Probing the mechanism of transport and compartmentalisation of polyamines in mammalian cells

Paul M Cullis, Ruth E Green, Louise Merson-Davies and Natalie Travis

Background: Many mammalian cells possess an active polyamine uptake system but little is known about the molecular mechanism of this transporter. The fate of polyamines taken up from the medium and the relationship to polyamine homeostasis remains to be fully established. The aim of this study was to develop a range of modified polyamines, particularly ligands incorporating a fluorophore, to explore the structural tolerances of the polyamine transport system and to probe the intracellular location of polyamines acquired from the medium.

Results: We synthesised a wide range of polyamine analogues incorporating cytotoxic agents, fluorescent chromophores and bulky substituents. All of these analogues have been shown to be good competitive inhibitors of spermidine uptake in a range of mammalian cells. Direct evidence for uptake of the fluorescent polyamine analogues and their subcellular distribution was obtained from confocal laser scanning fluorescence microscopy, which showed that they accumulated in granular structures within the cytoplasm and not in the nucleus. We demonstrated that their uptake is through the polyamine transport system by showing that pretreatment with DFMO, a potent inhibitor of polyamine biosynthesis, led to enhanced uptake, and cells deficient in the polyamine transport system did not accumulate these polyamine analogues.

Conclusions: The polyamine transport system has a surprisingly broad structural tolerance. Fluorophore-containing polyamine analogues derived from the extracellular pool are located in granular structures within the cytoplasm and not to any great extent in the nuclei of mammalian cells. These observations might be consistent with a mechanism involving receptor-mediated endocytosis, and the granular 'structures' seen might reflect polyamine compartmentalisation within vesicles.

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Introduction

Polyamines are ubiquitous components of all cells [1] and are required to maintain cell growth and differentiation. The critical role of polyamines in cell proliferation has stimulated considerable interest in polyamine metabolism as a new target in chemotherapy [2,3]. Mammalian cells possess an efficient biosynthetic pathway to the three common polyamines, putrescine, spermidine and spermine; polyamine levels are tightly regulated by control of the biosynthesis and degradation of these polyamines. In addition to the biosynthesis of polyamines, many cells have been shown to be capable of taking up polyamines from the extracellular medium. In particular, a number of mammalian tumour cell lines have been shown to contain elevated polyamine levels and high levels of an active polyamine uptake system have been characterised in a number of cells including rat prostatic tumour cells [4], neuroblastoma cells [5], B16 melanoma cells [6], human colonic [7] and lung tumour [8] cell lines, cultured human lymphocytic leukemia cells [9], Erhlich ascites tumour cells [10], L1210 cells [11] and ADJ/PC6 plasmacytoma cells [12].

Notwithstanding an incomplete understanding of the physiological role of polyamines, their association with proliferative responses, cell differentiation and tumour promotion has identified polyamine regulation and biosynthesis as an attractive target for a number of quite different anticancer chemotherapeutic strategies [2,3,13–15]. In this regard we [12] and others [16] have attempted to exploit the polyamine transport systems to deliver and target cytotoxic agents selectively to rapidly proliferating cells (the Trojan horse approach!). The high specific activity of the polyamine uptake systems in tumour cells is thought to be associated with the inability of the biosynthetic enzymes to provide the polyamine levels needed to sustain the rapid cell division; this is partially off-set by scavenging polyamines from the extracellular pool [17]. It is probable that there are multiple polyamine transport systems in mammalian cells with

differing specificities and, furthermore, these systems, in addition to transporting the natural polyamines, are thought to be capable of transporting a significant range of analogues, including methylglyoxal bis(guanylhydrazone) (MGBG) [18], paraquat [19] and N^1,N^{12}-bis(ethyl)spermine [20]. The success of this approach depended on two key components, the ability of the polyamine transport system to recognise and transport significantly modified polyamines, and the ability of these polyamine derivatives to target DNA once internalised within the cell.

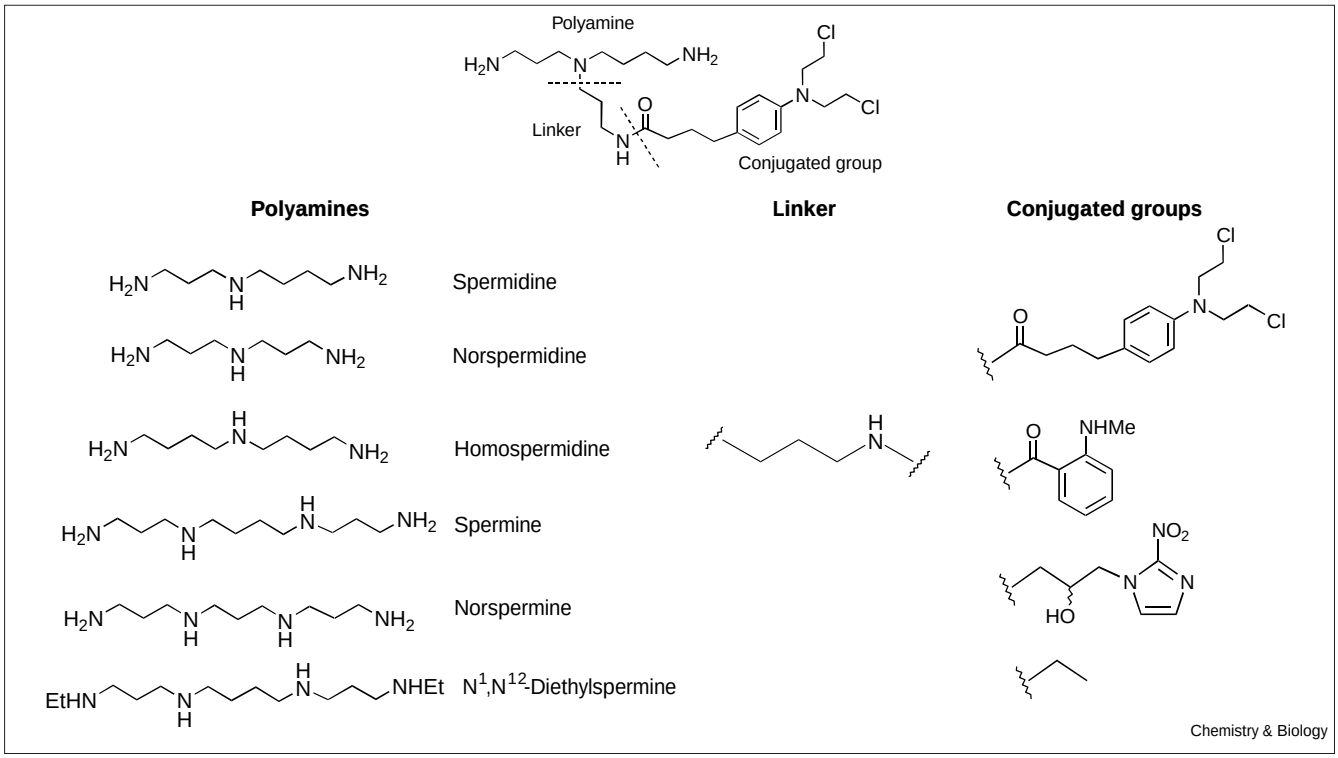
The N^4-spermidine-chlorambucil conjugate **1** [21], proved to be extremely active in *in vitro* experiments. It showed a 10^4-fold increase in cross-linking activity with naked DNA as compared with chlorambucil [22], a 200-fold increase in cytotoxicity in polyamine-depleted cells in culture, but a rather smaller enhanced activity in *in vivo* tumour studies [12]. The attentuation of activity *in vivo* could have a number of explanations: the polyamine transport system could fail to interact with these modified polyamines or transport them rather inefficiently; the polyamine-drug conjugates could be lost through competing hydrolysis [23] or reaction with cellular components other than DNA; the binding of **1** to DNA in cells might be weaker than to isolated DNA; the polyamine-drug conjugate might be prevented from reaching the nuclear DNA and so on. In this study we provide clear evidence that a range of polyamine analogues bind to the polyamine receptor and are transported, which might have implications in terms of the mechanism of the membrane translocation. In order to address the possible intracellular binding and localisation of modified polyamines we have exploited a number of analogues that incorporate the fluorophore N-methyl anthranilic acid (MANT), in conjunction with confocal fluorescence microscopy.

