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Polyamine Analogs with Xylene Rings Induce Antizyme Frameshifting, Reduce ODC Activity, and Deplete Cellular Polyamines

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Numerous studies have correlated elevated polyamine levels with abnormal or rapid cell growth. One therapeutic strategy to treat diseases with increased cellular proliferation rates, most obviously cancer, has been to identify compounds which lower cellular polyamine levels. An ideal target for this strategy is the protein antizyme—a negative regulator of polyamine biosynthesis and import, and a positive regulator of polyamine export. In this study, we have optimized two tissue-culture assays in 96-well format, to allow the rapid screening of a 750-member polyamine analog library for compounds which induce antizyme frameshifting and fail to substitute for the natural polyamines in growth. Five analogs (MQTPA1–5) containing xylene (1,4-dimethyl benzene) were found to be equal to or better than spermidine at stimulating antizyme frameshifting and were inefficient at rescuing cell growth following polyamine depletion. These compounds were further characterized for effects on natural polyamine levels and enzymes involved in polyamine metabolism. Finally, direct measurements of antizyme induction in cells treated with two of the lead compounds revealed an 8- to 15-fold increase in antizyme protein over untreated cells. The impact of the xylene moiety and the distance between the positively charged amino groups on antizyme frameshifting and cell growth are discussed.

Key words: antizyme, drug screen, frameshift, ornithine decarboxylase, polyamine analog.

Abbreviations: AG, aminoguanidine; DENSPM, N^1,N^{11}-diethylnorspermine; DFMO, Difluoromethylornithine; FBS, fetal bovine serum; GRR, growth rescue ratio; ODC, ornithine decarboxylase; %FS, percent frameshifting; %RF, percent relative frameshifting; SAMDC, *S*-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase.

Polyamines (putrescine, spermidine, and spermine) are essential for cell growth and cell cycle progression (reviewed in Ref. *1*), and intracellular levels are highly regulated through complex feedback mechanisms involving synthesis, catabolism/interconversion, import, and export (Fig. 1) (reviewed in Refs. *2* and *3*). Based on the observation that increased polyamine levels are correlated with neoplastic growth (reviewed in Refs. *4* and *5*), it has been proposed that reducing or depleting intracellular polyamine levels would be an appropriate therapy, or preventative, for diseases involving undesired cell proliferation such as cancer.

Early efforts at modulating polyamine levels focused on reducing synthesis through inhibition of the major polyamine metabolic enzymes, ODC and SAMDC. DFMO is an irreversible inhibitor of ODC (*6*) that induces growth arrest and decreases the intracellular content of putrescine and spermidine in normal and malignant cells (*7*). Despite its early promise, DFMO had slow uptake and rapid excretion from the body (*8*), lacked the ability to reduce spermine levels, and led to an up-regulation of polyamine import (*9*). Since extracellular polyamines are readily available from our diet and gut flora, inhibition of polyamine synthesis with DFMO alone has shown limited chemopreventive (*10*, *11*) and therapeutic success (*12*, *13*). Likewise, SAM486A (CGP48664), a potent inhibitor of SAMDC (*14*, *15*), showed mixed results in phase II clinical trials (*16*, *17*). As with DFMO, growth inhibition by SAM486A can be prevented by exogenous spermidine or spermine (*14*). Clinical success with these and other polyamine biosynthetic enzyme inhibitors is likely to be compromised by the up regulation in polyamine transport and the compensatory responses by enzymes involved in the homeostatic control of cellular polyamine levels.

Combination therapies with more than one target have been developed to affect multiple polyamine maintenance pathways. For example, polyamine transport inhibitors used in conjunction with DFMO have shown promise in cell culture studies (*18*, *19*) and in tumor inhibition studies

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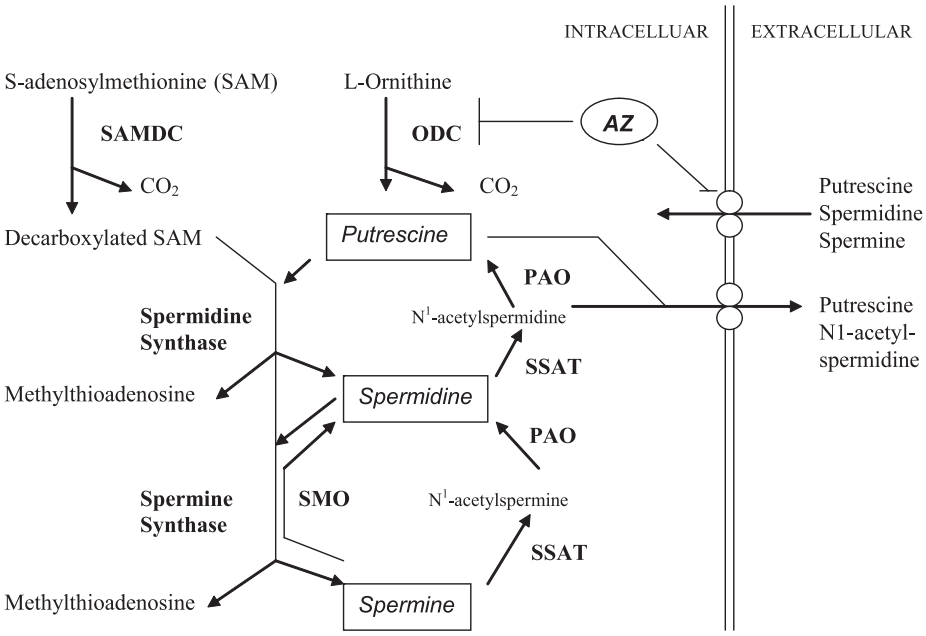

Fig. 1. **The polyamine metabolic pathway.** AZ = antizyme, PAO = polyamine oxidase, and SMO = spermine oxidase.

in nude mice (*20*). Similarly, polyamine analogs have been designed with the intent of affecting multiple targets in the polyamine pathway. The polyamine analog DENSPM induces the catabolic enzyme SSAT, down regulates ODC and SAMDC, suppresses polyamine uptake, and inhibits cell growth (reviewed in Ref. *21*). While preclinical trials showed promise for DENSPM as an anticancer drug (*22*, *23*), clinical trials uncovered gastrointestinal toxicities that compromised the dosing and selectivity (*24*, *25*).

