Antitumor Activity and Bone Marrow Toxicity of Aminoglucose Mustard Anticancer Agents in Mice

James E. Cantrell, Jr., Dianna Green, and Philip S. Schein

Division of Medical Oncology, Vincent T. Lombardi Cancer Research Center, Georgetown University School of Medicine, Washington, DC 20007

ABSTRACT

In previous structure-activity studies, we have demonstrated that attachment of a glucose molecule to the chloroethylnitrosourea cytotoxic group produces a compound with reduced murine bone marrow toxicity and retention of full antitumor activity. To further define this protective role conferred by the glucose moiety in bone marrow cells, we have replaced the nitrosourea cytotoxic group with another class of alkylating agent, a bifunctional nitrogen mustard. In a detailed structure-activity analysis, we have now characterized four analogues, with the mustard cytotoxic group positioned at carbon 2 [1,3,4,6-tetra-O-acetyl-2-(di-2-chloroethyl)aminolo-2-deoxy-D-glucopyranosylamine (TGM)], carbon 6, or carbon 1 (D- and L-isomers) of the aminoglucose molecule. On a molar basis, TGM was most toxic to normal BALB/c × DBA/2 F1 mice, with a 10% lethal dose (LD10) of 3.8 μmol/kg. The D- and L-isomers of 2,3,4,6-tetra-O-acetyl-N,N-bis(2-chloroethyl)glucopyranosylamine (C-1) were the least toxic, with an LD10 of 73 μmol/kg for both. Optimal antitumor activity against the murine P388 leukemia (single i.p. administration of the LD10) did not differ significantly among the four analogues, with increased life span ranging from 83–86%. P388 antitumor activity for nitrogen mustard (HN2) was significantly less, 60% increased life span (P = 0.01), while p-di(2-chloroethyl)aminolo-1-phenylalanine produced an increased life span of >101%. An LD10 of 6-bis-(2-chloroethyl)aminolo-6-deoxy-D-glucose (C-6) or TGM produced significantly less depression of WBC counts than did an equitoxic dose of the C-1 isomers, HN2, or p-di(2-chloroethyl)aminolo-1-phenylalanine. The mean nadir WBC count for C-6 equaled 86% of control, and for TGM, 80% of control. Consistent with this sparing effect on the peripheral WBC, C-6 and TGM produced significantly less in vivo murine bone marrow DNA synthesis depression, 77 and 64% of control, respectively, as compared to the depression nadir produced by HN2 (27% of control), the D-isomer of C-1 (17%), the L-isomer of C-1 (18%), and p-di(2-chloroethyl)aminolo-1-phenylalanine (2%). These structure-activity studies demonstrate that conjugation of the mustard cytotoxic group to carbon 6 or carbon 2 of glucose produces an analogue that retains P388 antitumor activity significantly greater than that of HN2, with a concomitant reduction in murine bone marrow toxicity.

INTRODUCTION

In previously reported structure-activity analyses of chloroethylnitrosoureas, our laboratory has demonstrated that conjugation of the cytotoxic moiety to a specific site on an aminoglucose molecule can alter its effects on normal and tumor tissues (2–5). We have identified the attachment of the nitrosourea cytotoxic group to a glucose molecule as a specific structural modification that can be correlated with reduced bone marrow toxicity. One such compound, 2-3-(2-chloroethyl)-3-nitrosoxadiazole-β-glucopyranosylamine (chlorozotocin), has significantly reduced murine myelotoxicity with full retention of antitumor activity, as compared to 1,3-bis(2-chloroethyl)-1-nitrosoourea or 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosoourea (2, 3); 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosoourea is a second glucose-containing nitrosourea with reduced bone marrow toxicity (6). The clinical application of this phenomenon, however, is limited by the innate therapeutic effectiveness of the chloroethylnitrosoureas for human cancers. To further evaluate the protective role conferred by glucose in bone marrow cells, we have replaced the nitrosourea cytotoxic moiety with another class of alkylating agent, a bifunctional nitrogen mustard. Preliminary studies with one compound, TGM, revealed that the concepts of molecular pharmacology and drug structure remain valid: as compared to nitrogen mustard, an LD10 of TGM produced greater in vivo antitumor activity for the P388 murine leukemia, while the marrow toxicity was significantly reduced (7).

