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Antitumor Effect of a New Multienzyme Inhibitor of Polyamine Synthetic Pathway, Methylglyoxal-bis(cyclopentylamidinohydrazone), against Human and Mouse Leukemia Cells

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ABSTRACT

Methylglyoxal-bis(cyclopentylamidinohydrazone) (MGBCP) has been synthesized as a multienzyme inhibitor for the polyamine-synthesizing pathway. This drug inhibited *S*-adenosylmethionine decarboxylase (EC 4.1.1.50), spermine synthase and spermidine synthase activities, competitively with *S*-adenosylmethionine, spermidine, and putrescine, respectively. MGBCP inhibited the growth of human leukemia Molt 4B and K 562 cells at 10 to 100 μM concentrations. Spermidine and spermine levels were markedly depressed in these MGBCP-treated leukemic cells, and the synthesis of protein, but not of DNA or RNA, was significantly diminished. In *in vivo* experiments, MGBCP depleted spermidine and spermine in the P388 leukemic ascites cells, and prolonged the survival time of mice bearing P388 leukemia.

The *S*-adenosylmethionine decarboxylase-stabilizing effect of MGBCP in mouse liver, Molt 4B and K 562 cells was much less than that of the parent inhibitor methylglyoxal-bis(guanylhydrazone). Induction of ornithine decarboxylase activity by MGBCP in the cultured leukemic cells was also much less than that by methylglyoxal-bis(guanylhydrazone).

INTRODUCTION

The polyamine biosynthesis pathway is likely to be an important target for the design of chemotherapeutic agents (1–5). The concept of inhibition of polyamine biosynthesis as treatment for cancer is based on a greatly increased production of the polyamines, spermidine and spermine, in various types of cancer (6–8). The biochemical and biological consequences of polyamine deficiency induced by inhibitors of polyamine-synthesizing enzymes include arrest of DNA synthesis and of tumor growth (9, 10). Efficient retardation of tumor cell growth has been obtained by the inhibitors to block the syntheses of spermidine and spermine (11, 12). Among these inhibitors, MGBG[1] is a well-known potent competitive inhibitor of AdoMetDC, depleting dec AdoMet as a substrate for spermidine and spermine synthases (13, 14). However, this compound has turned out to possess paradoxical diamine oxidase-inhibiting and AdoMetDC-stabilizing activities and severe undesirable side effects *in vivo* (15–19). These additional properties of MGBG hamper its wide range use in *in vivo* and clinical studies.

Moreover, owing to the complex compensatory reactions in polyamine biosynthetic pathway, efficient deprivation of cellular polyamines, particularly of spermine, has hardly been achieved by administration of an inhibitor having the monofunctional property (20, 21). In an effort to accomplish the satisfactory depletion of spermidine and spermine to inhibit tumor cell growth without any side effect, we have synthesized and investigated the compounds having the MGBG core structure and other inhibitory moieties in a single molecule (22, 23). These compounds showed multifunctional properties to inhibit AdoMetDC, spermidine synthase, and other enzymes, simultaneously. Intracellular polyamine contents were also decreased by the compounds, but the inhibition of spermine synthase and depletion of spermine were still insufficient.

In the present study, we have synthesized a new compound MGBCP to inhibit AdoMetDC, spermine synthase, and spermidine synthase, simultaneously. The compound effectively depressed cellular contents of spermine as well as those of spermidine, and exhibited relatively good antitumor effect *in vivo*.

MATERIALS AND METHODS

Chemicals. MGBCP was synthesized according to the methods previously published for the syntheses of guanylhydrazone analogues (22). Impurity was not detected by nuclear magnetic resonance and infrared spectrophotometric analyses. The chemical structure of MGBCP is shown in Fig. 1. MGBG was purchased from Aldrich Chemical Co. (Milwaukee, WI). DL-[1-^{14}C]Ornithine (48.8 mCi/mmol), [*carboxy*-^{14}C]*S*-adenosyl-L-methionine (58.9 mCi/mmol), [6-^{3}H]thymidine (15 Ci/mmol), [5,6-^{3}H]uridine (35 Ci/mmol) and [*U*-^{14}C]leucine (329 mCi/mmol) were obtained from New England Nuclear Corp. (Boston, MA). dec AdoMet was prepared by the action of AdoMetDC obtained from *Escherichia coli* (strain B) and purified by chromatography on Dowex-50-H^{+} and paper electrophoresis as described previously (24). All other chemicals were products of Nacalai Tesque, Kyoto, Japan.

