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COMMENTARY

ASPECTS OF THE BIOCHEMICAL PHARMACOLOGY OF METHYL GLYOXAL BIS(GUANYLHYDRAZONE)

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In 1958, Freedlander and French [1] reported that methyl glyoxal bis(guanylhydrazone) (MGBG) inhibited the growth of L1210 leukemia in mice. MGBG and some of its congeners depress the proliferation of a variety of malignant tumors in several mammalian species [2]. Clinical studies disclosed that MGBG could induce remissions in some patients with acute myelocytic leukemia, malignant lymphoma, and certain other neoplasms [2]. The severe toxicity of this drug towards many normal as well as malignant cells has set limitations on its effectiveness in cancer chemotherapy [2, 3]. However, more recent investigations involving variations in dosage schedules have reawakened interest in the potentialities of MGBG as an anti-cancer agent in man [4–7].

French *et al.* [8] were the first to point out that MGBG had structural resemblances to spermidine (Fig. 1) and they suggested that MGBG might interfere with the biological functions of this naturally occurring polyamine. Mihich [2, 9] found that administration of spermidine to mice inoculated with L1210 leukemia cells counteracted the inhibition of tumor growth by MGBG, but that hepatotoxic, cardiotoxic, and hypoglycemic effects of the drug were not antagonized by spermidine. That the antitumor actions of MGBG might be related to polyamines was further suggested by two other sets of observations. First, Field *et al.* [10], Block *et al.* [11] and Dave and Caballes [12] noticed that accumulation of MGBG by L1210 leukemia cells *in vitro* was inhibited by exogenous spermidine or spermine, and they obtained other evidence that MGBG and polyamines shared a common membrane transport system. Second, Williams-Ashman *et al.* [13, 14] found that MGBG in micromolar concentrations inhibited the putrescine-activated *S*-adenosylmethionine decarboxylase (AdoMetDC) of various normal and malignant tissues. The decarboxylated AdoMet produced by the action of AdoMetDC functions virtually exclusively as an aminopropyl group donor for the enzymic synthesis of spermidine and spermine, which in higher animals is catalyzed by separate enzymes [15]. Inhibition of AdoMetDC by MGBG is selective inasmuch as the three other mammalian enzymes of *de novo* polyamine synthesis [ornithine decarboxylase (ODC), and spermidine and spermine synthases] are not directly affected by MGBG even at millimolar concentrations [14]. These findings prompted a gamut of investigations in which MGBG was used as a tool to decrease intracellular spermidine and spermine levels in attempts to gauge the significance of polyamines as regulators of cell proliferation and functions, and the replication of certain viruses. Interpretation of many of these studies is complicated by the fact that MGBG influences certain other biochemical processes besides the production of decarboxylated AdoMet. These include bioenergetic functions of mitochondria, the polyamine N^1-acetyltransferase that participates in the conversion of spermidine back to putrescine, and also various polyamine-oxidizing enzymes.

Two general considerations must be raised before proceeding with this critical discussion of the biochemical basis of the actions of MGBG on various normal and malignant animal cells. It is astonishing that, in nearly all accounts of studies on tissues and body fluids taken from organisms to which MGBG was administered, no attention is given to the possibility that the drug might disturb the outputs, and/

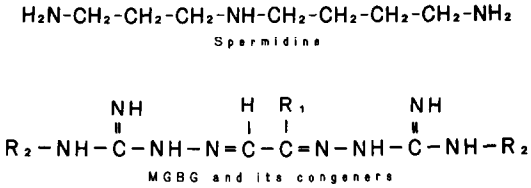

Fig. 1. Structures of spermidine and of MGBG and its congeners. The substituents of MGBG and its congeners, on the general formula depicted, are as follows: MGBG, $R_1 = CH_3$, $R_2 = H$; EGBG, $R_1 = C_2H_5$, $R_2 = H$; GBG, $R_1 = R_2 = H$; MBAG, $R_1 = CH_3$, $R_2 = NH_2$. The chemical name of MGBG is 1,1′-[(methylethanediylidene)-dinitrilo]-diguanidine and of MBAG is 1,1′-[(methylethanediylidene)-dinitrilo]-bis(3-aminoguanidine).

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or the receptor-mediated intercellular messenger functions, of countless hormones and neurotransmitters, thus ignoring the eventuality that some of the effects of MGBG on certain cells may be only indirect. Also, by selective depression of the development of antibody-forming cells, MGBG can exert profound immunosuppressive actions [2, 16], which frequently are not appreciated in evaluations of the effects of the drug on the growth of various tumors in living animals.

