consequently, studies have investigated the efficacy of inhibiting these molecules with the aim of reducing tumour growth. Some of these targeted inhibitors which have shown potential as a treatment are shown here in grey.*

Conclusion

MRT is a highly aggressive cancer which mainly affects infants and young children. Inactivation or reduced expression of SMARCB1 is the main driver of its growth. Consequently, SMARCB1 has been identified as a critical tumour suppressor gene and many different pathways have shown to be affected by its inactivation. The results of some novel treatments targeting these pathways

are encouraging, such as the Aurora A inhibitor alisertib, the EZH2 inhibitor EPZ-6438, arsenic trioxide which targets GLI1 expression, the CDK4/6 inhibitor LEE011 and many more which are still in clinical trial phases (Figure 3). While rhabdoid tumours still remain devastating, the prognosis for patients with these tumours has improved in the last decade. The lessons that can be learned from the therapeutic approach to MRTs could have significant effects both for patient cohorts and for research efforts in finding a cure for malignant rhabdoid tumours.

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Long Non-Coding RNA: the Regulatory Web of Genome Regulation

Jack Schofield
Senior Freshman
Science

Long non-coding RNAs (lncRNAs) have emerged as prolific regulators of gene expression. lncRNAs are RNA transcripts which do not code for proteins like "conventional" genes. lncRNA was once presumed to be non-functional genomic noise and biologically irrelevant. Recent work, however, has shown that lncRNAs are spatiotemporal 'master regulators' of the genome. Unlike double stranded DNA, single-stranded lncRNA folds internally to assume complex structures which allows it to recruit protein complexes such as Polycomb Repressive Complex 2 (PRC2) and repress genes. This lncRNA regulation was first shown in X inactivating specific transcript (Xist) in mammalian females, which inactivates one of two X chromosomes to prevent X gene and subsequent protein double-dose. Such whole-chromosome inactivation may also be applied in treatments for chromosome disorders such as Down's syndrome. HOX transcript antisense RNA (HOTAIR) broadens the extent of lncRNA gene regulation, controlling hundreds of genes around the genome. HOTAIR's widespread control has implications in cancer, as HOTAIR regulates tumour suppressor genes. lncRNA control also regulates immune system responses and initiated pathogenic infection. lncRNA regulation provides 'fine control' of genes, and a full understanding of lncRNA may improve diagnostic and therapeutic approaches to disease in the future.

Introduction

Most of the human genome is transcribed into RNA, but never translated into protein (Carninici *et al.* 2005,Flicek et al 2014). This non-translated DNA is not, as was once thought 'junk' or functionless. Instead, it produces a diverse array of regulatory non-coding RNAs (Martin & Chang 2012,Brosnan & Voinnet, 2009). Long non-coding RNAs (lncRNAs) are those longer than 100 nucleotides that do not code for a protein. Instead, lncRNAs have functions 'distinct' from protein coding (Fitzgerald & Caffrey 2014). They assemble into structures and work as guides and modulators of protein complexes which regulate when and at which chromosomal point e genes are expressed (Mercer and Mattick 2013). One such complex is the histone modifying protein complex Polycomb Repressive Complex 2 (PRC2). Histones are the proteins which package and organise DNA and PRC2 modifications to histones can repress gene expression. lncRNAs evolved as PRC2's spatiotemporal directors (Lee 2012), 'master' overseers of genomic regulation (Nie *et al.* 2012). An LNCipedia database has been assembled for these 'master' regulators, cataloguing their characteristics. (Volders *et al.* 2012), (Volders *et al.* 2015), (Nie *et al.* 2012). The evolution of lncRNA explains some of the 'fine control' of gene expression (Luco 2013).

One of the first notable demonstrations of lncRNA genome regulation was the X inactivating specific transcript (Xist) (Brown *et al.* 1991a), (Brown *et al.* 1991b), (Borsani *et al.* 1991), (Brockdorff *et al.* 1991a). In mammalian females, one of the two X (sex) chromosomes are silenced during development to prevent double-dosage of X chromosome genes. Xist is a conserved lncRNA (Brockdorff *et al.* 1991) transcribed from the X chromosome to be silenced, which co-ordinates the repression of the entire chromosome in X inactivation (Brockdorff *et al.* 1992), guiding PRC2 to repress X genes.

Xist provided the initial evidence of lncRNA's regulatory significance. Genomic studies have shown that such RNAs are widespread within the genome (Bertone P. *et al.* 2004), (Bernstein *et al.* 2006). With Xist demonstrating such powerful control over entire chromosomes, research into its application in silencing extra chromosomes in Down's syndrome has begun (Jiang *et al.* 2013).

Investigation into other lncRNAs that could regulate gene expression led to the discovery of HOX transcript antisense RNA (HOTAIR) lncRNA, regulating hundreds of genes in a wide regulatory network (Rinn *et al.* 2007). HOTAIR regulates many genes involved in cancer development and metastasis (Gupta *et al.* 2010). lncRNAs also activate host immune system responses as well as allow pathogens to initiate infection (Carpenter *et al.* 2013). Biological complexity arises not from sheer quantity of genes, but the finer control of when and where they are expressed. lncRNAs have emerged as critical regulators of this control and have the potential to affect all areas of gene expression (Necsulea *et al.* 2014), (Kogo *et al.* 2011).

lncRNA Lessons in X-Inactivation

Analysis of complete mammalian genome has shown an abundance of non-coding RNA (Carninci *et al.* 2005). lncRNA relevance was first shown in the lncRNA orchestration of the X inactivation centre (Xic). Mammalian females have two X chromosomes, whereas males have one. To ensure equal X gene expression in males and females, approximately 1000 X-linked genes (Brown *et al.* 1991a) on one of the two, randomly selected X chromosomes are repressed in females. Such dosage compensation was first observed by Mary Lyons (Lyons 1961). The inactivated chromosome is turned into a compact "Barr Body" incapable of gene expression (Walker *et al.* 1991).

Protein complexes catalyse X-inactivation, but require lncRNAs to be directed to their targets. lncRNAs recruit and coordinate the activity of the repressive PRC2 protein complex to "turn off" one X chromosome. PRC2 is a multi-subunit protein complex (Margueron et al 2011) which, by adding repressive (methyl) marks to the histone proteins (package and organise DNA), can inhibit gene expression (Clapier & Cairns 2009). In the Xic, at least seven lncRNAs coordinate the actions of PRC2 to control X chromosome inactivation (Lee 2009). Xic demonstrates the ability of lncRNA to orchestrate regulation of gene expression (Figure 1).

This ability of RNA to form complex structures is essential to their ability to recruit proteins and guide them to control gene expression (Sharp *et al.* 2009). Xist is a 17 kilobyte lncRNA transcribed exclusively from the inactive X chromosome and it does not code for protein translation like conventional transcriptional RNA (Brown *et al.* 1992). Instead, it folds into a complex secondary structure which allows Xist docking to the repressive PRC2 complex. It loads PRC2 with a distinct structural motif, Repeat A (RepA), a tetra-loop loading platform for this repressive complex (Zhao et al 2008), (Duszczyk *et al.* 2011). This lncRNA folding is significant as complex folded structures are hallmarks of functional biological molecules. Once loaded to the chromosome by Xist, PRC2 adds methyl groups to the histone proteins to epigenetically repress expression of X genes. The bases in lncRNAs, unlike those in double stranded DNA, can fold in on each other and form stable structures, such as the tetra-loop RepA.

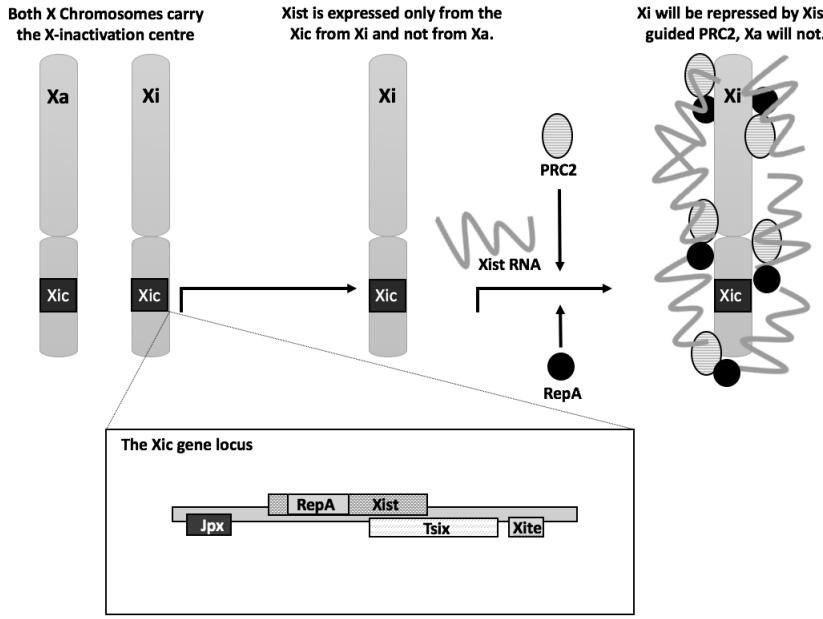

Figure 1. *X-Chromosome Inactivation. The X-Inactivation centre (Xic) is present on both the active X chromosome (Xa) and the inactive X chromosome (Xi). Xic encodes for Xist and when expressed Xist RNA binds PRC2. PRC2 facilitates the initiation of inactivation of the X chromosome destined to be inactivated; Xi, through the direction of Xist RNA. Xist then propagates and through maintained interactions with PCR2 keeps Xi inactive.*

Xist is the master lncRNA, initiating the process by spreading across the entire 3-D structure of the inactive chromosome (Clemson *et al.* 1996). It guides the repressive PRC2 which modifies the histone proteins packaging the DNA. Xist performs this job locally repressing the chromosome from which it is transcribed and regulation of the inactivation is provided by other lncRNAs.

The 40 kilobyte lncRNA Tsix is transcribed from and negatively regulates Xist on the active X chromosome, allowing gene expression (Lee *et al.* 1999). Xist is activated on the inactive chromosome by another lncRNA, Jpx. Jpx activates Xist allowing it to repress the inactive chromosome (Tian *et al.* 2010). Tsix and Jpx act as lncRNA 'switches' with opposing controls over Xist on either chromosome (Figure 2).

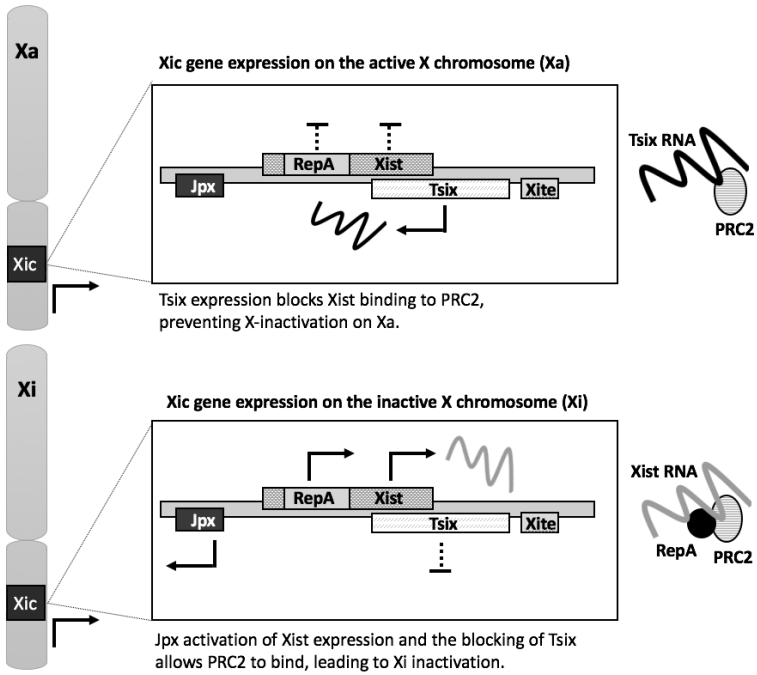

Figure 2. *X-Inactivation centre (Xic) gene expression on Xi and Xa. Jpx and Tsix are the positive and negative regulators of Xist repression on either chromosome. Xa will produce Tsix. Jpx activates Xist on Xi. The RepA motif from Xist recruits the repressive PCR2 complex. The RepA motif then binds PRC2 and together with Xist will catalyse the repression of the Xi chromosome. Tsix on the other hand prevents PRC2 binding Xist and thus ensures the Xa remains active.*

The designation of chromosomes as either 'active' or 'inactive' X's is also controlled by lncRNA and is thought to be clonally maintained. Initially identical chromosomes become active or inactive after making physical contact. The "choice" of which X chromosome is to be silenced is made by Xite. Xite is an RNA element which enhances Tsix on the active chromosome alone leaving Xist to repress the inactive chromosome (Ogawa & Lee 2003).

Xic is illustrative of extensive, chromosome-specific lncRNA regulation. lncRNAs either repress chromosomes (Xist, Jpx) or activate them (Tsix). The chromosomes 'choose' which lncRNAs they will express by communicating through physical contact between Tsix and Xite RNA (Xu *et al.*2006), (Lee 2009), (Ogawa & Lee, 2003), while ultimately repression of inactive X chromosome is performed by proteins (PRC2), spatiotemporal control of these proteins is lncRNA driven.

Applying Xist Lessons to Trisomy Disorders

Trisomy disorders develop in patients with three copies of any chromosome instead of the usual two. Down's syndrome (DS) is a chromosomal disease caused by trisomy of chromosome 21 (Chr21). Xist's ability to repress an entire chromosome could be applied in possible chromosomal therapies to turn off the supranumery DS chromosome. Jiang *et al.* (2013) tested this approach, applying Xist to cells derived from DS patients. Xist RNA "territories" were established in 85% of cells. 95% of Chr21 genes were repressed, bringing gene expression levels closer to normal, two-chromosome cells. This chromosome inactivation was maintained after three weeks, similar to inactive X chromosomes. This demonstrates that Xist lncRNA can silence extra chromosome in DS cells as it does to the inactive X. Most notably, Xist inactivation of Chr21 introduces the tentative possibility of corrections, or at least therapeutic options, for chromosomal disorders involving lncRNAs in the future (Disteche 2013).

HOTAIR broadens lncRNA influence

X inactivation's demonstration of powerful lncRNA genetic regulation led to research into other functional lncRNAs controlling gene expression. Functional lncRNA is identified by demonstrating interactions with regulatory complexes. The RIP-seq technique developed by Zhao et al (2010) identified thousands of lncRNAs which bind to and control PRC2, thus repressing genes. Such widespread lncRNA regulation was previously suggested by Khalil *et al.* (2009). With thousands of lncRNAs guiding and modulating protein complexes, they have since been dubbed genomic 'master regulators' (Nie *et al.* 2012)

One such master regulator is the lncRNA HOTAIR identified by Rinn et al (2007) and Woo & Kingston (2007) regulating thousands of genes across the genome in an expansive regulatory network (Lee et al 2012).HOTAIR folds into a more elaborate structure than Xist, acting as a scaffold for multiple protein complexes which control gene expression (Tsai et al 2010). Further studies of HOTAIR identified a specific 89-nucleotide binding site for PRC2 (Wu *et al.* 2013). A precise structural analysis of HOTAIR revealed multiple motifs (helical sections, terminal loops, internal loops, and junctions) in four domains, some binding PRC2 (Somarowthu *et al.* 2015). Such folding was previously thought characteristic of proteins but lncRNAs can self-assemble into similarly complex structures. Structure is essential to function in biomolecules and is another demonstration of lncRNA biological significance.

lncRNA – Implications In Disease

lncRNAs are widespread gene expression controllers and biological complexity comes not from increasing the number of genes, but in precise control of when, where, and for how long they are expressed. This control is important in organising immune responses to pathogens and its dysregulation is involved in carcinogenesis.

Immune function and immunopathology

lncRNAs are important regulators of immune system genes controlling both pathogenic and host responses (Yu *et al.* 2015) and many immune genes are X-linked. lncRNAs activate host immune responses to pathogens by controlling expression of hundreds of immune system genes (Fitzgerald & Caffrey 2014), (Heward & Lindsay 2014). Immunity lncRNAs include 'Nettoie Salmonella pas Theiler's' (NeST), which activates Interferon-γ (IFN-γ), a cytokine involved in defence against pathogenic infection (Gomez *et al.* 2013), (Baccala *et al.* 2005), (Hertzog *et al.* 2011). Toll-like receptors (TLRs) recognise pathogen-associated molecular patterns (PAMPs), molecules characteristic of pathogenic micro-organisms, helping to initiate inflammatory responses (Janeway & Medzhitov 2002). TLR4, after recognising Gram negative bacterial component lipopolysacharride, induces Cox-2 lncRNA expression. Cox-2 regulates hundreds of immune genes, repressing some and activating others to coordinate immune response (Guttman et al 2009), (Carpenter *et al.* 2013), (Li & Rana 2014).

TNFα and hnRNPL related immunoregulatory LincRNA (THRIL) activates tumour necrosis factor α (TNF-α) as well as other genes involved in the immune response (IL-8, CSF1, & CCL1) (Li *et al.* 2014). Genes encoding IL-8 and CCL5 are also activated by another lncRNA, nuclear enriched abundant transcript 1 (NEAT1) (Imamura *et al.* 2014).

Pathogens can exploit host lncRNA to infect their cells. HIV-1 viruses upregulate host cell NEAT1 to increase viral replication (Zhang *et al.* 2013), (Atianand & Fitzgerald 2014). A second host RNA, noncoding repressor of Nuclear Factor of T-Cells [NFAT] (NRON), is upregulated by HIV to control viral activity at specific stages during its life cycle (Imam et al 2015). The range of host lncRNAs exploited by HIV are reviewed by Lazar *et al.* (2016)

Pathogens express their own lncRNA during infectious attack. Kaposi's sarcoma-associated herpes virus (KSHV) expresses polyadenylated nuclear (PAN) RNA, which enhances viral activity and inhibits host immune response during infection. (Rossetto & Pari 2011). Human cytomegalovirus (HCMV) uses the lncRNA β-27 to prevent apoptosis in HCMV-infected cells, keeping them alive and protecting the virus, to permit persistent infection (Zhang & Jeang 2013), (Tycowski *et al.* 2015). Through their regulation of gene expression lncRNAs control both pathogenic infection and the host immune response, however notably the pathogens themselves can also use lncRNAs to evade the immune system.

Cancer

As HOTAIR regulates hundreds of genes, including tumour suppressors, loss of its control results in cancer development and metastasis (Lee *et al.* 2006) (Zhao *et al.* 2010), (Esteller 2011), (Wapinski & Chang 2011). Understanding HOTAIR's role in cancer may improve diagnosis and provide therapeutic targets (Zhang *et al.* 2014)

HOTAIR expression is significantly increased in breast cancer epithelial cells (Gupta *et al.* 2010). Experimental overexpression of HOTAIR guides PRC2 to repress 854 genes including tumour suppressors such as PCDH and JAM2, inducing breast cancer development(Gupta *et al.* 2010), (Novak *et al.* 2008), (Naik *et al.* 2008). HOTAIR repression of tumour suppressors removes the safe-guards against breast cancer. This control over tumour suppression also applies to other cancer types. Overexpression of HOTAIR increases metastatic and invasive capability of colorectal cancers through inhibition of genes which suppress tumour growth such as cadherin, which normally maintains cellular adhesion, preventing metastasis (Jeanes *et al.* 2008), (Berx & van Roy 2009). HOTAIR overexpression is also associated with hepatocellular carcinoma, upregulating MMP-9 and VEGF, genes which promote metastasis (Geng *et al.* 2011). In gastric cancers, HOTAIR overexpression results in dysregulation of metastasis-associated genes (ICAM-1, MMP1, MMP3 & MMP9)(Xu *et al.* 2013), (Emadi-Andani *et al.* 2014), (Endo *et al.* 2013). As a result of HOTAIR's wide regulatory reach, many tumour-related genes become dysregulated in HOTAIR overexpression, leading to cancer (Cai *et al.* 2014).

Conclusion

The fine spatiotemporal control that lncRNAs provide to the genome demonstrates the regulatory significance of lncRNA. lncRNA 'master' regulation is an elaborate, widespread mechanism for controlling when and where genes are expressed. lncRNAs in the X-inactivation centre allows chromosomes to communicate with each other, establish correct expression profiles (Tsix, Xite vs Jpx, Xist), and repress the X chromosome appropriately. Xist chromosome inactivation can be applied to extra chromosomes in Trisomy conditions such as Down's syndrome, potentially implicating chromosomal therapies for this disorder. HOTAIR extends the influence of lncRNA to hundreds of genes across the genome (Lee, 2012). The dysfunction of HOTAIR regulation leads to cancer because lncRNAs control wide regulatory networks, which include tumour suppressors. lncRNA regulation is also used by host immune system responses as well as pathogens infection.

lncRNA gene regulation remains poorly understood. Chromosome inactivation and lncRNA regulation of cancer and immunity are interesting , however many precise details of lncRNA function remain unclear. Further investigation will undoubtedly reveal more uncharted non-coding RNA as only 25 years have passed since Xist's characterisation. With improved sequencing and structural studies, the hidden complexity of genomic silencing may become understood.

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Microglial Polarization States: Implications for Astrocytes

George Timmons
Senior Sophister
Human Health and Disease

Microglia are the primary resident immune cells of the central nervous system. Astrocytes also contribute to central nervous system immunity; however, this role is often overshadowed by their broad range of non-immunological functions. Microglia are classified into functional subsets based on their altered expression profiles following activation. Microglial subsets are commonly described in terms of pro-inflammatory M1 microglia and anti-inflammatory M2 microglia – this M2 subset then diverging further into M2a, M2b, and M2c states. It has also been suggested that microglia do not maintain these subset populations strictly, but rather lie upon a dynamic spectrum where presence or absence of certain characteristics defines the microglia's main functional role. Upon activation astrocytes can also demonstrate pro- or anti-inflammatory phenotypes. They have shown to be integral to the pathology of certain neurodegenerative diseases, having a role just as or more important than that of microglia, such as their role in the progression of multiple sclerosis. It has recently been suggested that astrocytes also display polarization states similar to the microglial M1/M2 profiles, however these have not yet been defined in any great detail. Delineation of microglial polarization states has improved the understanding of functional microglial subsets and has allowed for the characterisation of the role of M1 and M2 microglia in physiology and pathology respectively. It therefore may be beneficial to delineate these polarization states in astrocytes also. Identifying the functional role of different astrocyte subsets would allow greater understanding of their contribution to neurodegenerative diseases. The idea of characterizing functional astrocyte polarization states has great potential which has yet to be truly realized.

Introduction – Macrophages, Microglia and Astrocytes

Macrophages are a leukocyte subset with phagocytic ability and are derived from circulating peripheral blood monocytes. The roles of the macrophage are primarily host defence, wound healing, and immune regulation functions (Mosser & Edwards 2008). Mosser and Edwards seek to ascribe each function to a specific macrophage subset, subset divergence being dependent on humoral factors acting to modify the macrophage phenotype. Different humoral factors affect macrophages in different ways by changing their functional role (Sica & Mantovani 2012).

These functional changes in response to humoral factors also occur in microglia, the macrophages of the central nervous system (CNS). Neural cells with similar characteristics to macrophages were identified in 1880 during histology experiments and these cells were first described as microglia by Río Hortega, the "Father of Microglia" in the 1920s, who was also the first to postulate that these cells may function similarly to macrophages. The main difference between macrophages and microglia lies in their ontogeny. Macrophages undergo traditional derivation from haematopoietic stem cells whereas microglia are derived from yolk sac stem cells (Prinz & Priller 2014). However, despite these ontogenetic differences, the phenotype and functional polarization states of macrophages have been shown to translate into microglia, at least *in vitro*. The vast range of markers identified which signify different macrophage states correlate with those described in microglia (Colton & Wilcock 2010). However it must be stated that despite similarities to extra-CNS macrophages, microglia remain a distinct cell type, maintaining their own specific markers and displaying unique expression patterns (Yamasaki *et al.* 2014). Overall the literature accepts that macrophage phenotypes translate well into microglia, as will this review, however the above caveats must be taken into consideration.

Neurons in the CNS are supported by several different types of neuroglia such as microglia, oligodendrocytes, and ependymal cells. The astrocyte is another important type of glial cell and our understanding of its functions has developed greatly in recent years. We have progressed from the long-held idea that these glia act as mere support cells, to the belief that they possess a more dynamic role in the CNS, this being supported by a plethora of evidence (Bayraktar *et al.* 2015, Khakh & Sofroniew 2015). The abundance and organized dispersal of these glial cells indicates a role more important than previously thought. The non-immunological roles of astrocytes in the CNS are broad and include a role in both foetal development, via synaptic pruning and trophic factor secretion, and in adulthood, contributing to ionic homeostasis, neurotransmitter metabolism, glutamatergic signalling, and structural contribution to the blood brain barrier (Sofroniew & Vinters 2010). However, astrocytes additionally have both pro- and anti-inflammatory roles in immune modulation (Min *et al.* 2006, Jang *et al.* 2013). Similar to macrophages, characterization of astrocyte polarization states relies on different cells having distinct immunological phenotypes. It may therefore be possible to move towards classifying and discussing astrocytes in terms of polarization states similar to the M1/M2 classification of macrophages.

Evidence is mounting showing that astrocytes contribute to many neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) – all of which affect members of the Irish population (Avila-Muñoz & Arias 2014, Meyer *et al.* 2014, Correale & Farez 2015). Over 700,000 people in Ireland are affected by a neurological condition with MS accounting for approximately 8,000 of these conditions and approximately 250 new MS cases being diagnosed each year (Neurological Association of Ireland 2014, Multiple Sclerosis Ireland n.d.). MS is a great burden on the exchequer with up to 80% of those diagnosed stopping work within 15 years and losing an average of 18 working years (The Work Foundation, 2011). Further research into the biology of astrocyte polarization states may help to further elucidate the mechanisms behind these debilitating neurodegenerative diseases and help to identify novel therapeutic avenues.

The Past – Classification of Macrophage Polarization States

Work on macrophage biology first looked towards defining two specific macrophage subsets: the classically activated pro-inflammatory M1 state macrophage and the alternatively activated anti-inflammatory M2 state macrophage. The M1 macrophage is activated by Toll-like receptor (TLR) agonists and interferon gamma (IFN-γ), and subsequently becomes pro-inflammatory. This inflammation is characteristic of the anti-microbial nature of M1 macrophages which was first described by Dalton in 1993 (Dalton *et al.* 1993). IFN-γ allows upregulation of the major histocompatibility complex II (MHC-II) and inducible nitric oxide synthase (iNOS) which signifies the priming of these M1 macrophages. MHC-II is a molecule which allows presentation of antigen to cells of the adaptive immune system, CD4+ T cells and iNOS is an enzyme that catalyses the production of nitric oxide, a reactive compound which can kill invading pathogens. MHC-II and iNOS are now seen as the archetypal markers of M1 macrophages in additional to a plethora of other receptors and pro-inflammatory secretions. The M1 macrophage amplifies the inflammatory response by production of cytokines, such as interleukin-1 (IL-1) or IL-12, chemokines, and free radicals, and by increasing antigen presentation to adaptive immune cells (Mosser & Edwards 2008).

Classification of the M1 state is straightforward when compared with the classification of M2 macrophages. It was first suggested in 2004 that M2 macrophages can be classified further into M2a, M2b, and M2c states (Mantovani *et al.* 2004). The first M2 state macrophage to be described was initially done in terms of IL-4 mediated upregulation of macrophage mannose receptor (MRC) and MHC-II (albeit a more restricted expression than that of M1) and was termed an M2a macrophage (Stein *et al.* 1992). It was then noted that IL-13 also modulated this macrophage subset, which is now known to occur through a common receptor chain, IL-4Rα (Doherty *et al.* 1993). The role of the MRC is the clearance of pro-inflammatory glycoprotein ligands, such as lysosomal hydrolases and tissue plasminogen activator (Gordon 2003). The

anti-inflammatory, resolving nature of M2a macrophages is also shown by their ability to express soluble factors which counteract pro-inflammatory mediators, for example arginase counteracts iNOS. Furthermore, M2a macrophages contribute to the repair of the extracellular matrix via factors such as collagenases and chitinases (Gordon 2003, Mosser & Edwards 2008).

M2a macrophages can be described as actively anti-inflammatory whereas M2c macrophages, which are also known as regulatory macrophages, have a deactivating function, comprehensively inhibiting production of, and antagonising pro-inflammatory factors and providing overall immunosuppression – a state termed "acquired deactivation" (Luo & Chen 2012, Fleming & Mosser 2011). Polarization to this state occurs primarily in response to IL-10 and transforming growth factor beta (TGF-β) (Colton 2009). IL-10 is also produced by M2c macrophages which has important contributions to immune tolerance/suppression, such as decreasing production of pro-inflammatory cytokines such as IFN-γ, and enhancing B-cell proliferation (Wang *et al.* 1995). This immunosuppressive state also causes tightening of vascular endothelial barriers thereby preventing leukocyte recruitment to sites of inflammation), increased growth factor production, and promotion of anti-apoptotic pathways; these homeostatic functions return the microenvironment to its normal physiological state.

M2b macrophages were originally described as type-II activated macrophages in reference to their ability to preferentially induce adaptive T-helper 2 cell responses (Mosser 2003). In contrast to the cytokine polarization signals of M2a and M2c macrophages, the M2b state is induced by engagement of the Fc gamma receptor on the macrophage surface by immunoglobulin G. Its functions are primarily mediated by IL-10 functions, similar to those carried out by M2c macrophages as mentioned above, but to a much greater extent. Interestingly M2b macrophages also secrete some of the same pro-inflammatory cytokines produced by classically activated M1 macrophages. Despite being considered an M2 macrophage, the M2b state shows distinct phenotypic differences in comparison to the other more traditional alternatively activated macrophages.

The Present – Accounting for Macrophage Plasticity in a Dynamic *in vivo* Environment

The current paradigm of M1/M2 macrophage polarization states was suggested by Mantovani *et al.* in 2004 and while the fundamentals of this paradigm are still viewed valid, there are growing concerns about the use of this information without considering its *in vivo* translation, and without immunological context (Mantovani *et al.* 2004, Sica & Mantovani 2012, Xue *et al.* 2014). Those suggesting a reassessment of how we consider macrophage states refer to the fact that an in vivo setting is a dynamic environment which will not maintain distinct macrophage types. Mosser and Edwards' spectrum of macrophage activation (Figure 1) takes this into account, suggesting that macrophages will not polarize into subsets with

explicitly defined features but rather gain or lose characteristics which deem them a macrophage primarily of a certain nature – be it host defence, wound healing, or immune regulation (Mosser & Edwards 2008). This spectrum also allows for the classification of "hybrid" macrophages, those which display characteristics from more than one subset. This spectrum is important in accounting for the ability of a macrophage to dynamically alter its expression of specific receptors and cytokines, i.e. its plasticity.

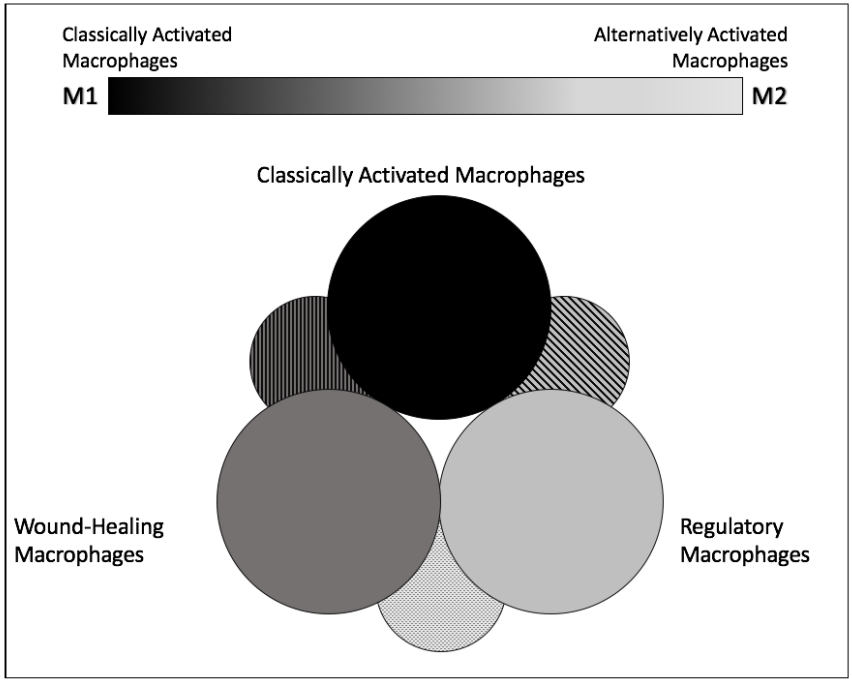

Figure 1. *The traditional linear M1/M2 model of macrophage polarization and the concept of a new polarization spectrum. The three primary colours represent the main groups of macrophage phenotype. The secondary colours in between groups illustrate the macrophage's potential to display characteristics from macrophages with different overall functions. For example, a macrophage falling into the orange area of the spectrum would be said to display functional characteristics from both classically activated and wound-healing macrophages. Adapted from Mosser & Edwards (2008).*

Recently, Martinez and Gordon (2014) identified what they believe to be the key limitations of the current M1/M2 model of macrophage activation namely; its ignorance of the source and context of polarization stimuli, its lack of consideration of co-existing M1/M2 polarization stimuli, and that the model doesn't account for the fact that macrophages may not differentiate into set subsets. While they do not officially propose a new model, the authors argue that future models should

consider the polarizing stimuli present at different levels of immunity in an *in vivo* environment. It suggests that these stimuli should be grouped based on their overall immunological role (Figure 2) instead of individual stimuli being directly compared with their antagonistic molecule. Doing so would permit a more comprehensive portfolio of polarizing stimuli to be created, thereby allowing identification of more specialized macrophage populations.

Growth and Survival Factors Maturation and Differentiation • Macrophage Colony Stimulation Factor Survival and Recruitment • Adhesion Molecules, Chemokines Other • Vitamin D3, Retinoic Acid	**Cytokines** Classic Activation • IFN-γ Alternative Activation • IL-4, IL-13 Pro-Inflammatory • IL-1β, IL-6 Anti-Inflammatory • IL-10, Tumour Growth Factor β
Pathogen Interaction Mechanisms Cell Receptors • TLRs, Nucleic Acid Sensors, NOD-like receptors Humoral Factors • Immunoglobins – IgA, IgE, IgG • Complement, Lectins, Ficolins	**Resolving Factors** Systemic • Glucocorticoids Local • Proteoglycans • Fatty Acid Derivatives i.e. Resolvins, Maresins

Figure 2. *The four hypothesised levels of immunological macrophage polarization stimuli. The different levels, and they subcategorisations, seek to provide a more comprehensive classification of macrophage polarization stimuli. Adapted from Martinez and Gordon (2014).*

Having described the functional polarization subsets of macrophages, it is important to reiterate how these phenotypes have been translated into microglia. While the two cell types may be ontogenetically distinct, the polarization states and specific subsets described for macrophages are now routinely referred to in microglia and both generally correlate in terms of M1 and M2 markers (Wilcock 2012, Chhor *et al.* 2013, Colton & Wilcock 2010). Macrophage polarization states can therefore, for the most part, be considered applicable to microglia meaning that we can use our understanding of macrophages to improve how we understand CNS immunity.

M1 microglia are generally seen as the primary mediators of neurodegenerative diseases such as AD, ALS, and Parkinson's disease while M2 microglia are usually downregulated during pathologies (Tang & Le 2015, Qin *et al.* 2015). Potential therapeutic avenues that have been identified include inhibiting the M1

polarization of microglia or re-inducing the expression of M2 microglia (Kobayashi *et al.* 2013, Cherry *et al.* 2014). Classification of the microglial polarization states allowed these avenues to be explored. Translating these states into astrocytes may therefore further progress our understanding of the role of the astrocyte in neurodegenerative diseases

The Immunological Role of Astrocytes in the CNS

Evidence is mounting illustrating the important immunological role of this multifunctional glial cell (Jang *et al.* 2013, Jensen *et al.* 2013). The immune response of the astrocyte is considered to be mainly pro-inflammatory. A hallmark reaction of this pro-inflammatory state is reactive astrogliosis, which can be defined as a spectrum of changes that occur in astrocytes in response to all forms of CNS perturbations, with the degree of change correlating with the severity of perturbation (Sofroniew 2009). Reactive astrogliosis can lead to the upregulation of many pro-inflammatory genes, leading to enhanced production of chemokines, cytokines, and growth factors. Other molecular changes include aberrant neurotransmitter synthesis/release and alterations in fluid/ion homeostasis, as well as morphological changes which prevent the spread of any threats to tissue integrity (Sofroniew & Vinters 2010). A pro-inflammatory astrocyte can help to further evolve the immune response by signalling for the recruitment of adaptive immune cells. A recent study has also suggested that astrocyte activation may cause the activation of microglia, the opposite of what is currently considered normal (Jang *et al.* 2013). The pro-inflammatory state of astrocytes is well classified as an evident component of several neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis, Alzheimer's Disease, and Parkinson's Disease (Maragakis & Rothstein 2006). However, perhaps less well described is the anti-inflammatory component of astrocyte responses. While their immune function is seen to be primarily pro-inflammatory, anti-inflammatory cytokine secretions have also been reported (Jensen *et al.* 2013). Studies have identified known mediators, such as the complement-5a fragment, and unknown mediators which exert anti-inflammatory activity (Gavrilyuk *et al.* 2005, Min *et al.* 2006). This activity however tends to be overshadowed by the pro-inflammatory role of astrocytes, despite providing novel therapeutic opportunities.