Another strategy to productively alter polyamine homeostasis has been to enhance the cell's innate ability to reduce polyamine levels through induction of antizyme. Antizyme serves as the cell's sensor and regulator of polyamine levels, maintaining homeostasis and preventing toxicity from excess polyamines. Antizyme binds ODC and targets it for proteasomal degradation (*26–28*), inhibits polyamine import (*29–31*), and enhances polyamine export (*32*). A number of studies have looked at both transient and inducible over-expression of antizyme in cell lines and animal tumor models (*33–36*). The results demonstrate that deliberate antizyme induction can effectively inhibit cell growth and reduce tumor formation in mice.

A family of three human antizyme genes has been identified. Antizyme 1 (*37*) and 2 (*38*, *39*) show nearly ubiquitous tissue expression, whereas antizyme 3 (*40*, *41*) is only expressed in developing male germ cells. All three antizymes use the same unique method of protein expression that requires +1 translational frameshifting (*42*, *43*). mRNA sequences 5′ and 3′ of the frameshift site (UCC UGA) have been identified as important stimulators of the frameshift event in both antizyme 1 and 2 (*42*, *43*). In addition to these *cis*-acting mRNA sequences, frameshifting is stimulated by polyamines, thus creating an autoregulatory circuit for controlling intracellular polyamine pools. The mechanism by which polyamines stimulate frameshifting is unknown, although the regulatory 5′ sequence and the ORF1 stop codon, but not the 3′ sequence, are required (*44*, *45*).

In the first published assessment of antizyme induction using polyamine analogs, a class of oligoamines was identified that could induce antizyme up to two-fold better than spermine (*46*). Four classes of analogs structurally similar to spermine and spermidine (total of 24 analogs) were characterized for their ability to induce antizyme and inhibit cell growth. Despite wide variation in cell growth inhibition after 3 days with the analogs, there was a general correlation with the highest antizyme inducers showing potent and rapid growth inhibition.

High throughput screens have proven to be a valuable and essential tool identifying potential hits among chemical libraries. We have developed two 96-well assays as a primary screening strategy to identify antizyme-inducing molecules in a 750-member polyamine analog library. A quantitative cellular antizyme frameshifting reporter assay (*44*, *47*) was adapted to a 96-well format providing a method to screen for antizyme frameshift-inducers. A growth-rescue assay, also in a 96-well format, was used to identify those molecules that have limited ability to function like natural polyamines in cell growth. Compounds were identified that were more potent antizyme frameshift inducers than spermidine, with little ability to substitute for polyamines in maintaining cell growth. Cells treated with five of the lead compounds, each containing xylene (1,4-dimethyl benzene), showed reduced polyamine levels, reduced ODC activity, were growth inhibited without induction of SSAT or inhibition of SAMDC, signifying that endogenous antizyme was being induced. As predicted, antizyme induction was confirmed for two of the lead compounds.

MATERIAL AND METHODS

Cell Culture and Reagents—The human embryonic kidney HEK293 and the Chinese hamster ovary CHO cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM media (Mediatech, Inc.; Herndon, WA), 10% FBS (Gibco BRL; Gaithersburg, MD), 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM l-glutamine (Cambrex Bio Science, Walkersville, MD) at

37°C in an atmosphere of 5% CO_2. When cells were cultured with polyamines or polyamine analogs, 1 mM AG (Sigma) was included to inhibit serum amine oxidase activity. DFMO was obtained from Advanced ChemTech (Louisville, Kentucky). DENSPM was obtained from Pfizer Pharmaceuticals (Ann Arbor, MI). Agmatine was obtained from Aldrich (Milwaukee, WI).

In Vitro *Frameshifting Assay*—Compounds were screened for their ability to induce frameshifting using a dual luciferase reporter assay (p2luc) (*47*). HEK 293 cells were plated in white sided, clear-bottomed 96-well assay plates at 15,000 cells per well in 100 μl of medium (DMEM supplemented with 10% FBS, 1% penicillin, streptomycin and L-glutamine) containing 2.5 mM DFMO. The cells were incubated for two days at 37°C in an atmosphere of 5% CO_2. Cells were transfected overnight with 100 ng of plasmid DNA and 0.3 μl lipofectAMINE reagent (Life-Technologies) in 50 μl of serum-free DMEM containing 2.5 mM DFMO. After transfections, compounds (diluted in either water or medium) were added at a concentration of 25 μM. The positive control contained 25 μM spermidine, and for the negative control no spermidine or compound was added. The cells were incubated overnight at 37°C in an atmosphere of 5% CO_2, washed once with 1× PBS, lysed with 50 μl of passive lysis buffer (Promega) and assayed for *Renilla* and firefly luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) (*47*). Plates were read using a Thermo Labsystems luminescence plate reader.

The %FS was determined by dividing the ratio of the firefly luciferase to *Renilla* luciferase activity in cells transfected with the inducible p2Lucaz1 construct by the ratio of the firefly luciferase to *Renilla* luciferase activity in cells transfected with the control, in frame vector p2Lucaz1-IF.