Results

Synthesis of polyamine conjugates

A series of polyamine analogues have been synthesised that contain either the cytotoxic agent chlorambucil or the MANT fluorophore, conjugated to the natural polyamines spermidine and spermine or non-natural structural variants of these (Figure 1). A total of 14 polyamine analogues are considered here that have been generated using six polyamines, a single aminopropyl linker and four different conjugated groups. The conjugates are named as derivatives of the polyamine where the same charged state as the parent polyamine is preserved, and the point of attachment of the linker is signified by the prefix. The structures of these conjugates were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy,

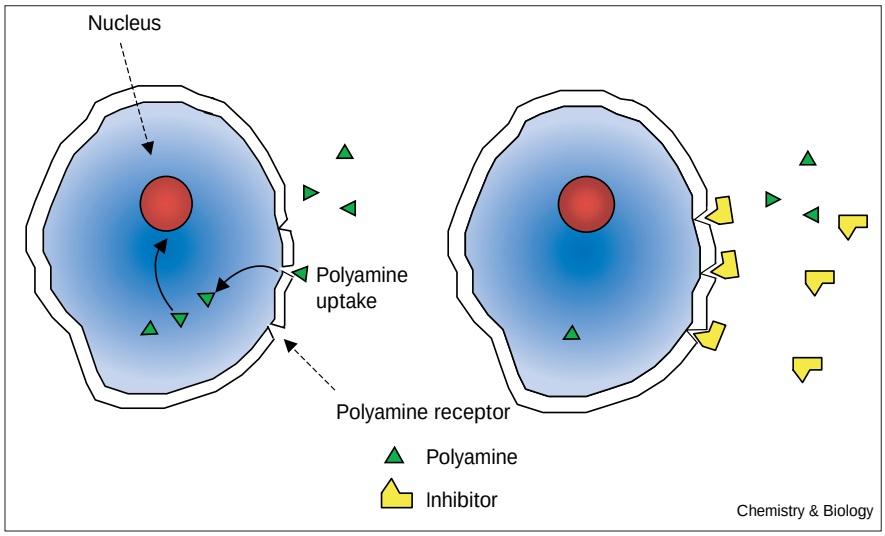
Figure 1



Structures of the various combinations of polyamines, linker and conjugated groups that have been used in the syntheses of 14 different polyamine probes.

Figure 2

Schematic representation of the assay for spermidine uptake in cells and the measurement of competitive inhibition.



high resolution mass spectrometry and the purities confirmed by high-performance liquid chromatography (HPLC) analysis. Full details of the synthetic routes will be published elsewhere.

Inhibition of spermidine uptake

The polyamine transport system in a number of cells has been well characterised in terms of the kinetics of spermidine uptake. Polyamine uptake can be easily monitored by measuring the amount of radioactivity retained in the cellular pellet by scintillation (Figure 2). The uptake of spermidine in A549 cells is linear over 1–2 hours at 37°C after which it rapidly plateaus (Figure 3a). Although nothing is known at the molecular level about the structure of the polyamine receptor, the kinetics of uptake can be analysed by simple Michaelis–Menten kinetics consistent with a single affinity, saturatable polyamine-binding site [12] (Figure 3b). From the data shown in Figure 3b the spermidine transporter in A549 human lung epithelial cells is seen to have a K_M for spermidine of 0.5 μM (standard error 0.076) and a V_{max} of 17 pmol/min/10^5 cells (standard error 0.96). From the radioactivity incorporated into cells at saturation it is possible to estimate that the intracellular concentration of spermidine accumulated from the medium is approximately 1.9 mM.

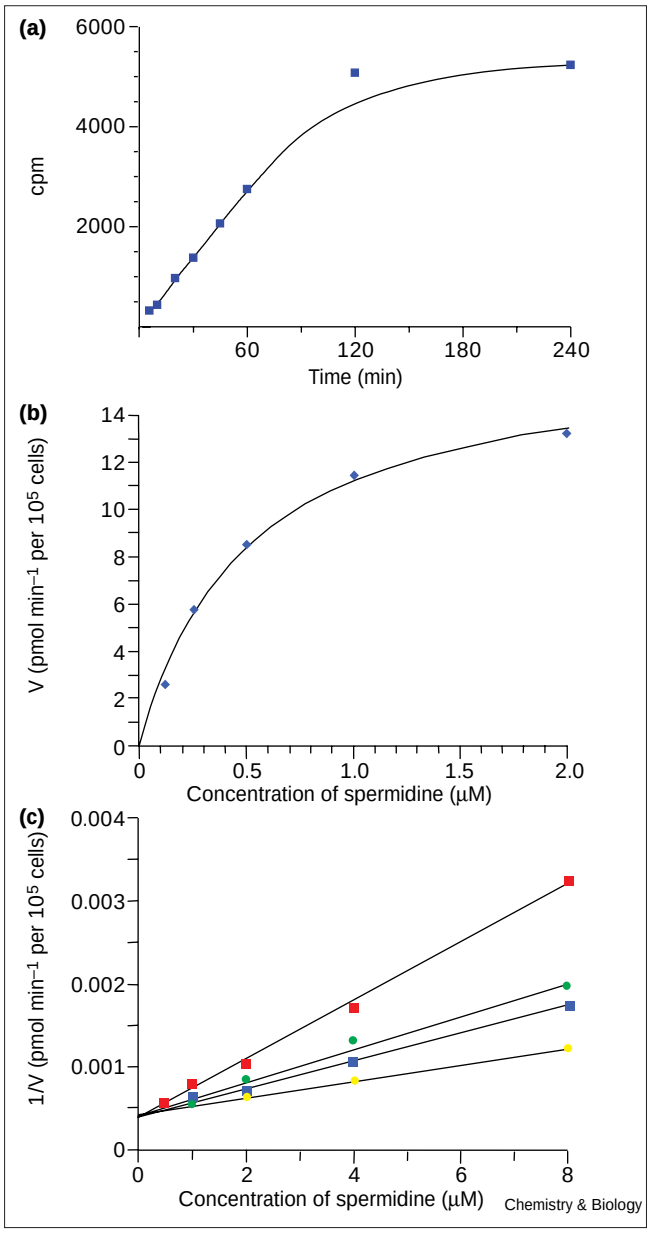
Binding constants for the binding of polyamine analogues to the polyamine receptor can be determined from the inhibition of uptake of ^{14}C-labelled spermidine (Figure 2). Figure 3c shows a typical set of data for the inhibition of spermidine uptake by the N^4-spermidine–MANT conjugate, which corresponds to a K_i for **5** of 5.9 μM. All the polyamines and polyamine conjugates shown in Figure 1 were competitive inhibitors of spermidine uptake with K_i values in the range 0.02–80 μM (Table 1) in A549 human lung carcinoma epithelial cells, L1210 mouse leukemia cells and Chinese hamster ovary (CHO) cells (Table 1). As with simple enzyme inhibitors, the lower the K_i value the more potent the inhibition of spermidine uptake.

