Biochemical Action of the Antineoplastic Agent 2-Formylpyridine
N-oxide Benzenesulfonylhydrazone

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ABSTRACT

Studies conducted with the relatively new antineoplastic agent 2-formylpyridine N-oxide benzenesulfonylhydrazone (2-PH) showed in vitro production of single-strand breaks in DNA of Sarcoma 180 cells exposed to this agent and inhibition of cell growth. The 50% growth-inhibitory concentration of freshly prepared 2-PH for Sarcoma 180 cells in culture after 24 hr of exposure was 100 µM. This level of drug caused damage to DNA, detected by alkaline sucrose gradient centrifugation within 15 min of treatment; drug-induced degradation of the DNA of Sarcoma 180 cells was not detected with neutral sucrose gradients. 2-PH was found to decompose rapidly in aqueous solution. Thus, the aging of 100 µM solutions of 2-PH by incubation at 37°C for 60, 120, and 180 min prior to cell exposure resulted in a progressive loss of growth-inhibitory activity to 33, 16, and 9% inhibition, respectively. A similar aged preparation of 2-PH did not induce the degree of breakage of DNA of Sarcoma 180 cells caused by nonaged drug. The majority of the damage to DNA induced by 2-PH did not appear to be repaired, even as long as 24 hr after removal of the agent from the cellular environment. Electrophoresis on agarose gels of purified DNA from PM2 phage grown in Pseudomonas thymidine auxotroph BAL-31 exposed in vitro to 2-PH demonstrated a significant conversion of supercoiled DNA to the nicked form under alkaline but not neutral conditions. The findings indicate that 2-PH causes damage to DNA in the neoplastic cell line used, which results in the production of single-strand breaks. In vitro studies with purified DNA from PM2 phage grown in Pseudomonas thymidine auxotroph BAL-31 imply that 2-PH induces damage to DNA which renders these macromolecules labile to alkaline hydrolysis. The damage to DNA produced by this agent correlates with cell cytotoxicity, suggesting that the lesions in DNA produced by 2-PH are responsible for its anticancer activity.

INTRODUCTION

Arylsulfonylhydrazones of 2-formylpyridine N-oxide represent a relatively new class of compounds possessing antineoplastic activity against a spectrum of transplanted murine neoplasms (1, 16); the prototype member of this series, 2-PH,2 was shown to be among the most active representatives of this group of agents. This compound, 2-PH, was shown to cause relatively weak inhibition of the incorporation of radioactive thymidine and uridine into the DNA and RNA, respectively, of Sarcoma 180 cells in vivo (16). The magnitude of the interference with the biosynthesis of nucleic acids by 2-PH did not appear to be consistent with the potency of this agent as an antineoplastic agent in this tumor system. Thus, the biochemical basis for the growth-inhibitory activity of this class of agents against experimental neoplasms is unknown.

The present report is concerned with our initial studies on the mechanism of action of the arylsulfonylhydrazones of 2-formylpyridine N-oxide. The findings demonstrate that 2-PH induces the formation of single-strand breaks of DNA, which correlates with the cytotoxic effects of this agent on Sarcoma 180 cells in culture; a preliminary report of portions of this work has been presented (9).

MATERIALS AND METHODS

Effects of 2-PH on Cell Cultures. Exponentially growing Sarcoma 180 cells were maintained in Fischer's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% horse serum and were grown at 37°C in an atmosphere containing 5% CO2. Growth rates were measured at various times of incubation by cell counting using a ZB1 Coulter particle counter.

Stock solutions of 2-PH in PBS were freshly prepared immediately before each experiment, except in those studies concerned with the aging of this compound. For cell growth studies, the drug solution was sterilized by filtration through 0.20-µm Nalgene filters (Nalgene Co., Rochester, N. Y.). Five hundred µl of either stock or diluted solutions of drug were added to 4.5 ml of cell suspension in Fischer's medium; control groups received an equal volume of PBS.

