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REVIEW ARTICLE

The polyamine transport system as a target for anticancer drug development

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Abstract The vast majority of anticancer drugs in clinical use are limited by systemic host toxicity due to their non-specific side effects. These shortcomings have led to the development of tumour specific drugs which target a single-deregulated pathway or over expressed receptor in cancer cells. Whilst this approach has achieved clinical success, we have also learnt that targeting a single entity in cancer is rarely curative due to the large number of deregulated pathways, receptors and kinases which are also present, in addition to the target. An attractive alternative to improve targeting would be to harness the already established activity of known anticancer drugs by attaching them to a molecule that is transported into cancer cells via a selective transport system. One possibility for this approach is the polyamine pathway. This review provides a brief overview of the polyamine pathway and how, over the years, it has proved an exciting target for the development of novel anticancer agents. However, the focus of this article will be on the properties of the polyamine transport system and how these features could potentially be exploited to develop a novel and selective anticancer drug delivery system.

Keywords Polyamine transport system · Targeted drug delivery · Anticancer therapy

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Abbreviations

DFMO	α-Difluoromethylornithine
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
ODC	Ornithine decarboxylase
PTS	Polyamine transport system

Introduction

Despite most anticancer drugs possessing potent cell killing activity in vitro, the vast majority in clinical use are limited by systemic host toxicity due to their non-specific actions. This results in a narrow therapeutic index and a limited therapeutic window and, as a consequence, their use in man is restricted. Thus, most anticancer drugs are used as an adjuvant, with few on their own resulting in cure.

These limitations have given momentum to the development of tumour specific drugs. The clinical success of targeting specific receptors or other markers that are over expressed on the surface of human cancer cells has validated this approach. Examples of successful targeting molecules include monoclonal antibodies, peptides, folic acid, hormones and growth factors (Chari 2008).

Whilst this approach has demonstrated that specific targeting of cancer cells is feasible, few of these therapies have resulted in the 'magic bullet' it was hoped this field would deliver. With most of these drugs blocking only one specific receptor, pathway, molecule or kinase, most are cytostatic and not cytotoxic. With it estimated that as many as 300–500 genes are deregulated in human cancer, it is unlikely that targeting a single entity will have a significant effect on the natural history of the disease. An attractive

alternative would be to harness the already established activity of known anticancer drugs by attaching them to a molecule which is transported into cancer cells via a selective transport system. One possibility for this approach is the polyamine transport system (PTS).

Polyamines and cancer

The polyamines consist of spermidine, spermine and the diamine precursor, putrescine (Fig. 1). This small family of low molecular weight aliphatic amines are found in all eukaryotic cells, and are essential for cell growth (Wallace et al. 2003). With this in mind, it is perhaps not surprising that increased polyamine concentrations and deregulated polyamine metabolism are known to play an important role in the development of cancer at all stages from initiation through to maintenance of the transformed phenotype (Martinez et al. 2003). As such, over the years, a number of different agents have been developed which aim to decrease the intracellular concentration of polyamines and produce cell stasis and/or cell death (Casero and Marton 2007). First, several agents that prevent polyamine synthesis through inhibition of the enzymes involved in their synthesis have been found to lower intracellular polyamine concentrations below those values required to support cell growth (for a review, see Wallace and Fraser 2004 or Seiler 2003a). As a means of providing potential anticancer agents, this method yielded limited success because cancer cells corrected for a decrease in polyamine synthesis by increasing uptake of exogenous polyamines via their PTS. Despite this disappointment in terms of cancer chemotherapy, one of these agents, namely α-difluoromethylornithine (DFMO), has been shown to be highly effective in the primary prevention of colorectal cancer, both alone (Love et al. 1998; Meyskens et al. 1998) and in combination with sulindac, a non-steroidal anti-inflammatory drug (Meyskens et al. 2008).

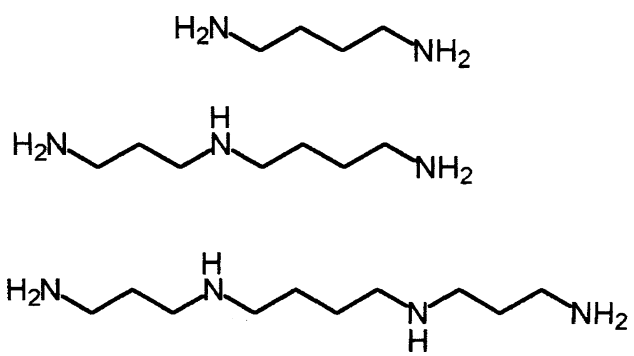

Fig. 1 The polyamines: (*from top to bottom*) putrescine, spermidine and spermine

