Hypothetical Mechanism of Therapeutic Synergism Induced by the Combination of 6-Thioguanine and 3-[(4-Amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea Hydrochloride

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ABSTRACT

Comparative studies were carried out using various antimetabolites in order to determine whether the synergistic cell-killing effect on L1210 murine leukemia cells induced by a treatment combining 6-thioguanine (6-TG) and 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (ACNU) resulted from "complementary inhibition." The combinations of ACNU plus 5-fluorouracil and ACNU plus 5-bromo-2'-deoxyuridine induced 31- and 18-fold greater cell killing of L1210 cells in vitro over the expected additive survival (the product of the observed survival for each drug), respectively, whereas the cytotoxic activity of the combinations of ACNU plus methotrexate and ACNU plus 6-methylmercaptopurine riboside was less than 2-fold greater. Moreover, therapeutic synergism defined by two parameters (survival time and/or cure incidence) against iv. L1210 leukemia was clearly elicited only by the combinations of ACNU plus 5-fluorouracil, 5-bromo-2'-deoxyuridine, or 6-mercaptopurine. As a result, the following possibilities that the 6-TG incorporated into the DNA in place of guanine increases the amount of the double-stranded DNA fraction and the amount of binding of [ethylene-14C]ACNU to the DNA and in the amount of the DNA interstrand cross-linking determined by the hydroxylapatite technique were observed with the increase in the 6-TG dose and, maximally, approximately 6-fold greater binding and 6- to 12-fold increases in the double-stranded DNA fraction over those of control DNA were obtained by 4- and 2-hr incubations at 37°, respectively.

INTRODUCTION

We have shown in a series of studies that a combination of 6-TG and ACNU induced therapeutic synergism against various murine tumors (12) and that these drugs in combination showed a synergistic cytotoxic activity against L1210 cells in vitro (10) but a less than additive effect with respect to lethal toxicity on host animals (11). Thus, these findings appear to suggest that the therapeutic synergism elicited by the combination of 6-TG and ACNU results from an increment in the selective toxicity towards tumor cells compared with vital organ cells of host animals. As a result, an analysis of the mechanism of synergistic cytotoxic activity towards tumor cells has become a major object of study in efforts to clarify the mechanism of the enhancement in selective toxicity.

With respect to biochemical aspects, 3 major concepts have been put forward to interpret therapeutic synergism, i.e., sequential blockade (27), concurrent inhibition (7), and complementary inhibition (30). Since the combination of 6-TG and ACNU appears to be the combination of an antimetabolite and an alkylating agent (9), the concept of complementary inhibition appears to be the most feasible of the 3 (31). Meanwhile, it has also been reported that 6-TG is incorporated, in nucleotide form, into nucleic acids (22) and that the incorporation of this drug into cellular DNA may be critical for the expression of cytotoxicity (21, 26, 37). Thus, comparative studies were carried out using various antimetabolites to examine which action of 6-TG is a dominant function in inducing the synergistic activity against L1210 cells in vitro and in vivo when given in combination with ACNU.

MATERIALS AND METHODS

Tumors and Animals. L1210 murine leukemic cells were supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and were maintained by continuous i.p. passage in female C57BL/6 × DBA/2 F1 (hereafter called BD2F) mice. Female BD2F mice weighing about 22 g were used in the chemotherapy experiments. They were supplied by Simonsen Laboratories, Gilroy, Calif., and Laboratory Supply Co., Indianapolis, Ind., and were given water and pelleted food ad libitum. Groups of 8 to 10 BD2F mice were given i.v. implants of 10⁶ L1210 cells, and drugs were given i.v. at the indicated times thereafter.

Evaluation of Therapeutic Response. Therapeutic response was evaluated by the prolongation of the life span of leukemic mice and the incidence of 60-day survivors. The results were analyzed for significance by Student's t test for prolongation of life span and by Fisher's exact test for 60-day survival incidence. The term "therapeutic synergism" is used in this study according to the definition advanced by Venditti et al. (40). This term refers to a situation in which a combination of drugs provides a therapeutic effect better than the maximum effect of either drug alone.