Measurement of the Biosynthesis of DNA, RNA, and Protein. Exponentially growing Sarcoma 180 cells were suspended in 1.0 ml of Fischer's medium containing 10% horse serum at a concentration of 1 × 106 cells/ml; 25 µCi of either [methyl-3H]thymidine (24 Ci/mmol), [U-3H]uridine (13.8 Ci/mmol), or L-[4,5-3H]leucine (20 Ci/mmol) (New England Nuclear, Boston, Mass.) were added, and the suspension was incubated at 37°C in the presence or absence of 2-PH. Cells were collected at the end of incubation by centrifugation at 12,000 × g and were washed 4 times with ice-cold 0.2 n perchloric acid. The cell pellet was hydrolyzed with 1.0 ml of 1.2 n perchloric acid and 0.1 n NaOH for nucleic acids and protein, respectively, for 30 min at 85°C, and radioactivity therein was determined with a Packard Tri-Carb liquid scintillation spectrometer.

Sucrose Gradient Sedimentation. Thirty ml of a suspension of Sarcoma 180 cells in Fischer's medium (1 × 105 cells/ml) were labeled with [methyl-3H]thymidine (13 nmol; 2.4 Ci/mmol)
for 6 hr in 100-ml Gibco bottles (Grand Island Biological Co., Grand Island, N. Y.). The medium containing radioactive thymidine was replaced with fresh nonradioactive medium and reincubated for 15 hr, followed by treatment with 2-PH. Cells were collected by centrifugation 1 hr later, and, using the method of Walters and Hildebrand (21), 4 to 5 x 10^4 cells in 0.1 ml of Tris-HCl-buffered NaCl solution (0.02 M EDTA, 0.075 M NaCl, and 0.05 M Tris-HCl, pH 7.4) were layered onto an alkaline sucrose gradient [5 to 20% sucrose containing 0.4 M NaOH, 0.1 M EDTA, and 0.1% Sarkosyl (Geigy Pharmaceuticals, Ardsley, N. Y.)] with a cell-lysing solution (0.4 ml of 1% Sarkosyl and 0.4 mg heparin). Neutral gradients (10 to 30% sucrose) were similarly prepared with the omission of NaOH. All gradients contained 2.0 ml of a 50% sucrose solution at the bottom of polyallomer tubes (% x 3¾ inches; Beckman Instruments, Inc., Fullerton, Calif.) to serve as a cushion.

Centrifugation was performed in a Beckman SW 40 Ti rotor at 86,000 x g for 2 and 3 hr at 5° for alkaline and neutral sucrose gradients, respectively. The gradients were fractionated by collection from the bottom of tubes using a micropipet (Rochester Scientific Co., Rochester, N. Y.) inserted through the tube. Either 20- or 30-drop fractions were collected in scintillation vials and were mixed with 2.0 ml of 0.25 N HCl and 10 ml of Aquasol (New England Nuclear). DNA sedimenting to the bottom of the tubes was recovered by washing the tube bottoms with 1.0 ml of 0.1 M NaOH-0.3% Sarkosyl; these washings were plotted in graphs as the initial bottom fraction (4). The radioactivity in the collected fractions was measured by liquid scintillation spectrometry.

Agarose Gel Electrophoresis of PM2 DNA. PM2 DNA was generously donated by Dr. R. J. Legerski of the University of Houston (Houston, Texas). Electrophoresis of the DNA was conducted essentially as described by Helling et al. (5). In brief, 1.25% agarose (Sigma Chemical Co., St. Louis, Mo.) in 50 mM Tris-20 mM sodium acetate-2 mM EDTA buffer titrated to pH 8.05 with glacial acetic acid was used to form 10- x 0.6-cm gels in 15-cm-long glass tubes. One-fourth volume of 0.1% bromophenol blue (Fisher Scientific Co., Pittsburgh, Pa.) in a 50% solution of sucrose was added to [methyl-3H]thymidine-labeled PM2 DNA after treatment with 2-PH to give a final sample volume of 100 to 130 µl. Electrophoresis of samples was conducted for 5 hr at 8 V/cm of gel (80 V); both buffer chambers contained 50 mM Tris-20 mM sodium acetate-2 mM EDTA buffer. Gels were fractionated into 3-mm lengths, and each fraction was heated at 100° for 10 min after the addition of 0.5 ml of formamide. Ten ml of Aquasol were added to each fraction, and radioactivity was determined using a liquid scintillation spectrometer.