Properties and pharmacokinetics of MGBG

X-ray and neutron diffraction studies on the corresponding crystalline dihydrochloride monohydrate salt disclosed that the dispositive ion of MGBG has a completely *trans*-configuration of the chain [17]. Presumably, MGBG as usually synthesized from methyl glyoxal and aminoguanidine (i.e. guanyl hydrazine) represents only one of the four geometrical isomers that could result from *syn*- and *anti*-configurations with respect to the two —N=C— bonds in the asymmetric molecule. In aqueous solution, MGBG exhibits two pK_a values of roughly 7.5 and 9.2 at 25° [18], presumably due to resonance stabilization of the protonated forms of the aminoguanidine moieties at both ends of the molecule. Spermidine, whose molecular contours are quite similar to those of MGBG in the most extended conformations of these substances, can become triply protonated with pK_a value of about 8.4, 9.8, and 10.8 at 25°. The two pK_a values for putrescine are 9.04 and 10.5 at 30°. The pK_a values for diamines, polyamines, and MGBG would be expected to decrease significantly with rise of temperature over the range of 25° to 37° (cf. Refs. 19 and 20). Thus, at physiological pH and temperature, MGBG would not be completely protonated, and the ratio of monoprotonated/diprotonated species would change considerably with variations in the ambient hydrogen ion concentration in the range of pH 7 to 8.

The disposition of MGBG in mammalian organisms [2, 6] depends on the species and route of administration of the drug. No metabolites of MGBG have ever been detected in urine, feces, or various tissues, and virtually no radioactive CO_2 was expired after *in vivo* administration of [^{14}C]MGBG. For these reasons, it is commonly held that MGBG does not undergo biotransformation in higher animals. However, that MGBG might be metabolized by certain cells to a small extent in a pharmacologically significant manner has never been rigorously overruled. Noteworthy in this regard is that MGBG is readily mono-ADPribosylated on both amidine groups by the "arginine-specific" ADPribosyltransferase of cholera toxin [18], and that comparable enzymes are present in some mammals [21]. The observation of Wallace *et al.* [22] that exposure of cultured baby-hamster fibroblasts to MGBG increased the ADPribosylation of cellular macromolecules almost 3-fold is conceivably of interest in the aforementioned context, although the investigators considered that depletion of cellular spermine and spermidine levels by MGBG might be related to the increase in ADPribosyltransferase activity.

Effects of MGBG and its congeners on S-*adenosylmethionine decarboxylase*

MGBG and some of its analogs are potent inhibitors of AdoMetDC [13, 14]. Yet paradoxically, treatment of animals or cultured cells with MGBG brings about dramatic increases in AdoMetDC levels in many tissues [23–25]. The mechanistic basis of these phenomena must be overviewed before considering the effects of MGBG on other enzymes of polyamine metabolism and the *de novo* synthesis of spermidine and spermine in living cells.

Enzymological studies on AdoMetDC and its inhibition by drugs have been reviewed extensively [15, 24, 26–28], so that references to only very recent reports will be cited in the following summary of salient features of this topic. In mammalian tissues, AdoMetDC is a cytosolic enzyme that contains 1 mole of pyruvate bound covalently to the N-terminus of each apparently identical subunit. In the homogeneous state, the catalytically active linear enzyme has been variously reported to have a molecular weight of 155,000 (tetramer) or 68,000 (dimer). Decarboxylation of AdoMet is enhanced more than 10-fold by saturating levels of putrescine (K_a about 25 μM), and to a lesser extent by a few other short-chain aliphatic diamines. Spermidine is only a weak activator and spermine is inhibitory. Much evidence suggests that the covalently-bound pyruvoyl moieties serve a prosthetic group function by forming a Schiff base with the AdoMet substrate. This bound azomethine intermediate can be reduced by sodium cyanoborohydride with resultant irreversible inhibition of the enzyme. The mechanism of activation of AdoMetDC by putrescine is mysterious. The diamine decreases the K_m for AdoMet and increases the V_{max}, but the kinetics of putrescine stimulation are not characteristic of classical homotropic or heterotropic subunit allosteric interactions.

Inhibition of putrescine-activated AdoMetDC by MGBG is competitive with respect to AdoMet (K_i below 1 μM) and seemingly uncompetitive with respect to putrescine; in the absence of putrescine, the diminished enzyme activity is much less susceptible to inhibition by MGBG. The inhibition by MGBG is reversible since it is overcome by removal of the drug by dialysis, or even by dilution. EGBG, the ethyl analog of MGBG (Fig. 1), is an even more potent inhibitor than MGBG [14, 29, 30], whereas the parent compound glyoxal bis(guanylhydrazone) (GBG, Fig. 1) is considerably less potent than MGBG [31]. The derivative of MGBG, usually abbreviated as MBAG, which as shown in Fig. 1 has additional amino groups added to both ends of the MGBG molecule, irreversibly inhibits AdoMetDC with a swift rate of inactivation; the inhibition by MBAG is competitive with respect to AdoMet with a K_i slightly higher than that of MGBG. MGBG and EGBG are the most potent inhibitors of AdoMetDC known at present. Aminoguanidine, the elements of which are present at both ends of the MGBG molecule, is only a feeble inhibitor of AdoMetDC. The mechanism by which MGBG and its congeners inhibit AdoMetDC remains foggy and is unlikely to be understood until the structure of the AdoMet binding site, as well as the mode of activation of

AdoMetDC catalysis by putrescine, are fully elucidated. Formation of bound azomethine adducts between the α-amino group of AdoMet and the ketone moiety of the pyruvoyl prosthetic group at the N-terminus of each of the enzyme's subunits is implicated as an intermediate in the enzymic decarboxylation of AdoMet [15, 24]. MGBG itself contains two azomethine linkages. But lucubrations about stereochemical resemblances between MGBG and separated segments of the AdoMet molecule that were construed as germane to the MGBG inhibition of AdoMetDC being competitive with AdoMet [28, 31] are hardly credible. It is worth noting that no simple correlation between direct inhibition of AdoMetDC and antileukemic activity *in vivo* is evident for a series of MGBG analogs [2, 14, 28].

