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Antizyme and antizyme inhibitor activities influence cellular responses to polyamine analogs

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Summary. Close structural analogs of spermidine and spermine, polyamine mimetics, are potential chemotheraputic agents as they depress cellular polyamines required for tumor growth. Specific mimetic analogs stimulate synthesis of the regulatory protein antizyme (AZ), which not only inactivates the initial enzyme in polyamine biosynthesis but also inhibits cellular uptake of polyamines. The role of AZ induction in influencing cellular uptake of representative analogs was investigated using three analogs produced by Cellgate Inc., CGC-11047, CGC-11102, and CGC-11144, which exhibit markedly distinct AZ-inducing potential. An inverse correlation was noted between the AZ-inducing activity of a compound and the steady-state levels accumulated in cells. As some tumor cells over express AZI as a means of enhancing the polyamines required for aggressive growth, analog sensitivity was examined in transgenic CHO cells expressing exogenous antizyme inhibitor protein (AZI). Although AZI over expression increased cell sensitivity to analogs, the degree of this affect varied with the analog used.

Keywords: Polyamines – Antizyme – Antizyme inhibitor – Polyamine mimetics – Polyamine analogs

Abbreviations: AZ, antizyme; AZI, antizyme inhibitor; CHO, Chinese Hamster Ovary cells; DFMO, difluoromethylornithine; HTC, rat hepatoma cells; ODC, ornithine decarboxylase; SPD, spermidine; SPM, spermine

Introduction

Polyamines are essential for normal mammalian cell physiology and, while deficiencies lead to growth inhibition, excessive cellular polyamine levels may contribute to hyperplastic growth. To maintain appropriate polyamine concentrations, there are multiple polyamine sensitive feedback systems. Paramount among these is a polyamine-induced regulatory protein called ODC-antizyme (AZ) (Matsufuji et al., 1995; Hayashi et al., 1996; Pegg, 2006). Abnormal elevations in free cellular polyamines stimulate an unusual translational frameshift that allows the synthesis of AZ from its constitutively abundant mRNA. AZ, in turn, binds with high affinity to the initial enzyme in polyamine biosynthesis, ornithine decarboxylase, and targets it for rapid degradation by the 26S proteasome. In addition to disrupting polyamine synthesis, AZ also reversibly inhibits the cellular transport system that normally allows uptake of polyamines from extracellular sources (Mitchell et al., 1994). When cells are stimulated to proliferate by hormones or growth factors, then a second regulatory protein is rapidly synthesized to sequester the AZ and allow for the polyamine increases required for cell growth (Nilsson et al., 2000; Kim et al., 2006). Little is known about this antizyme inhibitor protein (AZI), but it is notable that cells over expressing this gene demonstrate enhanced tumorigenic potential, and specific overexpression of this gene has been observed in several human cancers (Jung et al., 2000; Keren-Paz et al., 2006).

The elevated requirement for polyamines by invasive tumors has made this an obvious target for disruption by chemotherapy (Marton and Pegg, 1995; Cohen, 1998; Seiler et al., 1998; Seiler, 2005). Effective inhibitors of several key polyamine biosynthetic enzymes have been created, but appear of limited use in vivo as their blockade is easily circumvented by cellular uptake of polyamines derived from copious dietary sources and gastrointestinal flora. As an alternative approach to the disruption of tissue polyamines, many structural analogs of the polyamines have been created with the expectation that they may displace the native polyamines in at least some critical cell functions (Kramer, 1996; Casero and Woster, 2001; Seiler, 2005). Fortuitously, the polyamine uptake system is quite

tolerant to a wide spectrum of structural changes, and a large variety of polyamine analogs are readily taken up by this system and promote diverse physiological responses. In a review of cellular responses to these analogs, Seiler observed that many of these appear to be polyamine mimetics in that they induce feedback inhibition of the synthesis of native polyamines, causing polyamine depletion (Seiler et al., 1998; Seiler, 2005). Since the mimetics displace, but do not substitute functionally for, native polyamines, they can be cytotoxic. Seiler noted that others, among the analogs, appear to disrupt specific cell functions and interrupt cell growth even in the absence of polyamine deprivation. These have been termed polyamine antagonists. Many analogs, of course, exhibit both types of effects to varying degrees.

