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Evidence of a Role for Antizyme and Antizyme Inhibitor as Regulators of Human Cancer

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Abstract

Antizyme (AZ) and its endogenous inhibitor (Antizyme inhibitor or AZI) have recently emerged as prominent regulators of cell growth, transformation, centrosome duplication and tumorigenesis. Antizyme was originally isolated as a negative modulator of the enzyme ornithine decarboxylase (ODC), an essential component of the polyamine biosynthetic pathway. Antizyme binds ODC and facilitates proteasomal ODC degradation. Antizyme also facilitates degradation of a set of cell cycle regulatory proteins including cyclin D1, Smad1 and Aurora A kinase as well as Mps1, a protein that regulates centrosome duplication. Antizyme has been reported to function as a tumor suppressor and to negatively regulate tumor cell proliferation and transformation. The antizyme inhibitor binds to antizyme and suppresses its known functions, leading to increased polyamine synthesis, increased cell proliferation and increased transformation and tumorigenesis. Gene array studies show AZI to be amplified in cancers of the ovary, breast and prostate. In this review, we summarize the current literature on the role of AZ and AZI in cancer, discuss how the ratio of AZ to AZI can influence tumor growth, and suggest strategies to target this axis for tumor prevention and treatment.

Keywords

Antizyme; antizyme inhibitor; cell proliferation; cell transformation; centrosome amplification

Review

Regulation of the Polyamine Pathway by Antizyme Inhibitor and Antizyme

Antizyme inhibitor (AZI) and antizyme (AZ) are critically important for maintaining polyamine homeostasis within the cell. Polyamines are multivalent, organic cations and adequate polyamine levels are necessary for optimal cell growth. Within the cell, polyamines function in diverse processes including regulating chromatin condensation, stabilizing the double helical structure of DNA, regulating cell differentiation, and regulating translation through a unique post-translational modification known as hypusination (1). Polyamines are produced from the amino acid ornithine in a rate-limiting reaction catalyzed by the enzyme ornithine decarboxylase (ODC), as shown in Figure 1. ODC is only functional as a homodimeric complex, and individual ODC monomers have no enzymatic activity (2). ODC monomers bind to each other with relatively low affinity, and under physiological conditions ODC dimers are in rapid equilibrium with inactive ODC monomers.

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ODC is negatively regulated by antizyme (AZ). High intracellular polyamine levels induce a +1-frameshift that bypasses a stop codon and results in production of full-length antizyme protein. Depending on the start codon used, full-length antizyme protein can be either 29.5kD or 24kD in size. AZ decreases polyamine levels through three mechanisms, as summarized in Figure 1. First, antizyme disrupts active ODC dimers, resulting in decreased polyamine synthesis. Second, AZ targets ODC for degradation by the 26S proteasome in a reaction that does not require ubiquitin. ODC is already one of the most rapidly degraded proteins in mammalian cells, with a half-life of only 1-2 hours in the absence of antizyme. When antizyme is present, however, this half-life decreases to only a few minutes (3). Third, AZ inhibits polyamine uptake from the microenvironment through a mechanism that has yet to be completely characterized. Thus, an increase in antizyme protein is associated with lower levels of ODC, decreased polyamine synthesis, and reduced rates of cell growth.

AZ activity is further regulated by a protein called antizyme inhibitor (AZI). AZI is highly homologous to ODC, retaining 47.4% identity and 63.5% similarity at the amino acid level between human AZI and human ODC. Although AZI was originally thought to be a derivative of ODC (4), subsequent cloning and sequencing demonstrated that AZI is a distinct protein (5, 6). AZI acts as a positive regulator of the polyamine pathway by binding to AZ and preventing AZ-mediated ODC degradation (Figure 1). High levels of AZI correlate with increased ODC protein, increased polyamine synthesis, and greater cell proliferation. Whether AZI functions as a monomer or as a dimer has been examined in some detail. Seven out of the eight residues (87.5%) involved in dimer formation in ODC are also conserved in the AZI sequence, including a critical glycine residue (G387) necessary for ODC dimerization (5, 7). A comparison of the structure and conserved amino acids in AZI and ODC is shown in Figure 2. Although early studies reported that AZI could form homodimers that lack ODC enzymatic activity (4, 8), more recent evidence from size exclusion chromatography and crystallography indicates that AZI functions as a monomer (9).

Isoforms of Antizyme and Antizyme Inhibitor

The most predominant antizyme protein is antizyme 1 (AZ-1), although there are multiple antizyme genes and at least four members of the antizyme family. All antizyme proteins inhibit ODC activity (10). Antizyme 2 (AZ-2), like AZ-1, has a wide tissue distribution in vertebrates, though it is expressed at much lower levels than AZ-1 in most tissues. AZ-2 is more conserved evolutionarily than AZ-1, suggesting it may have an important physiological role.

Although AZ-2 is structurally similar to AZ-1, AZ-2 does not promote proteasomal degradation of ODC *in vitro*, even though it does inhibit both ODC activity and polyamine uptake. *In vivo*, however, AZ-2 promotes ODC degradation and inhibits both ODC activity and polyamine uptake (11). The difference in antizyme activity *in vitro* was subsequently mapped to two Asp amino acids in AZ-2 replacing Arg^{131} and Ala^{135} in AZ-1 (12). The physiological role of AZ-2 in facilitating protein degradation *in vivo* is not yet well understood, though it can promote ODC degradation in human embryonic kidney cells (13).

Expression of antizyme 3 (AZ-3) is testis specific and is restricted to a late stage in sperm production. This highly restricted expression suggests that AZ-3 is necessary to abruptly alter polyamine levels during sperm morphogenesis (3, 14). This is supported by reports that animals overexpressing ODC in the testes that have defects in spermatogenesis, perhaps because the high level of ODC overwhelms the levels of AZ-3 (15). AZ-3, like AZ-2, has the ability to inhibit both ODC activity and polyamine uptake, but does not target ODC for degradation (13). By yeast two-hybrid screen, AZ-3 was found to interact with

gametogenetin protein-1 (GGN-1), a germ cell-specific protein, although the functional consequences of this interaction are not known (16).

A putative fourth member of the antizyme family (AZ-4) was originally isolated from a human brain cDNA library but has not been well characterized. Yeast two-hybrid assays showed that AZ-4 can also bind to ODC and inhibit ODC enzymatic activity (17). The ability of AZ-4 to promote ODC degradation or inhibit polyamine uptake has not yet been examined.