Pösö and Pegg [32] found that the AdoMetDC of rat psoas and diaphragm muscles is significantly more sensitive to inhibition by MGBG than is the enzyme from rat liver, heart, and kidney. AdoMetDC was purified to homogeneity from rat liver and psoas to yield preparations with identical specific catalytic activities. The two enzymes appeared to be dimers of subunits of the same molecular weight (32,000), but could be separated by isoelectric focussing (the isoelectric points for the psoas and liver decarboxylases were 5.3 and 5.7 respectively). In comparison with liver AdoMetDC, the psoas enzyme exhibited a significantly higher K_m for AdoMet, the same K_a for activation by putrescine, and a greater extent of maximal enhancement by putrescine. As might only be expected from the relative differences in the K_m for AdoMet, the concentrations of MGBG, EGBG, and dimethyl glyoxal bis(guanylhydrazone) required to evoke 50% inhibition of the psoas enzyme were significantly lower than was evident with the liver AdoMetDC. Thus, there appear to be different forms of AdoMetDC in muscle versus liver, kidney, and heart that are differentially sensitive to MGBG; whether this reflects post-translational modification of a single gene product or, alternatively, the existence of at least two AdoMetDC genes that specify the production of enzymes with different primary structures remains to be worked out.

AdoMetDC is present in strikingly low abundance in normal and malignant mammalian cells. Contrary to asseverations frequently propounded in the literature, the activity of AdoMetDC is probably even more rate-limiting for spermidine and spermine synthesis *in vivo* than is ODC activity in many tissues. (Spermidine and spermine synthase levels are usually much higher than those of ODC or AdoMetDC.) Like ODC and also polyamine N^1-acetyl transferase (see below), but in contrast to spermidine and spermine synthases, AdoMetDC is rapidly degraded as well as synthesized in animal tissues, so that the apparent half-life of the enzyme (determined after application of protein synthesis inhibitors such as cycloheximide) is remarkably short ($t_{\frac{1}{2}}$ less than 60 min, with considerable variation with the type and metabolic status of the particular cell). As previously mentioned, treatment of animals or cultured cells with MGBG leads swiftly to massive enhancement of AdoMetDC activity (as estimated after dialysis to remove the drug) often as large as 25-fold or greater. These MGBG-induced increases in AdoMetDC activity are accompanied by marked prolongation in the apparent half-life of the enzyme. The big enhancement of AdoMetDC activities due to MGBG administration entails parallel increases in immunoreactive enzyme protein, whereas the irreversible inhibitor MBAG elicits a comparable increase in immunoreactive AdoMetDC protein which is, however, catalytically inactive even after dialysis. It is thought that three factors might contribute to the rise in tissue AdoMetDC levels brought about by MGBG *in vivo*. (1) Binding of MGBG to the enzyme may stabilize the enzyme against intracellular degradation, and hence prolong the half-life of AdoMetDC. (2) Inhibition of AdoMetDC by MGBG in intact cells results in accumulation of putrescine because utilization of this diamine for spermidine and spermine synthesis is attenuated as a result of depression of decarboxylated AdoMet production. Increased intracellular putrescine concentrations may also protect AdoMetDC against proteolytic destruction. As discussed below, putrescine may also accumulate in cells exposed to MGBG because the drug (a) is a powerful inhibitor of putrescine oxidation by diamine oxidases, and (b) provokes large increases in the activity of the polyamine N^1-acetyltransferase that is involved in the pathway for conversion of spermidine back to putrescine. (3) The fall in cellular spermidine levels caused by MGBG under some biological circumstances may facilitate AdoMetDC accumulation. As Mamont *et al.* [33] first proposed, spermidine exercises a strict negative control over AdoMetDC and decreases the half-life of the enzyme in living cells, an effect that seems to operate largely at a post-translational level, perhaps because a complex between free spermidine and AdoMetDC may be formed and is especially rapidly degraded [34]. However, the drop in tissue spermidine content elicited by MGBG may not always contribute to the paradoxical elevation of AdoMetDC because the direct inhibition of the enzyme by MGBG may quickly wear off as the drug is excreted. In fact, Shirahata and Pegg [35] showed that AdoMetDC activities, and also the levels of enzyme active site determined by radiometric titration, as well as immunoreactive AdoMetDC, were increased by about 12- and 25-fold in rat liver and ventral prostate 1 day after injection of MGBG under conditions where there was no significant drop in the spermidine or spermine content of either organ. Although direct or indirect stabilization of AdoMetDC seems to play a big role in the large increases in enzyme activity due to MGBG acting *in vivo*, whether the drug additionally influences the transcription and/or translation of AdoMetDC mRNA, or even evokes amplification of the AdoMetDC gene, is not known.