Drugs and Radiative Compound. [ethylene-14C]ACNU (4.0 mCi/ mmol) and unlabeled ACNU were kindly supplied by Sankyo Co., Ltd., Tokyo, Japan. 6-TG and 6-MP were provided by the Division of Cancer Research, National Cancer Institute, Bethesda, Md., and were maintained by continuous i.p. passage in female C57BL/6 × DBA/2 F1 (hereafter called BD2F) mice. Female BD2F mice weighing about 22 g were used in the chemotherapy experiments. They were supplied by Simonsen Laboratories, Gilroy, Calif., and Laboratory Supply Co., Indianapolis, Ind., and were given water and pelleted food ad libitum. Groups of 8 to 10 BD2F mice were given i.v. implants of 10⁶ L1210 cells, and drugs were given i.v. at the indicated times thereafter.

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Treatment, National Cancer Institute. MMPR and BrdUrd were purchased from Sigma Chemical Co., St. Louis, Mo.; 5-FUra was from Kyowa Hakko Co., Ltd., Tokyo, Japan; and MTX was from Takeda Chemical Ind., Osaka, Japan. All the drugs were dissolved in 0.85% NaCl solution and were freshly prepared prior to use. In the chemotherapy experiments, drugs were administered i.v. to tumor-bearing mice at a volume of, unless otherwise specified, 0.01 ml/g body weight by a single injection. In 2-drug combinations, each drug was administered by a separate injection with indicated time intervals including simultaneous administration.

Cell Culture. L1210 cells were collected from 6-day-old ascites of BD2F, mice (10^3 inoculation) and suspended at 5.5 x 10^7/ml in RPMI Medium 1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.), sodium bicarbonate (0.12%; Wako Pure Chemical Ind., Ltd., Osaka, Japan), 2-mercaptoethanol (10 μM; Wako Pure Chemical Ind., and kanamycin (100 μg/ml; Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; hereafter called RPMI medium). The cell suspensions (4.5 ml/tube) were incubated at 37° and in a humidified 5% CO2 atmosphere in stationary cultures. Under these conditions, the cells grew exponentially with a doubling time of approximately 10 hr between cell densities of 2 x 10^5 and 5 x 10^5/ml. A cell density of 5 x 10^5/ml was used in this study, since L1210 cells grew with a doubling time of 18 hr at this cell density and the 6-day-old L1210 cells in the peritoneal cavity of BD2F mice have a mass-doubling time of 25 hr (15).4

Drug Exposure. Unless indicated otherwise, drug concentrations were determined on the basis of TMC (34) calculated from the previously determined optimal dose (dose producing a maximum antitumor activity in vivo) of each drug except for ACNU (10). Drugs were dissolved and diluted to give stock solutions having concentrations 20 times greater than the final test concentrations. From the stock solutions, 0.25 ml of one drug was added in combination with 0.25 ml of the other drug or 0.85% NaCl solution in the cases of a combination group and a single-drug-treated group, respectively, to 4.5 ml of cultures to give the desired final concentrations. One-half ml of 0.85% NaCl solution was added to the control cultures. In general, the cell cultures were treated with drugs for 1 hr the day after initiation of culture. The cultures were then centrifuged at 1000 rpm for 3 min, and the cells were washed once and resuspended in 5 ml of the RPMI medium. The surviving cells were assayed by the soft-agar cloning assay.

Soft-Agar Cloning Assay. The soft-agar cloning assay was carried out by the method of Himmelstrand et al. (18) with minor modifications as described previously (10). Briefly, the cell suspensions were serially diluted in the RPMI medium and the final 10-fold dilution was made in double-strength RPMI Medium 1640 containing 40% fetal bovine serum, 40 μM 2-mercaptoethanol, 0.24% sodium bicarbonate, and kanamycin (200 μg/ml) to give the final test cell densities. This cell suspension (2.5 ml) was added to 2.5 ml of 0.3% Noble agar (Nakarai Chemical, Ltd., Kyoto, Japan) in deionized and distilled water (autoclaved at 120° for 30 min) maintained at 38° in 16- x 150-mm glass tubes (6 tubes/group) and mixed gently with a pipet. The control group was then centrifuged at 40 and 20 cells/tube, and the drug-treated groups were cloned at 3 different cell densities (usually, 10-fold dilutions). The tubes were incubated at 37° and in a humidified 5% CO2 atmosphere for 10 to 12 days. Only colonies more than 0.5 mm in size were counted macroscopically. The eligible colony numbers were generally unchanged by extension of the incubation time for 1 to 3 more days.

