A Comparison of the Biological and Biochemical Properties of 1-(4-Amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea and 2-[3-(2-Chloroethyl)-3-nitrosoureido]-D-glucopyranose

Robert A. Nagourney, Patricia Fox, and Philip S. Schein

Division of Medical Oncology, Georgetown University Hospital, 3800 Reservoir Road, N.W., Washington, D.C. 20007.

ABSTRACT

1-(4-Amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU) is a water-soluble nitrosourea that has produced delayed hematological toxicity in man during Phase 1 clinical trials. ACNU has in vitro alkylating activity 40% less than that of 2-(3-(2-chloroethyl)-3-nitrosoureido)-D-glucopyranose (chlorozotocin) but shares the property of negligible carbamoylating activity with the latter compounds. ACNU is highly active against murine L1210 leukemia. However, the maximum therapeutic dose, 30 mg/kg (a dose lethal to 10% of the animals), produced a 64% reduction in peripheral WBC and an 85% decrease in circulating neutrophils in normal mice. This was correlated with a 76% inhibition of normal mouse bone marrow DNA synthesis within 24 hr after treatment, followed by full recovery within 48 hr. In contrast, DNA synthesis in L1210 cells was suppressed to less than 10% of control for a minimum of 72 hr.

While ACNU, a pyrimidine analog, possesses many of the chemical properties of chlorozotocin, it does not share with the latter compound its reduced myelotoxicity at therapeutic doses. The glucose carrier of the chlorozotocin molecule appears to impart the selective sparing of normal bone marrow.

INTRODUCTION

The principal limitation of the effective clinical application of the chloroethynitrosourea antitumor agents has been delayed and cumulative myelosuppression (5, 7). Attention has recently been focused upon the property of water solubility and its potential for reducing nitrosourea bone marrow toxicity (9). Preclinical studies of the glucose-containing analogs, 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose (chlorozotocin) and 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea, have demonstrated curative activity against murine L1210 leukemia and reduced bone marrow toxicity at 10% lethal doses (1, 6).

ACNU1 is a pyrimidine analog that has also demonstrated curative activity against L1210 leukemia in mice (2). Recently completed Phase 1 clinical trials in Japan demonstrated activity for a range of human tumors (4). Nevertheless, at therapeutic dose levels, ACNU has produced delayed and cumulative bone marrow toxicity consistent with what has been observed previously with CCNU (12).

This report describes the chemical and biological properties of ACNU that are compared to those of chlorozotocin and CCNU. The delayed and cumulative bone marrow toxicity observed in the Japanese Phase 1 clinical trials are correlated with the acute bone marrow toxicity of this compound in mice. The results demonstrate the value of the murine model for predicting nitrosourea myelosuppressive toxicity in man and the specificity of the glucose carrier for the reduced myelosuppressive activity of chlorozotocin.

MATERIALS AND METHODS

Male BALB/c × DBA/2 F1 (hereafter called CD2F1) mice weighing 17 to 25 g, obtained from Hazelton Laboratories, Vienna, Va., were used throughout the experiment. The animals were maintained on Lab Blox laboratory chow pellets and water ad libitum. Chlorozotocin (NSC 178248), ACNU (NSC D 245382), CNU (NSC 47547), and CCNU (NSC 79-037) were kindly supplied by Dr. Harry Wood, Drug Development Branch, National Cancer Institute, Silver Spring, Md. Chlorozotocin and ACNU were dissolved in 0.01 M citrate buffer, pH 4.0. CCNU was suspended in an aqueous solution of 5% polyethoxylated vegetable oil (Mellphil; Antara Chemicals, New York, N.Y.) and 5% ethanol. All drugs were injected i.p. at a volume of 0.1 ml/10 g body weight. The mouse L1210 leukemia system was utilized to assess antitumor activity. Drugs were injected on the second day following i.p. implantation of 1 x 10⁶ L1210 cells. The percentage of ILS was calculated from the survival of tumor-bearing control animals that received appropriate volumes of the vehicles alone (10).

