New S-Adenosylmethionine Decarboxylase Inhibitors with Potent Antitumor Activity

Urs Regenass,2 Giorgio Caravatti, Helmut Mett, Jaroslav Stanek, Peter Schneider, Marcel Müller, Alex Matter, Paula Vertino, and Carl W. Porter


ABSTRACT

Methylglyoxal bis(guanylhydrazone) (MGBG) has been studied clinically as an antitumor and antileukemic agent and is recognized as a potent but nonspecific inhibitor of the key polyamine biosynthetic enzyme, S-adenosylmethionine decarboxylase (SAMDC). A series of four SAMDC inhibitors with structural features similar to MGBG have been found to have improved potency and specificity toward the target enzyme, SAMDC. Relative to MGBG, the new derivatives were much more effective in inhibiting partially purified preparations of SAMDC (50% inhibitory concentration, 10 to 100 nM), much less effective at inhibiting diamine oxidase, and inactive toward ornithine decarboxylase. The inhibitors varied relative to MGBG in their ability to compete with spermidine for uptake, with two being similar and two being less effective. Against L1210 leukemic cells and T24 bladder carcinoma cells, the compounds were slightly less effective than MGBG at inhibiting cell growth, with 50% inhibitory concentration values of 1 to 10 μM as compared with 0.5 and 1.1 μM, respectively, for MGBG. Under 50% growth-inhibitory conditions, the inhibitors decreased SAMDC activity, increased ornithine decarboxylase activity and putrescine pools, and markedly depleted spermidine and spermine pools of L1210 cells. At the same time, mitochondrial integrity as assessed by whole-cell pyruvate oxidase activity and mitochondrial DNA content was not affected as it was with MGBG. At doses less than one tenth that of the maximally tolerated dose, all of the new inhibitors strongly suppressed the growth of B16 melanoma in vivo with minimal weight loss or toxicity. At doses less than one sixth the maximally tolerated dose, they effectively inhibited the growth of T24 human bladder carcinoma xenografts. In these same systems, MGBG showed only marginal antitumor activity. These studies identify two potent and efficacious inhibitors of SAMDC as potential antitumor agents and reaffirm the importance of SAMDC as a target in anticancer drug discovery.

INTRODUCTION

Although polyamines are known to affect a variety of cellular functions, their involvement in the initiation and maintenance of proliferative states seems to be most critical (for review, see Ref. 1). It is well established that the activities of the polyamine biosynthetic enzymes, ODC3 and SAMDC, are regulated during the cell cycle and induced by various trophic influences (1–6). Polyamine biosynthesis may, therefore, be considered as a messenger system which facilitates and/or effects the transmission of stimuli for proliferative responses. As such, it may represent a potentially important target for chemotherapeutic intervention.

MGBG was the first identified inhibitor of polyamine biosynthesis and remained among the most potent inhibitors of SAMDC (7). In the past, MGBG has demonstrated potent and broad-spectrum antiproliferative activity in vitro (reviewed in Ref. 8). In vivo model systems, it has shown significant antitumor activity against murine leukemias but only marginal activity against solid tumors (9). When tested in humans on daily or weekly schedules, MGBG proved to be prohibitively toxic (reviewed in Ref. 10).

