Mechanism of Action of Arenesulfonylhydrazones of 2-Pyridinecarboxaldehyde 1-Oxide in L1210 Cells

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ABSTRACT

The arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide represent a relatively new class of anticancer agents. The biochemical alterations responsible for antineoplastic activity were investigated using the most potent member of this class synthesized to date, the 3,4-dimethoxybenzenesulfonylhydrazone of 2-pyridinecarboxaldehyde 1-oxide (3,4-DSP), as the prototype compound. The primary biochemical lesion observed was the production of DNA single-strand breaks, which were analyzed using alkaline elution methodology. This production of DNA damage required the spontaneous chemical formation of a reactive species; thus, "aging" of a solution of 3,4-DSP prior to exposure of L1210 leukemia cells in culture markedly decreased the production of DNA single-strand breaks. The chemical production of an alkylating species from 3,4-DSP has been proposed to occur by the intramolecular abstraction of the nitrogen proton by the 1-oxide group, followed by release of arenesulfonic acid to form the potent alkylating species, 1-oxidopyridin-2-ylidiazomethane. Replacement of the proton by a methyl group, lack of the 1-oxide group, or replacement of the aldehyde proton by a methyl group increases the chemical stability of the arenesulfonylhydrazones. These modifications have been shown in a previous publication (D. A. Shiba, J. A. May, Jr., and A. C. Sartorelli, Cancer Res., 43: 2023–2029, 1983) to lead to (a) an elimination of alkylating activity and (b) a decrease in in vitro cytotoxicity and in vivo antineoplastic activity. These effects are also accompanied by an inability to produce detectable DNA single-strand breaks. 3,4-DSP caused little or no inhibition of the biosyntheses of DNA, RNA, or protein, as measured by the incorporation of radiolabeled thymidine, uridine, or leucine, respectively, into acid-insoluble material; however, consistent with the production of DNA damage, 3,4-DSP inhibited the normal progression of L1210 cells through the cell cycle after a single treatment in vivo with drug (100 mg/kg). Cells were blocked in the G2-M phase of the cell cycle for 6 to 24 hr after exposure to 3,4-DSP; the cell population recovered by 48 hr after exposure and appeared to be progressing normally through the cell cycle.

INTRODUCTION

The arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide have demonstrated potent activity against a wide spectrum of transplanted murine tumors (1, 5–7). Previously, our laboratory observed that the benzenesulfonylhydrazone derivative of 2-pyridinecarboxaldehyde 1-oxide produced DNA single-strand breaks, which were not repaired during a 24-hr period (4). Recently, we also reported that members of this class of agents, including the most active compound synthesized to date, 3,4-DSP, underwent spontaneous chemical decomposition to generate a potent alkylating species, 1-oxidopyridin-2-ylidiazomethane. Production of the reactive species correlated with in vitro cytotoxicity and in vivo antineoplastic activity (8).

This report presents (a) data which demonstrate that 3,4-DSP produces DNA single-strand breaks in neoplastic cells but does not markedly affect DNA, RNA, or protein biosynthesis and (b) findings which suggest that formation of a reactive species by arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide is required for the creation of DNA lesions. The damage to DNA produced by these agents correlated with their capacity to induce cytotoxicity in vitro and antitumor activity in tumor-bearing animals. These actions of 3,4-DSP were accompanied by inhibition of the progression of L1210 leukemia cells through the cell cycle.

MATERIALS AND METHODS

3,4-DSP, the N-methyl-p-toluenesulfonylhydrazone of 2-pyridinecarboxaldehyde 1-oxide, and the p-toluenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide, 2-pyridinecarboxaldehyde, and 2-acetylpyridine 1-oxide were prepared in this laboratory using procedures described previously (1, 5, 7).