The "synergism index" was defined as the ratio of the expected additive survival to the observed fractional survival of L1210 cells, and the expected additive survival was taken as the product of the fractional survival obtained for each drug alone. A combination with a synergism index of more than 10 was tentatively considered as a synergistic one (6).

Extraction of DNA. Groups of 8 to 15 BD2F, mice were given 10^6 L1210 cells i.p. on Day 0 and a single dose of 6-TG (30, 3, or 0.3 mg/kg) on Day 6. Control L1210 cells were harvested on Day 6, and 6-TG-treated cells were harvested 5 hr after drug administration. The cells were washed twice with approximately 40 ml of cold 0.15 M NaCl:0.1 M EDTA (pH 8.0) and suspended in 25 ml of the same buffer solution.

Each DNA specimen was isolated by the method of Marmur (24). Briefly, the cell suspension was made 1.8% in sodium dodecyl sulfate, incubated for 10 min at 60°, and was then made 1 M in sodium perchlorate. After the mixture was shaken for 5 min at room temperature, an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, and the mixture was shaken vigorously for an additional 30 min. It was then centrifuged at 3000 rpm for 10 min, and the upper aqueous phase was carefully pipetted off from the intermediate solid material and the lower chloroform phase. The DNA was precipitated by adding 2 volumes of cold ethanol and dissolved in 0.015 M NaCl:0.0015 M sodium citrate (pH 7.0) by gentle mixing with a magnetic stirrer at 4°. Then, the DNA solution was adjusted to standard concentration (0.15 M NaCl:0.0015 M sodium citrate) by adding 0.1 volume of 1.5 M NaCl:0.15 M sodium citrate (pH 7.0), made 1 M in sodium perchlorate, shaken as before with an equal volume of chloroform:isoamyl alcohol for 15 min, and centrifuged, and the supernatant was removed. This deproteinization procedure was repeated until no protein was seen at the interface after centrifugation. The deproteinized DNA dissolved in the 0.15 M NaCl:0.015 M sodium citrate was treated with RNase A (Sigma Chemical Co.; type X-A, 50 μg/ml) for 30 min at 37°C. Following the digestion of the DNA, the solution was made 1 M in sodium perchlorate, and the deproteinization procedure was repeated until no protein was seen at the interface. The DNA was precipitated with ethanol and dissolved in 18 ml of the 0.015 M NaCl:0.0015 M sodium citrate, to which 2 ml of 3 M sodium acetate:0.001 M EDTA (pH 7.0) were added. While the solution was rapidly stirred with a glass rod, 0.54 volume (10.8 ml) of isopropl alcohol was added dropwise. The precipitated DNA was washed successively with 70, 80, and 90% ethanol, and the dried DNA was dissolved in 0.05 M sodium cacodylate:0.1 M NaCl buffer (pH 7.0).

In Vitro Binding of [ethylene-14C]ACNU to DNA. The DNAs of L1210 cells untreated or treated with various doses of 6-TG in vivo were isolated as described above. To each DNA specimen (526 μg) dissolved in 0.95 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer were added 50 μl of [ethylene-14C]ACNU (240 or 24 μg/ml). The mixture was incubated at 37°, and 50-μl aliquots in triplicate were withdrawn at appropriate time intervals. The aliquots were applied to 2.5-cm discs of Whatman GF/C paper and washed free of unbound radioactivity. The paper discs were placed in scintillation vials, and 1 ml of Protosol (New England Nuclear, Boston, Mass.) was added to each vial to ensure the solubilization of the DNA. Ten ml of liquid scintillation cocktail (Econofluor; New England Nuclear) were added, and the radioactivity was determined in a Beckman Model LS-7500 liquid scintillation counter.

