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Targeting Ligands and Nanovehicles: A Novel Approach to Lung Cancer Therapy

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Huge advances made in the field of nanotechnology over the past twenty years have opened a wide range of possibilities for innovative new cancer treatments. Ligand-decorated drug delivery nanovehicles are a novel treatment that can offer both therapeutic and diagnostic effects simultaneously. Not only this, but they also bypass the healthy cells due to the active targeting caused by the ligands, and so one does not experience the negative side effects associated with traditional chemotherapy. In this review, a variety of these novel drug carrier systems are examined and compared, as well as another possible diagnostic use of ligands in lung cancer – as a liquid biopsy to quantify circulating tumour cells. This review summarises the effect of the targeting ligand, the synthesis of the nanovehicles, liquid biopsies and the dual purpose of inorganic cores in these nanoparticles, illustrating their potential as viable cancer treatment options in the near future.

Introduction

Lung cancer is the most common cause of cancer death in both Ireland (O'Regan 2014) and the UK (Cancer Research UK 2014). There are two main types of lung cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) – the second of which is

more common (87%). The high mortality rate is primarily due to the difficulty in effective treatment methods currently available. This review explores an innovative form of lung cancer treatment, which involves the inhalation of drug-loaded nanoparticles directly into the lungs. In conventional cancer treatment, when the drug is administered it can attack both cancer and healthy cells without specificity, resulting in the side effects one might ordinarily associate with chemotherapy, such as hair loss, nausea and fatigue. However, the drugs encapsulated in nanovehicles will still be internalised by both cancer and healthy cells. It was in the late 1890's that Paul Ehrlich (Nobel Prize for Physiology 1908) proposed the idea of 'magic bullets' – drugs that have a special affinity for the disease cells they are designed to treat. Combining Ehrlich's idea with the recent advancements in the field of nanoscience, it is now possible to accessorise drug loaded nanovehicles with ligands that will bind preferentially to the cancer cells. This process is termed 'active-targeting'. It is the means by which these ligand decorated drug loaded nanoparticles accumulate and bind to tumour cells only, thus preventing the unwanted side effects observed in traditional chemotherapy (Ehrlich 2014).

This review examines the use of the epidermal growth factor receptor ligand as an active targeting moiety on the surface of the nanovehicles, the nanoparticles chosen as drug delivery systems and the use of the folate receptor ligands as a liquid biopsy in the diagnosis of NSCLC. Lastly, the new and innovative manipulation of nanovehicles as a theranostic device will be reviewed. In this case, theranostics refer to the means by which a nanovehicle can act as both a drug delivery system, and a biological imaging agent.

The use of active targeting are two vital components – the first is the attachment of an active targeting ligand, to the surface of the nanoparticles, such that it will bind selectively to receptors present on cancer cells. The second step of this treatment involves the internalisation of the nanoparticles into the cancer cell, whereupon it releases the drug it has been carrying in order to kill the cell itself.

The ligand most commonly used in the design of nanoparticles for the treatment of lung cancer are EGFR (Epidermal Growth Factor Receptor) ligands, as it is the EGFR that is expressed at significantly

higher levels in NSCLC cells compared to healthy lung cells, according to Fujino *et al.* (1996) and Rusch *et al.* (1996). The EGFR ligands are covalently bound to the outer surface of the nanoparticle drug vehicle so that the drug carriers will only interact with tumour cells. The use of other ligands, such as the Folate Receptor (FA) and its use in capturing circulating tumour cells (CTC) in the early stages of NSCLC will also be examined. Similar to EGFR, the folate receptor is produced at high levels on lung cancer cells, and at much lower levels on healthy cells (Thomas *et al.* 2013).

Nanoparticles can be used as drug delivery systems (DDS) in the treatment of lung cancer, and can be administered intravenously or directly into the lungs by inhalation. The use of inhalation as a drug delivery method is suited especially to lung cancer, as the drugs are being directly applied to the area they are required to treat. However, the toxicity of the nanoparticles themselves presents an issue. Silica, a material used in a number of the nanovehicles discussed in this review, is toxic at both high dosages and high concentrations (Lin *et al.* 2006). Problems may arise with residual nanoparticulate material, in which silica nanoparticles remain in the system and prove toxic to healthy cells. Long term effects of the nanoparticles in the body have not yet been addressed, and long running clinical trials are needed in this area.

