Antizyme affects cell proliferation and viability solely through regulating cellular polyamines

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Running title: regulation of cellular proliferation by antizyme

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Antizymes are key regulators of the cellular polyamine metabolism that negatively regulate cellular proliferation and are therefore regarded as tumor suppressors. While the regulation of Az synthesis by polyamines and its ability to regulate cellular polyamine levels suggest the centrality of polyamine metabolism to its anti-proliferative function, recent studies have suggested that antizymes might also regulate cellular proliferation by targeting to degradation proteins that do not belong to the cellular polyamine metabolic pathway. Using a co-degradation assay we show here that while they efficiently stimulated the degradation of ODC, Az1 and Az2 did not affect or had negligible effect on the degradation of Cyclin D1, Aurora-A and a p73 variant lacking the N-terminal trans activation domain whose degradation was recently reported to be stimulated by Az1. Furthermore, we demonstrate that while Az1 and Az2 can not be constitutively expressed in transfected cells, they can be stably expressed in cells that express trypanosome ODC, a form of ODC that does not bind Az and therefore maintains a constant level of cellular polyamines. Taken together, our results clearly demonstrate that Az 1 and 2 affect cellular proliferation and viability solely by modulating cellular polyamine metabolism.

The polyamines regulate fundamental cellular processes, most profoundly those supporting cellular proliferation (1,2). For maximal effectiveness, polyamines must be kept within a narrow optimal range. Deviation from the optimal level results at the lower end in inhibition of cell proliferation, and at the upper end in cytotoxicity and cell death.
death (3,4). It is therefore not surprising that optimal levels of cellular polyamines are maintained by tight regulation at several control levels. The central player of the main regulatory circuit is a small polyamine-induced protein termed antizyme (Az) (5-7). Polyamines promote Azs expression by stimulating programmed +1 ribosomal frameshifting that combines two different open reading frames to produce a full length functional protein (8,9). Az, whose affinity to ODC subunits is greater than the affinity these subunits display toward each other, binds to transient ODC monomers preventing their re-association to form active ODC. It then targets them to ubiquitin-independent degradation by the 26S proteasome (10). In addition, Az inhibits uptake and stimulates excretion of polyamines via a yet unresolved mechanism (11). Az inhibits cellular proliferation and displays anti-tumor activity, and therefore it is regarded as a tumor suppressor (12-15).

Mammalian cells express three characterized members of the Az family of proteins (6,16). While one of them, Az3 is testis-specific, observed only in haploid germinal cells (17,18); the other two, the prototypical Az1, and Az2 are ubiquitously expressed, with Az1 being expressed to much higher levels (5,6,19). The tight regulation of Azs synthesis by polyamines, together with its ability to regulate cellular polyamine levels, suggest that Az might exert its anti-proliferative effect exclusively by regulating the cellular polyamine metabolism. However, several recent studies put forward an alternative possibility, suggesting that Az might also inhibit cellular proliferation by targeting to ubiquitin-independent degradation growth regulating proteins that do not belong to the cellular polyamine metabolic pathway. These include Smad1, a key transducer of the bone morphogenetic proteins (20-22), the cell cycle regulators Cyclin D1 and Aurora-A (23,24), and the anti-apoptotic N-terminally truncated form of p73 (ΔNp73) (25).

In the present study we have tested these two alternative possibilities. First, we compared the ability of Az to target these proteins to degradation, relative to its ability to stimulate ODC degradation. Second, we monitored the effect of Az in cells displaying constant levels of polyamines due to the expression of trypanosome ODC, an ODC variant that is refractory to the destabilizing effect of Az (26,27). We show here that while greatly stimulating ODC degradation, Az1 and Az2 do not stimulate or exert negligible effect on CyclinD1, Aurora-A and ΔNp73 degradation under the same experimental conditions. Moreover, we show that both Azs can be efficiently expressed in trypanosome ODC expressing cells without exerting an antiproliferative effect, nor affecting the viability of these cells. Our results therefore demonstrate that the polyamine metabolism represents the only cellular target for the antiproliferative effect of Az.

**Experimental Procedures**

**Cell Culture Conditions and Transfections.** NIH3T3 mouse fibroblast and HEK-293T cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Biological Industries). NIH3T3 cells were transfected using JetPEI (Source BioScience AUTOGEN) following the manufacturer's instructions. HEK-293 cells were transfected using the calcium phosphate method (28). NIH3T3 cells expressing the tetracycline responsive repressor were grown and stimulated for the expression of Az as described (29).