Determination of Cross-Links by the Hydroxylapattite Technique. The isolated DNAs (250 μg) in 1 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer were incubated with unlabelled ACNU (120 μg/ml, 50 μg) or 0.85% NaCl solution (50 μl) at 37° for 2 hr. The reaction was terminated at ice water temperature, and the DNAs were precipitated by adding 2 volumes of cold ethanol with the aid of a Vortex mixer. The mixtures were kept at ice water temperature for 30 min and centrifuged at 5000 rpm for an additional 30 min at 5° in a Sorval HS-4 rotor. The precipitated DNAs were dissolved in 2.2 ml of 0.01 M potassium phosphate buffer (pH 6.9), and the molecular weights of the DNAs were reduced by sonication (30 sec at Output Control Setting 50 of a Sonic Dismembrator with microtip, Artek System Corp., Farmingdale, N. Y.). After sonic disruption, an aliquot (0.2 ml) from each DNA solution was determined spectrophotometrically for the concentration so as to ensure calculation of the recovery into the double-stranded DNA solution.
DNA fraction following the hydroxylapatite analysis (see below). The remaining DNA solutions (2 ml) were placed in boiling water for 10 min and rapidly cooled at ice water temperature in order to prepare the assay samples for determination of cross-linking.

Separation of the single-stranded and the double-stranded DNAs was essentially performed by the method described by Rydberg (28), except that a stepwise extraction method was used in the present study. An approximately 1.0-ml bed volume of hydroxylapatite (BioGel HY; BioRad Laboratories, Richmond, Calif.) that had been washed 3 times in 0.01 M potassium phosphate buffer (pH 6.9) was prepared in siliconized centrifuge tubes. The heat-denatured DNAs (2 ml), which were prepared as described above, were added to these tubes, and the adsorption of the DNA to hydroxylapatite was accomplished by incubating the mixtures at 60°C for 30 min in conjunction with several mixings by a Vortex mixer. The tubes were centrifuged at 1500 rpm for 1 min, and the supernatant of each group was separated by decantation. Then, the bed of hydroxylapatite was washed once in 2 ml of 0.01 M potassium phosphate buffer at 60°C for 10 min with 5 to 6 mixings, and the supernatant after centrifugation was separated. No DNA-related absorption spectrum was observed in these supernatants of each group. The single-stranded DNA was extracted twice by 2 ml of 0.125 M potassium phosphate buffer (pH 6.9) at 60°C for 10 min, and the double-stranded DNA was then extracted twice by 2 ml of 0.5 M potassium phosphate buffer (pH 6.9) at 60°C for 10 min. The net increase in the double-stranded DNA fraction by treatment with ACNU was obtained by subtraction of the recovery into the double-stranded DNA fraction of each ACNU-untreated DNA (i.e., nonspecific cross-linking).

RESULTS

Antitumor Activity of Combinations of ACNU and Various Antimetabolites. The following drugs were used in these comparative studies: (a) 6-MP, 5-FUra, and BrdUrd as the antimetabolites which may also function directly at the level of DNA and/or RNA by being incorporated into its (their) structure(s) (5, 17, 38); and (b) MTX and MMPR as the antimetabolites which have no such function (3) or have not been shown clearly to have such a function (23), respectively. Antitumor experiments using combinations of ACNU and these antimetabolites against i.v. L1210 leukemia were carried out by administering these antimetabolites with various treatment schedules (i.e., 48-, 24-, 16-, and 0-hr intervals) since the combination of ACNU and 6-TG induced synergistic antitumor activity against this tumor at a specific time interval (16 hr (10)).

From titration experiments, the optimal dose of 6-MP administered i.v. was determined to be 200 mg/kg against i.v. L1210 leukemia. Similarly, that of MMPR was determined to be 240 mg/kg. On the other hand, an apparent highest nontoxic dose (900 mg/kg) was used for BrdUrd since this drug was found to be ineffective against this tumor. The doses of 5-FUra and MTX were the therapeutic doses described previously (12). The results of the maximum therapeutic effects with a specific treatment schedule for each combination of drugs are shown in Table 1. The combination of ACNU and 5-FUra showed synergistic antitumor activity against i.v. L1210 leukemia with respect to 2 parameters (i.e., survival time and cure incidence). Synergistic antitumor activity was also observed with the combinations of ACNU and 6-MP or BrdUrd, but with respect to only one parameter. The combination of ACNU and MTX showed a significant but minor prolongation of the survival time of leukemic mice compared with that of the group treated with ACNU alone in one of 2 experiments; no synergistic antitumor activity was observed in the other. No advantageous antitumor effect could be observed with the combination of ACNU and MMPR.