Animals. Female mice of BDF1 hybrid strain (C57BL/6, female × DBA/2, male) weighing 23 to 25 g were obtained from Shizuoka Cooperative Association for Experimental Animals (Hamamatsu, Japan). The animals were given food and water *ad libitum*.

Enzyme Preparations. ODC (EC 4.1.1.17) from Ehrlich ascites tumor cells (25) or cultured leukemic cells (26), AdoMetDC from rat liver (27) or cultured leukemic cells and spermidine synthase and spermine synthase from rat ventral prostate (28) were prepared as described in a previous publication and used for *in vitro* experiments. Protein was determined by the method of Bradford (29) using bovine serum albumin as a standard.

Enzyme Assays. The activities of ODC (30), AdoMetDC (31), spermidine synthase, and spermine synthase (32) were assayed as described earlier.

Cell Culture and Determination of Macromolecule Syntheses. Human lymphoid leukemia Molt 4B cells and human erythroid leukemia K 562 cells were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) with 10% fetal calf serum, penicillin G (50 IU/ml) and streptomycin (50 μg/ml) at 37°C under humidified 95% air 5% CO_2 atmosphere. For the determination of intracellular DNA, RNA, or protein biosyntheses, radiolabeled thymidine, uridine, or leucine were added to the culture medium, respectively, and the radioactivities in trichloroacetic acid-insoluble materials were counted by a liquid scintillation counter.

Determination of Intracellular Polyamine Contents. Polyamines (putrescine, spermidine, and spermine) were determined by HPLC as described previously (33). Molt 4B and K 562 leukemic cells were harvested by low speed centrifugation (1,000 × *g* for 5 min), washed with cold 0.15 M NaCl, suspended in 0.4 N perchloric acid and disintegrated by freeze-thawing three times. The samples were centrifuged at 10,000 × *g* for 30 min, and the supernatants were analyzed directly

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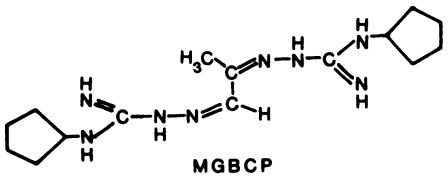
[1] The abbreviations used are: MGBG, methylglyoxal-bis(guanylhydrazone); MGBCP, methylglyoxal-bis(cyclopentylamidinohydrazone); AdoMet, *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; dec AdoMet, decarboxylated *S*-adenosylmethionine; ODC, ornithine decarboxylase; HPLC, high-performance liquid chromatography.



Methylglyoxal bis(cyclopentylamidinohydrazone)

Fig. 1. Structure of MGBCP.

by HPLC (Shimazu LC-5A) using a ISC-0.5 column. For the determination of polyamines in P388 leukemic cells, all the ascites tumor cells in the peritoneal cavity were harvested by rinsing with cold 0.15 M NaCl and sedimenting by centrifugation at 650 × *g* for 10 s. The pellets were suspended in 4 volumes of 0.15 M NaCl and centrifuged again as before to prevent the contamination of blood cells. Then the sedimented cells were resuspended as above and centrifuged at 1,000 × *g* for 5 min. The resulting pellets were suspended in 0.4 N perchloric acid and disintegrated by freeze-thawing three times. The samples were centrifuged at 10,000 × *g* for 30 min, and the supernatants were analyzed by HPLC as described above.

