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Review

Antizyme inhibitor: mysterious modulator of cell proliferation

U. Mangold [a, b]

[a] Program in Vascular Biology, Children's Hospital, Karp Family Building, 1 Blackfan Circle, Boston, Massachusetts 02115 (USA), Fax: +1 617 730 0268, e-mail: ursula.mangold@childrens.harvard.edu
[b] Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115 (USA)

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Abstract. In contrast to the considerable interest in the oncogene ornithine decarboxylase (ODC) and in the family of antizymes with regard to cell proliferation and tumorigenesis, the endogenous antizyme inhibitor (AZI) has been less well studied. AZI is highly homologous to the enzyme ODC but does not possess any decarboxylase activity. Elevated ODC activity is associated with most forms of human malignancies. Antizymes bind ODC, inhibit ODC activity and promote the ubiquitin-independent degradation of ODC. Consequently they are proposed as tumor suppressors. In particular, the most studied member of the antizyme family, antizyme 1, has been demonstrated to play a role in tumor suppression. AZI inactivates all members of the antizyme family, reactivates ODC and prevents the proteolytic degradation of ODC, which may suggest a role for AZI in tumor progression.

Keywords. Antizyme inhibitor, antizyme, polyamines, ODC, proteasome, cancer.

Introduction

In 1976 Canellakis and his co-workers found a unique inhibitor of the enzyme ornithine decarboxylase (ODC) in various cells. They named this protein antizyme since its synthesis is induced by the product of the enzyme [1]. Since then antizymes have been identified in a wide variety of organisms from yeast to man. Antizymes represent an important class of proteins. They regulate cell growth and metabolism by a diverse set of mechanisms that include ubiquitin-independent protein degradation, as well as inhibition of ODC enzyme activity and small molecule transport. In humans, antizymes comprise a family of at least three members with different activities and tissue distribution [2–4]. Since elevated ODC activity is associated with most forms of human malignancies [5], it was suggested early on that antizymes may function as tumor suppressors. In support of this hypothesis, it has been shown that overexpression of the most-studied member of the antizyme family, antizyme 1, leads to cell cycle arrest, apoptosis and reduced cell proliferation [6, 7]. Moreoever, it was demonstrated that antizyme 1 overexpression inhibits tumor growth *in vivo* using different mouse models [7–10]. Today, the role of antizyme 1 is seen in a considerably broader context, as antizyme 1 has been shown to influence the stability of several additional proteins besides ODC [11, 12].

Antizyme expression and stability are subject to a complex auto-regulatory feedback. Their expression is induced by an unusual polyamine-dependent mechanism [13, 14]. Polyamines such as putrescine, spermidine and spermine stimulate a programmed +1 frameshift during translation of the antizyme messenger RNA (mRNA), which allows the functional, full-length expression of antizymes and causes a rapid rise in antizyme levels. Once antizymes are expressed, they inhibit ODC and promote the proteolytic degradation of ODC in a ubiquitin-independent manner.

In parallel, the stability of antizymes is also regulated by a specific feedback mechanism. Though antizymes mediate ubiquitin-independent degradation, their own degradation is ubiquitin-dependent. Recent data suggest that high polyamine levels interfere with antizyme ubiquitination, thereby preventing their proteolytic degradation [15, 16].

Both mechanisms allow cells to increase antizyme activity and respond to high polyamine levels. But what kind of mechanisms do cells have to inactivate antizymes?

In 1982, a 'macromolecular factor' that inhibits the activity of antizyme 1 was found in rat liver extracts and named antizyme inhibitor (AZI) [17]. AZI proved to be a heat-labile molecule with a molecular weight similar to ODC and an affinity for antizyme 1 that was higher than that of ODC [17]. When equal amounts of AZI and ODC were applied to an antizyme 1 affinity column, most ODC passed through but AZI almost completely bound to the column [18]. This suggested a model in which AZI restores ODC activity by forming a tight complex with antizyme 1, thereby releasing ODC from antizyme inhibition. The binding constant between ODC and antizyme 1 is as high as 1.4×10^{11} M^{-1} [19]. Since AZI binding to antizyme is much stronger, it would be interesting to see what the affinity for this interaction is and whether it is close to strong interactions such as biotin-streptavidin, which has a binding constant of 10^{14} M^{-1} [20]. Biochemically, AZI proved to be very similar to ODC, which led to the question whether AZI was a protein distinct from ODC. *In vitro* experiments, however, showed that AZI has no cross-reactivity with polyclonal anti-ODC antibodies [18]. The development of a monoclonal antibody against AZI further ruled out that AZI is simply an ODC splice variant, a posttranslationally modified version of ODC or an intermediate formed in the process of ODC degradation [21]. Cloning and sequencing of mammalian AZI finally showed that AZI is highly similar but distinct from ODC [22–24]. Evolutionarily, the AZI gene might be the result of ODC gene duplication. Despite the high homology between both proteins, AZI, in contrast to ODC, does not exhibit any decarboxylase activity [23].