Of related interest is that 2-difluoromethylornithine (DFMO), an irreversible suicide inhibitor of ODC that provokes marked lowering of putrescine and spermidine (but usually not spermine) levels in cells, also increases AdoMetDC activities in many tissues. This effect of DFMO on rat ventral prostate is associated with increases in the half-life of AdoMetDC (as estimated by catalytic measurements or radiometric titration of the enzyme's prosthetic group), and even greater prolongation of the half-

life of AdoMetDC immunoreactive protein, under conditions where the DFMO treatment resulted in a marked decline in cellular spermidine content [35]. Treatment with a combination of DFMO and MGBG was somewhat less effective in enhancing AdoMetDC in both organs than was MGBG alone (which in these circumstances did not lower spermidine levels). It must be remembered in this context that intracellular decarboxylated AdoMet concentrations are depressed markedly by MGBG but can be raised many hundred-fold by DFMO in a variety of tissues, and also that treatment with MGBG leads to several-fold increases in cellular ODC levels, although not to as great an extent as AdoMetDC [36].

Inhibition of diamine and polyamine oxidases by MGBG

Hölttä *et al.* [37] found that low concentrations of MGBG inhibited the oxidation of putrescine by mouse thymus extracts. Using purified pig kidney diamine oxidase, Pegg and McGill [38] showed that putrescine oxidation was inhibited non-competitively by MGBG (K_i 0.1 μM) and competitively by MBAG (K_i 0.02 μM). The inhibition by MGBG was only partially reversed by prolonged dialysis. Administration of MGBG or MBAG to rats profoundly attenuated the conversion of [^{14}C]putrescine to expired $^{14}CO_2$. MGBG also inhibits oxidation of putrescine, spermidine, and spermine by human seminal plasma [39]. These effects of MGBG and MBAG are probably related to their aminoguanidine moieties since it is well known that aminoguanidine inhibits all reactions catalyzed by copper-containing amine oxidases [40], including the classical diamine oxidase (DAO). By contrast to the strong and roughly equipotent inhibition of DAO by aminoguanidine, MGBG, and MBAG, the intracellular flavoprotein polyamine oxidase (PAO) that oxidatively degrades spermidine and spermine to 3-aminopropionaldehyde (and for which the N^1-acetyl derivatives of spermidine and spermine are much more superior substrates) is inhibited significantly only by millimolar concentrations of MGBG [41]. Administration of aminoguanidine to rats indeed evokes moderate increases of the concentration of putrescine (but not of spermidine or spermine) in a variety of rat organs under some conditions [40], so it would be expected that inhibition of DAO by MGBG might contribute to the elevation of putrescine levels by the drug, although this notion has been disputed [42]. Moreover, since DAO activity in the gastrointestinal tract is very high, inhibition of putrescine and polyamine oxidation might facilitate increased uptake of polyamines from the gut, which could then be transferred to other tissues and accumulated therein [42]. Consideration of potential metabolic consequences of DAO inhibition by MGBG must also take into account that other substances besides aliphatic diamines (e.g. histamine) are effective substrates for this enzyme.

Effects of MGBG on spermidine/spermine N^1*-acetyltransferase and polyamine interconversions*

Conversion of spermine to spermidine and of spermidine to putrescine takes place in mammalian tissues, but not via reversal of the spermine and spermidine synthase reactions. Rather, the back conversion of polyamines to their biosynthetic precursor amines is due to the combined action of two enzymes: spermidine/spermine N^1-acetyltransferase, and the flavoprotein PAO previously mentioned. N^1-Acetylspermidine formed by action of the acetyltransferase is rapidly oxidized by PAO to yield putrescine and 3-acetylaminopropionaldehyde; initial N^1-acetylation of spermine is involved in a comparable pathway for formation of spermidine from spermine [36, 43]. The spermidine/spermine N^1-acetyltransferase is rate-limiting for these polyamine interconversions, since in comparison with the unconjugated polyamines the N^1-acetyl derivatives of spermidine and spermine are much more effective substrates for PAO, and the latter enzyme is present in tissues to excess. MGBG is actually a competitive inhibitor of the N^1-acetyltransferase with respect to the polyamine substrates (K_i 8 μM) [42] but MGBG inhibits the latter enzyme much less effectively than AdoMetDC or DAO. However, in intact rats, treatment with MGBG elicits a more than 10-fold rise in N^1-acetyltransferase activities and immunoreactive enzyme protein levels in liver, and somewhat smaller elevations in kidney, spleen, and lung; moreover the normally very short half-life of the N^1-acetyltransferase is greatly increased by MGBG [42, 44, 45]. The huge elevation in N^1-acetyltransferase levels induced by MGBG would probably counteract the direct inhibition of the enzyme by MGBG *in vivo*, so that N^1-acetylation of spermidine and spermine is likely to be enhanced in animals treated with the drug. It seems likely, therefore, that MGBG acting on intact cells would greatly facilitate the operation of the N^1-acetylase/PAO pathway for polyamine interconversions, and thereby enhance the production of putrescine from spermine and especially from spermidine. Observations of Pegg *et al.* [42] on effects of DFMO and/or MGBG administration on rat liver polyamine levels are consistent with this hypothesis.