Cytotoxicity studies

It is widely assumed that the cytotoxicity of nitrogen mustards such as chlorambucil is associated with their ability to cross-link DNA [24]. The polyamine conjugates that incorporate chlorambucil are expected, therefore, to be cytotoxic provided that they can gain access to cells and to nuclear DNA. The IC_{50} values were determined for each of the chlorambucil-containing compounds shown in Figure 1 by determining the rate of incorporation of [^{3}H]-thymidine into cells following pretreatment with the cytotoxic agent (Table 2). Within this small group of compounds it generally appears that more potent inhibitors of polyamine uptake show greater cytotoxicity, which would be consistent with the conjugates gaining access to the nuclear DNA via the polyamine transport system.

It has been shown that inhibition of polyamine biosynthesis leads to induction of the polyamine uptake system. Thus, for example, pretreatment with difluoromethyl ornithine (DFMO), a potent irreversible inhibitor of the first step in polyamine biosynthesis, leads to a significant increase in polyamine uptake. This effect can be used to provide further evidence for the polyamine conjugates being taken up by the specific polyamine transporter. Figure 4 shows the effect of DFMO pretreatment on the cytotoxicity of N^4-spermine–chlorambucil in A549 cells. Clearly there is a marked increase in cytotoxicity for a number of the chlorambucil conjugates but not for chlorambucil itself (Table 2). This provides strong supporting evidence for dependence on the polyamine transporter.

Figure 3



Kinetics of spermidine uptake in A549 cells. (**a**) Time course for uptake of [^{14}C]-spermidine. (**b**) Saturation kinetics for uptake of [^{14}C]-spermidine; average of ten independent data sets analysed by Grafit [46] K_m 0.5 μM (standard error 0.08); V_{max} 17 pmol min^{-1} per 10^5 cells (standard error 1). (**c**) Competitive inhibition of [^{14}C]-spermidine uptake by the N^4-spermidine–MANT conjugate (**5**), K_i value of 5.9 ± 0.6 μM. Inhibitory concentrations 2 (yellow), 5 (blue), 10 (green) and 20 (red) μM.

Uptake studies by flow cytometry

The fluorescent-labelled polyamines **5**, **6**, **8** and **9** are reasonably good surrogates for the cytotoxic polyamine conjugates and were designed as probes for monitoring polyamine uptake. In a related study Aziz *et al.* [25] have recently reported the synthesis and studies on the distribution of transported fluorescein-labeled polyamines in cultured pulmonary artery smooth muscle cells. In order to minimise complications from possibilities of nonspecific hydrophobic interactions with the lipid bilayer, for example, that could occur with a large polyaromatic fluorophore we have been anxious to select a fluorophore that is as small as possible, and hence less likely to perturb the interactions and subsequent distribution of the polyamine analogue. Thus using spermidine–MANT **5** the uptake of the conjugate itself in A549 cells could be conveniently followed by flow cytometry. The uptake kinetics showed the expected characteristics of a receptor-mediated process: it was saturatable, with an approximate K_m of 20 μM (Figure 5a); it showed approximately linear kinetics over 24 hours (Figure 5b); and the uptake was inhibited by spermidine (IC_{50} 27 μM). These characteristics are somewhat different to those for spermidine itself in the same cell line, for which the uptake kinetics are linear for only 1–2 hours after which it reaches a plateau, and shows a K_m of 0.5 μM and V_{max} of 17 pmol min^{-1} per 10^5 cells (Figure 3). The plateau seen in the time course for uptake of polyamines via the polyamine transporter signifies feedback inhibition of the uptake through induction of antizyme [26]. Given that uptake of spermidine–MANT **5** is linear over 24 hours it might be argued that the uptake differs from that of the natural polyamines because this feedback inhibition is not seen. The significantly higher K_m value for conjugate **5** compared with spermidine, however, would be consistent with slower uptake of **5** in this cell line such that after 24 hours the intracellular concentration of **5** might not have reached a sufficient level to induce antizyme production. In support of this conclusion are results from studies on the uptake of **5** in CHO cells and in the related CHO–MG cells that are resistant to MGBG by virtue of being deficient in polyamine uptake [27]. Interestingly, the uptake of N^4-spermidine–MANT (**5**) in CHO cells was rapid and reached a plateau within several hours, very similar to that seen with natural polyamines, and indicates that accumulation of N^4-spermidine–MANT (**5**) in CHO cells does show feedback inhibition. In contrast, very slow, nonspecific uptake was observed in CHO–MG cells under identical conditions (Figure 6).

We have also been able to demonstrate that the conjugate is intact within the cell and to estimate the intracellular concentration of N^4-spermidine–MANT (**5**) using HPLC, both of which have implications for exploitation in the context of drug delivery. Incubation of A549 cells with compound **5** for 24 hours followed by washing and extraction of the cells in acid to disrupt the membranes led to recovery of the N^4-spermidine–MANT (**5**) conjugate. The amount of **5** recovered was determined by HPLC using UV detection and calibrated against standard solutions of N^4-spermidine–MANT and MANT alone (data not shown). Using this method it was

Table 1

Inhibition of uptake of spermidine in cells.

	K_i values in μM*		
Conjugate	A549 cells	L1210 cells	CHO cells
Putrescine	17.2 ± 1.4		
Spermine	0.02 ± 0.0006		
N^1,N^8-Diethyl spermidine	5 ± 1		
N^1,N^{12}-Diethyl spermine	0.95 ± 0.37		
N^4-Spermidine–chlorambucil **1**	2.8 ± 1	1.9 ± 1.3	3.0 ± 0.8
N^4-Spermine–chlorambucil **2**	0.4 ± 0.2	0.57 ± 0.25	0.74 ± 0.014
N^3-Norspermidine–chlorambucil **3**	10 ± 5	3.5 ± 0.2	
N^5-Homospermidine–chlorambucil **4**	0.7 ± 0.3	1.07 ± 0.13	
N^4-Spermidine–MANT **5**	5.9 ± 0.6	23.4 ± 4	
N^4-Spermine–MANT **6**	0.4 ± 0.2		
N^1-Spermidine–chlorambucil **7**	0.4 ± 0.2		
N^1-Spermine–MANT **8**	0.11 ± 0.03		
N^1,N^{12}-Diethyl-N^4-spermine–MANT **9**	7 ± 3		
N^1,N^8-Spermidine-*bis*-2-nitroimidazole **10**	0.6 ± 0.2		
N^1,N^7-Norspermidine-*bis*-2-nitroimidazole **11**	5 ± 1.6		
N^1,N^8-Diethyl-N^4-spermidine–chlorambucil **12**	9.0 ± 2		

*Data analysed by Grafit [46].

possible to determine an approximate concentration of 2×10^{-15} moles per cell of spermidine–MANT, which, assuming a mean cell volume of 3×10^{-12} l, corresponds to an intracellular concentration of 0.7 mM. The errors on these measurements are probably quite large but it is clear that the intracellular concentration is at least an order of magnitude higher than the extracellular concentration, as expected for an active uptake system. The corresponding intracellular concentration of spermidine taken up from the medium calculated from the radioactivity that is retained in the cell pellet at saturation is 1.9 mM. The fact that the concentration of spermidine at saturation is almost three times higher than the concentration of N^4-spermidine–MANT (**5**) after 24 hours incubation would also be consistent with the suggestion above that uptake of **5** in A549 cells is significantly slower than for spermidine and that the concentration of **5** might not have reached a sufficient level to induce feedback inhibition within the 24 hour incubation period. Control experiments showed that MANT itself does not diffuse into cells and furthermore no significant amount of the liberated fluorophore was detected in the cellular extracts, confirming that the conjugate is not extensively metabolised.