While examining cellular responses to an assortment of structurally restricted polyamine analogs produced by the SLIL Corp., now Cellgate Inc., we observed that almost all of the tested analogs stimulated cells to produce AZ (Mitchell et al., 2002). Further, those that induced the most AZ were also the most effective at inhibiting growth of mammalian cell cultures exposed to equal concentrations of the compounds in 96-well proliferation studies. It appears that the obligatory polyamine sensing reaction in AZ translation must also be quite permissive to a range of structural variations. The AZ induced by accumulated analogs could certainly account for the observed inhibition in native polyamine synthesis. Such elevated AZ, however, would also be expected to inhibit the very polyamine transport system required for continued uptake of the analog. Recently we have reported that, despite the observed feedback inhibition of mimetic incorporation, the maintenance of enhanced AZ does appear sufficient to sustain cytotoxic polyamine depletion (Mitchell et al., 2004). In the present report we have extended these studies to demonstrate how the apparent AZ-inducing potential of an analog controls the cellular concentration attained by the analog. Further, evidence is presented to suggest that AZI over expressing cells, as a model for certain tumors, may be differentially sensitive to some of the polyamine analogs.

Materials and methods

Rat hepatoma (HTC) cells were grown in monolayer and suspension cultures in Swim's 77 medium containing 10% (v/v) calf serum as described previously (Mitchell et al., 1994). In the experiment where spermidine and spermine were added to the medium, horse serum was substituted for calf serum in order to minimize degradation of the added polyamines by amine oxidases of calf serum. Gene Switch Chinese Hamster Ovary cells (GS-CHO; Invitrogen) were maintained in RPMI-1640 medium supplemented with 5% fetal and 5% (V/V) calf serum containing 100 μg/ml hygromycin B in a 37 °C incubator with 3% CO_2. GS-CHO cells were stably transfected with a regulated expression vector that allows expression of a mouse AZI-V5-6H fusion protein in response to the addition of 10 nM mifepristone. The construction of this plasmid, and the production of GS-CHO-AZI-V5-6H clones has been detailed previously (Mitchell et al., 2004).

Cellular concentrations of the polyamines and the shorter polyamine analogs were determined by HPLC analysis of dansylated derivatives, as described previously (Mitchell et al., 2002). The dansylation of CGC-11144 was the same as for the polyamines and smaller analogs, however, the dansylated oligoamines were suspended in a 20:80 (v/v) mixture of tetrahydrofuran in isopropanol instead of methanol. Further, the HPLC chromatography was altered for samples containing this oligoamine. Specifically, after the polyamines and shorter analogs were eluted by an acetonitrile gradient from 33–100%, the peak of CGC-11144 was eluted by a 50:50 (v/v) mixture of isopropanol in acetonitrile introduced in a 4 ml linear gradient.

Three conformationally-restricted polyamine analogs that are symmetrically ethylated on their primary amino residues were used in this study. CGC-11047 is essentially bisethylspermine with a double bond between the central carbons. Similarly, CGC-11102 is bisethylhomospermine but with a double bond between its central carbons. Preparation of CGC-11047 and CGC-11102 were described previously in Reddy et al. (1998) and Valasinas et al. (2001) respectively. The structure and synthesis of the oligoamine CGC-11144 have also been described (Mitchell et al., 2002; Valasinas et al., 2003). The designation "SL" in the analogs SL-11047, SL-11102 and SL-11044 used in the referenced articles has been changed to "CGC" to reflect the merger of SLIL Biomedical Corp. with Cellgate Inc., Redwood City, CA.

Antizyme activity was determined by the ability of cell homogenates to inactivate a known amount of partially purified ornithine decarboxylase. The detailed assay conditions are described elsewhere (Mitchell et al., 2002).