Not only are there multiple isoforms of antizyme that contribute to the complex regulation of the polyamine pathway, there are also multiple isoforms of antizyme inhibitor. The most predominant antizyme inhibitor is antizyme inhibitor 1 (AZI-1/AZIN-1), which is ubiquitously expressed at high levels and has been the most studied. Antizyme inhibitor 2 (AZI-2/AZIN-2) was first identified in 2001 as an ODC paralogue and termed ODCp or ODC-like (18). Subsequent studies established that ODCp lacked enzymatic activity and appeared to function as a tissue-specific antizyme inhibitor in the brain and testes, where it is expressed at 6-fold or 23-fold greater levels than AZI-1, respectively (19, 20). Human AZI-2 retains 45% identity and 66% similarity to AZI-1 at the amino acid level, (21) and has been shown to interact with all three characterized antizymes (19, 22, 23). Similar to AZI-1, overexpression of AZI-2 has been shown to increase growth of NIH3T3 cells (23). In the future, it will be interesting to determine whether this growth advantage is mediated primarily through the polyamine pathway and is dependent on an intact antizyme-binding domain in AZI-2. To date, the majority of studies regarding the role of antizyme inhibitor in tumors have been conducted on AZ-1 and AZI-1. For the remainder of this article, AZ refers to antizyme 1 (AZ-1) and AZI refers to antizyme inhibitor 1 (AZI-1).

Polyamines and Cancer

Based on its key role in promoting cell proliferation, ODC is considered a potential oncogene. ODC is downstream of Myc and is one of the most rapidly induced genes upon growth stimulation (24). Elevated levels of ODC and polyamines have been associated with numerous types of neoplastic transformation, and ODC overexpression alone can induce cell transformation and *in vivo* tumor growth in NIH3T3 cells following subcutaneous implantation in nude mice (25). ODC activity is induced by a wide range of chemical, environmental, and genetic cancer risk factors, including ultraviolet light, asbestos, and exposure to chemical agents that induce skin carcinogenesis (26). ODC levels are also upregulated in intraepithelial neoplasias (IENs) – the non-invasive precursors of epithelial cancers. ODC expression is regulated by androgens in the prostate gland, and ODC levels are highly elevated in human prostate cancer (27) and numerous other cancer types (28-30). Furthermore, a meta-analysis of multiple microarray data sets established that the polyamine pathway is often highly altered in human prostate cancer (31).

Although high levels of ODC and polyamines are associated with numerous types of neoplastic transformation, the role of antizyme and AZI in this process is just emerging. It is clear from *in vitro* studies that increasing the ratio of AZI relative to AZ within the cell favors growth activation, while a decrease in the AZI:AZ ratio favors growth repression. The pathways directly upstream of AZI expression are largely unknown, and the AZI promoter has not yet been studied in detail. Treatment with mitogens or growth factors leads to a very rapid increase in AZI, prior even to the increase observed in ODC, an immediate early gene (6). The AZI gene has been localized to chromosome 8q22.3 in human cells, a region previously recognized as a frequently amplified hotspot associated with decreased overall survival and decreased time to distant metastases in primary breast tumors (32). AZI has been shown to be highly expressed in gastric cancer (33), as well as in tumors of the lung, liver, and ovary (33-35). In NIH-3T3 cells, overexpression of AZI alone was sufficient

to transform cells and promote tumor growth *in vivo* (36). Using siRNA to suppress AZI levels in A549 cells led to decreased cell proliferation and decreased polyamine levels; however, the ability of these cells to form tumors *in vivo* was not determined (37). These results suggest that decreasing the ratio of AZI to AZ within the cell could have a significant inhibitory effect on tumor growth.

Evidence from Transgenic Mice Suggests that Elevated Polyamine Levels are Associated with Tumor Development

Numerous transgenic mouse models are available to study the polyamine pathway and were summarized in a recent review (38). Several different mouse models with elevated ODC expression have been described. Transgenic mice overexpressing a C-terminally truncated ODC from the bovine keratin 5 (K5) and keratin 6 (K6) promoters have elevated ODC activity, as well as elevated levels of the polyamines putrescine and spermidine (39). These mice developed hair loss, formed dermal follicular cysts, and older mice frequently developed spontaneous squamous neoplasms. These experiments were performed, however, on founder and F1 mice with a mixed genetic background, and subsequent breeding to either a B6 or FVB background did not result in spontaneous tumor formation (40). K6/ODC mice do develop tumors following treatment with carcinogens, exposure to UV-radiation, or by breeding to transgenic mice that carry another oncogene. K5/ODC and K6/ODC mice were much more susceptible to tumor formation following a single treatment with the carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA), and treatment with the tumor promoter PMA (Phorbol 12-Myristate 13-Acetate) was not required (40). Subsequent treatment of existing tumors in the K6/ODC mice with the irreversible ODC inhibitor DFMO led to rapid tumor regression (41). Tumor initiation in K6/ODC mice was not limited to DMBA treatment, suggesting that these mice may be a useful model to identify potential human carcinogenic compounds (42). Tumor development in K6/ODC mice was not limited to treatment with chemical carcinogens, since crossing K6/ODC mice with mice overexpressing v-Ha-ras led to development of spontaneous squamous cell skin tumors. Consistent with results from previous models, these tumors also regressed upon ODC inhibition with DFMO (43, 44). These studies show that ODC overexpression is sufficient to increase tumor development in multiple models. Furthermore, the ability of DFMO to reverse tumor growth suggests that interfering with polyamine homeostasis may prove to be an effective therapeutic strategy for tumor treatment. This is supported to some extent by results from clinical trials in which DFMO has been used in cancer therapy (27, 45).

The ability of antizyme to influence tumor growth *in vivo* has also been examined in some detail. AZ has been shown to have tumor-suppressive activity in numerous mouse models. In one model, AZ expression was targeted to the skin using either the bovine keratin 5 or keratin 6 promoter (46). Transgenic K6/AZ and K5/AZ mice developed normally, but showed delayed tumor onset and decreased tumor numbers relative to wild-type controls after exposure to the chemical carcinogen DMBA (7,12-dimethylbenz(*a*)anthracene) and the tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate) (46). Expression of the keratin 5 and 6 promoters can sometimes occur in other epithelial cells, and K6/AZ and K5/AZ mice were also shown to have a decreased incidence of forestomach tumors induced by N-nitrosomethylbenzylamine (NMBA) (47).

