Potentiation of the Antimitochondrial and Antiproliferative Effects of Bis(guanylyldrazones) by Phenethylbiguanide

Janusz Z. Byczkowski, Lech Zychlinski, and Carl W. Porter

ABSTRACT

The ability of methylglyoxal-bis(guanylyldrazine) (MGBG) and 4,4'-diacetylphenylurea-bis(guanylyldrazine) to interact with the hypoglycemic agent, phenethylbiguanide (DBI), in affecting the bioenergetic functions of isolated rat liver mitochondria was studied. DBI was found to increase markedly the inhibitory effect of either 4,4'-diacetylphenylurea-bis(guanylyldrazine) or MGBG on respiration of isolated rat liver mitochondria. Conversely, these bis(guanylyldrazones) enhanced the inhibitory potency of DBI and increased the apparent affinity of mitochondria for the drug. As with MGBG and 4,4'-diacetylphenylurea-bis(guanylyldrazine), the potassium cationophore, valinomycin, increased the sensitivity of mitochondrial respiration to DBI. It is suggested that the enhancement of bis(guanylyldrazine) inhibition of mitochondrial respiration by DBI involves inhibition of proton fluxes across the inner mitochondrial membrane and the subsequent alkalinization of the mitochondrial matrix. This drug interaction was extended to the level of antiproliferative activity in which DBI was found to potentiate the growth-inhibitory effects of MGBG on murine L1210 leukemia in vivo.

INTRODUCTION

Several of the bis(guanylyldrazones) have gained recognition for their antiproliferative activity in experimental and clinical settings (22). The best known in this regard is the aliphatic derivative, MGBG (Chart 1), which has been used clinically in the treatment of lymphoma and leukemia (15, 16, 36). Although the aromatic bis(guanylyldrazine), DDUG (Chart 1), displayed a 20 times greater therapeutic index than did MGBG against animal tumors, it demonstrated limited activity in clinical trials (22).

At present, the molecular mechanism responsible for the antiproliferative action of these bis(guanylyldrazones) remains uncertain. The drugs differ in their effectiveness at certain intracellular sites with growth-inhibitory potential. MGBG is a potent inhibitor of S-adenosylmethionine decarboxylase, a key enzyme in spermidine and spermine biosynthesis, while DDUG is without effect (8). By contrast, DDUG binds tightly to linear DNA and inhibits DNA polymerase while MGBG is only weakly active in these effects (10, 11). Recently, it has been found that both MGBG (25, 28) and DDUG (24) have in common the ability to interfere profoundly with structure and function of mitochondria. With both drugs, this action is apparent at growth-inhibitory drug concentrations and occurs prior to detectable inhibition of growth or decrease in cell viability (29, 31). On the basis of these and other considerations (30), it is proposed that the mitochondrial effects caused by MGBG and DDUG contribute significantly to the antiproliferative action of these drugs.

The effects of MGBG and DDUG on the bioenergetic functions of isolated rat liver mitochondria have been characterized recently (6). At molar concentrations, MGBG inhibits markedly State 4 respiration but only slightly affects State 3 or uncoupled respiration. In terms of concentration, DDUG is 18 times more effective than MGBG in this action. On the basis of permeability studies and microelectrophoresis of isolated mitochondria, it was concluded that MGBG and DDUG act by neutralizing the surface potential of the inner mitochondrial membrane and by interfering with cation fluxes across that membrane. The basis for the differential effect of MGBG and DDUG on mitochondria according to their metabolic state is thought (6) to be due to electrophoretic attraction of the drugs to the mitochondrial membrane during State 4 respiration by the transmembrane potential. According to the chemiosmotic theory (26), this effect would only exist during State 4 respiration and not State 3 or uncoupled respiration.

The overall effects of bis(guanylyldrazones) on isolated mitochondria are comparable in many respects to those obtained with the biguanides, particularly the hypoglycemic agent DBI (12, 14, 32, 33). It seems probable, therefore, that an interaction might exist between these agents at the level of the mitochondrion. The present data indicate that the effects of MGBG and DDUG on the respiration of isolated rat liver mitochondria are, in fact, potentiated by DBI. Moreover, DBI was found to enhance the antileukemic action of MGBG in an additive fashion. While the findings could have clinical relevance, they also support our contention (30) that mitochondrial effects may be responsible for the antiproliferative action of MGBG and DDUG.

MATERIALS AND METHODS

MGBG was obtained from the National Cancer Institute (Bethesda, Md.). DDUG and DBI were obtained from Ciba Research Laboratories (Basel, Switzerland) and Ciba-Geigy (Summit, N. J.), respectively. Mitochondrial substrates were obtained from Sigma Chemical Co. (St. Louis, Mo.).