As a second approach, structural analogues of the natural polyamines were developed (for reviews, see Wallace and Niiranen 2007 or Seiler 2003b). These agents were recognised by cancer cells as polyamine-like (acting as polyamine mimetics or polyamine antimetabolites), and as such, depleted intracellular polyamine content. This was a consequence of both inhibition of biosynthesis and induction of polyamine breakdown and removal. However, because the analogues failed to substitute for the natural polyamines in terms of function, this led to apoptosis; and, in vitro, many of these agents possessed potent cytotoxic actions. One analogue was tested in man, but central nervous system toxicities resulted in the trial being stopped (Creaven et al. 1997).

Whilst these two approaches have yet to deliver an effective anticancer drug, they have provided proof of concept that the polyamine pathway is valuable vehicle for cancer therapy. Despite de novo synthesis being the major mode of polyamine production, transport of preformed polyamines into the cell from the extracellular environment also has a vital role to play.

The polyamine transport system

We all consume large quantities of polyamines everyday. For example, broccoli and cauliflower are high in spermidine, and citrus fruits are rich in putrescine, whilst all three polyamines are found in high concentrations in meat (Eliassen et al. 2002). In addition, the microbial flora of the gastrointestinal (GI) tract also provides an important source of polyamines (Wallace et al. 2003). Indeed, the important nature of transport from these sources in cancer is shown in animal cancer models (Quemener et al. 1994). When exogenous polyamines are eliminated by administration of a polyamine-free diet and by decontamination of the GI tract, biological parameters return to normal along with a significant reduction in metastases in the treated animals. In addition, recent studies with refractory prostate cancer in man have shown that even alone, a simple low polyamine diet can result in improved survival, a better quality of life and a significant decrease in pain scores (Cipolla et al. 2006). The necessity of uptake to cancer cells is further underlined in isogenic cell lines with or without a polyamine transport system (PTS). One of the problems with the aforementioned, DFMO is that it is cytostatic and not cytotoxic; however, in PTS-deficient cell lines, DFMO is cytotoxic as these cell lines are unable to utilise extracellular polyamines to compensate for inhibited biosynthesis (Wallace and Fraser 2004; Wallace and Niiranen 2007). With the polyamine analogues, the converse of this is true, as analogues which are dependent on the PTS for uptake are far less toxic in

the PTS-deficient cell line, when compared to the normal cell line (Le Roch et al. 2002).

Although the importance of polyamine uptake in cancer cells has been known for some time, the mammalian PTS has remained something of an enigma with both the gene and its molecular structure yet to be identified. Despite this obvious drawback, from various kinetic and pharmacological studies, certain parameters are known about the PTS (Seiler 2003b; Seiler et al. 1996). Due to the positive charge they carry, all cells must contain a transport system for exogenous polyamine uptake. It is also known that polyamine transport is carrier mediated, time-, temperature- and concentration-dependent, energy requiring, and saturable. A number of cell types, including L1210 murine leukaemia cells, have a single transport system for uptake of all three polyamines (Porter et al. 1984). However, in most cell types, two transport systems have been recognised (Seiler and Dezeure 1990; Seiler et al. 1996). The first is sodium dependent, and has preference for putrescine, although it is capable of transporting all three polyamines. The second is sodium independent, and is capable of transporting spermidine and spermine (being more selective to spermine), but not putrescine.

Recently, two different models regarding the mechanism of polyamine transport in mammalian cells have been described. The first model describes receptor-mediated endocytosis where polyamines bind to heparan sulphate on a molecule of glypican-1, before being taken into the cell by endocytosis. This is closely followed by a rapid accumulation into polyamine sequestering vesicles (Belting et al. 1999, 2003). The second model proposes that the polyamines are taken into the cell by a membrane transporter requiring an electronegative membrane potential (Soulet et al. 2004).

The important nature of the PTS in cancer cells led to the idea that one could exploit the transport system to improve the accumulation of toxic compounds in tumour cells; and, there are a number of features of the PTS that make this an attractive idea.