To date, only a few studies have addressed the role of AZI *in vivo*, and many of these reports have focused on expression of AZI during normal development (48). Attempts to make AZI knockout mice established that homozygous AZI deletion results in neonatal lethality (49), and $AZI^{-/-}$ embryos are currently being characterized to better understand normal AZI function (50). $AZI^{+/-}$ heterozygote mice are fertile, have no morphological abnormalities, and have a normal lifespan, although they do display lower ODC protein levels in the liver (49). One critical test of whether AZI functions as an oncogene *in vivo* will be to determine

whether transgenic mice with tissue-specific AZI overexpression have an increased rate of tumor incidence, with or without prior carcinogen treatment. For direct comparison to previous results from ODC transgenic animals, such mice could be generated using either the keratin 5 or keratin 6 promoters to explore the role of AZI in skin carcinogenesis. It is likely that, similar to K6/ODC mice, K6/AZI mice would display elevated levels of ODC and polyamines, and may develop skin tumors following treatment with carcinogenic compounds. Furthermore, it will be beneficial to determine whether treatment with DFMO would reverse tumor growth in K6/AZI mice. Generation of conditional mouse models for genes in the polyamine pathway would contribute to our understanding of how the balance between AZI, AZ, and ODC influence tumor growth *in vivo*.

Expression of AZI and AZ in Human Cancer

Genomic studies have previously determined that AZI expression is upregulated in human gastric cancer, ovarian cancer, breast cancer, and prostate cancer (32-35). Current data from the Oncomine database (http://www.oncomine.org) also suggests that AZI copy number is elevated in cancers of the bone marrow, breast, and prostate (Figure 3A). Furthermore, studies collected in the Oncomine database suggest that AZI expression is elevated in cancers of the breast (ductal invasive breast carcinoma), testis (seminoma), liver (hepatocellular carcinoma), lung (adenocarcinoma and squamous cell carcinoma), skin (melanoma), and prostate (carcinoma). Studies of AZI expression in cancers of the brain (oligodendroglioma), and bladder (superficial bladder cancer) were inconclusive, with some arrays showing moderately increased expression and others showing moderately decreased expression. Since AZI is known to promote cell proliferation, increased AZI levels may directly enhance tumorigenesis.

Antizyme has been shown to function as a tumor suppressor *in vivo*, and it is possible that decreased AZ levels may also contribute to tumor development. A meta-analysis of current data in Oncomine suggests that AZ copy number is slightly decreased in several types of breast tumors compared to normal tissue (Figure 3B). AZ copy number may also be decreased in tumors of the lung, ovary, and prostate, although there is significantly more variation in these data sets.

Expression of AZI and AZ in various cancers can also be explored using SAGE Genie Digital Northern analysis provided through the National Cancer Institute's Cancer Genome Anatomy Project (CGAP). As summarized in Table 1, AZI levels were substantially elevated in cancers of the prostate, brain (ependymoma), breast, and liver. Moderate increases were seen in cancers of the lung and pancreas, and AZI levels also may be increased in tissues from the stomach and kidney, although data from normal tissues was not available for comparison. Although AZ expression is regulated primarily at the level of translation, a decreased antizyme mRNA transcription may result in decreased levels of AZ protein and, consequently, increased tumorigenesis. As shown in Table 1, AZ transcript levels appeared to be decreased in tumors of the bone marrow, muscle, and lung, and moderately decreased in tumors of the liver and prostate.

Although gene expression data imply changes in the AZI:AZ ratio in many human cancers, these data cannot be directly applied to protein levels, especially for antizyme which is subject to post-transcriptional regulation. Confirmation of these results by immunohistochemical staining in tumor tissue microarrays would not only validate the electronic gene expression analysis, and allow us to better understand whether misregulation of the AZ/AZI pathway is a common feature of numerous tumor types, or is more specific to certain cancers.

Functions of AZ and AZI Outside of the Polyamine Pathway

Intracellularly, the ratio of AZI to AZ is critically important for determining whether conditions favor cell growth repression (high AZ) or growth activation (high AZI). Thus far, we have primarily focused on how changes in AZI or AZ levels affect ODC and polyamine levels; however, both AZ and AZI have also been shown to have additional functions outside of the polyamine pathway, which are discussed in detail below. This is particularly intriguing since the therapeutic potential of targeting AZ and AZI may be directly influenced by these additional functions.

Antizyme-Mediated Degradation of Cyclin D1 and Other Proteins

The realization that AZ has roles other than regulating polyamine levels came with the observation that AZ can degrade proteins other than ODC. The first demonstration of this was the finding that antizyme can degrade cyclin D1 in a ubiquitin-independent reaction, although cyclin D1 can also be degraded through the ubiquitin-mediated pathway. Antizyme was found not only to immunoprecipitate with cyclin D1, but also to mediate cyclin D1 degradation in an *in vitro* system using purified proteasomes (51). Because cyclin D1 activity is required for cell cycle passage, this effect provides a novel polyamine-independent mechanism for the growth repression observed in numerous cell lines upon antizyme overexpression. Since this initial report, antizyme has been found to regulate degradation of numerous other proteins involved in cell cycle regulation, including Smad 1 (52), Aurora A (53), and Mps1 (54, 55), a protein that regulates centrosome duplication. The effect of antizyme and AZI on centrosomes will be discussed in detail in the following section. It is likely that additional targets of AZ-mediated degradation remain to be identified. It is possible also that AZ binds to ODC with higher affinity than to cyclin D1 or other cell cycle mediators. Precise measurement of the binding constants between AZ and its interacting proteins, for example, by surface plasmon resonance, would greatly enhance our understanding of the relative importance of these interactions within the cell. Furthermore, under conditions where AZ levels are elevated, degradation of proteins other than ODC may exert a greater cellular effect.

Antizyme and AZI Function at Centrosomes

In addition to cytoplasmic and nuclear sites, AZ and AZI localize, in part, to centrosomes (56). These results suggest that centrosomal AZ and AZI may have an important role in regulating normal centriole duplication. This was confirmed by studies in which AZ overexpression caused a decrease in the number of cells with excess centrosomes in a variety of cell types, whereas AZI overexpression stimulated excess centrosome duplication. Centrosome amplification is a common feature of numerous solid tumors and appears to be an early event in tumorigenesis. These results suggest that centrosome duplication may be mediated in part by antizyme. This provides a novel mechanism for changes in the AZI/AZ axis to affect additional components of tumor behavior. Thus far, antizyme has been reported to be involved in degradation of Aurora A, and Mps1, two kinases that promote centrosome duplication. It is likely that antizyme and AZI are involved in directly regulating ubiquitin-independent degradation of multiple proteins that regulate centrosome duplication, and that this takes place directly at the centrosome. It is possible that altered polyamine levels affect centrosome duplication, but this has not yet been demonstrated.