The %RF compares the induced frameshifting of the test compound to 25 μM spermidine. The %RF was calculated by the following method. The background percent frameshifting activity determined from the 2.5 mM DFMO negative control was subtracted from the percent frameshifting activity for all compounds, including the spermidine control. The background-corrected frameshifting activity of each compound was then divided by the background-corrected frameshifting activity induced by 25 μM spermidine and multiplied by 100. Averages were determined from two to four experiments (n = 6–12).

Growth Rescue Screens—To determine the IC_{50} value for DFMO and the growth rescue ability of spermidine, cells were plated in 96-well plates such that they would be in log growth for the duration of the assay. Twenty four hours after plating, DFMO or spermidine was titrated onto the cells, and growth, if any, was permitted to continue for three days in the presence of 1 mM AG. At the end of the three days, cell growth was measured by MTS/PMS dye assay (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). OD values are an average of one to two experiments (n = 3–6). The IC_{50} is the concentration of DFMO that results in 50% of maximum cell growth inhibition.

For the growth rescue assays, HEK293 cells were plated in 96-well assay plates at 1,000 cells per well in 100 μl of medium (DMEM supplemented with 10% FBS, 1% penicillin, streptomycin and L-glutamine). The cells were incubated overnight at 37°C in an atmosphere of 5% CO_2. Compounds were added along with 1 mM AG and 2.5 mM DFMO in a final volume of 200 μl medium. The positive control contained 25 μM spermidine. The cells were allowed to incubate for six days before cell growth was measured by MTS/PMS dye assay. The rescue values were expressed as the GRR [(absorbance at 490 nm of cells treated with compound and DFMO)/(absorbance at 490 nm of cells treated only with DFMO)].

Intracellular Polyamine Levels and Enzyme Activities—Intracellular levels of putrescine, spermidine, spermine, N^1-acetylspermidine, and MQTPA1–5 were quantified using reverse phase high-performance liquid chromatography (*48*) with modifications as previously described (*49*). Following exposure to 10 μM of each analog for 24 h, cell pellets were extracted with 0.6 N perchloric acid, followed by dansylation, and peaks of interest were identified and quantified using purified standards. The results were expressed as pmol/10^6 cells. Results are an average of two experiments (n = 6), except spermidine which is from one experiment (n = 3). Cells were also collected to assay for ODC, SSAT, and SAMDC activities as previously described (*50*, *51*). Polyamine enzyme activities were expressed as pmol of N^1-acetylspermidine generated per min/mg of protein for SSAT and as nmol CO_2/h/mg protein for ODC and SAMDC. ODC activity is the average of two experiments (n = 6). SSAT and SAMDC activities are from one experiment (n = 3).

Western Blot Analysis—Chinese Hamster Ovary (CHO) cells were chosen to demonstrate AZ induction *in vivo* since their low steady state levels of AZ provide a clean system for quantifying fold increases. Cells were treated for 24 h with 10 μM of either putrescine, spermine, MQTPA3, or MQTPA5. Cells were harvested and total lysates (60 μg per lane) were run on a 10% Bis-Tris Criterion XT precast gel system (Bio-Rad Laboratories, Hercules, CA) using XT MES running buffer. Gels were transferred to a nylon membrane and immunoblotted with affinity-purified polyclonal rabbit antibody specific for antizyme (AZ; 24 kDa) generously provided by Dr. John Mitchell at Northern Illinois University, DeKalb, IL (*29*, *46*, *64*). Detection was performed with enhanced chemiluminescence from Amersham Pharmacia Biotechnology (Arlington Heights, IL). Protein was determined by the standard Bio-Rad assay, and β-actin (Sigma) was used to equalize for variations in loading. Fold increase values are the ratio of treated to untreated cells using ImageQuant volumes for AZ relative to β-actin.

Analog Synthesis—The synthesis of the xylene-polyamine analogs followed a solid-phase route involving the exhaustive reduction of resin-bound bisamides. Synthesis of the bisamides followed standard peptide coupling methodology on trityl-chloride substituted polystyrene. The borane-mediated reduction technique was slightly modified from previously described methods (*52*, *53*). The analogs, MQTPA1 *N*-(4-amino-1-butane-*N*-(4-aminobutyl-))-xylylenediamine; MQTPA2 *N*-(4-amino-1-butane-*N*-(5-aminopentyl-))-xylylenediamine; MQTPA3 *N*-(4-amino-1-butane-*N*-(6-aminohexyl-))-xylylenediamine; MQTPA4 *N*-(3-aminopropyl)-*N*'-(6-aminohexyl-)-xylylenediamine; MQTPA5 *N*-(3-aminopropyl)-*N*'-(6-amino-1-hexane-*N*-(6-aminohexyl-))-xylylenediamine, showed greater than 95% purity by a combination of ^{1}H NMR, TLC, LC/MS

and elemental analyses. A manuscript describing the full experimental procedures and characterization is in preparation. The proprietary polyamine library is owned by MediQuest Therapeutics, Inc., Bothell, WA USA. Questions about its nature and availability can be directed to MRB.

RESULTS

Screening for Frameshift Stimulators—Compounds in a 750-member polyamine analog library were screened for their ability to induce antizyme frameshifting using a 96-well plate format dual luciferase reporter assay in cultured mammalian cells (*44*, *47*). Cells were transiently transfected with p2Luc reporter plasmids. This reporter contains the *Renilla* luciferase gene separated from the firefly luciferase gene by a multiple cloning site. The *Renilla* luciferase protein acts as an internal control for transfection efficiencies, translation initiation, and mRNA stability (*47*). The portion of the human antizyme 1 gene known to be important for frameshifting was cloned between the two reporter genes such that the firefly gene is in the +1 frame (to monitor frameshifting) relative to the *Renilla* luciferase gene (*44*). The amount of frameshifting is then determined by comparing the ratio of firefly to *Renilla* luciferase activity in the test (+1) construct normalized to a control construct in which the firefly gene is in the zero frame relative to the *Renilla* luciferase gene. In order to reduce baseline levels of endogenous antizyme, HEK293 cells were incubated with 2.5 mM DFMO for two days prior to transfection of reporter plasmids and addition of polyamine analogs.