Fluorescence microscopy

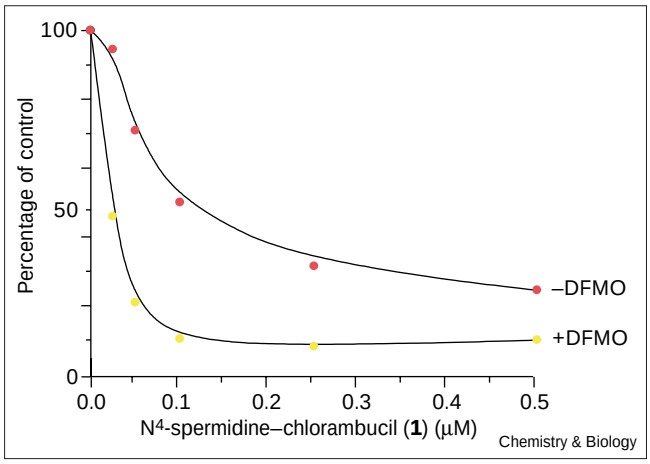
In addition to allowing the direct monitoring of polyamine uptake, the fluorescently labelled polyamines offer a

Table 2

***In vitro* cytotoxicity (IC_{50} μM) of polyamine conjugates in A549 cells.**

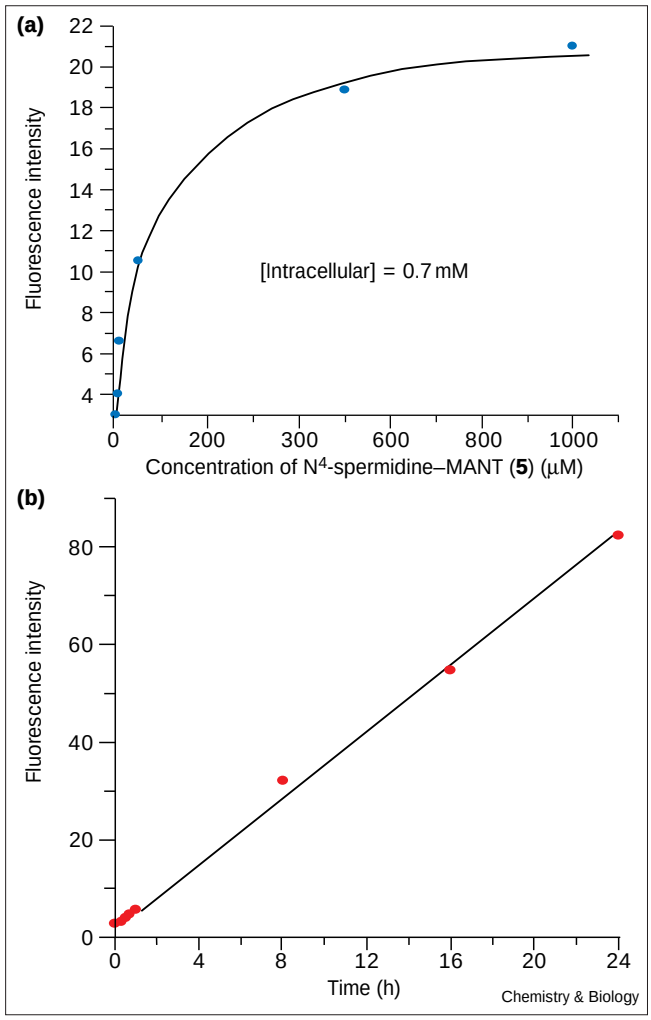
Compound	Cytotoxicity IC_{50} (μM)	Cytotoxicity after DFMO pretreatment IC_{50} (μM)	K_i (μM)
Chlorambucil	4.2	6.1	No effect on uptake at 500 μM
N^4-Spermidine–chlorambucil **1**	39	0.6	2.8 ± 1
N^4-Spermine–chlorambucil **2**	0.1	0.03	0.4 ± 0.2
N^1,N^8-Diethyl-N^4-spermidine–chlorambucil **12**	33	35	9.0
N^5-Homospermidine–chlorambucil **4**	0.5	–	0.7 ± 0.3
N^3-Norspermidine–chlorambucil **3**	81.5	–	10 ± 1
Chlorambucil-N^4-spermidine (no linker) **13**	1.7	–	16.5
N^1,N^{12}-Diethyl-N4-spermine–chlorambucil **14**	0.5	0.1	2.8

Figure 4



Cytotoxicity assay based on the incorporation of [^{3}H]-thymidine into A549 cells following exposure to N^4-spermidine–chlorambucil (**1**) expressed as a percentage of control cells. The figure shows the concentration dependence of cytotoxicity and the effects of pretreatment of the cells with DFMO (yellow circles) which enhances uptake via the polyamine transporter.

Figure 5

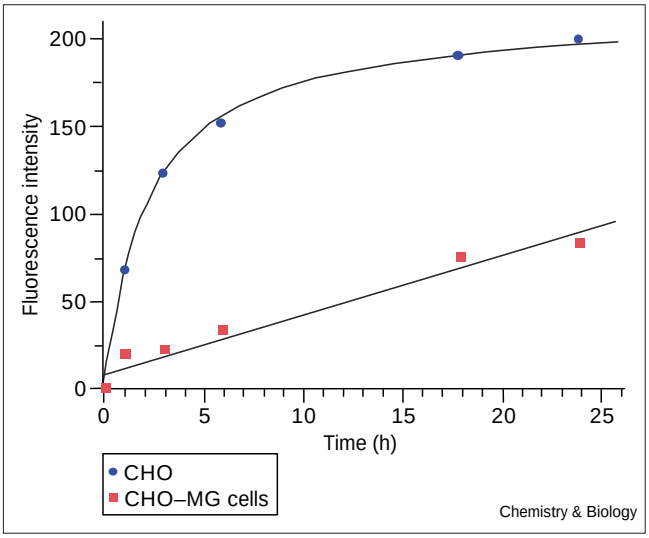


Flow cytometric monitoring of uptake of N^4-spermidine–MANT **5** in A549 human lung epithelial cells. (**a**) Concentration dependence of uptake of N^4-spermidine–MANT **5** monitored by flow cytometry showing rate saturation. (**b**) Time course for uptake of N^4-spermidine–MANT **5** monitored by flow cytometry.

potentially new way to map the intracellular distribution of polyamine analogues derived from the medium. Figure 7 shows an image of A549 cells that have been incubated with **5** for 24 hours taken from a conventional fluorescence microscope. The purple fluorescent shadow that is seen in all of these adherent cells corresponds to the fluorescent emission from the MANT fluorophore providing unambiguous evidence of internalisation of the polyamine conjugate. Using conventional fluorescence microscopy it is difficult to be confident of the internal location of the polyamine analogue. For some individual cells the fluorescence appears to coincide with the outline of the nucleus, whereas for other cells it appears to be outside the nucleus. It is clear, however, that the fluorescence is neither uniformly distributed within this cell, nor tightly concentrated in the nucleus.