Evaluation of Antitumor Activity. P388 leukemic cells (1×10^6) were inoculated i.p. into BDF1 mice. MGBCP was dissolved in 0.15 M NaCl solution and administered i.p. into the leukemic mice at a dose of 0.01 ml/g body weight starting at 24 h after tumor inoculation. The same volume of 0.15 M NaCl was given by i.p. injections to the control mice. Antitumor activity is expressed by $T/C \times 100(\%)$, where T is the mean survival time of treated animals and C is that of control animals.

RESULTS

Effect of MGBCP on Polyamine Synthesizing Enzymes *in Vitro*. MGBCP inhibited AdoMetDC, spermine synthase, and spermidine synthase activities. ODC and diamine oxidase activities were not inhibited by this compound at all. Nature of the inhibition of AdoMetDC, spermine and spermidine synthases by this drug, and relevant data such as K_i and K_m are presented in Table 1. The inhibition of AdoMetDC by MGBCP was competitive with AdoMet, and the calculated K_i for MGBCP was 57 μM. The K_m value for AdoMet was estimated to be 53 μM. On the other hand, the K_i value for the parent inhibitor MGBG was 5 μM and the inhibition by MGBG was competitive with AdoMet using the same AdoMetDC (data not shown). Thus, MGBCP was found to be a slightly weaker inhibitor of AdoMetDC than MGBG.

Two substrates, spermidine and dec AdoMet, are involved in the reaction of spermine synthase forming spermine and methylthioadenosine. The inhibition of spermine synthase by MGBCP was competitive with spermidine. The K_i value for MGBCP and K_m for spermidine were calculated to be 78 and 28 μM, respectively. The effect of the concentration of dec AdoMet on MGBCP inhibition of spermine synthase showed noncompetitive inhibition in terms of dec AdoMet. The K_i and K_m values for MGBCP and dec AdoMet were 82 and 6 μM, respectively.

In the reaction of spermidine synthase, two substrates of putrescine and dec AdoMet are involved to form spermidine and methylthioadenosine. Effect of the concentration of putrescine on the inhibition of spermidine synthase by MGBCP showed that the inhibition was competitive with putrescine. The K_i and K_m values for MGBCP and putrescine were 112 and 55 μM, respectively. Effect of the concentration of dec AdoMet on MGBCP inhibition of spermidine synthase showed noncompetitive inhibition in terms of dec AdoMet. The K_i and K_m values for MGBCP and dec AdoMet were 102 and 8 μM, respectively.

Inhibition of Growth of Molt 4B and K 562 Cells by MGBCP. Decreasing rates of cell proliferation were observed with increasing concentrations of MGBCP in Molt 4B (Fig. 2*A*) and K 562 cells (Fig. 2*B*). At 100 μM, MGBCP completely inhibited the growth of these cell lines. The inhibition of cellular growth was partially prevented by the addition of spermidine to the culture medium (data not shown).

Effect of MGBCP on Polyamine Contents, and ODC and AdoMetDC Activities in Molt 4B and K 562 Cells. MGBCP inhibiting multiple steps in the polyamine biosynthetic pathway

Table 1 *Effect of MGBCP on polyamine-synthesizing enzymes in vitro*

Effects of the concentration of substrates on MGBCP inhibition of AdoMetDC, spermine synthase, and spermidine synthase were analyzed, and K_i and K_m values were estimated from Dixon plots and double reciprocal plots, respectively.

Enzyme	Nature of inhibition	K_i (μM)	K_m (μM)	
AdoMetDC	Competitive with AdoMet	57	53	For AdoMet
Spermine synthase	Competitive with Spermidine	78	28	For spermidine
Spermine synthase	Noncompetitive with dec AdoMet	82	6	For dec AdoMet
Spermidine synthase	Competitive with putrescine	112	55	For putrescine
Spermidine synthase	Noncompetitive with dec AdoMet	102	8	For dec AdoMet

Table 2 *Comparison of the effects of MGBCP and MGBG on polyamine contents, and ODC and AdoMetDC activities in Molt 4B (A) and K 562 cells (B)*

These cells were exposed to MGBCP or MGBG at the indicated concentrations for 4 days (for polyamine contents) and for 1 day (for ODC and AdoMetDC activities), and harvested to determine polyamines, ODC, and AdoMetDC activities. The percentages of the control (without treatment) are shown in parentheses. Each value is the mean of triplicate experiments.