Previous studies on antizyme induction have shown that the natural polyamines putrescine, spermidine, and spermine have the ability to induce antizyme frameshifting in cultured cells (*44*). Using this assay, spermine and spermidine showed a similar result inducing frameshifting to 34% and 29%, respectively (Fig. 2). Spermidine was chosen as the internal control for all screening experiments. Initial screening of compounds was done at 25 μM, and the percent frameshifting relative to 25 μM spermidine, %RF, was calculated. The well characterized molecules agmatine and DENSPM were also tested in our screening assays. While agmatine is known to stimulate antizyme frameshifting (*54*), relative to spermidine it is a poor antizyme frameshifter (29% RF) when tested at the same concentration (Fig. 2). The polyamine analog DENSPM is recognized as a potent SSAT inducer as well as an inhibitor of ODC and SAMDC (reviewed in Ref. *21*). As has been demonstrated previously (*46*),

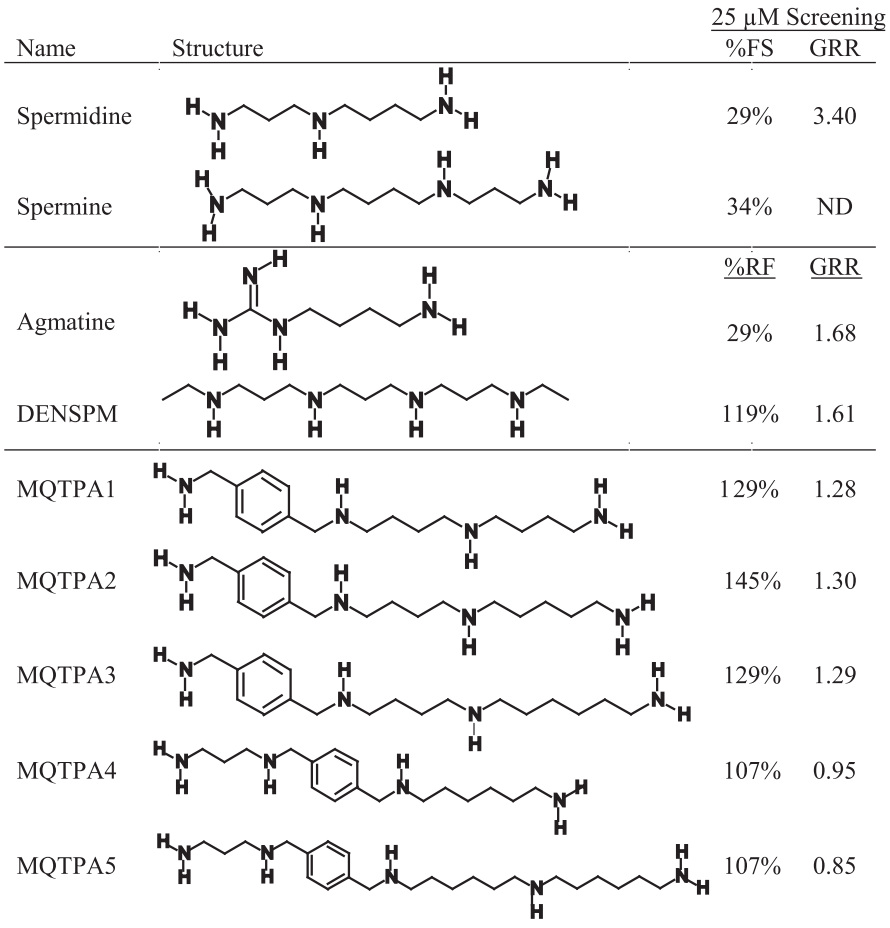

Fig. 2. **Structure, frameshifting, and growth rescue of the natural polyamines and select analogs.** %FS is the percent frameshifting, %RF is the percent relative frameshifting to 25 μM spermidine, and GRR is the growth rescue ratio of a six day assay in HEK293 cells with 2.5 mM DFMO. GRR of 1.0 equals cell growth with DFMO alone. ND is not done.

DENSPM also induces antizyme frameshifting (119% RF, Fig. 2). Out of the 750 compounds screened for antizyme frameshifting, 104 (14%) showed ⩾80% RF (data not shown). Within this group, MQTPA1–5 had relative frameshifting values of 107–145% (Fig. 2). These analogs were chosen for further analysis based on their inability to rescue growth, as described below.

Screening for Growth Rescue—The structural features of the natural polyamines that are essential for antizyme frameshift induction are unknown, but are likely different from those required for growth. The target of polyamines in antizyme induction could be the mRNA, the ribosome, or other molecules. Polyamines most likely play a role in growth regulation through multiple targets, affecting transcription, translation, mRNA splicing, chromatin condensation, and apoptosis (*4*, *55*, *56*). To be therapeutically useful, a polyamine analog must activate antizyme induction without functionally replacing the natural polyamines in growth regulation.

Compounds were screened for the inability to rescue HEK293 cells that were growth inhibited by the polyamine synthesis inhibitor, DFMO. DFMO shows a growth inhibition IC_{50} of 150 μM in a three day assay on HEK293 cells (Fig. 3A). With 1 μM spermidine included in the media, growth inhibition due to polyamine reduction was completely prevented because of uptake of exogenous spermidine (Fig. 3B).