MS, for example, is an autoimmune disease whose detrimental effects are mainly mediated by T-cells. However new research has suggested that astrocytes may also contribute to this chronic, demyelinating disease (Brosnan & Raine 2013, Correale & Farez 2015). The majority of current therapies are immune modulators or monoclonal antibodies which target T-cells. Therapies in general are expensive and impractical with regards to method and frequency of dose (Goldenberg 2012). Astrocytes may present a new therapeutic target. The active role of astrocytes in MS is only recently becoming a research focus and elucidation of their role in MS pathology indeed their role in any of the many other neurodegenerative diseases could benefit from an understanding of astrocyte polarization states.

The Future – Emerging Astrocyte Immune Functions & The Importance of Defining Astrocyte Polarization States

Classification of macrophage subsets relies on their different immune phenotypes. Astrocytes also express different immune phenotypes, pro- and anti-inflammatory, yet despite this, their specific polarization states have not been defined (Jensen *et al.* 2013). Data has indicated that astrocytes can also affect immunity indirectly by modulating pro- and anti-inflammatory microglial polarization states via inducing or inhibiting the release of certain inflammatory mediators (Bianco *et al.* 2005, Aloisi *et al.* 1997). Astrocytes may also exert this effect by upregulating microglial antioxidant enzymes such as heme oxygenase-1 (Min *et al.* 2006). TGF-β was discarded as a potential mediator of this microglial change by Min *et al.* in 2006 however recent new evidence exists which disputes this claim (Norden *et al.* 2014).

Irrespective of whether astrocytes affect CNS immunity directly or indirectly via modulation of microglia, it is clear that classification of specific astrocyte polarization states is the next logical step in elucidating the extensive role of astrocytes in neurodegenerative diseases. The role of astrocytes has been proven to cause immunopathology in many neurodegenerative diseases and delineation of their subsets would potentially allow for more focused research into their physiology and pathophysiology to take place . The changes in the expression profiles of pro- vs anti-inflammatory astrocytes could be determined, helping to identify either the specific molecules contributing to pathologies, or the defensive mediators present in normal physiology which become downregulated. Our knowledge of the astrocyte's contribution to CNS immunity has developed greatly over the past decade. We have moved from considering this cell to have only a filler function to a recently hypothesis suggesting that astrocytes have specific polarization states similar to those of macrophages (Jang *et al.* 2013). Mainly focusing on the pro-inflammatory astrocyte state, a basic M1 astrocyte molecular profile was postulated. Further studies in the area remain to be carried out, however may hold great potential for the field of neuroimmunology.

Conclusions

It is now clear that microglia are not the only important CNS immune cell. While the immune function of astrocytes in the CNS is still seen as secondary to that of microglia, the importance of these dynamic, multifunctional glia has seen more acceptance in recent years. There now exist a multitude of studies illustrating the pro- and anti-inflammatory roles of astrocytes, as previously described. Our knowledge of the astrocytic contribution to neurodegenerative diseases continues to grow and is helping to make the idea of defining specific astrocyte polarization states all the more relevant.

The M1/M2 macrophage paradigm has been suggested as a framework to aid the classification of astrocyte polarization states, important consideration must be made. The hypothesis that astrocytes "exhibit M1/M2-like functional polarization" similar to that of macrophages may technically be correct, however one must remember the recent paradigm shift towards a macrophage spectrum and one must consider the modulating effects of an *in vivo* environment (Jang *et al.* 2013). Astrocyte subsets may also exist on a similar spectrum and be affected by different levels of immune stimuli. It may prove beneficial for research into astrocyte polarization to consider a spectrum right away, rather than discuss a linear scale which may soon become obsolete.

Astrocytes are often seen as secondary CNS immune cells in comparison to microglia. Classifcation of astrocyte polarization states would help delineate the specific functional astrocyte populations involved in different neurodegenerative diseases. Combined with increased knowledge of their molecular profiles, astrocytes may provide a novel target in neurodegenerative diseases whereby targeting astrocytes modulates microglia, instead of targeting microglia directly. This knowledge may also help to clarify further the mechanisms by which these diseases manifest.

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The role of Receptor Interacting Protein Kinases in Necroptosis and Inflammation

Grainne Delaney
Senior Sophister
Immunology

Inflammation is a defensive response against pathogens. During the initial inflammatory response, white blood cells are recruited to the site of infection to aid elimination of the pathogen. However, any dysregulation in the immune response pathways can lead to severe acute, or chronic inflammatory diseases. Intracellular pathogens, those that reproduce within the host cell, can induced cell death by apoptosis, which destroys their replication niche. Some microbes exhibit evasive strategies to avoid this form of cell death. When this occurs the host cell can employ alternative countermeasures of programmed inflammatory cell death. Programmed necrosis, or necroptosis is one such mechanism. The important roles of the RIP kinase family and the defensive strategy of necroptosis in relation to the inflammatory response they induce relies on interconnected molecular pathways, which can be disrupted in pathology.

Introduction

Necrosis is a form of cell death defined by characteristic changes to cell morphology, including disruption of the plasma membrane and cytoplasmic swelling. Historically, necrosis is associated with cellular damage due to physical stress or other extracellular conditions, such as osmotic change, strong acidity, severe temperature and depletion of nutrients and oxygen (Vaupel *et al.* 1989, Boujrad *et al.* 2007). All of these observations resulted in the assumption that necrosis is an unregulated and passive form of cell death. However, in recent years necrosis has been conditionally shown to be elicited by specific signalling pathways (Chan 2012). This regulated type of necrosis is called programmed necrosis or 'necroptosis'.

Cellular stresses that can induce cell death and inflammation include cytokine stimulation, inflammation or DNA damage (Labbe *et al.* 2008). Pattern recognition receptors (PRRs) are protein receptors expressed on the surface of cells which identify molecular structures found on invading pathogens known as pathogen-associated molecular patterns (PAMPs). PAMPs induce the production of pro-inflammatory cytokines and can trigger apoptosis. PRRs also recognise danger-associated molecular patterns (DAMPs) which are produced by damaged cells in response to pathogens or by self-reactive cells (Mills *et al.* 2011). Recognition initiates a number of cascades resulting in the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP-1), the transcription of suitable pro-inflammatory genes and members of the interferon regulatory factor (IRF) gene family. (O'Neill *et al.* 2013, Akira *et al.* 2006). Types of PRRs include toll like receptors (TLRs) which are transmembrane protein receptors, RIG-I-like receptors (RIGs), Nod like receptors (NLRs) and also DNA sensors (O'Neill *et al.* 2013, Kawai *et al.* 2008, Creagh *et al.* 2006, Khanna *et al.* 2001). The successful activation of the transcription factor NFκB leads to the secretion of numerous inflammatory cytokines, including tumour necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), that recruit immune cells to inflammatory site. Receptors of these cytokines can further activate NFκB, to produce more pro-inflammatory cytokines, which in turn recruit leukocytes (neutrophils, monocytes and macrophages etc.) to the site of infection and subsequent elimination of the pathogen.

Cell death is also an important cell defence which integrates closely with inflammation within a multi protein oligo structure called the inflammasome. Initiation of inflammatory cascades by the inflammasome act as a driving force behind some forms of inflammatory defence (Vandenabeele *et al.* 2010). The nature of cell death can have varying effects on the inflammatory response (Bergsbaken *et al.* 2009). Programmed cell death by apoptosis is mainly regarded as a less disruptive, safer form of cell death. It is a highly regulated process where the cell membrane remains intact and the apoptotic bodies are cleared by phagocytosis, resulting in lower amounts of extracellular inflammation. By contrast, necrosis is highly disruptive, resulting in the cell membrane rupturing and release of cell contents (adenosine triphosphate (ATP), nuclear fragments, mitochondrial proteins, lysosomal digestive enzymes, high mobility group box 1 protein (HMGB1) etc.). These endogenous compounds are danger signals, which enhance inflammation. The receptor-interacting protein (RIP) kinase family have emerged as important sensors of both internal and external cellular stress signals and therefore play an essential role in amplifying inflammation, immune responses and also in death-inducing processes (Declercq *et al.* 2009). RIP1 is of particular importance as it's kinase activity is crucial in triggering apoptosis while both RIP1 and RIP3 are required in conjunction with each other to elicit cell death through necroptosis.

Both necroptosis and inflammation have closely interconnected signalling pathways and thus exhibit a level of co-ordination between them. Maintaining a tightly controlled balance is vitally important as exaggerated cell death can lead to tissue damage and a great loss in the number of immune cells. These pathways are thus crucial as they dictate the magnitude and duration of inflammation while also being responsible for the fate of the cell (Humphries *et al.* 2014). This is furthermore significant because an increased level of inflammation is the basis for characterisation of inflammatory diseases such as rheumatoid arthritis (Epstein *et al.* 2001). Therefore the kinase family of RIPs are critical to the necroptotic and inflammatory pathways, their significance lying in their determination of the fate of a cell: death via necroptosis or survival.

Inflammation or apoptosis? RIP1s crucial deciding role in Tumour Necrosis Factor (TNF) signalling

Receptor-interacting protein 1(RIP1) kinase activity is instrumental in TNF signalling. RIP1 mediates NFκB activation in response to a number of death receptors such as Fas, TNF-receptor 1 and TNF-related apoptosis-inducing ligand receptor-1 (TRAIL1) (Kreuz *et al.* 2004, Kelliher *et al.* 1998, Ting *et al.*1996, Lin *et al.* 2000). However, because of the important role of TNF in initiating the inflammatory and cell death pathways and ultimately in inflammatory diseases, the downstream signalling pathway of TNF and the TNF receptor 1 have been one of the most studied (Figure 1). TNF binding to the TNF-R1 initiates the extrinsic apoptotic signalling pathway, this interaction recruits TNF receptor-associated death domain protein (TRADD) and RIP1 to the cell membrane where they form a complex. Cellular inhibitor of apoptosis proteins (cIAPs) (1 and 2) and TNF receptor-associated factor (TRAF2) (2 or 5) join the complex and catalyse the polyubiquitination of RIP1. Recognition of the ubiquitinated RIP1 by a complex containing a ubiquitin-binding domain composed of TAK1 and IκB kinase (IKK) enables TAK-1-mediated phosphorylation which then activates the IKKs. IKK phosphorylates the IκB proteins which usually sequester NFκB in an inactive state in the cytoplasm. This results in further ubiquitination and degradation of IκB, which frees NFκB allowing it to relocate to the nucleus. NFκB, acts as a transcription factor by inducing the expression of pro-inflammatory genes such as IL-1 and TNF (Han at al. 2011).

There are many intrinsic regulatory components in this pathway. NFκB can act as an inhibitory regulator of apoptosis by promoting the expression of anti-apoptotic genes including cIAPs. These genes exert a positive feedback control on NFκB via an IκB targeting mechanism and supress TNF activity (Chu *et al.* 1997). NFκB can also induce the expression of a cellular fas-associate death domain (FADD)-like interleukin-1 beta-converting enzyme (FLICE) inhibitory protein (c-FLIP) which is a potent inhibitor of death receptor induced apoptosis, thus promoting a pro-inflammatory phenotype, as opposed to an anti-inflammatory one (Kreuz *et al.* 2004). Alternatively, it was shown that a member of the Pellino E3 ubiquitin ligase family was able to target RIP1 in

such a way that would prevent complex 2 formation hence suppressing cell apoptosis (Yang *et al*. 2013, Tenev *et al*. 2011).

Contrastingly, NFκB acts as part of a negative feedback loop by promoting deubiquitinating enzymes A20 and CLYD to remove the ubiquitin chains from RIP1, preventing further activation of NFκB (Declercq *et al*. 2009,Wilson *et al*. 2009). In this form RIP1 associates with FADD and pro-caspase8 forming a death inducing signalling complex: complex 2 (Figure 2) (Wang *et al*. 2008, Declercq *et al*. 2009). Complex 2 and the ripoptosome induce the processing of pro-caspase-8 to its active form, leading to a caspase cascade that results in apoptosis (Wilson *et al*. 2009). The loss of cIAP proteins would similarly drive the pathway in this direction (Tenev *et al*. 2011). Therefore the RIP1s ubiquitination and kinase activity status decides whether TNF signalling enters the inflammatory or apoptotic pathways. Intriguingly, both branches of the pathway counter-regulate each other.

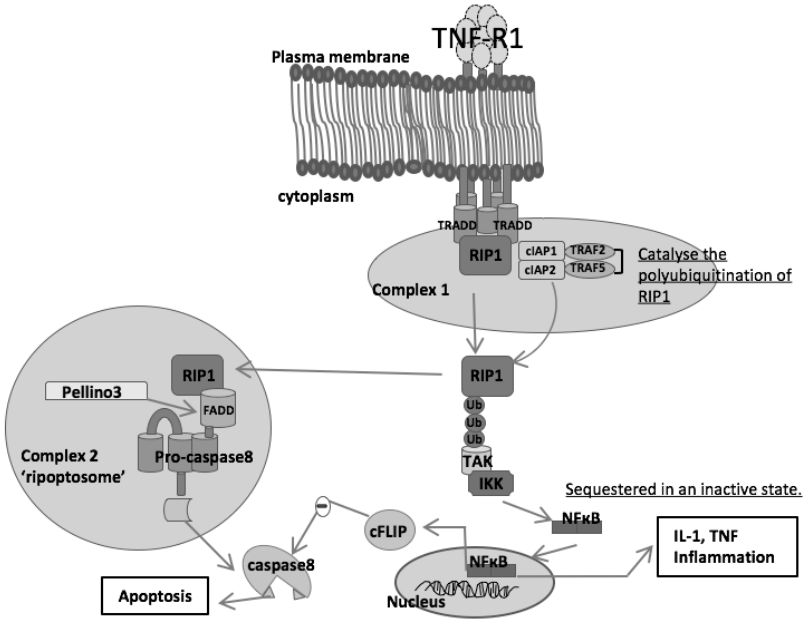

Figure 1. *The functions of RIP1 in TNF in signalling. TRADD and RIP1 are recruited to the receptor and form complex 1. RIP1 is polyubiquitinated in complex 1, which also contains the catalysing agents, cIAP1, cIAP2, TRAF2 and TRAF5. Phosphorylation of RIP1 is mediated by TAK which activates IKK resulting in NFκB activation and expression of pro-inflammatory genes in the nucleus. Inflammatory cytokines such as IL-1β and TNF are secreted along with the anti-apoptotic protein cFLIP. The formation of the complex 2 (known as the ripoptosome in the presence of IAPs) occurs after the deubiquitination of RIP1, along with FADD and pro-caspase 8. Processing of caspase 8 triggers downstream signals which results in apoptosis. Adapted from (Han et al. 2011).*

RIP1-RIP3s Role in Driving Necroptosis

Although apoptosis is a crucial host defence strategy against pathogen replication, other cell death mechanisms are required, as some pathogens have evolved evasive mechanisms to avoid this defence system. One example is cytomegalovirus which encodes a number of viral inhibitors to numerous key regulators in the apoptotic pathways, a specific example being an inhibitor against the caspase8 complex (Zhang *et al.* 2009).

Unlike necrosis driven by a physical trauma, one way to initiate necroptosis is by an underlying pathway composed of the kinases RIP1 and RIP3 (Han *et al.* 2011). RIP1 and RIP3 share a conserved kinase domain in their amino termini. They also contain a unique protein-protein interaction motif called the RIP homotypic interaction motif (RHIM) which is not present in other kinases within the RIP family (Sun *et al.* 2002). Upon stimulation of the TNF receptor, signalling for necroptosis is initiated. TRADD signals to RIP1 which recruits RIP3 (Figure 2). RIP1 is known to bind to RIP3 through their RHIM domain to form a heterodimeric filamentous scaffold, known as the necrosome (Wu *et al.* 2014, Orozco *et al.* 2014). Wu *et al.* 2014 report, using an inducible dimer system, that a signal cascade resulting in necroptosis cannot occur if there is not subsequent recruitment of additional RIP3 proteins to the RIP1-RIP3 heterodimer. This further recruitment of RIP3s is promoted by the initial RIP1-RIP3 interaction. In addition, when necrosis is triggered by death receptors, mediation of RIP1 requires, ripoptosome assembly, caspase 8, as well as inhibition of apoptosis (Feoktistova *et al.* 2011, Chan *et al.* 2012).

The necrosome then phosphorylates Mixed lineage kinase domain like (MLKL) which is a protein classified as a pseudokinase (Murphy *et al.* 2013), MLKL is a vital downstream component of RIP3, for inducing TNF-induced necroptosis (Cai *et al.* 2014). MLKL phosphorylation promotes oligomerization and translocation of the protein towards the plasma membrane. Once MLKL reaches the membrane, it interacts with phospholipids and reduces the integrity of the membrane resulting in cell rupture (Cai *et al.* 2014, Wang *et al.* 2014). MLKL also generates the expression of reactive oxygen species (ROS); the increase in ROS (especially from the mitochondria) is strongly linked with mediating TNF-induced necroptosis (Vandenabeele *et al.* 2010). RIP3 additionally activates a number of enzymes which metabolically contribute to the TNF-induced production of ROS (Zhang *et al.* 2009). These metabolic enzymes include, glutamate dehydrogenase 1 (GLUD 1), glutamate ammonia ligase (GLUL), and glycogen phosphorylase (PYGEL). An alternative mechanism of necroptosis results from stimulation of phosphoglycerate mutase family member 5 (PGAM5), causing PGAM5 to associate with Drp1 (a mitochondrial fission factor) resulting in fragmentation of the membrane (Murphy *et al.* 2013).

It was subsequently shown that RIP3s catalytic ability facilitates the switch between necrosis and TNF-induced apoptosis (Zhang *et al.* 2009). This was demonstrated by RIP3 deficient embryonic fibroblasts, from mice, surviving and reaching maturity, despite TNF exposure, due to TNF-induced necrosis resistance (He *et al.* 2009).

Further regulation is provided by caspase 8, which can block RIP1-RIP3 mediated necrosis by regulating RIP1-RIP3 complex formation with the aid of cFLIP and FADD (Osborn *et al.* 2010, Dillon *et al.* 2012). Caspase 8 also has the ability to deactivate the deubiquitinating enzyme CLYD, that functions by removing the ubiquitin chains from RIP1. When this enzyme is deactivated RIP1 no longer has the ability to activate NFκB, thus repressing necrosis (Wilson *et al.* 2009, O'Donnell *et al.* 2011)

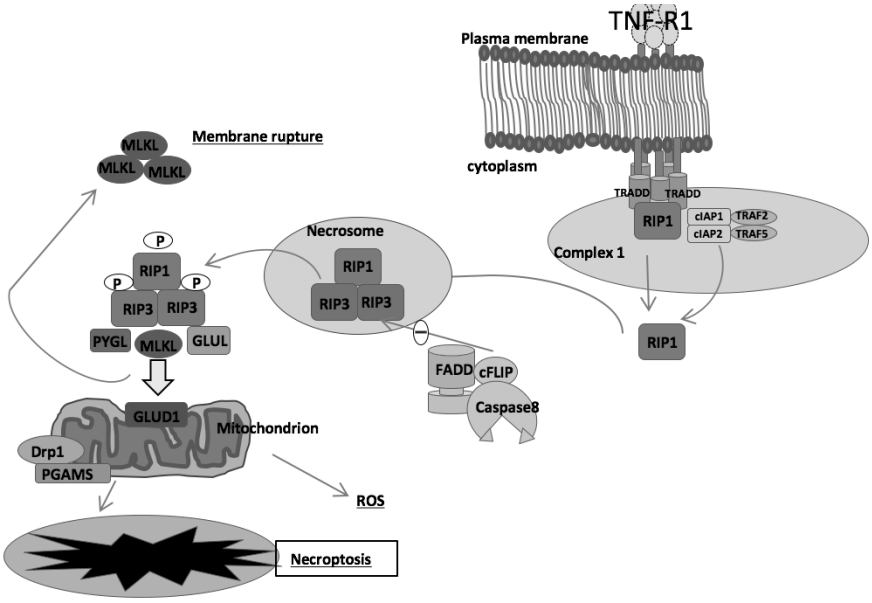

Figure 2. *Upon TNF-R1 stimulation, TRADD, RIP1 and RIP3 form the active necrosome complex. After RIP3 dimerisation, RIP3 intra-molecule auto-phosphorylation occurs. This phosphorylation is required for the recruitment of MLKL which triggers the downstream signalling necessary for necroptosis. It is the signalling events following the RIP1–RIP3 amyloid complex aggregation which are essential in executing necroptosis, RIP3 in the assembled amyloid scaffold recruit the free RIP3 followed by auto-phosphorylation of RIP3 and the subsequent recruitment of MLKL, are all key signalling events. It is proposed the formative events in the interaction between the RIP1-RIP3 heterodimeric amyloid fibril assembly are unlikely to directly instigate necroptosis because the heterodimer itself cannot induce necroptosis. However, they are a key part of the signalling cascade which results ultimately in necroptosis. Adapted from (Han et al. 2011).*

RIP1-RIP3 in a Pathological Context

As a result of necroptosis, the rupturing of the plasma membrane, as well as the lysosomal, and/or the mitochondrial membranes, leads to intracellular danger signals leaking into the extracellular space. These DAMPs include nuclear fragments, ATP and lysosomal enzymes, spilling into the extracellular space which activates PRRs. This promotes further tissue damage and inflammation. Due to the inflammatory nature of necroptosis it is no surprise that RIP1-RIP3 kinases participate in the pathogenesis of many inflammatory diseases and are attractive therapeutic targets. An example of such disease is retinitis pigmentosa (RP).

RP is a progressive genetic condition which results in the degradation of photoreceptor cells and retinal pigment epithelia cells, and eventual loss of vision (Murakami *et al.* 2012). RP can be inherited in an X linked, autosomal dominant, or autosomal recessive manner. It is primarily rod photoreceptors which harbour this deleterious gene, and these cells die by apoptosis, mediated through RIP1 kinase activity in humans (Hartong *et al.* 2006). Furthermore, Murakami *et al.* 2012 showed that in rd10 mice (endogenous mouse model of retinitis pigmentosa), cone cell death can occur through RIP3 mediated necrosis - RIP3 knockout mice thus showed significant preservation of cone cells accordingly. Other visual conditions linked to RIP1-RIP3 kinase activity in humans include, macular degradation and retinal detachment (Hanus *et al.* 2013, Trichonas *et al.* 2010).

Necroptosis plays a major role in the pathogenesis of ischaemic injury, including cerebral and retinal ischemia (Mehta *et al.* 2007, Rosenbaum *et al.* 2010). When the role of necroptosis in neuronal cell death and functional impairment in retinal ischemia was examined in rats, the use of the RIP1 kinase inhibitor nerostatin1 (nec-1) successfully reduced degradation of the inner retina, and induced a functional improvement (Rosenbaum *et al.* 2010). This demonstrates the significance of RIP1 in the impairment of vision in retinal ischemia and suggests exploring this kinase as a possible therapeutic target for treatment. The RIP1/RIP3 mediated necroptotic pathway has been shown to have dominance over apoptosis in kidney injury and has a pivotal role in kidney ischemia (Linkermann *et al.* 2012) and also neurodegeneration in brain ischemia. Mitochondrial dysfunction, excitotoxicity and oxidative stress all contribute towards necroptosis, and are implicated in brain ischemia as well as Alzheimer's and Parkinson's disease (Lin *et al.* 2006). Increased oxidative stress results from the accumulation of copper, iron and zinc as the brain ages (Vandenabeele *et al.* 2010).

Programmed necrotic cell death is thought to be the mechanism by which macrophages present in atherosclerotic lesions die (Figure 3). In a mouse model of early atherosclerosis, aortic lesions in RIP3 deficient mice showed no apparent differences, by comparison to aortic lesions from wild type mice (Lin *et al.* 2013). However, in advanced atherosclerosis, it was shown that significantly lower amounts of RIP3 were present in mice indicating RIP3 is important in preventing progression to advanced plaques (Lin *et al.* 2013).

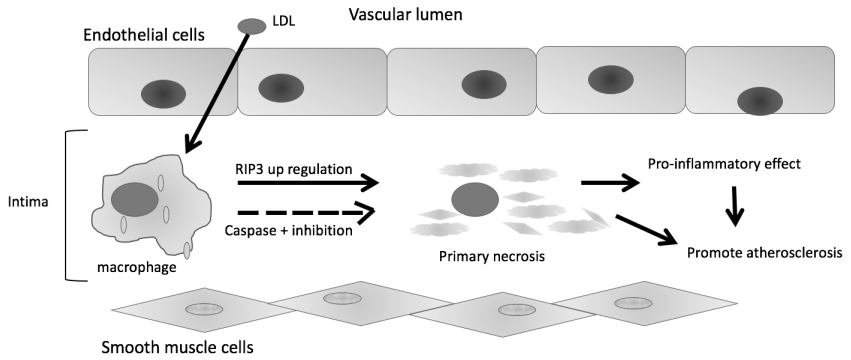

Figure 3. *schematic of macrophage death in atherosclerosis. Increased levels of RIP3 promotes the conversion from apoptosis to programmed necrosis in macrophages. The resulting inflammatory responses promote the development of advanced atherosclerotic lesions in blood vessels. Adapted from (Lin et al. 2013).*

The proteins which inhibit apoptosis are of new interest in cancer treatment. Preclinical drug therapies are trying to induce apoptosis in pre-apoptotic cells, suppressing the overall growth of cancerous cells (Hu *et al.* 2003). IAPs can play a deciding role in regulating whether a cell undergoes TNF-induced apoptosis, or production of pro-inflammatory cytokines. X-linked inhibitor of apoptosis (XIAP) is one of the most potent caspase and cytochrome c inhibitors in mammals, and has been linked with acquired resistance to inflammatory breast cancer (IBC), an extremely aggressive cancer with a very high mortality rate of up to 49% depending on tumour grade and stage of progression (Allensworth *et al.* 2013, http://www.cancer.net/cancer-types/breast-cancer-inflammatory/statistics). Second mitochondrial activator of caspase (Smac) protein is a potent antagonist of IAPs and is the target of Smac mimetic drugs in inflammatory conditions (Allensworth *et al.* 2013). Smac mimetic Birinapant is a drug currently in phase 2 in clinical trials that reduces formation of refractory solid tumors, inflammatory breast cancer cells and lymphomas by priming the cancer cells for death in an IAP dependant mechanism (Amaravadi *et al.* 2015).

Conclusion

Historically our understanding of inflammation and cell death overlap, but there is a necessary divergence between the two pathways that helps improves our molecular understanding of the underlying signalling pathways which control them. However, owing to the inherent complexity of the interconnected pathways

between the pro-inflammatory response and the cell death response to a pathogen, it is necessary to acknowledge both pathways within the same context. Progress has been made in understanding the biological roles of RIP kinases, specifically RIP1 in relation to its inflammatory role in intracellular death receptor signalling. RIP1 is a key regulator of necroptosis, it dissects a delicate balance between its role in complex 1 RIP1 kinase activity, its scaffolding forming function in the necrosome, and TNF-dependent inflammation which is crucial for maintaining cell homeostasis, and is regulated with the help of inhibitors.

Recent discoveries have further elucidated RIP3s functionality and exactly how it induces cell death through pro-inflammatory signalling, but like RIP1 it must also be tightly controlled, with failure to do so leading to hyper-inflammation (Yabal *et al*. 2014).

Most of the studies thus far have examined the role of receptor interacting proteins (RIPs) in cell death, and it is evident that they have many pathophysiological functions. Our current understanding of the these kinases and their role in biological functions implicates them as a potential therapeutic target for treatment of neurodegenerative, inflammatory, and ischemic diseases. Future research efforts should strive to increase our knowledge of RIP kinase biology into targeting and developing RIP derived therapeutic treatments for inflammatory disease.

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Animal, Plant and Earth Sciences

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Letter from the Editor

We live in a world of enormous complexity of organic design and diversity. The natural world is a source of both wonder, and of the most interesting questions on earth. The astonishing diversity of forms found in the plant and animal kingdoms, is mirrored in the diversity of the academic reviews here in this section. From plants, animals, and their environment; these areas are examined here at a genetic, behavioural and ecosystem level. Spanning the breadth of the natural world, the pieces in this section together show how these levels are integrated to produce the remarkable panoply of life on earth.

Our best in section piece by Andrew Mooney submerges us in the world of whales, dolphins, and other cetaceans, giving an exposition on one of the many ways human activity is affecting and shaping the natural world.

The extinction of native species poses a unique threat to the Irish ecosystem, leading to the loss of biodiversity, adverse reshaping of ecosystems and disruption of food webs. The reintroduction of extinct species is emerging as an important concept in Irish ecology and conservation. An examination of candidate species for reintroduction, and the benefits, pitfalls and challenges this poses are explored in this section.

Cannibalism in the animal kingdom has long been the fascination of Zoologists and the wider public alike. How these behaviours evolved and are maintained, and the advantages and disadvantages of conspecific consumption, are here insightfully surveyed. Finally the fascinating topic of plant fasciation is explored, detailing and analysing the molecular and pathogenic causes of this deformity.

The second edition of the TSSR marks the inauguration of the Animal, Plant and Earth Sciences section. Dedicated to the natural world, the study of whole organisms, their interactions, and the planet that they live on; the section provides a platform for the community of question-askers for whom the natural world is a source of the most interesting questions on earth. Overall, the quality of reviews both summited and published, reflect the high standard of natural students in Trinity, and I thank them for the time and effort invested in the writing of the pieces.

It remains for me to thank the wonderful team who edited and reviewed the pieces, including Darren O' Connell, Deirdre McClean, Dermot McMorrough, Aoibheann Williams, Bennet Thomson, and Maureen Williams. I'd like to also extend my sincere gratitude to Dr. Andrew Jackson, the academic supervisor of this section, for the generosity of his advice and time throughout the process, and for his constant support of the TSSR.

Finally, it has been a sincere privilege to serve as editor of this section, I hope you will read these wonderful pieces with pleasure.

Sarah Deegan
Animal, Plant and Earth Sciences Editor
Trinity Student Scientific Review 2016

Deafening Silence: The Impact of Naval Sonar Activity on Cetaceans

Andrew Mooney
Senior Sophister
Zoology

The ban on commercial whaling by most countries in the 1970's paved the way for the co-existence of humans and cetaceans i.e. modern whales and dolphins. Despite this advance, cetaceans still find themselves unintentionally in conflict with humans through the presence of anthropogenic acoustic noise in the oceans, particularly through the use of naval sonar. The use of naval sonar has been associated with both behavioural changes, such as modified dive behaviour, and physiological changes, such as Gas and Fat Embolic Syndrome, across many cetacean species. As a result of such changes, the lethal mass strandings of several cetacean species has occurred globally. High levels of public support and scientific data have prompted recent changes to naval sonar usage including complete moratoriums and limited activity in areas frequented by cetaceans. This contemporary issue and our response, demonstrates a greater understanding of the impacts human activities have on other species.

Introduction

The attitude of Humans towards other animals is constantly changing and nowhere is this clearer than with our attitude toward cetaceans. Humans and cetaceans have shared a long and often exploitative history, with cetaceans being harvested for various body products for thousands of years (Zelko, 2012). The expansion of the whaling industry throughout the 20th century ultimately resulted in the depletion of many whale populations, by up to 99% (Freedman, 1995). Thankfully, due to work by the International Whaling Commission and other organisations, the harvesting

of many cetaceans has been prohibited by most countries since 1975, allowing cetacean populations to recover (Clark and Lamberson, 1982). In fact, cetaceans are now worth more alive than dead with the global whale-watching industry currently generating $2.1 billion in revenue annually (O'Connor *et al.,* 2009).

Despite this enhanced legal protection, many cetaceans still find themselves subject to injury and stress caused unintentionally by humans. One of the main ways this occurs is through the presence of anthropogenic acoustic noise within their environments, particularly through the use of active naval sonar, which ultimately impacts upon their reproduction and survival (Sivle *et al.,* 2012).

Cetaceans are particularly well adapted to the low-light environment of the ocean, utilising acoustic signals for many basic functions, including feeding, communication, and reproduction. This is termed biosonar or echolocation (Surlykke *et al.,* 2014). As a result, they possess a heightened sensitivity to acoustic signals. The frequency that toothed whales (Infraorder Odontoceti) use ranges from 40 Hz to 325 kHz, while the frequency that baleen whales (Infraorder Mysticeti) use ranges from 10 Hz to 31 kHz respectively (Richardson *et al.,* 1998). As a comparison, humans are most sensitive to frequencies between 2,000 Hz and 5,000 Hz only. The broader frequency spectrums of cetaceans makes them more susceptible and sensitive to anthropogenic sounds produced as part of naval sonar activity.

Naval sonar employs both mid frequency active sonar (MFS) of 1kHz-10kHz, and to a lesser extent low frequency active sonar (LFS) of 100Hz-500Hz in order to determine the size, distance, and speed of objects within the sea through the principles of sound propagation and reflection (Friedman, 1997), similar to the way cetaceans use to find food. The use of sonar is often vital to the correct functioning of naval units (Friedman, 1997).

Almost all of the sound produced by naval sonar activity is inaudible to humans, however the use of sonar has been shown to elicit behavioural and physiological changes in cetaceans, particularly the toothed whales, impacting their overall fitness (Parsons *et al.,* 2008, Mooney *et al.,* 2009). Naval sonar activity has been associated spatially and temporally with the lethal mass strandings of several toothed whale species also (Filadelfo *et al.,* 2009). Here, I review the current known impacts of naval sonar activity on cetaceans.

Impacts: Behavioural

The impacts of naval sonar use on cetaceans vary according to species, age, location, time of year etc. However a general avoidance of the sonar by rapid movement away from the source is a common characteristic across many species (Miller et. al., 2012). This can be seen in Beaked Whales (Family *Ziphiidae*), where Tyack et al. (2011) reported a cessation of deep diving, which is associated with foraging, and echolocating in the presence of MFS and avoidance of the source with a prolonged ascent.

Such activity has also been shown to significantly influence the diving behaviour of Killer Whales (*Orcinus orca*), with an abrupt change from deep dives to shallow diving at sonar onset (Sivle *et al.*, 2012). This can impact the fitness of the individuals and the population as a whole, as deep diving has been shown to be associated with feeding (Sivle *et al.*, 2012). Other, more severe behaviour alterations have also been observed in Killer Whales, such as the temporary separation of a calf from its pod, cessation of feeding/resting, and the continuation of avoidance movements after the sonar had stopped being emitted (Miller et. al., 2012). However a study by Kuningas et al. (2013) demonstrated that the level of reaction to sonar by Killer Whales can be influenced by multiple factors, concluding that the availability of prey was the main factor dictating the movements of the Killer Whales.

Contrastingly, Long-Finned Pilot Whales (*Globicephala melas*) have been observed as increasing their vocalisations and huddling together in response to MFS, as opposed to the typical avoidance behaviours mentioned above (Rendell and Gordon, 1999).

Although the majority of studies carried out have been on toothed whales, baleen whales have shown behavioural modifications in response to naval sonar activity also. Grey Whales (*Eschrichtius robustus*) have shown an avoidance of sonar and travel displacement during seasonal migrations (Tyack, 1990). Humpback Whales (*Megaptera novaeangliae*) have also shown a cessation of 'singing' when exposed to prolonged sonar activity (Tyack, 1999). A study by Goldbogen et al. (2013) showed behavioural responses of Blue Whales (*Balaenoptera musculus*) to naval sonar ranging from cessation of deep feeding to an increased swimming speed and directed travel away from the sound source. This disruption of feeding and displacement from potentially high-quality feeding grounds could have important and previously undocumented impacts on the foraging ecology, individual fitness and overall population health of baleen whales (Goldbogen *et al.*, 2013). These behavioural alterations caused by naval sonar activity can also have more important physiological effects on cetaceans.

Impacts: Physiological

Physiologically, the use of prolonged sonar has been shown to induce temporary hearing loss in species such as the Bottlenose Dolphin (*Tursiops truncatus*) in addition to mild behavioural alterations and disorientation (Mooney *et al.*, 2009). The loss of hearing and subsequent loss of echolocational abilities in cetaceans likely impairs the orientational abilities and maintenance of ordinary dive behaviour in cetaceans (Talpalar and Grossman, 2005).

However of greater concern is that the modified diving behaviour as a result of naval sonar activity has been suggested as the cause of Gas and Fat Embolic Syndrome in cetaceans (Fernandez *et al.*, 2005). Gas and Fat Embolic Syndrome is the formation of gas bubble-associated lesions and fat embolism within the vessels

and tissues of vital organs. These form due to the modified diving behaviour caused by naval sonar, resulting in nitrogen supersaturation above a level normally tolerable in tissues. This is similar to the decompression sickness or 'bends' seen in humans, a condition resulting when decompression occurs too quickly, causing nitrogen bubbles to form in the tissues of the body. It had previously been thought that cetaceans did not suffer from such conditions (Fernandez *et al.*, 2005). This particular syndrome is induced by behavioural alterations associated with exposure to MFS and particularly affects deep-diving Beaked Whales, such as Cuvier's Beaked Whales (*Ziphius cavirostris*) and Blainville's Beaked Whales (*Mesoplodon densirostris*). The reason behind the heightened sensitivity of deep diving species to sonar is believed to be due to the fact that under high pressures at depth, sonar activity may stimulate more sensory fibres than at the surface water pressures, due to increased dendritic conduction and excitability as part of the adaptation of the CNS to high pressures (Talpalar and Grossman, 2005).