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5 The abbreviations used are: MGBG, 1,1'-(methylethanediylidene)dinitrilo diguanidine (also known as methylglyoxal-bis(guanylyldrazine)); DDUG, 4,4'-diacetylphenylurea-bis(guanylyldrazine); DBI, 1-phenethylbiguanide (also known as phenerfin).; DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide-m-chlorophenylhydroxyladrazone.

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Interactions of Bis(guanylhydrazones) and DBI

Chart 1. The structural formulas for the aliphatic bis(guanylhydrazone), MGBG, for the aromatic bis(guanylhydrazone), DDUG, and for the hypoglycemic agent, DBI.

Louis, Mo.). All other reagents used were of analytical grade. Water was twice distilled from an all-glass apparatus and deionized before use.

Isolation of Mitochondria and Respiration Measurements. Rat liver mitochondria were prepared by the method of Weinbach (37) essentially as described elsewhere (4, 5). The medium used for homogenization and centrifugation contained 250 mM sucrose (BHD Chemical Ltd.), 1 mM ethylene glycol bist(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (Sigma), and 10 mM Tris-HCl, pH 7.2.

Respiration of mitochondria was measured polarographically as described previously (6) with a Clark oxygen electrode at 25°C. The reaction mixture (final volume, 1.5 ml) contained 15 mM KCl and 50 mM Tris-HCl, pH 7.2, according to the method of Slater and Holton (35). In this half-isotonic medium, the respiratory measurements were essentially the same as those made in isotonic medium except that the rate transitions were more much more sharply demarcated. Oxygen consumption was recorded after the addition of 50 μl of mitochondrial suspension (3.5 mg of protein) to the electrode chamber containing medium and 5 mM glutamate, 5 mM succinate (plus glutamate), or 1 mM ascorbate (plus 0.1 mM N,N,N',N'-tetramethyl-p-phenylenediamine) as substrate. Two min after the addition of mitochondria, DDUG or MGBG was added in a volume of 10 to 20 μl using a Hamilton microsyringe. Relative oxygen consumption was calculated by assuming that the respiration rate before the drug addition was 100% of activity. Protein concentrations were estimated by the method of Lowry et al. (17).

L1210 Leukemia System. L1210 leukemia cells (10⁶) obtained from the peritoneal cavity of female DBA/2-Jax mouse donors, 5 to 6 days after implantation, were inoculated i.p. into test mice. Under these conditions, the average survival time of untreated animals is 8 to 9 days. DBI (40 or 80 mg/kg) and/or MGBG (12.5, 25, or 50 mg/kg) was injected i.p. into leukemic mice for 6 days, starting on the day after tumor inoculation. Each drug or drug combination was tested on a group of 10 mice, 5 mice/cage. Cages were monitored twice daily for dead mice.

RESULTS

State 4 respiration of isolated rat liver mitochondria was inhibited by DDUG, DBI, or MGBG (given in order of effectiveness, see Table 1). We have shown previously (6) that DDUG at low concentrations (<0.2 mM) stimulates State 4 respiratory activity, while at higher concentrations (>0.3 mM) it strongly inhibits respiration. DBI, by contrast, has no effect at concentrations lower than that required to inhibit respiration (1.4 mM) and is similar to MGBG in this respect. When stimulatory concentrations of DDUG were added to mitochondria pre-treated with slightly inhibitory (1.4 mM) or even noninhibitory (1 mM) concentrations of DBI, a marked inhibition of State 4 respiration occurred (Chart 2, A and B). This inhibition was not influenced by the subsequent addition of either DNP or CCCP (Chart 2A), whereas respiratory inhibition imposed by DBI alone was reversed by the uncoupling agent (Chart 2C). When the drug sequence was reversed (i.e., stimulatory concentrations of DDUG followed by DBI), an inhibition of respiration occurred which was not reversible with DNP (Chart 2D). The addition of either DDUG or DBI to mitochondria uncoupled with DNP or CCCP had little effect on oxygen utilization (Chart 2, F and G). However, when both DBI and DDUG were present, marked inhibition of uncoupled respiration was apparent (Chart 2, F and G). Qualitatively, similar effects were obtained with either succinate (plus glutamate) or glutamate alone as substrate.

Table 1 summarizes the various dose-dependent interactions of DDUG, MGBG, and DBI on the inhibition of State 4 respiration. At noninhibitory concentrations, both MGBG and DDUG enhanced the inhibitory effect of DBI on respiration with DDUG being more effective in this regard. From the double-reciprocal plot of DBI inhibitory ability versus concentration (Chart 3), it seems that MGBG and DDUG increased significantly the inhibitory potency of DBI and the apparent K_i at high concentrations (>2.0 mM). Similarly, pretreatment of mitochondria with even noninhibitory concentrations (1 mM) of DBI enhanced the inhibitory effects.