Cellular accumulation of MGBG and its congeners

Field *et al.* [10, 11] demonstrated that normal and malignant cells possess high-affinity and saturable transport systems that promote intracellular accumulation of MGBG, putrescine, spermidine, and spermine. Extensive investigations by Dave, Mihich, and their co-workers (for references see [28]) defined the substrate specificity and other characteristics of the polyamine/MGBG transport system in L1210 leukemia cells, which was defective in certain sublines that had developed resistance to growth inhibition by MGBG but were not deficient in AdoMetDC activity that was sensitive to inhibition by MGBG. Defective polyamine and MGBG transport was also found by Mandel and Flintoff [46] in mutant Chinese hamster ovary and rat myoblast cells that were resistant to MGBG. Polyamine and/or MGBG transport has also been studied in suspensions of neuroblastoma [47], fibroblast [48, 49], and Ehrlich ascites carcinoma [50–54] cells, and in mammary gland explants [55], slices of rat lung and other tissues [56, 57], and rat lung perfused *in situ* [58]. In general, it appears that putrescine, spermidine, spermine, and MGBG are transported by common membrane

transport systems that are (i) distinguishable from all other known transport systems (e.g. for various amino acids); (ii) saturable and temperature-dependent, with maximal rates evident at 37°; (iii) inhibited by uncouplers of oxidative phosphorylation and certain respiratory poisons; (iv) dependent on the proliferative status of the cells and in some instances subject to hormonal regulation; and (v) dependent on Na^+ in neuroblastoma and Ehrlich ascites tumor cells but not in lung slices. However, though many phenomenological accounts of their properties have been documented, virtually nothing is known about the biochemical mechanisms responsible for these transport processes. Whether polyamine/MGBG transport actually occurs via the same mechanisms in all types of cells in any particular organism is also unclear.

If cellular spermidine and putrescine levels of various types of cultured cells are depleted by prior exposure to DFMO, the uptake of MGBG and of labeled polyamines is increased [50, 52, 59, 60]. Preincubation of Ehrlich ascites carcinoma with MGBG also enhances intracellular accumulation of polyamines [54]. In neuroblastoma cells, the effects of preincubation with MGBG on putrescine uptake are stimulatory after 1 day of exposure to the drug but inhibitory after long intervals of exposure to the drug [47]. Certain tumor cells incubated with 5 μM MGBG can accumulate MGBG in concentrations as high as a few mmoles per kg of cells, and to an even several-fold greater extent if the cells are treated previously with DFMO. In studies on cultured L1210 leukemia cells, Alhonen-Hongisto *et al.* [61] found that the K_m for labeled spermidine uptake was 0.125 μM, and that the K_i value for MGBG as a competitive inhibitor was 5.2 μM. The K_i values for the congeners GBG (3.7 μM) and EGBG (6.5 μM) were not very different from that of MGBG. Exposure of the cells to DFMO for 24 hr increased the accumulation of spermidine, MGBG, and GBG more than 2-fold, but had little effect on EGBG concentration. In control cells not treated with DFMO, the relative accumulation of transported substances over a 1-hr period was GBG > spermidine > MGBG > EGBG. When the cells were preloaded with spermidine, or MGBG or its congeners, and then transferred to a polyamine- and drug-free medium, there was essentially no efflux of spermidine, and the efflux of MGBG and GBG was much slower than that of EGBG. The rates of efflux of GBG, MGBG, and EGBG were substantially less in cells that had been pretreated with DFMO. It was concluded that the poor cellular accumulation of EGBG in comparison with GBG or MGBG reflected the more rapid exit of EGBG from cells that had taken up the drug. The order of inhibitory potency of these substances towards mammalian AdoMetDCs is EGBG > MGBG > GBG [14, 29–31]. The relatively poor net accumulation of EGBG in certain tumor cells perhaps could account for early reports [2] that this drug is devoid of antileukemic action, although other pharmacokinetic factors might play a role.

Of related interest are the observations of Wiseman *et al.* [62] on four varients of a human transformed cell line that were highly resistant to the growth-inhibitory actions of MGBG. Although net accumulation of MGBG by the mutant cells was much less than that seen in the wild type cells in fairly long-term incubations, the apparent K_m and V_{max} values for initial linear rates of MGBG and spermidine uptake observed in short-term incubations hardly differed between the wild type and MGBG-resistant cells; however, the resistant mutant cells after preloading with [^{14}C]MGBG released the drug more rapidly. Results of investigations on MGBG uptake and accumulation have sometimes been viewed in terms of potential differences in the availability and affinities of putative intracellular binding sites that might react with both MGBG and its congeners and also polyamines. Seppänen [51] isolated subcellular fractions from osmotically shocked Ehrlich ascites carcinoma cells that had been pretreated with DFMO and then allowed to take up MGBG. He found that the relative content of MGBG per mg of protein for various fractions was: nuclear (24%); mitochondrial (20%); microsomal (39%); and cytosolic (17%). But one cannot conclude from these results that microsomal material in intact cells bound more MGBG than other subcellular fractions unless data on the total amount of protein in each subcellular fraction are available, and this was not documented. Moreover, one cannot overrule in these experiments the occurrence of redistribution artifacts resulting from the osmotic shock and homogenization procedures employed in the fractionation process. Because it is relatively poorly protonated at physiological pH and temperature, MGBG would not be expected to interact as firmly as spermidine or spermine with polynucleotides, anionic phospholipids, or many other intracellular biomolecules that could avidly bind to MGBG and polyamines. The integral of what we know about major binding sites for MGBG inside cells is zero (the extremely low abundance of AdoMetDC, DAO, and spermidine/spermine-N^1-acetyltransferase obviously would preclude these enzymes from binding more than a negligible fraction of the MGBG that is readily transported into cells).