RESULTS

The effects of 2-PH on the growth of Sarcoma 180 cells in culture were measured, and the results are shown in Chart 1; cell replication was inhibited by a dose-dependent manner by 2-PH during the first 72 hr of exposure to this agent. A 50% inhibition of cell growth was caused by 100 µM 2-PH in 24 hr; exposure of Sarcoma 180 cells to less than 1.0 µM drug produced virtually no effect on cell number.

To determine the relationship between the action of this agent on cell proliferation and macromolecular synthesis, the effects of the 50% growth-inhibitory concentration of 2-PH on the biosynthesis of DNA, RNA, and protein of Sarcoma 180 cells was measured by ascertaining the rate of incorporation of radioactive thymidine, uridine, and leucine, respectively, into acid-insoluble material; the results obtained are shown in Chart 2. The formation of both DNA and RNA was minimally inhibited during the initial 30 min of exposure to 2-PH, and the rate of protein synthesis was unaffected by this agent.

Since preliminary tests indicated that other biochemical processes, such as the rate of O2 consumption, cell agglutination by concanavalin A, and ribonucleotide reductase activity, in Sarcoma 180 cells appeared to be unaffected by 2-PH (data not shown), the possibility was considered that the slight inhibition of DNA and RNA biosynthesis observed in vivo (16) and in vitro might be due to direct damage to DNA produced by this agent. This possibility was examined by ascertaining whether 2-PH induced DNA strand-breakage, using both alkaline and neutral sucrose gradient centrifugation as described by Walters and Hildebrand (21). These measurements were conducted by labeling Sarcoma 180 cells with [3H]thymidine, followed by exposure of Sarcoma 180 cells to 2-PH for a 1-hr period. Chart 3 shows the effects of such treatment of Sarcoma 180 cells under 2 different conditions of DNA sedimentation. In alkaline conditions (Chart 3A), Sarcoma 180 cells treated with 100 µM 2-PH showed a significant reduction in the rate of sedimentation of DNA. Thus, more than 90% of the total radioactivity on the gradient was recovered from the top of the tube in the drug-treated samples, whereas the highest radioactive peak (about 50% of the total radioactivity) of control cells was found at the bottom of the tube. This shift in the sedimentation profile could be detected after the relatively short period of exposure of Sarcoma 180 cells to 2-PH of 15 min and with a drug concentration as low as 1 µM. In contrast, 2-PH did not alter the sedimentation profile of DNA under non-denaturing neutral conditions (Chart 3B).

To ascertain the potential for repair of 2-PH-induced damage to DNA, Sarcoma 180 cells were exposed to 100 µM 2-PH for 1 hr, collected by centrifugation and washed with fresh me-
The control reactions showed linear incorporation of each radioisotope into acid-insoluble material (Table 1). However, when the cells were further supported by experiments in which cells were treated with hydroxyurea were also exposed to agents that take place. Thus, drug-damaged DNA persisted for a relatively long period of time. The possibility that 2-PH induces non- or poorly repairable damage to the DNA of Sarcoma 180 cells was further supported by experiments in which cells treated with hydroxyurea were also exposed to agents that damaged DNA in a manner similar to that used by Lieberman et al. (11). Hydroxyurea (10 mM) was used to suppress DNA biosynthesis in those cells present in the S phase of the cell cycle, markedly decreased the rate of incorporation of [³H]thymidine into acid-insoluble material (Table 1). However, when this agent was used in combination with 0.01 mM nitrogen mustard, a 2.4-fold increase in the utilization of radioactive thymidine over that of hydroxyurea alone was observed. In contrast, the degree of incorporation of [³H]thymidine into the acid-insoluble fraction of cells treated with a mixture of 2-PH and hydroxyurea was identical to that of exposure to hydroxyurea only.

2-PH was found to have a short half-life in aqueous solution, which could be detected by a spectral decrease at 340 nm. Aging of solutions of 2-PH was accompanied by loss of the cytotoxic activity of this agent. Thus, preincubation of 2-PH in PBS at 37° for 60, 120, and 180 min prior to cell exposure resulted in a gradual decrease in growth-inhibitory potency from 50% by a fresh solution of 100 μM 2-PH to 33, 16, and 9% of control, respectively, as measured by cell number 24 hr after exposure to this agent (Table 2). Furthermore, a marked decrease in the capacity of 2-PH to alter the sedimentation profile of DNA of Sarcoma 180 cells on alkaline sucrose gradients was also observed with an aged solution of drug.