A variety of these nanovehicles are described in this review, each with similar biochemistry, showing the versatility in this method of treatment. An interesting subset of these nanoparticles that feature inorganic cores which have imaging capabilities will also be looked at. These special nanoparticles can be used in a new branch of medicine – theranostics – in which a drug may be administered to treat a disease, and simultaneously track the progression and effectiveness of the drug.

The Use of EGFR as an Active-Targeting Moiety

Epidermal Growth Factor Receptor is overexpressed on the surface of NSCLC cells, and only in small quantities on healthy cells, (Fujino *et al.* 1996). In designing ligands that will bind selectively to EGFR

and attaching them to nanovehicles containing an anticancer drug, such as paclitaxel or doxorubicin, cancer cells alone can be targeted. The use of the targeting ligands prevents drug expulsion occurring at any other site in the lungs or body – the result of a random drug release could be that the cancer cells will develop resistance to the anti-cancer agent, through a mechanism known as multi-drug resistance (Persidis 1999). There are a number of variations of the ways in which EGFR can appear as an active targeting ligand on the surface of nanoparticles.

In a study by Tseng *et al.* (2007), biotinylated EGF is used on a gelatin nanoparticle to target A549 adenocarcinoma cells in mice. The GP-Av-bEGF drug was nebulised to generate aerosol particles which were then inhaled. The mice were then sacrificed at different time points and their lungs examined using Xenogen IVIS. Tseng *et al.* (2009) conducted a further study in which they attached the same EGF ligands to gelatin nanoparticles; however, in this case they investigated the capabilities of the nanovehicle to deliver cisplatin (CDDP) via aerosol to the lungs of nude mice. Both experiments drew the same conclusion – the cancer cells alone were targeted and the nanoparticle was internalised into the cell, which was easily observable using bioimaging techniques (Fig. 1).

In another study conducted by Sundaraaj *et al.* (2014), a variation of the EGFR receptor ligands were bound to silica nanorattles. The ligands in this case were generated by using anti-EGFR in EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) to activate the carboxylic acid groups in the epidermal growth factor receptor antibody (EGFRAb). These were consequently bound to the porous gelatin surface of the nanorattles. In addition to these ligands, pyrrolidine-2 was loaded into the pores of the nanoparticles, which acts as a cPLA2α inhibitor (cPLA2α is an enzyme secreted primarily by cancer cells (Sundaraaj *et al.* 2014).) with the intention of more accurately targeting cancer cells. The effect of these ligands was observed, with the H460 lung cancer cells internalising significantly more than the healthy L-132 lung cells. This was determined using confocal microscopy (Fig. 2). As is evident from the results in each of these studies, and similar experiments, the use of targeting ligands, such as EGFR, greatly enhances the specificity of cells attacked.

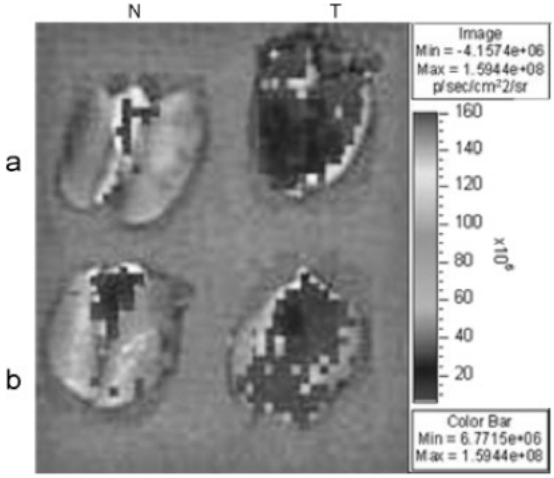

Figure 1: The effect of the targetiang EGF ligand on the lungs of healthy (N) SCID mice, and mice inoculated with A549 tumour cells (T). *a and b correspond to different time points (0.5hr and 24hr incubation respectively). The uptake of the drug by A549 cells is clearly illustrated by the spread of the fluorescence throughout the lung. As is seen in the healthy cells, the nanoparticles stay localised in the central cavity of the lung and do not get internalised. (Tseng et al. 2009)*