Uptake and accumulation (of variable transiency) of MGBG take place in many normal as well as malignant cells in living animals and are often enhanced by prior administration of DFMO via the drinking water. In studies on L1210 leukemia cells growing in mouse ascites fluids, Kramer *et al.* [63] reported that uptake of intravenously injected [^{14}C]MGBG by seven normal organs was in every instance lower than that of the tumor cells, and that providing the animals with DFMO in drinking water for 3 days prior to the MGBG uptake determinations increased the accumulation of the drug by the leukemia cells to no greater extent than occurred in various other tissues. Sequestration of MGBG in these experiments by the tumor in the absence of DFMO was more than 4-fold greater than in small intestine, the most active of the normal organs that were examined. However, the finding that MGBG is accumulated under certain experimental conditions to a greater extent in cancers as compared with normal tissues (cf. also Refs. 64 and 65) may not necessarily be an attribute of malignancy *per se*, but perhaps may

relate more to the proclivity of rapidly dividing cells to concentrate MGBG more extensively than slowly growing or resting tissues. Kallio *et al.* [66] showed that previous treatment of mice with DFMO increased the accumulation of MGBG by bone marrow and small intestine (in which rapidly proliferating tissues DFMO evoked decreases in putrescine and spermidine content), whereas DFMO did not enhance MGBG concentrations in brain, skeletal and cardiac muscle, liver, and kidney. Other investigations by the Helsinki group [67] indicated that preceding administration of DFMO markedly enhanced the uptake of subsequently administered MGBG by circulating leukemia cells in children. Sequential administration of DFMO and MGBG was reported to induce rapid though temporary therapeutic responses in five leukemic children.

Influence of MGBG on mitochondrial structure and bioenergetic functions

Pioneering studies by Dave, Porter, and their coworkers [68–70] disclosed that MGBG evoked mitochondrial aberrations (swelling, damage to the cristae and decreased matrix density) in L1210 leukemia and several other types of cultured cells, with no concomitant changes in nuclear ultrastructure. Similar effects of MGBG were also observed *in vivo* in L1210 leukemia [70] and crypt cells of intestinal epithelium [71]. The mitochondria of normal cells were damaged by MGBG only if the cells were proliferating, possibly because dividing cells accumulate MGBG most effectively. Injury to mitochondria by MGBG in living cells appeared to precede alterations in intracellular polyamines, or inhibition of cell growth or nuclear DNA synthesis, but was associated with reduction of cellular ATP pools and pyruvate oxidation, and depression of mitochondrial DNA replication [72–74]. Although it was reported that ultrastructural abnormalities were observed in mitochondria of rat 9L brain tumor cells that had been depleted of spermidine by prior treatment with DFMO [75], the latter drug did not elicit defects in mitochondrial morphology in concanavalin A-stimulated lymphocytes (in this lymphocyte system, EGBG, which was even more effective than MGBG in lowering cellular spermidine and spermine, did not bring about the mitochondrial injury wrought by MGBG) [76].

MGBG apparently damages mitochondria only after it becomes concentrated intracellularly to millimolar levels. The mechanisms by which MGBG affects mitochondria are obscure. There are a number of reports (see Refs. 2 and 28) that MGBG, like various alkyl guanidines, can depress cell respiration and uncouple mitochondrial oxidative phosphorylation. Recently, Byczkowski *et al.* [77] found that MGBG at 2.5 to 10 mM progressively inhibited state 4 respiration but had lesser effects on state 3 (i.e. uncoupled) respiration by rat liver mitochondria. MGBG caused mitochondrial aggregation, but actually inhibited non-specific mitochondrial swelling induced by very low concentrations of Triton X-100. From these and other results, it was concluded that MGBG directly binds to sites (perhaps phospholipids) on the inner mitochondrial membrane. However, the specificity of these effects of MGBG on isolated mitochondria was not examined in depth [77], and their pertinence to mitochondrial damage evoked by MGBG *in vivo* is uncertain, considering that spermidine and spermine at unphysiologically high concentrations have actions similar to those of MGBG on isolated mitochondria [78]. It was suggested that MGBG [77] and polyamines [78] in the 2.5 to 20 mM range modulate K^+ fluxes across inner mitochondrial membranes, but is is noteworthy that much lower concentrations of spermine directly increase Ca^{2+} uptake and fluxes by mitochondria [79].