The effects of 3,4-DSP on the cell cycle progression of L1210 cells were analyzed by fluorometric measurements of the relative DNA content of individual cells. BALB/c x DBA/2 F1 males (hereafter called CD2F1) mice given implants i.p. of 10⁶ L1210 leukemia cells received a single i.p. dose of either 3,4-DSP (100 mg/kg) in 0.4% N-methyl-o-glucamine in PBS or 0.4% N-methyl-o-glucamine in PBS 72 hr after tumor implantation. After 1, 6, 24, and 48 hr, L1210 cells were removed and stained with acriflavin-Feulgen according to the procedure of Gill and Jotz (2). Cells were fixed with 10% formalin for at least 12 hr at 4°C prior to staining. After centrifugation at 80 x g for 5 min, the cell pellet was sequentially washed with (i) 0.4% A/-methyl-D-glucamine in PBS; (ii) PBS; (iii) 0.1% A/-methyl-D-glucamine in PBS; and (iv) water at a final concentration of 10⁶ cells/ml. The cells were kept in the dark and refrigerated until analysis. After repeated pipetting the cells 10 times to break up any clumped cells, the suspension was filtered through a 37-µm nylon mesh (Small Parts, Inc., Miami, FL) and analyzed using a Becton-Dickinson FACS IV flow cytometer equipped with an argon-ion laser operated at a wavelength of 457 nm. Analyses were conducted using the Becton-Dickinson CellQuest database to determine the cell cycle phase and DNA content of individual cells. The mean DNA content was determined for individual cell populations (G1, S, or G2+M) and compared with the DNA content of controls. The DNA content was determined using a flow cytometer equipped with an argon-ion laser operated at a wavelength of 457 nm. Analyses were conducted using the Becton-Dickinson CellQuest database to determine the cell cycle phase and DNA content of individual cells. The mean DNA content was determined for individual cell populations (G1, S, or G2+M) and compared with the DNA content of controls.
MECHANISM OF ACTION OF 3,4-DSP ANALOGUES

The effects of 3,4-DSP on the biosyntheses of DNA, RNA, and protein were monitored by measuring the effects of this agent on the incorporation of radioactive thymidine, uridine, and leucine into acid-insoluble material. Exponentially growing L1210 leukemia cells were maintained in Fischer's medium containing penicillin and streptomycin (Grand Island Biological Co., Grand Island, NY) and supplemented with 10% horse serum (Flow Laboratory, McLean, VA). One-ml aliquots containing 7.5 x 10^6 cells were incubated at 37°C in microcentrifuge tubes containing 150 μM 3,4-DSP. Two μCi of [methyl-^3H]thymidine (50 Ci/mmol; Moravek Biochemicals, City of Industry, CA), 2 μCi of [5-^3H]uridine (20 Ci/mmol; Moravek Biochemicals), or 8 μCi of [L-4,5-^3H]leucine (58.5 Ci/mmol; New England Nuclear Corp., Boston, MA) were added and, at various times thereafter, samples were collected and centrifuged for 15 sec at 9,000 x g using an Eppendorf microcentrifuge. The resulting cell pellet was washed thoroughly 4 times with ice-cold 0.2 N perchloric acid, and the cell pellet was hydrolyzed for 30 min at 85°C with 1.0 ml of 1.2 N perchloric acid or 0.1 N NaOH for nucleic acids or protein, respectively. A 500-μl aliquot was combined with 5 ml of Aquasol (New England Nuclear). The protein aliquot was neutralized with 50 μl of 1.2 N perchloric acid prior to counting to prevent chemiluminescence. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer.

DNA single-strand breaks were evaluated using a modification of the alkaline elution methodology of Kohn et al. (3), except that the elution rate was 4 times the reported speed due to the unavailability of a pump capable of producing a flow rate of 0.04 ml/min. The DNA of exponentially growing L1210 leukemia cells was labeled for 20 hr in culture with either [methyl-^3H]thymidine (0.05 μCi/ml; 59 Ci/mmol; Schwarz/Mann, Orangeburg, NY) or [methyl-^14C]thymidine (0.02 μCi/ml; 51 mCi/mmol; New England Nuclear) and chased with 10^-3 M nonradioactive thymidine. The ^14C-labeled cells were treated with various concentrations of drug dissolved in Fischer's medium for 1 hr at 37°C. In other experiments designed to study the effects of "aging" of the drug solution on the production of DNA single-strand breaks, solutions of 3,4-DSP which had been incubated at 37°C with shaking for 0 min, 20 min, or 3 hr were used. ^3H-Labeled cells were treated with an equivalent volume of the tissue culture medium. Cells were then centrifuged at 140 x g for 5 min, the medium was removed, and the cells were resuspended in serum-free medium at a concentration of 1 to 2 x 10^6 cells/ml.