We used CD2F1 mice 4 days after implantation of 1 x 10⁶ L1210 cells to investigate the effect of ACNU upon DNA synthesis. The animals received a single i.p. injection of ACNU, 30 mg/kg, or the citrate buffer. One hr prior to sacrifice, 100 μCi of [methyl-5-3H]thymidine (specific activity, 19 Ci/m mole; Amersham/Searle Corp., Arlington Heights, Ill.) were given i.p. to each animal. The mice were sacrificed by cervical dislocation at 0, 8, 24, 48, or 72 hr posttreatment. The ascites tumor was aspirated from the abdominal cavity into 0.01 M sodium phosphate-buffered 0.85% NaCl solution, pH 7.4, at 4°C. Both femurs were removed, and the bone marrows were expressed with sodium phosphate-buffered 0.85% NaCl solution. We have previously determined that the bone marrows of mice,

Received July 25, 1977; accepted September 27, 1977.
examined after 4 days of i.p. L1210 growth, are not replaced by tumor (14). The DNA content of the ascites and bone marrows at each point was extracted with the use of a modification of the method of Schneider (11). A 0.5-ml aliquot of the final supernatant was added to 10 ml of liquid scintillation fluid (Scintiverse; Fisher Scientific Co., Pittsburgh, Pa.) and counted in a Searle Mark III liquid scintillation spectrometer with automatic quench correction and a counting efficiency of 35% for $^3$H. An additional 0.5-ml aliquot of the supernatant was used for the measurement of DNA by the method of Burton (3). Results as dpm/µg DNA were compared with the uptake in 0 time controls and are expressed as the mean percentage of control.

Serial peripheral WBC were performed with a 20-µl sample of retroorbital sinus blood from normal CD2F₁ mice; this was placed in 9.98 ml of Isoton (Curtin-Matheson Scientific, Houston, Texas). WBC were counted in a Model ZBI Coulter Counter, after lysis of RBC with Zapoglobin (Curtin-Matheson).

Differential WBC were performed on Wright-stained smears of blood taken on the third day following treatment, which was found to be the nadir of WBC depression. Statistical significance was determined by the Dünnet test at $p < 0.05$ (8).

We determined in vitro alkylating activity by a method previously described by Wheeler et al. (13). Each nitrosourea was dissolved in 200 µl of vehicle, in concentrations ranging from 0.20 to 2.0 µmoles. The vehicle for ACNU and chlorozotocin was 0.01 M sodium citrate buffer, pH 4, and that for CNU was acetone. The drug solutions were mixed with 350 µmoles of 4-(p-nitrobenzyl)pyridine (Fisher Scientific) in 1.5 ml aceton, then mixed with 4 ml of 0.025 M sodium acetate buffer (pH 6), and incubated at 37° for 2 hr. At the end of the incubation, 2 ml of aceton and 3 ml of ethyl acetate were added to the mixtures. In subdued light, the mixture was made alkaline by the addition of 1.5 ml of 0.25 N NaOH, shaken vigorously, and immediately centrifuged at 3000 rpm for 15 sec. The colored ethyl acetate layer was removed, and its absorbance at 540 nm was determined. The relative alkylating activity is expressed as a percentage of the activity of CNU, the nitrosourea of greatest in vitro alkylating activity in this series.

The in vitro carbamoylating activity was determined by incubation of the nitrosoureas with radiolabeled lysine (9). One-tenth µCi of L-[^14]C]lysine (specific activity, 300 mCi/m mole; New England Nuclear, Boston, Mass.) was added to 4.2 µmoles of unlabeled lysine in 120 µl of 0.1 M sodium monophosphate buffer to yield a final specific activity of 24 µCi/m mole at pH 7.3. The lysine solutions were mixed with 4.2 µmoles of the nitrosourea contained in 160 µl of ethanol:phosphate buffer (1:1), pH 7.3. As controls, the reaction mixtures with vehicle but without nitrosourea were used. The reaction mixture was incubated at 37° for 6 hr; then 10 µl were spotted on Whatman No. 3 chromatography paper. The reaction products, all containing carbamoylated lysine, were separated from unreacted lysine and parent drug by high-voltage electrophoresis at 3000 V for 75 min in a Savant high-voltage flat-plate tank with 0.1 M sodium phosphate buffer, pH 6.0. The dried paper was cut into 1-inch squares, immersed in 10 ml of scintillation fluid, and assayed for radioactivity in a Mark III liquid scintillation counter with 92% efficiency for $^1$C. The carbamoylation reaction products corresponded to the distinct peak of radioactivity present on the paper readily distinguished from[^14]C]lysine. Carbamoylation activity is expressed as a percentage of the activity of CNU.