Formation of the active site: a comparison between ODC and AZI

Mammalian ODC has been extensively characterized through the analysis of point mutations, sequence comparisons and structure analysis [25, 26]. As a result, several amino acid residues were identified that contribute to the enzymatic activity of ODC. In particular, the core of the active site of eukaryotic ODC is highly conserved between human, mouse and trypanosomal ODC [26]. ODC is only enzymatically active as a homodimer, and the active site is formed at the interface between the two monomers [27]. In addition, ODC is dependent on the cofactor pyridoxal 5′-phosphate (PLP). Therefore, it is not surprising that amino acid residues, which are important for ODC activity, are involved in dimer formation, binding of the cofactor PLP, formation of the active site and substrate binding. In total, 27 amino acid residues have been characterized that are critical for ODC enzymatic activity [25–31]. These residues are summarized in Table 1.

When mammalian AZI and ODC are compared, 6 out of 27 residues that are important for ODC enzymatic activity are not conserved within the AZI sequence (residue N398 is present within murine and human AZI but is not conserved in the rat sequence). These divergent residues are involved in formation of the active site, stabilization of the cofactor PLP (D332, Y389, D88, R154, R277) and dimer formation (Y331). The absence of these residues might explain why AZI is enzymatically inactive. But it is imaginable that other, not yet characterized residues might contribute to the enzymatic activity of ODC as well. It would therefore be informative to test whether mutating these residues back to the corresponding ODC amino acid residue could render AZI enzymatically active.

Residues C360 and D361 of mammalian AZI are involved in binding of the substrate ornithine and are conserved within all AZI sequences. Another well-conserved residue of mammalian AZI that contributes to substrate binding is K69. Both K69 and C360 have also been identified as part of the covalent attachment site of the irreversible ODC inhibitor α-difluoromethylornithine (DFMO) [28]. Despite the differences between ODC and AZI with regard to their active site, it might therefore be possible for AZI to bind these small molecules. Since AZI is enzymatically inactive, binding of ornithine or DFMO would not influence any en-

Table 1. Comparison of amino acid residues between mouse ODC and AZI which are important for ODC enzymatic activity.

Functional role	ODC (mouse numbering)	AZI (mouse numbering)	Reference
Involved in dimer formation	D134, K169*, K294, Y323, **Y331**, D364*, G387, F397	D134, K169, *K291*(K294), *Y321*(Y323), *D362*(D364), *G385*(G387), *F395*(F397)	[25–27, 29, 31]
Form interactions with the cofactor PLP	K69 (Schiff base), G235, G236, G237, E274, G276, **R277**, **Y389**,	K69 (Schiff base), G235, G236, G237, *E271*(E274), *G273*(G276)	[25–30]
Contribute to the active site	**D88**, E94, **R154**, H197, **D233**. F238, Y278, D332, N398	E94, H197, D233, F238, *Y275*(Y278), *N396*(N398)	[25–27, 29]
Involved in substrate binding and active site formation	C360, D361	*C358*(C360), *D359*(D361)	[25–29]

Residues marked with an asterisk are also part of the active site. Residues highlighted in bold are present in ODC but not conserved within the AZI sequence. AZI residues that have a different number than the corresponding ODC residue are marked in italic, with the ODC number in parentheses. Residues underlined are not conserved within human ODC-p.

zymatic activity, but might affect overall AZI conformation or its affinity to target proteins such as the antizymes.

Recently, a novel human ODC-like protein, ODC-p, was identified, which is expressed only in brain and testes [32]. ODC-p is 54% identical to ODC and 45% identical to AZI, and at least eight alternatively spliced forms of ODC-p have been cloned [32]. Most of the residues associated with the catalytic activity of ODC are conserved in human ODC-p, whereas other residues, importantly D88 and C360, are not. As a consequence ODC-p does not possess decarboxylase activity and is similar to AZI in this respect. Table 1 summarizes amino acid residues important for active site formation that are not present within human ODC-p. Since its affinity to antizymes has not yet been reported, it is not known whether ODC-p has AZI-like properties, and its overall function is unclear.