This is supported by the stranding and death of fourteen Beaked Whales in the Canary Islands in September 2002, close to the site of an international naval exercise. Strandings commenced approximately four hours after the onset of MFS. In this case no inflammatory or neoplastic processes were recorded, and no pathogens were identified. However the whales showed severe congestion and haemorrhage in areas such as the acoustic jaw fat, ears, brain, and kidneys due to gas-bubble associated lesions (Fernandez *et al.*, 2005). The mass stranding of cetaceans, and particularly Beaked Whales, in response to naval sonar activity is now believed to involve Gas and Fat Embolic Syndrome (Fernandez *et al.*, 2004)

The Result: Mass Strandings

Naval sonar activity has been associated both spatially and temporally with the lethal mass strandings of several whale species, particularly toothed whales. Historically, mass strandings of Beaked Whales having been reported in the scientific literature since 1874. During the period 1874-2004, 136 Beaked Whale mass strandings were recorded, and of these, 126 occurred between 1950 and 2004, after the introduction and application of contemporary, high-powered sonar (D'Amico *et al.*, 2009). However due to a lack of historical records, only 12 (9.5%) can be proven to coincide spatially and temporally with MFS naval activity.

Perhaps the most important mass stranding event to coincide with naval sonar activity occurred on March 15th 2000 in the Northern Bahamas. This event consisted of the stranding of 17 cetaceans, mainly Beaked Whales, within a 36 hour period following US naval activity in the area. Of these, seven died and were shown to have inner ear and brain damage. In a joint report by the National Ocean and Atmospheric Administration (NOAA) and the United States Navy it was agreed that naval sonar activity operating in the area was the most likely cause of the acoustic trauma and mass stranding (NOAA *et al.*, 2001).

The modern consensus regarding mass strandings and naval sonar activity is that sonar causes behavioural alterations in cetaceans, such as rapid ascent from depths while diving, which can in turn cause disorientation and physiological changes. These alterations are believed to be the principle cause of mass strandings (NOAA *et al.,* 2001, Cox *et al.,* 2006). In this way it is the combination of many factors operating together which ultimately cause the lethal mass strandings that have recently become popularised by the media.

The input of numerous factors influencing mass strandings is shown by the fact that the susceptibility of cetaceans, and particularly Beaked Whales, to naval sonar is not uniform. A study by Filadelfo et al. (2009) showed that naval sonar activity is not causing the mass stranding of Beaked Whales everywhere, but that certain populations are more susceptible due to particular bathymetric (ocean floor topography) conditions that are problematic. This finding was supported by the fact that strandings have been significantly correlated with naval sonar activity in areas such as the Mediterranean and Caribbean Seas where there is steep ocean bathymetry directly adjacent to the coastline, but not off the coasts of Japan or California where bathymetric conditions show broader shelves adjacent to the coastline (Filadelfo *et al.,* 2009).

It is also important to note that mass strandings are not exclusively as a result of naval sonar activity. Seasonal patterns of stranding have been observed for Beaked Whales (MacLeod *et al.,* 2004) and climatic factors such as weather and animals simply 'taking the wrong way' have also been suggested as likely causes of mass strandings (Mazzariol *et al.,* 2011). A worrying study by Madsen et al. (2006) has also suggested that acoustic disturbance due to the presence of wind turbines in the marine environment can alter the behaviour of cetaceans in a similar way to naval sonar, and this has been put forward as a possible cause for the deaths and strandings of 30 Sperm Whales (*Physeter macrocephalus*) in the UK and Western Europe between January and February 2016.

The mass stranding events that have occurred are atypical and further research is required in order to understand the exact cause and mechanism behind them and to limit any future events. However if it were not for the occurrence of such events, the discovery of important physiological responses of cetaceans to sonar such as Gas and Fat Embolic Syndrome (Fernandez *et al.,* 2005) would probably have been greatly delayed. The stranding of Beaked Whales particularly has also provided us with huge insights into the ecology and feeding patterns of these elusive species. The inspection of the stomach contents of stranded individuals allows us to infer information about their lifestyles and feeding patterns by the presence of freshly ingested food in their stomachs. The 2002 stranding of Beaked Whales in the Canary Islands allowed Santos et al. (2007) to show differential prey and thus feeding styles between Beaked Whale species. This rare ecological insight demonstrates the information we can garner and the learning opportunities available from these unfortunate lethal incidents.

The Future: A Quieter Ocean?

The solution to this problem seems simple, reduce or discontinue MFS usage. A complete moratorium on naval exercises in the waters off the Canary Islands employed by the Spanish government in 2004 has resulted in a complete cessation of mass strandings. This area had previously been considered a 'hotspot' for cetacean strandings (Fernandez *et al.,* 2013). However such extreme measures are not the global norm.

Current mitigation methods used during naval exercises focus on preventing auditory damage (Parsons *et al.,* 2008). This is achieved through the timing and location of naval exercises, the gradual increasing of sonar intensity over time, termed 'soft-start', and through monitoring the presence of animals in order to maintain an exclusion zone surrounding the sites (Dolman *et al.,* 2009). However behavioural changes, which are associated with mass strandings, often occur at lower sonar levels than auditory damage and can be just as damaging (Parsons *et al.,* 2008).

Although alternatives to the use of active sonar have been suggested, their effectiveness in contemporary situations is much more limited. The use of passive sonar (the detection of acoustic signals produced by an external source) rather than active sonar (producing your own signal and analysing its reflection) has been employed for many years (Horwitz, 2015), and unlike active sonar, passive sonar has no impact on cetaceans. In fact, passive sonar has even been used to study the movements of deep-diving cetaceans, such as Sperm Whales (Zimmer *et al.,* 2003). However the use of silent machinery in modern submarine vehicles greatly limits the future prospects of passive sonar (Horwitz, 2015).

A complete ban on naval sonar activity is unlikely to be the most viable solution currently, due to the extensive use of active sonar by naval units globally. Perhaps the way forward is simply a more responsible use of naval sonar in areas where the impacts it may have are greater due to natural ocean bathymetry conditions and species occurrence. In order to move forward, a balance between naval security and cetacean conservation must be made.

Conclusion

Due to the wealth of evidence available, there is now a generally accepted link between naval sonar activity and cetacean behaviour, physiology and strandings. This has resulted in an array of legal cases and formal statements of concern by international bodies and organisations seeking to limit the use of naval sonar to environmentally acceptable levels (Parsons *et al.,* 2011). This has been largely supported by the public, with a study by Parsons et al. (2011) showing that 51.3% of people surveyed believing that naval sonar impacted cetaceans and 75.8% believing that sonar use should be moderated if it impacts cetaceans. Increased

public awareness combined with growing scientific data have prompted recent changes to the use of MFS. This is shown by the recent agreement of the U.S. Navy to limit its use of sonar in the waters off California and Hawaii, areas considered cetacean 'hotspots'.

Such advancements in the conservation of cetaceans provide a solid grounding for future conservation efforts for many species, not just cetaceans, and demonstrate a realisation of the unintentional impacts that human activities have on the natural world. However as we enter the Anthropocene, a period where many species will face the risk of extinction, it is important to reiterate that commercial whaling still operates in countries such as Japan and Iceland and that true cetacean conservation and protection has yet to be achieved. However if it is to be achieved an understanding of all the threats cetaceans face is required.

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Reintroductions in Ireland: Restoring our Biodiversity

Cian White
Junior Sophister
Zoology

Reintroductions look to restore a species, lost originally due to human action. This relatively new branch of ecology lies squarely at the cross roads between biology and sociology, with economics and law increasingly more involved. Reintroductions are increasing in popularity based on the acceptance that they are a valuable conservation tool. The reasons for reintroducing a species are often complex. They should be objective and well defined, but any discussion on the matter usually involves a subjective element, especially when charismatic or culturally important species are considered. Of course all reintroduction schemes should primarily and directly benefit the target species and take precedence over any human desire. This review will focus on reintroduction in an Irish context; extinct native and naturalised species, the legislation regarding the reintroduction of species and review three reintroduction programmes taking place in Ireland today. A review of their success and failures will help inform future reintroduction policy among the conservation and governmental bodies. Numerous future reintroduction projects are considered.

Introduction

The reintroduction of species is increasingly seen as an important conservation tool that helps in the restoration of an ecosystem (Wilson, 2004; Manning *et al.*, 2009). For a synopsis of this topic see Estes et al. (2011). However the reintroduction of species, especially large carnivores, can create human-wildlife conflict, through increased contact (O'Rourke, 2014). If all stakeholders aren't involved early

on in the programme, this may jeopardise the success of the reintroduction. Reintroductions also tend to involve the ethical rationale of correcting the errors of the past and creating benefits for the local economy from eco-tourism (O'Rourke 2014). This review paper will review the legislation regarding reintroductions in Ireland, consider the ongoing debate of what a native species is and summarise the ongoing reintroductions of the Golden Eagle (*Aquila chrysaetos*), White-tailed Sea-eagle (*Haliaeetus albicilla*) and Grey Partridge (*Perdix perdix*) in Ireland.

The International Union for Conservation of Nature, IUCN, define a reintroduction as 'an attempt to establish a species in an area which was once part of its historical range, but from which it has been extirpated or become extinct' (IUCN/SSC 2013). It should also be noted that they state that a reintroduction of a species should only be considered as the last option to conservationists, must undergo strict justification, and adhere to all the guidelines outlined the IUCN (IUCN/SSC 2013).

When a reintroduction is considered, two things must be taken into account: the legislation regarding the reintroduction of species into the proposed country, and whether the species in question was a native to or ever naturalised in the country in question.

Legislation

There are two conventions and directives surrounding the reintroduction of species that apply to Ireland or which the Irish government has ratified. The UN Convention on Biological Diversity (UN Convention on Biological Diversity, 1992) creates an obligation to reintroduce threatened species, with Article 9 stating that each signed party shall 'adopt measures for the recovery and rehabilitation of threatened species and for their reintroduction into their natural habitats under appropriate conditions'. However, these obligations to reintroduce species are not replicated in European law. Article 22 of the Habitats Directive declares that Member states shall 'study the desirability of re-introducing species in Annex IV, that are native to their territory' (Council Directive, 1992). The obligation to reintroduce species was replaced by the obligation to study the desirability of reintroducing a species. The inconsistency in International and European law confuse the matter, however conservation organisations clearly do have some legal precedence for reintroduction.

The very word reintroduction implies that the species must have been present at some stage, but the specifics of what constitutes an applicable species are confusing. The distinction between native and historically naturalised becomes blurred in a temporal context. Is a species that arrived on the island alongside man introduced or native? Crees and Turvey (2015) define a native species as one that has been present continually since the late Pleistocene, or naturally colonised during the Holocene, roughly 12,000 years ago. By that definition, of the twenty nine species of fish to inhabit Irish freshwater only sixteen are considered native (Kelly *et al.*,

2007). This excludes the pike, perch, roach and bream, some of the commonest species without which our ecosystems would be radically different. Indeed by this definition, much of the species found in Ireland today would be considered non-native (Stokes *et al.*, 2006). Crees and Turvey (2015) propose a realistic solution to the dichotomy of native and non-native by providing a continuum between the two.

In their guidelines on reintroduction, the IUCN avoid using the word native, instead their phrase of choice is 'indigenous range' defined as 'the known or inferred distribution generated from historical (written or verbal) records, or physical evidence of the species' occurrence' (IUCN/SSC 2013). This a more satisfactory way of determining whether a species qualifies for reintroduction. The range of a species is ever changing based on biotic and abiotic factors (Chuangye *et al.*,. 2015), of which human influence, as the most dominant species on the planet, has the most effect (Chuangye *et al.*, 2015). The argument about whether a species was once native or not, in the context of a potential reintroduction, is not that useful and should be replaced by indigenous range as defined by the IUCN.

Extinct Species of Ireland

The Irish Red List lists ten angiosperm species, forty bryophytes, one mammal, three species of bee, one butterfly, two non-marine molluscs and eight species of water beetle, all of which are extinct (Marnell *et al.*, 2009; Foster *et al.*, 2009; Fitzpatrick *et al.*, 2006; Curtis & McGough, 1988; Byrne *et al.*, 2009; Regan *et al.*, 2010; Lockhart *et al.*, 2012). There were inconsistencies in the criteria necessary to declare a species 'extinct', with some authors satisfied if there had been no recordings in the last 100 years while the authors of the Terrestrial Mammals report excluded mammals extinct before 1500 (Lockhart *et al.*, 2012). They therefore excluded the Eurasian Brown Bear (*Ursus arctos arctos*) and Irish Elk (*Megaloceros giganteus*). The Wild Boar (*Sus scrofa*) was considered a post 1500 AD introduction and so was not taken as an extinct native (Marnell *et al.*, 2009).

The red list for Irish birds is published by Birdwatch Ireland. However this list does not include extinct species, so extinct birds in this review are based upon the book 'Ireland's Lost Birds' by Gordan D'Arcy (1999). This book states that, among others, Ireland has lost a type of grouse, the Capercaillie (*Tetrao urogallus*), and also six raptors including the Osprey (*Pandion haliaetus*), Marsh Harrier (*Circus aeruginosus*), Goshawk (*Accipiter gentilis*), Red Kite (*Milvus milvus*), White-tailed Sea-eagle and the Golden Eagle (D'Arcy, 1999). The latter three have been the subject of ongoing reintroductions in Ireland in Wicklow, Kerry and Donegal respectively, run by the Golden Eagle Trust, in partnership with National Parks and Wildlife Service (NWPS).

Golden Eagles Reintroduction

The Golden Eagle (GE) became extinct in Ireland at the beginning of the 20th century (Whilde, 1993). The two main reasons for extinction were persecution, consisting of poisoning, shooting, trapping and egg collecting, in addition to habitat change, due to the increasing rural population (O'Toole *et al.*, 2002). In 2001 the first chicks from Scotland were reintroduced to Glenveagh National Park by the Golden Eagle Trust. The first GE to fledge for over a century did so in Glenveagh, Donegal in 2007 (Sarr *et al.*, 2014). Eleven chicks reared in the wild have fledged since 2007. However in 2015, after two years, with only one chick fledging, the Golden Eagle Trust explained that the poor health of the upland habitat was reflected in the poor record of chicks fledging. With three to five chicks fledging a year being the estimated number to keep this small population viable, the current average of 0-3 is not sufficient. The Golden Eagle Trust called for a review of the management of the uplands of Donegal, in order to save the Golden Eagle population (L. O'Toole 2016 pers. comm. 7 January).

White-tailed Sea-eagle Reintroduction

White-tailed Sea-eagles (WTSE) were reintroduced to Killarney National Park in 2007 after an absence of over 100 years (D'Arcy, 1999). Over the next five years (2007-2012), one hundred chicks were reintroduced, the donor stock coming from Norway. In 2014 there were fourteen pairs and one chick fledged, changing to thirteen pairs and four fledged chicks in 2015 (Goldeneagletrust.org 2015). However this reintroduction programme, in contrast to the reintroduction of the GE, became a highly politicised conflict between the stakeholders involved; namely the hill famers in Kerry, the conservationists, and the tourist lobby in Killarney (O'Rourke, 2014). The danger posed to this reintroduction project does not seem to be the ecology of the habitats, but the stakeholders involved. This is reflected in the fact that there have been thirteen recorded cases where a WTSE has been killed by poisoning or shooting in Ireland, while only two GEs have been killed by human persecution (O'Donoghue, 2015). This is even more concerning given the fact that GEs have been reintroduced for approximately 50% longer, and the laying of poison for any animal other mice or rat is illegal. However, the WTSE is doing much better than the GE. Being an upland specialist, the GE feeds on hare and grouse, both of which are in short supply, after centuries of hill sheep farming having degraded the ecosystem. The WTSE is more of a generalist, taking fish, small mammals, birds and carrion. While the WTSE experiences more persecution, it is actually better suited to the current Irish landscape.

The Grey Partridge reintroduction

The Grey Partridge is a red listed game bird that has historically declined in Ireland with only one naturally occurring population confined to the Lough Boora peatlands in Co. Offaly (Lynas *et al.*, 2007). The Irish Grey Partridge Conservation Trust breeds wild partridge and releases them back into the Boora peatlands. Since

the trust started its breeding programme, the number of partridge has grown from a population of just 17 to 317, in 2010 (Buckley *et al*., 2011). A measure that will supplement a farmer's income if they provide a suitable habitat on their land for the Grey Partridge, has been included in the Green Low carbon Agri-environment Scheme, GLAS, part of the Rural Development Programme 2014 - 2020 (Martyn, 2014). This is a landmark project as it specifically awards farmers for helping to save an endangered species. A reintroduction of Grey Partridge from Boora to Fingal, North Co. Dublin is concluding this year (RTE 2011).This reintroduction scheme involved all stakeholders early, benefited each one, and is succeeding as a result.

Future possible reintroduction projects in Ireland

In Ireland the main stakeholders are the farmers, conservationists, tourist lobby, and the general public (O'Rourke, 2014). In any new reintroduction programme, all stakeholders should be involved from an early stage, as exemplified by the partridge reintroduction. It should also be noted that the reintroduction of a species should have a clear and definable benefit to the species in question, and not all extirpated species should necessarily be reintroduced. However there is an obligation stemming from the Habitats Directive to study the desirability to do so.

The Grey Wolf (*Canis lupus*), a recently extirpated species is one candidate for future reintroduction. Contrary to the prevalent view that wolves need huge wild expanses with no human contact to survive, they are thriving in human dominated landscapes in Europe outside of protected areas (Chapron *et al*., 2014). The coexistence model, where humans live alongside large carnivores in heavily modified managed habitats, is possible (Linnell *et al*., 2001), which will hopefully continue the current stabilisation and expansion of large carnivores in Europe (Chapron *et al*., 2014).

But whether or not the people of Ireland are ready to see a return of large mammals is currently a largely moot point. A report to the Convention on Biological Diversity on the state of Irelands protected habitats showed that of 'the 58 habitats assessed, 5 habitats were assessed as favourable, 29 as inadequate and 24 as bad' (Dahg, 2014). The habitats in Ireland are not in good enough health to support a large apex predator such as the wolf, as highlighted by the Golden Eagle in Donegal. As the condition of Ireland's habitats improve, and education changes public opinion, the wolf, as well as the boar, should be reassessed for an experimental reintroduction.

Another long term reintroduction project could be the Capercaillie, an extirpated game bird (D'Arcy, 1999). The Cappercaillie is a habitat specialist and requires an open Scots Pine (*Pinus sylvestris*) and oak forest, similar to the ancient oak forest present in Killarney National Park. There is no such forest of sufficient size and structure present in Ireland today. However, the National Woodland Scheme aims to restore over 1900 hectares of native forest by 2020 (Department of Agriculture,

Food & the Marine 2015). If this were implemented in conjunction with the Wild Nephin project in Mayo, then a suitable habitat for Capparcaillie could be created in forty years. This should be a long term conservation objective.

A species that would benefit from the existing GLAS scheme is the Common Irish Quail (*Coturnix coturnix*). This is a red listed game bird (Colhoun & Cummins, 2013) that needs a survey of the breeding number. Their number is likely to be very small and should be an immediate reintroduction project.

Not confining reintroductions to the terrestrial environment, the European Sea Sturgeon (*Acipenser sturio*) is also a potential candidate. The extirpated fish (Kelly *et al.,* 2007) is listed as critically endangered throughout its range by the IUCN (Gesner *et al*., 2010). This fish lives most of its life in the Atlantic Ocean, returning to spawn in the rivers of Europe. There is an ongoing European Sea Sturgeon reintroduction programme in the Rhine estuary in the Netherlands (Houben, 2015). Although future reintroductions will be limited by the genetic diversity of donor stock (Houben, 2015), this should be a high priority reintroduction project if we are to see the survival of this species, with Ireland potentially having a leading role to play.

Conclusion

Considering the definitions provided by the IUCN, there are many species that could be potentially reintroduced into Ireland, with only a few high profile species having been highlighted here. Furthermore, Ireland has an obligation to study the desirability to reintroduce these extinct species. The restoration of a full diversity of habitats essential for reintroduction of species, will require long-term commitments to wildlife population management by Government, NGOs, farmers, and other stakeholders.

While we should continue to preserve our biodiversity - due to our dependence on it for food, medicine, building materials, fertile soil, flood control, breathable air, habitable climate and drinkable water - perhaps we should also strive to prevent the extinction of other species for ethical reasons. As the most intelligent and powerful species on Earth, and with the growing awareness of our interdependency with our environment, the imperative for the conservation and protection of wildlife becomes self evident, and critical to our survival. The reintroduction of species should be considered for their own sake, but also for the impact that the creation of their habitats could have. For example the creation of Scots Pine and oak forest for the reintroduction of the Cappercaillie, would act as a carbon sink and restore biodiversity. E.O Wilson states: 'Right now we're pushing the species of the world through a bottleneck. We've got to make it a major moral principle to get as many of them through as possible. It's a challenge for now and the next century. And there is one good thing about our species: We like a challenge!' (Wilson, in Campbell & Reece 2004)

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Dog Eat Puppy World: A Review of Juvenile and Filial Cannibalism in the Animal Kingdom

Laura Matthews
Senior Sophister
Zoology

Cannibalism is a widespread behavioural strategy in the animal kingdom, providing many advantages to its subscribers, along with many substantial disadvantages. Most surprising are the benefits of filial cannibalism, i.e. the eating of offspring and close relatives. Here the topic of filial cannibalism is covered in detail, exploring its advantages and disadvantages to male and female parents. While it is found to be a highly beneficial and advantageous method of feeding, providing nutrition when absolutely necessary, it has many deleterious effects. Avoidance of juvenile and filial cannibalism by adults is also reviewed, along with the avoidance of becoming conspecific prey by juveniles.

Introduction

Cannibalism, or intraspecies predation (defined as the catching, killing and devouring of an animal by a conspecific), is widespread in the animal kingdom with at least 3000 species, across 900 groups subscribing at least partially to this method of feeding (Polis, 1981; Fox, 1975). What was once considered 'an aberrant and occasional' phenomenon is actually relatively common in many species (Hunte and Myers, 1984). 'Cannibalism' has been occasionally found in human history, particularly in New Guinea (Venkatachalam, 1962; Dornstreich and Morren, 1974) and often poses the same theoretical advantages to that of subscribers in the animal kingdom (though usually is in fact intraspecific necrophagy; the eating of dead conspecifics, a behaviour closely linked to

cannibalism). Many kinds of cannibalism exist in the animal kingdom and have been studied in some detail, this review explores one of the darker forms of cannibalism; filial cannibalism, the killing and eating of one's own offspring

Cannibalism in general can be very advantageous. It is an effective method of feeding, with the prey's nutrient content matching closely what is required of the predator, thus removing the danger of a nutritional mismatch (Bobisud, 1976). For example, arthropods are often limited in somatic growth by abundances of specific rare minerals such as nitrogen, and so, predating conspecifics eliminates the need to search for such substances (Denno and Fagan, 2003). Intraspecies predation ensures that these nutrients are easy to encounter.

Intraspecies predation reduces the need to search for prey, and is particularly common in colonial species, where the availability of same-species prey is high, or in areas where other species to predate on are in low densities (Polis, 1981). Cannibalism is also more likely to occur in populations that have overlapping generations in time and space, and also have notable differences in size (Wissinger, 1992). It has an indirect fitness advantage of removing potential competitors, thus increasing your potential resources. For example, female Three-spined Sticklebacks (*Gasterosteus aculeatus*) cannibalise stickleback eggs, even when alternative food is superabundant (Fitzgerald, 1992).

Cannibalism can be mediated by many other factors such as food availability, habitat type, genetics, and parasites. Females have a tendency to be more cannibalistic than males (Polis, 1981; Fox, 1975), as the majority of cannibalistic activity involves females eating males post copula (Cordoba-Aguilar and Contreras-Garduño, 2006), or mothers eating suboptimal offspring (Elwood, 1992). There is an evident genetic component to cannibalism; for example, rates of litter cannibalism in mothers remain the same for at least 13 generations in studies of laboratory mice (Hauschka, 1952). Poor quality habitats can mediate the incidence of cannibalism, with adult *Gammarus* having to turn to cannibalism due to lack of food and juveniles having to forage more widely to find food, increasing the risk of predation (MacGrath *et al*, 2007). Cannibalism can be mediated by parasitic infection., For instance, *Gammarus* infected with *Pleistophora mulleri* showed an increase in cannibalistic activity (Bunke *et al.*, 2015). Behavioural polyphenism or 'animal personality' can also mediate cannibalism (Poulin 2012). For example, in a study of a colony of 900 Herring Gulls (*Larus argentatus*), 23.3% of all eggs and chicks were eaten by conspecifics with 4 individual adults responsible for 2-5% of this figure (Parsons, 1971). Such specialist cannibalistic behaviour is also found in Smallmouth Bass (*Micropterus dolomieu*) (Clady, 1974) Californian Newts (*Taricha torosa*) (Kaplan, 1980), Chimpanzees (*Pan spp.*) (Goodall, 1977), among other species.

Why Juveniles?

In many species, smaller individuals in a population are usually cannibalized by larger individuals, known as size asymmetric cannibalism (Ebenman and Persson, 1988). Larger animals are usually the older, more fit individuals, while the smaller animals tend to be weaker or juvenile individuals. Size asymmetric cannibalism is particularly common in all species of amphipods (MacNeil *et al*., 1997) and teleost fish (Manica, 2002), with juveniles often being prey for adults. Sexton (1928) noted in his analysis of laboratory *Gammarus* that individuals don't prey on conspecifics unless they are at some disadvantage, such as being of smaller size or weakened from a recent moult.

Studies of cannibalistic tendencies in a wide range of species indicate that there is a 'cannibalism threshold', that an individual should be a certain percentage larger than its prey to cannibalize it (Polis, 1981). For example, conspecifics are only eaten in piscivorous fish if the predator-to-prey ratio exceeds the threshold of 80-100% (Popova, 1967) i.e predatory should be 80-100% larger than their prey. The danger of cannibalism decreases with age (Polis, 1981). By this generality, newborns are particularly vulnerable to attack and cannibalism, with predation of newborns by conspecifics found in over 80 species (Hardy, 1977). This can be a major source of juvenile mortality, for example 8% of all young Belding's Ground squirrels (*Urocitellus beldingi*) born each year are cannibalised by conspecifics (Sherman, 1980).

Infanticide is common in many species (Hardy, 1977) for example lions (*Panthera leo*) (Pusey and Packer, 1994), Belding's Ground squirrels (*Urocitellus beldingi*) (Sherman, 1980), and chimpanzees (*Pan spp.*) (Goodall, 1977), males are often found eating conspecific infants and in many, but not all cases, the infant will be eaten. In polygynous species (those in which one male receives exclusive mating rights with multiple females), an invading male will kill the progeny of the previous patriarch and the offspring will often be eaten, for instance Bertram (1975) reports that 25% of all lion cubs killed are cannibalised in this way.

Why not Cannibalise?

While cannibalism can present many advantages, and for many species may just be considered an extension of normal predation behaviours (Hardy, 1977), it can also often be disadvantageous. Cannibals and their conspecific prey (or their preys guardians (Sherman, 1981)) may be evenly matched in fighting ability, and the likelihood of injury to the cannibal may be high (Dawkins, 1976), although this is usually combatted by adhering to its cannibalism threshold. Cannibalism and intraspecific necrophagy can also increase the likelihood of disease or parasite transmission (Rudolph and Antonovics, 2007). For example, cannibalistic Tiger Salamanders (*Ambystoma tigrinum*) that ate diseased conspecifics were found less

likely to survive to metamorphosis (Pfenning *et al.,* 1998) than non-cannibalistic individuals, and that the frequency of cannibalistic tiger salamanders is negatively correlated with the bacterial density in their habitat, to avoid the predation of infected conspecifics (Pfenning, 1991). Overall, cannibalism can create a net decrease in inclusive fitness if one is likely to eat close relatives, a behaviour known as filial cannibalism. Therefore, cannibalism may be highly deleterious to subscribers.

Maternal Filial Cannibalism

While some species have developed behavioural methods to avoid eating close relatives (see Avoidance of Cannibalism, below), which may be highly deleterious, many species actively partake in this method of feeding known as filial cannibalism, the purposeful catching killing and devouring of progeny, along with filial necrophagy; the eating of dead progeny. In many circumstances, it can be a highly beneficial and common behaviour. Because the investment in young is inherently different between males and females (due to energy costs of gamete production, mating and raising the clutch), the benefits of filial cannibalism vary between the sexes.

Maternal filial cannibalism occurs in species where maternal parental care is the preferred method of care (Gubernick, 1991). This is particularly common in mammals, where 90% of species are raised exclusively by their mothers (Kokko and Jennions, 2008). Mothers invest a lot of time and energy into producing their offspring and losing them may seem like an obviously deleterious event, although through filial cannibalism this loss can be advantageous. Offspring can be a beneficial food source when other food sources are in short supply, or when the survival potential of the mother is low. Cannibalism provides very high direct benefits in this instance; a mother's current survival is more beneficial than survival of the offspring, if the reproductive potential for future clutches is high (Sargent, 1992). Wholly or partially eating a current clutch increases survival and future reproductive potential, at a cost to an individual's current potential. Rowher (1978) suggests that current offspring survival is traded off against feeding, and parents use their offspring as an alternative food source. This can present as whole clutch or partial clutch cannibalism.

Whole clutch cannibalism benefits the mother, whose survival potential increases instantly through eating her nutrient rich offspring. This can be viewed as an extreme form of brood termination, where the cost of caring for the clutch is higher than the expected benefits (Clutton-Brock, 1991). By no longer providing care, the parent can save its time and energy for searching for a new mate, or increasing survival (Alexander, 1974). In species with low survival rates without parental care i.e altricial young (born in a helpless state and requiring extended parental care to survive), it is beneficial to cannibalise the offspring, as their chance of survival

is already low. This behaviour can increase the mothers' potential to successfully raise another clutch in the future, when the offspring have a higher chance of survival. It will also benefit the potential future clutches to have a fitter mother than would be the case if the current clutch had been raised to adulthood (Clutton, 1991).

Partial clutch cannibalism involves the eating of some of the offspring in the clutch, and can benefit the mothers' current and future reproduction. Partial clutch cannibalism gives the mother immediate nourishment, enough to attend to her litter without starvation. Mothers guarding their clutch are usually near starving, and for many mammals, this presents a problem. Milk cannot be produced in starving animals ("Domestic Animal Behaviour And Welfare, 2008"). Filial cannibalism eliminates the need for the mother to leave the nest to forage for food. Leaving the nest leaves the young vulnerable to predation, and to the elements. There is also a possibility of the mother dying or deserting the clutch while away from the nest, which is common in rabbits (Dennenberg *et al.,* 1959). Thus, it is beneficial for the mother to stay in the nest and seek alternative food sources- i.e. her offspring.

Partial clutch cannibalism is common, particularly in animals that produce sizeable litters. Many animals produce more young per clutch than will survive to adulthood, to maximize the chance that some will survive and reproduce (Weir and Rowlands, 1973). Large litters inherently produce some offspring that will present low survival potential, often known as the 'runt of the litter', due to lack of growth space and resources *in utero* (Lodge and Lamming, 1968). These individuals can be viewed as an energy tradeoff, wherein the energy put into creating the individual can be returned to the mother at a later stage, when she is guarding her young and starving.

Some of these offspring are likely to be weak, deformed, sick or otherwise handicapped, especially in large litters. These sub-par offspring are usually the ones cannibalised by the mother (Elwood, 1992). Diseased or infected offspring are often eaten to prevent disease spreading through the rest of the clutch. Eating of diseased offspring occurs in many species, such as Californian newts (*Taricha torosa*) (Kaplan, 1980), Belding ground squirrels (*Urocitellus beldingi*) (Sherman, 1980), and rabbits (family *Leporidae*) (Dannenberg *et al.,* 1959).

These are also benefits for the young left in the clutch, by having better care and protection from their mother. This additional care and protection helps increase the fitness and survival of the remaining offspring, along with the mother. They also receive an indirect fitness benefit from the removal of their siblings, as they are potential future competition (Clutton-Brock, 1991), but may also sustain a disadvantage, by the reduction in their inclusive fitness from the offspring of their siblings.

Paternal Filial Cannibalism

Paternal cannibalism is more common in animals where the father provides the parental care. Whole clutch cannibalism is common in male teleost fish (Manica, 2002) for example in a study of damselfish, 28.3% of males ate the entirety of their clutch. Paternal care is high in this group, and the advantages of this method of feeding are similar to those of maternal cannibalism. The father may also parasitise the female, by using her nutrient-rich eggs as food (Rohwer, 1978). The cost of parental care is the same in small and large clutches, and so it benefits the parent to brood a large clutch. An unsuitably small clutch may be fully cannibalised, so the male may mate again, and produce a larger more beneficial clutch.

Partial clutch cannibalism occurs for many of the same reasons as in maternal cannibalism; mainly that it provides nutrition without having to abandon the clutch. In many fish species, males benefit from guarding their eggs straight after fertilizing them, to prevent other males sneaking in and fertilizing some (Gross, 1996). Fathers also guard the eggs from other predators. Leaving the clutch unguarded is dangerous, and so, eating part of the clutch to avoid this benefits the fathers' fitness, and the fitness of the surviving eggs. Klug and Lindstrom (2006) suggest that partial cannibalism may be necessary to reduce egg density and increase oxygen availability to remaining eggs, thus increasing the remaining offspring's fitness, as is the case in the Sand Goby (*Pomatoschistus minutus*). Klug and Lindstrom (2006) further suggest that partial clutch cannibalism may create no net losses in reproductive success.

Avoidance of Cannibalism

While the advantages of cannibalism have been outlined above, it is in no way advantageous to the individual being eaten. As such, many behavioural mechanisms have developed to avoid being eaten by conspecifics, above and beyond the usual predation avoidance tactics (Rudolf, 2007).

In many cannibalistic species, juveniles tend not to adhere to natal philopatry; the remaining in or returning to natal territory (Pearce, 2007), and quickly expand outwards from their native zone to create a separation between the vulnerable juveniles and cannibalistic adults. Members of the genus *Gammarus*, such as *G. tigrinis, G. mucronatus,* and *G. lawrencianus* exhibit changes in phototaxic behaviour (their movement towards or away from light) at an age that correlates to a reduction in vulnerability to predation. This movement causes a partial separation of adults and juveniles that reduces the likelihood of predation (Hunte and Myers, 1984). Smaller individuals and juveniles of *Gammarus* shift habitats to minimize the risk of predation by conspecifics. With larger conspecifics *in absentia*, juveniles of *G. pulex* will use larger pores in substrates to hide in whereas when there is predation danger, they only select smaller pores, regardless of food availability (MacGrath *et al.,* 2007). *Poeciliopsis* fish, a highly cannibalistic species, show a genetic propensity to avoid their parents from birth, with avoidance increasing as size does. (Lima and Vrijenhoek, 1996).

While juvenile and filial cannibalism presents the greatest disadvantages to juveniles, many adults have evolved behaviours to avoid filial cannibalism, to avoid the fitness costs. A temporal behavioural change occurs in relation to brood stage in female *G. pulex*, whereby the instances of cannibalism are significantly reduced concurrent to the time their own eggs are hatching, to prevent the likelihood of eating their own young (Lewis *et al*., 2010). It is unknown if other phenotypic recognition cues are utilized in this instance, or whether males subscribe to a similar temporal avoidance of cannibalism.

Flial cannibalism can be avoided in animals where both parents confer parental care on their offspring. This is the case in approximately 90% of bird species (Kokko and Jennions, 2008). One parent may guard the clutch, while another may forage for food for itself and its offspring, and roles may alternate. As such, filial cannibalism is not common in birds.

Conclusion

Cannibalism, in general, and more specifically the instances of juvenile and filial cannibalism present a fascinating insight into the survival strategies of many species in the animal kingdom. While it may seem deleterious for parents to eat their own young, it can present many advantages, and assist in survivorship of individuals and offspring, through additional nutritional intake when it is needed the most. The behaviour, aberrant to human society and considered unnatural in most societies (Dornstreich and Morren, 1974) is in fact in some instances a highly beneficial and well evolved strategy for many species as shown. Even so, it is perhaps not the best idea to eat your own children.

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The Genetics of Fasciation

Aimée Gilmartin
Junior Sophister
Genetics

Fasciation is a relatively rare plant deformity, which leads to the flattening of the stem bud by elongation, perpendicularly to the direction of growth of the flower. It can be caused by a range of different genetic factors such as inefficient repair of double stranded breaks, mutations and hormonal imbalances induced by fungal pathogens. The mutation of the breast cancer 2 gene (BRCA2), also present in plants, which is involved in double stranded break repair mechanisms, has been implicated in the progression of fasciation. Radiation has also been seen to induce an effect by increasing the rate of mutation. Gain of function mutations in the Arabidopsis thaliana (Arabidopsis) Fasciated Stem 4 gene (AtFAS4) during certain periods of development can lead to a fasciated stem and also the loss of function mutations in the Maintenance of Meristem genes (MAINS). Main mutants induce fasciation by causing premature differentiation of the meristematic stem cells or cell death by the loss of stem cell maintenance. Rhodococcus Fascians (R.fascians) releases methylated cytokinins (MeCKs) which mimic plant hormonal activity leading to fasciation in plants. Cytokinins induce fasciation in plants by regulating genes which control shoot apical meristematic activity such as the CLAVATA 1 gene (CLV1). Phytopathogens which induce fasciation in plants promote their own proliferation by decreasing host fitness. This is done by producing phytohormones which lead to unusual development of floral organs. They can also alter the main primary carbon metabolism which leads to increased disease establishment.