In an attempt to exploit this phenomenon, we have now completed a detailed structure-activity analysis of three additional aminoglucose mustard compounds. The nitrogen mustard cytotoxic moiety has been attached to carbon 6 or carbon 1 (D- and L-isomers) of the glucose molecule. Structures of the compounds are presented in Fig. 1.

MATERIALS AND METHODS

The four glucose mustards were provided for our studies by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Dr. John Montgomery of the Southern Research Institute, Birmingham, AL, synthesized the four compounds, which include: C-6 (NSC-340853), C-1D (NSC-342215), C-1L (NSC-342216), and TGM (NSC-344281). HN2 (NSC-762) and L-PAM (NSC-8806) were also kindly provided by the Drug Development Branch of the National Cancer Institute.

Estimation of LD10. Groups of 10 normal male BALB/c × DBA/2 F1 mice (hereafter called CD2F1) were treated with graded doses of each compound to determine a 10% lethal dose. C-6 and nitrogen mustard were dissolved in 0.9% sodium chloride (saline) at 4°C immediately prior to use. The tetraacetate compounds (TGM, C-1D, and C-1L) were dissolved in ethyl alcohol, and the resultant solution was added to hydroxypropyl cellulose (0.3% solution) to give a final concentration of approximately 4% ethyl alcohol and 96% hydroxypropyl cellulose. L-PAM was dissolved in ethyl alcohol containing less than 1% concentrated hydrochloric acid, and this solution was similarly added to the hydroxypropyl cellulose. Vehicle control mice received either saline or the ethyl alcohol-hydroxypropyl cellulose diluent.

Antitumor Studies. The murine P388 leukemia, maintained in female DBA/2 F1 mice, was used to evaluate antitumor activity. This tumor was selected because of its known sensitivity to nitrogen mustard and L-PAM (8). Male CD2F1 mice, weighing 18–24 g and maintained on Lab-Blox laboratory chow pellets and water, ad libitum, were used for all experiments.

Each drug was administered i.p. (0.1 ml/10 g body weight) to groups of 8 or 10 male CD2F1 mice on Day 1 after implantation of 1 × 106 P388 leukemia cells i.p. The mean survival for drug-treated animals was compared to the survival of control tumor-bearing animals that received vehicle.
received appropriate volumes of vehicle (8). Drug doses producing 10% lethality (single i.p. injection) were concurrently determined in normal CD2F1 mice.

Bone Marrow Toxicity Studies. Measurement of serial peripheral leukocyte (WBC) counts was performed using a 20-μl sample of retro-orbital sinus blood obtained from normal CD2F1 mice on Day 3, 4, or 10 following i.p. drug administration. Each sample was diluted in 9.98 ml of Isoton (Coulter Diagnostics, Hialeah, FL) and counted in a model ZBI Coulter Counter after lysis of erythrocytes with Zap-aglobin (Coulter Diagnostics). Mean WBC counts for drug-treated animals were compared to the values obtained for vehicle-treated mice.

Normal CD2F1 mice were used to investigate the comparative effects of the six drugs on in vivo murine bone marrow DNA synthesis. For each compound, animals received a single i.p. injection of the 1.1·mol/kg of drug, or an equal volume of vehicle. Serial WBC counts were performed over a 10-day period. The nadir of WBC count depression occurred on Day 4 for all the drugs tested, except nitrogen mustard in the hydroxypropyl-cellulose vehicle, which had a Day 3 nadir. These results, representing three replicate experiments, are summarized in Table 3. In comparison with the other compounds, C-6 and TGM produced a statistically significant decrease in myelosuppression. The mean nadir WBC counts for C-6 equaled 86% of control, and for TGM, 80% of control. The most myelosuppressive drug, L-PAM, produced a WBC nadir of 38% of

RESULTS

As summarized in Table 1, LD10s for the analogues varied widely despite similarities in chemical structure. On a molar basis, TGM was approximately 19-fold more lethal than were the least toxic C-1 isomers. Both the α- and β-isomers of C-1 had LD10s of 73 μmol/kg. The lethal toxicities of C-6 and L-PAM were not significantly different, with LD10s of 46 and 39.6 μmol/kg, respectively.