Nikula *et al.* [80] reported that [1-^{14}C]palmitate oxidation by rat heart muscle homogenates was inhibited progressively by MGBG, and also by putrescine, spermidine or spermine, at levels of 2.5 to 5 mM. Under the same conditions, palmitate oxidation was enhanced 5-fold by 1 mM L-carnitine. In the presence of L-carnitine, MGBG or putrescine hardly affected palmitate oxidation, which was inhibited by spermidine or spermine to a lesser extent than in the basal system without L-carnitine. MGBG appeared to act as a competitive inhibitor of palmitate oxidation (K_i 2.5 mM). Conceivably, MGBG and polyamines might directly influence the various long-chain fatty acid carnitine acyltransferases [81] that participate, via intermediate formation of fatty acid acylcarnitine derivatives, in transfer of fatty acid acyl-CoAs across the inner mitochondrial membrane, with subsequent regeneration of the fatty acid acyl-CoAs in the matrix compartment of mitochondria where oxidation takes place. Speculations [80] about the relevance of the L-carnitine-reversible inhibition of palmitate oxidation to the hypoglycemic effects and muscle dysfunctions evoked by MGBG *in vivo* have minimal experimental support. Nikula *et al.* [80] observed quite different actions of L-carnitine, MGBG, and polyamines on palmitate oxidation by cultured L1210 leukemia cells. In this case, overnight exposure of the cells to 1 mM L-carnitine increased palmitate oxidation about 1.5-fold. Prior exposure to 5 μM MGBG inhibited palmitate oxidation about 50% whether or not the cells had been incubated with L-carnitine, whereas the oxidation of palmitate was doubled by treatment with 5 μM spermine in the absence or presence of L-carnitine. Another contribution by Nikula *et al.* [82] describes how incubation of L1210 leukemia cells for 7 hr with 1 mM DL-carnitine increased, or with 5 μM MGBG decreased, subsequent pyruvate oxidation: treatment with both DL-carnitine and MGBG circumvented both the inhibition of palmitate oxidation and the mitochondrial damage due to MGBG solo (DL-carnitine did not influence the intracellular accumulation of MGBG). However, the mitochondrial-sparing effects of DL-carnitine were not apparent in other experiments in which MGBG concentrations had built up to values of greater than 1000 atamoles per cell. It is problematic that in these experiments [82] DL-carnitine was used, because D-carnitine inhibits certain carnitine acyltransferases competitively with respect to the natural L-carnitine substrate [83]. In the studies of Nikula *et al.* [80, 82] the specificity of the effects of L- or DL-carnitine was not determined.

Direct effects of MGBG on macromolecular biosynthetic reactions

MGBG interacts with naked DNA very weakly and is only a feeble inhibitor of DNA polymerase-α and thymidine kinase in cell-free systems [2, 27] even though DNA replication in intact cells can be profoundly attenuated by MGBG, as considered below. There are no indications that MGBG directly influences the activity of RNA polymerase II or leucyl tRNA synthetase [76, 84]. However, at high concentrations MGBG (but not EGBG or DFMO) was reported to cause profound inhibition of labeled leucine incorporation into protein by nuclease-treated rabbit reticulocyte lysates to which chick oviduct total RNA was added as a source of mRNAs [76]. This direct inhibition of protein biosynthetic reactions by MGBG was hardly reversed by addition of spermidine at 1.6 mM and apparently cannot be attributed to direct competition between polyamines and MGBG in polyribosomal systems. Pösö and Kuosmanen [85] observed that 4 mM MGBG or MBAG had no direct influence on reactions catalyzed by T4-DNA ligase from *Escherichia coli*, but that these drugs completely inhibited the stimulation of the reactions by 1 mM spermidine. Possible direct effects of MGBG on reactions catalyzed by other types of polynucleotide ligases and DNA topoisomerases *vis-à-vis* enhancement by polyamines merit further exploration.

Reinitiation of meiosis and maturation of starfish oocytes is promoted by low concentrations of MGBG in a manner similar to 1-methyladenine [86]. In starfish, 1-methyladenine liberated from ovarian follicular cells at spawning time acts as a hormone to reinitiate oocyte meiosis that had previously remained blocked at the first prophase stage. The hormone-like actions of MGBG on starfish oocytes do not seem to be related to effects of the drug on polyamine metabolism, and in view of the fact that MGBG is not cytotoxic to these invertebrate oocytes in levels as high as 10 mM, it seems improbable that the drug acts via deleterious effects on mitochondria. Meijer and Guerrier [86] propose that MGBG influences starfish oocyte maturation by interacting productively with the plasmalemma-bound receptor for 1-methyladenine. These interesting observations may not be relevant to effects of MGBG in mammals which are not known to utilize 1-methyladenine as a hormone.

It is noteworthy that MGBG (a) does not act like spermine or spermidine to accelerate phosphorylation of certain proteins by nuclear cyclic nucleotide- and Ca^{2+}-independent protein kinases [87], and (b) is neither a substrate nor an inhibitor of transglutaminase-mediated reactions* in which polyamines are attached covalently to proteins [88].

Suppression of cell division and DNA replication by MGBG

Application of MGBG to many types of cells in culture or whole organisms can strongly disturb progression through various phases of cell cycles and the synthesis of nuclear DNA [27, 28, 36, 89]. These effects of MGBG have often been related to mitochondrial damage or changes in cellular polyamines evoked by the drug. In our opinion, the available evidence considered above is too inconclusive to decide whether disruption of mitochondria in MGBG-treated cells generally represents a predominant cause of arrest of cell division and nuclear DNA replication. The remaining paragraphs discuss just a few aspects of depletion of spermidine and spermine in relation to inhibition of cell proliferation and DNA synthesis by MGBG.