Introduction

Fasciation is the elongation of the meristem tip which grows perpendicularly to the direction of growth of the plant. The tip which is usually concentrated at a particular point becomes elongated, flattened and cylindrical in shape (White, 1948). Fasciation is a relatively rare condition and can be caused by many different genetic factors such as double stranded breaks (Abe *et al*., 2009), point mutations in apical meristematic genes (Wenig *et al*., 2013), by phytopathogen infections e.g. *R. fascians* (Radhika *et al*., 2015) or by different types of radiation such as gamma radiation (Abe *et al*., 2009). Figure 1 shows wildtype daisies vs ones which are fasciated (this photograph went "viral" as it was falsely suspected to be attributed to radiation released from the Fukushima plant disaster). Fasciation has many contributing factors as will be discussed in this review along with the mechanisms of overcoming plant barriers to infection and issues which are unclear in the literature, requiring future research.

Fig 1. *Photograph of three fasciated daisies on the left vs two normal daisies on the right (Photograph courtesy of Kaido (2015). This photograph was taken 65 miles from the Fukushima plant (Harrington, 2015) and went viral because it erupted fears that these daisies were due to the radiation from the plant. However, the atmospheric dose of radiation (0.5 μSv/h at 1m above the ground) was not significantly higher than background levels and hence there was no evidence of an effect.*

What are the Genetic causes of Fasciation?

Fasciation can be caused by various different types of alterations in DNA including DNA damage and mutations. The most potent of these alterations are double stranded breaks (DSBs) as if not repaired, can lead to cell death

and if not repaired correctly, can lead to the formation of many more different types of mutations such as deletions, translocations or even fusions within the DNA. These DSBs are inefficiently repaired in plants containing what is known as a 'BRCA2 mutation' which have also been demonstrated to induce fasciation (Abe *et al.*, 2009). This mutation is more commonly known in humans where it is associated with increased risk of breast cancer (Wooster *et al.*, 1994), but also present in higher plants. In vertebrates the BRCA2 gene is involved in repair of double stranded breaks in two ways. The first and the most error prone mechanism used is known as non-homologous end joining (NHEJ). This is a pathway which involves the ligation of the break ends of the DNA without using the homologous chromosome as a template It is used at the beginning of cell division before the identical copies of the homologous chromosomes have been formed and made available for the second, less error prone mechanism, known as Homologous Recombination (HR). HR uses the homologous chromosome as a template for repair of the double stranded break and occurs towards the end of cell division (Abe *et al.*, 2009).

To identify the role of BRCA like genes in plants in DNA repair, transposon insertion mutants of the AtBRCA2a and AtBRCA2b genes were identified and characterised. The proteins encoded by AtBRCA type a and type b genes were found to be 94.5% identical (Abe *et al.*, 2009) and therefore hypothesized to be most likely the result of a recent genome duplication (Siaud *et al.*, 2004). It was thus believed that the two genes would perform a similar role or have redundant functions. To test this redundancy, genetic crosses were performed between homozygous single mutants to obtain double mutants. Previous studies in vertebrates showed that double mutants were more sensitive to genotoxic agents such as gamma radiation and cis-platin (an interchelating agent) and this was tested in Arabidopsis. It was shown as a result of these experiments that double mutants showed altered cell cycle progression. This suggested that there was inefficient repair of the double strand breaks in the double mutants which led to the disorganisation of cell cycle progression in apical meristems. In addition it was also found that mutants deficient in HR showed fasciation phenotypes and were also more sensitive to genotoxic agents.

One such example is the MRE11 gene. It was shown by Bundock and Hooykas (2002) that one of these mutants mre11-1 was hypersensitive to the alkylating agent Methyl Methane Sulfonate and also displays fasciation. However mutants deficient in NHEJ did not display this hypersensitivity to genotoxic agents and fasciation phenotype (Abe *et al.*, 2009). Examples of such mutants include: ku70 shown by Riha *et al.* (2002), ku80 shown by Friesner and Britt (2003); Gallego *et al.* (2003); West *et al.* (2002) and ligIV shown by Friesner and Britt (2003) and van Attikum (2003) . Indeed, it was confirmed that abnormal phyllotaxy (unusual arrangement of leaves around the stem) and stem fasciation were not increased in the ku80 mutant (WS ecotype background;(West *et al.*, 2002)) compared to the wild type, with or without

c-irradiation (short wavelength Ultra Violet germicidal radiation used to kill microbes by disrupting nucleic acids and breaking their DNA). Thus in contrast to the Homologous Recombination repair pathway, inefficient repair of DSB via NHEJ does not seem to induce disorganisation of apical meristem cells. As such the fasciation phenotype observed in atbrca2 single and double mutants may be linked to c-irradiation (Abe *et al.*, 2009).

Mutations in genes involved in specifying organ number such as the CLV3 gene leads to fasciation. CLV3 encodes a supposed ligand for a transmembrane receptor kinase, known as CLVI. Mutations in the CLV3 gene leads to an increase in the accumulation of undifferentiated stem cells in the stem tip (Clark *et al.*, 1996). It was also reported in another study that a CLV3 RNAi construct induced by dexamthamethasone also shows stem fasciation (Reddy and Meyerowitz, 2005). Many gene regulatory networks can be perturbed using this technique. The dexamthamethasone inducible RNAi construct enables the downregulation of CLV3 at will. Reports like this indicate that plant Stem Apical Meristem (SAM) developmental programmes can be changed by outside factors during development. This has been demonstrated using c-irradiation and will also be highlighted by discussing *R. fascians* which mimics plant hormonal activity.

Phytopathogens such as *R. fascians* produce phytohormones which alter plant development to facilitate their own development in the host plant. *R. fascians* is an aerobic, gram positive pathogenic bacterium which induces leafy gall disease and fasciation in plants. Leafy gall disease is the outgrowth of plant tissues which is analogous to benign tumours or warts in humans (Stes *et al.*, 2013). In order to achieve this *R. fascians* contains a *fas* locus. A *fas* locus is an operon which contains genes which are homologous to genes required for cytokinin (CK) biosynthesis and metabolism (Stes *et al.*, 2011). The phytopathogen produces methylated cytokinins (MeCKs) which inhibit root growth, which is a sign of CK action. MeCKs were however retained longer in the plant and showed signs of enhanced biological stability as they were not good substrates for oxidases and dehydrogenases. The MeCKs were shown to mimic cytokinins and play a role in plant-pathogen interaction (Radhika *et al.*, 2015).

Methylated cytokinins from the fungal pathogen R.fascians mimic plant hormonal activity leading to the induction of fasciation in plants by regulating genes which control shoot apical meristematic activity, such as the CLV1 gene discussed. When a cytokinin such as N6 -benzylaminopurine (BAP) is applied to the shoot apical meristem of Arabidopsis thaliana, floral organ number is increased, which is reminiscent of the CLV1 mutant phenotype (Radhika *et al.*, 2015). Transcriptome analysis reveals that exogenous cytokinin treatment of plants significantly reduces expression of CLV1, which a gene is encoding a receptor like kinase, which is involved in stem cell maintenance in shoot and floral apical meristems (Nikolaev *et al.*, 2007). Time course RTPCR of the BAP

treated plant transcript levels showed a decline and subsequent recovery of CLV1 and a concurrent increase in WUSCHEL (WUS). This is consistent with our knowledge that CLV1 suppresses WUS. WUS encodes a homeodomain which is known to be linked to shoot meristem proliferation. The fact that floral development is altered at the same time at which transcript levels of CLV1 and WUS change suggests that cytokinins regulate flower development through genes controlling shoot apical meristem activity (Radhika *et al*., 2015).

Gain-of-function mutations in the AtFAS4 gene are also shown to result in a fasciated stem. These gain-of-function mutations were introduced by inserting an overexpression construct of ATFAS4 into Arabidopsis. The analysis of the ATFAS4 gene by Pogorelko *et al.* (2008) showed that this gene is expressed at very low levels, if at all in the wildtype whereas it is constitutively expressed in the overexpression construct and highly expressed in the mutant plant. When they analysed the protein amino acid sequence, they located the presence of helicase domains on the N-terminal domain of the protein which are involved in DNA denaturation, which could possibly lead to the destruction of genes involved in apical stem cell maintenance, thus leading to symptoms of fasciation or leaf gall disease. These helicases can also lead to the activation of DNA and RNA substrates leading to fasciation. This is supported by the fact that a large quantity of peptides are made by ATFAS4 mutant DNA and also by their short life span, suggesting that they are only around during certain short periods of time and during certain environmental conditions such as hormonal exposure, such as what is expected during the development of the plant.

Loss-of-function mutations such as in MAIN gene, which encodes a protein DNA binding domains in transcription factors, can lead to altered stem cells which perform different functions, which leads to premature cell death (Wenig *et al*., 2013). MAIN encodes a nuclear localisation protein. Malfunction of MAIN leads to roots which are visibly shorter, leaves which are misshapen, reduced fertility and partial fasciation of stems as seen in Figure 2. These mutants become more sensitive to genotoxic agents which leads to the expression of genes involved in DNA repair, which may also be mutated in fasciated plants. The increased sensitivity was shown by analysing the expression of DNA repair genes (Deveaux *et al*., 2000; Lafarge and Montané, 2003; Zhu *et al*., 2011). Dead cells along with mutant DNA accumulate in the meristems of plants. MAIN maintains stem cell genome integrity. Descendant MAIN mutants have a disorganised shoot apical meristem (SAM) and show a fasciated phenotype.

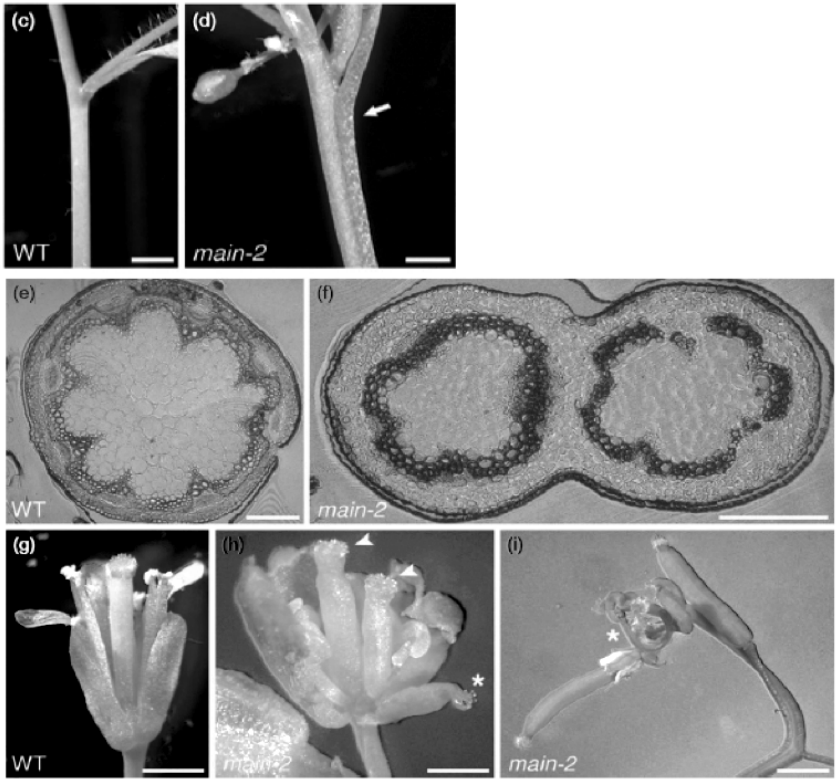

Figure 2. *Wildtype Arabidopsis vs Main mutants showing cell patterning defects in the cells in which MAIN is usually expressed (Root apical meristems and SAMs). Mutant MAIN phenotypes shown in Arabidopsis vs wildtype (g). Fasciation was observable in stems of both main mutants, but not in the wildtypes (WT). Cross section of main 2 mutant stem, indicated by arrow, in (d) is shown in (f). Cross section of wildtype stem shown in (c) is displayed in (e). (h) Shows how some of the main plants had more than one ovary, as shown by the arrows. The asterisk in (h) and (i) show how some main mutants have lost organ identity, which include papillae in the place where sepals should be. Fused flowers and pedicels are shown in (i) Scale bars 20 lm (e,f), 500 lm (h), 1 mm in (g) and 2 mm (all other images). Figure from Wenig et al. (2013).*

How is the immune response of the plant overcome by the pathogen?

For *R. fascians* and other pathogens involved in causing fasciation in plants, Cytokinin (CK) production is thought to be critical for virulence. *R. fascians* carries genes for CK homologue biosynthesis on a *fas* locus, as described previously. Since these genes are encoded on the *fas* operon, it is thought that these genes are essential for the pathogen-plant interaction. These genes are carried within a plasmid in the bacterium (Jacobsen *et al*., 1996). Phytopathogens promote their own virulence within the plant by producing phytohormones which lead to unusual development of floral organs. They can also alter the main primary carbon metabolism which leads to increased disease establishment (Robert-Seilaniantz *et al*., 2007). Bacterial pathogens such as the gram positive *R. fascians* develop gall structures (Sakakibara, 2006) which provides a better nutrient source environment for the bacterium.

Cytokinins (CKs) are detected by sensory Histidine kinases in Arabidopsis (arabidopsis his kinase 2 (AHK2) to AHK4. Once the cytokinins are detected, the sensory histidine kinases transfer a phosphoryl group from one Histidine kinase to another. This leads to the activation of direct target genes, e.g. type A ARABIDOPSIS RESPONSE REGULATOR (ARR) genes (Kieber and Schaller, 2010). Infection of Arabidopsis plants by *R. fascians* activates type-A ARR5 expression which leads to an increased expression of AHK3 and AHK4. This causes mitotic cell divisions which leads to infected cells being arrested in a meristematic state which establishes a nutrient-rich niche (Stes *et al*., 2011). This leads to the formation of a gall type structure. As the infection progresses, ISOPENTENYLTRANSFERASE (IPT) genes associated with cytokinin synthesis are turned off. However expression of all CYTOKININ OXYGENASE/DEHYDROGENASE (CKX) genes are highly upregulated in affected tissues (Depuydt *et al*., 2008).

FAS4 encodes an IPT which is involved in CK biosynthesis. The encoded IPT catalyses the rate limiting step of CK biosynthesis. This is vital for virulence (Stes *et al*., 2013). There are an additional 2 methyl transferase genes upstream of this FAS4 gene. The function of these is however unknown. Even though FAS genes are present in *R. fascians*, not many CKs have been detected compared to other bacteria which produce the leafy gall phenotype, such as *Agrobacterium tumefaciens* (Goethals *et al*., 2001). The virulence of *R. fascians* is thought to not be caused by CKs alone, as the leaf gall phenotype it produces is not caused by any one cytokinin in the bacteria containing the FAS genes (Goethals *et al*., 2001). CK analogues used by *R. fascians* to induce an effect are known as methylated cytokinins (Radhika *et al*., 2015). MeCKs are produced using two methyltransferases and the action of the AtFAS4 gene. MeCKs show CK-like activity and have a higher stability. Their higher stability suggests a role in co-ordinating efficient pathogenesis (Radhika *et al*., 2015).

Unclear in literature?

Despite several CKs having been isolated from R. fascian culture filtrates. There has of yet been no clear correlation with pathogenesis detected. This is due mainly to the low concentration of bacterial CKs (Eason *et al.*, 1996). It is thought that many CKs work together to produce an effect. When accumulated locally (Pertry *et al.*, 2009). No virulence-associated CK analogues have been identified that could contribute to the infection symptoms.

Conclusion

Fasciation is a relatively rare condition of plants which leads to the flattening and elongation of the stem tip. This disease can be induced by a wide variety of different factors such as double stranded breaks of DNA and mutations in critical genes involved in the development and maintenance of the plant, with cytokinin analogues released by fungal pathogens also being implicated. Different forms of radiation can also increase the rate of mutation in DNA imparing developmental, maintenance and repair pathways, thus inducing fasciation. The study of fasciation is fascinating as it provides insight into plant developmental mechanisms and how they can be manipulated in order to prevent such defects. This could lead to a minimization of major crop losses to affected plants.

Acknowledgements

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Chemistry

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Letter from the Editor

The multidisciplinary nature of Chemistry is something that is frequently commented on - encompassing all areas from medicinal chemistry, to environmental, nanoscience, biochemistry and much more. The second publication of the TSSR encompasses this perfectly, as well as showcasing the incredible scientific knowledge, ability, and creativity of Trinity students. The relevance of Chemistry in the lives of all, especially that of future generations, is undeniable. However one thing that always struck my interest in the TSSR publication, and is reflected again this year, is the contemporary nature of the research. Our goal as a review journal is to provide a picture of how Chemistry is evolving in the present day and to ignite students' passion for the possibilities of tomorrow.

The search for new drugs and drug delivery systems is naturally always one of high relevance and profile. With the potential of 4-aminoquinolines in the ongoing quest to combat malaria - a problem that affects 3.2 billion people in the world today - along with the emerging possibility of cell-SELEX procedures and aptamers for cancer cell treatment (as recent as 2015), it is clear that the way forward in treating human disease is through multidisciplinary, collaboratory and, most of all, creative research into all the facets that Chemistry encompasses. Moving from personal to collective issues, climate change is set to be one of the greatest challenges yet to face our generation and the generations to come. Environmental chemistry could provide an effective method to combat rising CO_2 emissions - one of the recent routes discovered through metal organic frameworks. It is inspiring to see the intense focus of Trinity students not only on their scientific discipline and knowledge, but in applying this knowledge and creativity to tackle real world situations and problems facing us all. Possibly one of the papers that epitomised most the direct interconnection of humans and Chemistry is that which discussed us - the very origins of life on this planet, and the building blocks of who we are today.

I have no doubt, with this focus and drive, that our generation will go far in finding solutions to these problems and more, and the TSSR provides an important outlet to build the skills of these scientists of the future. However, it could not go ahead without the help of some very important people that I would like to extend a sincere thanks to. Firstly Dr. Mike Southern, without whom none of this could have gone ahead, always available and generous with his time, feedback, and support of the TSSR. Secondly, all of the staff in the Chemistry department who gave up their time and effort to editing - Prof. Isabel Rozas, Dr. Wolfgang Schmitt and Dr. Rachel Evans - the students and publication are really appreciative of your input.

Lastly, I would like to thank all the students who submitted pieces and put in their own time and effort to investigate beyond their coursework. Whether successful or not, it is an exercise that showcases scientific passion and insight, and one that encompasses what science is all about - curiosity and knowledge. All submissions were of an incredible quality and I have no doubt that everyone who submitted will have fruitful scientific careers ahead of them. I leave you now in the capable hands of Chemistry's recent advances and some extremely talented Trinity undergraduate authors.

Kate Reidy
Chemistry Editor
Trinity Student Scientific Review 2016

Climate Change Mitigation Using Metal-Organic Frameworks for Direct Air Capture of CO_2

Dónal Ring
Junior Sophister
Chemistry

Rising atmospheric CO_2 levels must be addressed to avoid the inevitable environmental harms of climate change. Carbon capture and sequestration (CSS), separating CO_2 from other gases at anthropogenic point sources, has been a research interest in this area. Recently, carbon capture directly from the atmosphere, direct air capture (DAC) has been posited as a possible alternative. There are certain advantages to this, as only 47% of CO_2 emissions are addressable by conventional CCS, and it has the potential to decrease atmospheric concentrations, rather than simply slow the rate. There are unique challenges, however, as air concentrations are much more dilute, making the process more expensive. Currently, metal-organic frameworks (MOFs) seem the most promising sorbents for this process, although they face disadvantages such as high regenerability costs. This review will summarise progress in this area, focusing on chemisorption via alkylamine-appended MOFs and physisorption by MOFs bound with hexafluorosilicate anions, and will suggest further areas of research.

Introduction

CO_2 has been a primary target for climate change mitigation, having risen from pre-industrial levels of 280 ppm to current levels of 400 ppm (NOAA/ESRL), and will likely cause significant environmental harms if not addressed. An important mid/long term strategy is carbon capture and storage, or CCS (IPPC, 2014). This is gas separation of CO_2 from other gases such as N_2 and CH_4, and storage for further

use. Conventionally, this operates at large anthropogenic point sources to prevent the gas reaching the atmosphere – this area is where most of the research has been conducted and many argue the technology, though expensive, will soon become necessary to reach mitigation targets (Riahi *et al.,* 2015). However, it is estimated that only 47% of CO_2 emissions sources are addressable by conventional CSS (EEA, 2007), the rest coming from diffuse sources such as small isolated industry plants and transport. This has led some research interest into the more controversial field of CO_2 capture straight from the atmosphere, or direct air capture (DAC).

There are numerous recognised advantages to DAC. As well as capturing emissions from diffuse sources, air capture belongs to the negative emissions group of technologies, which can potentially *decrease* current CO_2 levels, in contrast to conventional capture, which can only slow the rate of release (Choi *et al.,* 2011). For this reason it has been posited as a fall-back option in case countries do not reach the necessary targets, and can be implemented anywhere, not just at selected point sources. Finally, it has applications beyond climate change mitigation, electrolyte function in fuel cells (Kordesch *et al.,* 2000), anaesthesia (Dosch, 2006), and air purification in space shuttles (Ernsting, 1999) being some examples.

There are, however, serious obstacles facing its implementation. The current process is very expensive (Socolow *et al.,* 2011), because atmospheric CO_2 levels of 400 ppm (0.04%) are hundreds of times lower than post-combustion flue streams of 10-15% and pre-combustion (for natural gas) of up to 35% (D'Alessandro *et al.,* 2010). The sorbent (material used to capture the CO_2) therefore faces larger obstacles than for conventional capture and must be extremely efficient to be practicable. It is estimated that for the current sorbents a 10-metre high DAC system would need to be 30 km long to capture the emissions from one 1000 mW coal power plant. Since it is thought that sorbent is the biggest contributor to the high cost (Socolow *et al.,* 2011), the need for new materials if the technology is ever to become usable is clear.

Among the most promising class of materials for future DAC is MOFs. These are highly crystalline, porous materials made of metal centres linked together by organic ligands, called "linkers", have very high surface areas and are easily tunable. Although MOFs began being used for DAC well after solid-supported amines, which have been discussed from at least 2009 (Lackner, 2009), the rate of progress has been quicker. They currently perform as well and sometimes better, although there has so far been no satisfactory study comparing performance in terms of overall cost. This review summarises the progress of MOFs for the purpose of DAC and will provide an explanation of the main properties that underlie their success. There are two broad classes – alkylamine-functionalized MOFs, which have been the main focus until recently, and MOFs that adsorb via physiadsorption, a more recent and very promising method.

There are a range of different properties important for assessing materials. The most important are identified by Huck *et al.,* (2014) in calculating parasitic energy (a cost-based metric), and include adsorption capacity (of CO_2, measured in mmol

of CO_2 adsorbed per gram of material), regenerability (related to the isoteric heat of adsorption, as desorption is essentially the reverse of this value), selectivity of CO_2 uptake versus other gases, and working capacity (measured by comparing uptake isotherms as the amount of CO_2 that can be released following adsorption). Each of these will be incorporated into the discussion as necessary.

Mechanism of Alkylamine-functionalized MOFs

The functionalization of MOFs was performed for other purposes, including post-combustion air capture before it was used for DAC, and the mechanism of functionalization has been long understood (McDonald *et al.,* 2011). Upon synthesis of certain MOFs, some solvent molecules, often DMF or H_2O, become coordinated to the metal centre. These can be removed, usually by heating around 400 °C (McDonald *et al.,* 2012), leaving what are called coordinately unsaturated sites (CUS), usually pentavalent metal centres. Sometimes entire one-dimensional pore channels can be lined with these CUS spaces, and in conventional MOF carbon capture these areas are the sites of adsorption, as the central metal is a strong lewis acid (D'Alessandro *et al.,* 2010). For DAC, these CUS have been exploited to allow alkylamine-functionalization. After expelling the solvent molecules the MOF is placed in an organic solvent with the desired alkylamine, and the CUS are functionalized, to varying degrees depending on the framework. The diamines chosen are highly basic and coordinate strongly to lewis acidic metal centres, leaving one amine end free.

The mechanism of reaction between the amine and CO_2 differs from that of the aqueous amines currently in use for carbon capture and from the solid-supported amines reported to work well for DAC. Early reports suggested a 1:1 amine:carbon reaction stoichiometry, whereas the usual mechanism forming a carbamate species showed a 2:1 ratio, where one nucleophilic amine attacks the partially positive carbon atom and another acts as a base to abstract the N-H proton (Planas *et al.,* 2013). This was originally thought to be due to a mechanism where a second CO_2 molecule was attacked by an amine hydrogen-bonded to the first carbamic acid, forming a bis-carbamic acid complex, giving a 2:2 stoichiometry. However, the evidence was indeterminate and this mechanism could not adequately explain the "step" in reported isotherms, giving a sigmoidal curve in contrast to the usual Langmuir-type isotherm (an important consequence of which is higher achievable working capacities – see figure below). Recently another mechanism was proposed, explaining the step and supported by DFT calculations and IR data. (McDonald *et al.,* 2015)

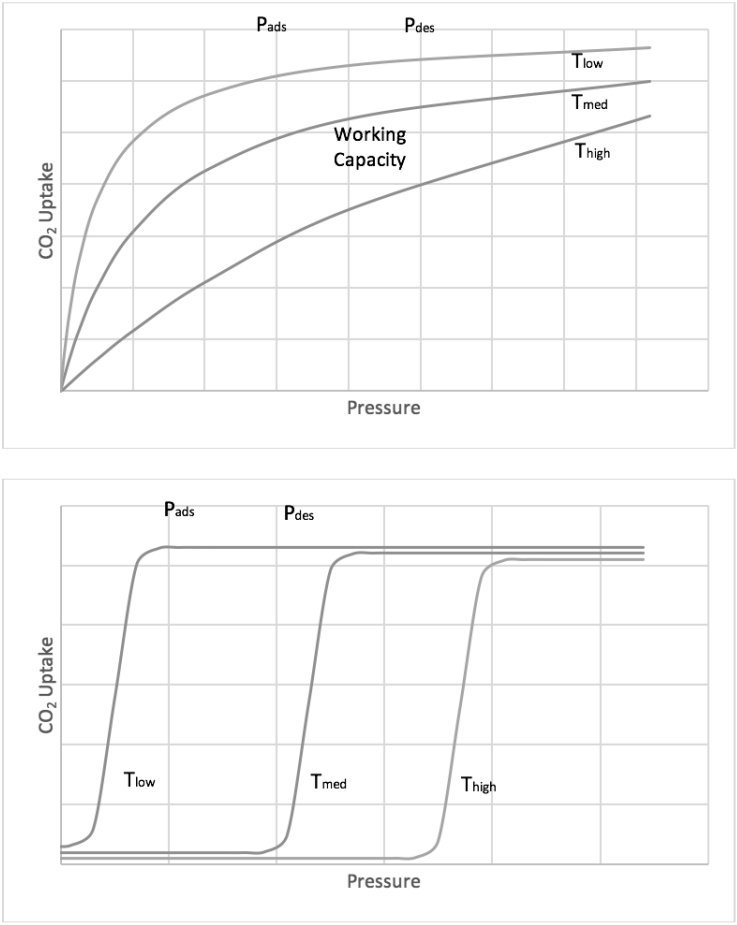

Figure 1. *Comparison of Langmuir isotherms (top) with sigmoidal isotherms(bottom). Adapted from McDonald et al., 2015*

In this proposed mechanism the incoming CO_2 molecule inserts into the amine-metal bond. In the presence of CO_2 an uncoordinated amine end will abstract the acidic N-H proton of a metal-bound neighbour along the c-axis (down the MOF channels), which then forms a carbamate with the incoming CO_2 molecule. This is stabilised by the recently protonated ammonium countercation. The M-N bond will break and the M-O bond will be formed. The rapid rise in uptake is now explained – the ion-pairing interaction destabilizes the mmen ligand (the

particular diamine ligand used for this study, and the mechanism is thought to be the same for other amine-appended MOFs) and weakens the M-N bond, leading to an increase in the rate of CO_2 insertion. This continues in a chain reaction across amines along the c-axis, each new interaction weakening the neighbouring M-N bond.

This proposed mechanism suggests some improvements and reveals limitations for such MOFs – amine bonds tethering it to the surface must be coordinate and not covalent, to allow for M-N breakage, so this mechanism is therefore likely to be unattainable in amine-functionalized silica sorbents, and neighbouring amines must be suitably aligned, so the mechanism will occur with only a limited set of MOFs with appropriate CUS placement. It is predicted that the position of the step, and therefore working capacity, may be changed by altering the linker ligands between each neighbouring amine group, the sterics of the M-N bond, and the strength of the M-N bond by changing the central metal.

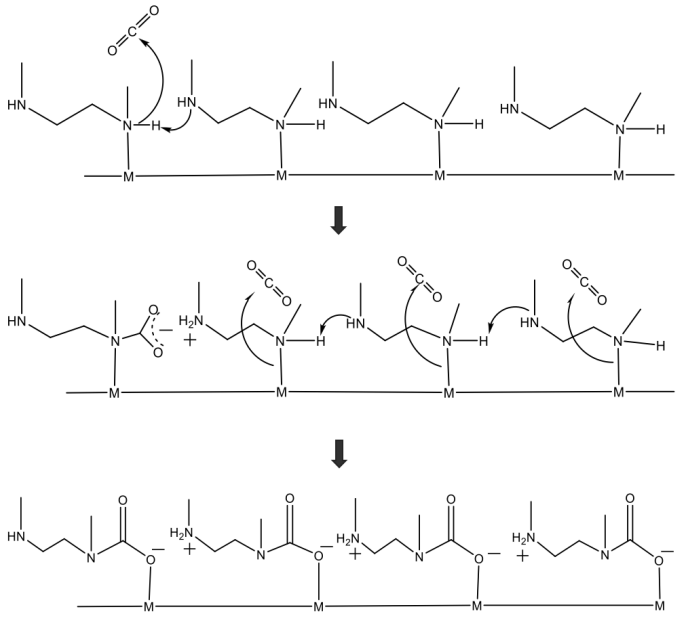

Figure 2. *Overview of proposed alkylamine-functionalized CO2 adsorption mechanism. Adapted from McDonald et al., 2015*

Improved performance from alkylamine-functionalization

Two such structures suitable for DAC were reported in 2012, one by Choi *et al.*, (2012), using Mg-MOF-74. This MOF is composed of Mg(II) metals centres linked with 2,5-dioxido-1,4-benzenedicarboxylate ligands. After synthesising Mg-MOF-74 and expelling the solvent molecules the MOF was functionalized with ethylene-diamine (en or ED) ligands. Analysis of this with dry simulated air streams with CO_2 concentrations at 390 ppm showed it to have exceptional properties, unprecedented for DAC with MOFs. Performance was measured over 4 adsorption-desorption cycles using temperature swing. ED-Mg-MOF-74 had an adsorption capacity from 1.51 mmol/g-1.55 mmol/g with no significant change over the four cycles, higher than non-functionalized Mg-MOF-74 (which also suffered a 20% drop in adsorption capacity) and close to the final value of PEI-silica at 1.65 mmol/g, which suffered a 29% drop from 2.36 mmol/g. There was no reported isoteric heat of adsorption, so it is difficult to assess the regeneration cost – however it is likely to be higher than the non-functionalized framework, as most other reports shows.

Even better results were reported for mmen-Mg_2-(dobpdc) (McDonald *et al.*, 2012). This MOF is an analogue of Mg-MOF-74 with extended linker ligands (4,4′-dioxido-3,3′-biphenyldicarboxylate) which increased the pore space from 11 Å to 18.4 Å. As with ED-Mg-MOF-74 the alkylamine was added via a post-synthetic pathway, giving the final structure, shown below. The material's adsorption capacity was measured using temperature swing over 10 adsorption-desorption cycles, giving an adsorption capacity of 2 mmol/g and no apparent degeneration of the MOF, close to the first use of PEI-silica gel of 2.4 mmol/g. The MOF has the advantage of better reaction kinetics – it takes PEI-silica 200 minutes to reach 4.6%$_{mc}$, while mmen-Mg_2-(dobpdc) reaches the same value after only 60 minutes. The material also performed well in purity of CO_2 sequestered and selectivity over N_2, with values of 96% and 49,000 respectively. However, the materials still suffer from a high isoteric heat of CO_2 adsorption at -71 kJ/mol compared to the value of -47 kJ/mol for the non-functionalized Mg-MOF-74, making the regeneration process energy-intensive – as noted earlier, cheap regenerability is key to the viability of new air capture materials.

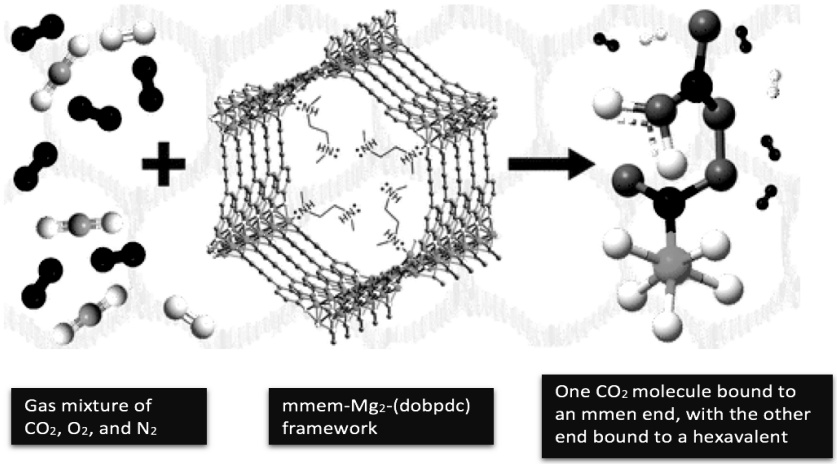

Figure 3. *Overview of CO2 adsorption by mmen-Mg2-(dobpdc). Adapted from McDonald et al., 2012*

The same strategy has since been used to the same effect on the same MOF framework, functionalized with different diamines. A very high-performing alkylamine-grafted MOF was reported by Lee *et al.,* (2014), called en-Mg_2(dobpdc). This is same MOF reported in McDonald *et al.,* 2012 but functionalized with en instead of mmen. This was measured to have a CO_2 adsorption capacity of 2.83 mmol/g, 1.4 times higher than that of mmen and with a lower isoteric heat of adsorption at -49-51 kJ/mol. It also showed superior selectivity over N_2 and purity of collected CO_2, at 70,000 and 97%. This study also reported that amine-functionalized MOFs are not greatly affected by humidity as was previously worried (Choi *et al.,* 2012 and McDonald *et al.,* 2012), showing both en-Mg_2(dobpdc) and mmen-Mg_2(dobpdc) having near perfect performance after capturing dilute CO_2 from humid air, while Mg-MOF-74 suffered a substantial decrease, probably because water molecules bound to CUS and were difficult to remove. However, on temperature swing analysis it showed a 6% reduction in adsorption capacity after 5 cycles – it is unsure why this degeneration occurred and is important to address.

MOF tuning for maximum physiadsorption

More recently a method of DAC using MOFs has been suggested that has departed from methods up to that point which warrants some attention – tuning the pore size and other properties of MOFs to allow for maximum physisorption. There are some advantages to physisorption over the more common chemisorption pathways (Nugent *et al.*, 2013). Capture occurs via weak van der Waals bonds and so should have a lower isoteric heat of adsorption and therefore less energy-intensive regeneration, and since the use of amines is not necessary it avoids some other disadvantages, such as sorbent degeneration. Also preparation costs should be reduced, as activation of CUS and functionalization of amines is often performed at extreme temperatures. However they usually have lower adsorption capacities, which must be addressed if they are to become a viable future option for DAC.

CO_2 has a high polarizability, which has been exploited before in carbon dioxide capture technology, for example in zeolites (Morris *et al.*, 2010). The most promising MOF for DAC of this class so far is called SIFSIX-3-Cu (Shekhah *et al.*, 2014) and was optimised by contracting the pores to increase electrostatic van der Waals interaction between CO_2 and the SIFSIX pillars. Metal-organic structures with SIFSIX anions are often classified as metal-organic-materials (MOMs), the difference being they are not necessarily infinite crystal structures and may be supramolecular finite structures (Cook *et al.*, 2013) – however in the original paper reporting this material it was classified as an MOF, as well as in much of the literature discussing it (Huck *et al.*, 2014, Shekhah *et al.*, 2015). SIFSIX-3-Cu is also structurally and functionally similar to MOFs discussed so far, having one-dimensional pore channels in an infinite lattice, and therefore it can be considered an MOF. The material has hexavalent Cu centres, bound to pyrazine to form two-dimensional sheets which are pillared together with SiF_6^{2-} (the "SIFSIX" pillars) to form the 3-dimensional structure with square pore channels. The pore size here has been contracted to 3.5 Å from structurally similar predecessors by using short pyrazine linkers and Cu, which has a d^9 electronic configuration and undergoes Jahn-Teller distortion. F-CO_2 distance is short and four fluorine atoms point directly into the square channels, allowing for high charge density.