A mechanistic analysis of the biological effects of MGBG led to the assumption that certain of the dose-limiting toxicities may be unrelated to polyamine depletion and due instead to other drug effects such as antimitochondrial activity (for review, see Ref. 11). Thus, the concept of using SAMDC inhibitors to deplete polyamines and control tumor growth could not be clearly evaluated with MGBG. To address this problem, several new SAMDC inhibitors have been synthesized as potential antiproliferative agents. Nucleoside analogues of the enzyme substrate, S-adenosylmethionine, have proven to be highly potent enzyme inhibitors but not particularly effective at inhibiting cell growth (12–14). Drug instability and cellular uptake are potential limitations to the effectiveness of these compounds. In contrast, MGBG is very stable in vivo and efficiently penetrates cells via the polyamine transport system (8). Other derivatives of this compound have been investigated. The ethyl derivative EGBG seems to be more selective for SAMDC than MGBG in that it exhibits diminished antimitochondrial effects (15). The derivatives EMGBG and DEGBG, are among the most potent SAMDC inhibitors described to date with Ki values in the range 10 nM (16, 17). Although these compounds also showed a decreased inhibition of diamine oxidase, they were only weakly at all active as antiproliferative agents in vitro, possibly because they differ from their parent compound in not penetrating cells effectively. Other MGBG derivatives were found to be less potent at the enzyme level than the parent compound. MCGA, for example, inhibited SAMDC with a Ki of 0.2 μM and cell growth with an IC50 between 1 and 5 μM (18). Similar results were obtained with MGBA (19). Although the analogues were better tolerated in vivo than MGBG, relatively high drug doses (50 to 100 mg/kg) were required to achieve antitumor effects.

In this report we describe a new series of four potent SAMDC inhibitors with structural elements similar to that of MGBG. Relative to MGBG, they displayed greater enzyme-inhibitory activity and a much improved antitumor activity and chemotherapeutic index.

MATERIALS AND METHODS

Compounds shown in Fig. 1 have been selected from a larger series of derivatives synthesized at CIBA-GEIGY Pharmaceutical Research Laboratories.4 MGBG was obtained from Aldrich Chemical Co. (Gillingham, Dorset, England). For in vitro experiments, all stock solutions

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: ODC, ornithine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; MGBG, methylglyoxal bis(guanylhydrazone); EMGBG, ethylmethylglyoxal bis(guanylhydrazone); DEGBG, diethylglyoxal bis(guanylhydrazone); MGBA, methylglyoxal guanylhydrazone-cyclo pentylaminodihydrazone; IC50, 50% inhibitory concentration; DAO, diamine oxidase; mtDNA, mitochondrial DNA; MTD, maximally tolerated dose; MGBA, methylglyoxal guanylhydrazone butylaminodihydrazone.


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Concentrations needed. and CGP-39'937) at 30 mM and diluted with culture medium. For in vivo experiments drugs were dissolved in 0.7% NaCl solution at the concentrations needed.

Measurements of Enzyme Activities. ODC and SAMDC were prepared and assayed as described previously (20–22). DAO was prepared and assayed as described by Seppänen et al. (23). Enzyme activities in cell extracts were determined as described by Porter et al. (24).

Antiproliferative Effects in Vitro. Antiproliferative effects on mouse L1210 leukemia cells and T24 human bladder carcinoma cells were analyzed as described elsewhere (25, 26). Culture medium was supplemented with Nu Serum (Collaborative Research, Bedford, MA) in the case of L1210 cells. The medium for T24 cells contained 5% fetal calf serum and 1 mM aminoguanidine (Fluka, Buchs, Switzerland) to inhibit serum oxidase activity (27).

Prevention of Antiproliferative Effects by Natural Spermidine. T24 human bladder carcinoma cells were seeded into microtitrator plates as described (26). Drugs were added to culture wells at the concentrations indicated in Fig. 2. Serial dilutions of spermidine (Sigma, St. Louis, MO; highest concentration, 16 mM) were added in parallel. Wells without drug and spermidine and wells with drug only served as controls.

Growth was assessed by staining cell layers with methylene blue, and absorbance values were determined using a Titertek microplate reader (26).

Polyamine Pools. Pools were determined by high-pressure liquid chromatography as described by Porter et al. (24) for L1210 leukemia cells.

Spermidine Uptake. Uptake of [3H]spermidine (NEN, DuPont, Boston, MA) was measured in L1210 cells as described previously (24). T24 cells were grown to confluency in 24-well plates and then used as attached cells for spermidine uptake. Cell numbers were determined by a Coulter Counter (Model ZB; Coulter Electronics). Data were analyzed by using Lineweaver-Burk and Dixon plots. For T24 cells, the K_m value for spermidine uptake was 0.23 ± 0.01 μM, and the V_max was 156 pmol/h and 10^6 cells. The kinetic data for L1210 cells were similar to those published previously (24).

