Polyamine Metabolism and Its Importance in Neoplastic Growth and as a Target for Chemotherapy

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Abstract

The polyamine-biosynthetic pathway represents an inviting target for the development of agents inhibiting carcinogenesis and tumor growth. Polyamines play an essential role in the proliferation and development of mammalian cells. Deranged polyamine metabolism may be an important factor in carcinogenesis. Depletion of polyamines inhibits growth of neoplastic cells in vitro and in animal models. Several different classes of other anticancer agents may under some conditions exert enhanced effects when polyamine levels are depleted. Some suitable inhibitors of polyamine production are currently available and other promising compounds are presently being tested. It should soon prove possible to block polyamine biosynthesis at every step in the pathway. The use of these inhibitors alone and combined either with each other or with other antitumor agents will enable a full examination of the potential of this approach.

The polyamines spermidine [H₂N(CH₂)₃NH(CH₂)₄NH₂] and spermine [H₂N(CH₂)₃NH(CH₂)₃NH(CH₂)₄NH₂] and the diamine putrescine [H₂N(CH₂)₄NH₂] are present in all mammalian cells. Recent reviews covering polyamine biochemistry and physiology include Refs. 1–7. Despite extensive research efforts, their functions in cellular physiology are not yet well understood. Studies in vitro with isolated enzymes, membranes, and subcellular organelles have revealed a vast and often bewildering array of responses to added polyamines. The fact that effects are observed in such experiments is not surprising since the basic polyamines are clearly capable of noncovalent interaction with biochemical macromolecules such as nucleic acids, proteins, and phospholipids and these interactions are very likely to influence the rate of biochemical reactions. Changes in the activity of such fundamental processes would be expected to have profound effects on cellular physiology, but it is very difficult to demonstrate clearly the biological relevance of these effects. However, the need for polyamines in order to maintain cell growth and function is firmly established. Many early studies indicated that polyamine synthesis was enhanced during growth and that growth-promoting stimuli led to an increase in polyamine biosynthesis. Studies with mutant microorganisms also supported the concept that polyamines were essential for normal growth processes (1–3, 8).

More recently, direct support for this hypothesis has been provided by experiments in which polyamine synthesis was prevented in mammalian cells in culture by mutations or by the application of inhibitors. This led to a virtual cessation of growth unless exogenous polyamines were provided. These findings have led to an increased interest in the possibility that inhibitors of polyamine biosynthesis might be useful therapeutic agents in a variety of diseases involving deranged cell prolifer-

1 The abbreviations used are: ODC, ornithine decarboxylase; AdoMet, S-adenosylmethionine; AdoMetDC, S-adenosylmethionine decarboxylase; DFMG, α-difluoromethylornithine; MAP, (R)-α-ethyl-(R)-α-methylputrescine [also described as (2R,5R)-α-methylacetylenicputrescine or (2R,5R)-6-heptyne-2,5-diamine]; MGBG, methylglyoxal bis(guanylylhydrazone); MAOE, 5′-deoxy-5′-[(N-methyl-N-[3-hydrazinopropyl)]aminoadenosine; MHTPA, 5′-deoxy-5′-[(N-methyl-N-[3-hydrazinopropyl)]aminoadenosine; AdoDat, S-adenosyl-1,8-diamino-3-thiooctane; AdoDatD, S-adenosyl-1,12-diamino-3-thiooctadecane; AdoSOH, S-adenosyl-5′-methylthioadenosine [the correct chemical name is dimethyl (5′-adenosyl) sulfonium perchlorate]; BCNU, 1,3-bis(2-chloroethyl)nitrosourea; ara-C, 1-β-D-arabinofuranosylcytosine; MTX, 5′-methylthioadenosine; cDNA, complementary DNA.

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2 In order to reduce the number of references, in most cases only the most recent primary papers have been cited and reviews are used for other citations. Full details of the primary investigators and papers are given in these reviews.

3 The chemical names throughout this paper are given in their IUPAC names. It should be noted that the polyamine putrescine is also known as (2Z,5Z)-1-methylacetylenicputrescine or (2Z,5Z)-6-heptyne-2,5-diamine.
arginine into ornithine (2, 4, 13). This fact may account for the apparent need for putrescine as a component of many media formulations used for cell culture in the absence of serum. This requirement is not due to an absence of ODC but rather to an absence of its substrate.

Putrescine is converted further to spermidine and spermine through the consecutive action of two distinct aminopropyltransferases, spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22). Both of the enzymes use decarboxylated S-adenosyl-L-methionine as an aminopropyl donor but are specific with respect to their acceptors (putrescine and spermidine, respectively). Thus, the aminopropyl groups of the polypeptide are ultimately derived from methionine. Once S-adenosylmethionine has been decarboxylated, it is committed to polyamine biosynthesis and is no longer available for methyl transfer reactions (4, 14). The content of decarboxylated S-adenosylmethionine is normally very low in mammalian cells and the activities of the aminopropyltransferases are regulated by the availability of this nucleoside substrate. AdoMetDC (EC 4.1.1.50), which produces it and is activated by putrescine, is therefore a key step in polyamine production.

Polyamines are interconverted and degraded back to putrescine by the action of two enzymes [polyamine oxidase (flavin adenine dinucleotide dependent) and spermidine/spermine N4-acetyltransferase]. After acetylation by the latter enzyme, the N4-acetyl derivatives of spermine and spermidine are substrates for polyamine oxidase which splits off 3-acetimidopropenal generating spermidine and putrescine, respectively. These acetylation reactions combined with relevant aminopropyltransferases account for the enhanced synthesis of ODC protein.

Polyamine oxidation proceeds through two distinct pathways, a rapid inactivation of ODC and AdoMetDC. The flux through the synthases is controlled by the supply of decarboxylated AdoMet. Whether the available decarboxylated AdoMet is used for the production of spermidine or spermine depends on the relative amounts of the two synthases and of their amine substrates.

Both ODC and AdoMetDC are very highly regulated in the cell and respond to a wide variety of stimuli affecting growth, and also to the cellular content of putrescine and polyamines (1–7). The rapid and profound increase in ODC activity in response to hormones, peptide growth factors, regenerative stimuli, and other drugs is very well documented. Recent studies using antibodies to ODC in Western blotting or radioimmunoassay techniques have shown that, in most cases, enhanced activity of ODC in these responses is due to an increased amount of ODC protein (4, 15). A part of the increase may derive from a stabilization of the protein but most of it is due to an elevated rate of synthesis. In some cases, this has been correlated with a greater content of mRNA measured using Northern blots hybridized to cDNA probes (4, 15, 16). However, it is not yet clear to what extent the greater mRNA content reflects changes in the transcription, processing, or degradation of the mRNA or whether the increase is entirely sufficient to account for the enhanced synthesis of ODC protein.

ODC levels are strikingly repressed by polyamines resulting in a profound decline in the amount of the protein. Conversely, the reduction of intracellular polyamine content by the addition of inhibitors leads to an increased content of ODC. These changes in ODC are not accompanied by changes in the content of the mRNA and appear to be mediated by alterations in its rate of translation and in the turnover of the protein (4, 15, 17, 18). Recently, direct evidence that the presence of spermidine or spermine inhibits the translation of ODC mRNA in reticulocyte lysates has been obtained (19).

The three key regulatory enzymes in the polyamine biosynthesis and interconversion pathway (ODC, AdoMetDC and spermidine/spermine N4-acetyltransferase) all have very rapid rates of turnover with half-lives of less than 1 h in many cells. This rapid rate of turnover allows their activity to be regulated by changes in the amount of the enzyme protein and still respond dramatically within a few hours (2, 4). Little is known about the mechanism of degradation of these enzymes but both ODC and AdoMetDC have sequences corresponding to the "PEST regions" postulated by Rogers et al. (20) to be important in rapid protein turnover. There is also some evidence that the turnover of ODC may be enhanced by its combination with an inhibitory protein discovered by Heller and Canellakis (21) and named "antizyme" by them. The amount of antizyme is increased in response to exposure to putrescine and other diamines (21) and the increased amount of antizyme:ODC complexes and the increased degradation of ODC may be major factors in the reduction of ODC in response to putrescine (4, 21, 22). However, it appears that spermidine and spermine are actually more important physiological regulators of ODC activity than putrescine itself (17–19) and the physiological significance of antizyme remains uncertain.