Results

When initially exposed to exogenous spermidine or spermine, mammalian cells will incorporate sufficient polyamine in 1–2 h to increase intracellular concentrations by 15–30%. As a consequence of this increase in cellular polyamines, an increase in AZ is observed within the first hour, and AZ protein and activity appear to be at their maximum by 3 h. Previously we demonstrated that exposure to structural analogs of the polyamines can elicit the same response, but differences were noted in the levels of AZ induced depending upon the analog used (Mitchell et al., 2002). Figure 1A illustrates the range of AZ induction noted for three of the polyamine analogs that we have chosen for further study. By comparing the amount of AZ induced following a 3 h exposure, we note that CGC-11047 is about as effective as the native polyamines, while CGC-11102 is slightly, but not statistically, less so, and CGC-11144 is much more effective at stimulating AZ synthesis. These differences in AZ-inducing potential are exploited in the following studies.

The AZ that is produced by a cell not only inhibits the first enzyme in polyamine synthesis, but it also serves to down-regulate the polyamine transporter through an, as

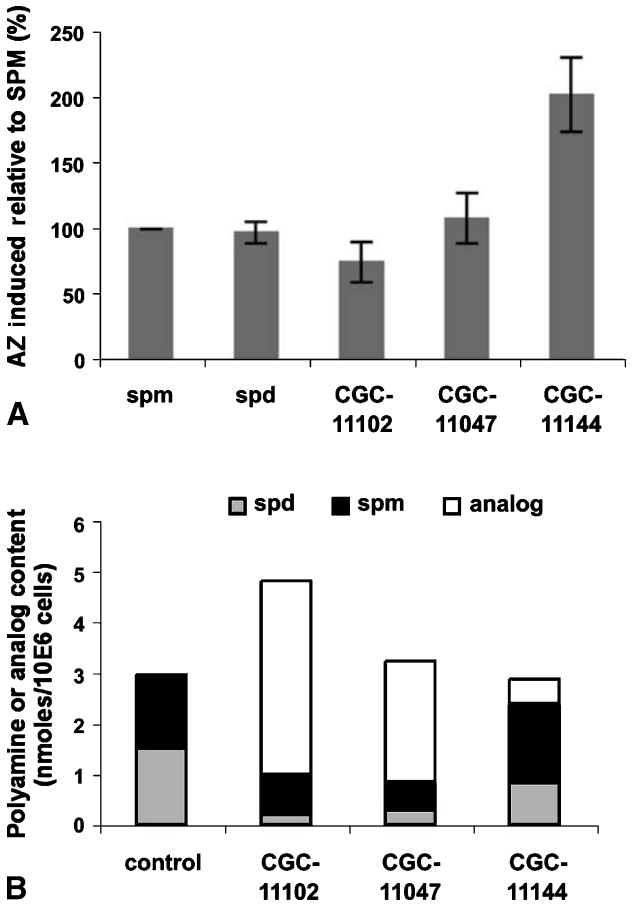

Fig. 1. Interrelation between propensity to induce AZ and cellular incorporation of representative polyamine analogs. **A** HTC cells were suspended in fresh medium containing 10% horse serum and incubated in a stirred flask for 20 h. Cultures were split into multiple small stirred flasks and the indicated polyamines or analogs were added at 10 μM. After 3 h exposure cell samples were withdrawn and analyzed for antizyme activity. The data represent antizyme activity, minus that of controls, relative to the antizyme induced by spermine (*SPM*). **B** The indicated analogs (1.0 μM) were added to exponential-phase monolayer cultures of CHO cells in 6-well plates. Cultures, in triplicate, were harvested after 24 h and cell pellets were counted by Coulter Counter and prepared for polyamine analysis. Shown are the cellular contents of spermidine (*spd*), spermine (*spm*) and respective analogs