Antizyme and DNA Methylation

In addition to direct effects of AZ on cell cycle progression, antizyme has also been suggested to act as a tumor suppressor by altering DNA methylation. Ectopic expression of AZ in the hamster oral keratinocyte cell line HCPC-1 led to a reduction in ODC activity and demethylation of 5-methyl cytosines (m5C) at CCGG sites (57). This intriguing result

suggests that AZ overexpression could lead to reactivation of cellular genes that had been previously silenced by hypermethylation during cancer development. Later mechanistic studies demonstrated that AZ overexpression was directly associated with hypomethylation of CpG islands in genomic DNA and histone H3 lysine 9 dimethylation (H3K9me2) (58). Furthermore, the protein levels of both DNA methyltransferase 3B (DNMT3B) and the histone H3K9me specific methyltransferase G9a were decreased upon AZ expression, providing additional evidence for a direct link between changes in AZ levels and methylation status. Whether the observed effect of AZ on gene methylation could be exploited for the therapeutic reactivation of silenced genes in tumors remains to be determined, but has intriguing implications for future cancer therapy.

Non-Polyamine Functions of AZI

AZI has also been shown to have additional functions beyond AZ binding and regulating AZ functions. When the AZ-binding region of AZI was deleted, overexpression of the mutant protein (AZIΔAZ) was still able to enhance cell proliferation, although not to the same extent as overexpression of wild-type AZI (59). This suggests that AZI has AZ-independent functions, perhaps mediated by preventing degradation of cyclin D1 or other cell cycle regulators. AZI also partially localizes to the centrosome in a variety of mammalian cells as described above, and overexpression of AZI can induce centriole amplification. Whether AZI directly promotes centrosome duplication by interfering with AZ-mediated functions or through some other mechanism has yet to be fully explored.

Can Altering the AZI:AZ Balance Promote Tumor Growth?

From the experiments described above, it is clear that alteration of the AZI:AZ ratio within the cell could have a direct effect on not only rates of cell proliferation, but also centrosome duplication and gene methylation. Alterations in the AZI:AZ ratio can also affect the uptake of extracellular polyamines. Since AZI inhibits antizyme, increased AZI expression would be expected to result in increased ODC activity and increased polyamine uptake. These effects are expected to be somewhat transitory, however, since increased polyamine uptake will induce expression of antizyme, which will inhibit polyamine transport. The ratio of AZ:AZI within the cell will determine the precise effect on polyamine transport following an increase in AZI levels (60). Numerous experiments thus far have shown that constitutive overexpression of AZI results in increased polyamine uptake and increased accumulation of polyamine analogs (36, 61). This may mimic the situations in some human cancers. Measurement of polyamine transport and accumulation of polyamine analogs following inducible expression of AZ or AZI would clarify the short and long term effects of the role of these proteins in polyamine transport.

The results described in this review argue that changes in cell proliferation following an increase in AZI levels would lead to cell transformation and increased tumor growth via decreased AZ-mediated degradation of ODC and other cell-cycle regulatory proteins. Although centrosome amplification is a common feature of numerous solid tumors, many cell lines have the ability to cluster extra centrosomes and still undergo a pseudo-bipolar cell division (62). Nevertheless, excess centrosomes can contribute to increased genomic instability and higher rates of aneuploidy, and may lead to other chromosomal changes that enhance cell transformation (63). We are only beginning to understand the effect of AZ overexpression on gene methylation, and further studies will be needed to determine the relative importance of those effects during tumor growth *in vivo*.

Could we Target AZI for Cancer Therapy?

Based on its critical role in regulating cell growth and transformation, there has been long-term interest in targeting ODC as a tumor treatment, and several ODC inhibitors are now in

clinical trials (45, 64-66). Despite dramatic effects in cell culture systems, early monotherapy trials with DFMO were largely unsuccessful, perhaps due to the ability of cells to enhance polyamine uptake in order to bypass the effect of DFMO treatment. It is possible that future combinations of DFMO with either polyamine transport inhibitors or cytotoxic drugs would provide more effective therapeutic treatments for many cancer types.

Considerable recent progress has been made in targeting the polyamine pathway in neuroblastoma (67). One frequent event in neuroblastoma development is Myc-N amplification (68), and ODC is known to be a downstream target of Myc. Tumor microarray analysis of 88 neuroblastoma tumors showed that elevated ODC mRNA levels were associated with highly undifferentiated stage 4 tumors and were predictive of decreased overall survival probability and poor disease prognosis (69). In contrast, elevated levels of antizyme 2 (AZ-2) were associated with several indicators of good prognosis (69). Treatment of neuroblastoma cells with DFMO induces G1 cell cycle arrest (70), and DFMO treatment impairs development of neuroblastoma in Eμ-Myc transgenic mice (71). Based on these results, an ongoing phase I clinical trial is being conducted to determine the efficacy of DFMO/etoposide in neuroblastoma (NCT01059071).

We believe that targeting AZI for cancer therapy may actually prove to be a better strategy than targeting ODC, since such a treatment would affect cell proliferation not only through the polyamine pathway but also through additional cell cycle mediators. As previously described, one of the limitations associated with using DFMO to inhibit ODC is that DFMO does not prevent cells from taking up polyamines from the microenvironment. It is possible that inhibiting AZI would have this same limitation, and therefore a combination of AZI inhibition and an agent to block polyamine uptake may have more effective therapeutic effects than simply AZI inhibition alone. Similar studies have already shown that combining DFMO treatment with polyamine transport inhibitors can deplete intracellular polyamine pools in cultured cells and can effectively inhibit tumor growth in the K6/ODC mouse model of squamous cell carcinoma (SCC) (72). The pathway involved in polyamine uptake in mammalian cells has not yet been fully characterized, and identifying the proteins involved could have a significant impact on the design of current and future therapeutics (73).