The compounds were evaluated against the murine P388 ascitic leukemia. Groups of 10 mice were treated with a single i.p. LD10 of each drug or vehicle on Day 1 following tumor implantation. For all the drugs tested, the LD10 proved to be the maximally effective single dose. The results, summarized in Table 2, represent three replicate experiments. Overall survivals did not differ significantly among the glucose-containing compounds; increased life span ranged from 83–86%. The increased life span produced by HN2 (60%) was significantly decreased as compared to any other single drug (P = 0.01). The only long-term survivors were 4 of 30 mice receiving an LD10 of L-PAM, and 2 of 20 mice receiving a 20% lethal dose, that were alive on Day 45.

The effect of each drug on serial peripheral leukocyte count was also determined. Groups of 10 normal CD2F1 mice received a single i.p. injection of the LD10 of drug, or an equal volume of vehicle. Serial WBC counts were performed over a 10-day period. The nadir of WBC count depression occurred on Day 4 for each drug, except nitrogen mustard in the hydroxypropyl-cellulose vehicle, which had a Day 3 nadir. These results, representing three replicate experiments, are summarized in Table 3. In comparison with the other compounds, C-6 and TGM produced a statistically significant decrease in myelosuppression. The mean nadir WBC counts for C-6 equaled 86% of control, and for TGM, 80% of control. The most myelosuppressive drug, L-PAM, produced a WBC nadir of 38% of

Table 1 Approximate LD10s (single dose, i.p. administration) in CD2F1 mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg/kg</th>
<th>μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>3.0</td>
<td>15.6</td>
</tr>
<tr>
<td>L-PAM</td>
<td>12.0</td>
<td>39.6</td>
</tr>
<tr>
<td>C-6</td>
<td>14.0</td>
<td>46</td>
</tr>
<tr>
<td>C-1D</td>
<td>34.5</td>
<td>73</td>
</tr>
<tr>
<td>C-1L</td>
<td>34.5</td>
<td>73</td>
</tr>
<tr>
<td>TGM (C-2)</td>
<td>1.8</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Table 2 Antitumor activity against murine P388 leukemia

Drugs were administered as a single i.p. dose on Day 1 following implantation of 10^6 P388 cells (i.p.) on Day 0.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (μmol/kg)</th>
<th>% of ILS* (range)</th>
<th>No. of 45-day survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGM</td>
<td>3.8*</td>
<td>86 (83–87)</td>
<td>0/30</td>
</tr>
<tr>
<td>C-6</td>
<td>46*</td>
<td>86 (83–89)</td>
<td>0/30</td>
</tr>
<tr>
<td>C-1D</td>
<td>73*</td>
<td>83 (81–85)</td>
<td>0/30</td>
</tr>
<tr>
<td>C-1L</td>
<td>73*</td>
<td>86 (82–88)</td>
<td>0/30</td>
</tr>
<tr>
<td>HN2</td>
<td>15.6*</td>
<td>60 (57–62)</td>
<td>0/30</td>
</tr>
<tr>
<td>HN2</td>
<td>18.2*</td>
<td>31</td>
<td>0/20</td>
</tr>
<tr>
<td>L-PAM</td>
<td>39.6*</td>
<td>&gt;101</td>
<td>4/30</td>
</tr>
<tr>
<td>L-PAM</td>
<td>48.8*</td>
<td>&gt;91</td>
<td>2/20</td>
</tr>
</tbody>
</table>

* ILS, increased life span.
* LD10, 20% lethal dose.
control on Day 4. Wilcoxon-Mann-Whitney U test statistical analysis contains the significance of these differences at a $P = 0.01$ level (Table 3).