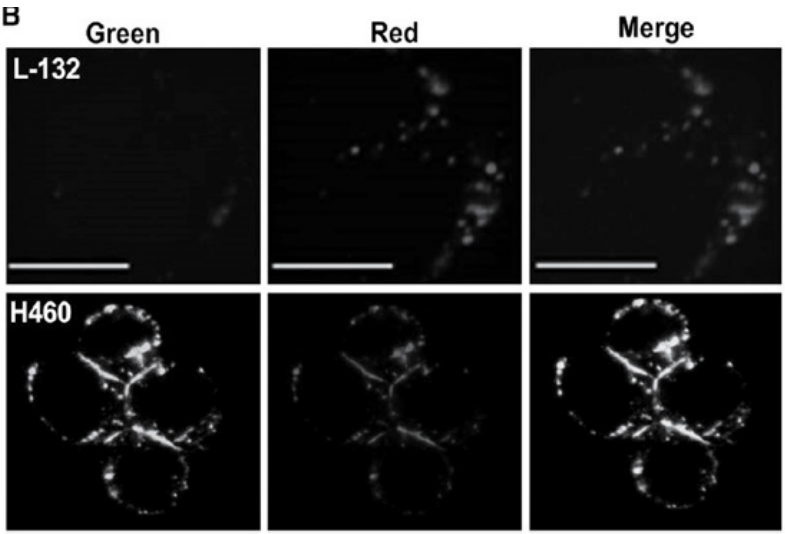

Figure 2: The internalisation of EGFRb/cPLA2α decorated nanovehicles into healthy L-132 cells vs H460 cells. *The nanovehicles were labelled with FTIC, RTIC and observed using confocal microscopy.* (Sundarraj *et al.* 2014)

Folate-Receptor Ligands as Bio-Markers for CTC in the Early Stages of Lung Cancer

In the early stages of any cancer, some tumour cells will be shed from the primary tumour and make their way into the bloodstream – these are known as circulating tumour cells (CTCs). Although these CTCs are relatively rare, they can be used as a 'liquid biopsy' – a means of diagnosing cancer non-invasively, resulting in less trauma for the patient (Alix-Panabie & Pantel 2013). This diagnosis can be achieved through the use of folate receptor ligands. Folate receptors are produced at high levels on NSCLC CTCs and at negligible levels on healthy cells (Parker *et al.* 2005, Thomas *et al.* 2013).

It was proposed that these FR-positive cells could be biomarkers in NSCLC (Yu *et al.* 2013), based on a polymerase chain reaction (PCR) that arises from targeting the ligand. It was found that this method was far more effective in diagnosing CTCs than the five more commonly used bio-markers, CEA, NSE, cyfra-21-1, CA125 and SCC Ag, and was second to only cyfra-21-1 in the detection of CTCs in the early stages of NSCLC. This illustrates that it is a satisfactory and viable biomarker. Similar results were found by Lou *et al.* (2013), although they also compared the results obtained with EpCam enrichment method of quantifying CTCs and found that the FR ligands had a significantly higher sensitivity. However, they also noted that Cyfra-21-1 was still the most sensitive detector of CTCs, although it is still not sensitive enough to quantify CTCs in the early stages of NSCLC. Discrepancies between different studies also came to light – while the study by Lou *et al.*(2013) found that folate was produced in similar levels across both squamous cells and adenocarcinoma cells. It was found in significantly higher levels on adenocarcinoma cells by O'Shannessy *et al.* (2012) and also by Nunez *et al.* (2012). This is indicative that greater research and broader based clinical trials are necessary in this novel method of diagnosis. However, these results illustrate the possibility of using and developing folate receptor ligands as biomarkers in NSCLC.

Ligand Decorated Nanovehicles as Drug Delivery Systems

Many of the side effects associated with chemotherapy are associated with the fact that it is administered freely (i.e. intravenously and not encapsulated) , and so has the capacity to attack and kill any fast growing cell it comes into contact with. One way to achieve the desired specificity and thus by-pass the unwanted side effects is to encapsulate the drug in ligand-decorated nanovehicles. The storage of the drugs within a nanoparticle is what enables the attachment of the ligands, and thus the active targeting. A number of possible nanovehicles have been investigated, including gelatin nanoparticles, silica nanorattles and gold nanoparticles. Gelatin is often chosen as a component of the nanoparticle due to its biocompatibility (Vandervoort & Ludwig 2004). Furthermore, treatment of the gelatin surface with Avidin enables the bonding of a number of biotinylated ligands to the surface of the particle (Coester *et al.* 2000).