A number of other mechanisms for the regulation of ODC activity have been proposed including a variety of posttranslational modifications [described in reviews (1–4, 23)] but none of these has been shown unequivocally to occur in vivo. There is little doubt that ODC can be phosphorylated, probably at the serine residue 303 (4, 24), but this phosphorylation has not yet been shown to lead to any significant change in the enzyme activity or stability.
Unlike ODC, which is a typical amino acid decarboxylase requiring pyridoxal phosphate as a cofactor, AdoMetDC contains a covalently bound pyruvate that functions as a prosthetic group (1–4, 14). It is synthesized as a precursor having an approximate molecular weight of 37,000 that is converted to the M₃2,000 enzyme subunit in a reaction which presumably generates the pyruvate group at the active site (25). It is possible that the small peptide released in this process remains associated with the enzyme as an additional subunit but this fragment has not yet been observed during analysis of the enzyme (14). AdoMetDC activity is elevated in response to a variety of growth-promoting stimuli including hormones and mitogens (2, 4, 14, 25–27). This increase is the result of an increased accumulation of the enzyme protein as measured by radioimmunoassay or Western blotting (27, 28). There is an increase in the mRNA for AdoMetDC under these conditions but this does not appear to be sufficient to account for the greater rate of enzyme synthesis; translational regulation may play an important role in this control (25–27). AdoMetDC is highly regulated by putrescine, spermidine, and spermine but, in this case, putrescine has effects quite different from those of the polyamines. Putrescine activates AdoMetDC directly (1–4, 14) and it also increases the rate of conversion of the M₃2,000 precursor to the enzyme subunit (27, 29). In these ways, a rise in putrescine levels increases the production of decarboxylated AdoMet, which can then be used by spermidine synthase to convert the putrescine into spermidine. The spermidine and spermine content of mammalian cells, which have this putrescine-activated AdoMetDC, is normally much higher than the content of the precursor putrescine (2, 4). In Escherichia coli, AdoMetDC is not regulated by putrescine, and the cellular content of putrescine usually exceeds that of spermidine (8).

Spermidine and spermine act to repress AdoMetDC (4, 14). There is a substantial increase in the amount of AdoMetDC protein when the polyamine content is reduced by application of inhibitors and AdoMetDC protein declines in response to exogenous polyamines (27). Part of this effect arises from an increased rate of degradation of the protein when polyamine levels rise but the major cause seems to be a change in the rate of synthesis (25, 27, 28). The content of mRNA for AdoMetDC appears to respond to the spermidine content but much of the effect of polyamines, particularly spermine, on the synthesis of AdoMetDC occurs at the translational level (25–30). When tested in reticulocyte lysates, AdoMetDC mRNA is much more sensitive than total mRNA or albumin mRNA to inhibition of translation by polyamines (29).

Spermidine/spermine N⁷-acetyltransferase activity can be induced by many fold by various toxic agents such as carbon tetra-chloride or by administration of spermidine or spermine, but it is unaffected by putrescine (4, 31). The increased acetyltransferase protein results from both an enhanced rate of synthesis and a decreased rate of degradation. A variety of polyamine analogues or derivatives also raise the content of this acetyltransferase in several tissues (4, 31, 32). The very large increases produced by some of these substances, including MGBG and \( N^7,N^{12}\)-bis(ethyl)sperrmine, appear to be due to the combined effects of an increased synthesis rate and a substantial inhibition of its degradation. Interference with the normal rate of degradation of this enzyme protein, which has a half-life of only 10–20 min, can lead to very large increases in the amount of enzyme within a few h. It appears that the binding of these polyamine analogues to the acetyltransferase stabilizes it against degradation (4, 31, 32).

Similarly, both ODC and AdoMetDC proteins are known to be degraded much more slowly when combined with competitive inhibitors such as \( \alpha \)-methylornithine and MGBG, respectively (1, 2, 14, 33, 34). These increases rapidly overcome the effects of the inhibitors. The short half-life of these enzymes and their repression by cellular polyamines therefore present a significant problem in the design of inhibitors which have sufficient potency and longevity to be useful drugs (1, 2, 4).

Direct Acting Inhibitors of Polyamine Biosynthesis

Inhibitors of Ornithine Decarboxylase (Fig. 2). Early studies identified a number of compounds as reversible inhibitors of ODC (1, 2, 35, 36). These fall into two classes: direct competitive inhibitors such as \( \alpha \)-methylornithine; and compounds reacting with the pyridoxal phosphate cofactor such as \( \alpha \)-hydroxynorornithine. Although these compounds did produce some reduction in putrescine production in cultured cells, they were not sufficiently potent or specific for most purposes. Their effects were overcome by the rapid turnover of ODC and the free exchange of the pyridoxal phosphate cofactor. It became clear that, because of the large compensatory increase in ODC when putrescine production is blocked, irreversible inhibitors of extremely high specificity and potency were required. Synthesis of such compounds, which is a major factor in the increased knowledge and interest in polyamines, was pioneered by Bey et al. and others who designed a series of enzyme-activated irreversible inhibitors (35, 37, 38).

Such inhibitors, often referred to as suicide inhibitors, \( k_{\text{cat}} \) inhibitors, or mechanism-based inhibitors, are chemically inert substrates for the enzyme which contain a group that is transformed into a reactive species as a result of their catalytic turnover. The active form of the inhibitors is therefore generated at the active site of the enzyme and reacts with the protein causing inactivation. Consideration of the mechanism of action of pyridoxal phosphate-dependent amino acid decarboxylases and the knowledge that \( \alpha \)-methylornithine did bind to the active site of the enzyme led to the synthesis of a series of compounds containing a leaving group on the \( \alpha \)-methyl substituent or an acetylene, ethylene, or aliene function in place of the \( \alpha \) hydrogen (35, 37, 38). Many of these compounds proved to be good inhibitors but most of the studies thus far carried out have utilized DFMO (eflornithine) (1–7, 37, 38).

DFMO (Fig. 2) does indeed act as an enzyme-activated irreversible inhibitor of ODC (35–37). Inhibition is time dependent showing pseudo first-order kinetics and shows saturation kinetics with increasing concentration of DFMO (\( K_i \sim 40 \mu M \)). Although the exact nature of the covalent adduct formed between DFMO and ODC has not yet been determined, studies
with labeled DFMO have shown that it was decarboxylated by ODC and that it bound stoichiometrically to the enzyme (4, 15, 39). The partition ratio of decarboxylation to inactivation was about 3.3. The binding of DFMO to ODC is highly specific and, even in crude extracts, titration with labeled DFMO provides a way to quantitate the number of molecules of the enzyme. The selective binding of labeled DFMO to ODC provides a useful way to localize the enzyme by autoradiography (4, 15).

Treatment of cells in culture or of whole animals with DFMO leads to a substantial fall in the content of cellular putrescine and spermidine (2, 4–7, 40–44). At the same time decarboxylated AdoMet levels rise tremendously (4, 14, 40, 41, 44). This increase is due to the increased level of AdoMet decarboxylase and the absence of putrescine and spermidine to act as amino-propyl acceptors. No other reactions except for acetylation are known to utilize significant quantities of decarboxylated AdoMet (4, 14) and the amount of this nucleoside and its acetylated derivative (44) therefore rise several hundred fold. In humans treated with DFMO, the increased excretion of decarboxylated AdoMet appears to be a useful way to evaluate the inhibition of ODC (45).

In contrast to the virtually complete reduction in putrescine and spermidine, spermine content is not much affected by application of DFMO (2, 4–7, 40–44). Several factors contribute to the lack of effect on spermine. (a) The inhibition of ODC activity by DFMO may not be complete. The half-life of the isolated enzyme in the presence of saturating amounts of DFMO is 3.1 min and the normal half-life of ODC in vivo is only about 20 min in many cells. Therefore, it is possible that a fraction of the cellular ODC may still be active. (b) l-Ornithine protects the enzyme from inactivation by competing for binding to the active site and this may also reduce the extent to which the enzyme is inhibited. (c) The existing putrescine and spermidine are efficiently converted into spermine in the presence of the excess decarboxylated AdoMet. (d) The inhibition of cell division and the lack of degradation of spermine results in the maintenance of the cellular spermine content when this is expressed on a per cell basis.

Application of DFMO to a wide variety of both normal and neoplastic cells in culture leads to striking inhibition of cell proliferation (2, 4–7, 40–43). The onset of growth inhibition requires a lag period during which a limited amount of cell replication helps to bring about the depletion of existing cellular putrescine and spermidine. Treatment with DFMO also has striking effects on differentiation and development in a number of systems (2, 46–49) but the effects are complex involving inhibition in some cases and stimulation in others. Treatment with DFMO may affect differentiation as a secondary consequence of the effects on cell growth but in some systems there is clear evidence for a distinct requirement for polyamines for differentiation (46–49). Inhibition of polyamine production can also induce terminal differentiation by interference with cell multiplication but again a specific role for polyamines is also by no means ruled out. The striking inhibition of embryonic development at the gastrulation stage brought about by application of DFMO should be emphasized in the light of the generally low toxicity of this drug (47–49). Administration of DFMO and other inhibitors of ODC to mice, rats, hamsters, or rabbits at the time of embryonic development corresponding to a rise in decidual ODC activity (immediately after implantation) leads to a complete inhibition of fetal progression (47, 49). This contragestational effect may have some potential for fertility control in animals since the effects are highly time dependent and fertility is restored at the next ovarian cycle, but, more importantly, they emphasize the possibility of severe toxicity if DFMO or other ODC inhibitors are administered to pregnant mammals.