To screen compounds for their inability to substitute for the natural polyamines in growth, HEK293 cells were incubated with 2.5 mM DFMO and 25 μM compound for six days. A GRR, the number of living cells with a given compound and DFMO normalized to the number of living cells with DFMO alone, was determined. Growth with DFMO alone was given a GRR of 1.0. Cell growth with DFMO plus 25 μM spermidine gave a GRR of approximately 3.4 (Fig. 2). If the drug had the desired effect of no rescue, then it had a value close to DFMO alone (1.0). If the value was less than one, then the compound was growth inhibitory. If the value was zero, the compound was cytotoxic. Compounds that rescued cells from DFMO-induced growth inhibition had a GRR greater than 1.0 and were less desirable. Of the 104 compounds that had shown the desired >80% RF, only 18 (17%) had acceptable GRRs of 0.5–1.5 (data not shown). This screening step was critical in identifying compounds that were not simply mimics of natural polyamines. Out of these 18 lead compounds, five structurally similar compounds (MQTPA1–5) were selected for further characterization. MQTPA1, MQTPA2, and MQTPA3 had GRR values of about 1.3 (Fig. 2). In contrast, MQTPA4 and MQTPA5 had GRR values less than 1.0, suggesting not only are these compounds unable to substitute for the natural polyamines, but they also further inhibit cell growth. Both agmatine and DENSPM could minimally growth-rescue DFMO-treated HEK293 cells (GRRs of 1.68 and 1.61, respectively). These GRRs are greater than those of MQTPA1–5, but much less than the GRR of spermidine.

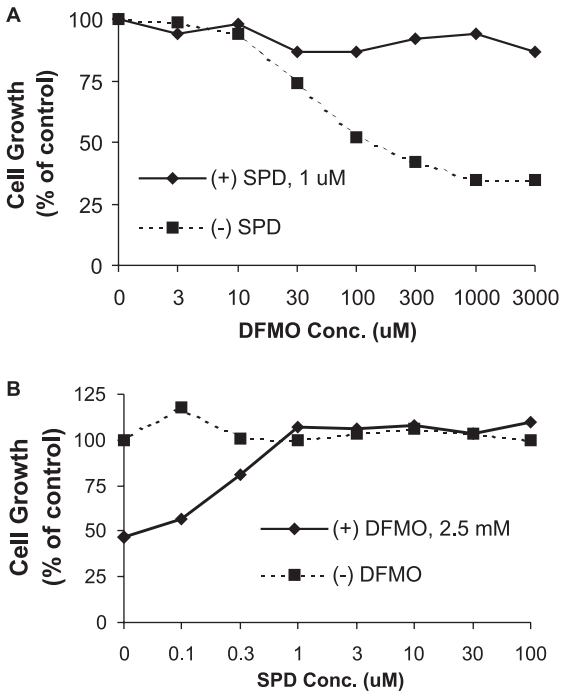

Fig. 3. **Three day growth assays on HEK293 cells.** (A) HEK293 cells were incubated for three days with 1 μM spermidine (SPD), 1 mM AG, and a titration of DFMO. (B) HEK293 cells were incubated for three days with 2.5 mM DFMO, 1 mM AG, and a titration of SPD. Cell growth was determined by colormetric MTS assays.

Frameshifting Potency and Growth Rescue Profile—MQTPA1–5 were tested at lower concentrations to determine frameshifting potency. Figure 4A compares the %RF of the five lead compounds and spermidine at 0.1–25 μM. Two of the five compounds (MQTPA1–2) showed greater frameshifting than spermidine at low concentrations (0.3–1 μM). The other three of the compounds (MQTPA3–5) showed a concentration-dependent frameshifting pattern very similar to spermidine. All compounds were more potent frameshift inducers than spermidine at high concentrations (10–25 μM).

At the same concentrations (0.1–25 μM) at which frameshifting was tested, the ability of compounds to rescue cells from DFMO-induced growth inhibition was determined (Fig. 4B). The HEK293 cells were treated for 6 days with spermidine or one of the five lead compounds. MQTPA1–5 showed a minor amount of growth rescue (GRR maximums of 1.2–1.7). When compared to spermidine, these compounds showed less ability to support cell growth at all concentrations tested. In contrast to spermidine, the maximum GRR was seen at the lowest compound concentrations, suggesting that as frameshifting increased with concentration, antizyme function (inhibition of ODC activity and polyamine transport) could negate contributions to growth due to polyamine substitution.

Intracellular Polyamines and Analog Concentrations—Increased frameshifting is predicted to lower polyamine levels due to increased expression of antizyme protein. Intracellular polyamines were measured in HEK293 cells that were treated for 24 h with 10 μM of each MQTPA compound. In all cases, there was a reduction in putrescine and spermidine (Table 1, compare MQTPA1–5 to control). Putrescine was reduced to less than 12% of the control cells, and spermidine reduction ranged from 7–55% of the control cells. The measurable levels of spermine were unchanged or increased. This