Hypothetical Mechanism of Synergism, 6-TG Plus ACNU

Mice were given 10^5 L1210 cells i.v. on Day 0 and a single i.v. dose of ACNU (25 mg/kg) or Day 5. A single dose of each antimetabolite, i.e., 5-FUra (0.5 mg/kg), 6-MP (200 mg/kg), BrdUrd (900 mg/kg), MTX (40 mg/kg), or MMPR (240 mg/kg), was given i.v. at 48 hr (Day 3), 24 hr (Day 4), 16 hr (Day 4), or 0 hr (Day 5, simultaneously) prior to ACNU administration, alone and in combination with ACNU.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Optimal time interval (hr)</th>
<th>Survival time (days)</th>
<th>% of increased life span</th>
<th>60-day survivors/total</th>
</tr>
</thead>
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<tr>
<td>1 Control</td>
<td>5-FUra</td>
<td>7.0</td>
<td>9.9 ± 0.3</td>
<td>41</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>ACNU</td>
<td>18.8 ± 4.2</td>
<td>169</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-FUra + ACNU</td>
<td>16</td>
<td>26.2 ± 6.9</td>
<td>274</td>
<td>4/10</td>
</tr>
<tr>
<td>2 Control</td>
<td>BrdUrd</td>
<td>5.6 ± 0.3</td>
<td>6.9 ± 0.1</td>
<td>13</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>ACNU</td>
<td>19.2 ± 3.4</td>
<td>215</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BrdUrd + ACNU</td>
<td>0</td>
<td>32.2 ± 6.5</td>
<td>428</td>
<td>1/10</td>
</tr>
<tr>
<td>3 Control</td>
<td>BrdUrd</td>
<td>5.6 ± 0.3</td>
<td>6.8 ± 0.4</td>
<td>5</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>ACNU</td>
<td>21.8 ± 2.4</td>
<td>235</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BrdUrd + ACNU</td>
<td>0</td>
<td>36.8 ± 13.1</td>
<td>463</td>
<td>1/6</td>
</tr>
<tr>
<td>4 Control</td>
<td>MTX</td>
<td>7.3 ± 0.5</td>
<td>10.5 ± 0.5</td>
<td>44</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>ACNU</td>
<td>16.6 ± 1.4</td>
<td>127</td>
<td>0/10</td>
<td></td>
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<tr>
<td></td>
<td>MTX + ACNU</td>
<td>0</td>
<td>18.5 ± 1.4</td>
<td>153</td>
<td>0/10</td>
</tr>
<tr>
<td>5 Control</td>
<td>MTX</td>
<td>7.0</td>
<td>9.5 ± 0.5</td>
<td>36</td>
<td>0/10</td>
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<tr>
<td></td>
<td>ACNU</td>
<td>31.4 ± 6.6</td>
<td>349</td>
<td>2/10</td>
<td></td>
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<tr>
<td></td>
<td>MTX + ACNU</td>
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<td>21.3 ± 4.7</td>
<td>204</td>
<td>0/10</td>
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<tr>
<td>6 Control</td>
<td>MTX</td>
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<td>9.7 ± 0.8</td>
<td>47</td>
<td>0/6</td>
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<td>28.0 ± 12.0</td>
<td>324</td>
<td>1/6</td>
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<tr>
<td></td>
<td>MMPR + ACNU</td>
<td>24</td>
<td>17.7 ± 2.8</td>
<td>168</td>
<td>0/6</td>
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</tbody>
</table>

| a | A solution of BrdUrd at a concentration of 30 mg/ml was prepared and administered at a volume of 0.03 ml/g body weight (900 mg/kg) because of its limited solubility. Similarly, a solution of MMPR at a concentration of 6 mg/ml was prepared and administered at a volume of 0.04 ml/g body weight (240 mg/kg).
| b | Excluding the survivors.
| c | p < 0.02 by Student's t test.
| d | p < 0.05 by Fisher's exact test.
| e | p < 0.01 by Student's t test.
| f | p < 0.001 by Student's t test.