**Cloning and construction of plasmids.** Az, trypanosome ODC, Cyclin D1, Aurora-A, ΔNp73 and the Tet-repressor were cloned into different variants of the bicistronic
pEFires vectors (30) which differ in their selectable markers, conferring resistance to puromycin, neomycin and hygromycin-B respectively. Thus we were able to achieve stable expression of three of these proteins in the same transfected cells. For inducible expression, Az cDNA was cloned downstream to a Tet-responsive promoter in a pCDNA3-based plasmid. A nucleotide was deleted from Az to permit expression of a full-length protein without requiring frameshifting. Mutations, deletions and tags were introduced using the overlap extension method (31,32).

**Western blot analysis.** Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol and a cocktail of protease inhibitors (Sigma). Cellular extracts containing equal amounts of protein were heated for 5 minutes in sample buffer and fractionated by polyacrylamide-SDS gel electrophoresis. The proteins were then electroblotted onto nitrocellulose membrane and the specific proteins were identified by incubation with the indicated antibodies followed by horseradish peroxidase-conjugated anti IgG antibodies. Signals were developed using “EZ-ECL” (Biological Industries), and the membranes were exposed to X-ray film.

**In vitro degradation assay.** ODC, Cyclin D1, Aurora-A and Az were translated in vitro using the TNT reaction mix (Promega) in the presence of [35S]-methionine. The synthesized proteins were resolved by electrophoresis, and the molarity of the synthesized proteins was normalized by dividing the radioactivity in the relevant band by the number of methionine residues in each of these proteins. Equal molar amounts of the tested proteins and of Az were then incubated in a degradation reaction containing 40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine, 1.6 mg/ml creatine phosphokinase, 6 μl reticulocyte lysate (Promega) at 37°C for the indicated times. The proteins were then resolved by electrophoresis, and the radioactivity present in individual bands determined using the Fuji Bas2500 phosphoimager.

**In vivo degradation assay.** Degradation rate was determined by adding cycloheximide (20 μg/ml) to the growth medium. Cells were harvested at the indicated times, cellular extracts prepared and the amount of the tested proteins determined by Western blot analysis. Degradation rate was quantified using ImageJ.

**ODC activity assay.** 200 μg of protein from cellular extracts or portions of reticulocyte lysate were brought to 100 μl with ODC assay buffer (25 mM Tris-HCl pH 7.5, 2.5 mM DTT, 0.1 mM EDTA, 0.2 mM pyridoxal phosphate, 33 mM L-ornithine), containing 0.5 μCi L-[14C] ornithine. The reaction was incubated at 37°C for 2 hours in a 96-well plate. The liberated [14C]-CO₂ was trapped in a covering 3mm paper soaked with saturated barium hydroxide solution. The paper was washed with acetone, dried and the results quantified using a Fuji Bas2500 phosphoimager.

**Polyamine uptake assay.** Cells (5x10⁴) were plated in a 96-well plate and treated with DFMO for 24 hours. Then the cells were washed once with serum-free growth medium and incubated for 15 minutes with 50 μl of 5 μM [3H]-spermidine (36 Ci/mmol). After extensive washing with phosphate buffered saline, the cells were dissolved in formic acid, transferred into 3 ml of UltimaGold LCS cocktail and radioactivity was determined using a liquid-scintillation counter.

**Polyamine analysis.** Cells grown in 10 cm dishes were harvested, sedimented and resuspended in 100μl PBS. The cells were lysed in 3% perchloric acid and
precipitated material was removed by centrifugation (5 minutes at 13,000 rpm). The supernatant was collected for polyamine analysis, while the pellet was used for normalization by DNA quantification (DNA was quantified by resuspension of the pellet in 400μl of 4% diphenylamine (Sigma) in acetic acid, 400μl of 10% perchloric acid and 20μl of 1:500 acetaldehyde (Sigma), followed by incubation for 16h at 30°C and OD determination at 595 and 700nm). For polyamine analysis, 100ul of the perchloric acid supernatant was mixed with 200ul of 6 mg/ml dansyl chloride (in acetone). After the addition of 10 mg of sodium carbonate, the mixture was incubated for 16h in the dark. To neutralize residual dansyl chloride, 50 μl of 100 mg/ml L-proline solution were added for 1h at RT. Dansylated derivatives were extracted into 250 μl toluene. Portions of 50-100ul were spotted on silica 60 F254 (Merck) TLC plate. Then the dansylated derivatives were resolved by thin layer chromatography using ethyl acetate/cyclohexane (1:1.5) as a solvent and, were visualized by UV illumination. Dansylated derivatives of known polyamines served as markers.