References

1. Morgan, DDL. Polyamine Protocols. Totowa New Jersey: Humana Press; 1998.
2. Kahana C, Asher G, Shaul Y. Mechanisms of protein degradation: an odyssey with ODC. Cell Cycle. 2005; 4:1461–4. [PubMed: 16205122]
3. Coffino P. Regulation of cellular polyamines by antizyme. Nat Rev Mol Cell Biol. 2001; 2:188–94. [PubMed: 11265248]
4. Murakami Y, Matsufuji S, Nishiyama M, Hayashi S. Properties and fluctuations in vivo of rat liver antizyme inhibitor. Biochem J. 1989; 259:839–45. [PubMed: 2730588]
5. Murakami Y, Ichiba T, Matsufuji S, Hayashi S. Cloning of antizyme inhibitor, a highly homologous protein to ornithine decarboxylase. J Biol Chem. 1996; 271:3340–2. [PubMed: 8631929]
6. Nilsson J, Grahn B, Heby O. Antizyme inhibitor is rapidly induced in growth-stimulated mouse fibroblasts and releases ornithine decarboxylase from antizyme suppression. Biochem J. 2000; 346(Pt 3):699–704. [PubMed: 10698696]
7. Tobias KE, Kahana C. Intersubunit location of the active site of mammalian ornithine decarboxylase as determined by hybridization of site-directed mutants. Biochemistry. 1993; 32:5842–7. [PubMed: 8504104]
8. Fujita K, Murakami Y, Hayashi S. A macromolecular inhibitor of the antizyme to ornithine decarboxylase. Biochem J. 1982; 204:647–52. [PubMed: 7126159]

9. Albeck S, Dym O, Unger T, Snapir Z, Bercovich Z, Kahana C. Crystallographic and biochemical studies revealing the structural basis for antizyme inhibitor function. Protein Sci. 2008; 17:793–802. [PubMed: 18369191]
10. Coffino P. Antizyme, a mediator of ubiquitin-independent proteasomal degradation. Biochimie. 2001; 83:319–23. [PubMed: 11295492]
11. Zhu C, Lang DW, Coffino P. Antizyme2 is a negative regulator of ornithine decarboxylase and polyamine transport. J Biol Chem. 1999; 274:26425–30. [PubMed: 10473601]
12. Chen H, MacDonald A, Coffino P. Structural elements of antizymes 1 and 2 are required for proteasomal degradation of ornithine decarboxylase. J Biol Chem. 2002; 277:45957–61. [PubMed: 12359729]
13. Snapir Z, Keren-Paz A, Bercovich Z, Kahana C. Antizyme 3 inhibits polyamine uptake and ornithine decarboxylase (ODC) activity, but does not stimulate ODC degradation. Biochem J. 2009; 419:99–103. 1 p following. [PubMed: 19076071]
14. Ivanov IP, Rohrwasser A, Terreros DA, Gesteland RF, Atkins JF. Discovery of a spermatogenesis stage-specific ornithine decarboxylase antizyme: antizyme 3. Proc Natl Acad Sci U S A. 2000; 97:4808–13. [PubMed: 10781085]
15. Hakovirta H, Keiski A, Toppari J, Halmekyto M, Alhonen L, Janne J, et al. Polyamines and regulation of spermatogenesis: selective stimulation of late spermatogonia in transgenic mice overexpressing the human ornithine decarboxylase gene. Mol Endocrinol. 1993; 7:1430–6. [PubMed: 8114757]
16. Zhang J, Wang Y, Zhou Y, Cao Z, Huang P, Lu B. Yeast two-hybrid screens imply that GGNBP1, GGNBP2 and OAZ3 are potential interaction partners of testicular germ cell-specific protein GGN1. FEBS Lett. 2005; 579:559–66. [PubMed: 15642376]
17. Mangold U, Leberer E. Regulation of all members of the antizyme family by antizyme inhibitor. Biochem J. 2005; 385:21–8. [PubMed: 15355308]
18. Pitkanen LT, Heiskala M, Andersson LC. Expression of a novel human ornithine decarboxylase-like protein in the central nervous system and testes. Biochem Biophys Res Commun. 2001; 287:1051–7. [PubMed: 11587527]
19. Lopez-Contreras AJ, Lopez-Garcia C, Jimenez-Cervantes C, Cremades A, Penafiel R. Mouse ornithine decarboxylase-like gene encodes an antizyme inhibitor devoid of ornithine and arginine decarboxylating activity. J Biol Chem. 2006; 281:30896–906. [PubMed: 16916800]
20. Lopez-Contreras AJ, Ramos-Molina B, Cremades A, Penafiel R. Antizyme inhibitor 2 (AZIN2/ODCp) stimulates polyamine uptake in mammalian cells. J Biol Chem. 2008; 283:20761–9. [PubMed: 18508777]
21. Lopez-Contreras AJ, Ramos-Molina B, Cremades A, Penafiel R. Antizyme inhibitor 2: molecular, cellular and physiological aspects. Amino Acids. 2010; 38:603–11. [PubMed: 19956990]
22. Kanerva K, Makitie LT, Pelander A, Heiskala M, Andersson LC. Human ornithine decarboxylase paralogue (ODCp) is an antizyme inhibitor but not an arginine decarboxylase. Biochem J. 2008; 409:187–92. [PubMed: 17900240]
23. Snapir Z, Keren-Paz A, Bercovich Z, Kahana C. ODCp, a brain- and testis-specific ornithine decarboxylase paralogue, functions as an antizyme inhibitor, although less efficiently than AzI1. Biochem J. 2008; 410:613–9. [PubMed: 18062773]
24. Pegg AE. Regulation of ornithine decarboxylase. J Biol Chem. 2006; 281:14529–32. [PubMed: 16459331]
25. Auvinen M, Paasinen A, Andersson LC, Holtta E. Ornithine decarboxylase activity is critical for cell transformation. Nature. 1992; 360:355–8. [PubMed: 1280331]
26. O'Brien TG, Simsiman RC, Boutwell RK. Induction of the polyamine-biosynthetic enzymes in mouse epidermis by tumor-promoting agents. Cancer Res. 1975; 35:1662–70. [PubMed: 48421]
27. Gerner EW, Meyskens FL Jr. Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer. 2004; 4:781–92. [PubMed: 15510159]
28. Hogarty MD, Norris MD, Davis K, Liu X, Evageliou NF, Hayes CS, et al. ODC1 is a critical determinant of MYCN oncogenesis and a therapeutic target in neuroblastoma. Cancer Res. 2008; 68:9735–45. [PubMed: 19047152]