Intracellular Concentrations of Compounds. Drug levels were determined as described by Seppänen et al. (23). In brief, T24 human bladder carcinoma cells were exposed to 1 or 10 μM test compound for either 1 h or 24 h. Cells were then lysed, and the extracts were heated to destroy endogenous SAMDC activity. Samples of extracts were then tested for their ability to inhibit exogenously added SAMDC, and the drug concentration per extract was deduced from a standard inhibition curve established for each compound.

Pyruvate Oxidation. As a means to evaluate mitochondrial function, pyruvate oxidation was quantified by the release of radiolabeled CO_2 from intact cells incubated in the presence of 21^4Clpyruvate (New England Nuclear, Boston, MA). Details of the methodology are described elsewhere (28). At a final concentration of 1 mM pyruvate and in the presence of 10^6 L1210 cells, the assay was linear up to 60 min. Treated cells were exposed to a drug concentration sufficient to produce an approximate 50% growth inhibition at 48 h prior to evaluating pyruvate oxidation.

Determination of mtDNA Content. Mitochondrial DNA content of treated and control L1210 cells was analyzed as described previously (29). Cells were treated with IC_50 drug concentrations (see Table 2) for 48 h. Total DNA was prepared and applied to nitrocellulose paper with the use of a dot blot apparatus. Filters were hybridized with a 35S-labeled dATP-translated probe which was made from mouse mtDNA (generously provided by Dr. D. A. Clayton, Stanford University, Palo Alto, CA). Hybridizable radioactivity was determined by densitometric scanning of a film autoradiogram of the filter.

Antitumor Effects in Vivo. For MTD determinations, three mice per dosage group were treated once i.p. The dosage was increased until animal death occurred within 10 days after treatment.

In vivo antitumor activity was tested in T24 human bladder carcinoma nude mouse xenografts performed as described previously (26). Tumor volumes were calculated as described by Evans et al. (30) using six animals per treatment and control group.

In vivo antitumor activity was also tested in syngeneic C57BL/6 mice implanted s.c. with B16-BL6 melanoma cells. The tumor was allowed to grow for 4 to 7 days to reach a size of up to 3-mm diameter before treatment was started. The size of the tumors was estimated by comparison with graded plastic balls and expressed in volume units. Treatment was once daily for 15 days. Six animals were used per treatment and control group.

RESULTS

Enzyme Inhibition. MGBG inhibited SAMDC with an IC_50 of 1 μM (Table 1). The series of inhibitors could be separated into two groups, the three monocyclic compounds showing IC_50 values of 0.1 μM or slightly less and the bicyclic compound CGP-39'937 showing IC_50 values of 6 nM, respectively (Table 1). MGBG inhibited partially purified preparations of SAMDC and DAO to approximately the same extent (Table 1). The derivatives CGP-33'829, CGP-35'753, and CGP-36'958 all showed 10- to 100-fold higher IC_50 values for inhibition of DAO than for SAMDC. This increased enzyme selectivity was greatest for CGP-39'937, which showed an IC_50 for DAO that was 1000-fold higher than the IC_50 for SAMDC. ODC activity was not affected by any of the compounds at concentrations up to 50 μM (Table 1).

Antiproliferative Effects in Vitro. Inhibition of cell growth was tested against cultured L1210 murine leukemia cells and T24 bladder carcinoma cells. Against L1210 cells (Table 2), MGBG was the most potent compound and CGP-39'937, the least potent. In contrast, against T24 cells, MGBG and CGP-39'937 were about equipotent. The other three compounds were similar in growth inhibition and were all less potent than MGBG in both systems (Table 2).

Spermidine Uptake Inhibition. Inhibition of [3H]spermidine uptake was evaluated in both L1210 murine leukemia cells and human T24 bladder carcinoma cells (Table 3). In L1210 cells, MGBG, CGP-33'829, and CGP-36'958 were approximately equipotent in this activity and inhibited spermidine uptake with K_ι values of 50 to 80 μM. CGP-35'753 and CGP-39'937 competed less effectively with K_ι values between 100 and 200 μM. A similar but not identical picture emerged with T24 cells.