The killing effect of BrdUrd, alone or in simultaneous combination with ACNU, was examined at concentrations ranging from 4.6 to 1170 µg per ml, which appeared to be the TMC of this drug attainable in the body fluids when 900 mg of this drug was administered per kg (10). As shown in Table 2, BrdUrd at a concentration of 293 µg/ml induced a synergistic killing effect with a synergism index of 30.6. 5-FUra at a concentration

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of 65 µg/ml (TMC) induced a similar effect with a synergism index of 17.6 when used in simultaneous combination with ACNU for 1 hr.

On the other hand, MMPR used in simultaneous combination with ACNU for 1 hr did not show any synergistic killing effect even at the concentration of TMC (410 µg/ml) which was obtained from the 10% lethal dose for BD2F, mice [315 mg/kg (33)]. Since it appeared that this was due to the low degree of killing potential of MMPR at the 1-hr exposure time, the exposure time of L1210 cells to this drug was extended for 19 hr. Although the killing effect of MMPR at the TMC was augmented by the extension of exposure time to a level similar to that attained by BrdUrd or 5-FUra alone at 1 hr of exposure time, neither an increment in the synergism index compared with that of the 1-hr exposure time nor a concentration-related change in the synergism index could be observed. Similarly, 6-MP used in simultaneous combination with ACNU for 1 hr did not induce any synergistic killing effect even at the TMC (260 µg/ml). By extending the exposure time of L1210 cells to this drug for 1, 2, 3, and 6 hr, a time-dependent augmentation of the killing effect of 6-MP occurred, and the killing effect of 6-MP at 6-hr exposure attained the fractional survival level of 0.150; however, the synergism index of the group treated with the combination of 6-MP for 6 hr and ACNU for 1 hr was only 2.8.

MTX used in simultaneous combination with ACNU for 1 hr did not induce any synergistic killing effect at the TMC (52 µg/ml). In this case, neither a time-dependent augmentation of the killing effect of MTX nor any increment in the synergism index of the combination with ACNU could be observed by extending the exposure time of L1210 cells to MTX for 1, 2, 3, and 6 hr.

In summary, the synergistic killing effect in vitro and the therapeutic synergism in vivo against L1210 leukemia cells were generally induced only by the combinations of ACNU and those antimetabolites which are known to be incorporated into the nucleic acids. Thus, we supposed such a hypothetical mechanism for the synergistic killing effect of the combination of 6-TG and ACNU on L1210 cells, i.e., that the 6-TG incorporated into DNA in place of guanine increased the amount of the DNA interstrand cross-links by ACNU. This possibility was examined as follows.

Increased Binding of 14C]ACNU to the DNA isolated from L1210 Cells Treated with 6-TG. The possibility of whether 6-TG incorporated into DNA increases the amount of 14C]ACNU binding was examined using DNA isolated from L1210 cells untreated or treated with 6-TG at the optimal therapeutic dose (30 mg/kg) in vivo. The isolated DNAs (500 µg) were reacted with 14C]ACNU (240 or 24 µg/ml) at 37° in 1 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer (pH 7.0). As shown in Table 3, the radioactivity in the aliquots increased with the extension of incubation time, and 3- to 8-fold greater radioactivity than that of the control DNA was observed in the reaction of 6-TG-treated DNA with both concentrations of ACNU. Table 4 shows a representative result from 2 experiments in which the correlations between the 6-TG dose and the extent of reaction were examined. A significant increase in the radioactivity with the increase in the 6-TG dose administered in vivo was observed at each incubation time. A similar result was obtained in the other experiment. These results may suggest that a dose-related increment in the incorporation of 6-TG into the DNA occurs.

Increased Recovery of 6-TG-treated DNA into Double-stranded DNA Fraction after Exposure to ACNU. The DNA interstrand cross-linking was measured by determining the fraction of the DNA that is un-denaturable by heat treatment. As shown in Table 5, a significant increase in the double-stranded DNA fraction was induced by the reaction of ACNU with the DNA isolated from L1210 cells treated with 6-TG (30 and 3 mg/kg) as compared with that of the control DNA.