29. Yoshida M, Hayashi H, Taira M, Isono K. Elevated expression of the ornithine decarboxylase gene in human esophageal cancer. Cancer Res. 1992; 52:6671–5. [PubMed: 1423312]
30. Hayes CS, DeFeo K, Lan L, Paul B, Sell C, Gilmour SK. Elevated levels of ornithine decarboxylase cooperate with Raf/ERK activation to convert normal keratinocytes into invasive malignant cells. Oncogene. 2006; 25:1543–53. [PubMed: 16278677]
31. Rhodes DR, Barrette TR, Rubin MA, Ghosh D, Chinnaiyan AM. Meta-analysis of microarrays: interstudy validation of gene expression profiles reveals pathway dysregulation in prostate cancer. Cancer Res. 2002; 62:4427–33. [PubMed: 12154050]
32. Chin SF, Teschendorff AE, Marioni JC, Wang Y, Barbosa-Morais NL, Thorne NP, et al. High-resolution aCGH and expression profiling identifies a novel genomic subtype of ER negative breast cancer. Genome Biol. 2007; 8:R215. [PubMed: 17925008]
33. Jung MH, Kim SC, Jeon GA, Kim SH, Kim Y, Choi KS, et al. Identification of differentially expressed genes in normal and tumor human gastric tissue. Genomics. 2000; 69:281–6. [PubMed: 11056045]
34. Schaner ME, Ross DT, Ciaravino G, Sorlie T, Troyanskaya O, Diehn M, et al. Gene expression patterns in ovarian carcinomas. Mol Biol Cell. 2003; 14:4376–86. [PubMed: 12960427]
35. van Duin M, van Marion R, Vissers K, Watson JE, van Weerden WM, Schroder FH, et al. High-resolution array comparative genomic hybridization of chromosome arm 8q: evaluation of genetic progression markers for prostate cancer. Genes Chromosomes Cancer. 2005; 44:438–49. [PubMed: 16130124]
36. Keren-Paz A, Bercovich Z, Porat Z, Erez O, Brener O, Kahana C. Overexpression of antizyme-inhibitor in NIH3T3 fibroblasts provides growth advantage through neutralization of antizyme functions. Oncogene. 2006; 25:5163–72. [PubMed: 16568078]
37. Choi KS, Suh YH, Kim WH, Lee TH, Jung MH. Stable siRNA-mediated silencing of antizyme inhibitor: regulation of ornithine decarboxylase activity. Biochem Biophys Res Commun. 2005; 328:206–12. [PubMed: 15670771]
38. Feith DJ. Carcinogenesis studies in mice with genetically engineered alterations in polyamine metabolism. Methods Mol Biol. 2011; 720:129–41. [PubMed: 21318870]
39. Megosh L, Gilmour SK, Rosson D, Soler AP, Blessing M, Sawicki JA, et al. Increased frequency of spontaneous skin tumors in transgenic mice which overexpress ornithine decarboxylase. Cancer Res. 1995; 55:4205–9. [PubMed: 7671221]
40. O'Brien TG, Megosh LC, Gilliard G, Soler AP. Ornithine decarboxylase overexpression is a sufficient condition for tumor promotion in mouse skin. Cancer Res. 1997; 57:2630–7. [PubMed: 9205069]
41. Peralta Soler A, Gilliard G, Megosh L, George K, O'Brien TG. Polyamines regulate expression of the neoplastic phenotype in mouse skin. Cancer Res. 1998; 58:1654–9. [PubMed: 9563478]
42. Chen Y, Megosh LC, Gilmour SK, Sawicki JA, O'Brien TG. K6/ODC transgenic mice as a sensitive model for carcinogen identification. Toxicol Lett. 2000; 116:27–35. [PubMed: 10906419]
43. Smith MK, Trempus CS, Gilmour SK. Co-operation between follicular ornithine decarboxylase and v-Ha-ras induces spontaneous papillomas and malignant conversion in transgenic skin. Carcinogenesis. 1998; 19:1409–15. [PubMed: 9744537]
44. Lan L, Trempus C, Gilmour SK. Inhibition of ornithine decarboxylase (ODC) decreases tumor vascularization and reverses spontaneous tumors in ODC/Ras transgenic mice. Cancer Res. 2000; 60:5696–703. [PubMed: 11059762]
45. Bailey HH, Kim K, Verma AK, Sielaff K, Larson PO, Snow S, et al. A randomized, double-blind, placebo-controlled phase 3 skin cancer prevention study of {alpha}-difluoromethylornithine in subjects with previous history of skin cancer. Cancer Prev Res (Phila). 2010; 3:35–47. [PubMed: 20051371]
46. Feith DJ, Shantz LM, Pegg AE. Targeted antizyme expression in the skin of transgenic mice reduces tumor promoter induction of ornithine decarboxylase and decreases sensitivity to chemical carcinogenesis. Cancer Res. 2001; 61:6073–81. [PubMed: 11507056]

47. Fong LY, Feith DJ, Pegg AE. Antizyme overexpression in transgenic mice reduces cell proliferation, increases apoptosis, and reduces N-nitrosomethylbenzylamine-induced forestomach carcinogenesis. Cancer Res. 2003; 63:3945–54. [PubMed: 12873989]
48. Murakami Y, Suzuki J, Samejima K, Oka T. Developmental alterations in expression and subcellular localization of antizyme and antizyme inhibitor and their functional importance in the murine mammary gland. Amino Acids. 2010; 38:591–601. [PubMed: 19997757]
49. Tang H, Ariki K, Ohkido M, Murakami Y, Matsufuji S, Li Z, et al. Role of ornithine decarboxylase antizyme inhibitor in vivo. Genes Cells. 2009; 14:79–87. [PubMed: 19077035]
50. Wan T, Hu Y, Zhang W, Huang A, Yamamura K, Tang H. Changes in liver gene expression of Azin1 knock-out mice. Z Naturforsch C. 2010; 65:519–27. [PubMed: 20737923]
51. Newman RM, Mobascher A, Mangold U, Koike C, Diah S, Schmidt M, et al. Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression. J Biol Chem. 2004; 279:41504–11. [PubMed: 15277517]
52. Gruendler C, Lin Y, Farley J, Wang T. Proteasomal degradation of Smad1 induced by bone morphogenetic proteins. J Biol Chem. 2001; 276:46533–43. [PubMed: 11571290]
53. Lim SK, Gopalan G. Antizyme1 mediates AURKAIP1-dependent degradation of Aurora-A. Oncogene. 2007; 26:6593–603. [PubMed: 17452972]
54. Kasbek C, Yang CH, Fisk HA. Antizyme restrains centrosome amplification by regulating the accumulation of Mps1 at centrosomes. Mol Biol Cell. 2010; 21:3878–89. [PubMed: 20861309]
55. Kasbek C, Yang CH, Yusof AM, Chapman HM, Winey M, Fisk HA. Preventing the degradation of mps1 at centrosomes is sufficient to cause centrosome reduplication in human cells. Mol Biol Cell. 2007; 18:4457–69. [PubMed: 17804818]
56. Mangold U, Hayakawa H, Coughlin M, Munger K, Zetter BR. Antizyme, a mediator of ubiquitin-independent proteasomal degradation and its inhibitor localize to centrosomes and modulate centriole amplification. Oncogene. 2008; 27:604–13. [PubMed: 17667942]
57. Tsuji T, Usui S, Aida T, Tachikawa T, Hu GF, Sasaki A, et al. Induction of epithelial differentiation and DNA demethylation in hamster malignant oral keratinocyte by ornithine decarboxylase antizyme. Oncogene. 2001; 20:24–33. [PubMed: 11244502]
58. Yamamoto D, Shima K, Matsuo K, Nishioka T, Chen CY, Hu GF, et al. Ornithine decarboxylase antizyme induces hypomethylation of genome DNA and histone H3 lysine 9 dimethylation (H3K9me2) in human oral cancer cell line. PLoS One. 2010; 5:e12554. [PubMed: 20838441]
59. Kim SW, Mangold U, Waghorne C, Mobascher A, Shantz L, Banyard J, et al. Regulation of cell proliferation by the antizyme inhibitor: evidence for an antizyme-independent mechanism. J Cell Sci. 2006; 119:2583–91. [PubMed: 16735445]
60. Mitchell JL, Thane TK, Sequeira JM, Thokala R. Unusual aspects of the polyamine transport system affect the design of strategies for use of polyamine analogues in chemotherapy. Biochem Soc Trans. 2007; 35:318–21. [PubMed: 17371269]
61. Mitchell JL, Thane TK, Sequeira JM, Marton LJ, Thokala R. Antizyme and antizyme inhibitor activities influence cellular responses to polyamine analogs. Amino Acids. 2007; 33:291–7. [PubMed: 17410332]
62. Kwon M, Godinho SA, Chandhok NS, Ganem NJ, Azioune A, Thery M, et al. Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. Genes Dev. 2008; 22:2189–203. [PubMed: 18662975]
63. Ganem NJ, Godinho SA, Pellman D. A mechanism linking extra centrosomes to chromosomal instability. Nature. 2009; 460:278–82. [PubMed: 19506557]
64. Bachrach U. Polyamines and cancer: minireview article. Amino Acids. 2004; 26:307–9. [PubMed: 15290334]
65. Meyskens FL Jr, McLaren CE, Pelot D, Fujikawa-Brooks S, Carpenter PM, Hawk E, et al. Difluoromethylornithine plus sulindac for the prevention of sporadic colorectal adenomas: a randomized placebo-controlled, double-blind trial. Cancer Prev Res (Phila). 2008; 1:32–8. [PubMed: 18841250]
66. Shantz LM, Levin VA. Regulation of ornithine decarboxylase during oncogenic transformation: mechanisms and therapeutic potential. Amino Acids. 2007; 33:213–23. [PubMed: 17443268]