Considerably greater resolution of detail is offered by confocal scanning laser fluorescence microscopy than by standard fluorescence microscopy, which, by virtue of the laser excitation, allows images to be taken as 'slices' through the cell. Figure 8 shows the confocal images of live A549 cells that have incubated with **5** for 24 hours then viewed directly on the cover slips on which they are grown. The blue fluorescence is the emission at 436 nm corresponding to the MANT fluorophore. The cells have been simultaneously stained with SYTO-13, a commercial nuclear stain for vital cells (Molecular Probes), and this shows up in Figure 8 as the intense green fluorescence at 488 nm clearly showing the nuclear bodies and the margins of the nucleus. The two fluorophores are excited separately and the images combined, and from the individual images it is clear that there is little of the 436 nm emission from the nucleus suggesting that the vast majority of the N^4-spermidine–MANT (**5**) is located in the cytoplasm and not in the nucleus. As is clearly visible in Figure 9, in which the two individual images are displayed side-by-side in enhanced colours, the N^4-spermidine–MANT fluorescence (green) appears to be located in granular structures that are capped on the nucleus (stained red by the SYTO-13). These granular structures persist through cell division with the fluorescent structures being shared between the daughter cells (Figure 10). Further confirmation that the subcellular distribution of N^4-spermidine–MANT (**5**) seen in A549 cells was not unique to this cell line was obtained by parallel confocal laser scanning microscopy studies on CHO cells. Once again the fluorophore is not located in the nucleus

Figure 6



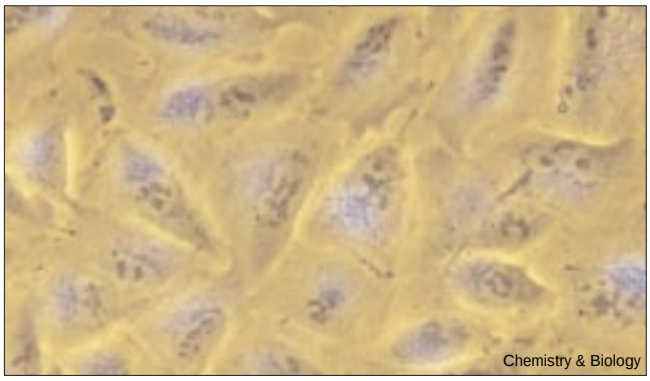
Time course for uptake of N^4-spermidine–MANT (**5**) in Chinese hamster ovary (CHO) cells (blue) and CHO–MG cells (red) monitored by flow cytometry.

and appears in granular structures in the cytoplasm (Figure 11). The corresponding images of CHO–MG cells incubated under identical conditions showed only low background fluorescence (data not shown).

Discussion

The first objective of this study was to probe the structural tolerance of the mammalian polyamine transport system. We have shown that a considerable range of polyamine analogues inhibit spermidine uptake. A number of earlier studies [28–31] had explored the effects of changes in the spacing between positive charges, conformational effects and so on and both MGBG and paraquat appear to be transported into cells, particularly pulmonary cells, via the polyamine uptake system. Hitherto there have been relatively few studies on uptake of significantly modified polyamines. All of the polyamines analogues that we have looked at to date have proved to be simple competitive inhibitors. Despite the considerable variation in structure, the inhibition constants vary over a range that is small when compared with typical enzyme–substrate specificity. From Table 1 it is clear that the number of positive charges is a major determinant of binding to the polyamine receptor, with spermine conjugates binding tighter that spermidine conjugates. Norspermidine and norspermine analogues bind more weakly than spermidine and spermine respectively, which might reflect some preference for a particular distance between positive charges. The N^1-linked conjugates were generally more tightly bound than the N^4-linked conjugates suggesting that there is no preference for structures with two primary amino groups.

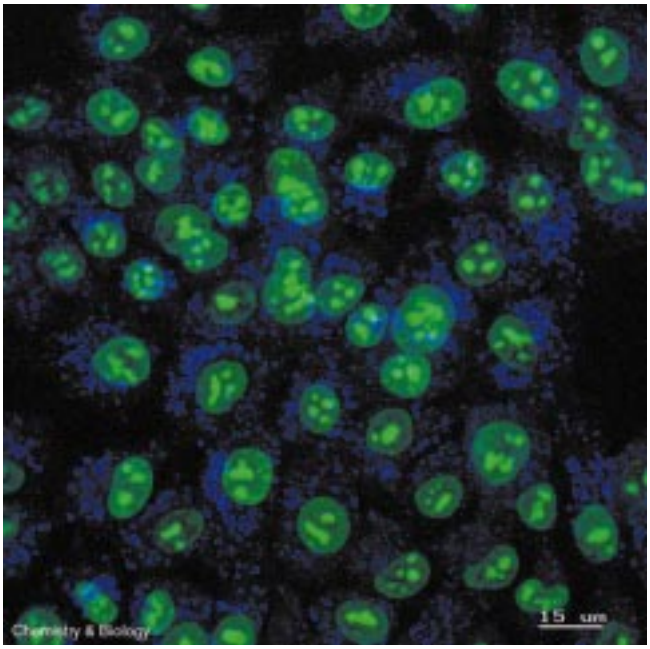
Figure 7



A549 human lung epithelial cells incubated with N^4-spermidine–MANT **5** and viewed under a Ziess Axiovert 135 inverted microscope, by phase contrast microscopy. The purple fluorescence corresponds to the emission wavelength of the MANT fluorophore.

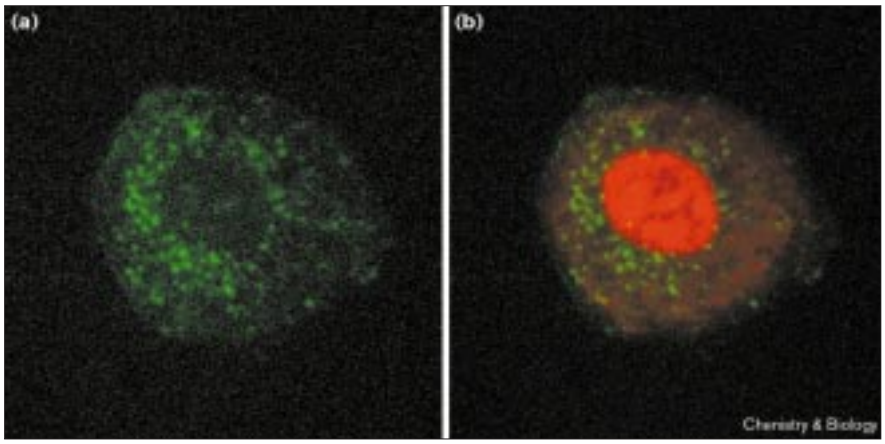
The demonstration that the polyamine analogues shown in Figure 1 are competitive inhibitors does not allow the conclusion that they are themselves translocated across

Figure 8



Confocal laser scanning images of A549 cells that have been incubated with N^4-spermidine–MANT **5** for 24 h and then viewed using a Leica TCS4D confocal laser scanning microscope, showing accumulation of **5** in the cytoplasm. The blue fluorescence is the emission at 436 nm corresponding to the MANT fluorophore and the intense green fluorescence at 488 nm corresponds to emission from SYTO-13, a commercial nuclear stain for vital cells (Molecular Probes). The two fluorophores are excited separately and the images combined.