The growth inhibition and the changes in differentiation and development after DFMO treatment can often be overcome by the provision of exogenous putrescine or polyamines. This indicates the specificity of the inhibitor and that its effects are due to interference with the polyamine-biosynthetic pathway. It is not ruled out that, in some cases, the rise in decarboxylated AdoMet and of acetylated AdoMet may also play a role in the toxicity of DFMO since these changes are also ablated by addition of putrescine or polyamines. However, as described below, the blocking of polyamine synthesis by inhibitors of AdoMet decarboxylase also leads to growth reduction that can be reversed by the addition of spermidine.

In most cells thus far examined, the effects of DFMO are cytostatic and can be reversed by the addition of putrescine even after an extended period in the presence of the inhibitor (2, 4–7, 40–43). However, some studies with small cell lung carcinoma cells revealed that these cells could not survive the chronic inhibition of ODC by DFMO (41, 50) and subsequent experiments have indicated a similar cytotoxicity towards several other tumor cell lines (41, 51–53). These results may be due to differences in both the cell type examined and the culture conditions (52, 53). Lung tumor cells grown as anchorage-dependent monolayers do not show the cytotoxicity in response to DFMO, whereas this effect is seen when they are grown as anchorage-independent spheroid aggregates (53).

The striking inhibition of cell proliferation seen in cell culture in response to DFMO led to a number of studies of the effects of DFMO on the growth of experimental tumors in vivo. Significant effects were obtained against a variety of chemically induced or implanted rodent tumors (5, 6, 38, 41) and human tumors (including small cell lung carcinoma) carried in nude mice (54–56). An impressive inhibition of tumor metastases was observed by Sunkara et al. (41, 57) and has been confirmed by others (41, 58). Continued study of the value of polyamine biosynthesis inhibitors in the prevention and control of metastases is clearly warranted.

In all of the experiments with DFMO as an antitumor agent it was necessary to use quite large doses and frequent doses because it has a rapid clearance in rodents [plasma half-life of about 80 min (40)]. However, the drug was not particularly toxic and can be given in the drinking water at levels of 1–3% so it was quite feasible to give the large doses needed to maintain inhibition of ODC.

Despite these promising results in some experimental models, clinical trials of DFMO as a single agent have not shown any lasting antitumor effects in humans although some cases of stabilization of the disease and a few apparent responses did occur (38, 59). The patients treated without significant effect included a group with lung small-cell carcinoma (60) that is particularly sensitive to DFMO in vitro and as xenografts in nude mice (50, 54). The reasons for the lack of activity of DFMO in vivo are not clear. Pharmacokinetic evaluations indicated that the plasma half-life of p.o. DFMO in humans was actually longer (around 200 min) than in rodents and that plasma levels equivalent to those in mice drinking 1% DFMO could be maintained in humans (38, 59). It is possible that the depletion of polyamines in the tumors was not sufficient for a major response because of the availability of exogenous polyamines in the diet (see below) and to the compensatory increases in other enzymes in the biosynthetic pathway. Another factor
that may come into play is the difference between human tumors, which at the time of diagnosis may frequently have already passed their most rapid growth phase, and the animal models. On the positive side, DFMO was quite well tolerated in the clinical trials (particularly when given i.v.) and the adverse effects of very high doses which include gastrointestinal effects, thrombocytopenia, anemia, and a bilateral hearing loss were generally reversible on reducing or discontinuing the treatment and were not life threatening (59–62). The possibility remains that under other treatment regimens, particularly in combination with other polyamine biosynthesis inhibitors or when combined with other therapies, DFMO will be a useful human antitumor agent. DFMO is unquestionably of great value in the treatment of certain protozoan diseases such as African trypanosomiasis and Pneumocystis carinii pneumonia (38, 59, 63). Much clinical experience with DFMO will be gained from its use in these conditions and may be useful in designating effective antitumor protocols.

It has been hypothesized that the lack of cytotoxicity of DFMO is due to the residual spermine content in the affected cells (2, 4–6, 40–43). Although this remains speculative, there is some experimental support for this idea. Mutant mammalian cells unable to synthesize polyamines lose viability quite rapidly if maintained in a polyamine-free environment until intracellular spermine is lost (64–67). Some preliminary evidence suggests that the combination of DFMO with inhibitors of other steps in polyamine production, or other maneuvers leading to increased spermine depletion, may be associated with an increase in cytotoxicity. It remains to be seen if increasing the extent of polyamine depletion and thus enhancing cytotoxicity will have any advantage in cancer chemotherapy but this possibility merits further attention if protocols can be devised to rescue or minimize the damage to nonneoplastic cells.

It is possible that one problem with the use of DFMO against malignancy is that it is not sufficiently active in blocking ODC activity in vivo. It suffers from the disadvantages that it is not taken up into cells particularly well (4, 36) and the \( K_f \) for ODC of about 40 \( \mu \)M (35, 38) is quite high, particularly in view of the fact that endogenous ornithine will compete with the inhibitor. A number of the other enzyme-activated irreversible inhibitors which have been described may represent improvements in these respects (35, 38). The introduction of an unsaturated function into the ornithine molecule favors a stretched conformation which seems needed for binding to the active site, and such unsaturation greatly increased the affinity of the resulting \( \alpha \)-fluoromethylornithine for the enzyme. The resulting \( \alpha \)-fluoromethyldehydroornithine has a \( K_f \) for ODC of 2.7 \( \mu \)M but was found to penetrate into cells even more poorly than DFMO (35, 38). However, this penetration can be facilitated by the synthesis of the methyl ester (Fig. 2). This penetrates readily through membranes and is hydrolyzed rapidly by esterases once inside the cell (68).

Another approach has been to make putrescine analogues containing the same groupings that were effective inhibitors as ornithine analogues (35, 38). Because of the microscopic reversibility principle, these product analogues are also enzyme-activated irreversible inhibitors (35). The most promising analogue found initially was \( \alpha \)-ethylidenputrescine but this compound was not useful in vivo because it is a substrate for monoamine oxidase. The oxidation can be prevented by adding a methyl group to the \( \delta \)-carbon atom. This addition leads to the presence of four isomers only one of which would be expected to be active and this was the case (35). The resulting \( R \)-\( \alpha \)-ethyliden-\( R \)-\( \delta \)-methylputrescine [also referred to as (2R,5R)-\( \delta \)-methylacetylenic putrescine or (2R,5R)-6-heptyne-2,5-diamine] has a \( K_f \) for ODC of 3 \( \mu \)M and a rate constant for inactivation such that the half-life for loss of activity was 1.7 min. MAP (Fig. 2) does not appear to enter cells by the polyamine transport system (42, 43) but it passed through the membrane sufficiently well to give a substantial inhibition of polyamine synthesis in HTC cells when added to the culture medium at only 1 \( \mu \)M (42). At 100 \( \mu \)M, MAP produced complete depletion of putrescine and spermidine (43, 44). In some (42, 43) but not all (69) experiments, treatment with MAP also reduced the spermine content in HTC, L5178Y, and L1210 cells. MAP can be modified from cells much more readily than DFMO by replacing the medium which also implies that it may pass more readily through the cell membrane (69).

Treatment with MAP or the methyl ester of \( \alpha \)-fluoromethyldehydroornithine produced inhibition of ODC in rat tissues at much lower doses than DFMO and the effects of the methyl ester were considerably more long lived (40). Both compounds were also more potent than DFMO in reducing the growth of transplanted tumors in animal models (38, 41, 70). It remains to be seen whether this improvement will lead to a more significant response in human malignancies.