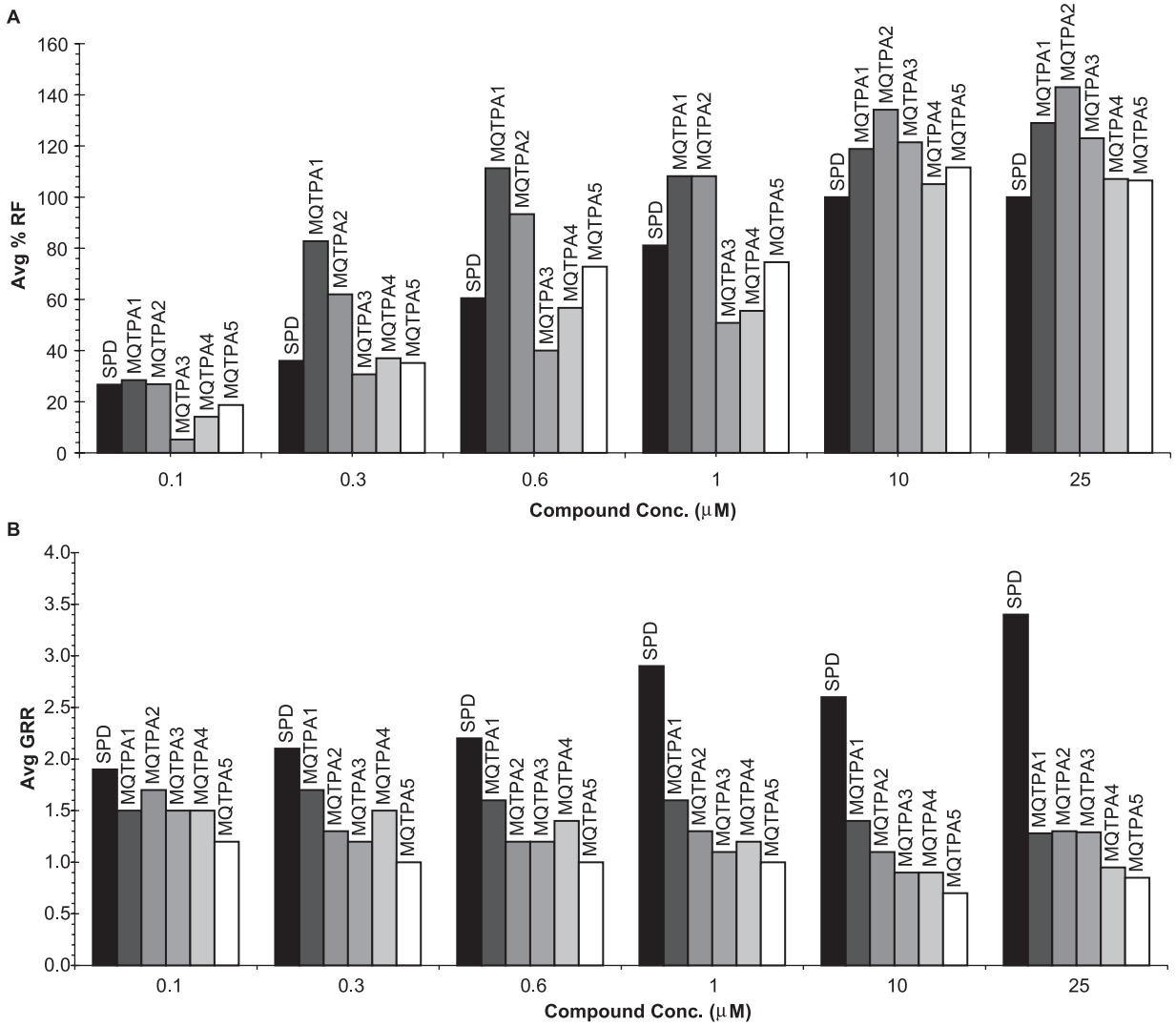

Fig. 4. **Percent relative frameshifting (%RF) and growth rescue ratios (GRR) of lead compounds and spermidine (SPD).** (A) Frameshifting was determined using the dual luciferase reporter assay in HEK293 cells that had been treated with 2.5 mM DFMO for 48 h before transfection of reporter plasmids. Cells were incubated with 0.1–25 μM of each compound for 24 h before luminescence was measured. Frameshifting relative to 25 μM SPD was determined for each concentration of compound. (B) HEK293 cells were grown for six days in the presence of 2.5 mM DFMO, 1 mM AG, and 0.1–25 μM of the MQTPA compounds. Cell growth was determined by colorimetric MTS assays. GRR of 1.0 equals cell growth with DFMO alone.

Table 1. **Polyamine reduction after treatment with MQTPA compounds. HEK293 cells were treated with DENSPM, spermidine (SPD), or MQTPA1–5 at 10 μM for 24 h.** Cells were counted and harvested. Proteins were acid precipitated, and the soluble polyamines (putrescine = PUT, spermine = SPM, N^1-acetylspermidine = N^1 AC-SPD) and analogs were derivatized and analyzed by HPLC.

	N^1 AC-SPD	PUT	SPD	SPM	Analog
	pmol/10^6 cells				
Control	<20	345	1,259	3,935	N/A
SPD	248	232	1,436	3,950	N/A
DENSPM	743	184	143	690	7,716
MQTPA1	<20	<20	510	4,751	540
MQTPA2	<20	<20	211	4,506	918
MQTPA3	<20	<20	83	3,673	1,224
MQTPA4	<20	<20	216	4,810	1,346
MQTPA5	<20	<20	112	3,910	586

pattern of decreased putrescine and spermidine and increased spermine is often seen with the inhibition of ODC by DFMO (*57*). It should be noted that treating cells with 10 μM spermidine or DENSPM does not give the same polyamine reduction pattern, suggesting that the pattern of polyamine reduction seen with MQTPA1–5 is specific to their mechanism of action. MQTPA1–5 entered and accumulated within the cells to various levels: 540–1346 pmoles/10^6 cells. There is no direct correlation of analog abundance with frameshifting potency at 10 μM (compare Fig. 4A and Table 1).

Polyamine Metabolism—Increased levels of antizyme should decrease cellular ODC abundance and activity due to antizyme's ability to bind and enhance the proteasome degradation of ODC (*26–28*). ODC activity was measured in HEK293 cells after 24 h of treatment with the MQTPA compounds at 10 μM. ODC activity

was decreased 63–77% by all five of the compounds (Table 2).