DISCUSSION

It has been reported that 6-TG, following conversion to its active metabolite, inhibits the activity of such enzymes as phosphoribosylpyrophosphate amidotransferase (32), inosine 5'-monophosphate dehydrogenase (25), and ATP:GMP phosphotransferase (25). Such multistep blockade of purine nucleotide biosynthesis should produce a remarkable decrease in the intracellular concentration of guanine nucleotides (31). However, 6-MP and MMPR, which are also known to be more potent inhibitors of purine nucleotide synthesis than is 6-TG (1, 4, 14, 37, 38, 42), failed to induce the synergistic cell killing in the combination with the alkylating agent ACNU (Table 2).
by sonication, one part (0.2 ml) of each DNA solution was used for determination

buffer (pH 7.0) was incubated with unlabeled ACNU (120 ng/ml) or 0.85% NaCl

recovery of the double-stranded DNA fraction was determined spectrophotometric
volumes of cold ethanol, centrifuged, and dissolved in 2.2 ml of 0.01 M potassium
solution (50 ng) at 37° for 2 hr. The DNAs were precipitated by addition of 2

ACNU (240 ng/ml) at 37° in 1 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer

0.30 Net Degree of reaction

0.30 Concentration of 6-TG administered in vivo and the extent of

Effect of 6-TG on DNA interstrand cross-linking by ACNU

Each group of DNA (250 ng) in 1 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer

were incubated for 1 to 2 hr, and the radioactivity of aliquots in triplicate was determined as described in “Materials and Methods.”

The extent of chloroethylation of guanine O-6 in the first step appears to depend on the alkylating activity of individual nitrosoureas, and the final extent of cross-linking was mainly determined by this initial extent of chloroethylation (20). In this regard, it is of interest that ACNU has a relatively high alkylating activity (16) and, more importantly, the mercapto group of 6-TG has a higher reactivity with alkylating agents than does the hydroxy group of guanine (29). Thus, the extent of the initial chloroethylation and, consequently, the final cross-bridge by treatment with ACNU may be increased by the 6-TG which was incorporated into the DNA in place of guanine (Chart 1). Promising results supporting this hypothesis could be obtained in the preliminary experiments (Tables 3 to 5).

The above hypothetical mechanism of synergism is compatible with the previous findings which showed the importance of prior or simultaneous administration of 6-TG to induce therapeutic synergism in vivo as well as synergistic cell killing in vitro against L1210 cells when used in combination with ACNU (10). In addition, this hypothetical mechanism appears to be compatible with the findings in the present study, i.e., that no synergistic cell-killing effects were observed with the combination of ACNU and 6-MP or with that of ACNU and MMPR (Table 2). Tidd and Paterson (38) showed that 6-MP produced delayed cytotoxicity towards L5178Y cells in culture after incorporation of 6-MP as 6-TG into DNA. Moreover, they showed that about a 100-fold higher concentration of 6-MP than 6-TG was required to attain similar levels of incorporation and to show similar levels of cytotoxicity. In the present study, however, the theoretical maximum concentration of 6-MP was only 10-fold greater than that of 6-TG, so that the incorporation of 6-MP as 6-TG appeared to be insufficient to induce a synergistic cell-killing effect in combination with ACNU. On the other hand, MMPR is a derivative of 6-mercaptopurine riboside and is believed to be an analog of adenosine (2). Thus, possible incorporation of this drug following conversion to its nucleotide levels is suspected (2). If this actually occurs, the chloroethylation due to ACNU of the sulfur group at position 6 of MMPR may not occur since the sulfhydryl group [although it exists largely in the thiolactam form at physiological pH (23)] of 6-mercaptopurine riboside is replaced by the methylmercapto

Thus, interference with the formation of guanine nucleotides (i.e., complementary inhibition) appears not to be the dominant function of 6-TG in eliciting the synergistic action on L1210 cells in the combination with ACNU.

The incorporation of 6-TG into DNA might be considered as an alternate mechanism of the synergistic action of 6-TG and ACNU. It has been clearly shown that the chloroethyl-nitrosoureas bearing a single alkylating function are active producers of DNA interstrand cross-links (8, 20), which is an attractive possibility for the origin of cytotoxicity.