Figure 9



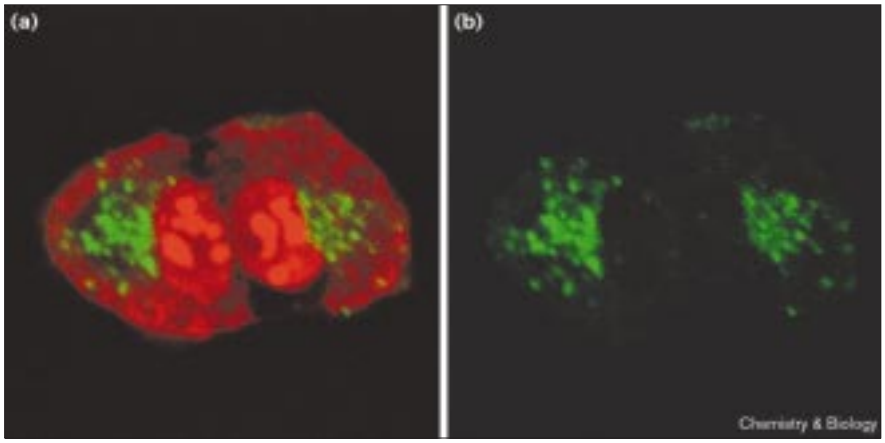
Confocal laser scanning image of a single A549 cell incubated with N^4-spermidine–MANT **5** and SYTO-13 showing the emission for (a) the MANT fluorophore at 436 nm (green) and (b) the superimposition of emission at 436 nm, the MANT fluorophore, and 488 nm, the SYTO-13 fluorophore (red; right image).

the membrane. As is implied in Figure 2, a competitive inhibitor could simply compete for the extracellular polyamine binding site but not be transported itself. Independent evidence must be sought to prove beyond a doubt that these polyamine analogues are indeed transported via the polyamine uptake system. For the chlorambucil-containing conjugates indirect evidence was obtained by showing that their cytotoxicity in a range of cells in culture correlated with the binding constant to the polyamine receptor, and that the cytotoxicity was enhanced by pretreatment of the cells with DFMO. The irreversible inhibition of the first step in polyamine biosynthesis by DFMO leads to a depletion of polyamine levels, which the cell attempts to compensate for by induction of the polyamine transport system. The fact that DFMO has no effect on the cytotoxicity of chlorambucil but increases the cytotoxicity of the polyamine–chlorambucil conjugates represents convincing evidence for specific uptake by the spermidine transporter.

The monitoring of uptake of the fluorescent polyamine conjugates by flow cytometry provides direct evidence that these conjugates are taken up by cells. Furthermore, four crucial observations confirm that this is via the polyamine transporter: firstly, the uptake is inhibited by spermidine and spermine; secondly, the uptake is enhance by pretreatment of the cells with DFMO (Figure 4); thirdly, uptake of the conjugates shows saturation kinetics (Figure 5a); and fourthly, uptake in CHO cells shows feedback inhibition (Figure 6). The demonstration that these polyamine conjugates are rapidly taken up by CHO cells but not by CHO–MG cells deficient in polyamine uptake, also provides strong support for the conclusion that all of these polyamine analogues are being taken into cells by the spermidine transporter.

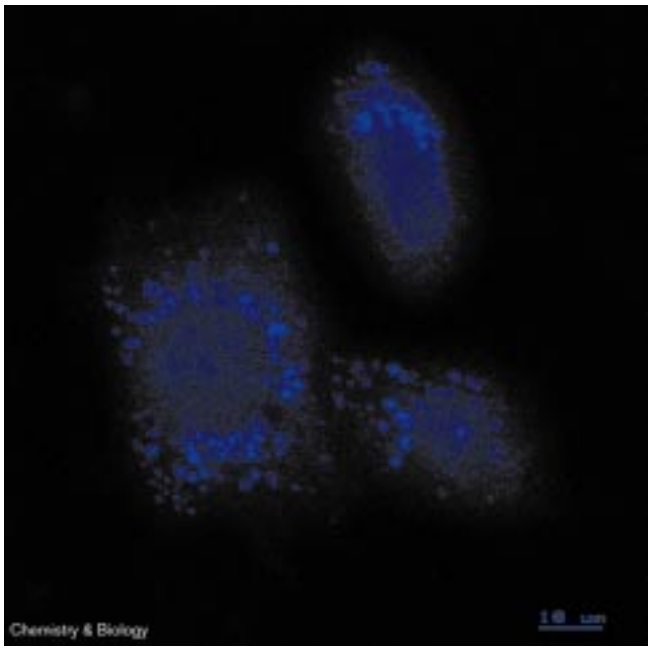
Figure 12 shows a comparison of the structures of spermidine, N^4-spermidine–chlorambucil (**1**) and N^4-spermidine–MANT (**5**) in space-filling representation. Clearly the polyamine-conjugates are substantially more bulky

Figure 10



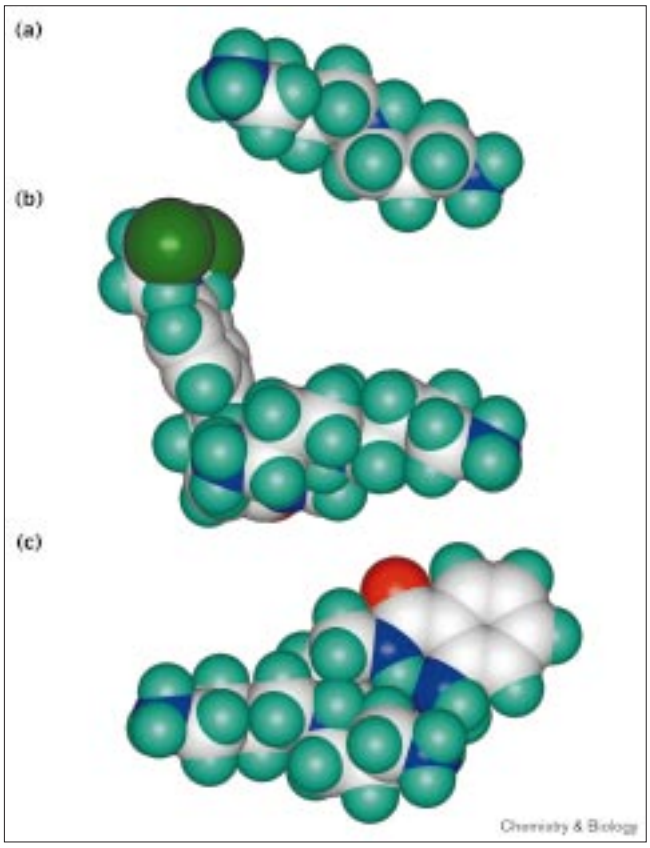
Confocal laser scanning image of an A549 cell that has been incubated with SYTO-13 and N^4-spermidine–MANT **5** undergoing division, showing (a) the superimposed emissions for the MANT fluorophore at 436 nm (green) and the SYTO-13 fluorophore at 488 nm (red), and (b) emission for the MANT fluorophore alone showing the distribution of the granular structures between the two daughter cells.

Figure 11



Confocal laser scanning image of CHO cells after incubation with N^4-spermidine–MANT (5) showing the polyamine conjugate located in granular structures in the cytoplasm in a similar pattern to that seen in A549 cells.

Figure 12

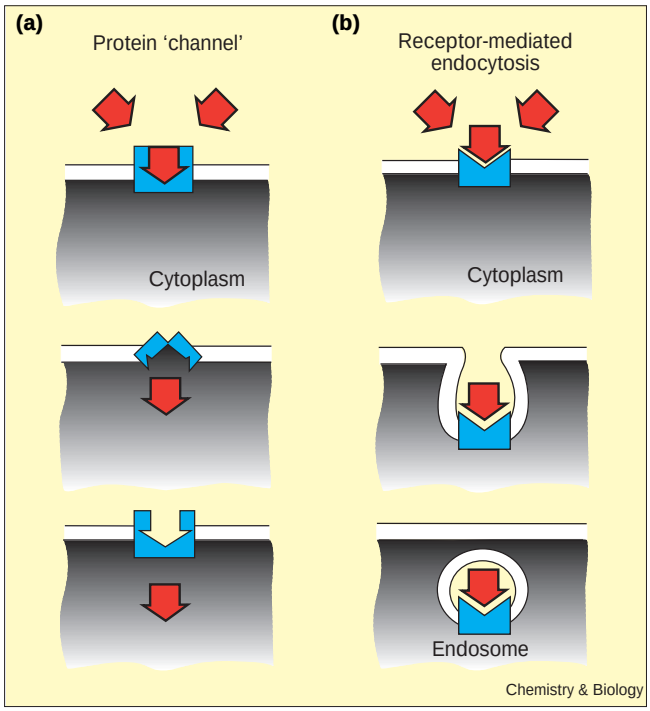


Space-filling models of (a) spermidine; (b) N^4-spermidine–-chlorambucil (1); and (c) N^4-spermidine–MANT (5).

than spermidine itself, and yet, as demonstrated above, they are still recognised and transported by the spermidine uptake system.