The effect of each drug on in vivo bone marrow DNA synthesis was determined by quantitation of $[^3H]$thymidine incorporation into extractable DNA following i.p. administration of an LD$_{50}$ of drug. Results as dpm/$\mu$g DNA were compared with the thymidine uptake in mice receiving vehicle only, and were expressed as the mean percentage of control (dpm/$\mu$g DNA). The data for TGM, C-6, HN2, and L-PAM are summarized in Fig. 2. The maximum level of DNA synthesis depression occurred at 8 h (for C-6, TGM, HN2, and L-PAM) or 24 h (for C-1D and C-1L). Consistent with their sparing effect on the peripheral WBC count, C-6 and TGM produced significantly less bone marrow DNA synthesis depression, 77 and 64% of control, as compared to the depression nadir observed with HN2 (27%), C-1D (17%), and C-1L (18%), or the most marked DNA synthesis depression with L-PAM, with a nadir of 2% of vehicle-treated control mice. Differences were also apparent in the length of the interval of recovery to the pretreatment DNA synthesis rate: bone marrows from C-6- and TGM-treated mice had recovered to 120 and 130% of control within 24 h. In contrast, the values for HN2, L-PAM, C-1L, and C-1D were, respectively, 88, 20, 18, and 17% of control at the 24-h time point. Bone marrows of animals treated with L-PAM, C-1L, or C-1D did not recover to the pretreatment DNA synthesis rate until 72 h post-drug administration.

An LD$_{50}$ of TGM did not produce hyperglycemia, as determined by blood glucose levels. As summarized in Table 4, an LD$_{50}$ of TGM produced no significant increase in blood glucose over vehicle-treated controls on Day 3 following drug administration. An LD$_{100}$ (3.6 mg/kg) similarly produced no increase in blood glucose levels.

**DISCUSSION**

In the past, nitrogen mustard analogues have been synthesized with modifications designed to increase tumor tissue specificity. L-PAM, a mustard compound with the cytotoxic moiety conjugated to phenylalanine, represented an attempt to synthesize a drug to exploit its potential preferential localization in melanoma cells, which utilize phenylalanine to synthesize melanin, and in rapidly growing tumor cells with a high requirement for amino acids. It is transported by carrier-mediated amino acid transport systems of the leucine type (12). However, L-PAM and other clinically available mustard congeners do not differ substantially in their major limiting toxicity, bone marrow suppression.

We have previously demonstrated that the bone marrow toxicity of nitrosoureas can be reduced by conjugation of the cytotoxic moiety to an aminoglucose molecule (2-5). Subsequently, we validated this concept with TGM, a C-2 amino-glucose mustard analogue (7). To investigate further this phenomenon of reduced myelotoxicity conferred by the glucose moiety, new analogues were synthesized with the mustard cytotoxic group positioned at the C-1L, C-1D, or C-6 of the aminoglucose molecule. Using a murine model, we have characterized these glucose-containing compounds in comparative studies with L-PAM and nitrogen mustard.

LD$_{10}$ studies utilizing normal mice demonstrated significant variations in dose lethality among the six compounds. TGM, the C-2 analogue, was the most toxic: on a molar basis, this compound was 12-fold more toxic than the C-6 analogue and 19-fold more toxic than the C-1 compounds. Lethal toxicities for the C-1 isomeric forms were comparable, with an LD$_{10}$ of 73 $\mu$mol/kg for each. These comparative studies, particularly the equivalent toxicities of the C-1D and C-1L isomers, demonstrate the importance of the position of the glucose carbon

**Table 4** Blood glucose concentration in CDF$_1$ mice, feeding ad libitum, after i.p. administration of TGM or HN2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg i.p.)</th>
<th>Blood glucose* (mg/100 ml)*</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>160</td>
<td>195</td>
<td>3</td>
</tr>
<tr>
<td>Vehicle*</td>
<td>180</td>
<td>195</td>
<td>3</td>
</tr>
<tr>
<td>TGM</td>
<td>1.8$^a$</td>
<td>180</td>
<td>5</td>
</tr>
<tr>
<td>TGM</td>
<td>3.6$^a$</td>
<td>129</td>
<td>5</td>
</tr>
<tr>
<td>HN2*</td>
<td>6$^a$</td>
<td>135</td>
<td>5</td>
</tr>
</tbody>
</table>

* Day 3 after drug treatment.
* Mean value.
* 5% vegetable oil, 5% ethanol, 90% saline (vehicle used for TGM).
* LD$_{10}$.
* LD$_{50}$.
to which the nitrogen mustard cytotoxic group is attached in determining the pharmacology of the compound.

