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Optimal Assay Conditions for ODC-Antizyme Interaction

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Polyamines are essential for life and are associated with cell cycle and development. Excess polyamine production occurs during tumor formation and is one possible target for chemopreventative and chemotherapeutic treatment. One enzyme, ornithine decarboxylase (ODC), plays a key role in polyamine biosynthesis. Among other regulators, the protein antizyme (Az) limits polyamine levels in the cell. It binds ODC and renders it unable to produce polyamines. The mechanism by which ODC and Az interact is unknown and is explored in this study.

It is known that ODC and Az have high affinity for one another, but the reason for the basal level of ODC activity that remains in excess Az in assays is unclear. Varying the temperature and coenzyme concentrations in an assay revealed that these factors successfully changed the level of ODC activity at which the two proteins ceased to bind. The persistence of ODC activity in excess Az under several assay conditions showed that the remaining activity is not a result of the methods by which the assay is prepared. The mechanism by which some ODC activity is allowed to remain in excess Az remains uncertain.

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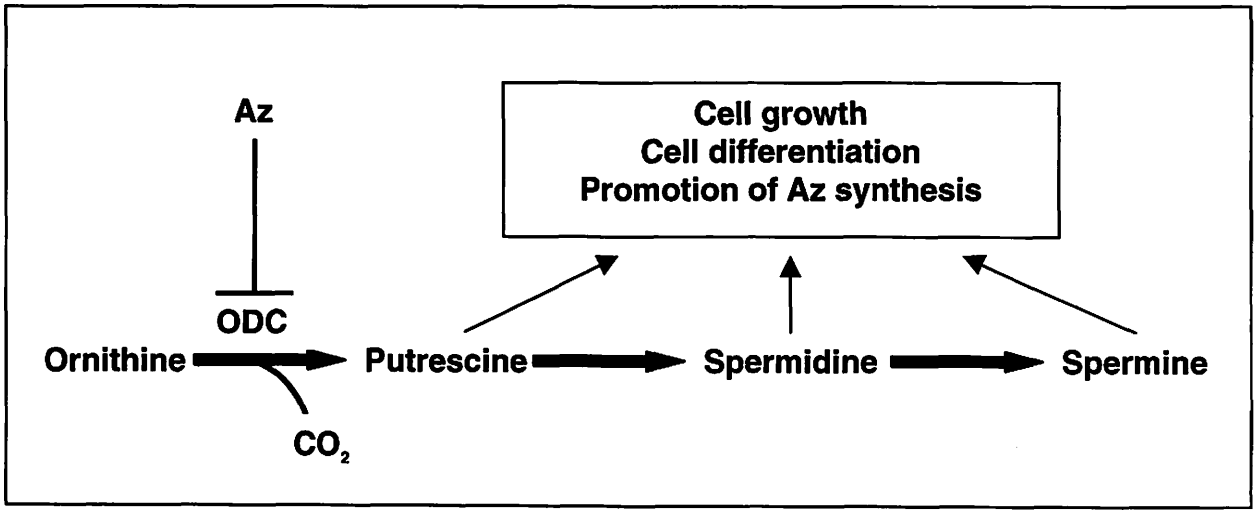
Modern cancer research explores a great diversity of biochemical pathways in the hopes of developing more effective chemotherapeutic and chemopreventative drugs. One promising area of research explores the roles of polyamines and the substances that regulate them as potentially effective mechanisms for treating cancer. Understanding their function, biosynthesis, transport, and catabolism in both healthy and cancerous cells is key. It is also helpful to investigate the ways in which some regulators affect the development and inhibition of these compounds in vitro and in vivo in clinical trials. Further exploration in the less understood mechanisms of polyamine control could yield improved chemotherapy for patients and chemoprevention in high-risk groups.