Table 1 Inhibition of partially purified SAMDC, ODC, and DAO preparations

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_50 (μM)</th>
<th>SAMDC (rat)</th>
<th>ODC (rat)</th>
<th>DAO (porcine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGBG</td>
<td>1.0 ± 0.48*</td>
<td>&gt;50</td>
<td>0.33 ± 0.024</td>
<td>&gt;50</td>
</tr>
<tr>
<td>CGP-33'829</td>
<td>0.062 ± 0.041</td>
<td>&gt;50</td>
<td>4.4 ± 0.07</td>
<td>&gt;50</td>
</tr>
<tr>
<td>CGP-35'753</td>
<td>0.12 ± 0.020</td>
<td>&gt;50</td>
<td>13.6 ± 1.16</td>
<td>&gt;50</td>
</tr>
<tr>
<td>CGP-36'958</td>
<td>0.099 ± 0.037</td>
<td>&gt;50</td>
<td>0.98 ± 0.035</td>
<td>&gt;50</td>
</tr>
<tr>
<td>CGP-39'937</td>
<td>0.006 ± 0.001</td>
<td>&gt;50</td>
<td>9.4 ± 0.40</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

* Mean ± SD of 3 independent determinations.
Polyamine Biosynthesis and Pools. Enzyme activities were measured in L1210 leukemia extracts after treatment of cells under conditions which produced a ~50% growth inhibition (Table 4). MGBG treatment led to an increase of both ODC and SAMDC activities: the former via a regulatory response; and the latter via enzyme stabilization due to reversible enzyme inhibition (31-33). All other SAMDC inhibitors increased ODC activity in extracts of treated cells, but decreased SAMDC activity by varying degrees. The most potent compound at the isolated enzyme level, CGP-39’937, inhibited SAMDC activity by >97% in L1210 cells (Table 4). In these same experiments, polyamine pools were affected as follows: putrescine levels increased 13- to 17-fold while spermidine and spermine levels were markedly lowered with all compounds. In this regard, the new SAMDC inhibitors, and in particular CGP-39’937, lowered spermine and spermidine pools to a much greater extent than did MGBG (Table 4).

Antimitochondrial Effects. The compounds were tested for antimitochondrial effects in L1210 cells (Table 5) under the same conditions used to examine polyamine enzymes and pools (i.e., Table 4). MGBG reduced whole-cell pyruvate oxidation to 25% of control values and decreased mitochondrial DNA content to 33% of control values. CGP-33’829 and, in particular, CGP-39’937 showed little, if any, effect on either mitochondrial assay, indicating a possible differential between drug concentrations required to inhibit cell growth and those required to affect mitochondrial function and/or mitochondrial DNA content. Drug Accumulation in Cells. T24 bladder carcinoma cells were treated with 1 and 10 μM test compound and incubated for 1 h and 24 h. Drug levels within cells were determined using the SAMDC enzyme assay after cell lysis (23). When compared with extracellular drug concentrations, MGBG accumulated inside cells by a factor of 4 after 1 h and by >100-fold after 24 h. CGP-35’753, CGP-36’958, and CGP-39’937 accumulated to a lower degree, particularly after 24-h incubation. CGP-35’753 showed the lowest drug accumulation of the compounds tested. The data indicate different pharmacokinetic behavior (Table 6).

Prevention of Antiproliferative Effects by Spermidine. When cells were treated with MGBG at doses which completely inhibited cell growth, spermidine was able to completely abolish the antiproliferative effect when given simultaneously (Fig. 3). The two compounds which inhibited cell growth without affecting mitochondrial function (CGP-35’753 and CGP-39’937) were chosen for determining whether their antiproliferative activity could be prevented by spermidine. Our data indicate that spermidine (17 μM) prevented the antiproliferative effects of CGP-35’753 and CGP-39’937, even when the compounds were applied to cells at doses as high as 100 μM (Fig. 3).