The second aspect of this study has been the related issues of the mechanism of polyamine uptake and intracellular localisation of the polyamines once internalised within the cell. There has been a considerable amount of effort put into localising the endogenous polyamines spermidine and spermine as part of the general attempt to delineate the precise physiological role of these ubiquitous molecules [32]. Cytochemical [33], immunological and subcellular fractionation methods [34,35] have all been developed and applied to a number of different tissue types but these have reached contradictory conclusions depending on the method of analysis. Subcellular fractionation of HeLa cells and chicken erythrocytes in oil (to minimise polyamine redistribution) demonstrated that spermidine and spermine are concentrated in the nuclei and chromosomes [33], which is in agreement with histochemical staining [36]. Cells containing uncondensed nuclei such as rat liver cells, however, stain for polyamines predominantly in the cytoplasm but on subcellular fractionation in organic solvents the nuclear polyamine levels appear to be equal or higher [34]. Unfortunately none of these methods is capable of studying viable cells and all are to a greater or lesser extent prone to either possible redistribution of polyamines during manipulations of the cells or inaccessibility of particular polyamine pools with respect to staining. In this present study we have looked directly at viable cells and no disruption of the cellular integrity was involved. On the basis of these results it is clear that the modified polyamines obtained from the extracellular pool are not located to any significant extent in the nuclei of these A549 human lung carcinoma cells (Figures 7–9) or CHO cells (Figure 11). The fluorescence intensity is seen in the cytoplasm of these cells rather than the nuclei and the images from confocal scanning laser microscopy clearly show that this fluorescence is not evenly distributed but is associated with granular structures. This distribution persists through many cell divisions suggesting that the polyamine pools that we are observing are not exchanging during the cell cycle. Interestingly we have noted that the polyamine-containing granular structures even appear to be shared between the two daughter cells on division (Figure 10). These results are very similar to those reported by Aziz *et al.* [25] using their fluorescein-spermidine conjugate in pulmonary artery smooth muscle cells which, taken together with this

Figure 13



Schematic representation of two alternative transport mechanisms: (a) transmembrane channel; (b) receptor-mediated endocytosis.

present study, demonstrates that this distribution of exogenously derived polyamines is seen in a diverse range of mammalian cells types.

The nature of these granular structures is unclear but on the basis of size and shape they would be consistent with vesicles. In an attempt to determine whether the polyamine **5** is initially transported into the free cytoplasm and subsequently the fluorescence becomes more readily visualised upon compartmentalisation within these vesicles we can compare the results from flow cytometry with the appearance under the confocal fluoresecence microscope. It is clear that the rate of uptake determined by flow cytometry closely matches the profile of appearance of the fluorophore within the vesicles. This would only be the case if transport and compartmentalisation were coupled or if transport across the membrane was slow but subsequent internal compartmentalisation was fast.

If these polyamines are indeed sequestered in vesicles this presents at least two paradoxes. Firstly, it is generally accepted that the N^4-spermidine–chlorambucil conjugate **1** owes its cytotoxicity to its ability to cross-link DNA and yet the bulk of the material would appear not to have access to the nucleus. Secondly, the feedback inhibition of polyamine uptake involves interaction of the exogenous polyamine with ribosomes to induce antizyme production. Clearly, in CHO cells the majority of the polyamine analogue is still associated with these granular structures and yet feedback inhibition is still observed. From the confocal laser scanning fluorescence microscopy, however, it would be impossible to exclude the possibility that although the majority of the polyamine analogue is sequestered ~10% might be released from these vesicles, which, in the case of conjugate **1**, would almost certainly be sufficient to be cytotoxic. Indeed, it can be seen from Figure 11 that there does appear to be a more uniform diffuse background blue fluorescence that could be 'free' polyamine–MANT conjugate.

Transport of specific molecules across the membrane is essential in all cells not only in terms of nutrient uptake and waste excretion but also to fulfil a multitude of regulatory and other functions [37]. Membrane transport is probably best characterised in prokaryotic systems and its importance is illustrated by the fact that in *E. coli* almost 20% of the genes so far identified appear to be associated with transport functions. In comparison only a limited number of mammalian transporters have been characterised, but efficient endocytosis in such cells might obviate the need for some of the highly specific protein transporters that are seen in prokaryotes [37].

Despite the considerable literature on active uptake of polyamines into cells, including expression of the polyamine receptor into CHO cells [38], little is known about the mechanism of transfer of polyamines across the cell membrane in eukaryotes. In terms of the polyamine transport processes observed in this study there are presumably two extreme mechanisms of uptake, either a specific protein transporter with characteristics of a membrane 'channel' or a receptor-mediated endocytosis (Figure 13). Fluid-phase endocytosis can be ruled out because the fluorescent conjugate **5** is accumulated intracellularly above the external concentration and the uptake is inhibited by a competing polyamine. Whatever mechanism is proposed it must be consistent with the following key observations: first, the extracellularly derived polyamine analogues are located in structures consistent with vesicles or secondary endosomes; second, **5** appears to co-localise with Lucifer yellow, a dye used as a marker for endosomes [39]; third, the mechanism of influx is tolerant of significant modification of the polyamine without major perturbation of uptake; fourth, the greater the positive charge the tighter the binding to the polyamine transporter; and fifth, the observed distribution of the fluorophore does not significantly change over 24 hours. As is implicit in the cartoons shown in Figure 13, we have assumed that the protein 'channel' will show a stricter structural requirement than receptor-mediated endocytosis (see below). In the case of endocytosis, if the interaction of the receptor is principally with the 'head' of the polyamine conjugate, extensive variations in the 'tail'

might well be tolerated without impeding the endocytosis step. There are examples of eukaryotic carrier-mediated transporters with apparently broad substrate tolerance, notably the multidrug resistance transporter [40–43]. In this latter case the substrates all tend to be lipophilic, which makes the task of translocation across the hydrophobic lipid bilayer inherently easier, and this might mean that it is sufficient to have a large, flexible hydrophobic binding pocket. In the case of translocation of more polar ligands, if a protein is to chaperone the ligand across the membrane (a 'flip-flop' mechanism) it will be necessary to 'mask' these polar groups (i.e. develop precise binding interactions) in order to overcome the high energetic cost of removing these polycations from the aqueous environment. A mechanism involving creation of an aqueous channel would get around this but would be expected to allow passage of the polyamine conjugates into the cytoplasm.