This was the premise of a study conducted by Tseng *et al.* (2007), who bound biotinylated EGFR ligands to the surface of a gelatin nanoparticle through the use of NeutrAvidin (Fig. 3). The internalisation of these nanoparticles into adenocarcinoma cells and healthy cells was then compared – it was found that the adenocarcinoma cells that over-expressed EGF (92%) had a much higher uptake rate than healthy lung fibroblast cells (2.2%), and that the internalisation occurred due to a receptor- mediated endocytosis pathway. However, this huge uptake of the nanovehicles by the adenocarcinoma cells may also be partially attributed to EPR (enhanced permeability and retention – a common occurrence in cancer cells). An issue with these nanoparticles is their relatively large size – 220 nm (ideally a nanoparticle must be 100nm or less in order to circulate longer and avoid being destroyed by macrophages), however, this problem can be overcome by delivering the drug via inhalation. The use of inhalation as a drug delivery method is suited specifically to lung cancer, as the drugs are being administered directly to their site of action. This was tested, and found to be as satisfactory as the internalisation achieved by administering the drug intravenously.

Gelatin nanoparticles were also used in a similar study conducted by Tseng *et al.* (2009), except in this study the nanoparticles were infused with CDDP (cisplatin). FTIR spectroscopy was used to examine the chemical composition of the encapsulated drug, and to verify it had not undergone polymorphism. These results indicate that although there is some bonding interaction between the cisplatin and the silica shell, the bond is easily reversible and does not affect the anti-cancer effect of the drug. It was ultimately found that this method of drug delivery using cisplatin was superior to the administration of the free drug (Fig. 1), illustrated by the IC_{50} (the concentration of the drug required to inhibit half of the cancer cell activity) being 2.1 times lower than that of the free drug. It is clearly evident that the encapsulation of anti-cancer agents has a variety of benefits, and, as such, deserves further investigation and clinical trials.

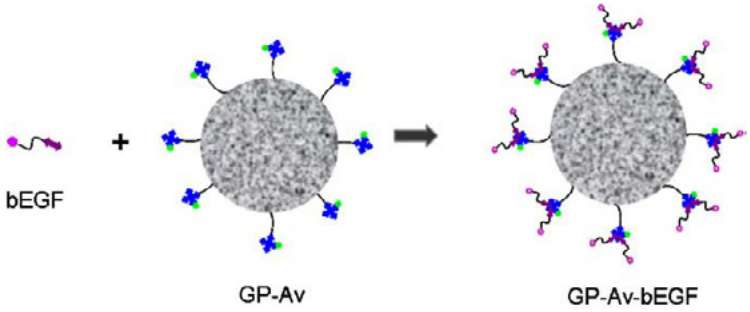

Figure 3: *An overview of the synthesis of EGF-decorated silica nanoparticles. (Tseng et al. 2007).*

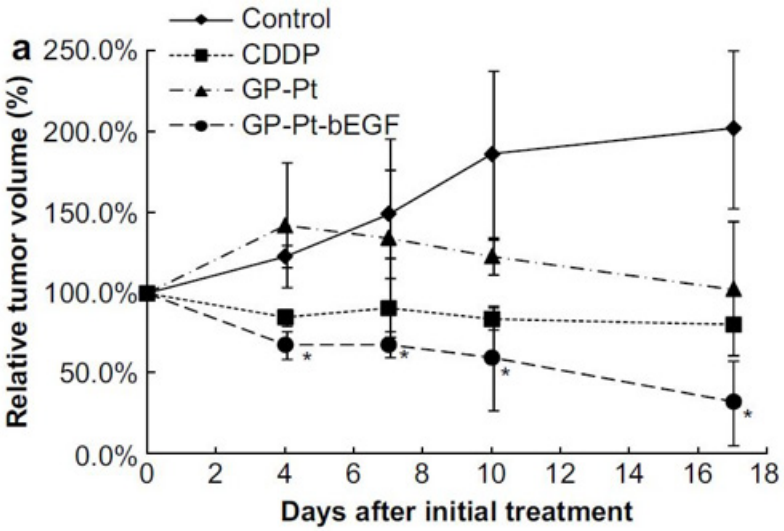

Figure 4: The superior anti-tumour effect of cisplatin encapsulated in an EGF decorated silica nanovehicle. (Tseng et al. 2009)

The Use of Nanoparticles as Imaging and Drug Delivery Agents

The use of an iron oxide core in the ligand decorated nanovehicles opens the door to a new realm of clever medicine – theranostics. Super paramagnetic iron oxide nanoparticles are an excellent MRI contrast agent, particularly in the diagnosis of solid tumours, due to their low cytotoxicity, high magnetic signal strength, low sensitivity to surrounding water molecules and long lasting contrast abilities (Rosen *et al.* 2012).