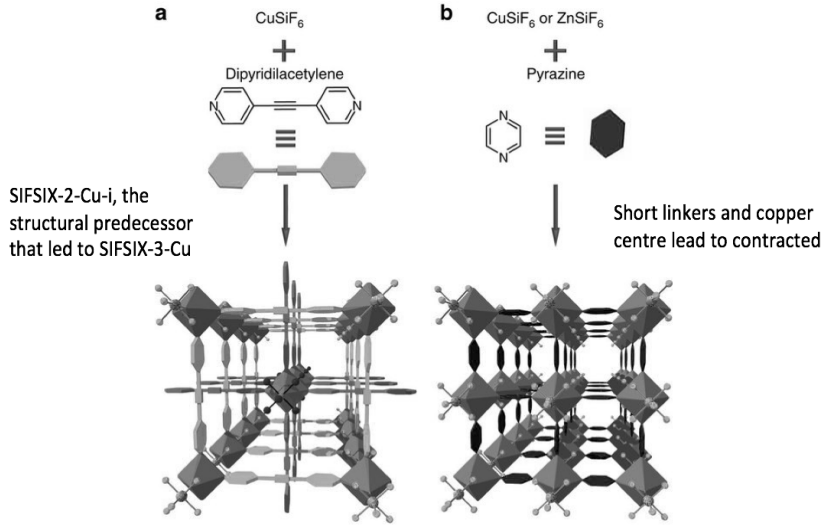

Figure 4. *SIFSIX-3-Cu and structural analogue. Adapted from Shekhah et al., 2014*

This MOF shows very good properties for adsorption. At 400 ppm CO_2 it had an adsorption capacity of 1.24 mmol/g, showed high selectivity of carbon over N_2 and CH_4, and was not affected by humidity. Although it has a reasonably high isoteric heat of adsorption at -54 kJ/mol which suggests energy intensive regeneration, desorption was easily done under the mild conditions of a vacuum at 323 K. Indeed, a recent review (Huck *et al.*, 2014) using parasitic energy as a metric showed this MOF as the second most promising material overall for DAC, lagging just being the top-performing silica-supported amine (although it is important to note that this review did not assess any alkylamine-functionalized MOFs). It is suggested that further work based on the same framework but with different metals will improve efficiency – this emerging method of DAC by MOFs looks like a promising alternative to amine-functionalized MOFs.

Conclusion

It is important to note that DAC is still unlikely to be implemented for use over other climate change mitigation strategies, such as alternative fuels and conventional CCS from anthropogenic point sources, as the cost will probably have to be higher to be equally effective (Socolow *et al.*, 2011). It will most likely be used for novel uses such as air purification in confined spaces and anaesthesia,

and possibly to decrease atmospheric levels of CO_2 if they become too high in the distant future. It is difficult to predict however as the area is still in its infancy, and technological advances may unexpectedly render it economical.

Technological advances so far have been impressive, particularly for MOFs. There is a good deal of further research necessary, however. First of all, all MOFs should be measured against each other and against other materials for DAC to see which is currently the most cost-effective and where further research should explore – a metric like parasitic energy used by Huck *et al*., (2014) would be suitable. There are some specific improvements necessary – the high regeneration energy and synthesis cost of alkylamine-functionalized MOFs need to be addressed, although improved performance is likely as different ligands are tested with different frameworks. The proposed mechanism helps guide this, predicting that changing the distance of neighbouring amines and changing the central metal may improve performance. Also, all materials using this method have been based on Mg-MOF-74, as it was the MOF which showed highest performance for post-combustion flue streams (Huck *et al*., 2014). However, trends of material efficiency often reverse for DAC (for example non-functionalized Mg-MOF-74 performs very poorly for DAC, with parasitic energies ten times higher than the best materials), so it is probable that another MOF not yet synthesised will perform better. For the physiadsorption method, fine-tuning the ligands and central metal again should improve performance – these materials need a higher working capacity to be viable. For both methods, future materials will probably not resemble current high-performing materials in terms of MOF used, but will function by the same principles and the research will rely on the lessons they offer to advance progress.

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Prebiotic Chemistry: Common Origins of Glycerol, Amino Acids, and Pyrimidines, and Cosmic Origin of Nature's Enantiomeric Excess of Amino Acids

Stephen Byrne
Senior Freshman
Science

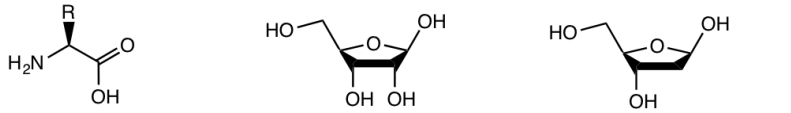
The origin of life on earth remains one of the greatest mysteries in scientific enquiry to date. Recent experiments support a model in which many fundamental biomolecules share a common chemical origin are a recent and major breakthrough in the field of prebiotic chemistry. The results of the experiments could direct researchers in the field to take a more systems chemistry approach. This is where complex mixtures of reactants and products are studied. It is opposed to the approach of assuming biomolecules originated individually from mutually incompatible syntheses. Though the model merely explains the potential origin of precursors and a fraction of amino acids, it does provide clues that could direct researches in the direction of a more encompassing model. Results of various experiments have pointed towards a plausible cosmic origin of the initial enantiomeric excess of amino acids on the prebiotic earth. A proposed cosmic model of the origin of enantiomeric excess of amino acids via the action of circularly-polarised light will be explored. The common origin of biomolecules, and the cosmic origin of enantiomeric excess have minor shortfalls. However, both are major developments in the field of prebiotic chemistry.

Introduction

The earth is approximately 4.55 x 10^9 years old (Patterson, 1956). Life originated nearly 3.8 x 10^9 years ago (Mojzsis *et al.,* 1996). Research into the origin of life seeks to discover what occurred in that first three quarters of a billion years that led to the emergence of cellular life. The physical and chemical conditions that existed on the prebiotic earth pose a dilemma for researchers. Researchers have to work with a multitude of different plausible physical and chemical conditions because the conditions on the early earth are not well understood. This makes it difficult to create a plausible model. A viable model must elucidate how fundamental biological molecules such as lipid bilayers, RNA/DNA, and amino acids originated. Additionally, the chronological order in which these fundamental components arose must be accounted for. Crucially, a successful model must be able to explain the transition from an abiotic world of simple racemic molecules, to the world where complex homochiral organic polymers compose cellular life.

Amino acids are quite easy to synthesise under prebiotically plausible conditions (Miller, 1953; Patel *et al.,* 2015). Ribonucleotides on the other hand are difficult to prepare (Orgel, 2004). The reaction of ribose with nucleobases is either inefficient or non-existent (Fuller *et al.,* 1972; Orgel, 2004). Lipids could have originated from complex metabolic pathways (Ourisson *et al.,* 2005), Lombard *et al.,* 2012). The major problem with all of these proposed chemical origins is that they are different. Patel *et al.,* (2015) have shown that this need not be the case and that these biomolecules may share a common chemical origin. Their findings link ribonucleotides, glycerol, and amino acids to a common chemical origin in what is referred to as a cyanosulfidic protometabolism.

Living things are chiral. Life is composed of polymers, and the monomers that compose those polymers are chiral. These monomers are enantiomerically pure. The origin of this "homochirality" is a mystery, and an important aspect prebiotic chemistry research. Amino acids are only found in the levorotatory form (Figure 1). Ribose and deoxyribose are only found in the dextrorotatory form (Figure 1). How such an asymmetry arose in our universe is perplexing. There are many plausible explanations for this imbalance. One model of the origin of nature's enantiomeric excess (ee) [ee = (R − S)/(R + S), where R and S are the concentrations of both enantiomers] posits a cosmic origin in which "chiral" photons induce an ee of amino acids. A model explaining the origin of a small initial ee has set the stage for models that could explain an enhanced ee via amplification processes analogous to natural selection (Klussmann *et al.,* 2006; Frank, 1953; Breslow *et al.,* 2006).



levorotatory amino acid dextrorotatory ribose dextrorotatory deoxyribose

Figure 1. *Schematic view homochiral biomolecules.*

Common Origin of Activated Pyrimidines, Amino Acids, and Glycerol

Patel *et al.,* (2015) have showed that various biomolecules could share a common chemical origin. This was merely a conceptual possibility, and had not been shown until now. (Miller, 1953), Oró's synthesis of nucelobases (Oró, 1960), and Butlerow's formose reaction (Butlerow, 1861). These syntheses are mutually incompatible. This proves problematic when trying to progress towards nucleotides, and a mix of nucleotides and α-amino acids. Assuming that these syntheses are how biomolecules arose on the prebiotic earth has led to many in the field operating under the assumption that one system preceded another. However, Patel *et al.,* (2015) presented syntheses of these biomolecules that are mutually compatible. They refer to it as a cyanosulfidic protometabolism, where lipid precursors (glycerol-1-phosphate), α-amino acids (e.g. glycine, threonine, valine), and ribonucleotides (β-ribocytidine-2′, 3′-cyclic phosphate, and uridine-2′, 3′-cyclic phosphate) all share a common chemical origin. Figure 2a shows part of the reaction network that relates the origin of the biomolecules mentioned. The chemistry of this network is based upon previous discoveries in the field (Powner *et al.,* 2009; Ritson *et al.,* 2012; Patel *et al.,* 2015). The network is a breakthrough in the field of prebiotic chemistry. It demonstrates the viability and feasibility of the hypothesis that biomonomers arose from mutually compatible syntheses.

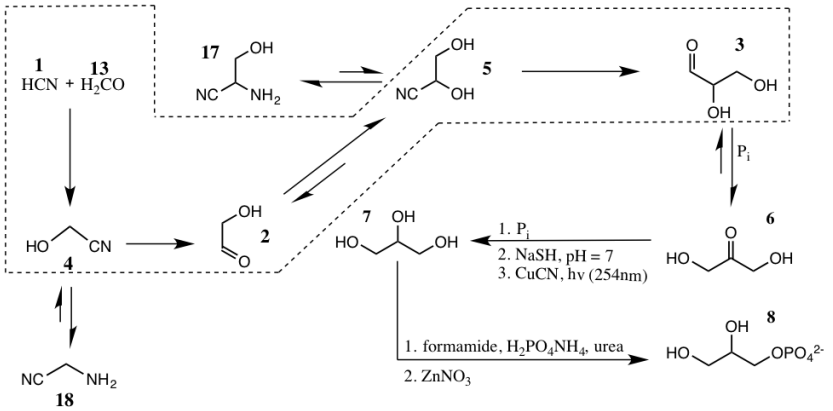


Figure 2a. *Schematic representation of cyanosulfidic protometabolism reaction network. .Pi (NaH2PO4). Source of Cu(I) for pathway in dashed lines: CuCN. Note: solvent is H2O in all reactions. Adapted from Patel et al., (2015)*

Part of the reaction network developed by Patel *et al.,* (2015) is shown in Figure 2a. A central feature of the network depicted in Figure 2a is the synthesis of glyceraldehyde **(3)** from the feedstock molecules **(1)** and **(13)** (dashed lines). **1** is thought to have formed on the early earth via the high temperature impact of carbonaceous meteorites with the earth (Kurosawa *et al.,* 2013). **1** and **3** have been detected on the LEMMON and ISON comets (Cordiner *et al.,* 2014). Analysis of the comet 67P/Churyumov-Gerasimenko via mass spectrometry by the Philae lander revealed the presence of H_2O and **1** (Goesmann *et al.,* 2015) The synthesis of 2-hydroxyaldehyde **(2)** and **3** proceeds by a Fischer-Kiliani-type homologation of **1** via Cu(I)-Cu(II) photochemistry to catalyse the reaction (Ritson *et al.,* 2013; Ritson *et al.,* 2014). The synthesis of **3** is a central feature because intermediates and the product of the pathway feed in to other pathways of the network. For instance, with hydrogen sulfide as a reducing agent 2-hydroxyacetonitrile **(4)** and 2,3-dihydroxypropanenitrile **(5)** are converted to the Strecker precursors of glycine **(18)** and serine **(17)**, respectively (Patel *et al.,* 2015). The Strecker precursors of threonine and alanine are produced as a consequence of the pathway to **3** (Patel *et al.,* 2015). The synthesis of **3** is the starting point for the preparation of glycerol **(7)**: depicted in Figure 2a. **3** interconverts to its more stable isomer dihydroxyacetone **(6)** (59% yield). **6** is then reduced to **7** (34% yield), which reacts with NaH_2PO_4 (Pi) to produce glycerol-1-phosphate **(8)** (31% yield) (Patel *et al.,* 2015). **8** is a lipid precursor. Cell membranes are the primary means of compartmentalization for organisms, and the major components of these cell membranes are esters and ethers of **8** (Lombard *et al.,* 2012). Most notably, intermediates of the synthetic pathway to **3** feed in to a synthetic route towards β-ribocytidine-2′,3′-cyclic

phosphate (9) and β-ribouridine-2′,3′-cyclic phosphate **(10)** (Powner *et al.,* 2009): Illustrated in Figure 2b. This pathway is a strong point of the model. It manages to integrate the prebiotically plausible synthetic pathway to activated pyrimidines developed by Powner *et al.,* (2009) by including **2**, **3**, and Pi (pathway in Figure 2a). **2** undergoes a condensation reaction with **12** to produce oxazol-2-amine **(11)**. **12** is also important because it undergoes hydrolysis to produce urea, which is incorporated into subsequent reactions. The final products are the activated pyrimidines **9** (96% yield), and **10.** UV irradiation of **9** resulted in a solution of an approximately 1:1 ratio of **10:9**. UV irradiation is prebiotically plausible because of the prebiotic earth's lack of O_3 to shield the earth's surface from UV radiation. As can be seen from the synthesis of **6, 7, 8,** and **9**, a major strength of the model is the relatively high yields of biologically relevant molecules, which makes this a kinetically feasible model.

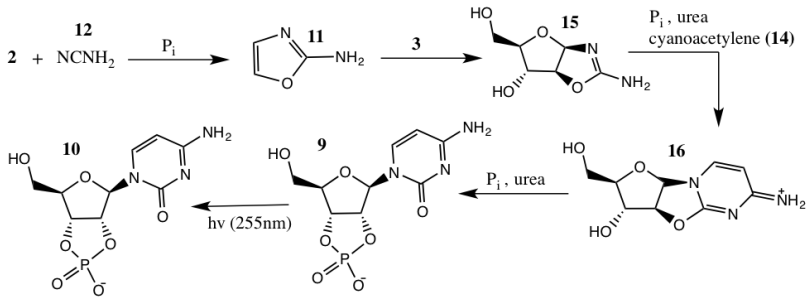


Figure 2b. *Prebiotically plausible synthetic pathway to activated pyrimidines. Adapted from Powner et al., (2009)*

The network requires different conditions for different reactions to progress. Patel *et al.,* (2015) proposed a geochemical model to account for this. It was suggested that different conditions could have existed along a river with tributaries and confluences. UV radiation from the sun could have allowed photochemical reactions to occur. A tributary could have provided isolation, while a confluence could have provided a way of mixing products of different reactions. The model is conceptually simple, but is difficult to recreate. Each synthesis was carried out step-wise with a lot of interference by the researches. Interference with experiments of this nature must be minimized (Bracher, 2015). The prebiotic earth produced life through geochemical processes, and experiments must mimic geochemical scenarios if they are to be successful in elucidating the origin of life. No precautions were taken to carry out the reactions under deoxygenated conditions. In the synthesis of **6** from **3** (50 mM), glycolate and formate were formed due to the presence of athmospheric O_2. These by-products were recognized and dismissed

as by-products that "would not be produced prebiotically because of the lack of oxygen in the atmosphere of the early Earth". These by-products along with O_2 could have influenced the outcome of the experiments. While it may be negligible, it would be good practice to repeat the experiments under deoxygenated conditions. The model is incomplete. It does not incorporate the purines and thymine, and various sulphur and aromatic amino acids e.g. cysteine and phenylalinine. This highlights the need to consider sulfur and aromatic reagents in future research. It is important to note that while a lot of the reaction network presented by Patel *et al.,* (2015) was not explored, the core aspects were emphasized i.e. the integration of various syntheses, the production of fundamental biomolecules, how it fits in to the geochemical and geophysical conditions of the prebiotic earth, and what could be done to improve the model.

Enantiomeric Excess of Amino Acids

Theories concerning the origin of homochirality can be separated into two categories: abiotic and biotic. A biotic origin of homochirality envisions a scenario in which homochirality comes about as a result of selection in early biological evolution. This idea is unlikely, as racemic biopolymers do not perform their function (Joyce *et al.,* 1984). This suggests that an abiotic origin of homochirality in biomolecules is more likely. Chemists have looked at meteorites in order to find a possible abiotic source of the enantiomeric excess (ee) of α-amino acids. Pizzarello *et al.,* (2000) showed that some α-amino acids in meteorites had an ee of (-)-amino acids of 1.0-9.2%. Of the amino acids that were found, valine and alanine were ubiquitous. Interestingly, both of these amino acids are included in the cyanosulfidic protometabolism (Patel *et al.,* 2015). It implies the possibility that the prebiotic earth's source of amino acids came at least in part from meteorites. Additionally, it implies that some sort of amplification process increased the ee, as the observed ee is too distant from homochirality.

A model of the cosmic origin of the ee of amino acids on earth is based on UV-circularly polarized light (UV-cpl). Interestingly, Pizarello *et al.,* (2000) suggest that the origin of the ee of valine and alanine on the Murchison meteorite was due to UV-CPL produced by a neutron star. Circularly polarized light is where the electric field vector describing the electromagnetic wave rotates clockwise (R-CPL) or anticlockwise (L-CPL) as it propagates through space from the perspective of the source. Experimental evidence shows that it is produced in space (Bailey *et al.,* 1998). There are three ways in which UV-cpl could have produced an ee in meteorites: photolysis, isomerization, or synthesis. Isomerization is where (R/L)-UV-cpl favors the conversion of one enantiomer in the racemate to its opposite. This is unlikely as there is little experimental evidence to support it (Inuoe *et al.,* 1996; Inuoe, 1992; Rau, 1983). Synthesis is where (R/L)-UV-cpl favors the production of one enantiomer over another. It has been shown that UV-cpl can induce asymmetry in products that are thought to be prebiotic gases (Takano *et al.,* 2007; Nuevo *et al.,* 2006; Marcellus *et al.,* 2007). The strongest argument rests with photolysis. This is the preferential destruction of one enantiomer induced by UV-

cpl. This was developed through quantum mechanics (Rosenfeld, 1929), which is beyond the scope of this review. Researchers have used this process for years as a way of achieving ee (Balavoine *et al.,* 1974; Nordén, 1977; Flores *et al.,* 1977; Nahon *et al.,* 2004; Meierhenrich *et al.,* 2005; Bailey *et al.,* 1998). Figure 3 depicts the results of an experiment by Flores *et al.,* (1977) that proves an ee of leucine can be produced via photolysis.

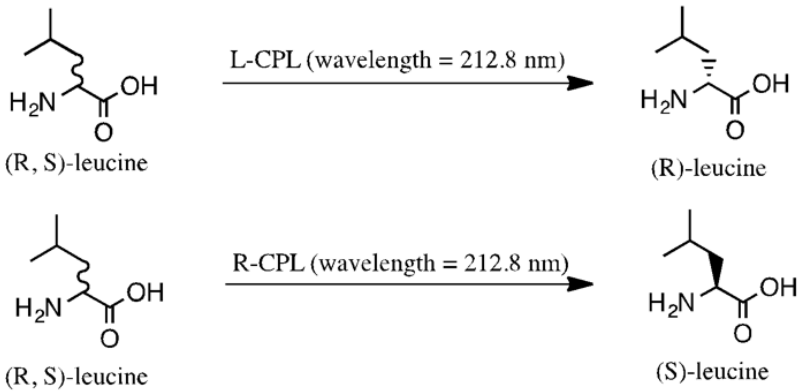


Figure 3. *Two racemates of leucine were treated with ultraviolet CPL (Right (R) or left (L)) When a racemate of leucine was treated with L-CPL an ee of (R)-leucine was produced. When another racemate was treated with R-CPL an ee of (S)-leucine was produced. Adapted from Flores et al., 1977*

The experiments of Pizzarello *et al.,* (2000), Bailey *et al.,* (1998), and Flores *et al.,* (1977) open up the possibility that the meteorites supplied the prebiotic earth with amino acids. One could argue that amino acids either came from meteorites or a synthesis of the sort proposed by Miller *et al.,* (1953) and Patel *et al.,* (2015). It is likely that it was both. Meteorites could have provided an initial ee, which combined with the already abundant amino acids from the synthesis proposed by Patel *et al.,* (2015). If UV-CPL did induce an ee in the prebiotic earth's source of amino acids, then the findings of Pizzarello *et al.,* (2000) suggest that the initial ee was only small. Life exhibits homochirality i.e. 100% ee. In order to explain this, an amplification process analogous to natural selection must be provoked (Klussmann *et al.,* 2006; Frank, 1953; Breslow & Levine 2006). This aspect of the problem is covered in a thorough review by Ruiz-Mirazo *et al.,* (2014). The methodology of Bailey *et al.,* (1998) and Pizzarello *et al.,* (2000) was sound. The only criticism is that the experiment carried out by Flores *et al.,* (1977) was conducted under conditions unlike the conditions present in a meteorite travelling in space.

To conclude that UV-cpl would produce such an ee under the conditions present on a meteorite would not be justified. To reach such a conclusion one would need to carry out the same experiment under conditions comparable to that of a meteorite travelling through the hostile environment of space. Additionally, only leucine was considered. The other amino acids were ignored. All 20 of the common amino acids should be considered in future experiments.

Conclusions

Patel *et al.,* (2015) have shown us that glycerol, various amino acids, and two activated pyrimidines are related through a network involving reagents such as cyanide, phosphate, and hydrogen sulphide. The model is a breakthrough and will likely guide future research. The geochemical scenario is prebiotically plausible. Subsequent research should aim to uncover a prebiotically plausible synthetic route to the purines, thymine, and the amino acids not included by Patel *et al.,* (2015). Future investigations should be carried out under deoxygenated conditions. More geochemical scenarios should be considered, and some effort needs to be made to experimentally justify the geochemical models. Regardless of its minor shortfalls, the cyanosulfidic protometabolism model is an important development in the field of prebiotic chemistry. Its novel syntheses and elaborate geochemical considerations make it a major development in the field. It is very likely to inspire future experiments, and bring us closer to a model of the origin of life on earth.

Pizarello *et al.,* (2000) have shown that ee can arise in abiotic environments. Bailey *et al.,* (1998) have shown a possible cosmic origin of nature's ee. Flores *et al.,* (1977) showed that UV-cpl can induce the enantioenrichment of a racemic leucine. These findings suggest that the earth's initial source of ee in amino acids could have been meteorites. This provides a possible starting point for subsequent research, which should aim to explain how a slight ee can be amplified under prebiotically plausible conditions. The findings of Flores *et al.,* (1977) may not be enough to conclude that UV-cpl produced the initial ee of amino acids supplied by meteorites to the earth. Only leucine was considered, more amino acids must be considered in future photolysis induced enantioenrichment experiments. The findings of Pizarello *et al.,* (2000) and Patel *et al.,* (2015) could lay the foundations for future experiments. These experiments should look to combine a racemic system with a system containing a small ee to produce a homochiral system analogous to life.

Prebiotic chemistry research has evolved a lot since Miller (1953) discovered that biomolecules could be produced under prebiotically plausible conditions. Patel *et al.,* (2015) have linked various biomolecules to a plausible common chemical origin. The discoveries of Pizzarello *et al.,* (2000), Flores *et al.,* (1977), and Bailey *et al.,* (1998) suggest that the earth's initial source ee could be from meteorites.

The links between the various fields of research in prebiotic chemistry are being elucidated as more discoveries are made. Once plausible routes to the synthesis of fundamental biomoleclues and their enantiopurification are uncovered, researchers will be equipped to explain the transition into the "RNA world". This would be a considerable step towards explaining the origin of life on earth.

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Aptamers: An emerging class of affinity reagents

Daniel Fortunati
Junior Sophister
Biochemistry

Aptamers are short nucleic acid oligomers selected from a random, chemically synthesised oligonucleotide library that are able to bind with very high affinity and specificity a wide range of molecular targets through a process called "Systematic evolution of ligands by exponential enrichment"(SELEX). Their binding capabilities are comparable to those of antibodies, making them a valuable alternative to the use of protein-based affinity reagents. Aptamers exhibit a variety of desirable characteristics as affinity reagents, but their main advantages over antibodies are their chemical synthesis (relatively easy, cheaper and exhibiting little batch to batch variation), their stability (which translates in longer shelf-life and less stringent storing conditions) and the potentially limitless amount of targets aptamers can be selected for. Despite this, some issues with both the isolation platform and the pharmacokinetic properties of aptamers exist, and only one aptamer-based drug has currently been approved for clinical use. The dawn of a variation in the aptamer isolation procedure called cell-SELEX is opening exciting new possibilities for therapeutic and diagnostic uses of aptamers.

Introduction

What are aptamers?

When one thinks of nucleic acids and their functions in a living cell, the first thing that comes to mind is their role (central to life) as carriers of heritable information that codes for proteins. However, this is not the whole story. Over two decades ago,

another interesting (and, as it turns out, extremely useful) property of nucleic acids was discovered: Nucleic acid molecules can fold into complex three-dimensional shapes that are capable of binding with very high affinity and specificity to a vast variety of molecular targets. This was first observed thanks to the development of an in vitro selection procedure called "Systematic Evolution of Ligands by Exponential enrichment", or SELEX (Tuerk and Gold, 1990, Ellington and Szostak, 1990). This essentially consists in the incubation of a chemically synthesised library of short, single stranded oligonucleotides (either ssDNA or ssRNA) with a target molecule and collecting those nucleic acid molecules that bind to the target. The binding oligonucleotides are then amplified (typically through PCR or RT-PCR) and the whole procedure is repeated on the new, enriched library obtained. Through the iteration of these steps eventually only ligands that bind very tightly and with a high degree of specificity remain (Steps are further explained in Figure 1) (Darmostuk *et al*., 2015). Oligonucleotide molecules obtained through this method are termed "aptamers" from the Latin "*aptus*" which means "to fit" and "*merus*", which means "particle". With binding constants (K_D) usually in the low nanomolar or high picomolar range (Keefe *et al*., 2010), aptamers exhibit binding properties comparable to those of antibodies and their potential as diagnostic, therapeutic and analytical tools was immediately recognized upon their discovery.

Aptamers and other affinity reagents

Antibodies are currently one of the most important and most widely used tools in the biomedical sciences, utilized for a wide range of applications for which specific molecular recognition is required. However, the isolation and production of novel antibodies is a difficult and expensive process, with some inherent limits due to their animal origins and chemical properties: Antibodies have a short shelf life, are hard to mass-produce and often give rise to immune reactions *in vivo*. Furthermore, as the antibody isolation procedure starts from an animal, antibodies capable of binding to molecules toxic or harmful to animals are difficult to isolate. This is also true for targets that exhibit intrinsically low immunogenicity (Jayasena, 1999). Another emerging issue with commercially available antibodies is that a significant amount of these (up to 51% according to a recent study (Berglund *et al*., 2008)) exhibit either non-specific binding or lack the ability to recognize the target altogether (Groff *et al*., 2015). On the other hand, aptamers are quicker and relatively cheaper to isolate, very stable chemically and can be readily modified through medicinal chemistry techniques. Their chemical stability, coupled with their *ex vivo* isolation procedure means that aptamers can be engineered to bind potentially any molecule over a much wider range of conditions than those accessible to antibodies. Because of these desirable characteristics, aptamers capable of binding many different molecular targets have already been isolated and tested. These range from proteins (Savory *et al*., 2010, Wang *et al*., 1993), low molecular weight metabolites (Miyachi *et al*., 2009), sugars (Boese and Breaker, 2007) and even a variety of different cell types (Li *et al*., 2014, Shangguan *et al*., 2006, Homann and Goringer, 1999).

Limitations exist

This being said, only one aptamer-based drug (pegaptanib, a vascular endothelial growth factor targeting drug developed for the treatment of age-related macular degeneration, the oligonucleotide was conjugated to polyethylene glycol to avoid renal filtration) has been approved by the FDA since aptamer discovery (Gragoudas *et al*., 2004, Ruckman *et al*., 1998). This because there are still some difficulties associated with the use of aptamers that need to be addressed: As aptamers are essentially low-molecular weight oligonucleotides, they are subject to renal filtration and degradation by serum nucleases, which means they have a short circulating half-life.

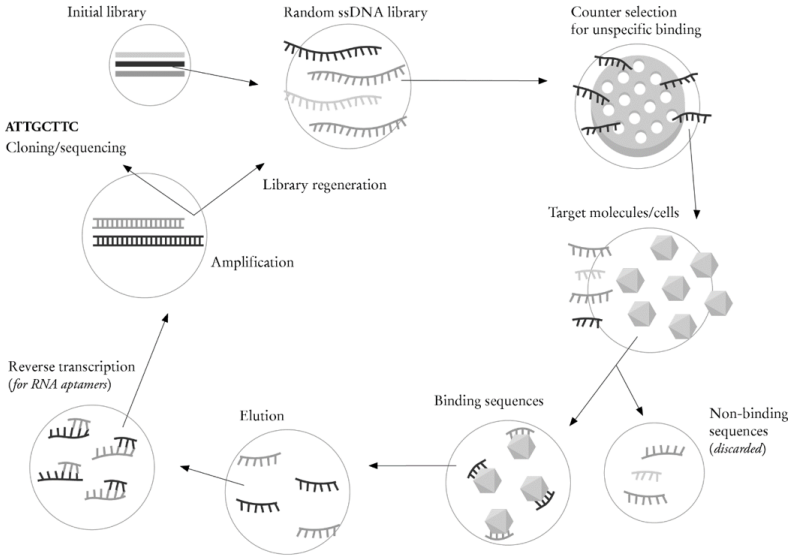

Figure 1. ***Schematic representation of the classic SELEX procedure***. *Often a counter-selection step is added (this step is especially necessary in some variants of the SELEX procedure, such as cell-SELEX), which is needed to ensure that the aptamers bind only to the target molecule. It consists in the opposite of the positive selection step, aptamers demonstrating non-specific binding towards molecules other than the target (what molecules are used for the negative selection step depends on the prospective use of the aptamer) are discarded, while unbound aptamers are collected. The rest of the steps are as illustrated: An oligonucleotide library is incubated with a target molecule, unbound aptamers are discarded, while oligonucleotides exhibiting selective binding properties towards the target molecule are isolated and amplified, in order to produce a new, enriched library for the next round of selection. Iteration of these step produces aptamers binding with the desired affinity and specificity, which are then cloned and sequenced. Adapted from: Darmostuk et al., 2015*

There are also some issues with methods for aptamer isolation, such as the use of PCR during the selection process, which is inefficient for the task of amplifying an extremely diverse library of different DNA templates, as it is biased towards certain sequences rather than others, which in turn can unnecessarily lengthen the SELEX procedure (Rozenblum *et al*., 2015). The focus of this review will be describe the main features of aptamers and outline the current prospects and challenges in their use as affinity reagents, comparing them with antibodies and highlighting pros and cons of both.

Structural features

Binding mechanisms

Aptamers are short nucleic acid molecules selected from an initial nucleic acid library typically consisting of oligonucleotides containing a random sequence of about 20-100 bases in length, with constant regions at the 3′ and 5′ ends needed to manipulate them enzymatically. A library will generally contain between 10^{13} and 10^{15} different individual sequences. For other nucleic acid-based therapeutic agents (such as antisense oligonucleotides and short interfering RNAs) the effect of the compound depends directly on the oligonucleotide sequence, as it must interact with other nucleic acids through classical Watson-Crick base pairing (Wagner, 1994, de Fougerolles *et al*., 2007). The mechanism of action of aptamers, instead, depends directly on their 3D structure and thus the aptamer's ability to bind with the target molecule through a combination of Van der Waals interactions, hydrogen bonding, classical base pairing mechanisms and electrostatic interactions (Rozenblum *et al*., 2015). This means that while other nucleic acid based therapies must necessarily act intracellularly, aptamers can act intracellularly, extracellularly or upon cell-surface receptors. Various common structural motifs involved in the binding of aptamers to specific ligands have been identified, including a variety of loops, stems, hairpins and some more specific structures like the pseudoknot and the G-quadruplex (Radom *et al*., 2013) (Figure 2). It is interesting to note that, although aptamers are synthetic molecules that were first isolated through combinatorial chemistry methods, oligonucleotides that function in a similar manner also exist in nature. Untranslated mRNA regions commonly found in eubacteria and termed riboswitches have been shown to regulate gene expression through their binding to metabolites. As the process does not require protein factors, riboswitches work essentially as natural aptamers (Tucker and Breaker, 2005).

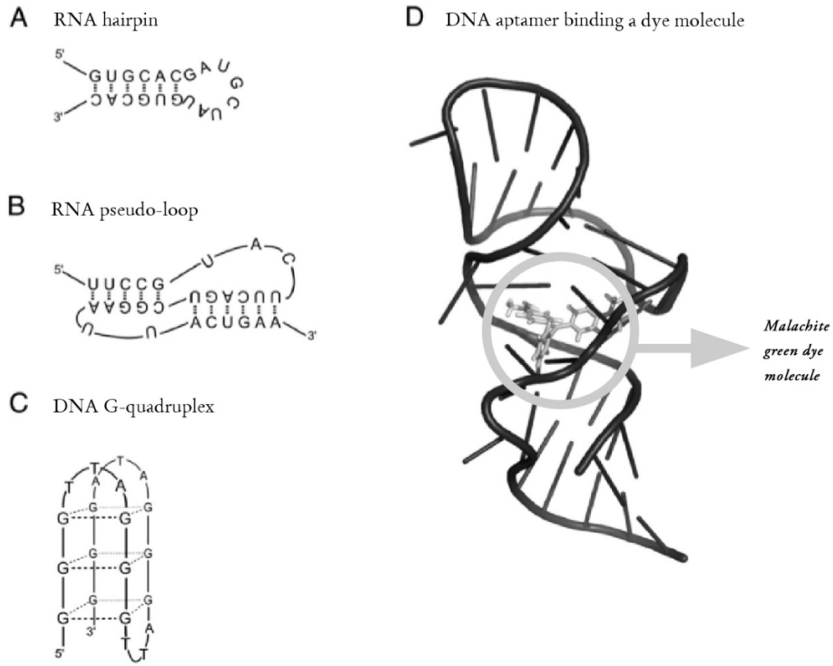

Figure 2. ***Some common structural motifs involved in the aptamer-ligand binding process.*** *It can easily be seen that single stranded nucleic acid structures fold following the same base-pairing rules which determine the structure of double stranded oligonucleotides, with the addition of some unconventional base-pairing. Typical RNA hairpin* ***(A)****, an RNA pseudo-knot* ***(B)****, and a more exotic DNA G-quadruplex* ***(C)*** *are shown.* ***(D)*** *Is a computer generated three-dimensional model of an aptamer binding its ligand (in this case malachite green), showing how the aptamer folds and snugly "fits" around the ligand (this if a small molecule such as a dye is the target, if the ligand is a large macromolecule like a protein, the aptamer may fit inside it's active site). Adapted from: Radom et al, 2013*

A wide range of structures is available.

Nucleic acid libraries can provide an immense amount of diversity, a typical 25 residue nucleic acid sequence made up by A, C, G and T (U if RNA is being used) will in fact give rise to 4^{25} possible combinations which means about $\approx 1.12 \times 10^{15}$ unique sequences, producing a vast array of different three dimensional structures. This means there is an astounding number of different structures available for selection during a typical SELEX experiment, more, in fact, than those of any other known combinatorial library (Gloekler *et al*., 2010).

Aptamers and antibodies

Monoclonal antibodies and aptamers

Monoclonal antibodies have, since their discovery (Kohler and Milstein, 2005), revolutionized research methods used in the biological sciences. Nucleic acid aptamers exhibit similar binding affinities and specificities to antibody based affinity reagents. Aptamers also have a variety of desirable qualities that make them an interesting alternative to antibodies for analytical, diagnostic and therapeutic applications. Possibly the most important advantage of aptamers over antibodies is that they are synthetized chemically, eliminating the use of animals from the isolation and production steps (Tombelli *et al*., 2005). This greatly extends the variety of targetable molecules, as animal-based antibodies cannot be isolated against toxic compounds or molecules with a very low native immunogenicity. Antibodies also work only at physiological conditions, while conditions in which aptamers are capable of binding can be modified and tweaked by changing SELEX parameters (This may be useful if an aptamer for an *ex vivo* assay is required). It must be noted though that the use of recombinant antibodies can also partly overcome these limitations, as animals are not directly involved in their production (Groff *et al*., 2015). Another advantage is that aptamer isolation through SELEX is cheaper and faster than monoclonal antibody isolation, and bulk production of aptamers can be achieved with relative ease through solid phase phosphoramidite chemistry (Caruthers, 1985) . Further limitations of antibodies are their short shelf-life (and careful storage is required, as they are susceptible to irreversible denaturation if measures are not taken to avoid this). High batch-to-batch variability in efficiency is another major limitation, as recent data suggests that about half of antibodies commercially available may not be capable of actually recognizing their targets with the required degree of affinity and specificity (Groff *et al*., 2015) . Aptamers can overcome these, as they are very stable molecules and can thus be stored with little precautions for long periods of time. Furthermore, as they are produced through chemical synthesis, there is virtually no batch-to-batch variation associated with aptamer production. The nucleic acid nature of aptamers also makes them relatively straightforward to modify through medicinal chemistry techniques, making conjugation to drugs and reporter molecules, as well as pharmacokinetic tailoring, possible. Interestingly, developing an antidote for a specific aptamer also appears to be very straightforward, as oligonucleotides with a complementary sequence to the aptamer have been shown to inhibit it's effects (Rusconi *et al*., 2002). Antibody modifications (such as humanisation to reduce immunogenicity, site specific modifications to enhance pharmacokinetics or antibody-drug conjugation) are instead non-trivial, require the investment of substantial time and effort and have some inherent limitations to the modifications achievable (Keefe *et al*., 2010). More specifically to *in vivo* therapeutic and diagnostic applications, antibodies are inherently immunogenic molecules, and the risk of adverse effects is always present, while aptamers have in general shown low or no immunogenicity (Rusconi *et al*., 2000, Martin *et al*., 2002). It must be noted

though that adverse effects observed during clinical trials of REG1, an aptamer based drug targeting coagulation Factor IXa as a reversible anticoagulation system, suggest that low immunogenicity may not be an intrinsic property of all aptamers (Rusconi *et al*., 2002, Rozenblum *et al*., 2015). Finally, the use of cell-SELEX offers the opportunity of creating aptamers for molecular targets of unknown structure by selecting the aptamers against whole cells rather than highly purified molecules, which may also aid in the identification of novel biomarkers.