Inhibition of S-Adenosylmethionine Decarboxylase (Fig. 3). In 1972, Williams-Ashman and Scherone (71) made an important finding that MGBG was a powerful inhibitor of AdoMetDC. A comprehensive review of work with MGBG is given in Ref. 33. MGBG is known to be an antileukemic agent and has been reported to have some activity against some solid tumors but its clinical use has been limited by its severe toxicity (5, 33, 34, 72, 73). The possible interaction of MGBG with the polyamine metabolism had been postulated earlier on the basis of its structure and studies showing that it shared a common transport system with polyamines (33). Much subsequent work has confirmed and extended the finding that MGBG inhibits AdoMetDC and many studies have been published in which this compound has been used as an inhibitor of polyamine metabolism (1–7, 14, 33, 72, 73). Exposure to MGBG does lead to an increase in putrescine and a decrease in polyamines and to a profound inhibition of growth and viability which in some cases was reversible by administration of spermidine. However, these studies must be interpreted with great care because MGBG is by no means specific. MGBG is also a potent inhibitor of diamine oxidase and a strong inducer of spermidine/spermine \( \beta \)-acyetyltransferase (31–33, 73–75). These factors contribute to the rise in putrescine brought about by

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**Fig. 3. Inhibitors of S-adenosylmethionine decarboxylase.** The inhibitors shown are: (1) 5′-deoxy-5′-N-Methyl-N-[2-(aminoxy)ethyl]laminoadenosine (MAOA); (2) 5′-deoxy-5′-[N-methyl-N-(3-hydrazinopropyl)]laminoadenosine (M2ZPA); (3) 5′-deoxy-5′-[(S)-methylthioethylhydroxylamine; and (4) methylglyoxal bis(guanylhydrazone) (MGBG).
MGBG and the induction of the acetylase/oxidase pathway may also contribute to the fall in polyamines. The blockade of AdoMetDC activity by MGBG in vivo may be transitory since MGBG greatly stabilizes the enzyme leading to an enormous increase in its amount (14, 28, 33). MGBG accumulates to very high levels in mammalian cells, causes severe damage to mitochondria, and has indirect effects on macromolecular synthesis including DNA replication (1, 2, 5, 6, 33, 73). These changes may be prevented by administration of polyamines but this could, perhaps, be due to the displacement of MGBG from intracellular binding sites rather than to the depletion of polyamines. More probably, the reversal of the effects of MGBG by spermidine may be brought about by the reduction in the intracellular MGBG content since MGBG and spermidine compete for the same transport system (5, 6, 33, 72, 73). On balance, it appears that the antitumor effects of MGBG are not mainly due to the inhibition of AdoMetDC.

However, more potent and specific inhibitors of AdoMetDC could have considerable potential for use in studies of polyamine function and as antineoplastic agents. A considerable number of MGBG congeners are known, some of which such as ethylyglyoxal bis(guanhydrizone) are more active as inhibitors of AdoMetDC (1, 14, 33, 72–74). These compounds may, at low doses, inhibit AdoMetDC without some of the side effects of MGBG but they are also not likely to be totally specific and are known to inhibit diamine oxidase (73, 74) and induce the acetyltransferase (31). Various other compounds which inhibit AdoMetDC have been described (reviewed in Refs. 14, 34, and 36) but the only promising candidates at present are some nucleotide analogues of AdoMet which contain reactive hydrazo or aminoxy derivatives (76–78). These compounds presumably bind to the active site of the enzyme and then react with the pyruvate-prosthetic group. Since the pyruvate is covalently linked to the enzyme, this reaction leads to permanent loss of activity. Artamnova et al. (76) have reported that S-(5′-deoxy-5′-adenosyl)methylthioethylhydroxylamine (Fig. 3) is an irreversible inhibitor of AdoMetDC, and Secrist (77) has synthesized a number of irreversible inhibitors including MAOEAm and MZHZA (Fig. 3). These compounds have been found to inhibit polyamine synthesis in L1210 cells leading to a massive accumulation of putrescine and a reduction of spermidine and spermine, decarboxylated AdoMet, and 5′-methylthioadenosine (78). All of these changes are consistent with blocking the polyamine biosynthesis pathway at the AdoMetDC step. Cell proliferation was inhibited once spermidine and spermine became depleted and could be restored by the addition of spermidine (78). These results show that putrescine cannot substitute for the function of polyamines essential for growth of L1210 cells. When DFMO and MAOEAm were given together, there was no detectable increase in the total putrescine and polyamine content of the culture for 48 h (78).

It appears unlikely that these nucleoside inhibitors of AdoMetDC will have sufficient stability in vivo for a prolonged period of inhibition to be achieved readily. However, their interesting effects in cell culture indicate the need for other specific inhibitors which would have better stability. One interesting possibility for the design of pharmacological agents which interfere with polyamine production would be to manipulate compounds which would block the synthesis of AdoMetDC at the processing steps which generate the pyruvate at the active site and convert the M37,000 precursor to the M32,000 enzyme subunit (25, 27, 29). This reaction can now be studied in vitro using mRNA copied from plasmids containing cDNA inserts in reticulocyte lysate translation systems (29) and the sequence of the precursor is available from the cDNA.4

These tools should enable the mechanism of this reaction to be established thus allowing the design of inhibitors. They also provide an in vitro system in which the putative inhibitors could be evaluated.

Inhibitors of Aminopropyltransferases (Fig. 4). The aminopropyltransferases appear to be present in excess and to be regulated by the availability of their substrates. This has led to a concentration of the efforts in the production of inhibitors of polyamine biosynthesis toward the decarboxylases, but there are some compelling reasons to attempt to produce inhibitors of spermidine and spermine synthases. The aminopropyltransferases do not turn over rapidly and do not show the extent of regulation that leads to the large compensatory increases in the amounts of the decarboxylases when polyamines are depleted. Also, as described above, it is difficult to achieve substantial depletion of spermine by the use of ODC inhibitors. The availability of reagents selectively blocking the synthesis of spermidine or spermine would enable the function of these polyamines to be studied independently of the regulation of ODC and AdoMetDC.

Inhibitors available at present are the multisubstrate adduct inhibitors synthesized by Coward (reviewed in Ref. 79). It was found that AdoDato (Fig. 4) was a very powerful inhibitor of spermidine synthase. More recently the synthesis has been accomplished of the analogous multisubstrate adduct AdoDada (Fig. 4) which was designed to inhibit spermine synthase. Preliminary studies confirm that this is the case (79). Both AdoDato and AdoDada are taken up by tumor cells in culture. AdoDato leads to the depletion of spermidine but to an increase in spermine (80). AdoDada leads to an increase in spermidine and a decrease in spermine.5 These results support the concept discussed above that the two aminopropyltransferases compete for the available decarboxylated AdoMet. Both compounds also caused an increase in the production of decarboxylated AdoMet because of the derepression of AdoMetDC. This increase tends to overcome the inhibition and the combination of these compounds with an AdoMetDC inhibitor such as MZHZA may prove to be a useful approach.

Fig. 4. Inhibitors of aminopropyltransferases. The inhibitors shown are: (1) S-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDada); (2) S-methyl-5′-methylthioadenosine [AdoS′(CH3)3]; (3) S-adenosyl-1,8-diamino-3-thiooctane (AdoDato) and (4) cyclohexylamine.

4 O. Jänne, A. Pajunen, B. Stanley, and A. E. Pegg, unpublished observations.
5 A. E. Pegg and J. K. Coward, unpublished observations.
The activity of spermine synthase is also strongly inhibited by AdoS'CH₂₂ (Fig. 4) (81, 82). This compound is somewhat less specific than AdoDadat since at higher concentrations it also inhibits AdoMetDC, but it is much easier to synthesize and this additional inhibition may be of little practical importance. Treatment of SV-3T3 cells with AdoS'CH₂₂ led to a large depletion of spermine but had no effect on cell growth (81). Preliminary results with AdoDadat give the same result. These experiments suggest that spermine synthesis is not necessary for a normal growth rate in these cells provided that a compensatory increase in spermidine occurs. It is possible that this will not prove to be the case in other cell types but the finding raises the interesting question as to why the SV-3T3 cells make spermine at all and retain spermine synthase to carry out this reaction.

Various other substances having some inhibitory action on the aminopropyltransferases have been identified, but only a few potent compounds are known (34). These include a natural product antibiotic (83). In view of the wide role that polyamines play in cell physiology, further search for natural product inhibitors may prove rewarding.

Cyclohexylamine [often incorrectly designated as dicyclohexylamine (4)] is an inhibitor of spermidine synthase, which does cause a reduction in spermidine levels in cultured cells (84-86) and has some effect on growth of tumors in rodents (84). It has the advantage of being inexpensive and readily available and gave results similar to those for AdoDadat in HTC cells (86) but a full study of its specificity has not been carried out. Recently, a derivative described as N-chlorosulfonyldicyclohexylamine has been synthesized and is a more potent inhibitor which reduces spermidine levels and retards the proliferation of human leukemia cells (87).