Treatment	Concentration (μM)	nmol/mg protein			Activity (pmol/30 min/mg protein) of	
		Putrescine	Spermidine	Spermine	ODC	AdoMetDC
A						
Control		0.15 (100)	4.47 (100)	7.23 (100)	9.5 (100)	2.8 (100)
MGBCP	25	0.18 (120)	4.41 (92)	5.72 (79)	10.0 (105)	3.2 (114)
	50	0.49 (326)	2.44 (51)	3.11 (43)	14.5 (152)	3.5 (125)
	100	0.41 (273)	1.53 (32)	1.74 (24)	13.0 (136)	3.6 (129)
MGBG	5	3.54 (2360)	2.41 (54)	3.68 (51)	65.8 (693)	25.0 (893)
	10	2.81 (1873)	1.96 (44)	3.04 (42)	62.9 (662)	26.5 (946)
B						
Control		0.15 (100)	6.84 (100)	9.04 (100)	9.8 (100)	3.0 (100)
MGBCP	50	0.14 (93)	4.98 (72)	3.67 (41)	9.8 (100)	3.3 (110)
	100	0.13 (86)	2.32 (34)	2.30 (25)	9.7 (99)	3.8 (126)
MGBG	5	3.63 (2418)	3.76 (55)	4.61 (51)	65.6 (669)	21.5 (717)
	10	2.80 (1867)	2.87 (42)	3.62 (40)	60.8 (620)	23.8 (793)

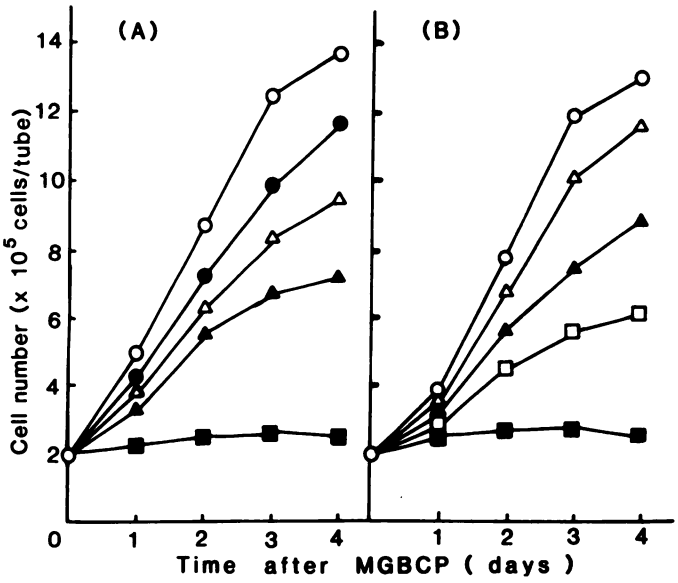


Fig. 2. Effect of MGBCP on the growth of Molt 4B (*A*) and K 562 (*B*) cells. These cells were diluted to an initial density of 2.0×10^5 cells/ml and grown in the absence (○) or presence of 10 μM (●), 25 μM (△), 50 μM (▲), 75 μM (□), and 100 μM (■) MGBCP for the time indicated.