The idea to incorporate iron oxide into ligand decorated nanovehicles was first tested by Chen *et al.* (2010). The use of iron oxide as a rattle-type centre in mesoporous silica nanoparticles enables the simultaneous delivery of drugs, and imaging of the tumour masses. The functional nanocore also introduces the possibility of magnetically targeting the tumour, further focusing the drug delivery on the cancer cells only. These nanoparticles exhibit a high loading capacity of about 20%, and, in this experiment, the mesopourous

nanoparticles were loaded with doxorubicin hydrochloride (DOX). The enhanced loading effect is due to electrostatic interaction between the silica and the DOX molecules, as well as the large pore size and large surface area. In the same way as many other of the nanoparticles observed in this manner of treatment, it is suspected that the NPs enters the cell via endocytosis, resulting in a higher accumulation of DOX within the cell than one would find from the administration of free DOX. The iron oxide NPs were found to be incredibly biocompatible.

In the same study, spherical gold nanoparticles replaced the ellipsoidal iron oxide nanoparticles in mesoporous silica structures, which were again loaded with DOX. These nanoparticles were also grafted with GD-SI-DTPA, and combining the optical properties of gold nanoparticles (surface plasma resonance enhanced light scattering) with those of GD-Si-DTPA (common MRI contrast agent), dark field light scattering cell imaging can be utilised, as well as MRI (Fig. 5). This was the first in a promising new world of lung cancer treatments.

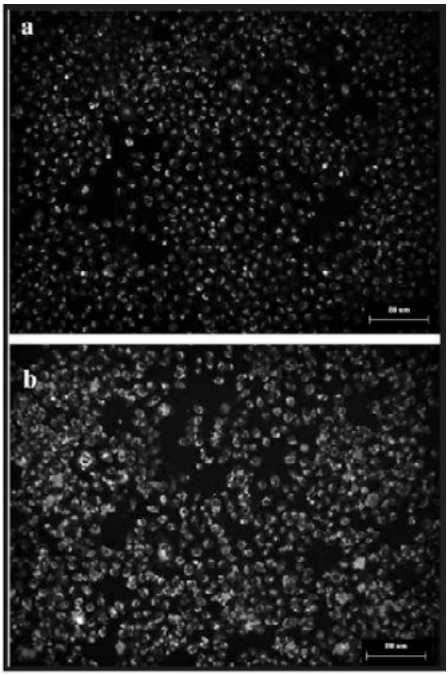

***Figure 5**: Dark field optical microscope images of HeLa cells before (a) and after incubation with Gd-Si-DTPA grafted gold nanoparticles (b). The optical scattering induced by the presence of the gold centred in the nanocapsules can be clearly seen in in second image, showing its possible use in theranostics. (Rosen et al. 2012)*

Conclusions

Ligand decorated nanoparticle drug delivery systems looks to be the future of lung cancer treatment. With the massive advancements made in the field of nanotechnology over the

past few years, drug vehicles such as these are becoming easier and easier to synthesise, and will one day hopefully lead to the effective treatment of lung cancer, and a sharp decline in mortality rates. The use of targeting moieties on the surface of the nanovehicle means that healthy cells are highly unlikely to be incorporate them, as they will bind to receptors present primarily on cancer cells. A number of these possible drug delivery systems also had the potential to be nebulised and administered via inhalation, thus minimising discomfort to the patient and targeting the lung directly. This method showed incredibly satisfactory results. A number of these drug delivery systems were rattle-type silica nanoparticles with a functionalised core, and this opened the door to a range of imaging possibilities. The use of iron oxide in these drug delivery systems means that the therapeutic effect of the drug could be measured as the drug was administered. Furthermore, there also exists a possibility that the drug could be further guided to the tumour masses using magnets. The light scattering properties of a functional gold nanocore were also examined, and demonstrated to be of great use. However, there are a number of issues associated with this form of treatment. There have been very few long term toxicity studies conducted with these DDSs, and there is a need for more clinical trials which test the action of the anti-cancer drugs after their encapsulation into the nanovehicle. A number of specifically acting drugs, such as Iressa and Erlotnib, which act as EGFR inhibitors, are known and widely used in the maintenance therapy of advanced lung cancer. However, resistance to these drugs is an issue (Pao *et al.* 2005). The problem of drug resistance is by-passed through the encapsulation of the drugs, and the decoration of the nanovehicles with targeting ligands ensures the drug will only be released to cancer cells, thus minimising the possibility of drug resistance taking place. The majority of recent research in this area has been conducted using nude mice or in vitro cell cultures, with very few clinical trials taking place. However, the future of ligand decorated nanovehicles for use as drug delivery systems looks promising, and will hopefully result in more efficient treatment and longer life-spans for lung cancer patients.

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