Table 1

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Inhibitor</th>
<th>I_50 for inhibitor * (mM)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>MGBG</td>
<td>6.00</td>
</tr>
<tr>
<td>0.3 mM DDUG</td>
<td>MGBG</td>
<td>1.50</td>
</tr>
<tr>
<td>1 mM DBI</td>
<td>MGBG</td>
<td>1.33</td>
</tr>
<tr>
<td>None</td>
<td>DDUG</td>
<td>0.33</td>
</tr>
<tr>
<td>1 mM DBI</td>
<td>DDUG</td>
<td>0.19</td>
</tr>
<tr>
<td>4 mM DBI</td>
<td>DDUG</td>
<td>0.17</td>
</tr>
<tr>
<td>2.5 mM MGBG</td>
<td>DDUG</td>
<td>0.18</td>
</tr>
<tr>
<td>None</td>
<td>DBI</td>
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</tr>
<tr>
<td>2.5 mM MGBG</td>
<td>DBI</td>
<td>0.85</td>
</tr>
<tr>
<td>0.3 mM DDUG</td>
<td>DBI</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*I_50 is calculated as the inhibitor concentration causing 25% inhibition of respiratory activity assuming 100% activity before inhibitor addition.

Table 1 Interactions of DDUG, MGBG, and DBI involving inhibition of respiratory activity of rat liver mitochondria at State 4

The incubation medium contained 15 mM KCl and 50 mM Tris-HCl (pH 7.2). Succinate (plus glutamate) was a substrate. In this medium, drug effects on bioenergetic functions were the same as in isotonic medium except that respiratory transitions were more sharply demarcated and therefore easier to measure. Mitochondria at respiratory State 4 were pretreated with DBI, DDUG, or MGBG, and 2 min later another inhibitor was introduced at different concentrations.

Chart 2. Synergistic inhibition of oxygen uptake by rat liver mitochondria with DBI and DDUG using glutamate (A to C) or succinate (plus glutamate) as substrate (D to G). Incubation medium contained 15 mM KCl and 50 mM Tris-HCl, pH 7.2.
litory activity of DDUG or MGBG (Table 1). Comparable interactions were also noted between DDUG and MGBG with either bis(guanylhydrazone) enhancing the potency of the other. By contrast, pretreatment of mitochondria with the polyamines putrescine, spermidine, or spermine actually protected mitochondrial respiration from the inhibitory actions of DBI, DDUG, or MGBG (data not shown).

The presence of valinomycin in the medium increases significantly the inhibition of mitochondrial respiration by DBI. From the double-reciprocal plot of DBI inhibitory action versus concentration (Chart 4), it appears that valinomycin enhanced the inhibitory potency of DBI without altering the apparent \( K_i \) for the drug.

In the studies testing the antileukemia action of DBI, MGBG, and their combination, the untreated mice bearing L1210 leukemia cells died 9 days after tumor cell inoculation (Table 2). Treatment of mice with MGBG alone resulted in a prolongation of survival time by 4.5 days with no evidence of toxicity at the maximum sequential dose of 50 mg/kg. DBI at 40 or 80 mg/kg increased the survival time by slightly more than 2 days. The drug combination of MGBG at 25 mg/kg and DBI at 80 mg/kg was the most effective among those tested and resulted in a 66% (6-day) increase in life span. When MGBG was used in the drug combination at 50 mg/kg, toxicity was encountered as evidenced by an increase in mortality. Overall, the combination of DBI and MGBG at the optimum drug concentrations appears to be an additive interaction with respect to antileukemic activity.

**DISCUSSION**

The present data demonstrate that, at the level of isolated rat liver mitochondria, DDUG and MGBG interact with DBI to inhibit State 5 respiration in a synergistic manner. Pretreatment of mitochondria with noninhibitory concentrations of either bis(guanylhydrazone) permits marked inhibition of respiration by concentrations of DBI that are otherwise ineffective when added alone. Similarly, when the reverse situation is applied, DBI permits inhibition of mitochondrial respiration by ineffective concentrations of DDUG or MGBG. The double-reciprocal plot of DBI dose-dependent inhibition suggests that this synergism is the consequence of both enhanced DBI inhibitory potential and greater binding of DBI to mitochondria, as indicated by the increased \( K_i \) (Chart 3).