Polyamines, including putrescine, spermidine and spermine, are essential for life and are associated with cell cycle and development. They are nutritive to normal cells and enable the increased growth of cancerous cells by promoting proliferation, differentiation and immortalization (Cohen 1998, Nishioka 1996). The polyamines are involved with ribosome function and protein synthesis, maintenance including apoptosis, and angiogenesis (Cohen 1998, Nishioki, et al 1996). In addition, spermidine is connected with the formation of hypusine, an amino acid that is necessary for protein synthesis in yeast and animal cells (Cohen 1998). It has also been discovered that reduced cellular polyamine levels decrease the rate of virus multiplication (Cohen 1998). Clearly, polyamines serve key functions in the growth and maintenance of both normal and unhealthy cells.

Polyamines become available to cells by two means: they are either synthesized within the cells or they are consumed by an organism and then transported into the

cells. As shown in Figure 1, ornithine decarboxylase (ODC) converts ornithine to the first polyamine putrescine in the rate-limiting step of polyamine synthesis. ODC is activated by GTP and is inhibited by a number of compounds including the protein antizyme (Az). ODC catalyzed biosynthesis of polyamines is limited by a feedback control mechanism. The presence of high levels of polyamines promotes translation of Az mRNA, creating a feedback loop in which polyamines that are synthesized inhibit further production of polyamines (Almrud 2000), as shown in Figure 1. ODC is a protein with an extremely short half-life, only about 20 minutes. Its short half-life and the diversity of conditions that strongly alter its activity indicate that its activity is highly controlled.

Figure 1: The Polyamine Biosynthetic Pathway



Az binds active ODC and renders it unable to produce polyamines. Az serves to inhibit ODC by binding it in its monomeric (unpaired) state, enabling conformational changes in ODC that expose its C terminus. The exposed C-terminus of the ODC allows it to be degraded by the 26S proteosome (Almrud 2000, Persson 1996). This

idea is supported by the fact that ODC truncated at the C-terminus has a significantly longer half-life (Persson 1996). Given that high levels of polyamines are present in cancerous cells, Az provides one opportunity for chemopreventive and chemotherapeutic research.

While Az inhibits excessive cell growth and differentiation by inhibiting polyamine synthesis within the cell, it may also serve to limit polyamine levels by interfering with the polyamine transporter. Polyamines originate from the food consumed by an organism are transferred from the gastrointestinal tract to the cell. They can supplement the needs of cells that have become unable to synthesize sufficient amounts of polyamines "due to lack of substrates or to inhibition of ODC" (Persson 1996). Transporter activity is stimulated by growth promoting agents, another group of compounds that are present in high levels in cancerous cells. Reducing the effects of growth promoting agents with Az in these cells could decrease the rate of cancerous growth.

The transporter is active during periods in which cells are deprived of polyamine synthesis. It is inactive when polyamines are present or in excess (Persson 1996). It is thought "that elevations in cellular polyamine levels stimulate the production of a labile protein that reversibly inactivates the polyamine transporter" (Mitchell 1994). Further evidence suggests that feedback response noted in polyamine transport may share a common intermediate with the response resulting in ODC instability (Mitchell 1994). This implies that Az is one mechanism of transporter control. The mechanism by which Az inhibits polyamine transport remains unknown.

While a cell can limit polyamine production and transport into the cell, it must also retain the ability to catabolize polyamines. Polyamine catabolism can consist of regression to a polyamine earlier in the metabolic pathway, acetylation for excretion, break-down into amino acids, or oxidation of putrescine for the formation of GABA, a neurotransmitter. Spermidine spermine acetyl transferase (SSAT), another well-regulated enzyme, acetylates its products and can convert spermine to spermidine and spermidine to putrescine. Polyamines "created" by SSAT can continue through metabolism or be excreted. The transacetylated product polyamines are far more easily excreted that their non-acetylated counterparts. SSAT is induced under conditions where a cell acquires high levels of polyamines due to toxins, hormones or the polyamines themselves (Persson 1996).

The polyamines may be catabolized into amino acids via a variety of pathways. One such example is the indirect relationship between putrescine, glutamate and ornithine, shown in Figure 2. Interestingly, putrescine catabolism to amino acids occurs more frequently in aged cells than younger ones (Cohen 1998). This infers that there is some relationship between polyamine control mechanisms and senescence. One possible explanation is that older cells have increased amine oxidases (Cohen 1998).