It has been shown that the polyamine analog DENSPM upregulates SSAT (reviewed in Ref. *21*). Since SSAT is responsible for the first step in the interconversion pathway for spermine to spermidine and spermidine to putrescine, treatment with an SSAT-inducer, like DENSPM, greatly reduces spermidine and spermine levels compared to untreated cells (Table 1). SSAT activity was measured in HEK 293 cells after 24 hr of treatment with 10 μM of compound. In contrast to DENSPM, the MQTPA compounds reduced SSAT activity to 20–84% of untreated cells (Table 2).

SAMDC activity was measured in HEK293 cells after 24 h of treatment with 10 μM of the MQTPA compounds. MQTPA1–5 did not inhibit SAMDC, in fact, the cells showed an increase of 126–250% of control SAMDC activity (Table 2). Previous studies have shown an increase in SAMDC activity when both putrescine and spermidine levels are decreased by inhibition of ODC (*58*, *59*). In contrast, DENSPM and spermidine treatment inhibit SAMDC activity.

Antizyme Induction—The properties of the lead compounds presented above (induction of antizyme frameshifting, inhibition of ODC activity, reduction of putrescine and spermidine levels, and inhibition of growth without induction of SSAT or inhibition of SAMDC) are the hallmarks of inducing antizyme protein expression. To directly confirm that antizyme protein was being induced, CHO cells were treated with 10 μM of either putrescine, spermine, or the polyamine analogs MQTPA3 or MQTPA5 for 24 h and then analyzed by Western for detection of antizyme (Fig. 5). CHO cells were chosen instead of HEK293 cells due to the high background of endogenous antizyme protein expression found in HEK293 cells in the absence of DFMO treatment (data not shown). Cells treated with putrescine and spermine revealed a slight 2–4 fold increase in antizyme expression. Whereas, treatment with MQTPA3 and MQTPA5 increased antizyme expression 8- and 15-fold, respectively. Although the relative frameshift levels were shown to be similar between these two compounds at 10 μM, at lower concentrations, MQTPA5 was a more potent antizyme frameshift inducer than MQTPA3 (Fig. 4); consistent with its ability to induce higher levels of antizyme protein in CHO cells. These Western results demonstrate that, as predicted by the screening assay results, endogenous antizyme expression is increased upon treatment with MQTPA compounds.

DISCUSSION

Many analogs of polyamines have been synthesized with the intention of finding one useful in the fight against cancer (*60*, *61*). As antizyme is a central regulator of polyamine biosynthesis (*26–28*), import (*29–31*), and export (*32*), analogs which are able to induce antizyme expression by stimulating +1 frameshifting, and are unable to mimic the cell growth functions of natural polyamines are potential therapeutic drugs. In the absence of detailed knowledge about the key polyamine structural features required for these functions, one way to identify such compounds is to screen large polyamine analog libraries. We have developed a sequential two assay approach to screen a 750-member polyamine analog library. Both assays have been optimized for a 96-well format. The first screen tests for the ability of the analog to stimulate the +1 frameshifting event required for antizyme expression. The second screen identifies compounds that are unable to rescue cell growth following polyamine depletion by DFMO.

Studies illustrating that over-expression of antizyme results in ODC inhibition, reduction in putrescine and spermidine levels, and suppression of tumor growth in mice (*33*, *34*, *36*) indicate that antizyme is a good target for cancer drug development. Cells treated with lead compounds induced antizyme frameshifting, inhibited ODC activity, reduced putrescine and spermidine levels, and were growth inhibitory without induction of SSAT or inhibition of SAMDC. These results are indicative of increased antizyme expression by all five lead compounds. Antizyme induction was subsequently directly confirmed by Western analysis of antizyme protein levels in cells treated with two of the compounds, MQTPA3 and MQTPA5 (Fig. 5). As expected, the results revealed a considerable (8 to 15 fold) increase in antizyme protein levels.

All MQTPA compounds described here are imported into the cell by the polyamine transporter based on the observation that they have higher IC_{50} values in the presence of a polyamine transport inhibitor or spermidine in the media (data not shown). The ability of the analogs to use the polyamine transporter is useful, because polyamine transport tends to be up-regulated in cancer cells (*21*, *62*), and the analogs can displace the uptake of the natural polyamines. However, because of antizyme's ability to inhibit polyamine import (*29–31*), the analogs which induce antizyme will inhibit the very transport system they

Table 2. **Polyamine metabolic enzyme activities in HEK293 cells.** HEK293 cells were treated for 24 h with compounds at 10 μM. SSAT activity is expressed as nmol of acetylspermidine synthesized/h/mg of protein. SPD = spermidine and SD = standard deviation.

Treatment	ODC nmol/h/mg (SD)	ODC activity % of control	SSAT nmol/h/mg (SD)	SSAT activity % of control	SAMDC nmol/h/mg (SD)	SAMDC activity % of control
Control	0.31 (0.04)		45 (2)		0.80 (0.06)	
DENSPM	<0.03 (<0.05)	<10	9,584 (525)	21,300	<0.05 (<0.05)	6
SPD	0.10 (0.02)	32			0.43 (0.05)	54
MQTPA1	0.12 (0.03)	37	38 (2)	84	1.10 (0.07)	138
MQTPA2	0.07 (0.02)	23	22 (4)	49	1.05 (0.06)	131
MQTPA3	0.08 (0.02)	26	22 (2)	49	2.00 (0.11)	250
MQTPA4	0.09 (0.03)	27	25 (1)	56	1.01 (0.07)	126
MQTPA5	0.07 (0.02)	23	9 (1)	20	1.40 (0.09)	175

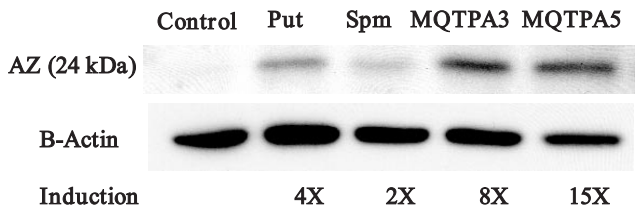

Fig. 5. **Western immunoblot analysis of antizyme expression in CHO cells.** CHO cells were either untreated (Control) or treated with 10 μM of putrescine (PUT), spermine (SPM), MQTPA3, or MQTPA5 for 24 h. Immunoblots of protein extracts (60 μg/lane) were probed with antibodies to β-actin and antizyme. Fold induction was determined as the ratio of antizyme (AZ; 24 kDa), normalized to β-actin, in extracts from treated versus untreated cells.