**Determination of growth rate.** Cells were plated in 12-well plates and grown in medium supplemented with 10% FBS, 0.5% FBS, or 10% FBS. At the indicated times following induction of Az expression, the cells were trypsinized and counted using a Bright Line Counting Chamber (Hausser Scientific, Horsham, PA, USA).

**Results**

**Az differentially stimulates ODC degradation.** Azs expression and function is tightly associated with the cellular polyamine metabolism. However, it was recently suggested that Az might exert its anti-proliferative effect not only through targeting ODC to ubiquitin-independent degradation and to inhibiting polyamine uptake, but also by stimulating the degradation of the growth regulating proteins CyclinD1, Aurora-A, and ∆Np73, that do not belong to the polyamine metabolism (23-25). However, in these studies Azs ability to stimulate the degradation of these proteins was not compared to its ability to stimulate ODC degradation. We therefore set out to compare the ability of Az1, the prototype and most studied member of this protein family, to stimulate their degradation to its ability to stimulate ODC degradation. We tested for its effect both in an *in vitro* reticulocyte lysate based degradation mix, and in transfected cells. For the *in vitro* degradation reaction, Az1 and the tested proteins were translated in reticulocyte lysate in the presence of 35S-methionine. Next, three degradation reactions were set containing CyclinD1 and ODC with or without Az1, Aurora-A and ODC with or without Az1, and ∆Np73 and ODC with or without Az1. In all three reactions, Az1 greatly stimulated the degradation of ODC, but failed to stimulate or had negligible effect on the degradation of CyclinD1, Aurora-A and ∆Np73 (Fig 1A, B and C). To test for the effect of Az1 in cells, constructs encoding Az1, each of the tested proteins and ODC were transiently co-transfected into 293T cells and cycloheximide was added to the growth medium 24 hours post tranfection. Cellular extracts were prepared at the indicated times, and the degradation rates of the tested proteins were determined by Western blot analysis. The results showed that, while dramatically stimulating ODC degradation, Az1 did not affect the degradation rate of co-transfected CyclinD1, Aurora-A or ∆Np73 (Fig 2A, B and C).

**Cells expressing trypanosome ODC tolerate constitutive Az1 expression.** Although the above results suggest that Az1 displays clear specificity towards components of the cellular polyamine
metabolism, we can not exclude the possibility that Az affects cellular proliferation by affecting other cellular proteins. We therefore wanted to test in a general, unbiased way whether Az1 affects cell proliferation solely through manipulating the cellular polyamine metabolism, or whether it does so also by affecting other growth regulatory proteins that do not belong to the polyamine metabolic pathway. In this respect it is important to note that we failed to stably express Az in transfected cells. We have therefore established a NIH3T3-derived cell line that stably expresses trypanosome ODC. This form of ODC does not bind Az, and therefore is not targeted to degradation in the presence of Az (26,27). Since the polyamine level in these cells is expected to remain steady, we inferred that if Az regulates growth solely through manipulating cellular polyamines, it should not exert any anti-proliferative effect in these cells. As expected, stable expression of Az1 in the trypanosome ODC expressing cells (Fig 3A 1) did not reduce the elevated ODC activity and polyamine levels attributed to the expression of trypanosome ODC (Fig 3A 2 and 3). Importantly, Az expression did not reduce the proliferation rate of these cells. The rate was practically identical to that of cells expressing only trypanosome ODC, which is higher than that of cells carrying empty vectors (Fig 3B). That the expressed Az1 was active was demonstrated by the ability of cellular extracts of the Az1 transfected cells to stimulate degradation of mouse ODC in an in vitro degradation reaction (Fig 3C).

**Inducible Az1 expression inhibits the proliferation of NIH3T3 cells, but not of trypanosome ODC expressing cells.** Although unlikely, it can be argued that during selection of stable transfectants, in addition to the adaptation of the polyamine metabolism, Az expression may also provoke adaptation through other cellular components. We therefore set out to test whether trypanosome ODC expression is sufficient to protect cells from the consequences of inducible expression of Az. For this purpose, Az1 was cloned downstream to a Tet-inducible promoter and the resulting construct was used to stably transform NIH3T3 cells already stably transformed with a trypanosome ODC encoding construct, or with an empty vector (control cells). As shown in figure 4, while induction of Az expression inhibited ODC activity and growth of control cells (Fig 4A), ODC activity and growth of the trypanosome ODC expressing cells remained unaffected (Fig 4B). This further supported the notion that Az1 affects cellular proliferation only by manipulating the cellular polyamine metabolism.