Limitations of aptamers and possible solutions

Renal filtration

For all their desirable properties as affinity reagents, aptamers also have some limitations, especially for *in vivo* applications. Due to their small size, aptamers are subject to renal filtration, which greatly reduces their circulating half-life. Rapid degradation by serum nucleases is also a problem, further reducing their availability in the bloodstream. Renal filtration can be efficiently avoided through conjugation with molecules of high molecular mass such as polyethylene glycol (the effects of PEG-conjugations are further illustrated in Figure 2) with aptamer circulating half-lives going from 5-10 minutes for unconjugated oligonucleotides up to a day for PEG-conjugated molecules (Healy *et al*., 2004, Boomer *et al*., 2005). Cholesterol can also be used for the same purpose, although it appears to be less effective then PEG (Keefe *et al*., 2010).

Nuclease attack

It is possible to reduce aptamer attack from serum nucleases by pre- or post-SELEX base modification. This is generally done through the addition of a variety of functional groups to the nucleotide bases (mostly on the pyrimidine bases, preferred target of serum nucleases), capping of oligonucleotide termini and the use of purine rich aptamers (Lapa *et al*., 2015). Further chemical base modifications can also enhance the aptamer's binding parameters (some functional group additions to oligonucleotides are shown in Figure 3). Other, more exotic methods are also being investigated, such as the use of spiegelmers, aptamers in which the sugars are the opposite enantiomer of the wild-type sugar (so RNA spiegelmers, for example, would be made using L-ribose rather than D-ribose), but there are some additional synthetic difficulties associated with these (Bilik *et al*., 2007).

Unknown properties, patenting and funding allocation.

Further problems are that the pharmacokinetic properties of a specific aptamer are hard to predict in advance, although they can be somewhat tailored during the SELEX process or through medicinal chemistry techniques. The extensive

patenting of SELEX technology is also a discouraging element, and may very well be the main reason why many companies choose to allocate financial resources towards the development of other types of affinity reagents, as the intellectual property of most monoclonal antibodies has instead expired by now (Keefe *et al*., 2010). This, coupled to the relative inefficiency of the SELEX procedure deriving from PCR bias may also be slowing down the development of aptamer technology (Rozenblum *et al*., 2015).

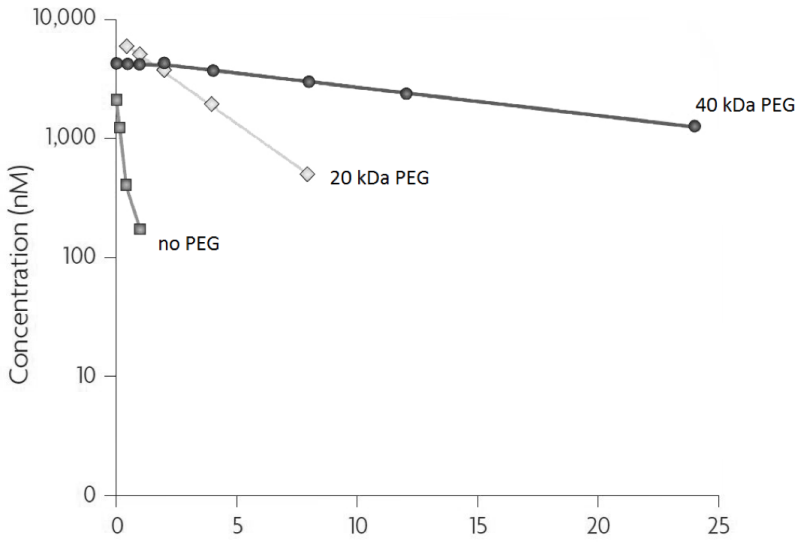

Figure 3. ***Plot illustrating the dramatic increase of the circulating half-life of Polyethylene glycol-conjugated aptamers when compared to non-conjugated oligonucleotides.*** *The aptamers used in this experiment are composed by 39 residues of 2'deoxypurines and 2'-O- methyl pyrimidines, conjugated (20 kDa PEG or 40 kDa PEG) and were injected in CD-1 mice (triplicate samples) in a concentration of 10 mg kg^{-1}. Adapted from: Keefe et al, 2010*

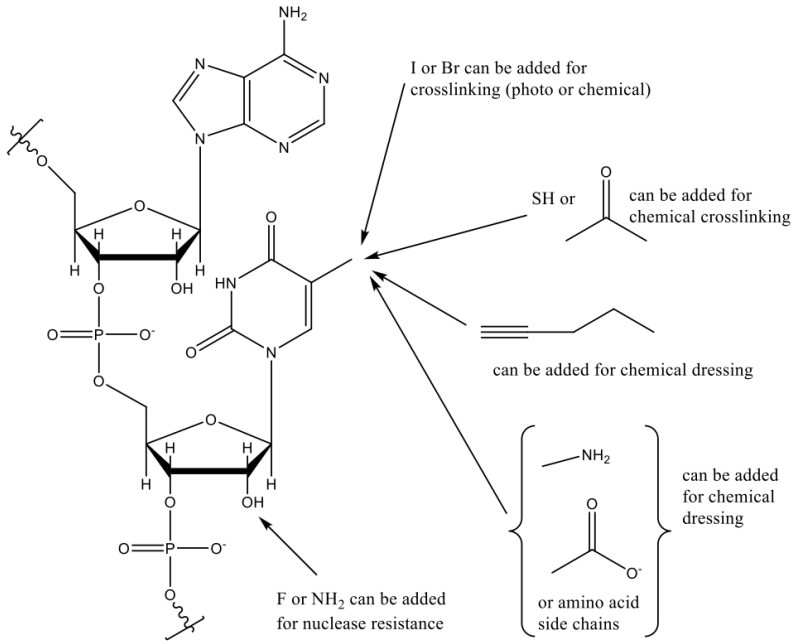

Figure 4. ***Summary of some common chemical modifications commonly carried out on nucleic acid aptamers, used to produce modified oligonucleotide libraries for the SELEX procedure.*** *Functional groups added to the 2′ sugar carbon are important for nuclease resistance, while additions made on the 5′ pyrimidine carbon can enhance binding of the oligonucleotide to its target or help crosslinking to it. Adapted from: Jayasena, 1999*

Conclusions

Great therapeutic and diagnostic potential

Since the possibility of cell-SELEX (a SELEX procedure in which whole live cells are used as targets for selection) was theorized (Vant-Hull *et al*., 1998) and subsequently proven possible through the isolation of aptamers targeting red blood cell ghosts (Morris *et al*., 1998), a plethora of SELEX experiments against targets of therapeutic interest has arisen. Just to cite a few notable examples, aptamers for pancreatic cancer cells (Champanhac *et al*., 2015, Li *et al*., 2014), prostate cancer cells (Kim *et al*., 2010), metastatic colorectal cancer cells (Li *et al*., 2014) and even live eukaryotic parasites (Goeringer, 2012) have been successfully isolated, demonstrating their therapeutic potential either by exhibiting intrinsic cytotoxic capabilities or through their use as vectors for cell-specific drug delivery. Cell-

SELEX, coupled with aptamer chemical modification techniques, has also caused a bloom of research into aptamer-based diagnostics, for example in the detection of circulating tumour cell and cancer biomarkers (Sun *et al*., 2015). As exemplified by these studies, aptamers show great potential as affinity reagents, possibly even surpassing antibodies: They are cheap, easy to store and readily produced on an industrial scale. More importantly, aptamers can go where antibodies cannot, with a much wider range of molecular targets available for selection.

Issues must be solved

But for aptamers to unleash their full potential as affinity reagents, some steps have yet to be taken: Synthetic methods for generating and producing aptamers need to be improved, as, although cheaper than antibodies to produce, they are still more expensive than classic small molecule pharmaceuticals due to their large size and complexity. To this end a variety of "one-round" SELEX procedures are currently under scrutiny (Darmostuk *et al*., 2015). Their pharmacokinetic properties also need to be extensively studied, as the current lack of knowledge in that respect greatly lengthens development times and costs. If these conditions are met, and unless unforeseen undesirable properties of aptamers are discovered, their use as therapeutic and diagnostic agents should flourish in the following years, hopefully fulfilling and exceeding expectations.

Acknowledgements

This work was adapted from a review: "Aptamers as diagnostic and therapeutic tools: Future prospects and challenges" for the course BI3020 (Research skills, academic year 2015/16). The author would like to thank Prof. Vincent Kelly for helpful appraisals and Ms. Laura Belli for assistance in image design.

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4-aminoquinolines as Antimalarial Drugs

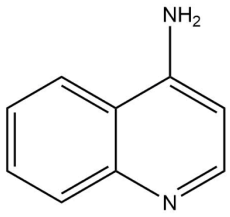
Dylan Lynch
Junior Sophister
Medicinal Chemistry

Malaria is a widespread parasitic infection with high mortality rates and a distribution which happens to correlate with the distribution of poverty. The class of drugs known as the 4-aminoquinolines has shown promise in treating malaria, especially infection by the Plasmodium falciparum parasite. While drugs such as chloroquine heralded the start of 4-aminoquinoline prophylaxis, resistance soon developed and became widespread. By investigating the structure-activity relationships of chloroquine, it was possible for medicinal chemists to devise novel compounds such as amodiaquine and isoquine for treatment of chloroquine-resistant strains of the parasite. These structural alterations ranged from compounds which could undergo bioactivation into toxic metabolites, to fluorinated alternatives and structural isomers. The present review aims to discuss the chemical investigation into chloroquine and its alternatives, and to examine the prospects of future antimalarial drugs of the 4-aminoquinoline class.

Introduction

Malaria is a blood-borne parasitic infection transmitted through the bite of a female mosquito of the genus *Anopheles*. There are around 30 species in this genus which act as malarial vectors, and around 3.2 billion people are at risk of this life-threatening disease (WHO, 2015). According to the WHO Malaria Fact Sheet (2015), 97 countries had ongoing malaria transmission in 2015 and between January and September 2015 there were an estimated 214 million clinical cases of malaria and over 400,000 deaths attributed to the disease. Malaria is caused by

protozoan (unicellular eukaryotic organisms) parasites of the genus *Plasmodium*, of which there are 5 species which infect humans (WHO, 2015) – *P. vivax, P. ovale, P. malariae, P. falciparum* and *P. knowlesi* (however *P. knowlesi* rarely causes disease in humans and only zoonotic transmission from monkey to human is known (Singh *et al.,* 2016). *P. falciparum*-induced malaria is widely regarded as one of the most dangerous forms of malaria, as it is most prevalent and cause the most deaths globally (WHO, 2015).



4-aminoquinoline

Figure 1. *The structure of 4-aminoquinoline, of which 4-aminoquinoline antimalarial are based.*

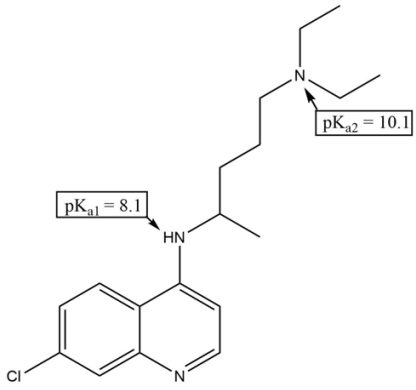
In 1934 the drug chloroquine was synthesised for the first time (Cook & Zumla, 2009), which became first in class of the quinine-based drugs termed the 4-aminoquinolines. The structure of 4-aminoquinoline (Figure 1) is based on the natural crystalline alkaloid quinine, found in Cinchona bark. While chloroquine was not the first synthetic antimalarial, it surpassed early compounds created by the company Bayer – such as mepacrine – and became a cost effective solution to falciparum malaria (that is, caused by *P. falciparum*). Clinical mepacrine use was discontinued due to reports of patients developing severe aplastic anaemia, psychotic reactions and exfoliative dermatitis. There were also minor adverse effects recorded, including yellow skin pigmentation (Peto, 1989). As mepacrine was declining in usage, chloroquine became available clinically, and this may have contributed to its widespread use. Resistance to chloroquine arose in four separate locations where falciparum malaria is endemic including the Thai-Cambodian border in the late 1950s (Harinasuta *et al.,* 1965), in Venezuela and Colombia strains of resistant *P. falciparum* were identified in 1960 (Moore & Lanier, 1961) and clinical cases of chloroquine resistance were detected in Papua New Guinea in the late 1970s (Grimmond *et al.,* 1976). Between 1978 and 1988, resistance to chloroquine was reported in all African countries, yet it remained as the first-choice treatment in many African countries into the early 2000s (Trape ,2001). As no effective malarial vaccines are yet available, health services rely on drugs for treatment and prophylaxis, however efficacy of antimalarial compounds has plummeted due to emerging *Plasmodium* resistance towards many different drug classes including first-line treatments such as chloroquine (Severini & Menegon, 2015). Drugs such as amodiaquine were developed, however they displayed adverse side effects and were redesigned as fluorinated derivatives.

Later, other 4-aminoquinolines such as isoquine were also synthesised. These compounds and their design are discussed in the main body of this review.

The current accepted mechanism of 4-aminoquinolines involves haemoglobin degradation by the *P. falciparum* parasite. During the red blood cell stage of *P. falciparum* infection (between the trophozoite and mature trophozoite stages), the parasite digests the haemoglobin of the human host as a source of amino acids through specialized processes (Elliott *et al.,* 2008). This degradation of haemoglobin releases the iron protoporphyrin "heme" as a by-product, which is toxic to the parasite – it can inhibit enzymes such as proteases and DNA polymerases and also damage the protozoan's membranes via lipid peroxidation (Aft & Mueller, 1983; Schmitt *et al.,* 1993; Vincent 1989; Orjih AU et. al, 1981). The *P. falciparum* parasite utilises a detoxification technique in which the monomeric heme (α-hematin) is converted into the crystalline polymer hemazoin (otherwise known as β-hematin), which is non-toxic to the parasite (Slater & Cerami, 1992). It is thought that this is where 4-aminoquinolines exert their activity, and this will be examined in the case of chloroquine, amodiaquine and isoquine.

Synthetic 4-aminoquinolines

Chloroquine



Chloroquine
N^4-(7-chloroquinolin-4-yl)-N^1,N^1-diethylpentane-1,4-diamine

Figure 2. *The structure of Chloroquine. Adapted from Vandekerckhove & D'hooghe (2015).*

Chloroquine was the first synthetic antimalarial drug and the first-in-class of the 4-aminquinolines, and it was synthesised in 1934 by chemists working for the German company Bayer (Cook & Zumla, 2009). The current predicted mechanism

of chloroquine is that the slightly basic chloroquine (pKa1 = 8.1, pKa2 = 10.1) (Olliaro 2001) accumulates down the pH gradient into the acidic digestive vacuole of the *P. falciparum* parasite, where the aforementioned haemoglobin digestion occurs. While the environment outside of the digestive vacuole is at physiological pH (pH = 7.4), the inside of the digestive vacuole has pH = 5.5 (O'Neill *et al.,* 2012). In this acidic environment the chloroquine structure become chloroquine^{2+}, and cannot readily diffuse back out of the digestive vacuole and instead begins to accumulate (Mushtaque & Shahjahan, 2015). In the digestive vacuole, chloroquine^{2+} inhibits heme crystallisation to hemazoin by intercalating between the polymeric crystal packing and forms a dimeric hematin complex through π-π stacking interactions with the heme protoporphyrin ring (Shelnutt, 1983; White, 1978). This form of heme-drug complexation therapy results in the steady increase in heme concentration within the acidic digestive vacuole, which is toxic to the parasite and will eventually lead to death of the *P. falciparum* (Hempelmann, 2007).

Regarding the structure-activity relationships (SAR), the terminal amino group was identified as essential for accumulation, as this is one of the sites where protonation in the digestive vacuole occurs. The two aromatic rings of the quinoline moiety also proved useful for the π-π stacking interactions and presence of a chlorine atom at the 7-position of the quinoline also correlates with higher antimalarial activity (O'Neill *et al.,* 2012; Mushtaque & Shahjahan, 2015). Based on these functionalities, Koh *et al.,* (1994) proposed a structure for the chloroquine binding sites on heme, which is shown in Figure 3. Heme is also known as α-hematin or ferriprotoporphyrin IX. The carbon chain between the tertiary amine and secondary amine is referred to as the 'linker'.

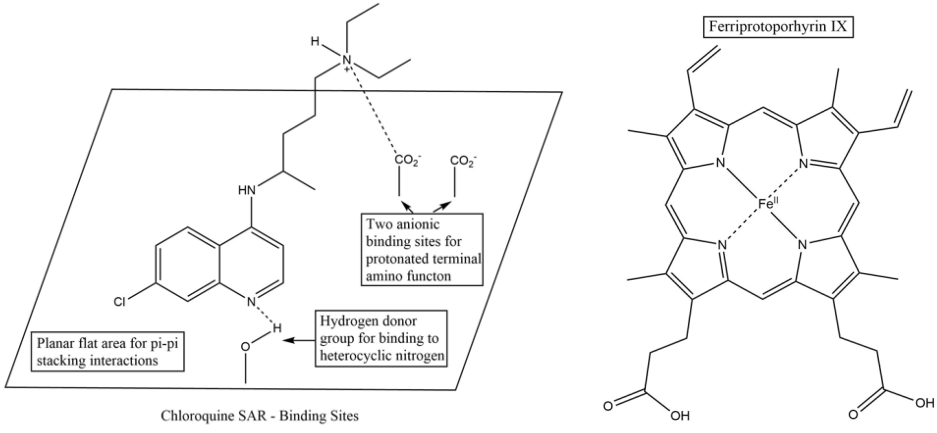


Figure 3. *Binding of chloroquine to the key features of the proposed receptor sites (top) in ferriprotoporphyrin IX (bottom). Adapted from O'Neill et al., (1997) and Koh et al., (1994)*

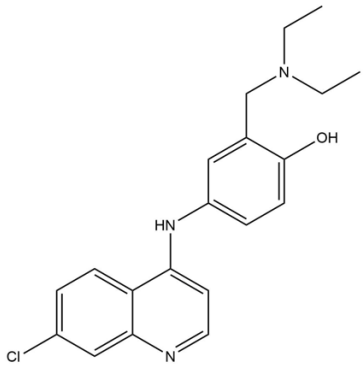
In the paper published by Koh *et al.* (1994), it was initially proposed that the central iron atom of ferriprotoporphyrin IX can bind to the aromatic quinoline nitrogen, and that the carboxylates could bind to the proton of the protonated nitrogen atom belonging to the 4-aminquinoline side chain. It should be noted however that Koh only accounted for the protonation of the terminal amino group and not the quinoline nitrogen, and so while the original binding model is reasonably accurate otherwise, it fails to account for any interactions the protonated heterocyclic nitrogen may have. This was highlighted in a publication by O'Neill *et al.* (1997). In this publication, O'Neill *et al.* (1997) proposed that the receptor proposed by Koh *et al.* was in fact ferriprotoporphyrin IX since it contains a planar flat region of 30 – 40 Å to interact with the aromatic quinoline moiety, two negatively charged carboxylates to bind to the protonated nitrogen of the terminal amino group of the alkyl side chain, and a central iron atom which can act as an acceptor site for the quinoline nitrogen. The distance between the quinoline nitrogen and the alkyl chain nitrogen was calculated as 8.30 Å (monoprotonated), which is quite similar to distances between the central iron atoms and the carboxylate oxygens in heme (calculated as 8.20 Å, analysed via X-ray crystallography) (O'Neill *et al.,* 1997).

Unfortunately, resistance to chloroquine gradually arose and has now become a major health concern for the developing world. As mentioned in the introductory section of this review, chloroquine resistance is now widespread everywhere that malaria is endemic. As the mechanism of action of parasite inhibition has still not been definitively proved, the mechanism of resistance is also debated. Chloroquine-sensitive parasites appear to accumulate much more of the drug in the digestive vacuole than resistant strains (Yayon *et al.,* 1984; Saliba *et al.,* 1998; Fitch, 1970). Research has identified point mutations in the gene encoding for *P. falciparum* chloroquine-resistance transporter (PfCRT) protein as a cause for the reduced drug accumulation (Bray *et al.,* 2005). A recent report (Chinappi *et al.,* 2010) stated that the PfCRT is found in the digestive vacuole and contains 10 predicted membrane-spanning domains (Cooper *et al.,* 2007). Furthermore, isolates exhibiting the chloroquine-resistant prototype not only carry the PfCRT point mutation but a charge-loss mutation K76T, often presented as two single mutations (K76N and K76I), which overall will affect the accumulation in the parasite. (Huaman *et al.,* 2004; Cooper *et al.,* 2005; Fidock *et al.,* 2000; Cooper *et al.,* 2002) The debate as to whether the point mutation in the gene encoding for PfCRT is the mechanism of chloroquine resistance in P. falciparum is ongoing, and the empirical data can be interpreted in numerous different ways. Several research groups are still devising novel approaches to pinpointing the mechanism of chloroquine resistance, yet there is no solid undisputed answer yet (Chinappi *et al.,* 2010).

Interestingly, long before PfCRT had been identified, it was observed that in some cases increasing or decreasing the length of the alkyl side chain could overcome the unknown resistance mechanism (Bray & Ward, 1998). This led some research groups to begin altering the properties of the N-alkyl amino side chain and later, the aromatic rings of the quinoline structure, which spurred on the discovery of new 4-amioquinolines.

Amodiaquine

The spread of chloroquine resistance acted as a catalyst for new research into the pharmacology and development of novel 4-aminoquinoline antimalarials. The structure of one of these compounds, amodiaquine, is shown as Figure 4. Amodiaquine contains many of the same structural features as chloroquine with the primary structural difference between them being the presence of a phenol group in the alkyl side chain.



Amodiaquine
4-((7-chloroquinolin-4-yl)amino)-2-((diethylamino)methyl)phenol

Figure 4. *The structure of amodiaquine. Adapted from O'Neill et al., (2003).*

Amodiaquine was predicted to exhibit many of the same structural interactions as those detailed for chloroquine, with the planar quinoline system engaging in π-π stacking interactions with the heme protoporphyrin ring (Shelnutt, 1983; White, 1978), and preventing heme crystallisation. Working from the receptor model suggested by Koh *et al.* (1994), work published by O'Neill *et al.* (1997) showed the possible interactions of amodiaquine with the receptor, which are obviously quite similar to that of chloroquine.

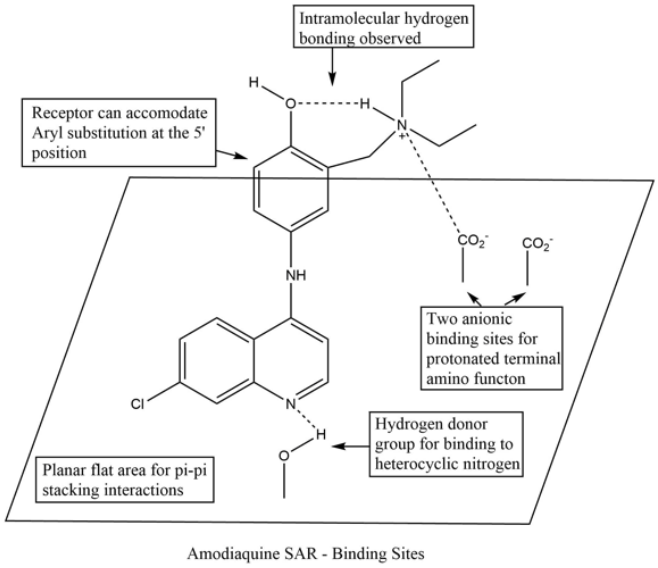


Figure 5. *Binding of amodiaquine to the key features of the proposed receptor sites in heme. Adapted from (O'Neill et al., 1997)*

Amodiaquine displayed very high efficacy in treating the chloroquine resistant strains of *P. falciparum*. In vitro assays against the chloroquine-sensitive strain HB3 and chloroquine-resistant strain K1 (Table 1) provided evidence that amodiaquine was a lead compound for treatment of chloroquine resistant *P. falciparum* malaria.

Table 1. *In vitro assay of antimalarial activity of chloroquine and amodiaquine, adapted from O'Neill et al., (2003). Amodiaquine tested as hydrochloride salt, whilst chloroquine tested as diphosphate. IC50 indicates concentration required for 50% inhibition in vitro.*

Drug	IC_{50} (nM) HB3 Strain	SD ± Mean	IC_{50} (nM) K1 Strain	SD ± Mean
Chloroquine	14.98 (6)	3.98	183.82 (6)	11.13
Amodiaquine	9.60 (9)	3.73	15.08 (9)	9.36

Whilst amodiaquine displayed exceptional activity against the chloroquine resistant K1 strain, the clinical use of the drug has been extremely restricted due to hepatotoxicity and agranulocytosis (Neftel *et al.,* 1986; Lind *et al.,* 1973; Bray & Ward, 1998). Although very seldom used due the adverse side effects, researchers began investigating how the toxicity of amodiaquine was exerted: the drug's efficacy and potential to become a first-line treatment for chloroquine-resistant malaria were high, and so if the mechanism of toxicity was understood it may be possible to prepare analogues without adverse side effects. Studies of amodiaquine metabolism in rats showed that the drug is excreted in bile as a 5′ thioether conjugate, with glutathione and/or cysteinyl forms (Harrison *et al.,* 1992). This excretion indicates that the compound must undergo some form of bioactivation to either an amodiaquine quinoneimine (AQQI) or an amodiaquine semiquinoneimine (AQSQI) with later conjugative addition (Maggs *et al.,* 1988). Work published recently has recently been ascribed the toxicity of amodiaquine to oxidation by cytochrome P450 enzymes (O'Neill *et al.,* 2012). The mechanism of this toxicity is shown in Figure 6. The adverse effects likely occur when glutathione stores have been depleted and the AQQI metabolite persists. Indeed this metabolism is quite similar to the metabolism observed in paracetamol (acetaminophen) overdose, in which conjugation of glutathione depletes stores until conjugation no longer occurs, and an electrophilic arylating compound which can covalently bind to macromolecules is formed, initiating the onset of hepatotoxicity (Beckett *et al.,* 1985).

Due to the side effects of amodiaquine, it is not often used in a clinical setting. Studies of the toxicity inspired the synthesis of *para*-fluorinated alternatives, where the hydroxyl group of the phenol is substituted by a fluorine have been prepared, as these compounds cannot undergo *in vivo* bioactivation to the toxic quinoneimines (Staines & Krishna, 2012). The current reasoning for why they do not undergo bioactivation is to do with the strength of the C-F bond and the high oxidation potential of these compounds (O'Neill *et al.,* 1994). The cost of the starting materials for fluorinated alternatives tends to be expensive compared to that of the parent compound, and when one is investigating drugs for a disease found primarily in the third world, cost must be considered. Hence in 2003, a reasonably cheap alternative to amodiaquine, with high efficacy against chloroquine resistant and sensitive strains was synthesised – isoquine (O'Neill *et al.,* 2003).

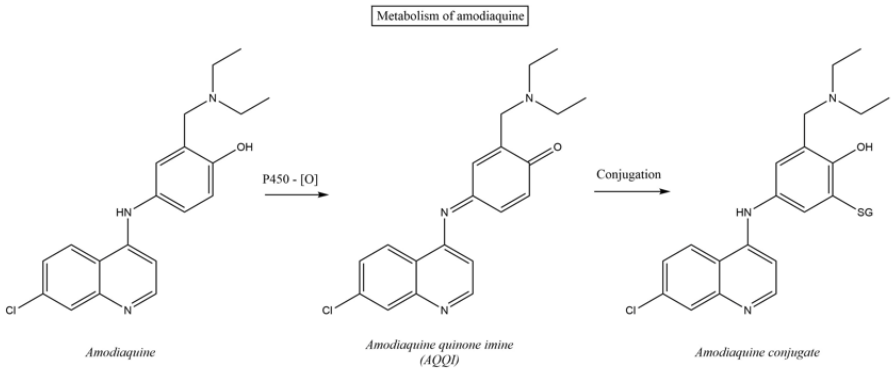


Figure 6. *Metabolism of amodiaquine by the enzyme cytochrome P450, showing the highly toxic metabolite AQQI which leads to agranulocytosis and hepatotoxicity. Adapted from O'Neill et al., (2003).GS indicates glutathione, an antioxidant.*

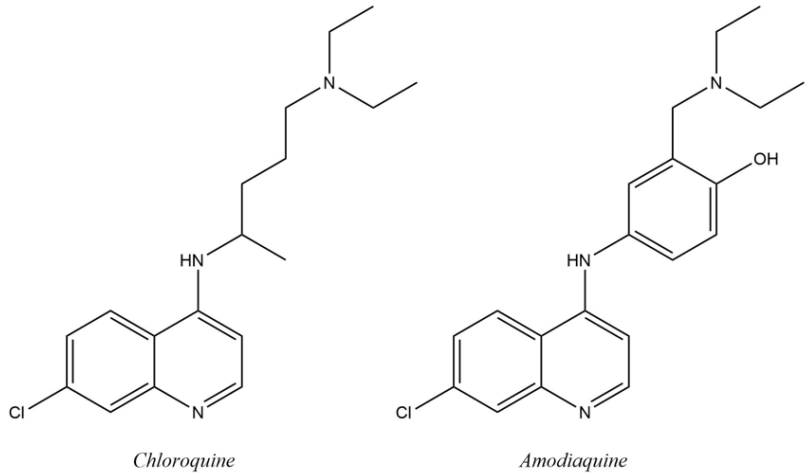


Figure 7. *Comparison of the structures of chloroquine and amodiaquine.*

Isoquine

Isoquine is a structural analogue of amodiaquine with high antimalarial activity and no possibility of toxic quinoneimine metabolite formation, as the *in vivo* bioactivation is prevented (O'Neill *et al*., 2003). Isoquine has been formulated for oral administration and displays high ED_{50} (median effective dose) activity of 1.6 and 3.7 mg/kg against the *P. yoelli* NS strain (a strain used for laboratory infection of mice) compared to 7.9 and 7.4 mg/kg for amodiaquine. Furthermore, isoquine provides a cost effective and potentially safer alternative to amodiaquine, as its metabolism is quite different; isoquine and its Phase I metabolites appear to undergo clearance by Phase II glucuronidation, evidenced by the lack of glutathione metabolites in the bile (O'Neill *et al.,* 2003). Isoquine is now being investigated further and is considered a lead compound for the treatment of chloroquine-sensitive and chloroquine-resistant *P. falciparum* malaria. It is currently reaching the end of Phase I clinical trials, conducted by GlaxoSmithKline in the UK (GlaxoSmithKline 2010-Present).

Future analogues

A huge field of currently active research deals with synthesising hybrid compounds of 4-aminoquinolines with other classes of antimalarial drugs. As it stands, conjugation of some of the 4-aminoquinolines to natural product antimalarials has proved useful – for example, the synthesis of many different artemisinin-quinoline hybrid dimers was recently published by the Lombard group, displaying reasonably high potency with low resistance occurrence (Lombard *et al*., 2011). In terms of other forms of modification, side chain modification with oxalimide linkers,α-ketoamide linkers coupled in indole and hybrid aminoquinoline-triazine derivatives have been reported, with some of the these compounds reporting quite high antiplasmodial activity (Sunduru *et al*., 2009).

Novel motifs for conjugation to quinoline backbones are constantly emerging from different research groups. One example are the 7-Chloroquinoline-chalcone hybrids which so far have shown somewhat low antimalarial activity (Sharma *et al.,* 2009) but are being researched further because they are cost effective and further modification could potentially boost their efficacy (Vandekerckhove & D'hooghe, 2015). Ultimately, the field of 4-aminoquinoline and 7-chloroquinoline modification could provide cost effective and potent antimalarial drugs, whether that is through conjugation with other natural antimalarials like artemisinin or Cinnamic acid (Wiesner *et al*., 2001) or through carbon linker modification, or some of the other many forms of modification published in recent years (Vandekerckhove & D'hooghe, 2015). These modifications show varying degrees of toxicity and potency, however what is quite interesting is that Wiesner et. al reported in 2001 that Cinnamic acid derivatives show markedly low toxicity (Wiesner *et al*., 2001). As Cinnamic acid is a water-soluble natural product obtained

from oil of cinnamon (and also shea butter), perhaps natural product conjugation to the quinoline moiety should be focused on for the future of cheap antimalarial conjugates with low toxicity.

Table 2. *In vitro assay of antimalarial activity of chloroquine, amodiaquine and isoquine, adapted from O'Neill et al., (2003). Amodiaquine tested as hydrochloride salt, whilst chloroquine tested as diphosphate. Isoquine tested as free-base. IC50 indicates concentration required for 50% inhibition in vitro.*

Drug	IC_{50} (nM) HB3 Strain	SD ± Mean	IC_{50} (nM) K1 Strain	SD ± Mean
Chloroquine	14.98 (6)	3.98	183.82 (6)	11.13
Amodiaquine	9.60 (9)	3.73	15.08 (9)	9.36
Isoquine	12.65 (9)	4.75	17.63 (9)	7.00
Isoquine diphosphate	9.02 (3)	4.06	6.01 (3)	8.00

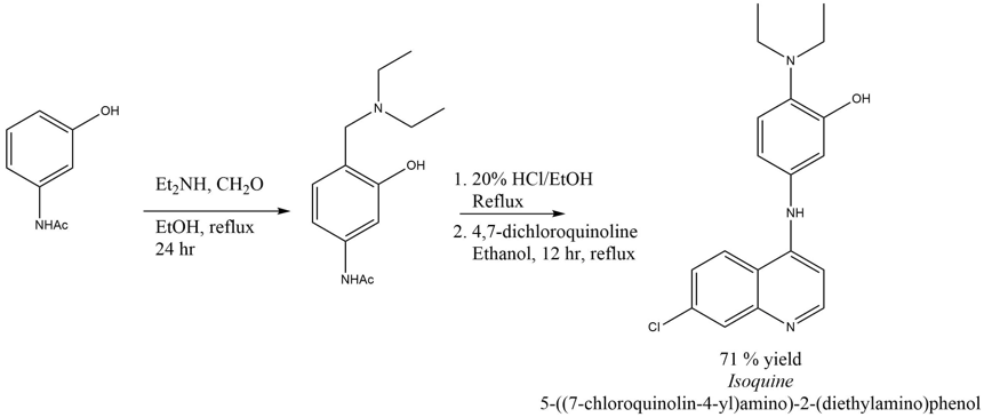


Figure 8. *Synthesis of isoquine. Adapted from O'Neill et al., (2003)*

Discussion and Conclusions

Malaria is a widespread disease, with a distribution close to the distribution of poverty over the world. Reliable drugs for parasite prophylaxis are of immense value, and while the first of the 4-aminoquinolines, chloroquine, showed high efficacy and prophylaxis, resistance soon arose and became widespread. Drugs based off this early 4-aminoquinoline show similar degrees of activity and in some cases, structural analogues display higher efficacy than previously encountered. The scope of 4-aminoquinoline modification is huge – conjugation to natural product antimalarials which show reasonably low toxicity and promising potency. Modification of the length and nature of the carbon linker is shown in the case of amodiaquine and isoquine, however that appears to only be the beginning. In amodiaquine, the carbon linker transformation to a phenol proved useful in increasing potency, yet induced adverse side effects such as hepatotoxicity. The regioisomer isoquine was hence designed based on amodiaquine, and proved to be an effective antimalarial with low toxicity and IC_{50} values similar to that of amodiaquine for the treatment of chloroquine-resistant malaria. In isoquine, rational drug design with respect to Phase I metabolism was implemented, and it is this kind of rationale which may lead to further advances in reducing the toxicity of quinoline-based antimalarials.

In terms of the future of synthetic 4-aminoquinolines and their derivatives, there are a wide range of conjugates and modified structures being researched, other than amodiaquine and isoquine. Functionalisation and/or interconversion of the tertiary and secondary amine component in chloroquine has proved beneficial, showing high potency. Furthermore, natural product derivatives, especially those from Cinnamic acid, have shown very low toxicity in studies published in the last six to seven years. In the future of antimalarial research, it is likely that cheap natural product conjugates and dimers based off the 4-aminoquinoline backbone structure or even the isoquine structural motif will find clinical use in plasmodia prophylaxis. Indeed, the area of antimalarial drug design requires careful navigation around toxicity and indeed with novel analogues such as isoquine and its potential for modification, we very well could be on the brink of discovering a safe and cheap alternative for treating resistant strains of *Plasmodium* parasites.

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All structures drawn using ChemBioDraw Ultra 14.0.

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Physics

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Letter from the Editor

We are moving towards an era where interdisciplinary collaboration is crucial to carry out successful scientific investigation. Researchers in many fields are thinking beyond the traditional boundaries that define the specific disciplines, combining their efforts and expertise to investigate nature and develop new technologies. The modern facet of scientific research requires thinkers capable of challenging the established boundaries and eager to join international collaborations. I, thus, applaud the authors published in the Physics section of the Trinity Student Scientific Review. They have demonstrated the skills and the attitude crucial to producing successful reviews, thereby holding the promise of becoming researchers capable of tackling the challenges of modern scientific investigation. By expanding on applications of Quantum Mechanics and Particle Physics to Biology, cancer treatment, information transmission and novel renewable energy infrastructures, the reviews showcase the state-of-the-art advancements in some of the most exciting interdisciplinary areas of research.