**RESULTS**

**Biochemical Activities.** The in vitro carbamoylating activity of ACNU, 3.5% of CNU, is similar to that of chlorozotocin (4% of CNU), whereas CCNU demonstrated a high level of

<table>
<thead>
<tr>
<th>Drug</th>
<th>Alkylating activity (% CNU)</th>
<th>Carbamoylating activity (% CNU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNU</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ACNU</td>
<td>38</td>
<td>3.5</td>
</tr>
<tr>
<td>Chlorozotocin</td>
<td>64</td>
<td>4.0</td>
</tr>
<tr>
<td>CCNU</td>
<td>10</td>
<td>94</td>
</tr>
</tbody>
</table>

Table 1

*Comparison of chemical structures and in vitro biochemical activities of CNU, ACNU, chlorozotocin, and CCNU*
carbamoylating activity with 90% of the CNU.

The relative in vitro alkylating activity of each compound is presented as a percentage of CNU (Table 1). ACNU and chlorozotocin demonstrated alkylating activities of 38 and 64%, respectively, which are significantly greater than the 10% of CCNU.

**Bone Marrow Toxicity.** Groups of 10 normal CD2F1 mice received single graded i.p. doses of ACNU, ranging from 10 to 50 mg/kg. Serial WBC were performed on Days 3, 7, and 14 postinjection. The results were compared with those obtained from animals receiving single i.p. doses of CCNU and chlorozotocin at their respective 10% lethal dose levels. The nadir of WBC occurred on the third day following treatment with all drugs tested, consistent with results previously obtained in this species (Chart 1).

ACNU administered at the highest nonlethal dose (20 mg/kg) produced a 43% reduction in WBC and reduced the absolute neutrophil count to 21% of control (Table 2). A 10% lethal dose, 30 mg/kg, produced a 64% reduction in WBC and an 85% reduction in neutrophils. A 100% lethal dose, 50 mg/kg, produced a 76% reduction in WBC with a resultant relative lymphocytosis. The degree of WBC depression produced by each dose was significantly different from control values (p < 0.05). Recovery was established by Day 14 postinjection and sustained throughout the 28-day period of observation.

Chlorozotocin administered at a 10% lethal dose, 20 mg/kg, produced a mean WBC reduction of 18% with no decrease in absolute neutrophil count compared to pretreatment controls (Table 2). CCNU administered at a 10% lethal dose, 40 mg/kg, produced a 62% reduction in mean WBC with a 75% decrease in circulating neutrophils (Table 2). These values are both significantly different from pretreatment controls (p < 0.05).

**Activity against L1210 Leukemia.** Groups of 10 mice bearing L1210 leukemia received graded single i.p. doses of ACNU on Day 2 following tumor implantation. The 10% lethal dose in normal mice, 30 mg/kg, produced a 492% ILS compared to vehicle-treated controls, with 100% of the tumor-bearing animals surviving 45 days. Chlorozotocin administered at a 10% lethal dose, 20 mg/kg, resulted in a 282% ILS compared with controls, with 40% of the mice surviving the 45-day period of observation. CCNU administered at a 10% lethal dose, 40 mg/kg, resulted in a 364% ILS compared with controls with 70% of the mice surviving 45 days (Table 3).