Table 3. **Characterization and comparison of the lead MQTPA compounds and spermidine (SPD).** %RF is the average percent relative frameshifting in HEK293 cells compared to 25 μM spermidine. GRR is the growth rescue ratio at 10 μM in a six day assay with 2.5 mM DFMO. ODC inhibition was measured in HEK293 cells treated with 10 μM of analog or spermidine for 24 h. (+) indicates the relative degree of drug favorable properties for each activity.

	0.3 μM		10 μM	
	%RF	GRR	%RF	GRR
SPD	36 (=)	2.1 (=)	100 (=)	3.4 (=)
MQTPA1	83 (+++)	1.7 (+)	119 (++)	1.4 (++)
MQTPA2	62 (++)	1.3 (++)	134 (+++)	1.1 (++)
MQTPA3	31 (=)	1.2 (++)	121 (++)	0.9 (+++)
MQTPA4	37 (=)	1.5 (++)	105 (=)	0.9 (+++)
MQTPA5	35 (=)	1.0 (+++)	112 (+)	0.7 (+++)

need to enter the cell (*63*). Nevertheless, uptake of the analogs in this study did occur (see Table 1), and enough analog enters the cell to stimulate antizyme frameshifting, inhibit ODC, and decrease cellular polyamines.

All the MQTPA compounds efficiently stimulated antizyme frameshifting to levels at or above those seen with spermidine. The para (1,4-dimethyl) position on a benzene ring was found to be favorable for inducing frameshifting. Comparison of MQTPA1–3 is informative as they differ only by the number of carbons separating the two terminal amines. At low concentrations (0.3 μM), the highest levels of frameshifting (Table 3) were observed with MQTPA1 (4 carbons). When the number of carbon atoms separating the terminal amines is increased to 6 (MQTPA3), although still efficient, a significant reduction in frameshifting is observed. Intermediate levels of frameshifting were observed with MQTPA2 (5 carbons). This result is consistent with a previous study indicating that 4 carbons (or less) separating the amino groups in diamines have the maximal effect on frameshift induction (*64*). One compound with a 3 carbon spacer separating the terminal amines was tested for RF and GRR (data not shown). This compound gave lower levels of frameshifting than that seen with MQTPA1 or MQTPA2 and was more efficient at growth rescue than compounds with 5 or 6 carbon spacing (see below). It should also be noted that the natural polyamines have 3 or 4 carbons separating the amines. These results suggest that the relative distance between the positively charged amine groups is important for stimulation of antizyme frameshifting.

The decrease in cellular polyamine pools caused by the five analogs described in this study may induce or contribute to cell death. For all compounds, putrescine and spermidine levels are reduced to less than 12% and 55% of untreated cells, respectively. Analogs which decrease cellular polyamine pools must not substitute for the natural polyamines in growth function if they are to be effective anti-cancer drugs. DFMO treatment inhibits cell growth by depleting polyamines, and spermidine rescues cell growth from this treatment. GRR is a measure of the ability of the analogs to rescue cells from DFMO treatment, i.e. substitute for the natural polyamines in growth. When comparing MQTPA1–3 (Table 3), the analog with 4 carbons between the terminal amines (MQTPA1) was observed to rescue cell growth better than those with 5 or 6 carbons (MQTPA2 and 3); although none of the analogs rescued as efficiently as spermidine. These results suggest that the analogs with amine groups separated by the same distance as the natural polyamines are better substitutes for the natural polyamines in antizyme frameshifting and in growth function. Identifying an optimal drug candidate requires a separation of these polyamine functions.

The effectiveness of an analog at low concentrations is one important consideration for its potential as a drug candidate. While all five analogs have favorable properties, MQTPA2 is notable as it stimulates significant levels of antizyme frameshifting at both high and low concentrations (Table 3). In addition, MQTPA2 has little ability to growth rescue cells at both high and low concentration. The five carbons between the terminal amine groups of MQTPA2 appears to be similar enough to the natural polyamine structure to function in frameshifting, but different enough to prevent rescue of cell growth from polyamine depletion. MQTPA2 is also one of the best inhibitors of ODC activity (Table 2), and it reduces both putrescine and spermidine to less than 20% of the control (Table 1). Further studies are needed to fully access whether the properties identified here for MQTPA2 and the other analogs result in inhibition of cancer growth *in vivo*.

Frameshifting and growth rescue were more effective with MQTPA1–3 than with MQTPA4–5 (Table 3). It is interesting to note that MQTPA4 and 5 have their xylene rings in a different location than the other four analogs, though other differences prevent a direct functional comparison between analogs with regard to this structural feature. The ability of all five analogs to induce frameshifting at levels equal to or greater than spermidine suggest that the xylene moiety near the end is not detrimental to its role in stimulating antizyme production. However, the presence of the xylene ring does appear to interfere with the ability of these analogs to substitute for the function of natural polyamines in cell growth. These features may explain why five of the eighteen favorable analogs identified by this screen contain xylene rings. The compounds identified here are candidates of high interest for therapeutic development, and provide a rationale for future polyamine analog structural design aimed at altering intracellular polyamine levels.

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