Transport of Polyamines

A wide range of mammalian cells contain uptake pathways for polyamines (5, 15, 72, 88-92) but these systems, which may be Na⁺ dependent in some cases but not in others, have not been fully characterized at the biochemical level. It is probable that there are multiple transport systems (91). The physiological utility of polyamine transport is not entirely clear since the polyamine-biosynthetic enzymes are ubiquitous and extracellular polyamine levels are usually very low. However, its potential significance is emphasized by the demonstration that the uptake is greatly stimulated in cells in which the synthesis of polyamines is prevented by DFMO (6, 72, 88). Furthermore, this transport system is clearly critical for the growth of cells in which polyamine biosynthesis is blocked. Studies with Chinese hamster ovary cells, in which the transport system had been inactivated by mutation (89), revealed that these cells could not be stimulated to grow in the presence of DFMO by either putrescine or spermidine at concentrations of up to 0.5 mm. The presence of the transport system may be a significant factor in ameliorating the effects of polyamine biosynthesis inhibitors given in vivo since substantial amounts of polyamines may be present in the diet.

Although the polyamine uptake system does not act on amino acids, it is not strictly specific for the naturally occurring di- and polyamines. A wide variety of analogues of these compounds are known to be transported by this system including MGBG (5, 6, 72, 89, 92-94). It appears that the uptake of the poisonous herbicide parquat occurs by means of a polyamine transporter (91). Since parquat is accumulated to high levels only in certain cell types (probably alveolar type II cells and Clara cells in the lung) it may be that these cells have a particularly active polyamine uptake system and would be particularly suitable for its purification. The powerful cytotoxic action of MGBG and its uptake via the polyamine transport system provide a convenient selection method for the isolation of mutants deficient in such transport (89) but it should be noted that not all MGBG-resistant mutants result from alterations in membrane transport (95). Changes in intracellular binding sites for MGBG were thought to be responsible for the reduced accumulation of the drug and resistance to it in some variant human cell lines (95), and the retention of drug rather than the rate of uptake may be responsible for differences in the intracellular accumulation of different MGBG congeners (5, 6, 72-74).

Additional evidence that the polyamine transport system may have physiological importance comes from studies in which the activity of this system has been shown to increase during hormonal stimulation and proliferation and to decrease during differentiation (reviewed in Ref. 90). Conversely, when cell proliferation is restricted by confluence, lack of growth factors or inhibitors, there occurs a substantial efflux of polyamines, particularly spermidine, from the cell (1-6). Studies with a variant DFMO-resistant L1210 cell line which contains very high levels of ODC and therefore produces very large amounts of putrescine indicate that a major fraction of this putrescine is excreted into the medium (96). It is clear that uptake and efflux are important ways in which intracellular polyamine levels can be controlled and require more detailed investigation at the biochemical level.

Further study of the regulation of the transport system and the development of specific transport inhibitors would therefore be a valid approach to complement the work on drugs blocking polyamine biosynthesis. Appropriate experimental systems are now available for the more detailed characterization of the polyamine transporters using MGBG as a nonmetabolized substrate, DFMO pretreatment to increase their activity, and the comparisons of cell types with high transport capacity to the mutants which lack the transport system. Isolation of the gene for the transport proteins may be possible by transfection of genomic DNA fragments from control cells into the mutant cells followed by selection for growth in medium containing DFMO and putrescine or spermidine.

Indirect Inhibitors and Analogues of Polyamines

A number of interesting studies have been carried out with a variety of polyamine analogues and derivatives. There are several underlying ideas behind the design of these experiments. Firstly, the analogues may be taken up into the cell by the polyamine transport system and such uptake may be enhanced by polyamine depletion. The ability of the polyamine transport system to facilitate the entry into L1210 leukemia cells of polyamine analogues has been demonstrated by Porter and colleagues (5, 92) who suggest that this property could be used to design anticancer agents. They have shown that a wide variety of N⁺ substituents on the spermidine molecule do not affect uptake. Therefore, this would be a good region in which substituents with anticancer potential might be placed (92, 97). In this way, the efficient uptake of derivatives with cytotoxic activity may be achieved. It is also possible that the use of such analogues with a chemically reactive group at this position might be used to interact specifically with the carrier protein.
Another interesting example of this approach is the work of Heston et al. (98) who synthesized monoaziridinylputrescine. This drug was found to enter prostatic cancer cells using the putrescine carrier and its cytotoxicity was enhanced by prior exposure to DFMO.

Secondly, some analogues may fulfill some but not all of the functions of polyamines. Such analogues may bind to the same sites as natural polyamines and prevent their normal function by displacing them from these sites. Alternatively, these analogues might release bound polyamines and thus allow a transient period of cell division in the presence of an ODC inhibitor which depletes the natural polyamines to a greater extent than can be achieved with the inhibitor alone. For example, Casero et al. (99) found that the use of DFMO plus a spermidine analogue such as aminopropylcadaverine or N2-methylspermidine led to a much greater depletion of spermine in L1210 cells than DFMO alone. McGovern et al. (100) confirmed an earlier report that 1,3,6-triaminohexane and 1,4,7-triaminoheptane could reverse the inhibition of cell growth by DFMO (101) but it was found that this effect was transient and that growth ceased when the cellular content of spermine was depleted to values below 20% of control. It may be possible to use this approach to reduce the normal intracellular content of polyamines to a point where cell death occurs when the analogue is withdrawn.

Thirdly, some analogues may act as polyamines with respect to their regulatory functions in the polyamine-biosynthetic pathway and thus repress the biosynthetic enzymes and induce the acetylation/oxidase pathway leading to the depletion of cellular polyamines. Provided that compounds can be found which have this function and lack the ability to mimic the function(s) which are needed for growth, these may prove to be very effective antimitotics. A particular advantage of such a strategy is that both ODC and AdoMetDC may be affected and that the compensatory increase in their activity which occurs when polyamines become reduced may not occur.

Porter and Bergeron and colleagues have obtained evidence that bis(ethyl) derivatives of spermidine and spermine may have these desirable properties (5, 97, 102, 103). Treatment of L1210 cells with N6,N8-bis(ethyl)spermidine led to a rapid fall in the activity of ODC and a complete decline in putrescine and spermidine. Spermine levels were also depleted by 40–50%. These changes were accompanied by an inhibition of cell proliferation similar to that produced by DFMO. However, N6,N8-bis(ethyl)spermidine had the advantages that it was active at lower concentrations, produced significant reduction of spermine, did not lead to an increased cellular uptake capacity for polyamines, and did not increase AdoMetDC activity (102). It also acts as an inducer of the acetylation/oxidase systems (31) and this may also contribute to the decline in polyamine levels. Further studies of the effects of N6,N8-bis(ethyl)spermidine have shown that it does inhibit the synthesis of ODC at the translational level (104) and also inhibits the synthesis of AdoMetDC in reticulocyte lysates. The lack of change of AdoMetDC at a time when polyamines are depleted implies that the compound is substituting for the polyamines in regulating this enzyme. Despite the inhibition of cell growth, N6,N8-bis(ethyl) spermidine was not cytotoxic towards L1210 cells (102) but it has since been found to be very effective in the reduction of ODC and of putrescine and polyamine levels in human large cell lung cancer cells (105). In these cells (NCI H157), which had been found to be quite resistant to DFMO, N6,N8-bis(ethyl)spermidine was cytotoxic at concentrations of 5 μM or higher (105).

More recently, N6,N12-bis(ethyl)spermine has been found to be even more active than the spermidine derivative in reducing the synthesis of putrescine and polyamines in L1210 cells (103). In the presence of 10 μM N6,N12-bis(ethyl)spermine, the activities of both ODC and AdoMetDC were greatly reduced. Within 48 h, all putrescine and spermidine and about three-fourths of the spermine had been depleted. As expected, the N6,N12-bis(ethyl)spermine was strongly inhibitory to growth of these cells. The greater potency of the N6,N12-bis(ethyl)spermine derivative compared to its spermidine equivalent is consistent with the fact that spermine is more active than spermidine in blocking the translation of the mRNA for ODC and AdoMetDC (19). This is particularly the case for AdoMetDC, and N6,N12-bis(ethyl)spermine was much more effective than N6,N8-bis(ethyl)spermidine in the inhibition of the synthesis of AdoMetDC in reticulocyte lysates. The additional reduction in AdoMetDC is therefore likely to be the reason for the enhanced depletion of spermine.

These bis(ethyl) analogues therefore have considerable potential and further work including preliminary clinical trials are clearly warranted. However, it should be noted that their effects may differ greatly from one cell type to another depending on the requirement of the cells for polyamines. Since the analogues clearly substitute well for the natural polyamines in the regulation of ODC and AdoMetDC, it is quite possible that they may also substitute for other functions including those needed for the growth and differentiation of some cells. Furthermore, the specificity of their effects towards the polyamine-biosynthetic pathway alone has not been fully investigated. Since they are transported by the same system as the polyamines, it is not possible to confirm the specificity of the effects by adding exogenous polyamines.