As discussed above, ODC is only active as a 106-kDa PLP-dependent homodimer, and the active site is formed at the interface between the two monomers. Despite the high homology between both proteins, no ODC/AZI heterodimers are formed [23]. There are, however, data indicating that AZI, like ODC, takes on the conformation of a homodimer [17, 23]. AZI was originally purified from rat liver extracts. In this preparation, the apparent molecular weight of AZI was determined to be about 105 kDa by gel filtration analysis, which would correspond to the approximate weight of an AZI homodimer [17]. However, the AZI homodimer might be less stable since residue Y331, which is important for ODC dimerization, is not conserved within the mammalian AZI sequence. If AZI dimerization is less stable than ODC dimerization, the antizyme binding site of AZI might be more accessible, which could contribute to the overall strong antizyme-AZI interaction.

Recently, the AZI sequence for the model organism zebrafish (*Danio rerio*) became available [33]. All 27 residues important for mammalian ODC activity are present within the zebrafish ODC sequence. Strikingly, 12 of those residues are not present in zebrafish AZI. In addition to the 6 residues that are divergent between mammalian ODC and AZI, residues K69, D134, K169, H197, Y275 and N396 are also not conserved. It is noteworthy that K69, which forms a Schiff base with the cofactor PLP, is replaced by a serine residue within the zebrafish AZI sequence. Dimerization of zebrafish AZI is also less likely since two additional residues, which are important for ODC dimer formation, D134 and K169, are not conserved in zebrafish AZI. Taken together, this suggests that zebrafish AZI evolved even further from its enzymatically active counterpart, ODC, than mammalian AZI.

Variation within: the AZI isoforms

Recently there have been new database entries which provide information about the canine (*Canis familiaris*), bovine (*Bos taurus*) and chicken (*Gallus gallus*) AZI sequences. In particular, the bovine and canine entries show a great diversity in transcript variants, which suggests the existence of several AZI isoforms. Some of these variants show differences in the non-coding region, whereas others affect the coding region and lead to truncated forms of AZI. These entries are, however, XM sequences, the majority of which are generated by computational prediction. Some may have been derived from aberrantly spliced transcripts or generated by incorrect prediction of intron-exon junctions in silico. For rat, mouse and human AZI there is, however, better evidence, demonstrating the existence of multiple AZI isoforms. Using Northern blot analysis, three mRNA species of approximately 2.2, 2.6 and 3.7 kb were detected in various rat tissues [23], and two AZI isoforms were found in mouse mRNA [24]. Interestingly, the relationship between the longer and the shorter AZI transcripts varied among cell lines [24]. In humans, a similar pattern was observed, although the smaller band was more diffuse and may consist of more than one mRNA species [22]. Several rat, mouse and human AZI isoforms have also been cloned. These isoforms proved to be the result of alternative splicing of the 5′- and 3′- UTR and the us of different polyadenylation signals. Despite major differences in mRNA size, the amino acid coding region of the corresponding AZI complementary DNA (cDNA) was unaffected [22–24].

There is, however, evidence that an AZI isoform exists that exhibits a difference within the AZI coding region. Mouse EST clone (AA162795) generates an AZI protein that has a C-terminal deletion and is 75 amino acids shorter than the full-length AZI protein [24]. This isoform was recently confirmed by database entries such as mouse EST clone AI386447 and human EST clone AW884549. It should be noted that the coding sequence of these EST

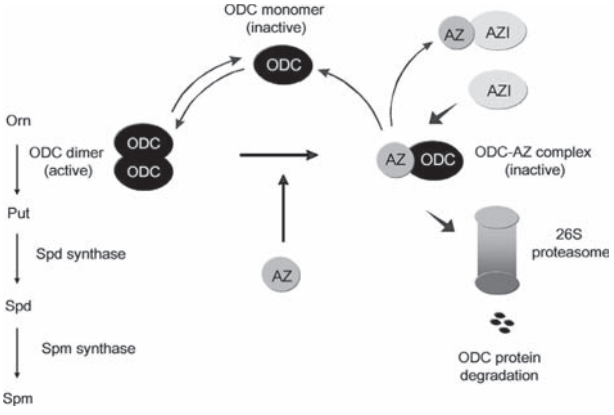

Figure 1. Schematic diagram showing antizyme (AZ) and antizyme inhibitor (AZI)-mediated regulation of ornithine decarboxylase (ODC). ODC, the key enzyme of polyamine biosynthesis decarboxylates ornithine (Orn) to the putrescine (Put), which is then further converted into the polyamines Spd (spermidine) and Spm (spermine).

clones is incomplete, and N-terminal sequences have to be added. Further studies should show whether this truncated isoform is functional and whether it is able to bind antizymes. Theoretically, the region necessary for antizyme binding is not affected by the C-terminal truncation, and therefore this particular AZI isoform would be predicted to bind antizyme.