Exploiting the PTS: a 'Trojan horse' approach for drug delivery

First, in comparison to normal cells, it has been shown that transformed cells accumulate exogenous polyamines at an enhanced rate (Bachrach and Seiler 1981; Wallace and Kerr 1982). In vivo, radiolabelled putrescine accumulates in brain tumours at a rate thirty times greater than the surrounding normal brain parenchyma (Volkow et al. 1983). Furthermore, human promyelocytic leukaemia cells have an affinity for putrescine, spermidine and spermine between 10 and 200 times greater than normal human polymorphonuclear leucocytes (Walters and Wojcik 1994). In addition, a range of tumour cell lines including rat prostatic tumour cells, neuroblastoma cells, B16 melanoma cells, human colonic and lung tumour cell lines, Ehrlich ascites tumour cells, L1210 murine leukaemia cells and ADJ/PC6 plasmacytoma cells have highly active PTS (Cullis et al. 1999). Second, and more importantly, the PTS is promiscuous. This enables the PTS to transport various polyamine-like molecules (Phanstiel et al. 2007).

Using these features of the PTS, it has been proposed that known cytotoxic drugs can be attached to polyamine vectors and targeted more selectively to cancer cells than they had been previously. As a result, such an approach has quite aptly been likened to the Trojan horse in Greek mythology (Cullis et al. 1999). The polyamine can be attached to the toxin to provide a vector for delivery through the PTS, and in doing so, reduce the toxic effect on normal cells associated with conventional chemotherapy. In addition, the polyamine vector may also enhance the delivery of cytotoxic drugs that act in the nucleus due to the polyamines affinity for DNA (Dallavalle et al. 2006).

DNA targeting vectors: delivery to the site of action

The polyamines are organic cations, and have many interactions with anions within the cell, particularly DNA. They exert this DNA-binding ability through strong, but reversible electrostatic interactions. This allows the polyamines to cause both condensation and important conformational changes within DNA (Pastre et al. 2006). As such, it is believed that cytotoxic drugs attached to polyamine vectors will have enhanced DNA-binding ability. This has been demonstrated with naphthoquinones (Esteves-Souza et al. 2008) and protoberberine (Pang et al. 2007) attached to polyamine vectors and other examples will be referred to later.

Established anticancer agents with polyamine vectors

Probably, the most cited example of a polyamine drug conjugate is work conducted by Cohen's group, who examined the attachment of the established anticancer agent chlorambucil to spermidine (Holley et al. 1992b). Chlorambucil is a nitrogen mustard alkylating agent which has been used in the treatment of chronic lymphatic leukaemia, lymphomas and advanced ovarian and breast carcinomas. Like many alkylating agents, the maximum therapeutic dose which can be administered is limited by haematological suppression. In order to show that this conjugate utilised the PTS, Cohen compared the cytotoxicity of the conjugate to chlorambucil in untreated and

DFMO pre-treated ADJ/PC6 plasmacytoma cells. DFMO is a suicide inhibitor of ornithine decarboxylase (ODC), the first and rate limiting enzyme in polyamine synthesis, and treatment of mammalian cells with DFMO has been shown to result in increased uptake of preformed polyamines via the PTS. Therefore, Cohen related any increase in cytotoxicity as indirect evidence for the conjugate using the PTS to gain entry to the cell. In comparison to non-DFMO-treated cells, pre-treatment of the cells with DFMO resulted in the conjugate being 35- and 225 times more cytotoxic, than chlorambucil alone. In addition, the conjugate, but not chlorambucil successfully competed with spermidine for uptake. The fact that the conjugate was 10,000 times more active than chlorambucil at causing DNA strand breaks demonstrated the ability of this conjugate to successfully target DNA. The enhanced ability of the conjugate to cause DNA strand breaks is the most likely reason for the conjugate being four times more potent than chlorambucil in its ability to inhibit ADJ/PC6 tumour growth in BALB/c mice. However, the therapeutic value was not increased because the conjugate was found to be 10 times more neurotoxic. The most likely explanation for this finding is that the spermidine moiety conferred increased lipophilicity upon chlorambucil due to the positive charge it carries; however, whilst this conjugate ultimately failed, it did demonstrate that the PTS could potentially be exploited for drug delivery.

Extending the polyamine conjugate paradigm

Cohen also examined the attachment of nitroimidazoles to polyamines (Holley et al. 1992a). Several nitroimidazole derivatives constitute the class of antibiotic used to treat anaerobic bacterial and parasite infections, the well-known and most widely used being metronidazole. Anaerobic cells selectively take up these antibiotics by diffusion where they are reduced to compounds which are toxic to cells. Again, Cohen used the subsequent increase in toxicity induced by DFMO as indirect evidence for increased transport of these conjugates into the cell via the PTS. The fact that DFMO had no effect on the equivalent antibiotic (metronidazole and misonidazole) without a polyamine vector provided further evidence for their transport by the PTS. In addition, the conjugate, but not the equivalent antibiotic was able to compete with its native polyamine for uptake. This study was particularly novel, as it demonstrated that toxic moieties not capable of gaining access to mammalian cells could be transported into these cells if attached to a polyamine vector. With many agents known to possess potent anticancer activity, this was particularly exciting.