As previously mentioned, putrescine can be converted into the inhibitory neurotransmitter GABA. The transformation can occur in both the brain and peripherally. The putrescine to GABA change is essentially the result of an oxidase, followed by an aldehyde dehydrogenase (Cohen 1998).

Figure 2: An Overview of Polyamine Metabolism

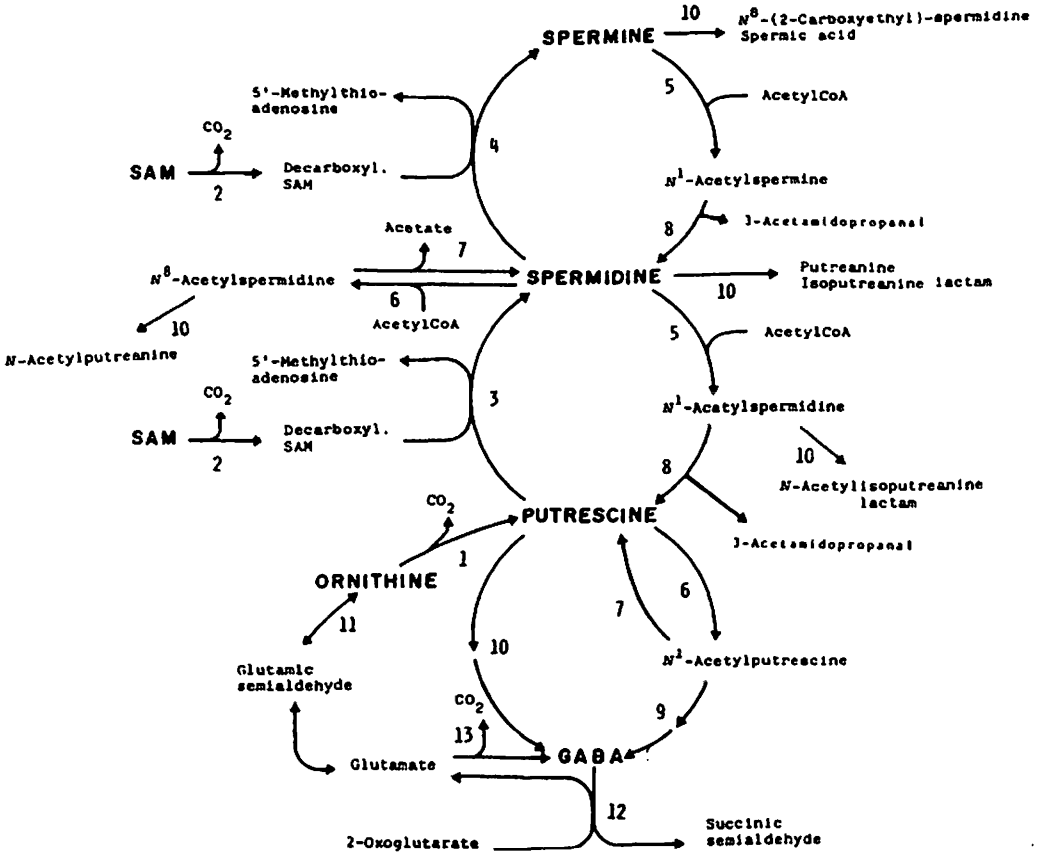


Figure from Cohen (1998).

Neoplastic transformation, or the immortalization of cells, is linked with abnormal polyamine metabolism. Changes in polyamine synthesis, degradation, and excretion can be demonstrative of cancer (Cohen 1998). Evidence indicative of this association includes:

1. Increased excretion of polyamines by many cancer patients
2. Correlation of ODC content to the growth rates of certain cancers
3. Correlation of cellular ODC and polyamines to the growth rates of some tumor cells
4. Some effective cancer therapies decrease "abnormally high rates of polyamine synthesis" (Cohen 1998).