yet, unknown mechanism. Since the polyamine analogs rely on this transporter for entrance to the cells, it is likely that there is a correlation between AZ-inducing potential and steady-state concentrations of the analogs accumulated in cells. To explore this, CHO cells were exposed to 1.0 μM concentrations of the analogs to be tested, and total polyamine levels were subsequently evaluated. Figure 1B compares the polyamine/analog profiles of these cultures 24 h after analog addition. The cells exposed to CGC-11102 accumulated a large amount of the analog such that the total cellular polyamine content (including spermidine, spermine and analog) was increased by about 65% relative to control cells. By comparison, the cells exposed to CGC-11047 exhibited only a small increase in total polyamines and those treated with CGC-11144 actually exhibited a slight decrease. Since incorporation of exogenous analogs will cease once a cell's polyamine content is sufficient to induce the synthesis of excess AZ, the results obtained are consistent with the differential AZ-inducing propensity demonstrated for these analogs. Accordingly, a relatively large amount of the weak AZ inducer CGC-11102 must be incorporated before an inhibitory level of AZ is synthesized, and much less of the analog CGC-11144 is required. Once the total cell polyamine levels are sufficient to induce synthesis of enough AZ to inhibit polyamine synthesis and uptake, then native polyamine levels should decline and additional uptake of the analog will be restricted. Clearly, the steady-state total concentration of the larger polyamines (native plus analog) is higher for analogs that are less adept at inducing AZ.

Similar studies have been conducted to evaluate polyamine/analog levels of cultures exposed to analogs for up to 4 days. In experiments with CGC-11047 and CGC-11102, where cell growth usually stopped between the second and third day of exposure, the cells maintained approximately the same level of total polyamine (plus analog) per cell for this 4-day period. Similar studies could not be conducted using CGC-11144 as this compound appears to be quite cytotoxic and very few cells remained attached after 24 h. The rapid inhibition of cell growth by CGC-11144 also contributes to the distinct polyamine/analog pattern of treated cells noted in Fig. 1B. In the cultures exposed to CGC-11047 and CGC-11102, the cell numbers continued to increase even though AZ blocked polyamine biosynthesis, resulting in obvious decreases in the native polyamine concentrations. Some loss of spermidine and spermine is also probably attributable to degradation and export. The cells exposed to CGC-11144 did not exhibit this rapid decline in natural cell polyamines over this time period. It is interesting to note that the decrease in native polyamine concentrations noted in the other cultures is compensated for by additional analog uptake, such that the total cell burden of polyamines plus analog remains the same. An example of this compensatory analog uptake to replace declining native polyamine levels can be seen in Fig. 5A.

A second consequence of AZ induction by polyamine analogs is demonstrated in the experiment shown in Fig. 2. CHO cells were exposed to concentrations of polyamine analogs over a 20-fold range from 0.25 to 5 μM, and the cellular contents of the analogs were evaluated after 24 h. Consistent with the previous study, very little CGC-11144, a good AZ inducer, was incorporated, while relatively

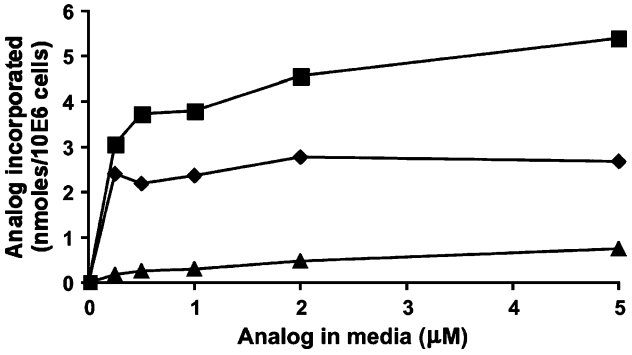

Fig. 2. Concentration of polyamine analog incorporated is largely independent of its concentration in the culture medium. CHO cell cultures as described in Fig. 1B were exposed to CGC-11102 (■), CGC-11047 (◆), or CGC-11144 (▲), at concentrations varying from 0.25 to 5 μM for 24 h. Cell pellets were subsequently counted and cellular analog concentrations determined by HPLC analysis

large amounts of the weak AZ inducer CGC-11102 were taken up. Remarkably, the amount of each of the polyamine analogs incorporated into cells was, for the most part, independent of their extracellular concentrations over this range. Cellular uptake of these polyamine analogs is limited by the activity of the polyamine transporter, which, in turn, is controlled by AZ. Once cells have achieved a total polyamine level sufficient to induce AZ, then heightened extracellular concentrations are of little consequence. On the other hand, by maintaining this slightly elevated AZ level the polyamine biosynthetic pathway of the cells remains effectively blocked, and essential natural cellular polyamines are depleted.