The ^3H-labeled cells were irradiated with 300 rads of X-ray using a Siemens Stabilipan X-ray machine calibrated with a Keithley Instruments Model 409 picocommeter. Equal numbers of ^14C-labeled drug treated cells and ^3H-labeled X-irradiated cells to serve as an internal standard were mixed, and 1 ml of this suspension was placed onto a PBS-washed 25-mm Nucleopore 2.0-μm polycarbonate filter (Nucleopore Corp., Pleasanton, CA). Cells were washed once with 6 ml of PBS, disrupted with 5 ml of lysing solution (2% sodium dodecyl sulfate, 0.025 M EDTA) containing proteinase K (0.5 mg/ml) at pH 9.7 to remove DNA-associated protein, and washed with 5 ml of 0.02 M sodium EDTA, pH 9.5. The DNA was eluted at a flow rate of 0.15 ml/min using 0.02 M EDTA (acid form) containing 0.1% sodium dodecyl sulfate and titrated with tetradoxypolyammonium hydroxide to a final pH of 12.1. Twenty 1.5-ml samples were collected during the elution. The filter was removed after collection of the last sample and hydrolyzed using 1.0 ml of Protosol (New England Nuclear). The filter holder was reassembled, and the elution apparatus was washed 3 times with 1.5 ml of eluting solution and twice with 1.5 ml of 0.4 N NaOH. A 1.5-ml aliquot of the pre- and postelution washes, the elution samples, and the filter hydrolysate were combined with 6 ml of Hydrofluor (National Diagnostics, Sommerville, NJ) containing 0.7% glacial acetic acid to prevent chemiluminescence of the alkaline solutions. The samples were counted using a ^3H/^14C dual-label counting program with automatic quench control on a Beckman LS 7500 liquid scintillation counter. Corrections for ^14C spillover were made for each sample, as well as for the fraction of counts remaining on the filter during the elution (DNA remaining on the filter). The data were plotted on a double-log graph, with the fraction of ^14C-labeled DNA remaining on the filter on one axis and the fraction of ^3H-labeled reference DNA remaining on the filter on the other. The later values were plotted on a reverse-log scale of from 1.0 to 0.0. The slopes of the linear portion of each elution curve were compared.

RESULTS

The effects of a single i.p. treatment of L1210 leukemia-bearing mice with 100 mg of 3,4-DSP/kg on the progression of the leukemia cells through the cell cycle was determined by fluorometric analysis. One hr following exposure to this agent, cells replicated normally, as demonstrated by the similarity of the DNA histograms from fluorometric measurements of L1210 leukemia cells treated in vivo with 3,4-DSP. CD2F1, mice given implants i.p. of 10^6 L1210 leukemia cells received a single i.p. treatment of either 3,4-DSP (100 mg/kg) in 0.4% N-methyl-o-glucamine in PBS or 0.4% N-methyl-o-glucamine in PBS (control) 72 hr after tumor implantation. After 1, 6, 24, and 48 hr, the L1210 cells were removed and stained with acriflavine-Feulgen as described in "Materials and Methods."
MECHANISM OF ACTION OF 3,4-DSP ANALOGUES

1.0-

Control

Fraction of 3H-Labeled Reference DNA Remaining on Filter

100 uM

200 uM

400 uM

Fraction of 3H-Labeled Reference DNA Remaining on Filter

Chart 3. Alkaline elution analysis of DNA single-strand breaks in cultured L1210 leukemia cells produced by various concentrations of 3,4-DSP. DNA of exponentially growing L1210 leukemia cells was labeled for 20 hr with either [methyl-3H]thymidine (0.05 ¿Ci/ml) or [methyl-14C]thymidine (0.02 ¿Ci/ml) and chased with 10^{-6} M nonradioactive thymidine. The 14C-labeled cells were treated for 1 hr at 37° with various concentrations of 3,4-DSP or were untreated. 3H-Labeled cells received 300 rads of X-irradiation. Equal numbers of 14C-labeled cells and 3H-labeled internal standard cells were mixed, and the resulting suspension was analyzed by alkaline elution analysis as described in "Materials and Methods." The radioactivity of the elution aliquots was determined, and the data were plotted on a double-log graph. Histogram to that of untreated control cells (Chart 1). Six hr after treatment with 3,4-DSP, however, few cells were present in the G1 phase, due to blockage of their progression through the G2-M phase. This G2-M block persisted for up to 24 hr after exposure to the single dose of this agent, but the population recovered by 48 hr and appeared to progress normally through the cell cycle.