Excess polyamine production occurs during tumor formation and slows with cancer remission. Because excessively high levels of polyamines have been associated with cancer, an opportunity has arisen for study into possible improvements in cancer diagnosis and chemotherapeutic or chemopreventative treatments involved with this pathway.

Increased ODC-catalyzed biosynthesis of polyamines can contribute to cancerous growth. The protooncogene c-myc enhances ODC transcription, resulting in increased levels of polyamine synthesis. This augmentation of polyamine synthesis is one characteristic of cancerous cells (Cohen 1998). ODC is associated with proliferation and hyperplasia (increased number of cells) (Cohen 1998) and its levels can correlate with malignancy. The strong correlation between ODC activity and cancer suggest that testing of ODC activity levels could be used to help diagnose cancers and that the polyamine biosynthetic pathway provides an excellent target for new chemotherapeutic and chemopreventative practices.

Regulation of polyamine levels must be highly controlled under normal conditions by the short-lived proteins ODC, SSAT and others. One mechanism by which excess polyamines contribute to cancerous growth is via constitutively active ODC. This ODC is no longer regulated, synthesizing polyamines without bound after the cell has been "transformed by carcinogens, viruses or oncogenes" (Almrud 2000). For this reason, substances that inhibit ODC activity could be productively explored in a laboratory setting. This inhibition could arise from "regulation of odc transcription, post-translational modification of ODC and negative feedback control" (Almrud 2000).

The close relationship between polyamine level control and cancer enable exploration into the development of clinically relevant improvements in the understanding of cancer. One such application is the advancement of clinical testing for cancer. Given the strong association of elevated polyamine levels in urine and red blood cells and cancer, it is possible that these levels could reveal the degree to which the cancer has advanced in a patient. (Cohen 1998). ODC activity could also be used to detect cancer. Increased activity was detected in "normal-appearing colonic mucosa . . . in patients with familial polyposis" over that which was present in controls (Nishioki, et al 1996). This clinical trial indicates that the characteristic can be used as an effective biological marker for cancer. The efficacy of testing ODC activity as a marker in other organs is supported by similar results (Nishioki, et al 1996).

Inhibiting polyamine biosynthesis may also be used in chemotherapy of cancer. A number of clinical studies targeting the pathway involved in polyamine biosynthesis have already begun, using polyamine analogs and other compounds. Eflornithine (DFMO) and methylglyoxyl-bis(guanylhydrazone) (MGBG) are two such potential chemotherapeutic agents. These agents are specifically targeted against ODC and a similar enzyme, AdoMetDC, respectively.

DFMO irreversibly inhibits ODC and leads to depletion of polyamines in the cells. Clinical studies involving DFMO have consisted of chemoprevention trials, anti-trypanosomal therapy, and treatment of brain tumors and cervical dysplasia. (Nishioki 154). While successfully reducing putrescine and spermidine levels, its effects on spermine levels are limited. "Limited clinical evaluation against solid tumors" in the 1980s were unsuccessful "due to a lack of potency" (Kramer 1996). Later studies have

established its ability to limit polyamine synthesis in vitro and in vivo. Patients' cancers did not enter remission, but some were stabilized and levels of circulating blast cells decreased in some of the patients (Nishioki, et al 1996). The side effects of DFMO treatment are nontoxic, relatively low and are reversible.

MGBG causes the inhibition of AdoMetDC, another polyamine biosynthetic enzyme. It serves to block spermidine synthesis, thereby reducing Az and subsequently increasing ODC activity. Clinical studies involving MGBG have dealt with non-Hodgkins lymphoma in AIDS patients. Clinical trials involving MGBG were initially unsuccessful because the compound remained in tissues for extended periods of time. Some of MGBG's undesired effects include disruption of mitochondrial structure and function (Kramer 1996). However, it can be very effective against certain cancers, including non-Hodgkins lymphoma (Nishioki et al 1996). MGBG analogs that are more specific, and are therefore less likely to produce toxic side effects, have also been developed. One example of an MGBG analog with reduced potential side effects is CGP-48664. It binds ODC as a competitive inhibitor using an alternative transport mechanism and provides another opportunity for improved chemotherapeutic treatment.