The natural enediyene antibiotics are one such group of agents and constitute one of the most potent anticancer agents ever discovered. However, a major obstacle to successful clinical application of these antibiotics is their lack of tumour selectivity. In fact, harnessing the powerful DNA-cleaving activity of these molecules is a highly active research area in anticancer drug design. The partial success of Gemtuzumab ozogamicin (marketed as Mylotarg), an anti-CD33 monoclonal antibody conjugated to an enediyene antibiotic (calicheamicin) in acute myeloid leukaemia has shown the potential of this approach (Stasi 2008). As a result, promising attempts to equip enediyenes with a polyamine as the DNA targeting vector have been made. In fact, enediyenes conjugated to all three polyamines have demonstrated potent DNA damaging ability in comparison with the equivalent antibiotic without a polyamine as the DNA-binding group (Suzuki et al. 2004). As expected, the DNA damage induced by these conjugates increased from the putrescine vector all the way through to the spermine vector. The attachment of a polyamine vector to camptothecin, another enediyene derivative, has added further weight to this mode of targeting (Dallavalle et al. 2006). In the human non-small-cell lung cancer cell line, H460, 11 of the polyamine–camptothecin conjugates were more than 90 times more potent (some in the nanomolar range) than topotecan; a camptothecin drug in clinical practice for both lung and ovarian cancer. Disappointingly though, neither group examined how their conjugates interacted with the PTS.

However, a study carried out with tetracyclic amidines conjugated to putrescine has added further weight to this Trojan horse approach for drug delivery (Mens et al. 1997). These compounds were found to have significant affinity for DNA, whilst two isomers of these compounds were found to inhibit tumour cell growth in the Lewis lung cancer cell line, 3LL. Like the previously mentioned studies, these compounds successfully competed with their native polyamine for uptake. In addition, in an isogenic cell line with and without a PTS, the cytotoxicity of these compounds was significantly reduced compared to wild-type cell line, demonstrating that the PTS increased the efficacy of these compounds. Furthermore, in vivo, prior polyamine depletion with DFMO enhanced the cytotoxicity of these compounds further still. The authors speculated that this increase in efficacy might be due to two different factors, either alone or in combination. First, depletion of the intracellular polyamine pools may reduce the number of interactions between the polyamines and DNA leading to increased accessibility for the DNA-binding conjugate; and second, DFMO enhances the polyamine uptake system in cancer cells, increasing the entry of the conjugate.

An intriguing approach employed by Eiseman and co-workers was to incorporate two small moieties of aziridine onto spermine (Eiseman et al. 1998). Azridines are electrophiles, and are attracted to endogenous nucleophiles

such as the nitrogenous bases in DNA base pairs. The addition of aziridine to the spermine molecule would therefore provide it with targeted DNA cross-linking ability, making it an ideal alkylating agent. This compound generates potent cytotoxicity via the induction of apoptosis in the human prostate cell lines, PC-3 and DU-145. Furthermore, treatment with the conjugate prior to radiotherapy sensitised the cells, resulting in a lowering of the apoptotic threshold to radiation.

Cullis and his group used a particularly elegant approach to look at the transport of polyamines and their related cytotoxic conjugates (Cullis et al. 1999). Returning to the study conducted by Cohen, they looked at the attachment of chlorambucil to polyamine vectors. In addition, they synthesised a parallel group of compounds which contained the same polyamine vector and linker, but instead of chlorambucil, they used an analogous fluorescent chromophore, N-methyl anthranilic acid (Fig. 2).