67. Evageliou NF, Hogarty MD. Disrupting polyamine homeostasis as a therapeutic strategy for neuroblastoma. Clin Cancer Res. 2009; 15:5956–61. [PubMed: 19789308]

68. Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, et al. Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. J Clin Oncol. 1991; 9:581–91. [PubMed: 2066755]

69. Geerts D, Koster J, Albert D, Koomoa DL, Feith DJ, Pegg AE, et al. The polyamine metabolism genes ornithine decarboxylase and antizyme 2 predict aggressive behavior in neuroblastomas with and without MYCN amplification. Int J Cancer. 2010; 126:2012–24. [PubMed: 19960435]

70. Wallick CJ, Gamper I, Thorne M, Feith DJ, Takasaki KY, Wilson SM, et al. Key role for p27Kip1, retinoblastoma protein Rb, and MYCN in polyamine inhibitor-induced G1 cell cycle arrest in MYCN-amplified human neuroblastoma cells. Oncogene. 2005; 24:5606–18. [PubMed: 16007177]

71. Rounbehler RJ, Li W, Hall MA, Yang C, Fallahi M, Cleveland JL. Targeting ornithine decarboxylase impairs development of MYCN-amplified neuroblastoma. Cancer Res. 2009; 69:547–53. [PubMed: 19147568]

72. Burns MR, Graminski GF, Weeks RS, Chen Y, O'Brien TG. Lipophilic lysine-spermine conjugates are potent polyamine transport inhibitors for use in combination with a polyamine biosynthesis inhibitor. J Med Chem. 2009; 52:1983–93. [PubMed: 19281226]

73. Palmer AJ, Wallace HM. The polyamine transport system as a target for anticancer drug development. Amino Acids. 2010; 38:415–22. [PubMed: 19956998]

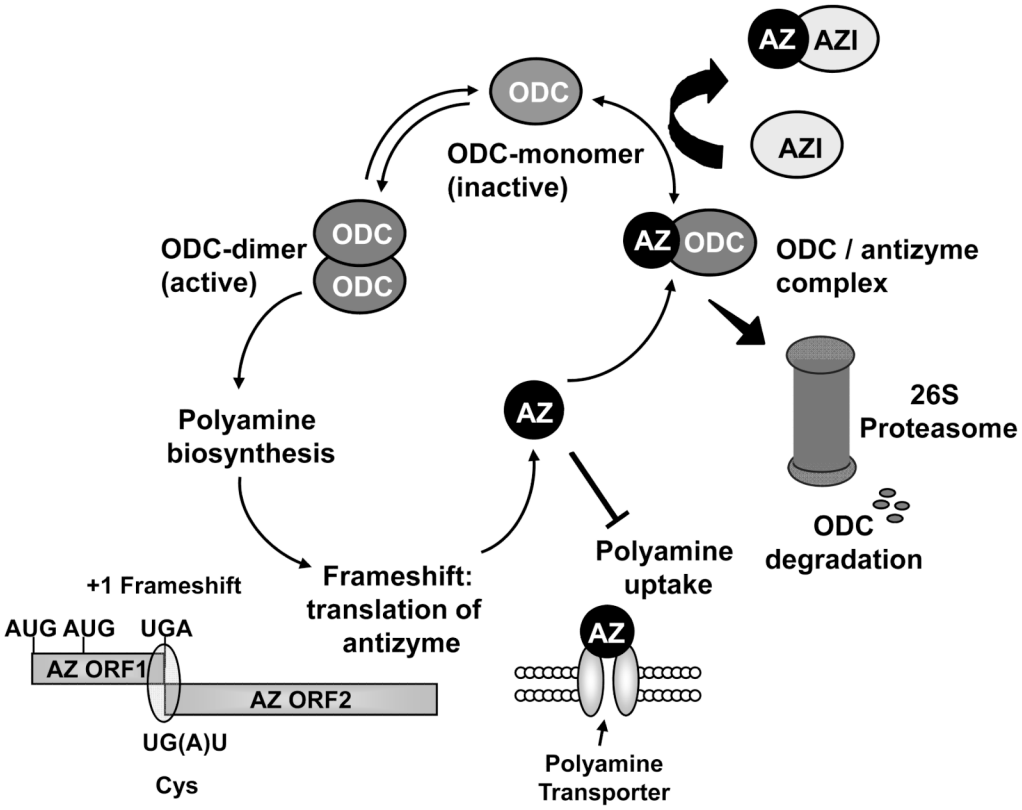

Figure 1. Antizyme Inhibitor (AZI) and Antizyme (AZ) Regulate Polyamine Biosynthesis Through Ornithine Decarboxylase (ODC)

Dimeric ornithine decarboxylase (ODC) catalyzes the first step in polyamine synthesis, converting ornithine into the polyamine putrescine. Antizyme (AZ) disrupts ODC homodimers, targets ODC for degradation by the 26S proteasome in a ubiquitin-independent manner, and inhibits polyamine uptake. Activity of AZ is further regulated by antizyme inhibitor (AZI). Antizyme inhibitor can displace ODC from the ODC-AZ complex, restoring polyamine biosynthesis by ODC.