The polyamine biosynthetic pathway has also been explored for the development of chemopreventative agents. Trials consisting of DFMO (the irreversible inhibitor of ODC) treatment to prevent cancer have been performed on animals that have been exposed to cancer-causing agents. The results were positive and clinical trials in humans have yielded similarly promising results with relatively low levels of side effects (Nishioki et al 1996). In a similar way, polyamine analogs may also prove to be effective in chemoprevention (Nishioki et al 1996).

Az provides another key opportunity for chemotherapeutic and chemopreventative treatment. Where DFMO only blocks ODC-catalyzed polyamine biosynthesis, Az would be expected to inhibit both ODC activity and polyamine transport into the cells. This indicates that Az could more efficiently cause polyamine deficiency in affected cells, creating an environment in which cancerous growth would be inhibited. Further research into the role of Az in ODC inhibition and polyamine transport will provide the knowledge necessary to develop Az into a clinically effective chemotherapeutic and/or chemopreventative agent.

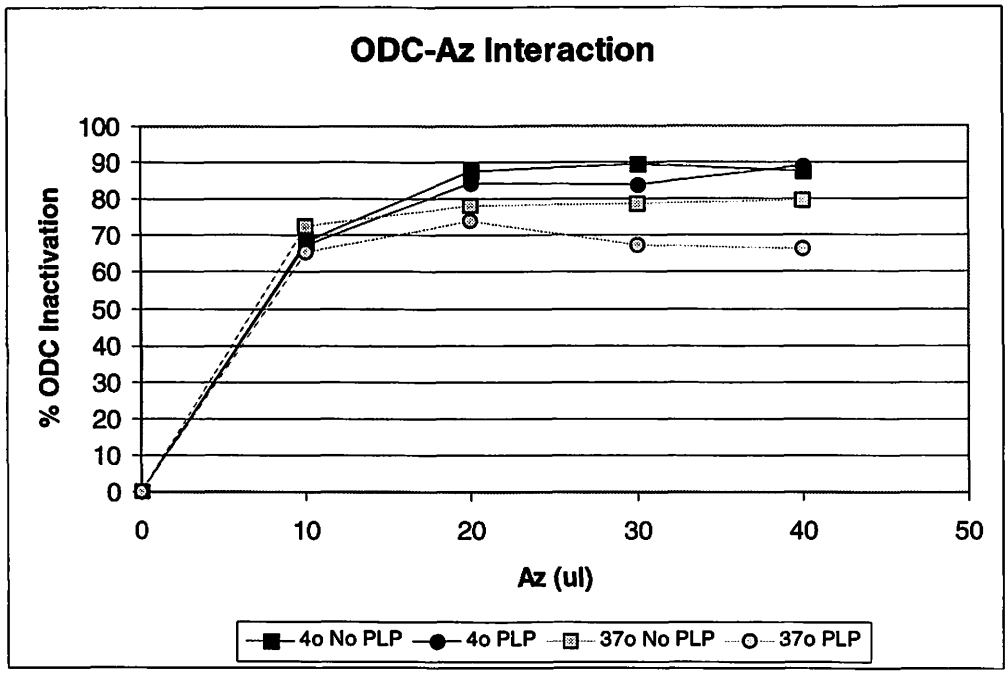
One aspect of Az inhibition of ODC that remains unclear is the mechanism by which Az binds ODC. While it is clear that Az and ODC have high affinity for one another, assays reveal a basal level of ODC activity that remains in excess Az. Understanding the mechanism by which some ODC activity is able to remain in excess Az could reveal new questions in the development of Az as a chemopreventative agent. Varying the conditions under which the assay is performed should elucidate the roles that different factors play in controlling the level of ODC activity at which ODC and Az cease to bind.