The Trinity Student Scientific Review acts as a great learning platform to acquire the necessary skills, for any aspiring researcher, that stretch beyond the scope of the undergraduate curriculum. Successful scientific enquiry requires equally committed scrutiny and effective communication. As Physics Editor I have had the chance to expand my knowledge of scientific communication and to work with brilliant students, eager to collaborate to put forward compelling articles. By experiencing the many steps leading to the second volume of the Trinity Student Scientific Review, we were all given the chance to get a powerful insight into the dynamics at play in the world of scientific writing. I firmly believe that this is a crucial set of skills for the upcoming researchers, and I am proud of the role Trinity Student Scientific Review plays in enabling such development. On behalf of the Editorial Team, I wish to thank all the candidate authors for their dedication and efforts. Without such a crucial contribution, the Review would not be able to move in the promising direction we are witnessing today.

As a closing note, I would like to express my sincere gratitude to Prof. W. Blau, Prof. S. Hutzler, Dr. S. Bergin and Mr. J. Magan for their assistance in the reviewing process and to the TSSR Team. What we have achieved this year is, in my opinion, something remarkable, and I encourage any interested undergraduate students in Trinity to take part in this fresh and much needed enterprise. I hope you will enjoy this section of the Trinity Student Scientific Review. We have tried our very best to illustrate some of the most enticing areas of current research in Physics in a clear fashion.

Blaise Delaney
Physics Editor
Trinity Student Scientific Review 2016

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The Nuclear Option: Advanced Radiotherapy Techniques for Cancer Treatment

Oskar Ronan
Senior Sophister
Physics

Conventional radiotherapy techniques for the treatment of malignant tumours are known to carry some risk as a method of combatting cancerous cells. The damage caused to healthy tissue as a part of these treatments poses a significant obstacle to overcome in the fields of radiobiology and oncology. The use of linear particle accelerators and cyclotrons to generate beams of charged radioactive particles for use in cancer therapy has been developed over recent years, and new and more effective cancer treatments such as hadron therapy have been made possible because of experimental particle physics and hold the promise of greater survival rates for patients. The effectivity of such particle physics based treatments is discussed here, and potential new treatments explored.

Introduction

Standard radiotherapy is carried out in hospitals as a means of combatting cancerous cells. This treatment consists of using ionising radiation to damage the DNA of the targeted cells, rendering the cells unable to reproduce. The radiation ionises molecules in the cells, creating free radical compounds that attack and damage DNA. In order to replicate itself, the cell must repair the damage done to the DNA. Many tumours and cancerous cells possess a greatly reduced ability to repair their damaged DNA compared to normal, healthy cells. This then allows for an enhanced tumour control method, such as chemotherapy or surgery to destroy the tumour (Warrell *et al*., 1983).

The dose of irradiation is defined as the amount of energy absorbed by biological tissue. This value has been standardised in the form of the Gray (Gy), a unit defined as the absorption of 1 joule of energy by 1 kilogram of matter. The effective dose of radiation is similar to the absorbed dose, however is corrected for factors such as radiation type, tissue susceptibility to radiation, and non-uniform dosage. The effective dose is measured in Sieverts (Sv), and has the same dimensions as Gy.

Typical therapeutic doses are in the order of 20 Gy to 60 Gy over the course of several weeks (Warrell *et al*., 1983), but can be higher for tumours that are resistant to radiation damage, such as epithelial tumours like skin and oesophageal cancers (Warrell *et al*., 1983).

Radiation is highly effective in killing tumour cells, but this effectivity can, and often does, come at the cost of damage to the surrounding tissue. Tissues comprised of fast-dividing cells, such as skin, mucosal linings, and bone marrow, are extremely susceptible to the damaging effects of radiation. Diarrhoea, nausea, and vomiting are common side effects in patients who receive radiation therapy to any organs in proximity to the gastrointestinal tract (Warrell *et al*., 1983). In the case of whole-body irradiation, the lymphocytes in the blood can be damaged as much as the intended cancerous cells, and the immune system can be suppressed, leading to dangerous secondary infections (Warrell *et al*., 1983).

The most common form radiotherapy involves the bombardment of the tumour with high energy photons, typically in the MeV range. These photons are usually either X-rays created *in situ*, or gamma rays (γ-rays) from a radioactive isotope source (most commonly ^{60}Co).

The problem with these methods of administering radiation is that they don't deliver their dose to just the cancer cells. Damage to surrounding cells can be quite significant. Over the years, there have been some inventive engineering solutions to this problem, most of which involve a rotating radiation source; this allows for the largest dose to be delivered at the point of intersection of the different beam directions, thus minimising surrounding tissue damage (Warrell *et al*., 1983).

Hadron Therapy

The alternative to more common radiation therapies being developed by particle physics laboratories around the world is known as hadron therapy. Hadron therapy is the use of subatomic particles composed of quarks to treat tumours.

Most hadron therapies exhibit distinctly different energy distributions through matter than photon radiotherapy. They possess a feature, known as a Bragg peak, made evident by plotting their dose against distance into tissue, known as a Bragg curve (Figure 1). Bragg curves can be used to show where the highest dose of radiation will be delivered, and are thus used in radiotherapy to assist in calculating

the desired position and exact energy of the radiation being administered (Figure 1). The curves are named after Sir William Henry Bragg, who along with his son William Lawrence Bragg, studied such data curves during their development of X-ray spectroscopy in the early 20th century.

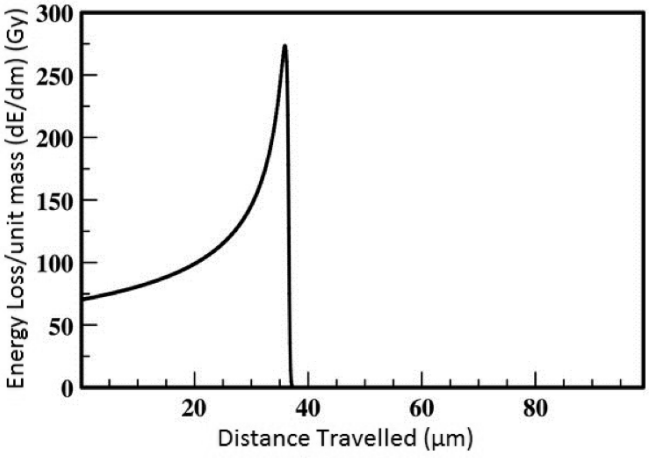

Figure 1. *A typical particle Bragg curve showing the loss of energy (dE/dx), or dose, as a function of distance travelled in water (adapted from Bevelacqua, 2015).*

The peak in the Bragg curve comes about due to Coulombic interactions between the ion particle beam and the atoms in the matter being penetrated. As the ions interact, the particles in the incoming beam have their velocities decreased by the interacting electric forces. This loss in kinetic energy is converted directly into a photon (eq. (1)), giving rise to its radioactivity. This is known as *bremsstrahlung*, or breaking radiation. The lost kinetic energy is converted directly into photons:

$$m(v_2 - v_1) = E_2 - E_1 = hf \qquad (1)$$

As the particles slow down, their interaction cross section, and thus the probability that they will have further interactions with the surrounding matter, increases (eq. (2)). The relationship between the differential cross section of an interaction, and the kinetic energy of the incoming particle can be derived to be (Rutherford, (1911)):

$$\frac{d\sigma}{d\Omega} = \frac{1}{T^2}\left(\frac{Z_1 Z_2 e^2}{16\pi\varepsilon_0}\right)^2 \left(\frac{1}{\sin\left(\frac{\Theta}{2}\right)}\right)^4 \quad (2)$$

Where $d\sigma/d\Omega$ is the differential cross section of interaction, T is the kinetic energy, given by $T = \frac{1}{2}mv^2$ for non-relativistic particles where m is the mass of the particle and v is its velocity, Z is the atomic number of the particles involved, e is the electron charge (1.6022×10^{-19} C), ε_0 is the permittivity of free space (8.85×10^{-12} F/m), and Θ is the angle of deflection.

Note the inverse relation of the cross section to the square of the kinetic energy (eq. (2)); this gives rise to the characteristic shape of the Bragg curve.

As the differential cross-section increases with decreased velocity, the incoming particles lose further kinetic energy as radiation, which leads to an exponential increase in energy lost to the surrounding tissue (eq. (2)). This exponential increase in interactions and energy losses gives the Bragg peak its characteristic exponential shape. Eventually, the particle will lose all of its kinetic energy, and this gives rise to the sharp cut-off point present on the Bragg curve of many hadrons (Figure 1).

The idea of using hadrons such as fast moving protons was first suggested just after the Second World War (Wilson, 1946). The most common form of Hadron Therapy is the use of accelerated proton beams directed at the tumour site to irradiate. Theoretically, the use of proton beams would allow for a maximum dose of radiation to be delivered at a specific point within the patient, and cause little to no permanent damage to surrounding tissue. This can be seen in Figure 2, showing the greatly reduced radiation exposure of surrounding healthy tissue after proton therapy. Such a treatment method adds additional controllability to treatment as penetration depth, position and dose targeting can all be easily manipulated in comparison to conventional photon radiotherapy (MacReady, 2012).

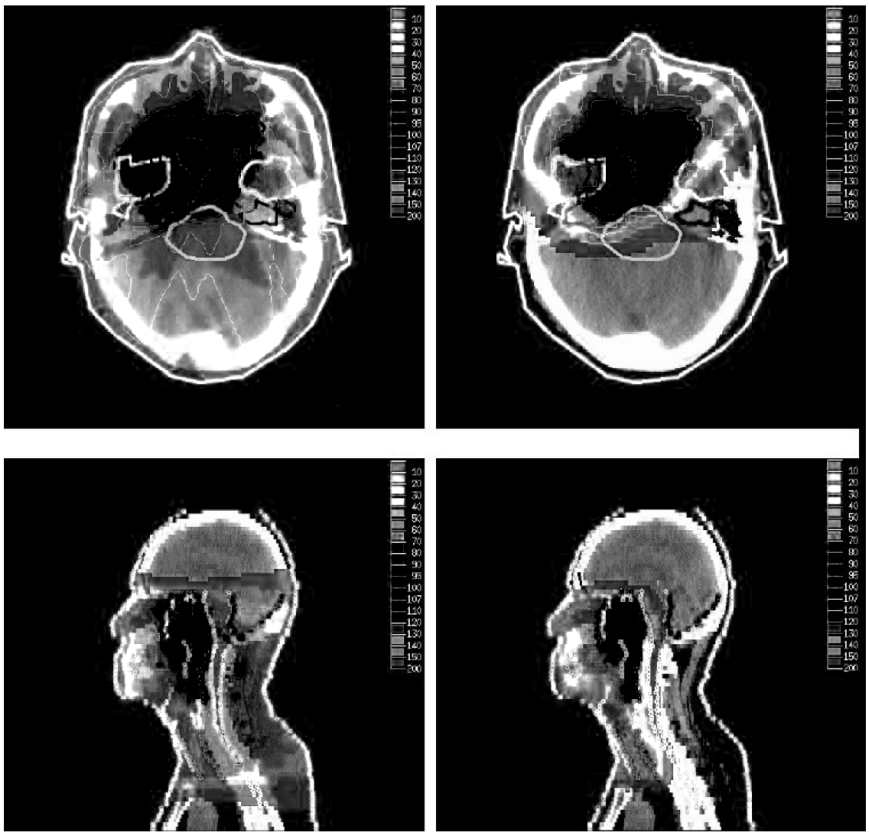

Figure 2. *Dose distribution comparison of conventional photon therapy (left) versus proton therapy (right) to treat nasopharyngeal carcinoma. The proton therapy clearly shows greater targeting of the cancerous cells, with less radiation exposure to surrounding tissue when compared to conventional photon therapy (Taheri-Kadkhoda et al., 2008).*

Proton beam therapy is already in use in cancer treatment centres across the world (*Where to get Proton Therapy*, 2014), however the facilities where such procedures are carried out are rare; only 54 such treatment centres capable of providing proton therapy exist worldwide, some of which have yet to begin treating patients (*Where to get Proton Therapy*, 2014).

This idea of using ion beams for cancer treatment is promising. There are, however, more effective ways in which the physics can be manipulated to augment treatment effectivity and lower the risk of excess damage further still. One such method is to increase the amount of energy available to be lost as *bremsstrahlung*. This can be

achieved by increasing the initial kinetic energy of protons. However, this would increase the penetration depth, and the target may be missed. A solution to this issue is offered by heavy ion beam therapy (Schlaff *et al*., 2014). This is the use of heavier ions (usually carbon) in place of protons. Increasing the mass of the ions leads to an increase in the kinetic energy when these are accelerated to similar velocities as the protons, thus leading to an increase in the amount of energy available to transfer to surrounding tissue.

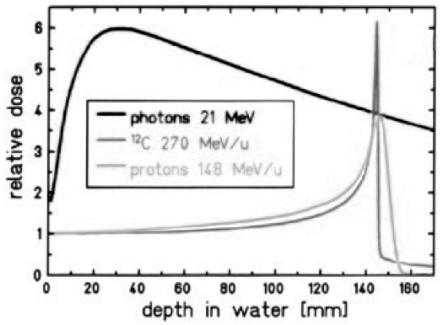

Figure 3. *Comparison of proton and C-ion beam Bragg curves to conventional photon therapy (adapted from Fokas et al., 2009).*

Carbon ions have similar characteristic Bragg curves to protons, exhibiting a much larger peak (up to 60% larger) just before coming to rest (Figure 3). A small 'tail' present in carbon ion curves can be noted. This is known as the fragmentation tail (Figure 4) (Suit *et al*., 2010). This is due to collisions of the carbon ions and target nuclei breaking into smaller radioactive particles, and contributing to the total dose (Amaldi *et al*., 2005).

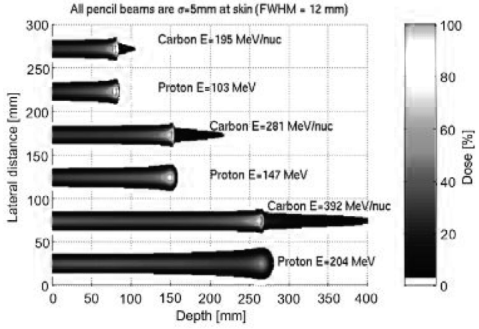

Figure 4. *Dose distribution comparison of proton and carbon ion beams. Note the fragmentation tail, and increased dose at peak for the carbon beams (Suit et al., 2010).*

There are a number of more exotic treatments under development in medical particle accelerator facilities. Hadrons consist of two main groups, baryons and mesons. Baryons are composed of three quarks (although theories suggest that exotic baryons consisting of up to nine quarks could exist, (Aaij *et al*., 2015) and make up most of the baryonic matter in the universe. Mesons are composed of one quark and one antiquark. The use of mesons for radiotherapy has similar advantages to the baryonic therapies discussed above. π-meson (or pion) beam therapy has been suggested as an alternative (Raju, 1971; Mokhovy *et al*., 1999) but is not a common treatment for cancer. The pions decay producing a number of secondary particles that in turn decay, releasing a much higher dose of radiation than from just the initial decay. Depending on the type of pion beam used in treatment, a number of decay routes are possible.

The simplest decay is that of neutral pions (π^0) into two photons (Khan, 2012). π^0 are composed of either an up and an antiup quark, or a down and an antidown quark. They thus have extremely short lifetimes (approximately 0.1 fs (Von Dardel *et al*., 1963)). However, this can prove problematic as a treatment, as there is currently no way of directly controlling a π^0 beam in a magnetic field due to its lack of electronic charge.

The more viable option, and also more complex, is charged pion decay. Charged pions have much longer lifetimes (approximately 26 ns (Perkins, 2003)) and can be easily accelerated, due to their charge. The primary mode of pion decay is a purely leptonic decay into a muon, and a muon antineutrino via a *W*-boson (Figure 5). Such particles can also decay into electrons and electron antineutrinos, or positrons and electron neutrinos, depending on the charge conservation. This decay is, however, far less probable.

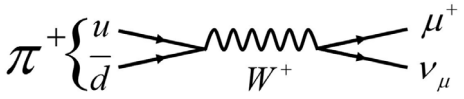

Figure 5. *Lowest order Feynman diagram showing the decay of the π^+-meson into muons and neutrinos via a W^+ boson. The decay for the π^--meson is similar, only with opposite charge conservation.*

Both the positive and negative pions can decay via this route, however the π^- has the additional option of binding to a nucleus in the tumour tissue, and interacting to the nucleus that captured it (Khan, 2012). If the nucleus in question is hydrogen, the π^- can interact with the proton, and produce a π^0 and a neutron, or a neutron and a photon (Mokhovy *et al*., 1999), as shown in eqs. (3, 4).

$$\pi^- + p \rightarrow \pi^0 + n \rightarrow n + 2\gamma \quad (3)$$

$$\pi^- + p \rightarrow n + y \quad (4)$$

The elementary muons produced in pion decay can also contribute to the total radiation dose. As the muons decay, the binding energy of the muon is released as radiation.

Figure 6. *Lowest order Feynman diagram of positive muon (μ^+) decay into a muon antineutrino, a positron and an electron neutrino via a W^+ boson.*

The exact dynamics of this decay change depending on what flavour of muon decays (Figure 6) (eqs. (5, 6)). When a μ^+ decays, it decays into a muon antineutrino, a positron and an electron neutrino via a W^+ boson (eq. (5)), releasing energy in the process. Further energy is released upon the subsequent positron-electron annihilation.

$$\mu^+ \rightarrow e^+ + \nu_e + \overline{\nu}_\mu + hf \quad (5)$$

However, if a μ^- is present, the μ^- can displace an electron in an atom, and bind to the nucleus, creating a form of exotic matter known as a muonic atom. The captured μ^- can then relax to the ground state from its excited state, releasing photons before decaying into a muon neutrino, an electron and electron antineutrino via a W^- boson (eq. (6)), along with more photons.

$$\mu^- \rightarrow e^- + \overline{\nu}_e + \nu_\mu + hf \quad (6)$$

If the μ^- is captured by a hydrogen nucleus in the tumour, further irradiation is caused by electron capture (or inverse β-decay) and the emission of a 5.1 MeV neutron (Mokhovy *et al*., 1999).

It should be noted that muon beam therapy could also be used as a cancer treatment, however, as such particles are technically not hadrons, they are simply discussed as a side-product of other hadron therapies in this review.

The most effective treatment in radiation oncology would need to be a combination of the methods previously discussed. Antiproton radiotherapy is a method that combines the dose distribution of proton therapy with the large energy releases of the other decay methods (Bassler *et al*., 2008; Kavanagh *et al*., 2013). Protons and antiprotons share similar interaction characteristics before the Bragg peak, exhibiting similar radiobiology and differential interaction cross-section. The difference between their radioactivity occurs when antiprotons reach the Bragg peak. As the antiproton comes to rest, proton-antiproton annihilation occurs. This is a complex process, involving some clean quark-antiquark annihilation, with the formation of new pions and kaons from the rearrangement of the remaining quarks of the collision (along with the gluon interactions forming strange quarks for kaon formation). The pions that are created would then decay as described above, releasing more energy, and the subsequent muon decay would release further energy still.

The decay of additional radioactive side-products of proton-antiproton annihilation increases the effective dose at the Bragg peak by about 20-30 MeV per antiproton (on average) when compared to the proton Bragg peak (Bassler *et al*., 2008). This treatment combines the increased effectiveness of heavy ion hadron therapy at the target site without the increased dose before the Bragg peak (Holzscheiter *et al*., 2006). The fragmentation tail is still observed due to the multitude of additional radioactive shrapnel particles ejected in the annihilation.

Discussion and Conclusions

While hadron therapy has many benefits over conventional radiotherapy, it is important to note some of its disadvantages. Studies have shown that while proton therapy can give far more targeted results by way of delivering the majority of radiation at the target site, the more powerful heavy-ion and antiproton therapies have drawbacks that become more noticeable when put to practical use. The fragmentation tail caused by secondary particles begins to become a serious issue when doses are applied across the entire tumour, causing a halo of damage to surrounding healthy cells (Paganetti *et al*., 2010).

Another issue surrounding these advanced tools for combatting cancer is the cost of implementing the facilities required to carry out treatments such as hadron therapy. Expensive linear accelerators, cyclotrons and synchrotrons have to be built *in situ* in order to provide these radiotherapies, and this is an expensive endeavour that not many hospitals are capable of funding. The capability of engineering cost-effective particle accelerators could enable such potentially highly effective treatments to become a more commonplace occurrence in the future.

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Hybrid Photovoltaic Thermal Cells: A Viable Solution to the Problem of Renewable Energy

Kyle Frohna
Junior Sophister
Nanoscience, the Physics and Chemistry of Advanced Materials

Hybrid photovoltaic thermal (PVT) cells represent a current frontier in renewable energy. These cells can harness both thermal and electrical energy from the sun resulting in large increases in net efficiency over traditional photovoltaic (PV) or solar thermal cells. High net efficiency PVT cells have already been demonstrated. However, the PV elements in these cells show poor electrical efficiencies when compared with traditional PV cells. The same can be said for the the thermal element. Further optimisation is needed. The current state of research in the field of PVT cells is presented, as well as potential avenues for future improvements. These avenues include the physical geometry of the cell, the material composition of both the PV cell and the heat exchanger and the coolant fluid. Current research is being conducted into nanofluids as an alternative to water or air cooling in PVT cells, which shows substantial increases in efficiencies of the cells. It is suggested in the literature that PVT cells could provide a viable alternative to centralised power generation by placing these cells on buildings. PVT cells may allow progress towards net zero energy buildings (nZEB).

Introduction

The scientific community, and indeed the global community, has turned its attention towards alternatives to fossil fuels in the generation of electricity. Photovoltaic (PV) cells are of particular interest. This clean and renewable source of energy has come to prominence in recent years as PV cells have become more popular among the general public. There are many clear advantages to PV cells: they provide power without the need for fossil fuels, they can be retrofitted for home use in attempts to reach an energy equilibrium or an nZEB. These are buildings where the power demands of the building are met entirely by power produced on site (Sartori *et al.*, 2012). They can also be scaled up to large scale photovoltaic plants such as the potential 10 MW power plant in Abu Dhabi discussed by Harder and Gibson (2011). Power plants producing greater than an order of magnitude more power have been discussed theoretically and have been installed in practice, such as the Solar Star I and II plants in California, USA.

There are some considerable drawbacks and limits to the effectiveness of PV cells. The Shockley-Quiesser limit is a fundamental limitation to the efficiency of traditional photovoltaic cells (Shockley and Quiesser, 1961). Most traditional PV cells consist of a p-n junction produced with either monocrystalline or polycrystalline silicon. The Shockley-Quiesser limit determines that the maximum possible efficiency for an ideal silicon p-n junction PV cell with a band gap of 1.1 eV is 30%. The experimental upper limit was predicted by Shockley and Quiesser to be in the region 27%. The primary reasoning behind the inefficiency of the PV cell is that the cell cannot efficiently absorb at all wavelengths as photons with energy less than the band gap will not excite electrons to the conduction band. The excess energy from photons with energy greater than the band gap will be converted into heat. There is, as a result, a large generation of heat in the cells. This problem is compounded by the fact that PV cells become less efficient at higher temperatures resulting in even lower efficiency (Skoplaki and Palyvos, 2008). Steps have been taken to increase the efficiency of the photovoltaic cells by increasing the number of terminals and the variety of materials to increment the range over which the cell will absorb efficiently. Steiner *et al.* (2015) produced a large-scale four-junction PV module with an efficiency of 36.7%. Efficiencies can be increased further by concentrating the light incident onto the cell (Guter *et al.*, 2009).

However, the problem of waste heat generated still remains and the cells have to be cooled to maintain optimal efficiency. Hybrid photovoltaic thermal (PVT) cells aim to harvest both the electrical energy and the thermal energy produced in the cells. These cells combine a photovoltaic cell and a solar thermal cell to produce solar electricity as well as either thermal electricity or usable heat. PVT cells have the potential to vastly increase overall efficiencies of the traditional PV cell. The research on this innovative technology is still in relative infancy although very high total efficiencies have already been reported (Dupeyrat *et al.*, 2011).

Basic Principles of PVT Cells

The most common form of PVT cell is the flat-plate PVT cell. The flat plate PVT cell consists of a PV element that is placed in thermal contact with a thermal collector. The methods of mounting the PV element onto the thermal collector can considerably effect the net efficiency of the cell (Dupeyrat *et al.*, 2011). The thermal collector is simply a material with a high absorbance with a cooling fluid passing through it to transfer heat from the cell. The cooling fluid, the most common being either water or air, is pumped through the system for active cooling of the cell and heat transport. Natural air convection can instead be used for passive cooling in some circumstances. There are several popular configurations as outlined by Chow (2010). The main competitor to the flat plate PVT cell is the concentrator type PVT (c-PVT). In this type of cell, light is concentrated onto the cell using an optical system, either reflectors or lenses, in order to increase the radiation intensity incident upon each cell. The specific designs of the systems vary depending on the cooling fluid but their basic structures are similar. The recent research in the area can be split into three main categories: air cooled PVT cells (PVTa), water cooled PVT cells (PVTw) and c-PVT cells.

PVTa Cells

The path a coolant takes through the thermal collector can drastically effect the performance of the cell. Hegazy (2000) compared four types of PVTa configurations. The 4 configurations involved were as follows:

1. Air flowing over the heat absorber.
2. Air flowing under the heat absorber.
3. Air flowing both over and under the heat absorber in a single pass.
4. Air flowing first over and then under the heat absorber in a double pass.

Hegazy (2000) found that the thermal efficiency (η_T) for configuration 1 was considerably lower than the others, showing η_T values 3-5% lower than the other configurations, at flow rates of air ranging from 0.005 to 0.04 kg s^{-1} m^{-2}. The electrical efficiency (η_e) was found to be very similar in all four cases, ranging from 0.068 to 0.081. A point Hegazy (2000) makes is that the power required to circulate the air with fans should be factored into any calculations regarding net power produced. It was found that the third configuration required the least amount of fan power and, as a result, produced the largest net available electrical energy of the four configurations. The net calculations for the configuration 3 PVTa cell show a net efficiency (η) of 0.55 at a mass flow rate of 0.03 kg s^{-1} m^{-2}.

Tripanagnostopoulos *et al.* (2002) tested both PVTa and PVTw cells outdoors under a variety of configurations. The bulk of the tests were carried out using polycrystalline Silicon (pc-Si) PV cells. The most notable inclusions are additional glazing in order to increase η_T of the cell and the inclusion of reflectors between cells, which increase the incident power onto the cells. It was found in both cases that the highest η_T was achieved when both the additional glazing and the reflectors were in place. At a cell coolant temperature equal to ambient temperature, η_T of 0.8 for the PVTw cell and 0.75 for the PVTa cell were observed. η_e for both cases were similar, between 0.1 and 0.15 respectively. The inclusion of the glazing notably lowered η_e, however the inclusion of the reflector effectively reversed this decrease. The maximum electrical efficiencies were found with reflectors without glazing.

Bambrook and Sproul (2012) attempted to optimise a PVTa system integrated into a home for use as an air heater. Building-integrated PVT (biPVT) cells are becoming of increasing interest in the attempt to reach a nZEB (Good *et al.*, 2015). Of particular relevance to this paper is the optimisation of the fans providing the cooling to the cell. It is noted in the paper that there is a delicate trade-off between increased fan speed and η. Although an increased mass flow rate of air will increase both η_T and η_e, the increased fan speed will, itself, require more power. At mass flow rates from 0.02-0.055 kg s^{-1} m^{-2}, the electricity gained from increased efficiency of the PV cell outweighs the power required to drive the fans. At higher flow rates, the fans power consumption outweighs the benefit provided. The maximum observed thermal efficiency was between 0.55-0.60 at a mass flow rate of 0.082 kg s^{-1} m^{-2}. The air was found, at these elevated flow rates, to only deviate from ambient temperature by 3-4 K. This temperature gradient is insufficient for effective heating of a household. Such issues lead the authors to suggest a moderate airflow of 0.04-0.05 kg s^{-1} m^{-2}.

Pathak *et al.* (2012) take a different approach to the PVT cell. They suggest that rather than trying to lower the temperature of the cell to increase η_e, operating at a higher cell temperature will increase η_T. This could also have benefits for biPVT cells used for heating purposes. They note that the η_e of amorphous silicon PV cells has a much lower dependence on temperature. Thus, operating at an increased temperature would result in minor decreases in η_e but would result in considerable increases of the η_T of the cell.

Parametric Optimisation of a PVTw System

Dupeyrat *et al.* (2011) discuss an optimisation process for a glazed flat plate biPVT used for hot water systems (biPVTw). The first point considered is the reflection of incident light by the glass coating. It is found that glass used in conventional PV cells has a global normal transmission coefficient of approximately 0.91. With the use of double-sided anti-reflective coated low iron glass, this coefficient is

increased above 0.94. Single crystalline silicon (c-Si) is found to have a higher η_e than polycrystalline silicon (pc-Si). Dupeyrat *et al.* (2011) investigated η_T of pc-Si to determine its viability in comparison to c-Si. c-Si proved superior in this regard, showing an absorption coefficient of 0.90 compared with pc-Si of 0.85, resulting in higher η_e values for c-Si. The discrepancy is explained by the uniform surface texturing of c-Si compared to the pc-Si. The rough surface of pc-Si results in higher reflection of incident radiation compared to c-Si.

The PV packing factor, defined as the ratio of the surface area of the PV cells to the total surface area, was then investigated to determine a packing factor that produced optimum η. It was found that increasing the packing factor increased η_e and decreased η_T values (Dupeyrat *et al.*, 2011). The material composing the heat exchanger to which the PV cells are bonded generally has a correspondingly higher absorption coefficient than the cell itself, and this leads to the decrease in η_T. A packing factor of 0.8 was used by Dupeyrat *et al.* (2011). It was found that the method of adhering the PV elements to the heat exchanger can effect η_T. Early investigations by Zondag *et al.* (2003) and Ji *et al.* (2007) used epoxy glue and silicon adhesive respectively. Dupeyrat *et al.* (2011) used a lamination process which increased the thermal conductivity between the PV cell and the heat exchanger, with improvements in η_T of between 4-8% and improvements in η_e of roughly 1% compared to that used by Zondag *et al.* Combining the anti-reflective coating, c-Si PV cells, 0.8 packing factor and laminated adhesion of the cells to the heat exchanger produced a highly efficient cell. The cell with coolant at ambient temperature produced an η_e of 0.087 and a η_T of 0.790 for an overall η of 0.877. This result shows a marked improvement in efficiency over conventional PV cells as governed by the Shockley-Quiesser limit (Shockley and Quiesser, 1961). The coolant remaining at ambient temperature is an ideal case. However, the more realistic measurements provided show only minor decreases in performance over small temperature ranges. It should be noted that the η_e of this optimised cell is considerably lower than traditional PV cells which commercially show values of η_e of between 0.15-0.18 (Saga, 2010). Further work is needed to improve η_e. Changing the material composition of the PV cells could provide a solution.

Nanoparticle Suspensions as Coolant for Increased net PVT Efficiencies

The thermal conductivity of the coolant fluid can effect η of a PVT, as a coolant with higher thermal conductivity than the water coolants currently used will allow the PV cells to operate at lower temperatures, resulting in higher η_e. One of the most promising advances in the field is the use of nanofluids. These are fluids, most commonly water, which have a suspension of nanoparticles within them. It has long been known that the thermal conductivity of a suspension of nanoparticles is considerably higher than that of the original fluid (Choi and Eastman, 1995). Water has a thermal conductivity of 0.6155 W kg s^{-1} m^{-2} at 303K (Bashirnezhad *et*

al., 2015). Suspensions of 80 nm Cu nanoparticles at 3% volume were reported by Bashirnezhad *et al.* to increase the thermal conductivity of water by up to 33%. Similar values for other nanoparticles such as Al2O3 were also reported.

Selective Absorption of Radiation by MgO Nanoparticles

The optical properties of nanoparticles change with their size (Cui and Zhu, 2012). A suspension of MgO nanoparticles was used as a coolant in a PVT cell in an attempt to improve η. One of the key advantages of nanoparticles is the fact that their optical properties can be easily tuned. In the configuration used by the group, the nanofluid flowed between the PV cells and the source of radiation, rather than in a pipe underneath the PV cells. The ideal coolant in this instance would be one that is totally transparent in the region relevant to the band gap of silicon and absorbing all infrared (IR) radiation beyond the band gap of silicon (Cui and Zhu, 2012). In theory, the optical properties of the nanoparticles can be tuned to maximise the heat absorbed by these, ensuring high η_T while still allowing a large portion of the radiation to be harnessed by the PV cells to generate photocurrent. A film of MgO suspension of 0.02% by weight with thickness 10 mm showed transmittance in the region of 40-70% in the wavelength range of 400-1300 nm, corresponding well with the 1.1 eV band gap of silicon. The nanofluid was shown to be virtually opaque outside of this range. Increases in the concentration of the particles produced large decreases in the transmittance of the nanofluid. The thickness of the film was also varied. Thicknesses of 2mm showed marked improvements in overall transmittance, including in the 1500-1900 nm range which would result in excess heat being absorbed by the PV cell and not the coolant. The reported η of the solar cell is above 0.6 (Cui and Zhu, 2012). These particles, which can be tuned to absorb light across various regions of the spectrum, show promise. Nonetheless, further work must be done to increase the transmittance over the region of the spectrum corresponding to the band gap while maintaining opacity over other regions of the spectrum.

Increased η_T and System Control Through use of Nano Ferrofluids as Coolant

Other promising options for coolants include magnetic nanofluids, or nano ferrofluids. These are fluids in which the suspended nanoparticle is super paramagnetic. This is a property of nanoscale particles of ferromagnetic and ferrimagnetic materials in which the overall dipole moment of the particle undergoes random and rapid reversals over time (Papaefthymiou, 2009). Ferrofluids show increased thermal conductivity in agreement with other nanofluids, but their viscosity and thermal conductive properties can be changed by applying an external field (Papaefthymiou, 2009). Ghadiri *et al.* (2015) used a suspension of Fe_3O_4 particles in a more traditional configuration where the coolant flows through

pipes in thermal contact with the heat exchanger beneath the PV cell. They tested at both 1% weight and 3% weight concentrations of the ferrofluid and under a variety of magnetic conditions: firstly with no external field applied, then with a constant field applied of 0.03 T and finally with a field of 0.03 T alternating at 50Hz.

Under conditions of no magnetic field, η_T of 0.68 was reported for the cell containing the 3% weight ferrofluid, marginally higher than the 1% weight ferrofluid. The thermal efficiencies under a constant magnetic field are effectively unchanged. However, a field oscillating at 50Hz produced a marked improvement, particularly in the case of the 3% weight ferrofluid, increasing its η_T to 0.73. Ghadiri *et al.* (2015) suggest the reason for this increase in thermal performance lies in the tendency of the particles to form clusters in suspension. The clusters align in chainlike morphologies which increases their overall thermal conductivity. Ghofrani *et al.* (2013) suggest that the increase in thermal conductivity may be caused by convection of the nanoparticles due to the alternating magnetic field. The convection of the nanoparticles disturbs thermal boundary layers, which they highlight as being an important mechanism for heat transfer. A stark difference in efficiency is observed when a comparison is made with the same system cooled with deionised water. Efficiencies when the coolant is cooled with water are below 0.5. Ghadiri *et al.* (2015) note that the η_T of the system increased by 45% in the absence of a magnetic field and by 50% in the case of an alternating magnetic field when cooled with the ferrofluid instead of the water. One must question how much the benefit in thermal performance is offset by the electrical cost to power the alternating magnets. However, the notable η_T increase of the ferrofluid compared to water, even in the absence of an external field, is substantial.

Conclusions

Although work has been done on the subject of hybrid PVT systems since the 1970s, it is only recently that large scale attention has been turned towards this technology. As a result, the technology is still in its infancy. The electrical efficiency η_e of these hybrid cells is currently lower than that of the current PV cells. Additionally, their thermal efficiency η_T is lower than that of traditional thermal cells. However, net efficiencies of PVT cells are very high, such as the 0.877 net efficiency cell produced by Dupeyrat *et al.* (2011). The potential for large increases in the efficiency of solar component of the PVT cells is very promising. Current PVT cells tend to operate between 0.08 and 0.15 solar efficiency, whereas readily available commercial c-Si PV cells with solar efficiencies above 0.2 are widespread. Incorporating these more advanced technologies into the PVT cells is a potential next step in the field of PVT optimisation. The work on nanofluid coolants is extremely promising. Parametric analysis in the vein of Dupeyrat *et al.* (2011) is essential to combine the current advances into more efficient PVT cells.

The primary future application for PVT cells will most likely lie in distributed home use, rather than in large power plants. Much of the current research, including Bambrook and Sproul (2012) and Dupeyrat *et al.* (2011), is involved in home-integrated cells for combined heating and power use. Comparisons between solar thermal, PV and PVT regarding net usable power have been shown in recent literature (Good *et al.*, 2015). The analysis suggests that at present, a total PV array or a total solar thermal array may currently provide a greater overall benefit than a PVT in attempts to produce nZEB. PVT cells are not up to par with current PV and thermal technologies, however, there appears to be a considerable scope for improvement as this technology continues to mature.