Antizyme/AZI interaction and its consequences

The putative antizyme 1 binding element (AZBE) of ODC was proposed to lie within residues 117–140 of mouse ODC [34]. The AZBE is accessible in monomeric ODC but is buried in the dimeric, active form of ODC [26]. It was identified by comparison between mouse ODC, which binds antizyme 1, and trypanosomal ODC, which does not bind antizyme [34]. Since AZI also binds antizyme 1, it was suggested that the corresponding region within the AZI sequence may contain the AZBE of AZI [23]. Compared with the overall identity of 47.1%, between rat AZI and ODC this region is more conserved. Rat AZI and ODC share an identity of 63% within the 117–140 AZBE [23]. More recently it was shown that mutating several residues within the 117–140 potential antizyme binding region results in an AZI mutant that does not bind antizyme 1, demonstrating that this region is indeed involved in the AZI/antizyme 1 interaction [35]. The antizyme 1 binding region, however, might encompass additional amino acids. Structural comparison between human ODC and *Trypanosoma brucei* ODC suggests an even larger antizyme binding region, which includes residues 92–183 of human ODC [26]. Since the interaction between AZI and antizyme 1 is much stronger than the corresponding interaction between ODC and antizyme 1, AZI might have an even larger, more complex AZBE than ODC.

Interestingly, it has been shown that AZI acts as a general inactivator of antizyme function. Zebrafish has two antizymes, which bind and inhibit ODC and are both inactivated by AZI [33]. Human AZI can bind and inactivate four members of the antizyme family, though human antizyme 1 and 2 share only 55% amino acid identity, and antizyme 3 is even more divergent (26 and 29% identity to antizyme 1 and 2, respectively) [36]. In addition, it was shown that the interactions between AZI and members of the antizyme family were much stronger than the corresponding interactions between ODC and antizymes [36], which again suggests that additional residues of AZI contribute to the antizyme binding.

Although AZI and antizyme 1 interact closely, AZI degradation is not accelerated by antizyme 1 [23, 35]. This is in contrast to the antizyme/ODC interaction. Antizyme 1 binding is thought to cause a conformational change within ODC, exposing the C-terminal domain, which contains signals important for degradation [37]. Two PEST domains, regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T), are located within the C-terminal part of mammalian ODC. In particular, the PEST domain close to the carboxy terminus has been identified as necessary for antizyme 1-mediated proteasomal degradation of ODC [37]. AZI, however, does not contain such PEST domains, and the homology between ODC and AZI within the carboxy-terminal part is very low [23]. Taken together, this might explain why antizyme 1 is unable to promote AZI degradation. It was recently shown, however, that AZI has a very short half-life and that it is degraded even more rapidly than ODC, which is known to be one of the most labile proteins [35, 38]. The degradation of AZI is mediated by the proteasome, but in contrast to ODC, AZI is degraded in a ubiquitin-dependent manner [35]. Neither the AZI/antizyme 1 interaction nor the carboxy terminus of AZI is required for AZI degradation. Instead, the conjugation of ubiquitin to AZI is inhibited by antizyme 1, and AZI is stabilized by its interaction with antizyme 1 [35]. It was therefore suggested that antizyme binding might induce a conformational change that hides the AZI degradation signal, in contrast to ODC, where the degradation signal is exposed upon antizyme 1 binding [35]. AZI might therefore buffer antizyme 1, which increases upon elevation in the intracellular concentration of polyamines.

Both polyamine uptake and polyamine secretion are modulated by antizymes via an as yet unresolved mechanism [39–41]. In addition, antizyme-independent transport mechanisms exist, and cell-surface heparan sulfate proteoglycans may also play a role in the uptake of polyamines [42]. Since antizymes are able to inhibit polyamine uptake, it was postulated that AZI, which inhibits antizyme activity, should prevent antizymes from downregulating the polyamine transport system [43]. Indeed, it was reported that overexpression of AZI in CHO cells leads to a more efficient uptake of either spermidine or polyamine analogues [43]. The positive effect of AZI on polyamine uptake was, however, only modest compared with the negative effect antizyme has on polyamine uptake [41, 43]. More recently, it was shown that NIH3T3 fibroblasts overexpressing AZI demonstrate elevated spermidine uptake, indicating that AZI has a role in modulating polyamine uptake [44].