The attachment of the fluorescent chromophore allowed them to use confocal microscopy to examine uptake in greater detail. They reasoned that they could extrapolate their findings with the fluorescent conjugates back to the chlorambucil conjugates due to the similarities in structure. They demonstrated that these compounds were, indeed, taken up by the PTS. Furthermore, they showed that once inside the cell, these conjugates were not evenly distributed within the cytoplasm, but stayed within vesicle like structures, in line with the receptor-mediated endocytosis model of polyamine transport. In addition, they also found that these conjugates stay within the vesicle like structures and do not readily associated with the nucleus. Given that the increased cytotoxicity of this conjugate is believed to be due to its increased DNA-binding ability, this was an unexpected finding. However, it is interesting to note that internalisation by endocytic processes is the preferred delivery method for anticancer drugs, as this avoids the cellular components involved in degradation. Thus, a possible explanation might be that transport via the PTS is providing another benefit and offering the conjugate a degree of protection, which is not afforded to chlorambucil alone. Interestingly, vesicular internalisation of the conjugate does not fit with the feedback inhibition of polyamine uptake observed. The authors noted that inhibition of polyamine uptake is dependent upon free polyamines being detected by the ribosome in order to induce antizyme production. This illustrates the importance of molecular characterisation of the PTS, so that findings like this can be interpreted fully.

More recently, building on the approach used by Cullis, cytotoxic compounds which fluoresce have been attached to polyamine vectors. This has negated the argument that analogous fluorescent compounds, although similar, may behave differently to the cytotoxic conjugate. Phanstiel has examined both the attachment of aminoacridine (Phanstiel et al. 2000) and anthracene (Gardner et al. 2004; Phanstiel et al. 2000, 2007; Wang et al. 2001, 2003a, b) to polyamines, whilst Delcros et al. (2002) has examined the former. A derivative of aminoacridine, amsacrine, is used in the treatment of acute leukaemia and acts by inhibiting topoisomerase II. The three-ringed structure of aminoacridine is believed to be responsible for the ability of amsacrine to inhibit topoisomerase II. As such, the use of the linear three-ringed aminoacridine and anthracene was a rationale choice for the intercalator (Phanstiel et al. 2000).

Both aminoacridine (Delcros et al. 2002; Phanstiel et al. 2000) and anthracene (Phanstiel et al. 2000) demonstrated increased DNA-binding ability and inhibition of topoisomerase II (Phanstiel et al. 2000) when attached to polyamines. In addition, both have been shown to utilise the PTS for uptake (Delcros et al. 2002; Gardner et al. 2004; Phanstiel et al. 2000, 2007; Wang et al. 2003a, b). However, Delcros found that despite the spermine–aminoacridine conjugates having a high affinity for the PTS, uptake was poor. Phanstiel also demonstrated that the spermidine anthracene conjugates, despite being more cytotoxic, had a lower affinity for the PTS than the spermine anthracene conjugates (Wang et al. 2003a). However, Phanstiel used deconvolution microscopy to demonstrate the internalisation of the spermidine conjugates; whereas the spermine conjugates were shown to bind tightly to the cell, but not gain entry, thus explaining their apparent higher affinities for the PTS (Wang et al. 2003b). They reasoned that the increased positive charge of the spermine vectors resulted in tighter binding to other components of the cell membrane, resulting in slowing of their transport. Furthermore, deconvolution microscopy studies added to the growing body of evidence for the receptor-mediated endocytosis model of polyamine transport. More importantly, it also

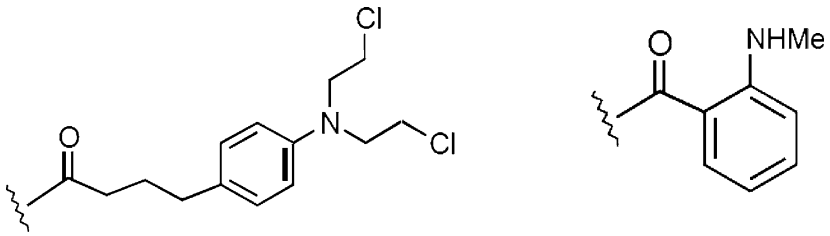

Fig. 2 Chlorambucil (*left*) and the analogous fluorescent chromophore, *N*-methyl anthranilic acid (*right*)

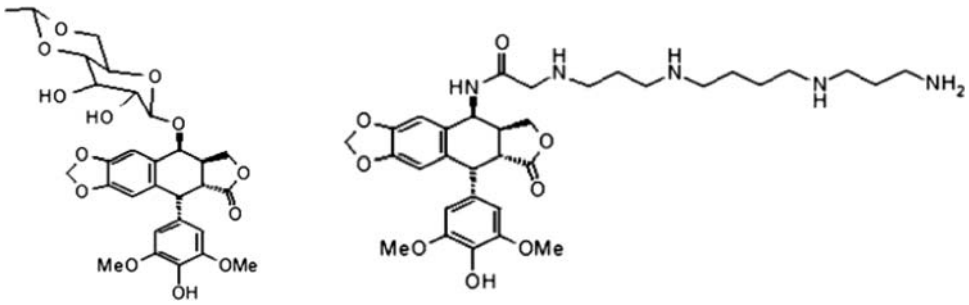

Fig. 3 Etoposide (*left*) and Barret's spermine–etoposide conjugate, F14512 (*right*)

demonstrated the conjugate dissociating from the vesicles, an effect not observed by Cullis. This study has also been particularly important in helping to identify the best vector for delivery and the size of drug that can be tolerated by the PTS.