**Az2 affects cellular proliferation solely by regulating the polyamine metabolism.** As mentioned above, Az1, the prototype of the Az family of proteins, is not the only form of Az expressed in mammalian cells. The other two forms are Az3, which is testis specific, and Az2, which is ubiquitously expressed like Az1, but to significantly lower levels (33). Since Az2 is evolutionarily conserved to an even higher degree than Az1 (19), its minority co-existence with Az1 in the same cells is of interest, and may suggest that it has a different cellular role. In support of this possibility is a recent demonstration that Az2 is predominantly nuclear, while Az1 is cytoplasmic (34). We have therefore set out to determine whether Az2 might regulate cellular proliferation by affecting proteins that do not belong to the polyamine metabolic pathway. The ability of Az2 to stimulate protein degradation was tested only in cells, as it was demonstrated previously that Az2 does not stimulate ODC degradation in an in vitro degradation reactions (29,35). Simultaneous cotransfection of Az with all four tested proteins demonstrated that like Az1, also Az2 did not stimulate the degradation of
CyclinD1, Aurora-A and ΔNp73, but did efficiently stimulate ODC degradation (Fig 5A). Furthermore, we demonstrated that as with Az1, stable expression of trypanosome ODC permitted the selection of cells that constitutively express Az2 without affecting cellular viability (Fig 5B). As with Az1, also Az2 did not affect growth rate when expressed in trypanosome ODC expressing cells (Fig 5C). Interestingly however, although expressed from an identical construct as Az1, Az2 was expressed to significantly lower levels compared to Az1 and amplification of its expression unit was required to achieve levels that equivalent those of Az1 observed in the initial transformants (Fig 5B). These results demonstrated that like Az1, also Az2 could be constitutively expressed in cells provided that supply of polyamines was preserved.

**DISCUSSION**

We provide here compelling evidence that Az1 and Az2 affect mammalian cell proliferation and viability solely through affecting the cellular polyamine metabolism.

Az is a small polyamine-induced protein that inhibits mammalian cell proliferation, and is therefore regarded as a tumor suppressor (12-15). Az binds to transient ODC monomeric subunits, preventing their association to form active dimers, and targeting them to ubiquitin-independent degradation by the 26S proteasome (7,10). In addition to stimulating ODC degradation, Az regulates polyamine transport across the plasma membrane through a yet undefined mechanism (11,36,37). Since the efficiency of Az synthesis is regulated by intracellular polyamine concentrations (8,9), while at the same time Az regulates cellular polyamine levels, it can be assumed that Az affects cellular proliferation by regulating the cellular polyamine metabolism. On the other hand, it can be argued that it is rather unlikely that such a system was evolutionary selected for the degradation of a single cellular protein (ODC), and for affecting a single metabolic pathway. Indeed, recent studies raised the possibility that Az might affect cellular proliferation and viability through stimulating the degradation of additional proteins such as Smad1, Cyclin D1, Aurora-A and ΔNp73, that can regulate growth independently of the cellular polyamine metabolism (20-25). However, these studies did not compare the efficiency of their degradation to the efficiency with which Az stimulates ODC degradation. This is particularly important as Az acts catalytically in stimulating ODC degradation, being effective even when present in inferior molar amounts (38). We show here that the two major forms of Az, Az1 and Az2, efficiently stimulate ODC degradation but fail to stimulate or negligibly affect the degradation of Cyclin D1, Aurora-A and ΔNp73. A reflection of the efficient stimulation of ODC degradation by Az is significantly reduced basal level of ODC in co-transfected cells (Fig 2 and 5). No reduction in the level of the three other tested proteins was observed.

The observation that both Azs did not stimulate Cyclin D1, Aurora-A and ΔNp73 degradation does not rule out the possibility that Az negatively regulates cell proliferation by affecting the expression or functionality of other growth regulating proteins. We therefore sought to determine in a general way whether Az1 and Az2 regulate cell growth solely by affecting the cellular polyamine metabolism, or whether they are doing so by also affecting other growth regulating proteins. For this purpose we have established NIH3T3 and HEK 293 cells stably expressing trypanosome ODC that is refractory to the deleterious effects of Az since it does not bind Az. Since the expression of trypanosome ODC provides steady supply of polyamines, in contrast to wild-types cells whose polyamines are
depleted by forcefully expressed Az, we inferred that these cells will survive stable Az expression if components of the polyamine metabolism are the only targets of Az, but will die or their proliferation will severely retarded if Az has targets outside the polyamine metabolic pathway that are crucial for cell proliferation and viability. Our results show that while it is impossible to stably express Az in wild-type cells, both Az1 and Az2 did not affect growth rate or viability of the trypanosome ODC expressing cells.