Table 3

Comparison of [ethylene-14C]ACNU binding to DNAs isolated from control and 6-TG-treated L1210 cells

Mice were given 106 L1210 cells i.p. on Day 0 and a single i.p. dose of 6-TG (30 mg/kg) on Day 6. Control L1210 cells were harvested on Day 6, and 6-TG-treated cells were harvested 5 hr after the administration of drug. DNA was isolated by the method of Marmur (24).

<table>
<thead>
<tr>
<th>Concentration of ACNU (µg/ml)</th>
<th>DNA specimen</th>
<th>Degree of binding (dpm/ aliquot) to DNA at the following time after initiation of incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Control</td>
<td>6-TG-treated</td>
</tr>
<tr>
<td>1</td>
<td>240</td>
<td>256 ± 221b</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>16 ± 11</td>
</tr>
</tbody>
</table>

* See “Materials and Methods.”

Numbers in parentheses, significance (p) between 2 individual groups by Student’s t test.

Table 4

Correlation between the dose level of 6-TG administered in vivo and the extent of DNA synthesized from 6-TG

Each isolated DNA (500 ng) was reacted simultaneously with [ethylene-14C] ACNU (240 µg/ml) at 37° in 1 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer (pH 7.0). The reaction mixtures were incubated for 1 to 2 hr, and the radioactivity of aliquots in triplicate was determined as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>DNA specimen isolated from cells treated with 6-TG at the dose level (mg/kg) of</th>
<th>Degree of binding (dpm/ aliquot) to DNA at the following time after initiation of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>1 hr</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

Numbers in parentheses, significance (p) between 2 individual groups by Student’s t test.

Table 5

Effect of 6-TG on DNA interstrand cross-linking by ACNU

Each group of DNA (250 µg) in 1 ml of 0.05 M sodium cacodylate:0.1 M NaCl buffer (pH 7.0) was incubated with unlabeled ACNU (120 µg/ml) or 0.85% NaCl solution (50 µg) at 37° for 2 hr. The DNAs were precipitated by addition of 2 volumes of cold ethanol, centrifuged, and dissolved in 2.2 ml of 0.01 M potassium phosphate buffer (pH 6.9). After reduction of the molecular weight of the DNAs by sonication, one part (0.2 ml) of each DNA solution was used for determination of the concentration, and the remainder (2 ml) was heat denatured and subjected to the hydroxylapatite analysis as described in “Materials and Methods.”

The recovery of the double-stranded DNA fraction was determined spectrophotometrical for each DNA specimen treated or untreated with ACNU, and the net increase in the double-stranded DNA fraction by treatment with ACNU was obtained by subtraction of the recovery of the ACNU-untreated group from that of the ACNU-treated group.

Thus, interference with the formation of guanine nucleotides (i.e., complementary inhibition) appears not to be the dominant function of 6-TG in eliciting the synergistic action on L1210 cells in the combination with ACNU.

On the other hand, the incorporation of 6-TG into DNA might be considered as an alternate mechanism of the synergistic action of 6-TG and ACNU. It has been clearly shown that the chloroethyl-nitrosoureas bearing a single alkylating function are active producers of DNA interstrand cross-links (8, 20), which is an attractive possibility for the origin of cytotoxicity.
alkylating the incorporated analogs themselves. In fact, these ble to alkylation and cross-linking by ACNU, possibly, without individual nucleic acids that would make these more suscepti
these incorporated analogs might alter the structures of the following conversion to their individual nucleotide forms, and incorporated into RNA (17), DNA (5), or both (38), respectively, as well as synergistic cell killing in vitro against L1210 cells. ACNU and Brd Urd could induce therapeutic synergism in vivo against L1210 cells and of hematopoietic stem cells by the spleen colony assay method, showed that 6-TG produced an approx-
ately 2000-fold greater cytoxicity towards L1210 cells but that 6-MP at a dose level 10 times greater than 6-TG produced only a 20-fold greater one. Thus, if the incorporation of 6-TG or 6-MP into DNA is critical for the expression of cytoxicity, the significant difference in the selective toxicity of both agents is understandable and, more importantly, these results suggest that 6-TG was selectively incorporated into the DNA of L1210 cells. Therefore, the fatal cross-linking would occur with high selectivity in the tumor cells by treatment with a combination of 6-TG and ACNU, which by itself also has tumor selectivity.

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Hypothetical Mechanism of Synergism, 6-TG Plus ACNU


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