As an initial event in response to stimulation of proliferation, AZ is thought to be inactivated by the regulatory protein AZI. Microarray analysis has suggested that the gene for AZI is over expressed in certain human tumors, and cells transfected with AZI demonstrate increased tumorigenic potential (Jung et al., 2000; Keren-Paz et al., 2006; Kim et al., 2006). If the cytotoxicity of certain polyamine mimetics is dependent upon their propensity to induce AZ, and thereby induce deprivation of the native polyamines, then tumor cells that have up-regulated AZI may exhibit altered sensitivity to such analogs. Perhaps such cells will be less sensitive to polyamine mimetics because the extra AZ induced by the mimetic would be counteracted by the over expressed AZI, minimizing the effect of the mimetic on natural polyamine concentrations. Alternatively, such cells may be expected to be more sensitive in that the absence of AZ inhibition of the polyamine transporter will allow incorporation of even greater concentrations of a polyamine analog and this could enhance the displacement of the native polyamines.

To explore potential effects of excess cellular AZI on sensitivity to polyamine analogs we made stable transfectants of CHO cells that contain a mouse AZI gene controlled by a tightly regulated promoter. Upon induction with the synthetic hormone mifepristone these cells express mouse AZI at levels 10–20 times the native AZI (Mitchell et al., 2004). As anticipated, cells expressing this excess AZI do accumulate more polyamine analog than transgenic clones in which the AZI has not been induced. As shown in Fig. 3A, 24 h after exposure to 10 μM CGC-11102 transgenic CHO cells expressing mouse AZI

