Unusual aspects of the polyamine transport system affect the design of strategies for use of polyamine analogues in chemotherapy

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Abstract
One strategy for inhibiting tumour cell growth is the use of polyamine mimetics to depress endogenous polyamine levels and, ideally, obstruct critical polyamine-requiring reactions. Such polyamine analogues make very unusual drugs, in that extremely high intracellular concentrations are required for growth inhibition or cytotoxicity. Cells exposed to even sub-micromolar concentrations of such analogues can achieve effective intracellular levels because these compounds are incorporated by the very aggressive polyamine uptake system. Once incorporated to these levels, many of these analogues induce the synthesis of a regulatory protein, antizyme, which inhibits both polyamine synthesis and the transporter they used to enter the cell. Thus this feedback system allows steady-state maintenance of effective cellular doses of such analogues. Accordingly, effective cellular levels of polyamine analogues are generally inversely related to their capacity to induce antizyme. Antizyme activity is down-regulated by interaction with several binding partners, most notably antizyme inhibitor, and at least a few tumour tissues exhibit deficiencies in antizyme expression. Our studies explore the role of antizyme induction by several polyamine analogues in their physiological response and the possibility that cell-to-cell differences in antizyme expression may contribute to variable sensitivities to these agents.

Overview of polyamine transport and the use of polyamine analogues
Cancer cells frequently demonstrate an elevated requirement for cellular polyamines in association with their enhanced growth potential. As such, mechanisms of depressing cellular polyamines are of acute interest as an anti-tumour strategy [1–4]. To this end, several elegant inhibitors of polyamine biosynthetic pathways have been created and tested. Unfortunately, these have had limited success in whole animal studies and in clinical trials, as tissues can easily obtain needed polyamines from the diet and those released by microbes in the gut. Tumour cells seem to be especially well adapted for survival, as many show elevated polyamine uptake activity.

An alternative approach to depressing cellular polyamines involves the creation of polyamine analogues that resemble the native compounds sufficiently to repress polyamine production or incorporation yet, by themselves, are incapable of supporting cell growth. Many such compounds have been synthesized and tested for this purpose, and several are currently in clinical trials as potential anti-tumour agents (see reviews [4–6]). In most cases the analogues appear to utilize the natural polyamine transport system to enter cells, which suggests a potential for specific targeting of tumours that have enhanced polyamine transport activity. Those analogues that are structurally quite similar to spermine or spermidine, sometimes called polyamine mimetics, generally will reduce natural polyamine levels due to activation of normal feedback systems [3,4]. Since the natural polyamines have many critical activities within a cell, it is not surprising that some of the analogues also appear to affect specific, yet diverse, cell targets. Compounds that have cytotoxic effects not clearly attributed to their depletion of the native polyamines have been termed polyamine antagonists [3,4]. Many analogues, of course, exhibit both types of effect to varying degrees. Some close structural analogues of spermine, for example, feedback inhibit polyamine biosynthetic pathways as well as increase the activity of SSAT (spermidine/spermine N1-acetyltransferase), resulting in enhanced depletion of extant polyamines. Other analogues appear to affect calmodulin activity or aspects of mitochondrial function. Longer oligoamines have been found to have high binding affinity for DNA and have been reported to inhibit various aspects of its function. Possible specific cellular actions of the diverse polyamine analogues are grist for further investigations. In the current review, however, we will focus on more common aspects of the cellular response to structural analogues of spermine, namely the implications of the use of the polyamine transport system and its feedback regulation by antizyme. This analysis could help in understanding cell-to-cell differences in sensitivity to growth inhibition by polyamine analogues, differential dose requirements and variable sensitivity of cells to structurally similar analogues.

Key words: antizyme, antizyme inhibitor, cytotoxic activity, polyamine analogue, polyamine mimetic, transport.

Abbreviations used: AzI, antizyme inhibitor; CHO, Chinese-hamster ovary.

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The polyamine transporter

All mammalian cells appear to have an aggressive transport system capable of incorporating spermidine and spermine and, less efficiently, putrescine [7,8]. In some cells there may also be a second transporter more specifically for putrescine. As anticipated, close structural mimetics of spermidine or spermine were found to utilize this polyamine transport system for their uptake. Of great surprise, however, is the lack of specificity demonstrated by this transporter. A very wide range of polyamine-based compounds, some containing very complex side chains, have been shown to rely upon this transporter for their entrance into cells [9–11]. This has led to the design and testing of novel strategies for delivery of various toxic, fluorescent or growth-regulatory agents to targeted cells [9,12,13]. Although the physiology of the polyamine transporter has been extensively studied, very little is actually known about the biochemical components involved in the mammalian transporter and there remain diverse models for the uptake mechanisms [14–16].

Three important aspects of the polyamine transport system are of special interest in attempting to understand polyamine analogue effects on mammalian cells. The transporter has high affinity for spermidine and spermine, the velocity of uptake is quite substantial and the transport system is feedback-regulated. The implications of each of these attributes are discussed below.