AZI and tumorigenesis

In contrast to the considerable interest in the oncogene ODC and in the family of antizymes with regard to cell proliferation and tumorigenesis, the endogenous antizyme inhibitor has been less well studied. AZI, however, which inactivates antizymes and increases ODC activity, may play a prominent role in cell proliferation and tumor progression. Congruently, it has been reported that down-

regulation of AZI is associated with inhibition of cell proliferation and reduced ODC activity, presumably through modulation of antizyme function [45]. More recently, it was shown that overexpression of AZI increases cell proliferation and promotes cell transformation [44, 46] and that AZI-overexpressing NIH3T3 cells give rise to tumors when injected into nude mice [44]. Whether the growth promoting role of AZI is due solely to the neutralization of antizymes must be further tested since it has also been reported that AZI has antizyme-independent functions that may be linked to cyclin D1 stability [46].

The human AZI gene is located on chromosome 8q22.3, and amplifications of this region have been associated with prostate cancer and ovarian carcinoma [47, 48]. More recently it was shown that Ras transformation, which induces ODC activity, also stimulates AZI expression [44, 49]. In addition, AZI expression is induced by the following factors:

The non-metabolizable amino acid α-aminoisobutyric (AIB) acid potently increases hepatic ODC activity [50] and stimulates AZI expression in rat liver [21]. Treatment with α-hydrazino-δ-aminovaleric acid, a competitive inhibitor of ODC which augments the induction of ODC, was also successfully used to induce AZI expression [17]. Treatment with the beta-adrenergic agonist isoproterenol increased the amount of AZI in rat heart [21, 23].

Importantly, mitogens such as thioacetamide, which is a hepatocarcinogen that causes oxidative stress, hyperproliferation and fibrosis in rat liver, lead to the induction of AZI [17, 18, 21]. Moreover, AZI is rapidly induced after serum stimulation or treatment with the phorbol ester PMA (phorbol 12-myristate 13-acetate) [24]. PMA activates protein kinase C, rapidly increases the gene expression of several oncogenes such as ODC, *c-fos* and *c-myc*, and is a potent tumor promoter in skin carcinogenesis [51]. The accumulation of AZI transcripts after serum stimulation occurred several hours before ODC transcripts reached their maximum [24]. In addition, the overall expression level of AZI mRNA was much lower than that of ODC, suggesting that the promoter region of both genes must have deviated markedly to explain their different expression patterns. But so far, not much is known about the AZI promoter. ODC is a well-characterized target of the oncogenic transcription factor c-Myc [52]. The AZI promoter, in contrast, has not been characterized, and it would be informative to determine whether c-Myc also plays a role in the regulation of AZI expression.

The hypothesis that AZI might play a role in tumorigenesis was recently supported by tumor patient data. A search for differentially expressed genes in gastric cancer showed that AZI is highly upregulated in cancer compared with matched normal gastric tissue [53]. High-level expression of AZI was also reported in different tumor cell lines, suggesting another possible correlation between AZI and malignancy [53].

Interestingly, it was recently demonstrated using a microarray analysis approach that AZI may also have a role in angiogenesis [54]. ODC and AZI were among genes highly induced in capillary endothelial cells after prolonged stimulation with the known angiogenic factor FGF-2 (fibroblast growth factor 2) or the hyaluronan fragment HA12 [54]. It has been proposed for some time that ODC is angiogenic since ODC overexpression is associated with downregulation of the anti-angiogenic factors endostatin and thrombospondin (TSP-1 and TSP-2) [55, 56]. The coordinated strong upregulation of ODC and AZI suggests that AZI might be important for endothelial cell sprouting and angiogenesis as well.

Still, the overall role of AZI remains to be investigated. The extremely low content of AZI protein and AZI transcripts compared with ODC raises the question of its role in the regulation of ODC activity [18, 24]. Antizymes, however, are recycled, and the inactivation of one molecule of antizyme by AZI could prevent the degradation of several molecules of ODC. The role of AZI might also be seen in a broader context. Perhaps reactivation of ODC is not the only AZI function. Since antizyme 1 has been shown to have other targets besides ODC, AZI could potentially also influence the stability of additional proteins. Moreover, AZI increases the level and activity of the oncogene ODC and inhibits the activity of the potential tumor suppressor antizyme 1. If AZI is confirmed to be pro-proliferative and pro-angiogenic, it might be a good target for anti-cancer therapy itself. Taken together, there are a lot of exciting questions still waiting to be explored, and more investigations will be needed to unravel the function of this interesting protein.

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