To determine the metabolic basis for the inhibition of cellular replication of the L1210 leukemia cells by 3,4-DSP, the effects of this agent on DNA, RNA, and protein synthesis were measured by exposure of exponentially growing cells in culture to this agent for 1 hr, followed by pulse labeling with [3H]thymidine, [3H]uridine, or [3H]leucine. The concentration used in these investigations was 100 ¿M 3,4-DSP, a level that inhibits L1210 cell replication in culture by 50%. As shown in Chart 2, 3,4-DSP, under these conditions, did not significantly inhibit the synthesis of DNA, RNA, or protein, as measured by the incorporation of the radiisotopically labeled precursors of these macromolecules into acid-insoluble material. Exposure of L1210 cells to 3,4-DSP for 6 hr, however, produced a 30% inhibition of DNA synthesis, while RNA and protein biosynthesis continued to be unaffected (data not shown).

DNA single-strand breaks produced by 3,4-DSP and other related arylsulfonylhydrazones in culture were demonstrable by alkaline elution analysis. Despite the decreased sensitivity of the modified alkaline elution analysis because of increased mechanical shearing resulting from the relatively fast elution rate, as shown in the untreated control sample, the alkaline elution curves, presented in Chart 3, demonstrated clearly that 3,4-DSP induced extensive DNA single-strand breaks in L1210 leukemia cells and that the DNA damage was dose-dependent over the range of 100 to 400 ¿M 3,4-DSP.

To examine the relationship between the spontaneous generation of an alkylating species by 3,4-DSP and the production of DNA single-strand breaks, solutions of 300 ¿M 3,4-DSP were "aged" at 37° for 20 min or 3 hr prior to addition to suspensions of [methyl-14C]thymidine labeled L1210 leukemia cells. Alkaline elution analysis curves demonstrated that a 20-min "aged" solution produced 22% fewer breaks than did a corresponding "non-aged" control solution, and a 3-hr "aged" solution of 3,4-DSP did not produce a significant number of detectable breaks (Chart 4).

Further support for a correlation between the spontaneous chemical production of a reactive species from arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide, the generation of DNA single-strand breaks, and both in vitro cytotoxicity and in vivo anticancer activity in tumor-bearing mice was obtained using the series of arenesulfonylhydrazones analogues shown in Chart 5.
MECHANISM OF ACTION OF 3,4-DSP ANALOGUES

Chart 5. Chemical structures of a series of arenesulfonylhydrazone analogues used to study the correlation between the capacity to spontaneously generate a reactive species and to produce DNA single-strand breaks.

sulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide (Compound II), 2-pyridinecarboxaldehyde (Compound III), 2-acetylpyridine 1-oxide (Compound IV), and the N-methyl-p-toluenesulfonylhydrazone of 2-pyridinecarboxaldehyde 1-oxide (Compound V) were assessed by alkaline elution analysis after a 1-hr exposure of L1210 leukemia cells in culture to 300 μM concentrations of each agent. The alkaline elution curves, shown in Chart 6, for 3,4-DSP (Compound I) and the p-toluenesulfonylhydrazone analogue (Compound II) were similar whereas, in contrast, compounds either lacking the 1-oxide group (Compound III) or containing a methyl group in place of the aldehydic proton (Compound IV) or nitrogen proton (Compound V) did not produce detectable DNA single-strand breaks.

DISCUSSION

Arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide spontaneously generate through chemical rearrangement a potent alkylating species; production of this cytotoxic species correlated with both in vitro cytotoxicity and in vivo antitumor activity (8). The most active compound of this relatively new class of antineoplastic agents, 3,4-DSP, was chosen as a prototype compound in studies designed to elucidate the metabolic lesion(s) responsible for cytotoxic activity.