Antitumor Effects in Vivo. MTDs were determined to obtain a baseline for therapy experiments. MGBG and the monocyclic compounds CGP-33’829, CGP-35’753, and CGP-36’958 showed MTD values of 63 mg/kg when given i.p., whereas the bicyclic compound, CGP-39’937, showed a MTD value of about 30 mg/kg. The therapeutic doses used in human T24 human tumor xenografts were one sixth of the MTD given i.p. As shown in Fig. 4, MGBG was only marginally effective in inhibiting the growth of T24 tumor. The monocyclic and the bicyclic compounds were about equally active, inhibiting by about 40 to 50%. Different doses of compounds were tested for inhibition of growth of B16 melanoma. Again, MGBG showed only marginal antitumor effects at a dose of 5 mg/kg. In contrast, at the same dose, the new inhibitors were very potent antitumor agents (Fig. 5), with up to 85% growth inhibition. A dose dependence of the antitumor effect was observed in all cases.

CGP-39’937 was found to be the most potent compound in this system with a 60% growth inhibition at doses as low as 100 μg/kg (Fig. 5). Typical tumor responses to the SAMDC inhibitors are shown for the B16 melanoma in Fig. 6.
Fig. 2. Dot blot analysis of mtDNA content of L1210 cells treated for 48 h with IC50 concentrations of MGBG and related SAMDC inhibitors. By dilution analysis, analogues 33'829, 39'958, and MGBG all deplete mtDNA. By contrast, analogues 35'753 and 39'937 have no apparent effect on the mtDNA content. These findings are quantitated and compared with data from the pyruvate oxidation assay in Table 5.

Table 6 Intracellular accumulation of MGBG and related SAMDC inhibitors in T24 bladder carcinoma cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>External concentration (μM)</th>
<th>Exposure (h)</th>
<th>Approximate intracellular concentration (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGBG</td>
<td>1</td>
<td>24</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1</td>
<td>15.8</td>
</tr>
<tr>
<td>CGP-35'753</td>
<td>1</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td>CGP-36'958</td>
<td>1</td>
<td>24</td>
<td>2.8</td>
</tr>
<tr>
<td>CGP-39'937</td>
<td>1</td>
<td>24</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Means of 2 to 3 independent experiments.

Fig. 3. Prevention of antiproliferative effects by coincubation with spermidine (spd). T24 human bladder carcinoma cells were coincubated with 10 or 100 μM drug and 17 μM spermidine in microtiter plates. Plates were incubated at 37°C, and cell growth was assessed at the end of the 5-day incubation period. The percentage of control growth represents mean values of double determinations of two separate experiments.

DISCUSSION

MGBG was tested in clinical trials (34) before being recognized as a potent inhibitor of SAMDC (7). Subsequent studies seemed to indicate that, while antitumor activity might be attributable to inhibition of SAMDC, prohibitive host toxicities might be related to other drug actions (35, 36). Although several attempts have been made to synthesize new SAMDC inhibitors with greater enzyme specificity (see "Introduction"), MGBG and its congeners remained the most potent class of SAMDC inhibitors with respect to antiproliferative and antitumor activity (37). It has been previously shown that derivatization of MGBG could lead to compounds with increased potency (16, 19). Most of these compounds, however, either lacked meaningful activity in cell culture or potency in in vivo models. In addition, the majority of MGBG derivatives have not been thoroughly evaluated with respect to antitumor efficacy and pharmacological behavior.
This report describes four new SAMDC inhibitors containing structural elements common to MGBG. The most potent compound, CGP-39'937, is about 100-fold more potent as an inhibitor of SAMDC than MGBG. As a bicyclic compound, CGP-39'937 was clearly superior to the monocyclic types. Derivatization of MGBG also led to increased enzyme specificity as indicated by an increased differential between concentrations required to inhibit SAMDC and DAO. Among the new series of compounds, CGP-35'753 and CGP-39'937 showed the largest DAO/SAMDC IC50 ratios. It has been previously shown that phenylated bis(guanylhydrazones) lost their ability for potent SAMDC and DAO inhibition (38, 39). If, as hypothesized by Balana-Fouce et al. (39), DAO inhibition by MGBG is responsible for hypoglycemic effects which are seen clinically and in animals (10), the new SAMDC inhibitors might be devoid of this important toxicity at therapeutically active doses.