Covariantly closed, supercoiled DNA, such as PM2 phage DNA, is a sensitive tool for the detection of strand breaks; cleavage of any one of the phosphodiester bonds results in a relaxed form. Furthermore, the supercoiled DNA and the nicked form can be readily resolved by agarose gel electrophoresis (5). To provide information on whether the fragmentation of DNA was due to a direct action of the drug or was the result of enzymatic action on 2-PH-induced DNA damage, electrophoresis of PM2 DNA exposed directly to 2-PH was performed on agarose (Chart 5). Significant conversion of supercoiled DNA to the nicked form (SSC and SSL) was observed only when freshly prepared 2-PH was used, and the sample of DNA was subsequently exposed to alkaline conditions. Thus, treatment

![Chart 2: Effects of 2-PH on the synthesis of DNA, RNA, and protein. Exponentially growing Sarcoma 180 cells were exposed simultaneously to 100 μM 2-PH and either [methyl-³H]thymidine, [U-³H]uridine, or L-[4,5-³H]leucine at 37°. At various times thereafter, acid-insoluble radioactivity was measured. @, [³H]thymidine incorporation; @, [³H]uridine incorporation; ©, [³H]leucine incorporation. The control reactions showed linear incorporation of each radioisotope into acid-insoluble material for at least 30 min, and 7670, 4900, and 1300 cpm were incorporated per 10⁶ cells in 30 min into DNA, RNA, and protein, respectively.

![Chart 3: Sucrose gradient analysis of DNA of Sarcoma 180 cells treated with 2-PH. Sarcoma 180 cells suspended in Fisher’s medium containing 10% horse serum were labeled with 13 nmol [methyl-³H]thymidine (2.4 Ci/mmol) for 6 hr. Cells were washed, suspended in fresh medium for 15 hr, and then incubated with 100 μM 2-PH for an additional 1-hr period. Cells (5 x 10⁶) were collected, resuspended in 0.1 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.02 M EDTA, and layered directly onto the top of a sucrose gradient along with a cell lysing solution consisting of 0.4 m mol of heparin in 1% Sarkosyl. Velocity sedimentation through 5 to 20% alkaline sucrose (A) and 10 to 30% neutral sucrose (B) at 5°. The total radioactivity applied on each gradient was approximately 1 x 10⁶ cpm/ml with 70 to 75% recovery after fractionation. @, control; ©, 2-PH.

![Chart 4: Alkaline sucrose gradient analysis of the repair of DNA damaged by exposure to 2-PH. Sarcoma 180 cells treated with 100 μM 2-PH for 1 hr were subjected to alkaline sucrose gradient centrifugation 24 hr after incubation in Fisher’s medium plus 10% horse serum in the absence of drug (see legend for Chart 3 for experimental details). @, control; ©, 2-PH.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mM)</th>
<th>cpm</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td>54,870</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td></td>
<td>750</td>
</tr>
<tr>
<td>Hydroxyurea + nitrogen mustard</td>
<td>10 + 0.01</td>
<td>1,860</td>
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<tr>
<td>Hydroxyurea + 2-PH</td>
<td>10 + 0.05</td>
<td>630</td>
</tr>
<tr>
<td>Hydroxyurea + 2-PH</td>
<td>10 + 0.01</td>
<td>640</td>
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</tbody>
</table>
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Table 2

Growth inhibition of Sarcoma 180 cells by aged solutions of 2-PH

<table>
<thead>
<tr>
<th>2-PH preincubation (min)</th>
<th>Cell no./ml × 10⁻⁸</th>
<th>% of control</th>
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<tr>
<td>30</td>
<td>1.2 ± 0.01</td>
<td>60</td>
</tr>
<tr>
<td>60</td>
<td>1.3 ± 0.03</td>
<td>67</td>
</tr>
<tr>
<td>90</td>
<td>1.5 ± 0.03</td>
<td>73</td>
</tr>
<tr>
<td>120</td>
<td>1.7 ± 0.08</td>
<td>84</td>
</tr>
<tr>
<td>150</td>
<td>1.7 ± 0.06</td>
<td>88</td>
</tr>
<tr>
<td>180</td>
<td>1.8 ± 0.16</td>
<td>91</td>
</tr>
<tr>
<td>210</td>
<td>2.2 ± 0.09</td>
<td>111</td>
</tr>
</tbody>
</table>

a Starting cell number, 0.9 × 10⁶ cells/ml.
b Control (untreated) cell number at 24 hr, 2.0 × 10⁶ cells/ml.
c Mean ± S.D. from 6 different samples.