Based on the data we presented here, we conclude that both Az1 and Az2 affect growth and viability of mammalian cells solely by targeting components of the cellular polyamine metabolism. We can not however, rule out the possibility that Az might regulate the degradation, or influence in a different way, other cellular proteins that do not affect cell proliferation and viability.

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LEGENDS TO FIGURES

Figure 1. Az1 stimulates ODC but not Cyclin D1, Aurora A and ΔNp73 degradation in-vitro in a reticulocyte lysate based degradation reaction. Az1, ODC, Aurora-A, Cyclin D1 and ΔNp73 were synthesized in vitro in reticulocyte lysate. Three degradation reactions were set as described in Materials and Methods containing Az, ODC and Aurora-A (A), Cyclin D1 (B) and ΔNp73 (C). A reaction lacking Az was set as a control. Following the indicated time of incubation at 37°C, the material was resolved by SDS-PAGE and the protein bands were visualized and quantified using a Fuji Bas2500 phosphoimager. Each of the above experiments was repeated twice, yielding similar results.

Figure 2. Az1 stimulates ODC but not Cyclin D1, Aurora A and ΔNp73 degradation in-vivo in transfected NIH3T3 cells. Constructs encoding Az1 and ODC were transfected into HEK-293T...
cells together with a construct encoding Aurora-A (A), CyclinD1 (B) or ΔNp73 (C). Cycloheximide (CHX) was added to the growth medium 24 hours post-transfection, the cells were harvested at the indicated times, and the level of the individual tested proteins determined by Western blot analysis using anti-FLAG antibodies. Anti-Actin antibodies were used to normalize the amount of protein loaded in each lane. The presented experiments were repeated three times, yielding practically identical results.

Figure 3. Expression of trypanosome ODC enables constitutive Az1 expression. NIH3T3 mouse fibroblasts were stably transfected with constructs encoding trypanosome ODC (TrypODC), TrypODC + Az1 or with the compatible empty vectors (control) (A1). ODC activity (A2) and cellular polyamines (A3) were determined as described in Materials and Methods. The growth rates of the three cell lines were determined by daily counting of cells as described in Methods (B). * P<0.02 denotes significant difference from control cells using student’s t-test. The functionality of the transfected Az1 was determined by mixing cellular extract prepared from the three cell lines in an in vitro degradation reaction using in vitro synthesized [35S]-methionine labeled mouse ODC as a substrate (C).

Figure 4. Trypanosome ODC protects against the anti-proliferative effect of induced Az. Construct encoding Az1 cDNA cloned downstream to a Tet-responsive promoter was transfected into NIH3T3 cells previously transformed with a plasmid encoding Tet controlled transactivator and trypanosome ODC encoding construct (B) or the compatible empty vector (A). Successful induction of Az was demonstrated by inhibition of ODC activity in cells transformed with the empty vector (see insert). Cell counting at days 7, 8 and 9 following seeding and induction determined the growth rates of the various cells. * P<0.02 denotes significant difference from control cells using student’s t-test.

Figure 5. Az2 stimulates ODC but not Cyclin D1, Aurora A and ΔNp73 degradation and can be stably expressed in trypanosome ODC expressing cells. (A) HEK-293T cells were cotransfected with constructs encoding flagged Az2, ODC, Aurora-A, CyclinD1, and ΔNp73. Cycloheximide (CHX) was added to the growth medium 24 hours post-transfection, cellular extracts were prepared at the indicated times, and the amounts of the individual tested proteins were determined by Western blot analysis using anti-FLAG antibodies. The presented data are representative of three repetitions. * denotes a non specific band (B) 293T cells stably expressing trypanosome ODC were transfected with Az1 or Az2 expressing constructs. Focuses were initially selected with 1mg/ml puromycin followed by amplification of the expression unit by increasing puromycin to 5, 10 and 20 mg/ml. Cellular extracts were prepared, and the expression levels of trypanosome ODC, Az1 and Az2 were determined by Western blot analysis. (C) The growth rate of cells expressing Trypanosome ODC and Az2 was compared to that of control cells transfected with two empty vectors by daily counting of cells as described in methods. * P<0.02 denotes significant difference from control cells using student’s t-test.
Fig 1

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ODC Deg %
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Fig 2
Fig 3
Fig 4

A

B

trpODC
Az-Induction
ODC activity

Days after seeding

No. of cells (x10000)

uninduced
Induced

trpODC
Az-Induction
ODC activity

Days after seeding

No. of cells (x10000)

uninduced
Induced
Fig 5
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