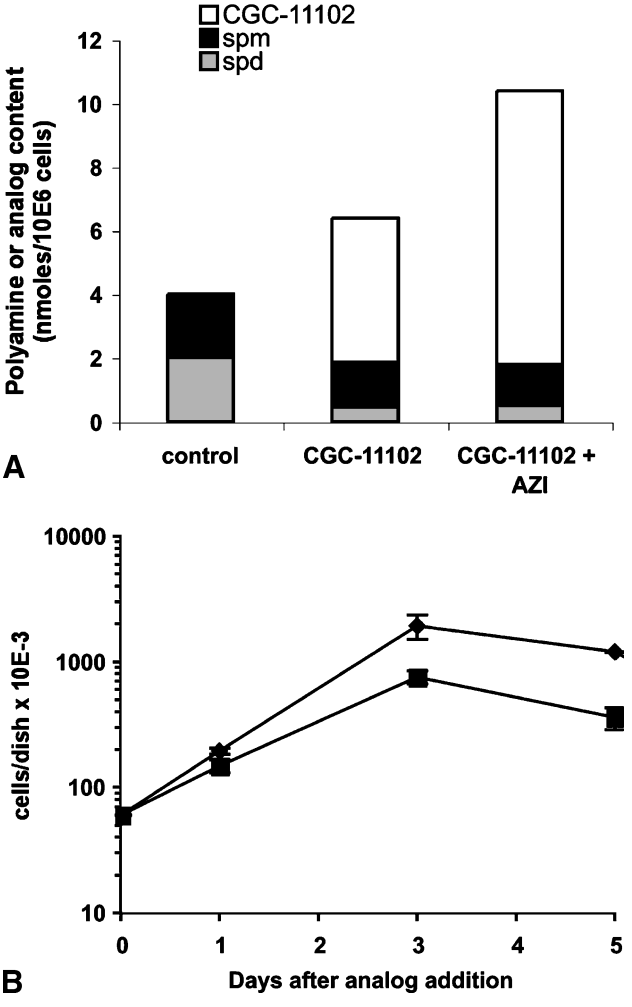

Fig. 3. Effect of exogenic AZI expression on cellular uptake of CGC-11102 and its effect on cell proliferation. GS-CHO(AZI-V5-6H) cells were seeded at 2.5×10^4 cells/dish in 6-well tissue culture plates. Some of the cultures were treated with 10 nM mifepristone to induce exogenic AZI. After 24 h, 1.0 μM CGC-11102 was added to some of these and triplicate cultures were harvested after 1, 3 and 5 days of exposure. Cell pellets were subsequently counted by Coulter Counter and analyzed for polyamine and analog contents. **A** The average polyamine/analog contents of cells 24 h after addition of analog. **B** Growth curves of cultures exposed to CGC-11102 either with (■) or without (◆) the induction of exogenic AZI

contained almost twice as much of the analog as control cells in which the AZI was not induced. Further, the burden of this additional polyamine analog appears to enhance the cytostatic effect of this mimetic, as the AZI-expressing cells show more profound growth inhibition than the control cells which are also exposed to this mimetic (Fig. 3B). Normally, cells exposed to CGC-11102 will show extensive polyamine depletion after 2–3 cell doublings, at which time the cells stop replication. This timing is reminiscent of difluoromethylornithine (DFMO) induced polyamine deprivation and growth inhibition. In the cells expressing exogenous AZI, cell division generally ceases somewhat earlier, after only 1–2 cell doublings.

The increase in sensitivity to polyamine analogs noted in cells over expressing AZI appears to vary somewhat with the analog tested. CGC-11047 only differs slightly from CGC-11102 (the former is a 3-4-3 analog while the latter is a 4-4-4), yet AZI over expressing cells find CGC-11047 much more toxic than CGC-11102. As with CGC-11102, exposure of CHO cells not over expressing AZI to CGC-11047 will result in polyamine deprivation and growth inhibition after 2–3 cell doublings. Surprisingly, the same mimetic induces extensive cell detachment and apparent cell death in just a few hours in cells over expressing AZI (Fig. 4). Detachment starts between 6 and 8 h of exposure, and 80–90% of the cells are lost by 24 h. The reason for this greatly enhanced cytotoxicity is clear upon consideration of the cellular polyamine/analog contents of the cultures during the course of this experiment (Fig. 5). The cells expressing normal AZI levels incorporated only enough CGC-11047 to increase the total cellular concentration of polyamines plus analog slightly, at which time ample AZ was produced to inhibit further uptake. As indicated earlier and in Fig. 1, additional analog did enter the cell over this 24 h period in proportion to the loss of native polyamines, thereby maintaining a rather consistent total cellular polyamine (including the analog) concentration (Fig. 5A).

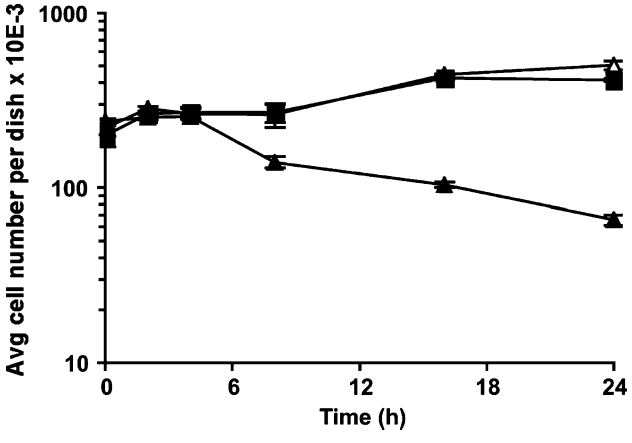

Fig. 4. Effect of exogenic AZI expression on CHO cells exposed to CGC-11047. GS-CHO(AZI-V5-6H) cells were seeded at 2.5×10^4 cells/dish in 6-well tissue culture plates. After 24 h some of the cultures (△, ▲) were treated with 10 nM mifepristone to induce exogenic AZI expression. After an additional 24 h, 1.0 μM CGC-11047 was added to cells that either had been induced for AZI (▲) or not (■). Triplicate cultures of each treatment were harvested and cell counts determined by Coulter Counter at the indicated time periods ending at 24 h