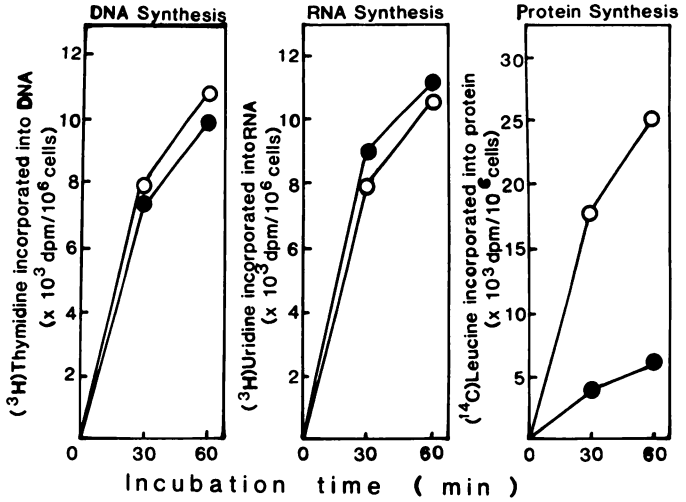


Fig. 3. Effect of MGBCP on DNA, RNA, and protein syntheses in Molt 4B cells. The cells at initial density of 2×10^5 cells/ml were grown in the absence (○) or presence (●) of 50 μM MGBCP for 2 days before radiolabeled thymidine, uridine, or leucine was added to the medium. At the times indicated the trichloroacetic acid-insoluble radioactivities were counted.

was expected to be more effective in depleting polyamines in the tumor cells than monofunctional inhibitors. As shown in Table 2, MGBCP decreased the levels of spermidine and spermine in these cell lines. The level of putrescine was rather increased slightly in MGBCP-treated Molt 4B cells, but not in K 562 cells. Increase of putrescine level in Molt 4B cells was considered to be a result of the slight induction of ODC by MGBCP in Molt 4B cells. Slight induction of AdoMetDC activity by MGBCP was observed in both cell lines. On the other hand, the parent inhibitor MGBG markedly induced ODC and AdoMetDC activities and increased putrescine content 20-fold or more over the control levels with depletion of spermidine and spermine contents in both cell lines. Spermidine/spermine N^1-acetyltransferase activity was not affected by MGBCP in both cell lines (data not shown).

Effect of MGBCP on DNA, RNA, and Protein Syntheses in Molt 4B Cells. As shown in Fig. 3, protein synthesis in Molt 4B cells was markedly inhibited by MGBCP added to the culture medium, but DNA and RNA syntheses were not inhibited.

Antitumor Effect of MGBCP on P388 Mouse Leukemia. MGBCP administered daily starting at 24 h after the tumor inoculation prolonged the survival time of mice bearing P388 leukemia. The percentage of mean survival days of treated relative to control mice (*T/C%*) were 136.6, 172.3, and 167.3% at MGBCP dosages of 20, 30, and 40 mg/kg, respectively (Table 3). 30 mg/kg was the most effective dose. MGBCP was not toxic at all at the doses of 30 to 40 mg/kg administered daily for 10 days.

Effect of MGBCP Administration on Polyamine Contents in P388 Leukemic Ascites Tumor Cells. As shown in Table 4, the doses of 30 and 40 mg/kg i.p. having antitumor effect (Table 3) caused the decrease in contents of spermidine and spermine with little effect on the putrescine content in the tumor cells.

Comparison of Stabilizing Effect of MGBCP and MGBG on Mouse Liver AdoMetDC. Six-week-old mice were given each single injection of MGBCP (30 or 50 mg/kg i.p.) or MGBG (50 mg/kg i.p.) 24 h before sacrifice as shown in Table 5. AdoMetDC activity in the livers of MGBCP-treated animals was not so different as compared with the control (1.22-fold increase), whereas a 9.54-fold increase of enzyme activity was observed in the livers of MGBG-treated animals as has been reported previously (15). Thus MGBCP did not produce signif-

Table 3 *Antitumor effect of MGBCP on P388 mouse leukemia*

Groups of each 10 female BDF1 mice were inoculated i.p. with 1×10^6 P388 leukemic ascites cells on Day 0, and given i.p. daily at the indicated doses of MGBCP on Days 1 to 10. Control mice received 0.15 M NaCl instead of MGBCP solution.

Treatment	Dose (mg/kg)	Survival time[a] (days)	T/C[b] (%)
0.15 M NaCl		10.1 ± 1.34	100
MGBCP	10	11.5 ± 1.45	113.9
	20	13.8 ± 1.92[c]	136.6
	30	17.4 ± 2.84[d]	172.3
	40	16.9 ± 2.51[d]	167.3

[a] Mean ± SD.
[b] The percentage of mean survival days of treated relative to control mice.
[c] $P < 0.05$ compared to 0.15 M NaCl-treated control.
[d] $P < 0.01$ compared to 0.15 M NaCl-treated control.