The primary biochemical alteration produced by 3,4-DSP in L1210 leukemia cells appears to be the production of DNA single-strand breaks. Another member of this class of arenesulfonylhydrazones, the benzenesulfonylhydrazone derivative, also produced DNA single-strand breaks, which were not repaired during a 24-hr period when analyzed by alkaline sucrose gradient analysis (4).

The DNA breaks produced by these agents probably result from alkylation of DNA by the spontaneously chemically produced alkylating species. We have provided evidence that the arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide undergo chemical decomposition to form a potent alkylating agent, 1-oxidopyridine-2-yl diazomethane (8). Chemical production of this reactive species and the capacity to alkylate several nucleophilic trapping agents was shown to correlate with both cytotoxicity in vitro and anticancer activity in vivo. Furthermore, "aging" of solutions of 3,4-DSP prior to evaluation of activity showed a corresponding decrease or absence of both alkylating activity and cytotoxic capacity. The present study shows that a decrease in the capacity of 3,4-DSP to produce DNA single-strand breaks occurs following the "aging" of solutions of this agent, implying that the spontaneous chemical formation of a reactive species was a prerequisite for DNA damage.

The mechanism responsible for the chemical production of an alkylating agent by members of this class has been postulated...
to result from intramolecular abstraction of the nitrogen proton by the 1-oxide group. Arenesulfonylhydrazone analogues (a) containing a methyl group which might function by sterically preventing this abstraction, (b) containing a replacement of the nitrogen proton by a methyl group, or (c) lacking the 1-oxide function all had greater chemical stability than did 3,4-DSP; these derivatives lost the capacity to alkylate 4-(4-nitrobenzyl)pyridine in vitro and to inhibit the replication of L1210 leukemia cells in culture (8). These inactive analogues also did not produce detectable DNA single-strand breaks when analyzed by alkaline elution methodology, further supporting the concept that the arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide require spontaneous chemical activation to exert anticancer activity and that this activity is dependent on the generation of lesions at the level of DNA.

The extensive damage to DNA by 3,4-DSP was not accompanied by major inhibition of the biosyntheses of DNA, RNA, or protein. Thus, the incorporation of \(^{3}H\)-radiolabeled thymidine, uridine, and leucine into acid-insoluble materials of L1210 leukemia cells in culture was not affected significantly by relatively short-term exposure to 3,4-DSP (i.e., 1 hr) at a concentration which inhibited cellular replication under these conditions by 50%. A similar lack of inhibition of nucleic acid and protein biosynthesis has been reported previously for the related benzenesulfonylhydrazone of 2-pyridinecarboxaldehyde 1-oxide in Sarcoma 180 cells in culture (9) and the \(p\)-toluenesulfonylhydrazone derivative in Sarcoma 180 ascites cells treated in vivo through exposure of tumor-bearing mice (7), indicating the lack of a primary metabolic block on the biosynthetic pathways leading to the formation of these macromolecules.

The extensive DNA damage produced by the arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxidre corresponded to specific effects on the normal progression of L1210 leukemia cells through the cell cycle, with accumulation of cells in the G2-M phase occurring by 6 hr and persisting for up to 24 hr after the treatment of leukemia-bearing animals with a single dose of this agent. By 48 hr, the DNA histograms appeared to be normal. Since these cell cycle studies were measured on the total cell population, the normal cell cycle distribution which occurred at 48 hr after drug may not only represent possible recovery of drug affected cells but also the outgrowth of any unaffected or minimally affected members of the population.

In summary, the arenesulfonylhydrazones of 2-pyridinecarboxaldehyde 1-oxide appear to require spontaneous chemical production of a cytotoxic alkylating species, 1-oxopyridin-2-ylid azomethane, to exert anticancer activity. Unlike most alkylating agents, inhibition of nucleic acid and/or protein synthesis does not appear to be important for the expression of cytotoxicity by 3,4-DSP. The cytotoxicity of 3,4-DSP appears to be due to extensive DNA single-strand breaks and subsequent arrest of neoplastic cells in the G2-M phase of the cell cycle. These effects suggest that DNA is the biochemical target for these arenesulfonylhydrazones. Further studies are currently under way to define the specific lesions created by this agent at the level of DNA.

REFERENCES

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