**Combinations of Inhibitors**

**Combinations of Inhibitors of Polyamine Biosynthesis.** The combination of DFMO with AdoDato (80) or with MAOEA (78) leads to increased polyamine depletion in short-term studies. Further tests of such combinations, which will be feasible when larger quantities of these drugs become available, should indicate whether the increased polyamine depletion reproductively leads to a greater reduction in cell growth and to a significant loss of viability.

**MGBG and DFMO.** The combination of MGBG and DFMO has been examined in cultured cells, in animal tumor models, and in clinical trials (5, 6, 56, 59, 72). There are two reasons for testing this combination. One rationale is the obvious use of inhibitors of two steps in the polyamine-biosynthetic pathway. The second is that the uptake of MGBG occurs via the polyamine transport system and the activity of this system is therefore likely to be the reason for the enhanced depletion of spermine.

Some promising results were obtained in cell culture and in animal tumor models and some indications of clinical utility have been published, both the presently available clinical evidence and the results of the animal tests suggest that this combination must be approached circumspectly (59, 72). There are two major problems. One is that the earlier findings indicating that there may be some selective enhancement by DFMO of the uptake of MGBG into tumor cells (5, 88, 93) do not appear to be generally applicable (94). The toxicity of MGBG, which is already considerable, was enhanced by DFMO in a

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*A. E. Pegg, T. Kameji, R. Bergeron, and R. Madhubala. Effects of polyamine analogs on growth, ornithine decarboxylase and S-adenosylmethionine decarboxylase in L1210 cells resistant to α-fluoromethylnitrosourea, manuscript in preparation.*

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number of clinical studies (59). Secondly, in some cases, MGBG actually antagonizes the antitumor effects and depletion of polyamines by DFMO (72, 75, 106–108). This paradoxical finding is probably due to the inhibition of diamine oxidase in the tumor-bearing animal which increases the availability of putrescine to support the tumor growth in the presence of DFMO (75, 108). In fact, as discussed by Porter and Jänne (72), there is little evidence that the combination of MGBG and DFMO led to greater polyamine depletion than with either drug alone. Any enhancement of the antitumor activity of MGBG obtained in these studies by combination with DFMO appears to result from the increased uptake of the MGBG.

Nevertheless, there is some reason for continued investigation of this combination under carefully controlled conditions in which the dose schedule is adjusted to maintain acceptable levels of toxicity. Levin et al. (61) have found some beneficial effect of MGBG plus DFMO on recurrent primary brain tumors, particularly slow growing anaplastic gliomas.

Combinations with Other Drugs Not Directly Related to Polyamine Metabolism. In view of the lack of toxicity of DFMO and the evidence that it retards the growth of tumor cells, it is natural that attempts should be made to combine this inhibitor of polyamine biosynthesis with disparate drugs that do not affect polyamine dynamics directly. Some of these studies have quite well developed rationales. Others appear to be more based on the hope of useful interactions than any particular theme.

A particularly interesting combination of DFMO and certain anticancer agents has been suggested by the work of Marton, Seidenfeld, and colleagues who found that depletion of polyamines by DFMO potentiated cell killing by DNA-alkylating and cross-linking agents such as BCNU (52, 109–112). Similar results were obtained with other agents which give rise to lethal cross-links in DNA, including chlorozotocin, 1-(2-chloroethyl)nitrosourea, and 1-(2-chloroethyl)-3-trans-4-methylcyclohexyl-1-nitrosourea (52, 109–112). This potentiation, which occurs not only in the rat brain tumor cell line 9L (109) but also in L1210 cells and a variety of human carcinoma cells (111–113), may arise from the increased accessibility of the DNA when spermidine and putrescine are lowered. DNA damage and cross-link formation by BCNU was found to be enhanced by prior exposure to DFMO in some experiments in 9L cells (111, 114) but this does not appear to be the case in human colon tumor cells even though they show the synergistic response to BCNU and DFMO (115). These results suggest that mechanisms other than increased DNA interstrand cross-link formation may be responsible for the increased toxicity of BCNU and 1-(2-chloroethyl)nitrosourea in polyamine-depleted cells. Irrespective of the mechanism of action, it is possible that even greater effects could be achieved if agents which also lower spermine are used; similar studies with MHZPA, AdoDatad, and AdoS+(CH3)2 would be of much interest.

It should be stressed that depletion of polyamines does not necessarily increase DNA damage and cytotoxicity of drugs that interact with DNA. In 9L cells, pretreatment with DFMO actually reduced cross-linking and cytotoxicity of cis-platinum and reduced the toxicity of aziridinylbenzoxquinone (72, 111, 114). Other workers have observed increased killing of other cells when cis-platinum was combined with DFMO (72, 115, 117). (The temporal relationship may also be critical here since those studies showing reduction of toxicity were carried out using cells pretreated with DFMO whereas the increased toxicity has been seen in studies in which the two drugs are given together.) DFMO pretreatment protected against the toxicity of chlorambucil (111, 118). No effect on the response to N-methyl-N-nitrosourea, bleomycin, or N,N',N''-triethylenethio-phosphoramidate was observed (111, 118).

A number of workers have attempted to enhance the toxicity towards tumor cells of so-called S-phase cell cycle-specific agents by applying these compounds after exposure to MGBG or DFMO. This approach was partly based on some studies suggesting that nonneoplastic cells failed to enter S phase when treated with polyamine biosynthesis inhibitors whereas tumor cell lines accumulated in S phase (2, 41, 111). Although some success in obtaining selective killing of tumor cells in culture has been reported by this approach (summarized in Ref. 41), it does not seem to be generally applicable. The effects of DFMO on cell cycle traverse are not straightforward. They depend on the cell type and some tumor lines examined did not appear to be arrested at any particular phase (111, 119). Although the combination of ara-C and DFMO increased the survival time of mice inoculated with L1210 cells, DFMO pretreatment actually reduced the toxicity of ara-C in 9L cells (41, 72). It is apparent that the effects of DFMO and polyamine depletion on the antiproliferative effects of known anticancer agents depend both on the protocols and on the tumor cells examined. Although potentiation of the effects of 5-fluorouracil, Adriamycin, vindesine, mitomycin C, l-phenylalanine mustard, and ara-C have been reported (41, 56, 72, 107, 113, 116), opposing effects have been seen in other experiments with different cell types (41, 72, 120).

There does appear to be some potential for the use of DFMO to inhibit tumor development in between doses of a cytotoxic anticancer agent (110). However, much care will have to go into the scheduling of such treatments to ensure that the inhibition of cell growth is not deleterious to normal cells. Highly relevant to this is the observation that, although DFMO can inhibit intestinal tumor growth without drastically damaging the normal cells in the intestinal mucosa (55), DFMO treatment retards the recovery of the intestinal mucosa after damage by ara-C (121). The combination therapy may therefore have much more serious toxic effects.

The combination of DFMO with interferon was reported to be effective against the growth of s.c. B16 melanomas in mice (122). Further studies have confirmed this effect and indicated an apparent synergism between these agents in a variety of animal and human tumors grown in vitro or as transplants in nude mice (41, 72, 123, 124). At present the mechanism by which this combination is active is unknown. Clinical trials indicated some positive responses to DFMO plus interferon in phase I studies of patients with malignant melanoma and there were indications of activity in patients having colon or renal carcinomas (59, 62).

Another possible approach to the treatment of melanomas is suggested by the finding that exposure of B16 melanoma cells to DFMO leads to an increased tyrosinase activity (125). The catalytic action of tyrosinase converts 3,4-dihydroxybenzylamine to a cytotoxic quinone and combination of this drug with DFMO potentiated their cytotoxic effects (125).

The combination of DFMO and cyclosporin A inhibited the survival of H2T hamster pancreatic cells to a greater extent than either agent alone (126). The content of polyamines in the pancreas is among the highest of any tissue and inhibition of polyamine metabolism is a strategy which should be given more consideration in the therapy of pancreatic cancer.

Finally, Chinese hamster ovary cells in which polyamines have been depleted after exposure to DFMO become more sensitive to heat treatment (127–129). The sensitization requires a period after polyamine depletion before it becomes...
apparent, suggesting that some biochemical alteration in response to polyamine depletion mediates the effect. Paradoxically, cells can also be sensitized to hyperthermia by increasing the extracellular concentrations of polyamines (127).