The glucose analogues were compared for toxicity to normal murine bone marrow, as measured by depression of the peripheral WBC count as well as in vivo bone marrow DNA synthesis depression. When administered at an LD$_{10}$, C-6 and TGM were significantly less myelosuppressive than the C-1 isomers, L-PAM, and nitrogen mustard ($P < 0.01$).

Optimal P388 antitumor activity (for a single LD$_{10}$) was not statistically different among the four analogues, while the antitumor activity of nitrogen mustard was significantly less ($P = 0.01$). L-PAM produced P388 antitumor activity superior to that achieved with the glucose analogues, with 4 of 30 animals surviving beyond 45 days.

Overall, these results demonstrate that the activity of the bifunctional nitrogen mustard alkylating molecule is substantially altered by its conjugation to a glucose moiety. There are statistically significant differences in both lethality and myelosuppression among the four glucose analogues. Conjugation of the cytotoxic group to the C-2 or C-6 position of glucose produces an analogue that retains P388 antitumor activity significantly greater than that of nitrogen mustard, with a concomitant reduction in murine bone marrow toxicity.

For the chloroethylnitrosourea alkylating agents, studies reported by Anderson et al. (13) provide evidence that glucose-mediated active transport is not important for either the antitumor activity or the reduced myelotoxicity of D-chlorozotocin. In comparative studies with L-chlorozotocin, there was no significant difference in the dose-response curves for lethal toxicity in CD2F$_1$ mice. Further, L-chlorozotocin demonstrated antitumor activity against L1210 leukemia equivalent to that achieved with D-chlorozotocin, and the relative bone marrow sparing effects of the two isomers were comparable. These studies demonstrate that active transport of D-chlorozotocin via the D-glucose moiety is not important in determining its structure-activity characteristics. Similarly, we report here that in mice the lethal toxicities, bone marrow toxicities, and optimal antitumor activities for the D- and L-isomers of C-1 glucose mustard are equivalent. In vitro, Tew and Wang (14) have recently evaluated these D- and L-isomers using two Walker carcinoma cell lines, one sensitive to alkylating agents (WS), and the other a resistant line (WR) developed by sequential exposure of the parent WS line to chlorambucil. They report that for concentrations up to 10 $\mu$M, the D-isomer was slightly more toxic against WS cells (as measured by inhibition of colony-forming ability) than was the L-isomer. Neither isomer had significant activity against the WR cells at drug concentrations up to 50 $\mu$M.

Our laboratory has recently demonstrated that the reduced myelotoxicity of the glucose-containing chloroethylnitrosourea analogues, chlorozotocin and 1-(2-chloroethyl)-3-(6-D-glucopyranosyl)-1-nitrosourea, can be correlated with a decreased quantitative alkylation of DNA in murine hematopoietic as compared to L1210 leukemia cells (3, 5). We have also correlated this relative reduction in bone marrow toxicity with decreased alkylation of transcriptionally active regions of murine and human bone marrow chromatin, when compared to the more myelotoxic nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (4, 5). We are now determining the significance of these differing patterns of alkylation for the less myelotoxic glucose-containing mustard compounds, C-6 and TGM. These studies should further define, at a molecular level, the specific drug interactions that contribute to antitumor activity and to myelotoxicity.

**REFERENCES**

8. Screening Data Summary, Drug Evaluation Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, 1977.


Antitumor Activity and Bone Marrow Toxicity of Aminoglucose Mustard Anticancer Agents in Mice

James E. Cantrell, Jr., Dianna Green and Philip S. Schein


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/5/2340

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.