Transporter affinity for spermidine and spermine

Although there may be some cell-type variation, kinetic studies of mammalian polyamine uptake generally suggest an apparent $K_m$ of spermine for the transporter at 1 µM or less [7,17–20]. The triamine spermidine demonstrates almost the same affinity as spermine, while putrescine is found to be a poorer substrate with an apparent $K_m$ in the 5–10 µM range. $K_i$ determinations for several spermine mimetics suggest that their affinity for the polyamine transporter is closer to that of the larger natural polyamines [10,11,21]. Further, analyses of cellular effects induced by varying extracellular exposures to a variety of structural analogues of spermine have revealed apparent uptake $K_m$ values in the 0.5–0.8 µM range [22]. These data suggest that polyamine analogues compete very effectively with the natural polyamines for the transporter and effective doses of the analogues should easily be obtained in animal studies.

Polyamine transporter velocity

There are, of course, multiple physiological factors that will affect the initial velocity of the polyamine transport system. Values for this uptake velocity from a number of different mammalian cell lines suggest that 2–5 nmol/10^6 cells per h is a common range [7,17–19,23,24]. At this velocity the spermidine or spermine level of a cell could be doubled in 1 h! This ability has indeed been demonstrated in cells deprived of normal feedback regulation of the transporter [25]. If we calculate normal cellular polyamine contents relative to cell volume, we realize that polyamines are unusually prevalent cell molecules with a cell spermine or spermidine concentration in the region of 1 mM. Considering the micromolar $K_m$ for the uptake system, it is interesting to note that this very high cellular uptake velocity can occur against a 1000-fold gradient. What affect does this very aggressive polyamine transport system have on cellular response to polyamine analogues?

Since many of the polyamine mimetics act by displacing native polyamines in feedback systems, it follows that, to be effective, cellular levels of such analogues must be somewhat comparable with those of the native polyamines being displaced. Thus millimolar levels of the analogues are required in cells, a far greater concentration than generally required for physiologically important drugs. Yet, the very efficient polyamine transport system permits this accumulation. This aggressive transporter and the necessity for extremely high cellular accumulations of analogues can cause problems for unsuspecting investigators who are not accustomed to having cell cultures require so much of a test compound from their media in order for cellular effects to be noted.

Feedback control of polyamine incorporation into cells

The aggressive polyamine transport system would quickly upset cellular polyamine homoeostasis if it were not controlled by a rapid and sensitive feedback-inhibition system. Small increases in the levels of free cellular polyamines stimulate the immediate synthesis of a regulatory protein, antizyme [26,27]. This multifunctional protein not only prevents additional polyamine synthesis by inactivating ODC but it also reversibly inhibits the polyamine uptake system by some, as yet undetermined, mechanism [28]. The role of antizyme in maintenance of polyamine homoeostasis is illustrated in Figure 1. Owing to this elegant feedback system, cellular polyamine levels are only increased slightly when cultures or tissues are exposed to exogenous polyamines. Obviously, this feedback control of polyamine incorporation is very important in vivo as polyamines are constantly available in our diet and are carried to tissues by the circulatory system.

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This antizyme-mediated feedback inhibition of polyamine transport needs to be considered in evaluating cellular effects of polyamine analogues. Consider the response of PC-3 cells, which is a cell line derived from a human prostate tumour. These cells greatly overexpress a polyamine biosynthetic enzyme, ornithine decarboxylase, causing increased polyamine levels that, in turn, give these cells a distinct growth advantage ([29] and J.L.A. Mitchell, J.M. Sequeira and R. Thokala, unpublished work). Because of the increased polyamines, these cells exhibit elevated antizyme levels and, consequently, very limited polyamine uptake capacity. As anticipated, these cells are extremely resistant to polyamine analogues in their medium as they incorporate only very limited amounts of these compounds (J.L.A. Mitchell, J.M. Sequeira and R. Thokala, unpublished work).

A deficiency in antizyme expression may contribute to enhanced growth potential in a variety of tumours. Specifically, underexpression of antizyme activity has been demonstrated in oral tumours, gastric carcinomas and prostate cancers [30–32], and stimulated expression of antizyme has resulted in suppression of tumour growth in several systems [33–36]. Such antizyme deficiency most likely explains why polyamine synthesis and transport are up-regulated together in many cancer cells. In contrast with the desensitization to polyamine analogues seen with the PC-3 cells above, one would expect that a depression of antizyme activity should allow more cellular uptake of exogenous analogues and thus a greater growth inhibitory effect. This might allow a very desirable selective targeting of cytotoxic polyamine analogues to tumour cells. Unfortunately, there are several compounding factors to this simple model as discussed below.