As noted by others, SAMDC activity is typically increased in cells treated with MGBG (Ref. 31; Fig. 2). This has been attributed to stabilization of SAMDC by reversible enzyme inhibition and the subsequent accumulation of inhibited enzyme protein in cells (for review, see Refs. 10 and 36). The increased enzyme activity measured in MGBG-treated cells is artificially created during extraction of the enzyme, at which time reversible inhibitors disengage from the enzyme. It appeared that, because of their increased binding potency, the new compounds inhibited SAMDC in the cells and remained bound to the enzyme during the assay of cellular extracts. Alternatively, destabilization of the enzyme by the inhibitors seems unlikely. Extensive dialysis of extracts of cells pretreated with compounds allowed recovery of enzymatic activities. Similar to MGBG, the new inhibitors led to increased SAMDC activity after extract dialysis. The most potent inhibitor at the enzyme level, CGP-39'937, showed the greatest reduction in enzyme activity in cells. The potency in inhibiting intracellular enzyme activity was also reflected in the degree to which the new compounds depleted polyamine pools. All compounds appeared to be able to deplete spermidine and spermine pools to a greater extent than MGBG.

An MGBG derivative with increased potency at the enzyme level has been obtained previously (16), but it lacked cellular antiproliferative activity, most likely because of uptake problems. In contrast, all new SAMDC inhibitors showed meaningful antiproliferative activity in vitro. The potency of enzyme

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5 H. Metz, unpublished observation.
inhibition in vitro, however, was not necessarily reflected in the antiproliferative potency. A recent in vivo study using α-difluoromethylornithine as an inhibitor of polyamine biosynthesis indicated differences in the sensitivity of various human cancers to growth inhibition independent of endogenous polyamine levels (40). Furthermore, the catabolism and interconversion of polyamines could play an important role in determining cellular sensitivities to biosynthesis inhibition (41–43). The differential uptake and cellular accumulation as demonstrated in this report might also influence the antiproliferative potency. CGP-39'937 and CGP-35'753 showed a reduced affinity for the polyamine uptake system when compared with MGBG. Interestingly, CGP-39'937 accumulated in cells to a similar extent as did MGBG (23), whereas CGP-35'753 and the other new SAMDC inhibitors showed up to 10-fold lower accumulation levels. MGBG enters the cell exclusively via the polyamine uptake system (44). This remains to be demonstrated for the new inhibitors. The present data indicate that at least CGP-33'829 and CGP-36'958 behave very similar to MGBG with respect to spermidine uptake inhibition. Little is known so far concerning the polyamine uptake system (44). This remains to be demonstrated for the cellular targets. However, coinoculation of the inhibitors which lack mitochondrial effects, CGP-35'753 and CGP-39'937, with spermidine completely prevented growth inhibition by either of these compounds. While this may be due to the maintenance of intracellular spermidine pools by the exogenous polyamines and, therefore, besides an indication of inhibitor specificity, the possibility must also be considered that spermidine blocks inhibitor uptake into the cell and prevents growth inhibition by this means. The finding that growth inhibition by MGBG is also prevented even though it has potent antimitochondrial effects attests to this possibility.

MGBG has been described as an antileukemogenic agent with little activity against solid tumors (8) in animals. The lack of efficacy against solid tumors has never been studied in detail, but could be related to lack of bioavailability or the lack of persistent inhibition of polyamine biosynthesis. In contrast to MGBG, the new SAMDC inhibitors, in particular CGP-39'937 and CGP-35'753, showed high efficacy against solid tumors in vivo. The B16 melanoma proved to be particularly sensitive since high efficacy (i.e., >50% tumor growth inhibition) could be obtained with doses as low as 100 µg/kg. Additional studies are now needed to understand the high efficacy of the new SAMDC inhibitors in vivo. It will become important to determine whether antitumor effects correlate with the interference of the drugs with polyamine biosynthesis.

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