Resistance to Polyamine Biosynthesis Inhibitors

Isolation of a mouse lymphoma cell line resistant to the antiproliferative effects of DFMO following chronic exposure to increasing concentrations of the drug was first reported by McConlogue and Coffino (130). Subsequently, similarly resistant cells have been obtained in a number of other laboratories using similar protocols (4, 15, 96, 131, 132). This resistance appears to be mediated by the presence of elevated levels of ODC. In most cases thus far examined, the increase in ODC protein levels was due to an increased rate of synthesis. In some cases, this overproduction was produced by a massive increase in ODC mRNA which in turn was due to amplification of the ODC gene. Indeed, ODC gene amplification in response to chronic exposure to DFMO seems to occur remarkably readily. However, other examples in which there was an increase in the translation efficiency of the ODC mRNA or an increased rate of transcription of the gene have also been reported (15, 133). Another pathway to resistance appears to be an enhanced stability of the ODC protein which leads to an increased protein content (2, 134, 135). It is not yet known whether the underlying cause of the reduced rate of degradation resides in the ODC protein itself or to a change in the biochemical system responsible for its turnover. Overproduction of arginase leading to a high intracellular content of ornithine also appears to impart resistance to DFMO in human myeloma cell lines (136).

Although all of the cases of resistance to ODC inhibitors which have been described thus far occur in cultured cell lines, it seems quite likely that the phenomenon of resistance will appear in tumors in vivo particularly in view of the ease with which gene amplification occurs. However, one way in which this may be dealt with would be to use either the AdoMetDC inhibitors or the substances repressing ODC and AdoMetDC described above. Experiments with a resistant L1210 cell line, which grew at a normal rate in the presence of 20 μM DFMO and contained ODC levels several hundred fold higher than the original L1210 cells, showed that it was sensitive to growth inhibition by either MHZPA (96) or N4,N6-bis(ethyl)spermine.2 It therefore seems unlikely that the development of such resistant cells will prove to be a serious problem in the therapeutic use of polyamine biosynthesis inhibitors although the DFMO-resistant cell lines have proved to be extremely useful for studies of polyamine metabolism and of the molecular biology of ODC (4, 15, 130). However, the possibility that under strong selective pressures cells may arise which do not require polyamines for growth cannot be ruled out, and this situation may prove to be more difficult to handle. There is one preliminary report that both rodent and human tumor cell lines which could grow with very low intracellular polyamine levels and were resistant to DFMO were obtained after cycloheximide treatment (137).

Metabolism of 5′-Methylthioadenosine

The aminopropyltransferases produce MTA in stoichiometric amounts to the aminopropyl groups transferred. MTA (Fig. 5) is therefore formed in amounts equal to those of the aminopropyl groups in spermidine and spermine but in most cells it does not accumulate in more than trace amounts because of its rapid degradation to adenine and 5-methylthioribose 1-phosphate in a reaction catalyzed by MTA phosphatase. The kinetics of this reaction have been studied (4, 2.4.2.28) (138, 139). Some tumor cells lack MTA phosphatase and fail to degrade MTA which is excreted into the extracellular environment (127). In contrast to increasing concentrations of the drug was first reported by McConlogue and Coffino (130). Subsequently, similarly resistant cells have been obtained in a number of other laboratories using similar protocols (4, 15, 96, 131, 132). This resistance appears to be mediated by the presence of elevated levels of ODC. In most cases thus far examined, the increase in ODC protein levels was due to an increased rate of synthesis. In some cases, this overproduction was produced by a massive increase in ODC mRNA which in turn was due to amplification of the ODC gene. Indeed, ODC gene amplification in response to chronic exposure to DFMO seems to occur remarkably readily. However, other examples in which there was an increase in the translation efficiency of the ODC mRNA or an increased rate of transcription of the gene have also been reported (15, 133). Another pathway to resistance appears to be an enhanced stability of the ODC protein which leads to an increased protein content (2, 134, 135). It is not yet known whether the underlying cause of the reduced rate of degradation resides in the ODC protein itself or to a change in the biochemical system responsible for its turnover. Overproduction of arginase leading to a high intracellular content of ornithine also appears to impart resistance to DFMO in human myeloma cell lines (136).

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Fig. 5. Substrates and inhibitors of 5′-methylthioadenosine phosphorylase. The compounds shown are: (1) 5′-deoxy-5′-methylthioadenosine (MTA); (2) 5′-deoxy-5′-chloroformycin; (3) 5′-deoxy-5′-methylthio-9-deazaadenosine; (4) 5′-deoxy-5′-ethylothio-2-fluoroadenosine; (5) 5′-deoxy-5′-ido-inosine adenine; and (6) 5′-deoxy-5′-ido-2-fluoroadenosine.
amines is present in some bound form and is not available for below the control value. A possible explanation for these results to low levels of exogenous spermine, growth resumes at a reduce only a small enhancement of intracellular levels; (/>)when would be expected based on the amount of intracellular poly
repressed and spermidine/spermine W-acetyltransferase is in
amines normally present. For example: (a) ODC activity is
cells more dependent on the methionine supply ( 138,147,151 ).
pathway prevents the salvage of methionine and renders the
phosphate and 2-keto-4-S'-methylthiobutyrate (34, 138, 139,
148). This results in the conservation of the methylthio group
bases in a way in which they will be taken up by the cell and
metabolically converted to toxic ATP analogues. For example,
5'-deoxy-5'-ethylthio-2-fluoroadenosine (Fig. 5), which is de-
graded by MTA phosphorylase generating 2-fluoroadenine, was strongly cytotoxic towards tumor cell lines which contained both MTA phosphorylase and adenine phosphoribosyltransferase but had little activity towards cell lines lacking either of these enzymes. A second class of compounds has also been made which replace the 5'-methylthio group of MTA with a halogen such as 5'-deoxy-5'-iodoadenosine (Fig. 5). These are also substrates for the phosphorylase which liberates 5-halogen-
ated ribose 1-phosphates and these interfere with cell growth. Recently, the synthesis of derivatives containing both function-
alities such as 5'-deoxy-5'-ido-2-fluoroadenosine (Fig. 5) has been achieved (149). These compounds are much more toxic towards MTA phosphorylase-positive cells. Unfortunately, this strategy has not yet found any useful application in selective killing of tumor cells since all of the cells known to lack MTA phosphorylase are malignant and the enzyme appears to be ubiquitous in nonneoplastic cells. Selective killing of MTA phosphorylase-deficient cells can be achieved by placing the cells in a medium in which de novo
purine synthesis is prevented and MTA is added as the only potential source of adenine (150) but this strategy is not easy to apply in vivo.

The 5-methylthiouribose 1-phosphate produced by MTA phosphorylase is converted back to methionine via a series of reactions involving as intermediates 5-methylthioribulose 1-phosphate and 2-keto-4-S-methylthiobutyrate (34, 138, 139,
148). This results in the conservation of the methylthio group and maintains the cellular supply of methionine. The absence or inhibition of MTA phosphorylase or other enzymes in this pathway prevents the salvage of methionine and renders the cells more dependent on the methionine supply (138, 147, 151).

Compartmentation of Polyamines

A puzzling feature of many experiments in which exogenous polyamines have been applied to mammalian cells in culture is that these polyamines appear to have more striking effects than would be expected based on the amount of intracellular polyamines normally present. For example: (a) ODC activity is repressed and spermidine/spermine N\(^2\)-acetyltransferase is induced by quite low levels of exogenous polyamines which produce only a small enhancement of intracellular levels; (b) when DFMO-treated cells which are growing very slowly are exposed to low levels of exogenous spermine, growth resumes at a normal rate even though the total polyamine content is still far below the control value. A possible explanation for these results is that a substantial fraction of the normal intracellular polyamines is present in some bound form and is not available for regulation of polyamine synthesis or stimulation of growth.

Newly taken up or newly synthesized polyamines may enter this inert compartment relatively slowly and thus have a greater effect. Although this is entirely speculation, there is convincing evidence that such compartmentation occurs in Neurospora crassa where detailed studies by Davis et al. (152, 153) have shown that more than 70% of the total putrescine and spermidine is sequestered and is not available for spermine synthesis. Only a minor portion of this (28%) was present in vacuoles. There is no doubt that polyamines as small basic molecules are likely to be associated with subcellular fractions such as membranes, microsomes, or chromatin. It also appears that spermine may be transported into the mitochondrial matrix (154). Although there have been a number of attempts to study the compartmentation of polyamines in mammalian cells (see Refs. 1, 2, 4, and 155), none of these methods is entirely satisfactory, and the possibility of redistribution during cell fractionation is very difficult to rule out. A metabolic labeling method such as that used by Davis (152, 153) may be more helpful in providing an answer to this important question.

Importance of Putrescine and Polyamine Biosynthesis in Carci-
nogenesis

There is convincing evidence that a common property of many tumor promoters is the ability to induce increases in ODC activity (9-11, 156, 157). The resulting elevated production of putrescine appears to be critical for the induction of tumors. Tumor formation is prevented by the administration of substances which block the induction of ODC such as retinoic acid or inhibitors of adenosine metabolism including indomethacin or by direct inhibitors of ODC such as DFMO (9, 11, 157).