of PM2 DNA with 0.75 mM 2-PH for 30 min at room temperature and then exposure to alkaline conditions induced 1.3 breaks/molecule of DNA as calculated by the Poisson distribution (8), whereas nondrug-treated control DNA exposed to alkaline conditions had only 0.6 break/molecule. The number of DNA breaks/molecule induced by 0.75 mM 2-PH was increased to 1.9 when the sample was heated for 15 min at 70°C before alkaline treatment.

DISCUSSION

To characterize the cytotoxic mechanism of action of the arylsulfonylhydrazones of pyridine N-oxide, a newly developed class of antineoplastic agents, studies have been conducted in Sarcoma 180 cells in culture with 2-PH, a representative member of this series. The findings demonstrate that treatment of Sarcoma 180 cells in culture with a concentration of 2-PH that inhibits cell growth by 50% (100 μM) had minimal effects on the biosynthesis of DNA, RNA, and protein (Chart 2), suggesting that the major enzyme systems involved in the formation of these macromolecules are not directly affected by this agent.

The sedimentation rate of DNA of cells treated with the 50% growth-inhibitory level of 2-PH was greatly reduced under alkaline denaturing conditions in sucrose gradients (Chart 3A), indicating that this arylsulfonylhydrazone induces single-strand scission of the DNA of these neoplastic cells. That 2-PH or a metabolic product or products thereof directly produce single-strand breaks in DNA or create alterations that render DNA labile to alkaline hydrolysis is supported by the finding that the sedimentation of DNA from drug-treated cells on neutral sucrose gradients is not appreciably altered (Chart 3B).

It has been suggested that, unlike double-strand DNA breaks, a large number of single-strand breaks in DNA can be repaired rapidly (7, 17) and almost completely (10). However, if DNA breaks are not repaired, even a small number of these lesions might be expected to contribute significantly to the kill of cells (14). In contrast to compounds that induce single-strand breaks in DNA which can be repaired readily upon removal of the offending agent (6, 15), the majority of the damage to the DNA of Sarcoma 180 cells produced by 2-PH appears to persist for at least 24 hr after removal of the drug from the cellular environment (Chart 4). These results suggest that the cell cytotoxicity caused by 2-PH might well be due to its ability to induce DNA strand scission. This possibility is supported by experiments with aged, inactivated 2-PH, in which the capacity of this material to induce breakage of DNA is correlated with cell cytotoxicity.

2-PH did not induce breakage of PM2 DNA in vitro when added directly to these macromolecules unless the mixture was subsequently exposed to an alkaline environment. These findings suggest that an analogous mechanism exists in intact Sarcoma 180 cells (i.e., 2-PH produces damage to DNA which renders these macromolecules labile to alkaline pH) or that 2-PH is converted to an active form which exerts biological activity. 2-PH is rapidly inactivated at the relatively high temperature used and once inactivated by this technique is unable to induce DNA strand breaks using alkaline treatment. Thus, the damage to DNA induced by 2-PH was created before the heat and alkaline treatments.

There are a number of sites at which strand breakage can be introduced in DNA (3, 20). One of these is an apurinic-apyrimidinic site, which can serve as the substrate for a correct endonuclease (3, 12), and is also labile to alkaline hydrolysis (19). Slow depurination of DNA occurs spontaneously when purine bases are alkylated (2, 18). In addition, in vitro depurination of alkylated purine bases can be readily achieved by heating, since alkylated purines are heat labile (13). The lability of PM2 DNA treated with 2-PH to heat under alkaline conditions may indicate that treatment of DNA with 2-PH results in alkylated purines. To achieve such alterations, however, requires the conversion of this arylsulfonylhydrazone to a form(s) capable of covalent interaction with DNA.

REFERENCES

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