**Polyamine analogues also stimulate antizyme synthesis**

In recent investigations of cellular responses to a large selection of polyamine analogues we were surprised to find that, like the native polyamines, most of these were able to stimulate various levels of antizyme synthesis [22]. Although polyamine-stimulated steps in antizyme translation have been identified, the precise reaction responsible for polyamine sensing is still not known [37]. Regardless, this polyamine-dependent reaction is not very fastidious, as many of the over 25 different poly- and oligo-amines that we examined were equivalent to, or better than, spermine at stimulating antizyme [22]. Such antizyme synthesis by the analogues has major implications in considering cellular physiological responses. First, the induction of antizyme will inhibit the polyamine transporter and therefore the uptake of the analogues. Thus, polyamine analogue levels within cells will be limited and will depend inversely upon their capacity to induce antizyme. Accordingly, an oligoamine such as CGC-11144 (formerly SL-11144), which is very proficient at inducing antizyme [22], is limited to much smaller cellular levels than the homospermine analogue CGC-11102 (formerly SL-11102), which is much less proficient at antizyme induction [38]. This feedback inhibition of analogue uptake precludes cellular dosing in excess of a set amount, regardless of the extracellular exposures. If this set level is sufficient to exert the desired physiological effect, then this may be a positive attribute of analogues, as tissues would be expected to maintain a constant analogue level over a very large range of doses. We have shown, at least in a model cell system, that the allowed cellular analogue concentration is cytostatic for several polyamine mimetics [39].

Induction of antizyme by polyamine analogues may have other consequences for cellular responses to such analogues. Since antizyme down-regulates both polyamine synthesis and uptake, the induction of antizyme by analogues should be even more effective at depressing tissue polyamines than the polyamine biosynthetic inhibitor DFMO (difluoromethylornithine). Certainly, cells transfected to overexpress antizyme can show over a 90% inhibition in transport activity [39], but this level of repression may not be achieved with the lower amounts of antizyme stimulated by the steady-state levels of polyamine analogues. We have exposed several human tumour cell lines to mimetics such as CGC-11102, at the same time that the cells were exposed to a 10-fold excess of putrescine, and found that the extracellular putrescine was unable to rescue either the analogue-induced polyamine deprivation or cytotoxicity (J.L.A. Mitchell, J.M. Sequeira and T.K. Thane, unpublished work). Thus it seems that the antizyme levels induced by polyamine mimetics are sufficient to maintain polyamine starvation and inhibit cell growth. This effect is in addition to any other specific effect on a cell’s physiology an analogue may exhibit.

**Antizyme inhibitor activity may affect cell responses to polyamine analogues**

Cells of higher eukaryotes possess an antizyme-binding protein, termed AzI (antizyme inhibitor) that appears to be involved in mitigating antizyme activity as a prelude to activation of polyamine biosynthesis (Figure 1). Overexpression of the gene for AzI, with a resultant increase in cellular polyamine levels, has been suggested as a contributing factor in unregulated growth of certain tumours [31]. Further, cells in which this gene is overexpressed exhibit transformed properties and will form tumours when grown in test animals [40,41]. Since polyamine analogues depress polyamine synthesis and uptake by enhancing antizyme production, physiological responses to the analogues are likely to be altered in tissues overexpressing the antizyme inhibitory protein AzI. The exact nature of this effect depends upon the specific analogue and the degree of AzI overexpression.

First, it may be that the increase in antizyme stimulated by incorporated analogues would be obliterated by the extra AzI produced in such tumours, resulting in a decreased sensitivity of these cells to growth inhibition by polyamine analogues. On the other hand, a moderate increase in AzI, and the resultant decrease in antizyme activity, would allow cells to incorporate even higher levels of the polyamine analogues. This response was indeed demonstrated using a clone of CHO (Chinese-hamster ovary) cells that had been
stably transfected with a mouse AzI gene under an inducible promoter [39]. Considering that extra analogue will be incorporated until sufficient antizyme is induced to titrate the overexpressed AzI, there may be no change in cell sensitivity to polyamine analogues that act solely by feedback repression of native polyamines. However, as we and others have predicted, if the extra incorporation is of an analogue that has a specific cytotoxic cellular reaction, then we would expect cells overexpressing AzI would have increased sensitivity to such analogues [39,40]. Another scenario can be predicted, if the extra incorporation is of an analogue that has predicted in an instance where the overexpression of AzI is subject to disruption by the osmotic imbalance. We have shown this to occur upon exposure to 10 μM CGC-11047 by a stably transfected clone of CHO cells that have been induced for about a 20-fold excess of AzI (J.L.A. Mitchell and T.K. Thane, unpublished work). Clearly there are many factors that need to be considered in order to understand the effect overexpression of AzI may have on sensitivity to the polyamine analogues.

The use of mimetics to induce polyamine limitation and cytotoxicity appears to show great promise. As we have discussed, however, investigators must be aware that the polyamine transport system used by many of these analogues has several unusual features that must be taken into consideration when designing and testing polyamine mimetics as part of a therapeutic strategy.

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