Work in our laboratory with Phanstiel's anthracene conjugates has started to shed light on their mechanism of action and biological properties. For the first time with one of these conjugates, we demonstrated uptake via the PTS in human cell lines (Palmer et al. 2009). Interestingly, we have found these molecules to have additional effects outwith their original design (delivery) by depleting the polyamine pools (Ghani et al. 2009; Palmer et al. 2009). We believe that this may be due to polyamine analogue like actions, and work is ongoing to determine the effect of these conjugates on the enzymes involved in polyamine metabolism. In addition, we found them to trigger cell death via the induction of apoptosis (Ghani et al. 2009; Palmer et al. 2009), an effect with has been observed by others (Xie et al. 2008). Mechanistic studies revealed that this may be due to DNA damage and oxidative stress (Palmer et al. 2009).

Validation of the Trojan horse approach

Etoposide, discovered in 1961, is still one of the first line agents in the treatment of non-small cell lung cancer, testicular tumours and non-Hodgkin's lymphoma. It is a semi-synthetic derivative of podophyllotoxin, a substance extracted from the mandrake root *Podophyllum peltatum*. In fact, etoposide possesses potent anticancer activity, and is still one of the best inhibitors of topoisomerase II. Unfortunately, like most anticancer agents its promising activity in vitro is limited in vivo by its non-specific actions and side effects. However, Barret and co-authors have recently reported the successful design of a novel, highly potent spermine–etoposide conjugate (F 14512; Fig. 3; Barret et al. 2008). In fact, F 14512 seems to have all the properties hypothesised of the ideal polyamine drug conjugate: increased cytotoxicity and DNA-binding ability over its parent compound, uptake via the PTS and therefore, reduction in toxicity when used in vivo.

In an isogenic cell line with and without a PTS, the cytotoxicity of F 14512 was 73 times greater in the wild-type cell line. Compelling evidence that F 14512 utilised the PTS was demonstrated by it successfully competing with spermine for uptake. Furthermore, in the murine leukaemia cell line, L1210, addition of exogenous polyamines resulted in significant cell rescue. Cytotoxicity studies demonstrated that on average, F 14512 was eight times more potent than etoposide in 21 of the cell lines tested. This may be related to the increased DNA-binding ability of the conjugate, as the spermine vector was found to anchor F 14512 to DNA, a property not shared by etoposide in the absence of topoisomerase II. Ten times the concentration of etoposide was also needed to produce the level of topoisomerase II inhibition observed with F 14512. The potential of this compound in vivo was demonstrated using the MX-1 human breast tumour xenograft mouse model. A dose of 1.25 mg/kg/injection was found to result in partial or complete regression of the tumour. In comparison, etoposide needed to be dosed at 30 mg/kg/injection to achieve the same results; however, this dose also resulted in toxicity, as demonstrated by one-third of mice losing greater than 20% of their body weight. To the best of our knowledge, this is the first report of a polyamine drug conjugate being used successfully. It will be interesting to see how this drug develops in the future, and to see if these initial findings lead to a clinically useful anticancer drug.

The future

Identification of the mammalian PTS will greatly help in the development of polyamine drug conjugates in the future. Currently, in most cases evidence for their uptake via the PTS can only be obtained indirectly. As such, one cannot prove definitely that these conjugates are utilising the PTS for uptake.

However, with the human genome now sequenced, it can only be a matter of time before the mammalian PTS is identified. Even with this 'blind' approach, recent study with etoposide has demonstrated that improved therapeutic indices can be achieved. In addition, as we have seen with

the fluorescent conjugates, such compounds are helping to identify the best vector for delivery and establish the structural tolerance of the PTS.

In conclusion, the idea of selectively targeting cancer cells via the polyamine transport system shows great promise. At present there is no direct evidence for transport via the PTS. However, the mounting indirect evidence and the recent use of deconvolution microscopy to track the cellular localisation of polyamine drug conjugates suggest strongly this will be the case. We believe that, in the future, the idea of selective targeting of cancer cells with effective drugs via the polyamine transport system will lead to the development of a novel and selective anticancer therapy.

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