Inhibition of mitochondrial respiration by DBI was also increased by valinomycin, a cationophore specific for potassium (Chart 4). The nature of this drug interaction at the level of mitochondria was distinctly different from that of DBI with either bis(guanylhydrazone). The latter drug combination decreased markedly the apparent \( K_i \) for DBI (Chart 3), whereas the combination of DBI and valinomycin did not (Chart 4), suggesting different mechanisms for increased respiratory inhibition.

It is interesting that valinomycin enhanced the inhibition of respiratory by DBI while classical uncouplers, such as DNP or CCCP, did not (Chart 2). The latter act by dissipating the proton gradient across the inner mitochondrial membrane, while valinomycin increases potassium fluxes across the membrane which is usually followed by an equal and opposite movement of protons (27). These 2 processes, potassium fluxes and proton ejection, are believed to be inhibited by DBI (32). Thus, respiration stimulated by valinomycin appears to be more sensitive to DBI than does respiration uncoupled by DNP or CCCP, which apparently is not dependent on processes affected by DBI. It is suggested that the enhancement of bis(guanylhydrazone) inhibition of mitochondrial respiration by DBI involves inhibition of proton fluxes across the inner mitochondrial membrane with possible subsequent alkalization of the mitochondrial matrix.

From the antileukemic data, it is apparent that the interaction of DBI and MGBG in inhibiting mitochondria also exists with respect to antiproliferative activity, at least as additivity (Table 2). At optimum drug concentrations, DBI increased the antileukemic effects of MGBG by about 30%, which is not quite as much as that caused by pretreatment of mice with \( \alpha \)-difluoromethylornithine (2). The latter is an extremely specific inhibitor of ornithine decarboxylase (18), which has no significant mitochondrial effects (30). Unlike DBI, it enhances MGBG cytotoxicity by depleting tissues of spermidine and thereby increas-

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**Table 2**

<table>
<thead>
<tr>
<th>DBI Concentration (mg/kg/day)</th>
<th>None</th>
<th>12.5 mg/kg/day</th>
<th>25 mg/kg/day</th>
<th>50 mg/kg/day</th>
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<tr>
<td>Survival (days after inoculation)</td>
<td>11.0 ± 1.3</td>
<td>11.3 ± 0.7</td>
<td>11.8 ± 1.1</td>
<td>13.5 ± 0.8</td>
</tr>
<tr>
<td>40</td>
<td>11.2 ± 0.8</td>
<td>13.0 ± 1.4</td>
<td>13.3 ± 0.7</td>
<td>10.9 ± 1.0</td>
</tr>
<tr>
<td>80</td>
<td>11.4 ± 1.9</td>
<td>12.6 ± 0.9</td>
<td>15.2 ± 0.8</td>
<td>11.1 ± 1.4</td>
</tr>
</tbody>
</table>

* Treatments were given i.p. once daily for 6 days starting the day after tumor inoculation.

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**Chart 3.** The double-reciprocal plot of the dose-dependent inhibition by DBI of the respiratory activity of rat liver mitochondria with succinate (plus glutamate) as substrate in the presence of DDUG or MGBG. Percentage of inhibition was calculated as 100 minus relative respiratory activity. For other experimental details, see Chart 2 and Table 1.
ing their uptake of MGBG (1), which utilizes the serine dermidine carrier mechanism (9). We suggest that the increased uptake with α-difluoromethylornithine leads to increased inhibition of mitochondrial function.

Several of the biguanides, in addition to being hypoglycemic agents, have potential in cancer chemotherapy. DBI potentiates the antitumor effects of cyclophosphamide and hydrazine sulfate (13) and 1,3-bis(2-chloroethyl)-1-nitrosourea (7). The antidiabetic agent, decamethylenebiguanide (also known as Synthalin), inhibits the growth of L1210 leukemia and Sarcoma 180 cells in vivo (23), and the action of MGBG on transplanted mouse leukemia is potentiated by the biguanides, stilbamidine, hydroxystilbamidine, and decamethylenebiguanide (2, 3, 20, 21). Whether or not the DBI enhancement of antitumor activity of cyclophosphamide and hydrazine sulfate (13) or 1,3-bis(2-chloroethyl)-1-nitrosourea (7) are also related to drug interactions at the level of the mitochondrion seems unlikely since the action of these latter drugs is not thought to involve that organelle. In the case of the biguanides, however, there is increasing evidence (6, 29, 30) that the antiproliferative action may be related to their effects on mitochondria. Certainly, the data presented here showing synergistic drug effects at the level of the mitochondria and additive effects in antileukemic activity by the drug combination is supportive of a casual relationship. Moreover, recent studies (19, 30) indicate that the seemingly high concentrations of MGBG used in these experiments with mitochondria are actually comparable to those achieved intracellularly by a variety of cell types exposed to growth-inhibitory (10 μM) concentrations of drug (34).

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