**In Vivo L1210 and Bone Marrow DNA Synthesis.** The effect of in vivo treatment with ACNU (30 mg/kg i.p.) on DNA synthesis in L1210 leukemia and normal bone marrow is presented in Chart 2. For L1210 cells control

---

**Table 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (µmoles/kg)</th>
<th>% lethality in normal mice</th>
<th>Bone marrow toxicity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/kg)</td>
<td></td>
<td>Nadir WBC</td>
</tr>
<tr>
<td>ACNU</td>
<td>32</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>Chlorozotocin</td>
<td>162</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>CCNU</td>
<td>64</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>171</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (µmoles/kg)</th>
<th>% lethality in normal mice</th>
<th>L1210 activity (% ILS)</th>
<th>No. of 45-day survivors/no. treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACNU</td>
<td>32</td>
<td>10</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>20</td>
<td>0</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>30</td>
<td>100</td>
<td>492</td>
</tr>
<tr>
<td>Chlorozotocin</td>
<td>64</td>
<td>15</td>
<td>0</td>
<td>306</td>
</tr>
<tr>
<td>CCNU</td>
<td>64</td>
<td>20</td>
<td>100</td>
<td>282</td>
</tr>
<tr>
<td>Control</td>
<td>171</td>
<td>40</td>
<td>100</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/10</td>
</tr>
</tbody>
</table>

*Compared to untreated controls.*
The results of animal studies show a positive correlation between the acute hematological toxicity of ACNU in mice and the delayed and cumulative bone marrow toxicity observed in humans as has been previously demonstrated with 1,3-bis(2-chloroethyl)-1-nitrosourea and CCNU (1, 9). The delayed and cumulative leukopenia and thrombocytopenia produced by ACNU in clinical trials (4) indicate that the chemical properties of water solubility and low carbamoylating activity do not in themselves reduce the bone marrow toxic potential of nitrosourea agents in man. As a result, ACNU serves as an important positive control for future structure-activity studies that attempt to identify water-soluble nitrosourea agents with reduced myelosuppressive toxicity.

DISCUSSION

With the development of chlorozotocin and its subsequent introduction into clinical trials, attention has been focused upon water-soluble chloroethylnitrosoureas with reduced in vivo carbamoylating activity. ACNU, a compound with chemical properties similar to those of chlorozotocin, has shown curative activity against L1210 leukemia in mice (2). However, at therapeutic dose levels, ACNU is a potent toxin for the normal murine bone marrow as measured by peripheral neutrophil counts and [3H]thymidine incorporation into bone marrow DNA. The normal lymphocytes were relatively spared in contrast to the myelocytic series, which is consistent with our previous results with other myelotoxic nitrosoureas (1). Although ACNU, a pyrimidine analog, is comparable to chlorozotocin in water solubility and in vitro carbamoylating activity, it does not demonstrate the bone marrow-sparing characteristic of chlorozotocin. The glucose carrier of the chlorozotocin molecule appears to impart the selective sparing of normal bone marrow.

ACKNOWLEDGMENTS

We thank Dr. Joanna Heal for her help throughout these studies and Dianna Green and Faye Logan for their assistance in the preparation of the manuscript.

Addendum

Since this manuscript was prepared, Tanaka et al. (12) have identified a nonenzymatic intramolecular carbamoylation product of ACNU. This is the probable explanation for the low carbamoylating activity of ACNU, as determined by our [3H]thymidine assay.

REFERENCES


CANCER RESEARCH VOL. 38

R. A. Nagourney et al.

Chart 2. In vivo incorporation of [3H]thymidine into DNA of normal mouse bone marrow and L1210 leukemia after treatment with ACNU, 30 mg/kg i.p. Results are presented as a percentage of pretreatment (dpm/µg DNA). 

[3H]thymidine incorporation into DNA was 77,945 dpm/µg DNA; for bone marrow the control incorporation was 1085 dpm/µg DNA. ACNU produced a 92% reduction in L1210 DNA synthesis by 24 hr postinjection (Chart 2). This degree of inhibition was sustained through the 72-hr period of observation. An equivalent dose resulted in a 76% reduction in DNA synthesis in the normal bone marrow by 24 hr after treatment; however, this degree of inhibition was not sustained, and a rapid increase in DNA synthesis was demonstrated by 48 hr after treatment (Chart 2).
A Comparison of the Biological and Biochemical Properties of 1-(4-Amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea and 2-[3-(2-Chloroethyl)-3-nitrosoureido]-d-glucopyranose

Robert A. Nagourney, Patricia Fox and Philip S. Schein


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/38/1/65

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.