Acknowledgements

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Quantum Mechanical Navigation: The Avian Compass

Holly Herbert
Junior Sophister
Physics

The mechanism enabling the seasonal movement of birds between breeding and wintering grounds defies understanding. It is known that birds derive directional information from the stars, the Sun, and the topographical features which define the planet. Additionally it has been hypothesised that birds may navigate by virtue of ''sensing'' the magnetic field of the Earth. A chemical compass based on the radical pair mechanism has been proposed, and proven viable. Such a quantum mechanical model acts to explain the experimentally determined features of the avian compass and offers a mechanism which is viable in the context of biological systems. This does not imply however that the model is complete. The involved magnetoreceptor molecules remain unidentified, although the protein cryptochrome located in the eyes of birds does hold much promise. In addition, the manner in which the mechanism interfaces with the visual transduction pathway has yet to be understood. Magnetoreception is thus predicted to function in conjunction with other senses in order to facilitate avian navigation.

Introduction

The idea that birds utilise the geomagnetic field of the Earth as a source of directional information was hypothesised as early as 1859 by von Middendorf. The use of a magnetoreceptive compass was first demonstrated in the European robin and thereafter in 17 species of bird (Wiltschko *et al*., 1966). Despite growing evidence that magnetoreception plays a major role in enabling avian navigation, the biophysical mechanism behind such a compass remains elusive.

Any theoretical model must act to explain the experimentally determined features of the avian compass. Wiltschko *et al.* (1972) demonstrated that the employed compass is inclination based, i.e. it is sensitive to the axis but not the polarity of an external magnetic field. Wiltschko *et al.* (1993, 1999) demonstrated that the ability of a bird to orient itself is dependent on the ambient light conditions. European robins showed good orientation under green and blue light, but were disorientated under red. This suggests that the involved mechanism is photo-dependent. Finally, the mechanism responsible for the avian compass must be sensitive to very weak magnetic fields, as that of the Earth is of strength 50μT.

In an attempt to explain all such features, Schulten *et al.* (1978) first proposed a chemical compass based on the radical pair mechanism. They postulated that magnetoreception involved light induced biochemical reactions. The product yields of such reactions have been shown (Schulten *et al.*, 1976) to be influenced by an external magnetic field. Ritz *et al.* (2000) suggested that if such reactions were to take place in immobilised radical pairs (RPs) in the eye of a bird, the product yields may depend on the orientation of the birds head with respect to the geomagnetic field of the Earth. Product yield variations could then affect the sensitivity of the bird's vision and grant it a magnetic map of the Earth.

The Radical Pair Mechanism

A radical is any atom or molecule with one or more unpaired valence electrons. All electrons have intrinsic angular momentum or 'spin' (Pauli, 1924), quantised by the quantum number $S = \frac{1}{2}$. The electron may exist in one of two quantum states, dictated by the magnetic quantum number $M_S = \pm 1$, and regarded as spin up and spin down respectively. The spin of an electron can be represented by an angular momentum vector. Figure 1 illustrates such spin up and spin down states, with a magnetic field applied in the *z*-direction. The Uncertainty Principle dictates that no two directional components of the angular momentum of an electron may be known simultaneously. Thus, in knowing the length of the spin vector and its magnitude along the *z*-direction, the x and y components remain unknown. For this reason the spin vector is represented as precessing about the *z*-direction.

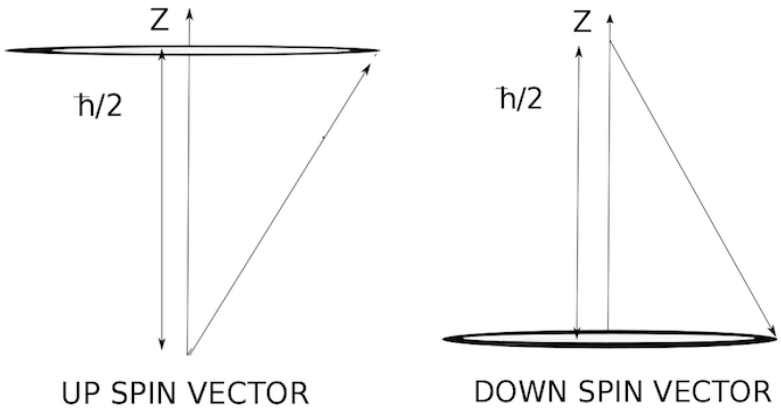

Figure 1. *A vector representation of the spin up and spin down states of the electron.*

A radical pair (RP) may be formed in a spin correlated singlet or triplet state. In the singlet state, the spin vectors of the unpaired valence electrons associated with each radical point in opposite directions. The total spin of the pair sums to zero. In the triplet state, the spin vectors point in the same direction and the net spin is one. Wigner (1927) first introduced the concept of spin conservation in elementary chemical reactions. Spin conservation has been proved (Guo *et al*., 2011) to be at play in chemical reactions such as electron transfer and bond cleavage and features as the key assumption (Tiersch *et al*., 2012) in explaining spin chemical effects. Thus, assuming spin is conserved in RP formation, the state in which the RP is generated will match the state of the precursor molecules. A simple radical pair reaction scheme was outlined by Rodgers in 2009 and may be understood in a number of simple steps. The involved magnetoreceptor molecules have yet to be identified and will be referred to as precursor molecules A (acceptor) and D (donor) for the purpose of this review.

Ground state precursor molecules A and D exist in either a singlet (*S=0*) or triplet (*S=1*) state. The photo-excitation of molecule D results in the transfer of a single electron to molecule A and the creation of a radical pair (RP). Each radical possesses one unpaired electron, and the pair is formed in either a singlet or triplet state, matching that of the precursor molecules.

Singlet and triplet radical pairs (RPs) are chemically different and react to produce different products with different reaction rates. Singlet RPs undergo spin-selective reactions to produce singlet products, with reaction rate K_S. The coherent evolution of the spin state of the RP competes with such reactions, and acts to interconvert singlet and triplet RPs. This singlet to triplet interconversion is driven by magnetic interactions within the RP and with an external magnetic field. Triplet RPs undergo

their own spin-selective reactions to produce triplet products with reaction rate K_T. The key feature of the radical pair mechanism in the context of magnetoreception is the singlet to triplet interconversion (Rodgers, 2009). An externally applied magnetic field has been shown (Schulten *et al*., 1976), to effect the singlet and triplet product yields by altering the rate of singlet to triplet interconversion.

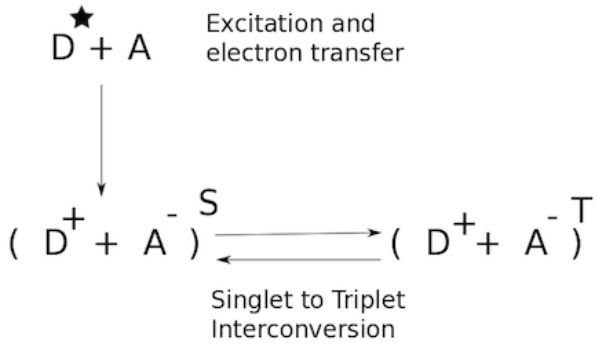

Figure 2. *Adapted from Ritz et al., 2000) A schematic representation of the radical pair mechanism, illustrating radical pair formation and singlet to triplet interconversion.*

Magnetic Field Effects

The manner in which an external magnetic field as weak as 50 μT may affect the spin evolution of a radical pair is one of the key features of the proposed chemical compass. Figure 3 provides a vector representation of the singlet and triplet states of a radical pair. For the purposes of this review, the respective radical spin vectors will be referred to as $\vec{S}_1$ and $\vec{S}_2$. In the presence of an external magnetic field $\vec{B}$, $\vec{S}_1$ and $\vec{S}_2$ precess about the *z*-component of the field at the Larmor frequency (Larmor, 1987).

$$\omega = g\mu_B B \qquad (1)$$

ω is the Larmor frequency, μB is the Bohr magneton, B is the magnitude of the magnetic field and g is the electron spin g factor. This is a dimensionless quantity

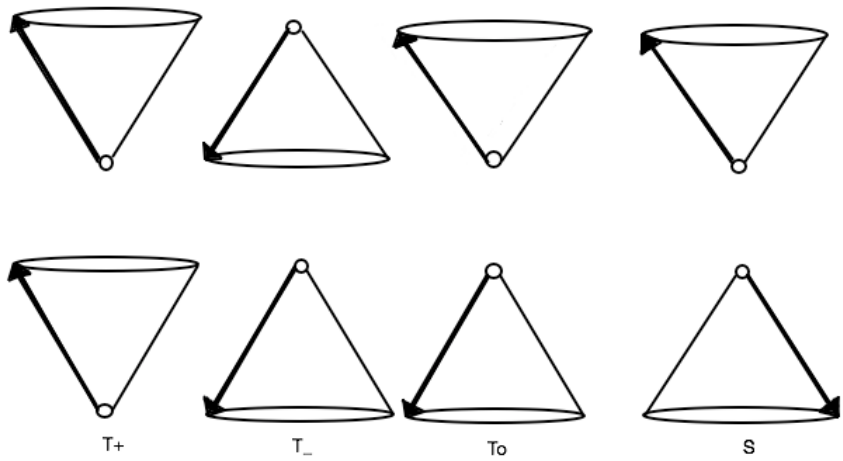

Figure 3. *(Adapted from Steiner, 2007) A vector representation of the radical pair spin states, illustrating the triplet states T_+, T_0, T_- and the singlet state S.*

The singlet state of the radical pair is understood to be the state in which $\vec{S}_1$ and $\vec{S}_2$ precess 180 degrees out of phase and point in opposite directions such that the state has a net spin of zero. With respect to the triplet state, the Zeeman effect lifts the three-fold degeneracy (Zeeman, 1897), and the spin vectors are quantised along the *z*-component of the magnetic field with spin quantum numbers $M_S = -1, 0, 1$ (T_-, T_0, T_+). The associated energy of interaction, ΔE, is given by

$$\Delta E = g\mu_B M_s B \tag{2}$$

The triplet states T_+ and T_- are understood to be the states in which $\vec{S}_1$ and $\vec{S}_2$ are coplanar and precess in phase. The Zeeman interaction shifts both states away from state T_0, with state T_+ increasing in energy and T_- decreasing in energy. State T_0 remains unshifted and so degenerate with the singlet state. The *z*-components of the spin vectors sum to zero, however a resultant spin vector of unit length in the *xy* plane exists, illustrated by noticing that the *x* and *y* components in Figure 4(a) do not sum to zero.

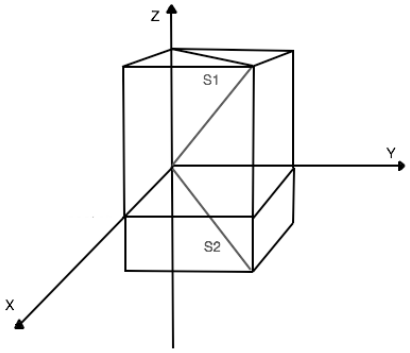

Figure 4a. *Illustation of the triplet state* T_0. *The z-components of the spin vectors may cancel, yet a spin vector of unit length in the xy plane may exist.*

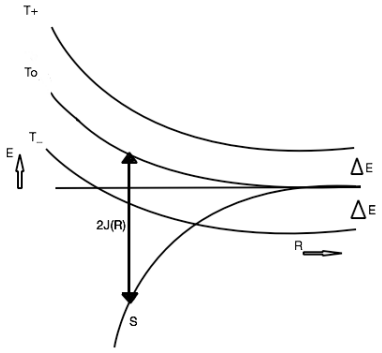

Figure 4b. *(Adapted from Valko, 1988) Illustration of the exchange energy as a function of radical pair separation.*

In order to assess the manner in which an external field may effect the radical pair (RP) mechanism, it is first assumed that the spin correlated valence electrons do not interact. The exchange interaction between two unpaired electrons, if included, would lift the degeneracy of the *S* and T_0 states, separating them by a large energy gap and thus inhibiting any singlet to triplet interconversion. The strength of this interaction falls off exponentially with increasing RP separation, as illustrated in Figure 4(b). The following model, in neglecting this effect, assumes that the RPs are appropriately separated such that interconversion may be facilitated and the exchange interaction may be neglected.

In the context of investigating weak field effects (50 μT), the spin coherent evolution of a radical pair (RP) occurs by virtue of the hyperfine mechanism (Brocklehurst *et al.*, 1995). Hyperfine coupling is an interaction between an electrons total angular momentum $\vec{J}$ and the nuclear spin $\vec{I}$ whereby the nuclear magnetic moment generates a local magnetic field. In the absence of an external field, spin vectors $\vec{S}_1$ and $\vec{S}_2$ precess about the *z*-component of this effective hyperfine field at the Larmor frequency. Each radical may contain several nuclei, and the effective hyperfine field is the vector sum of all individual hyperfine interactions.

It is often the case that the unpaired valence electron associated with each radical experiences a different effective hyperfine field. In this situation, the spin vectors $\vec{S}_1$ and $\vec{S}_2$ precess at different frequencies. Beginning with a singlet-born RP, after time Δt, a phase relationship is reached whereby $\vec{S}_1$ and $\vec{S}_2$ precess in phase. A time $\Delta t'$ later, $\vec{S}_1$ and $\vec{S}_2$ will precess completely out of phase. This process is referred to as spin dephasing, and the oscillation between the singlet state and triplet state T_0 is known as singlet to triplet interconversion, as illustrated in Figure 5(a).

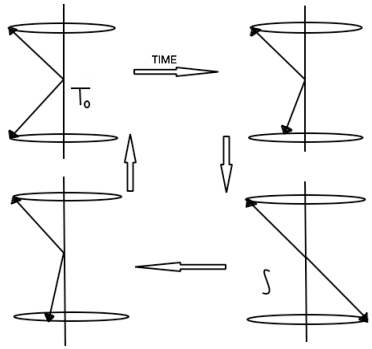

Figure 5a. *(Adapted from Steiner, 2007) Interconversion between the singlet state S and the triplet state* T_0. *Illustrated using the vector spin model in order to highlight the process of spin dephasing.*

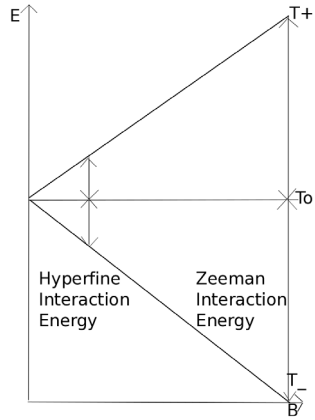

Figure 5b. *Zeeman splitting of the triplet states as a function of magnetic field strength. The extent of the energy shift experienced by states* T_+ *and* T_- *increases with increasing magnetic field.*

In the presence of a weak external field (strength less than that of the hyperfine field), the radical pair spin vectors $\vec{S}_1$ and $\vec{S}_2$ precess about the vector sum of the external and hyperfine field. The Zeeman effects lifts the degeneracy of the triplet states, shifting T_+ and T_- away from T_0. Provided that the interaction energy associated with hyperfine coupling is greater than that associated with the Zeeman effect, interconversion between the singlet state and all three triplet states is facilitated. As a consequence, singlet to triplet interconversion is enhanced and the triplet yield is maximised.

It should be noted that the interconversion between S and $T_\pm$ requires a simultaneous flip of the nuclear and electron spins. In order for this to occur, a magnetic field in the xy plane must act on the electron spin vector. Hyperfine interactions with surrounding nuclei often provide the necessary torque for such a spin flip. Additionally it should be noted that if both unpaired electrons experienced exactly the same local magnetic fields, no interconversion would be possible.

In addition, the spin coherent evolution of a radical pair may be effected by the Δg mechanism (Boxer *et al.*, 1982). The Δg mechanism enables interconversion between the singlet state and triplet state T_0 only. This mechanism relies on differences in g factors between the spin correlated valence electrons. Differences in g factor result in different precessional frequencies of the spin vectors $\vec{S}_1$ and $\vec{S}_2$ and thus facilitate spin dephasing. Differences in g factor are generally small (typically one part in

10^3 or 10^4 in organic free radicals (Brocklehurst, 1995)) and so strong magnetic fields are required for this mechanism to have a noticeable effect. As a result of the strong field requirement, the interaction energy associated with the Zeeman effect will be larger than that associated with hyperfine coupling, and interconversion between the singlet state and $T_{\pm}$ is inhibited. Strong fields thus act to minimise the triplet yield. Since this mechanism is known to dominate in large magnetic fields, the effects may be neglected in a model for the avian compass.

In order to describe the state of the radical pairs a spin Hamiltonian is constructed, assuming that the spatial and spin components of the radical pair wave function may be decoupled.

$$H_{RP}(\vec{B}) = g\mu_B \vec{B} \cdot (\vec{S}_1 + \vec{S}_2) + g\mu_B (\sum_i^a A_{1i} \vec{I}_1 \cdot \vec{S}_1 + \sum_j^b A_{2j} \vec{I}_{2j} \cdot \vec{S}_2) \qquad (3)$$

The first two terms account for the Zeeman interaction, and the final two terms for the hyperfine interaction. A_{1i}, A_{2j} are the respective hyperfine coupling tensors, a and b are the numbers of nuclei in each component of the radical pair and $\vec{I}_{1i}$ and $\vec{I}_{2j}$ are the respective nuclear spins of radicals 1 and 2. In weak external fields, the Δg mechanism may be neglected such that a single value for the g factor may be used.

Schulten *et al.* (1976) demonstrated that the radical pair (RP) mechanism is sensitive to the strength of an external magnetic field. However, in order to act as a source of directional information, a significant difference in the effects of an external field on RPs must exist, and have different orientations with respect to the field. Ritz *et al.* (2000) proposed and proved that if the hyperfine coupling tensor is anisotropic, the RP will be sensitive to different alignments of the magnetic field. This is unlikely to occur for solution based reactions, as tumbling tends to average any isotropic responses. It was thus concluded that the involved radicals should be oriented and immobilised. The dependence of the triplet yield on the angle between the *z*-axis of the RP and the axis of the magnetic field is illustrated in Figure 6(a).

The viability of the radical pair (RP) mechanism model in the context of a biological system must be evaluated. The energy of magnetic interaction per particle involved in the RP mechanism is much smaller than the average thermal energy per particle, $K_B T$. K_B is the Boltzmann constant of value 1.3806 x 10^{-23} $m^2\ kg\ s^{-2}\ K^{-1}$ and T is the temperature, on average circa 300K in biological systems. This does not imply that the effects of the magnetic field become undetectable, since the spins of the electrons bound to the biomolecules are not coupled strongly to the thermal bath. The lifetime of the RPs also plays an important role. Fast decay rates (>10μs^{-1} (Ritz *et al.*, 2000)) of the RPs will not allow sufficient time for the external field to effect the rate of singlet to triplet interconversion. The sensitivity of the RP mechanism increases as the lifetime of the RP increases, as illustrated by Figure 6(b). Given

the significance of the decay rates, the values assumed by decay rates in biological systems must be assessed. Decay rates as slow as 1μs-2μs have been realised in biological systems, as well as proved by Mohtat *et al.* (1998) to allow sufficient time for magnetic field induced singlet to triplet interconversion.

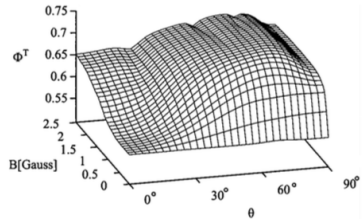

Figure 6a. *(Ritz et al., 2000) The dependence of triplet yield Φ_K on the orientation of the external field with respect to the radical pair is illustrated.*

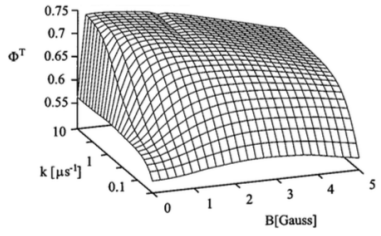

Figure 6b. *(Ritz et al., 2000) The dependence of triplet yield Φ_K on the decay rate k is illustrated.*

Finally, the manner in which the radical pair (RP) mechanism may interact with the visual pathway must be investigated. One suggestion put forth by Ritz *et al.* (2000) is that the RPs may be orientationally fixed on the retina of a bird's eye such that different head orientations of the bird would result in different orientations of the radicals with respect to the magnetic field of the Earth. Different head orientations would thus result in different product yields. Provided that the product yields may affect the sensitivity of light receptors in the eye, this modulation in sensitivity would result in a response pattern that varies over the hemisphere of the eye. A response pattern of light and dark from which the bird may gain directional information would thus be generated. Such a mechanism has been mathematically modelled (Ritz *et al.*, 2000), and could in theory produce a pattern (Figure 7(b)), which may be utilised as a "magnetic map". This suggestion is purely speculative as the manner in which the RP mechanism effects the visual transduction pathway is not yet fully understood.

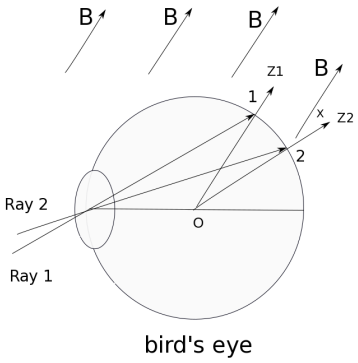

Figure 7a. *(Adapted from Ritz et al. 2000) Illustration of the manner in which radical pairs immobilised on the retina may be influenced by the orientation of the magnetic field of the Earth.*

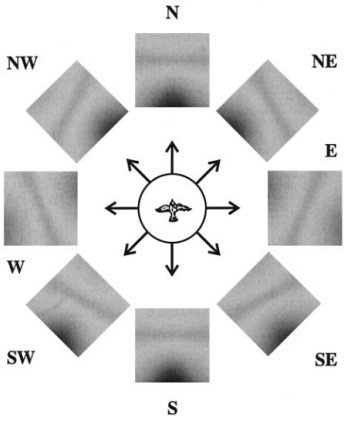

Figure 7b. *(Ritz et al. 2000) Visual representation of the modulation pattern which may be utilised by a bird as a 'magnetic map'.*

Conclusions

The quantum mechanical model of the avian compass has been gaining credibility as evidence mounts in its favour, however the model is by no means complete.

Firstly, the chemicals involved in the radical pair mechanism have yet to be identified, although the protein cryptochrome which has been located in the eyes of several species of migrating bird holds much promise for future research (Solov'yov *et al*., 2007).

Secondly, the point at which the radical pair mechanism is involved in the visual transduction pathway is not fully understood, and requires more research in the area of Physiology.

Additionally, as an inclination compass is unable to distinguish North from South, the radical pair mechanism alone would not be sufficient to enable navigation. It is likely that the radical pair mechanism is working in conjunction with other senses. For example, the beaks of many birds have been shown to contain the magnetic mineral magnetite, which has been suggested to be linked to magnetoreception (Kirschvink *et al*., 2001).

While still in its infancy, this model acts to explain many of the experimentally determined features of the avian compass and offers a viable mechanism by which a migrating bird may utilise the geomagnetic field as a source of directional information. A full understanding is within reach and will allow advancements in nanotechnology, as well as offer an insight into how weak magnetic fields influence biological systems, an area of concern in the health industry.

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TS
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Quantum Encryption: Unconditional Security for the Information Age

Jeffery Lyons
Junior Sophister
Physics

Current encryption methods such as public or asymmetric key encryption utilize the computational difficulty inherent in performing certain types of tasks to make third party decryption computationally impossible. However, the rise of quantum computing, which would render encryption methods such as asymmetric key distribution obsolete, necessitates the development of a new and perfectly secure method of encryption. Quantum encryption or quantum key distribution is a method of encryption which relies on fundamental quantum mechanical properties to ensure the unconditional security of information. Two main protocols have been developed by Bennett, Brassard and Ekert for the implementation of quantum key distribution. The details of these protocols are investigated as well as the practicalities of implementing them.

Introduction

Cryptology is defined as the science of rendering messages sent between two parties unintelligible to any external observers known as adversaries. Encryption refers to the exact process by which this is achieved. Specifically, it is the use of an algorithm to combine the original message with an additional piece of information common to both the sender and receiver, known as the key. The key allows the sender to encrypt the message for communication over unsecure channels assumed accessible to adversaries. The party for which the message was intended,

termed the receiver, can then use the key to decrypt the message. In cryptology the sender and receiver are often referred to as Alice and Bob respectively while any present adversary is known as Eve. This formalism will be used throughout the review.

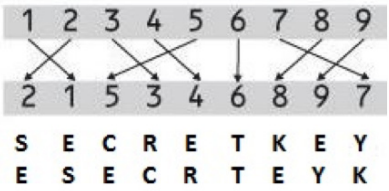

Figure 1. *(Adapted from Skelton, 2015) A transposition cypher. The key used to encrypt the message is the form of the transposition. If the receiver knows how the letters have been transposed the message may be decrypted.*

A cryptographic system where both Alice and Bob are in possession of the same key is known as symmetric key encryption (SKE). SKE is efficient for fast and secure communication of large amounts of information. Alice and Bob can only communicate securely through open channels if they share the same key. This process relies on the successful exchange of the key on which the encryption is based.

Figure 2. *(Adapted from Skelton, 2015) Symmetric key encryption. Both the sender and receiver are in possession of the same key and use this to encrypt and decrypt the message.*

The development of public key encryption and the RSA cryptosystem offers a solution to the problem of key distribution (Rivest *et al*., 1978). As opposed to SKE, public or asymmetric key encryption (AKE) uses two separate keys to construct a secure cipher. If Bob desires to communicate with Alice he chooses a private key which is then distributed throughout the open channels. Any message sent to Bob is then encrypted using his public key. Bob then decrypts the message using his private key. The asymmetry in this system arises from the 'one-way' nature of the public-private key relationship. The public key may be constructed

from the private key; however the reverse process is not computationally feasible. The security which AKE affords is based solely on the idea of computational complexity. A one-way-function is a function computationally feasible to compute given any input but computationally infeasible to invert given an output. The use of such functions ensures that any attempt to reverse the public key production process is computationally infeasible (Rivest *et al*., 1978).

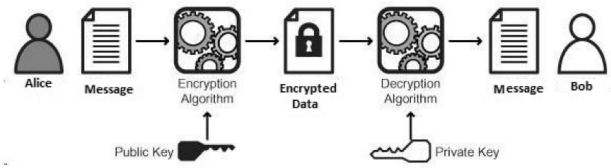

Figure 3. *(Adapted from Skelton, 2015) Public or Asymmetric key encryption. The sender encrypts the message using the receiver's widely available public key. The message may then be decrypted by the receiver using their private key.*

RSA encryption exploits two characteristics of prime products. It is trivial to compute the product of two prime numbers regardless of size. However, it is infeasible to compute the reverse process. This is due to computational time scaling exponentially with the length of the origin primes (Lenstra *et al*., 1992). Therefore, the successful decryption of an RSA cipher in the absence of the correct key would be practically impossible for adversaries. However, it has not been proven that an algorithm capable of completing this task on practical timescales does not exist. The absence of a mathematically rigorous underpinning to RSA asymmetric key encryption means that it is not unconditionally secure (Gisin *et al*., 2015).

Shor's algorithm is one such an algorithm, though it may only be run on a quantum computer (Shor, 1999), a theoretical computing system which makes use of quantum mechanical phenomena to perform operations on data. RSA encryption will be rendered obsolete when a quantum computer capable of running Shor's algorithm is built. A secure method of key distribution must be developed in advance of this eventuality. While quantum computation makes use the characteristics of quantum mechanics to undermine AKE, these same characteristics may provide a provably unconditionally secure method of key distribution known as Quantum Key Distribution (QKD) (Lo *et al*., 2001) (Quan *et al*., 2002).

The unconditional security of QKD arises when the operation of measurement on quantum system is considered. The Heisenberg Uncertainty Principle (HUP) places fundamental limits on the amount of information an observer can have about a quantum system (Heisenberg, 1927).

The HUP states that in performing a measurement on a quantum system only one property out of a pair of conjugate properties can be known with any certainty. It is therefore impossible to have perfect knowledge of the conjugate properties of a system simultaneously (Heisenberg, 1927).

The HUP is utilised by QKD to attain unconditional security. As a consequence of the HUP it is impossible for Eve to make a measurement on a key during distribution without altering it in a detectable way (Wooters *et al*., 2006). Once Bob detects that an adversary has attempted to make a measurement, the key is discarded and a new one is chosen for distribution.

The BB84 protocol

While the concept of QKD as an alternative to traditional key exchange methods was first introduced by Stephen Weisner in 1983, it was not until 1984 that Charles Bennett and Gilles Brassard developed the first practical quantum key exchange protocol.

Alice and Bob are connected by a quantum channel, a channel which preserves quantum states, assumed to be populated by adversaries. Photons are used as message carriers due to their ease of production, detection, and transmittance through optical fibre (Lodewyck, 2005).

The security of the BB84 protocol is based on the HUP. Information is encoded in conjugate states. As per the HUP, a measurement cannot be made on either of these states as this will cause a detectable change in the quantum system.

The BB84 protocol makes use of four non-orthogonal quantum states, which form two conjugate bases to transmit information. The first basis is rectilinear and the states can be represented by ↑ and →. The second basis is diagonal and can be represented by ↗ and ↘. These states correspond to the polarisation direction of the photons. Values are assigned to each state so that results may be tabulated and compared upon detection. The two bases and their corresponding bit values are illustrated in table 1.

To initiate quantum key distribution according to the BB84 protocol an information carrying photon must be prepared by the sender. To prepare a photon for transmission Alice generates a random bit (0 or 1) and randomly chooses a basis to transmit the bit value in (rectilinear or diagonal).

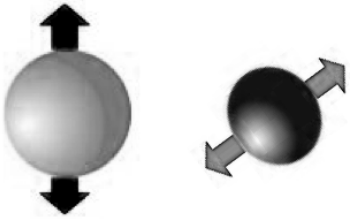

Figure 4. *Photon polarisation directions in the rectilinear and diagonal regimes respectively.*

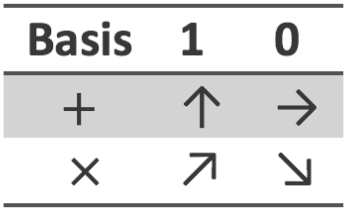

Figure 5. *Photon polarization direction according to chosen basis and bit value.*

Alice then selects a photon with a polarization state that corresponds to both the randomly chosen bit value and basis. The photon is then sent to Bob through a quantum channel assumed to be populated by adversaries. This process is repeated until the key has been exchanged with the details of each photon and the transmission times recorded by Alice.

As Bob has no knowledge of which emission basis Alice transmitted the photons in, he must attempt to detect them using a randomly chosen detection basis of his own. If he measures a vertically polarized photon sent by Alice with a rectilinear basis he will record a bit value of 1. However, on average, 50% of the time Bob will use the wrong measurement basis for the photon resulting in the receipt of a random bit. Since the bases are non-orthogonal, no measurement scheme would be able to effectively distinguish between all four quantum states (Gisin *et al*., 2015). If an observer attempted to measure a diagonally polarized photon with a rectilinear detector, the detector would return a random bit value, with a 50% probability of a 1 or 0.

After transmission and measurement has taken place Alice and Bob communicate openly over an unsecured channel assumed accessible to Eve. They compare the basis they used on each measurement and discard those measurements where Bob chose the conjugate basis to Alice. They do not mention any of the bit values at this point as this would compromise the security of the eventual key. The remaining string of bits is the shared key which is then used to transfer information using SKE.

Table 1. *The exact process by which the encryption key is derived from the transmitted photons is outlined.*

Alice's random bit	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**0**
Alice's random sending basis	+	+	×	+	×	×	+	×
Photon polarization direction	→	↑	↗	→	↘	↘	→	↘
Bob's random measurement basis	+	×	×	×	+	×	+	+
Photon polarization measured by Bob	→	↗	↗	↘	↑	↘	→	↑
Comparison of bases used.								
Shared key.	0	-	1	-	-	0	1	-

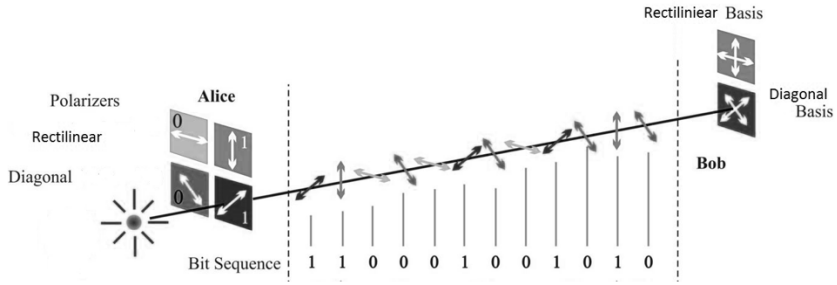

Figure 6. *(Adapted from Swiss Quantum Corp, 2015) The sender randomly chooses an emission basis (rectilinear or diagonal) and a bit value (0 or 1). A photon whose polarisation direction corresponds to the chosen values is sent to the receiver. The receiver then makes measurements using a randomly chosen basis as shown in the diagram.*

To verify the security of the encryption key Alice and Bob now compare a certain subset of the bit string. If they notice a discrepancy in the chosen section of the bit string they abort the key and try again. As a consequence of the HUP, any attempt by Eve to intercept a portion of the key and make a measurement on a certain proportion of the photons will change the nature of the system. Just as making a positional measurement on a quantum particle will cause a change in its momentum, so will a measurement of the photon by Eve cause a change in the photon polarisation received by Bob (Wootters *et al*., 2006). The errors that Eve's measurements introduce into Bob's bit string are easily detectable upon comparison with Alice (Bennett *et al*., 1984).

Through the use of the BB84 protocol, two friendly parties can share an encryption key over unsecured quantum (photon transmission phase) and classical (comparison phase) channels for use with SKE.

The E91 protocol

In 1991 Artur Ekert submitted a new method of QKD for consideration by the scientific community. The theoretical unconditional security of his protocol is entirely underpinned by another set of quantum mechanical phenomena entirely distinct from those utilized by Bennett and Brassard.

The E91 protocol makes use of quantum entanglement to exchange a key in a similar manner to that of the BB84 protocol (Ekert,1991). Instead of Alice sending photons to Bob, a central source creates a pair of entangled photons, sending one to Bob and one to Alice. In a fashion similar to the BB84 protocol, both Alice and Bob choose random bases with which to detect the photons. According to the characteristics of quantum entanglement, for each instance where they both choose the same base, opposite results will be recorded. The results of all measurements are perfectly random. Neither Bob nor Alice can predict whether they will measure horizontal or vertical polarization in the photons provided by the source (Ekert,1991). Both parties then communicate over an open channel and compare the bases chosen for each measurement. When both parties discard the measurements for which opposite bases were chosen, they are left with bit strings that are binary complements of each other. To assemble a private key one party must simply invert the respective bit string.

Any interference by Eve will be immediately detectable upon comparison of the measured polarization directions. A small subset of the key shared by both parties can be compared over unsecure channels. If the strict opposite correlations resulting from the nature of quantum entanglement are not preserved, then Eve has attempted to make a measurement on one of the photons.

Practicalities of implementation

A quantum channel preserves the quantum state of the photons used in the exchange of an encryption key. For both the BB84 and E91 protocols ordinary single mode optical fibre is appropriate as it is widely used and preserves the conjugate quantum states used in BB84 and E91. In practice there will be signal loss which will limit the number of measurable photons arriving at a detector. This directly curtails the key exchange, as the raw key rate is directly proportional to the probability of photon transmission (Hjelme *et al*., 2015). Typical optical fibre transmission loss rates can become problematic over large distances. A typical loss rate for widely used optical fibre is 0.2 dB/km at a wavelength of 1500 nm. At 15 km, a minute distance in the context of modern communication, at least 50% of the photons are lost. This increases to 99% at 100 km (Hjelme *et al*., 2015). Transmission losses of this magnitude limit the practical use of QKD.

Success has been reported with the use of open air as a quantum channel. The longest successful atmospheric transmission of information carrying photons to date is over 144 km between peaks in Gran Canaria (R. Ursin *et al*., 2007).

The practical application of the QKD protocols examined in this paper require reliable single photon sources. In reality single photon detectors represent a significant problem for QKD. Most extant systems rely on faint laser pulsing to produce single photons for transmission. The number of photons present in the pulse is governed by Poisson statistics (Hu *et al*., 2007), which, if examined with regards to the attenuated laser beam, reveal the difficulties inherent in single photon emission. If the beam is attenuated such that there is an average of 0.1 photons per pulse then there is 90% probability that an observer will not measure any photons in the pulse. In addition, there exists a 9% probability that the desired single photon will be observed. A risk is also posed to the security of the key by photon emitters with a high probability of emitting more than a unitary photon. Eve may be able to capture one of the extra photons and wait until the basis information is broadcasted on the open channel (Gisin *et al*., 2007). With knowledge of the sequence of bases used by both parties, Eve may then perform a measurement on the captured photon and reduce the security of the key without possibility of detection.

Single photon detectors are far easier to realise than emitters. Single photon avalanche photodiodes represent the most efficient single photon detection method available for QKD. They operate in a Geiger mode whereby photons entering the detector excite an electron due to impact ionization (Geiger *et al*., 1909). This triggers an avalanche of electrons until the initial excitation is amplified to a level that can be detected as a pulse of electric current by external circuitry.

Conclusions

The central tenet of modern telecommunications is the transfer of information quickly, efficiently and securely. While modern cryptographic methods such as symmetric and asymmetric key encryption currently provide the required security, the prospect of a quantum computer capable of running Shor's algorithm necessitates the development of a new system.

Quantum encryption and QKD promise to provide unconditional security for use with SKE. The quantum physical principles on which these are based cannot be circumvented by a potential attacker and are therefore ideal for use in cryptography. The practical implementation of such systems and the resultant problems constitute the biggest difficulties in the applications of QKD. Such systems are also vulnerable to attack using loopholes in detector design such as the continuous wave illumination attacks.

It is widely believed that these difficulties will be overcome (Jogenfors *et al*., 2015) and a viable QKD system will be built. However, to ensure the security of information, this must occur before RSA encryption is rendered obsolete by quantum computing.

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