Table 4 *Effect of MGBCP administration on polyamine contents in P388 leukemic ascites tumor cells*

Groups of each five female BDF1 mice were inoculated i.p. with 1×10^6 P388 leukemic cells on Day 0 and MGBCP was given i.p. daily at a dose of 30 or 40 mg/kg on Days 1 to 7. Control mice received 0.15 M NaCl instead of MGBCP solution. Polyamine contents were determined at Day 8 as described in the text. The percentage of the control is shown in parentheses. Each value is the mean ± SD of independent five experiments.

Treatment	Dose (mg/kg)	(nmol/mg protein) Putrescine	Spermidine	Spermine
Control		0.18 ± 0.02 (100)	7.80 ± 0.92 (100)	8.05 ± 0.78 (100)
MGBCP	30	0.19 ± 0.01 (105)	5.81 ± 0.72 (74)	5.91 ± 0.62 (73)
	40	0.21 ± 0.03 (116)	3.22 ± 0.35 (41)	3.17 ± 0.41 (39)

Table 5 *Comparison of AdoMetDC stabilizing effect of MGBCP and MGBG*

6-week-old mice were given a single injection of MGBCP (30 and 50 mg/kg, i.p.) or MGBG (50 mg/kg, i.p.) 24 h before being killed, and AdoMetDC activity of the liver was measured as described in the text. Each value is the mean ± SD of triplicate experiments.

Treatment	Dose (mg/kg)	AdoMetDC activity (pmol/mg protein/30 min)	-fold
Control		2.66 ± 0.32	1.00
MGBCP	30	2.89 ± 0.38	1.09
MGBCP	50	3.24 ± 0.41	1.22
MGBG	50	25.38 ± 2.87	9.54

icant stabilizing effect of AdoMetDC such as that shown by MGBG.

In Vivo Toxicity of MGBCP. We obtained the LD_{50} value (dosage level producing 50% lethality) of 270 mg/kg by a single i.p. injection of MGBCP to mice (data not shown). This value was significantly higher than that for a single i.p. injection of MGBG (120 mg/kg) previously reported by Mihich (34).

DISCUSSION

The present study shows that MGBCP is a multienzyme inhibitor for AdoMetDC, spermine and spermidine synthases. Results on enzyme kinetics that MGBCP inhibited AdoMetDC, spermine synthase, and spermidine synthase competitively with AdoMet, spermidine, and putrescine, respectively, suggest that MGBCP would bind to the sites for AdoMet, spermidine, and putrescine in these enzyme molecules. A similar multifunctional enzyme inhibitor of polyamine biosynthesis, 1-amino-3-aminopropane, has been reported by Khomutov *et al.* (35). The compound inhibited ODC and spermidine synthase competitively and AdoMetDC irreversibly, but not spermine synthase. MGBG, which had been described to exhibit antileukemic action by Mihich (34), was reported to inhibit strongly AdoMetDC (36) and diamine oxidase (37). In the early studies, we have shown that methylglyoxal-bis(butylamidinohydrazone) inhibits ODC, AdoMetDC, and spermidine synthase (22) and that methylglyoxal-bis(cyclohexylamidinohydrazone) inhibits AdoMetDC and spermidine synthase (23). These compounds also failed to inhibit spermine synthase, and showed little effects on intracellular contents of spermine. MGBCP, having a property to inhibit spermine synthase, distinctly lowered the concentration of spermine as well as that of spermidine in the tumor cells *in vivo*. This might be correlated with elongation of survival time of the tumor-bearing animals.

In conclusion, the data of this study show that advantageous features of MGBCP have no capacities to (*a*) stabilize AdoMetDC, and (*b*) accumulate putrescine which may be useful for tumor growth. These findings suggest MGBCP was a good antitumor agent.

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