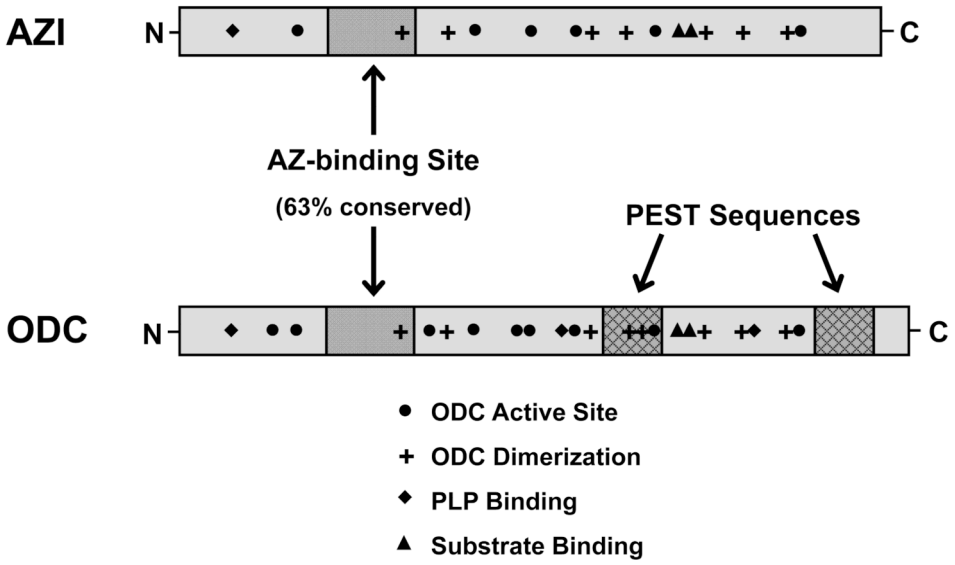

Figure 2. Comparison of Conserved Residues in AZI and ODC

Amino acids involved in formation of the ODC active site (circles), ODC dimerization (+), PLP binding (diamonds), and substrate binding (triangles) are shown in both AZI and ODC proteins.

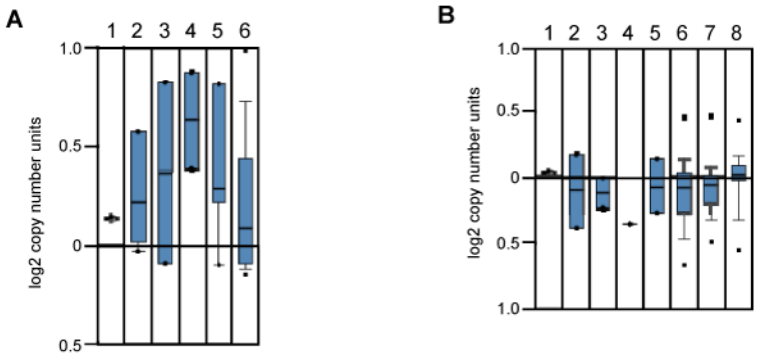

Figure 3. Comparison of AZI and AZ Copy Number in Normal vs. Cancer Tissue

Changes in AZI (A) and AZ (B) copy number were determined by meta-analysis of Oncomine data. For AZI, samples analyzed include the following: 1) normal, 2) acute myeloid leukemia, 3) breast adenocarcinoma, 4) breast carcinoma, 5) ductal breast carcinoma, and 6) prostate cancer. For AZ, the following tissue types were analyzed: 1) normal, 2) breast adenocarcinoma, 3) breast carcinoma, 4) dedifferentiated liposarcoma, 5) ductal breast carcinoma, 6) non-small cell lung carcinoma, 7) ovarian carcinoma, and 8) prostate carcinoma.

Table 1
SAGE Analysis of AZI and AZ Expression in Normal vs. Cancer Tissue

SAGE Genie Digital Northern analysis from the Cancer Genome Anatomy Project (CGAP) was used to assess AZI and AZ mRNA expression levels in normal vs. cancerous tissues from a variety of organs. The unique sequence TCTTTCACCC from the 3′ untranslated region (UTR) of AZI mRNA was used for analysis, and the sequence TTGTAATCGT was used for AZ. The number of times the gene-specific sequence was detected out of every 200,000 tags was determined, and then compared to the observed level in normal tissue. Values are given as average ± sd. ND, not determined.

Gene	Tissue	Normal	Cancer	Fold Change
AZI	Prostate	4.67 ± 2.89	16 ± 7.55	3.43
	Brain (Ependymoma)	4.33 ± 2.89	14.20 ± 4.80	3.28
	Breast	2.82 ± 2.38	7.05 ± 4.83	2.50
	Liver	3.00 ± ND	7.33 ± 4.51	2.44
	Brain (Meningioma)	4.33 ± 2.89	7.50 ± 4.95	1.73
	Lung	4.00 ± ND	6.67 ± 3.79	1.67
	Pancreas	6.00 ± ND	7.75 ± 3.10	1.29
	Brain (Glioblastoma)	4.33 ± 2.89	5.58 ± 3.63	1.29
AZ	Bone Marrow	263.67 ± 32.59	12.33 ± 14.43	.05
	Muscle	107.00 ± 5.66	42.00 ± ND	.39
	Lung	254.00 ± ND	140.67 ± 24.42	.55
	Prostate	187.67 ± 45.79	116.50 ± 65.76	.62
	Liver	199.00 ± ND	133 ± 15.87	.67
	Brain (Oligodendroglioma)	103.00 ± 56.95	78.00 ± 41.01	.76
	Brain (Ependymoma)	103.00 ± 56.95	89.64 ± 30.66	.87