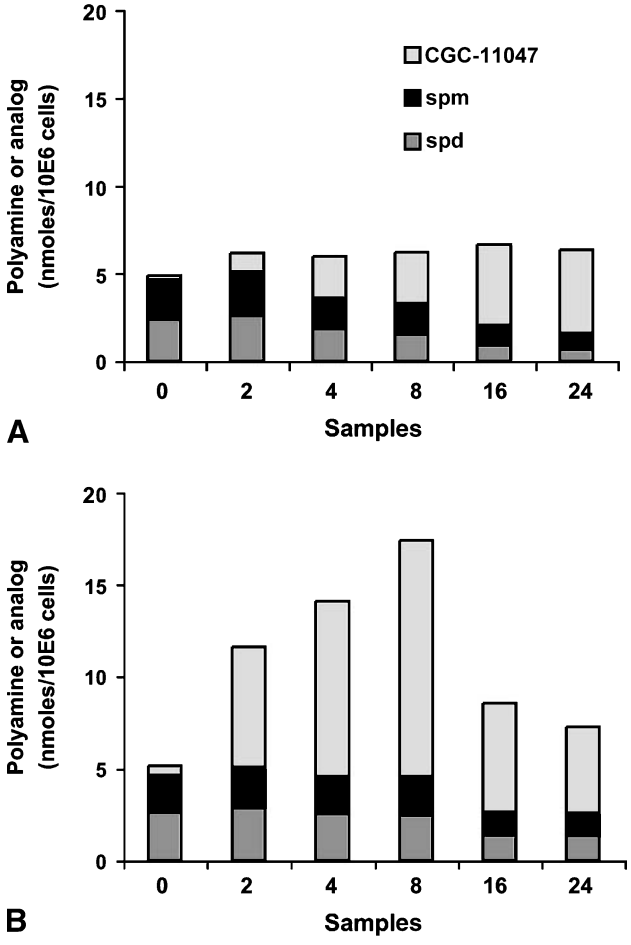

Fig. 5. The effect of exogenic AZI expression on uptake of CGC-11047 by CHO cells. Polyamine and CGC-11047 content of the cultures in Fig. 4 that were induced for AZI (**B**) or not (**A**) and subsequently exposed to 1.0 μM CGC-11047

By sharp contrast, feedback inhibition of CGC-11047 was not evident in the cells over expressing AZI, and by 8 h the total cellular polyamine burden was over 3-times that of control cells (Fig. 5B). The repression of AZ by the expressed AZI in these cells permitted the incorporation of CGC-111047 to continue, allowing concentrations that apparently are incompatible with cell viability. Such radical over accumulation and cell lysis was observed previously in cells made incapable of producing AZ by the

addition of cycloheximide during exposure to spermine (Mitchell et al., 1992). By preventing AZ-mediated feedback inhibition of polyamine uptake, cells containing an active transporter and exposed to spermidine, spermine or an efficiently transported mimetic, might be expected to accumulate toxic levels of these compounds. It should be noted that the decrease in analog burden in the cells at 16 and 24 h merely reflects the polyamine/analog contents of the 10–20% of cells that did not detach initially. These may be cells that had low transport activity at the start of the experiment and never achieved toxic polyamine concentrations.

Discussion

These studies demonstrate that the uptake of certain polyamine analogs into cells and their ability to induce polyamine deprivation, both are influenced by their propensity to stimulate AZ production. Further, the results show that tumor cells that over express AZI may have increased sensitivity to certain analogs because of their interference with the normal AZ-mediated feedback controls. We reported previously that the AZ induced when cells incorporate certain polyamine analogs is sufficient to prevent synthesis of the native polyamines (Mitchell et al., 2004). The current results extend these observations by showing that there is a clear, inverse correlation between the propensity of an analog to stimulate AZ production and the concentration of the analog a cell accumulates. Polyamine analogs are incorporated only until the total cell content of larger polyamines (spermine, spermidine and analog) stimulates sufficient AZ to inhibit the transporter. Subsequent analog entry appears to be allowed only to compensate for dilution, export or degradation of the native polyamines. Thus, the concentration of polyamine analog incorporated into cells is determined by its propensity to induce AZ and, once AZ synthesis is initiated, further incorporation is not elicited by increasing external doses.