Finally, it is necessary to address the question of whether or not the observations and conclusions made concerning the uptake and localisation of polyamine–MANT derivatives can be extrapolated to the natural polyamines themselves. It is clear that the fundamental properties associated with the transporting of these polyamine conjugates are remarkably similar to those for the polyamines themselves: firstly, K_m and V_{max} values for natural polyamines and polyamine conjugates are in tolerable agreement; secondly, the same enhancement of uptake by pretreatment with DFMO is seen for natural polyamines and a wide range of polyamine conjugates; thirdly, feedback inhibition of uptake of **5** in CHO cells is similar to that seen for spermidine; fourthly, direct competitive inhibition between spermidine and these polyamine analogues for uptake is observed; and fifthly, intracellular concentration of exogenous polyamines and polyamine conjugates at saturation are closely similar. If natural polyamines are playing a crucial role(s) in terms of regulation of cell growth and differentiation it will be necessary to tightly regulate the concentrations of 'free' polyamines. One way of doing this would be to sequester polyamine in vesicles from where they can be released in response to some appropriate cell signal. It would be extremely interesting if these granular structures are indeed polyamine pools, but such a conclusion would be premature at this stage. It is equally possible that the polyamine conjugates are processed by the cell in an entirely different manner to the natural polyamine.

Clearly the mammalian polyamine transport pathways are complex and as yet poorly characterised at the molecular level. This present study has led, however, to a number of key observations concerning the uptake and localisation of extracellularly derived polyamine analogues against which mechanistic proposals can be tested. Influx and efflux of polyamines might have a significant physiological role, as yet to be defined, and it has been suggested that endogenous and exogenously derived polyamines might exist in essentially nonexchangeable pools [1,44]. Our results provide some support for these suggestions. Finally, the probes described in this manuscript have many potential applications in looking at polyamine uptake generally and at changes that might occur to the polyamine compartmentalisation during the cell cycle or in response to external agents.

Significance

Polyamines play a crucial role in regulation of cell growth and differentiation. Mammalian cells satisfy this requirement via the *de novo* biosynthesis of polyamines and/or the exploitation of an efficient polyamine uptake mechanism that can acquire polyamines from the extracellular milieu. Despite a lack of full understanding of the role that polyamines play in controlling cell division, there has been considerable interest in polyamine regulation as a new target for chemotherapy. One approach has been to attempt to exploit the high specific activity of the polyamine transport system that is widely seen in tumour cells to deliver selectively cytotoxic agents. This present study has demonstrated a broad tolerance of the polyamine transporter of three mammalian cells (A549 human lung epithelial carcinoma cells, CHO cells and L1210 human lymphocytic leukemia cells) towards a wide range of polyamine conjugates. Fluorescently labelled polyamines have been developed to probe the uptake and intracellular distribution of polyamines using confocal laser scanning fluorescence microscopy. These studies have shown that exogenously derived polyamine analogues are localised in the cytoplasm of cells, in granular structures that resemble endosomes, and are not found to any significant extent in the nucleus. The mechanism of polyamine uptake in mammalian cells is poorly understood at the molecular level and these present observations might have important implications in advancing this understanding. These results also suggest that there is considerable potential for exploiting the polyamine transport system in the delivery of cytotoxic agents. Finally, this study has provided molecular probes that might prove useful in unravelling the role of polyamines in controlling cell proliferation and differentiation.

Materials and methods

Cell lines and bacterial strains

A549, human epithelial lung carcinoma cells were used throughout this study and were a gift from Dr. C. Courage, CMHT, Leicester University. Cells were seeded at $5–10 \times 10^4$ cells/ml and maintained in Nutrient Mix; F-12 (Hams) with Glutamax (Gibco BRL, cat 31765-027) supplemented with foetal calf serum (PAA Laboratories Ltd, UK), penicillin (100 iu/ml) and streptomycin (100 μg/ml).

CHO cells and CHO–MG cells were a gift from Prof. A.E. Pegg, Pennsylvania State University College of Medicine, Hershey, USA. Cells were maintained in MEM Alpha medium (Gibco BRL, cat 32561-021) supplemented with foetal calf serum (PAA Laboratories Ltd, UK), penicillin (100 iu/ml) and streptomycin (100 μg/ml). All cell lines tested negative for mycoplasm contamination.

Once cells approached confluence, spermidine–MANT (50 μM) was added to the media and the cells were incubated for a further 24 h. Cells were washed once in 0.9% sodium chloride containing 1 mM spermidine, then twice with phosphate buffered saline (PBS) and either viewed directly or collected for extraction of internal polyamines. In co-staining experiments using SYTO-13 (Molecular Probes), the DNA stain was added 1 h before the end of incubation with spermidine–MANT.

Determination of polyamine uptake

To study the uptake of [^{14}C]-spermidine A549 cells were seeded into 24- well tissue culture plates (Nunc –1×10^5 cells/well) and incubated for 16 h to form a monolayer. Cells were incubated with either [^{14}C]-radiolabelled spermidine (112 μCi/mmol, Amersham International) at various concentrations over periods up to 4 h. After incubation, the plates were placed on ice and the cells were washed with cold 0.9% NaCl plus 1 mM spermidine, to displace any radiolabelled spermidine still attached to the cell surface. Cells were disrupted by the addition of 1 M NaOH (400 μl) and incubation at 60ºC for 30 min–1 h. Samples were neutralised by the addition of an equal volume of 1 M HCl. Duplicate samples (400 μl) were added to 4 ml Optiphase 'Safe' and radioactivity determined in a Wallac scintillation counter. The results were expressed as pmol spermidine uptake/min/10^5 cells.

Cytotoxicity assay

A549 human epithelial lung carcinoma cells were seeded in 24-well plates at 500 μl of 1×10^4 cells/ml and cultured for 24 h. Polyamine conjugates were added to give the appropriate concentration in a final volume of 1 ml. Cells were cultured for a further 72 h. [^{3}H]-Thymidine (0.25 μCi) was added and the cells cultured for a further 2 h. The cells were washed twice with cold 0.9% NaCl solution containing 1 mM thymidine. To each well was added 1 ml cold trifluoroacetic acid solution (10%) and the wells left for 10 min. The resulting precipitate was dissolved in 400 μl NaOH solution (1 M), and neutralised with HCl solution (1 M) then the tritium content determined by scintillation counting. The [^{3}H]-thymidine incorporation is expressed as a percentage of the incorporation seen in the control wells (no polyamine-conjugate added). Each data point is the average of experiments carried out in triplicate.

Microscopy

Cells were viewed under a Ziess Axiovert 135 inverted microscope by phase contrast microscopy. A UVG 365 UV filter (Ziess) was used to observe the fluorescence of compounds with the MANT group and a Blue 450–490 filter (Ziess) was used to observe SYTO-13. Confocal images were obtained using a Leica TCS4D confocal laser scanning microscope, excitation was via a laser in the UV region (spermidine–MANT), or at 488 nm (SYTO-13).

Determination of intracellular polyamines

Cells (5–10×10^6) were scraped into 2 ml PBS and pelleted. The cell pellet was then resuspended in 0.2 M perchloric acid (200 μl) and incubated for 2 h at 4°C. The precipitate was removed by centrifugation and the supernatant neutralised by the addition of saturated sodium carbonate (10 μl) and saturated sodium hydrogen carbonate (120 μl). This method was modified from Chen *et al*. [45].

The extract was then separated by HPLC using a reverse-phase column (C18, 5 μ, BDS column, Hypersil) connected to a Gilson 715 system. A Dynamax absorbance detector (model UV-1, Rainin) was used to detect the spermidine–MANT compounds. The solvent system used was eluent A; 0.1 M ammonium acetate pH 6, eluent B; acetonitrile, gradient 0 min (20% B); 20 min (60% B); 23 min (80% B); 26 min (80% B); 28 min (20% B); 30 min (20% B); flow rate was 1 ml/min at room temperature. The elution was monitored and quantitated by UV at 340 nm.

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