As first shown by Pegg [90], MGBG blocks incorporation of labeled putrescine into spermidine and spermine in various tissues of animals treated with the drug and, likewise, in many types of normal and malignant cells in culture. However, changes in cellular polyamine concentrations evoked by MGBG in both biological situations can vary greatly with experimental conditions. We have already emphasized that increases in cell putrescine content brought about by MGBG may reflect not only lack of utilization of this diamine for *de novo* spermidine and spermine synthesis resulting from inhibition of AdoMetDC, but also inhibition of putrescine oxidation by DAO, and, perhaps more importantly, enhancement of the conversion of spermine to spermidine, and of spermidine to putrescine, by the polyamine N^1-acetyltransferase/PAO system. That MGBG, especially when acting on resting cells either in culture or *in vivo*, does not invariably diminish spermidine and spermine contents is not altogether surprising, considering indications that a substantial proportion of these polyamines may not be freely available to act either as substrates for enzymes that produce or degrade them, or as regulatory agents, because of tight binding of the polyamines to DNA, RNA, and possibly also membrane phospholipids. Germane to this is the incisive demonstration in the lower eukaryote *Neurospora crassa* of a multiplicity of intracellular polyamine pools that are not readily exchangeable [91].

Exposure of mitogen-activated lymphocytes to micromolar concentrations of MGBG depresses DNA synthesis [84, 92–94]. This effect can be reversed by exogenous spermidine or spermine under certain conditions but it must be remembered that polyamines interfere with cellular accumulation of MGBG. Igarashi *et al* [76, 84] have recently re-examined effects of MGBG, and also EGBG, on polyamine levels and macromolecular biosyntheses in concanavalin A-stimulated bovine lymphocytes, in which MGBG causes mitochondrial disruption whereas EGBG does not. Cellular accumulation of MGBG occurred to a greater extent than that of EGBG, but EGBG at relatively lower intracellular concentrations was just as effective as MGBG in lowering the spermidine and spermine, and increasing the putrescine, content of the lymphocytes. MGBG was more active than EGBG in lowering thymidine entry into cellular DNA, and MGBG evoked a relatively greater depression of labeled leucine incorporation into protein. Combinations of DFMO with either MGBG or EGBG were about equal in provoking a very marked decline in spermidine, and spermine levels. Yet with DFMO, EGBG was less active than MGBG in depressing

* H. G. Williams-Ashman, unpublished observations.

synthesis of DNA in either intact cells or isolated nuclei supplied with TTP. Moreover, upon addition of spermidine to cells rendered deficient in polyamines by prior treatment with DFMO and EGBG or MGBG, restoration of normal cellular spermidine levels occurred within 4 hr, whereas it was judged that complete recovery of DNA and protein synthesis took 10–20 hr [84].

MGBG has been applied, alone or together with ODC inhibitors, in many experiments aimed at analyzing the significance of polyamines for cells to progress through different phases of the cell cycle [27, 36, 89]. Although the results varied considerably with the cell type and the experimental conditions, in general the findings support the view that depletion of spermidine and spermine impedes the normal traverse of cell cycles, and that polyamines may be especially important for initiation of DNA synthesis. Given the powerful antiproliferative actions of MGBG, it is hardly startling that measurement of plating efficiency for cultured cells in the continuous presence of the drug reveals an almost complete absence of colony formation with as little as 1–2 μM MGBG [46, 63]. By contrast, when exponentially growing cultures are treated with as much as 40 μM MGBG for 1–3 days, and then harvested and replated in its absence, there is no more than a 20% reduction in plating efficiency relative to untreated control cells harvested and replated at the same time [95, 96]. Using the latter approach, Hung *et al.* [96] showed that rat brain 9L tumor cells pretreated with MGBG for 48–72 hr became more sensitive to the cytocidal action of the cross-linking agent 1,3-bis(2-chloroethyl)-1-nitrosourea. This sensitization was partially reversible by adding spermidine to the MGBG-pretreated cells and was comparable to that evoked by the ODC inhibitor DFMO [97].

Replication of certain DNA viruses (vaccinia, human cytomegaloviruses) in animal host cells is quite potently inhibited by MGBG [98, 99]. Although the MGBG inhibition was reversed by exogenous spermidine, neither viral nor host cell DNA synthesis was apparently affected by the drug, which nonetheless depressed formation of cytoplasmic DNA-containing inclusions and the yield of infectious progeny virus. Studies on the actions of MGBG and ODC inhibitors suggest that new polyamine synthesis is mandatory for replication of DNA viruses, possibly because spermidine and spermine produced by the host cells are needed for stabilization of the viral DNA or other processes involved in the maturation of viral particles [99].

Ohnishi *et al.* [100] recently reported that MGBG noncompetitively interferes with spermidine stimulation of polyphenylalanine and globin synthesis directed by appropriate mRNAs in cell-free translation systems. It was also shown that MGBG binds preferentially to ribosomal RNA and inhibits interactions of spermidine or spermine with ribosomal RNA but not with poly(U) or DNA. These findings suggesting that MGBG might directly depress the synthesis of certain proteins may be germane to the suggestion of Igarashi and Morris [84] that polyamines may specifically influence the biosynthesis of proteins that are components of DNA replication processes.

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