The inability to escalate cellular concentrations of a polyamine analog may be viewed as a negative attribute as it may make it difficult to force cells to incorporate sufficient amounts to cause a desired toxicity. Although this AZ-mediated restriction of analog uptake persisted in our cell culture experiments for at least the first few days of treatment, it is possible that longer studies may show an eventual loss of this feedback. Since AZ is a very labile protein and requires continuous synthesis, any decline or disruption in protein synthesis could prevent this uptake-limiting feedback reaction. The observation that uptake of many polyamine analogs appears to be limited by this natural transporter feedback system may need to be considered in evaluating polyamine antagonists, where the cellular concentration is likely to influence toxicity. On the other hand, for polyamine mimetics this may be a positive feature in that, over a large range of external concentrations, tissues are anticipated to self-dose precisely the amount of analog required to synthesize inhibitory levels of AZ. The polyamine analogs used in this study have a high affinity for the polyamine transporter, with an apparent Kt on the order of 1 μM, similar to that of spermidine or spermine (Mitchell et al., 2002, 2004). As such, circulating levels of these analogs in this range or above should suffice to maintain cellular levels of AZ sufficient to induce polyamine deprivation. It should be noted that in mouse xenograft studies there is a dose response curve for a number of the polyamine analogs that spans over at least a log concentration.

If the CHO cells that have been modified to express elevated AZI levels accurately represent model tumor cells, as has been suggested (Keren-Paz et al., 2006; Kim et al., 2006; Mangold, 2006), then it is of great interest that the over expression of AZI is associated with increased sensitivity to the tested analogs. The reasons for this enhanced sensitivity, however, may be somewhat complex. If a cell has sufficient AZI to completely prevent AZ activity, as with the CHO-AZI clones used in this study, then cytotoxicity might be expected due to osmotic imbalance caused by greatly elevated levels of cellular polyamines. The incorporation of cytotoxic levels of the larger polyamines in AZ-depleted cells can be caused by exposure to native polyamines (Mitchell et al., 1992) or a polyamine analog as shown here with CGC-11047. In light of this, it is odd that the cells over expressing AZI did not incorporate cytotoxic levels of CGC-11102. Although these two analogs are structurally quite similar, we have noticed that cellular uptake of CGC-11102 is generally much slower than the native polyamines or CGC-11047 [unpublished observations]. It is conceivable that such decreased uptake velocity, combined with efficient export or other possible limitations on import of this analog, may be sufficient to limit its further incorporation. Notably, even though only a two-fold increase in incorporated CGC-11102 was observed in cells over expressing AZI, this was sufficient to cause increased sensitivity to this compound. Such a moderate increase in cellular analog concentrations might be especially important for the physiological effects of polyamine antagonists, where higher concentrations would be expected to induce greater toxicity. Consistent with this concept, Keren-Paz et al. (2006) have shown that NIH3T3 cells transformed to over

express AZI were much more quickly growth inhibited by the cytotoxic polyamine analog CGC-11157 than was the parental NIH3T3 cell line. We considered extending these studies by exposing our AZI over expressing CHO clone to the toxic analog CGC-11144, however, even in the absence of AZI induction, the CHO cell line exhibited a considerable cell loss within the first few hours of treatment, and it did not appear that any significant increase in sensitivity would be detected.

These studies only examined a few representative polyamine analogs and, of course, observations on cells in culture do not necessarily correlate directly to in vivo physiological results. Further, the present studies have concentrated on evaluating short term responses, and longer time course experiments would clearly benefit our understanding of these responses. However, there does appear to be good evidence that AZ induction is an important factor in cellular uptake and the response generated by the less toxic polyamine analogs. If so, then it may be useful to consider AZ induction and abnormalities in AZI production in planning dosage and treatment strategies with such polyamine analogs.

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