Many experimenters have shown that appropriate application of DFMO prevents tumor promotion in a variety of systems (9-11, 156-159). The ODC inhibitor also blocks the retrovirus-induced transformation of mouse erythroid cell precursors (160). Treatment with DFMO was found to inhibit mammary carcinogenesis in rats treated with N-methyl-N-nitrosourea (161, 162), the development of tumors of the gastrointestinal tract in rodents treated with 1,2-dimethylhydrazine or azoxy-
methylene (159, 163-165), and the incidence of bladder tumors initiated by N-methyl-N-nitrosourea (166). In some of these experiments DFMO may act by inhibiting tumor promotion, but in others it may act to actually block tumor growth. Such inhibition at an early stage of tumor development may lead to the elimination of the tumor by host-mediated responses. Irre-
spective of the mechanism, a particularly interesting feature of this inhibition of carcinogenesis is that a significant effect is brought about by quite low levels of DFMO which have minimal toxicity (161) and that the combination with other substances which also act as chemopreventative agents such as retinoids (9, 11), butylated hydroxyanisole (156), tamoxifen (162), piroxi-
cam (165), and fish oil (165) may have additive or synergistic effects. There is, therefore, some possibility that prophylactic doses of DFMO might be used to reduce the incidence of tumors in high risk groups.

Since cell proliferation is clearly necessary for the expression of malignancy in initiated cells, it is perhaps not surprising that agents such as DFMO which reduce the rate of cell proliferation might inhibit carcinogenesis in these two stage models. How-
ever, it appears that increased ODC activity may be a more critical event in carcinogenesis than merely permitting the synthesis of polyamines needed for cell division. Work by Boutwell, O'Brien, Verma, and colleagues (9, 11, 167-170) has shown that the regulation of ODC in mouse epidermis appears

POLYAMINE METABOLISM AND NEOPLASTIC GROWTH

Summary and Conclusions

Work with the inhibitors of polyamine biosynthesis currently available shows clearly in animal models that these compounds have significant potential to block tumor growth and prevent metastases. Although the current preliminary results with DFMO as an antitumor agent in clinical trials have been less encouraging, there is good reason to continue and extend work using polyamine biosynthesis inhibitors as antitumor agents. Several reasons can be put forward for the lack of effect of DFMO in addition to the obvious one that the patients treated in phase I and II trials are clearly not optimal for testing this agent.

DFMO is quite strikingly nontoxic in view of the ubiquitous distribution of polyamines, the requirement for them for cell growth, and its blockade of cell division in cultured cells in vitro. In part, this may be because treatment with DFMO does not deplete polyamines to the same extent under in vivo conditions where competition with ornithine, its rapid excretion, and the availability of putrescine and polyamines from dietary sources tend to minimize its effect. Appropriate protocols to take account of these factors may be useful in extending the effects of DFMO. A greater extent of polyamine reduction may also be achieved by some of the second generation ODC inhibitors or by combination of DFMO with inhibitors affecting other steps in the polyamine-biosynthetic pathway. Moreover pronounced effects may also result as greater knowledge of the control of the biosynthetic enzymes by polyamines is used to devise protocols to reduce the extent of compensatory increases in their activity. The polyamine analogues that repress ODC and AdoMetDC may have particular value in such protocols although the possibility remains that they may substitute for natural functions of polyamines as well. Many other analogues can be made and tested for their ability to act as repressors without activity in support of cell growth. New or refined approaches to the production of inhibitors such as the development of substances interfering with the processing of AdoMetDC should lead to even more active compounds becoming available. A better understanding of the polyamine transport system may lead to substances or protocols which could improve the depletion of polyamines in response to these inhibitors by blocking uptake of exogenous polyamines. The ancillary pathways relating to polyamine metabolism such as MTA metabolism also provide opportunities for the design of anticancer agents that may have synergistic effects with inhibitors of polyamine biosynthesis.

The reactions forming polyamines are now well understood at the biochemical level and complete cDNA clones have been obtained for ODC and AdoMetDC (4, 15, 16, 26, 27, 130–132). Genomic clones will shortly become available and the chromosomal location of these genes has now been derived (174). These results raise the possibility that polyamine biosynthesis might also be influenced by the synthesis and application of appropriate “antisense” RNAs.

A problem, which has not yet received much attention, is the question of selectivity. Although there are some indications that there might be a distinct form of ODC in certain tumors, the polyamine-biosynthetic system is common to all cells and a more complete blockade of polyamine synthesis may not be to be altered under the influence of the phorbol ester tumor promoters. Application of these promoters gives rise to a transient increase in ODC activity that is mediated by activation of protein kinase C leading to an increased content of ODC mRNA and protein (11, 167, 168). However, in the resulting epidermal papillomas, there is a constitutive increase in ODC activity. This elevated ODC also appears to turn over more slowly than the activity in control mouse skin and appears to be relatively unresponsive to repression by putrescine.

Recently, some evidence has been obtained that the changes in ODC regulation may be accounted for by the induction of a form of ODC which differs significantly from that present in the normal epidermis. A form of ODC isolated from epidermal papillomas was more resistant to heat inactivation and had a higher $K_m$ for ornithine than ODC from control epidermis (169). The enzymes could also be distinguished on the basis of gel filtration, immunoblotting where they differed in isoelectric point but not molecular weight, and by the effects of GTP which activated the enzyme from the papillomas but not that from the normal epidermis (169, 170). The possibility that tumor ODC may actually be significantly different from that of the normal cells of origin is particularly exciting since the possibility of selective inhibition of the tumor-specific enzyme is raised, but much more work on the characterization of the enzyme from these sources is needed. It is not clear whether the changes observed are due to posttranslational modifications of the enzyme or to the expression of a gene coding for an enzyme with a different primary structure. Studies with highly purified enzyme preparations rather than crude extracts and isolation and sequencing of cDNA clones to ODC from these sources will be needed to establish the underlying biochemical mechanism.

The importance of ODC and putrescine production in tumor development and the possibility that different forms of ODC exist have led to some interest in the possibility that ODC activity might provide a marker for susceptibility to cancer (159, 171). No clear picture has yet emerged from these studies. Luk and Baylin (171) reported that ODC levels are higher than controls in the colonic mucosa of individuals with familial polyposis which is associated with a high incidence of carcinoma. All polyps biopsied also had high activity. Furthermore, ODC in colon extracts from first degree relatives of patients with familial polyposis showed a bimodal distribution with a peak in the normal range and a second peak corresponding to the higher level found in the patients. These results raise the possibility that ODC might be used to identify asymptomatic family members who carry the genotype for familial polyposis. Whether the high level of ODC relates directly to the formation of adenomatous polyps and their progression to adenocarcinoma has not been determined. Both ODC and AdoMetDC activities were reported to be greater in extracts from adenomatous polyps and carcinomas than those from normal colonic mucosa (172). However, ODC activity in rectal mucosa was actually significantly reduced in patients with colorectal adenomas and carcinomas (173). In view of the great instability of ODC and the possibility that different forms may exist and play a role in carcinogenesis as discussed above, it seems unlikely that the mere measurement of ODC activity alone will be sufficient to provide valuable diagnostic information. If this approach is to be useful at all, it will probably require more detailed studies using immunochemical techniques with suitable antibodies to quantitate the amount of ODC protein combined with activity measurements.

D. M. Radford, R. Eddy, L. Haley, W. M. Henry, A. E. Pegg, A. Pajunen, and T. B. Shows. Gene sequences coding for human S-adenosylmethionine decarboxylase are present on chromosomes 6 and X and are not amplified in colon neoplasia, manuscript in preparation.
beneficial if toxicity towards normal cells is increased along with a greater effect on the tumors. There is, however, good evidence that the effect of ODC inhibitors such as DFMO can rapidly be reversed by the provision of putrescine. Therefore, the possibility of rescuing cells by giving putrescine at a later stage is raised. Protocols in which polyamine biosynthesis inhibitors are combined with other known anticancer drugs, which are known to have some selectivity themselves, may also have some advantage in the killing of tumor cells. However, these combinations must be evaluated very carefully on an empirical basis since studies with tumor cells indicate that the effects of these combinations depend on both the agent and the cell type. Both synergism and antagonism have been observed.

The combinations of polyamine biosynthesis inhibitors with biological response modifiers may have application not only in cancer chemotherapy as in the combinations of DFMO and interferon but also in the interference with carcinogenesis and tumor promotion.

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POLYAMINE METABOLISM AND NEOPLASTIC GROWTH


Polyamine Metabolism and Its Importance in Neoplastic Growth and as a Target for Chemotherapy

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