In this study, the most favorable conditions for ODC-Az interaction have been explored. A fixed amount of ODC was prepared to which increasing amounts of Az were added to excess. The amount of ODC activity was recorded and losses in ODC activity were attributed to interaction with Az, thereby revealing Az activity. This Az activity is compared across variables (temperature and presence or absence of coenzyme), with the highest level of Az activity indicating optimal assay conditions for ODC-Az interaction.

To perform the assay, HTC (rat hepatoma) cells were first induced with methylglyoxyl-bis(guanylhydrazone) (MGBG) and dicyclohexylamine (DCHA). These cells were used to provide the ODC activity. Other HTC cells were induced for Az using putrescine, making use of the feedback repression of ODC by polyamines. Graded amounts of Az were incubated with the ODC on ice for 15 minutes in buffer. After this reaction period the level of active ODC was determined by a ^{14}C release assay. ^{14}C-labelled ornithine was added to the reaction vials. Filter paper that was treated with KOH was placed into the reaction vial stoppers. The KOH on the paper captured the ^{14}C-labelled product CO_2 during incubation. After the stopper had been placed onto the reaction vial, the mixture was incubated and shaken at 37°C for one hour. Citric acid was injected into the vials to stop the reaction. The mixture was then incubated and shaken for 30 minutes. Following the assay, the filter paper was placed in scintillation fluid. The disintegrations of the ^{14}C-label were expressed as the emission of light that was counted (in counts per minute) by a liquid scintillator. The radioactivity counted by the scintillator revealed the levels of ODC activity remaining and consequently the level of Az activity.The experimental conditions were varied in temperature (4° and 37°) and presence of absence of the coenzyme, pyridoxal phosphate (PLP). Temperature was expected to play a role in the level of Az activity because it affects the availability of ODC to interact. ODC remains homodimeric (paired ODC molecules) at 37°, but becomes monomeric (unpaired, open for interaction) at 4°. The addition of PLP, a coenzyme that promotes the formation of ODC dimers, should have decreased ODC inactivation by Az. A combination of these conditions should have revealed if the

remaining ODC activity in excess Az was a result of the methods by which the assay was prepared.

Figure 3: Levels of ODC Inactivation



Assay results revealed that the conditions under which the assay was prepared did play an important role in the level of ODC activity that remained in excess Az, as shown in Figure 3. The assays demonstrated that Az was able to inactivate more ODC at 4° than at 37°, as expected. Performing the assay at 4° allowed the ODC to remain in its monomeric state, which made it more readily available to promote polyamine synthesis. Under physiologic conditions (37°), the ODC should have been dimeric and would be expected to not be as susceptible to Az binding. The addition of PLP provided a similarly predictable result. Given that PLP is a coenzyme that encourages the formation of ODC dimers, it too decreased observed Az activity. While the variables

successfully changed the activity level at which ODC-Az interactions ceased, they could not entirely eliminate the enduring ODC activity.

The levels of ODC activity at which ODC and Az cease to bind varied from about 65% of original activity (for the assay performed with PLP at 37°) to about 90% (for the assays performed at 4°). The fact that at least 10% of ODC activity was able to remain under all of the assay conditions explored indicates that the remaining activity is not a result of the methods by which the assay is prepared. The mechanism by which some ODC activity is allowed to remain in excess Az remains unclear. Though it is clear that Az is unable to completely inactivate ODC under various assay conditions, its function under normal physiologic conditions remains very important. Further inquiry will be needed to reveal more about how Az binds ODC and to discover what enables ODC activity to remain in excess Az.

Continuing research of the interactions between Az and ODC should elucidate more about polyamine synthesis, inhibition and transport. Given the strong link between excessive polyamines and cell transformation and immortalization, repression of polyamine synthesis and transport into the cell could limit cancerous growth. Az serves as an effective inhibitor of both ODC-catalyzed polyamine synthesis and polyamine transport into cells. This combination is not shared by other compounds in the polyamine pathway, some of which have already proven successful in clinical trials. Using what is already known about these other regulators in the pathway as a background, learning more about Az inhibition of high levels of polyamines be helpful in establishing Az as a safe, effective chemotherapeutic and chemopreventative agent.

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