Protection against cis-Diaminedichloroplatinum Cytotoxicity and Mutagenicity in V79 Cells by 2-[(Aminopropyl)amino]ethanethiol

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ABSTRACT

The effect(s) of the radioprotector 2-[(aminopropyl)amino]ethanethiol (WR1065) on cis-diaminedichloroplatinum(II) (cis-DDP)-induced cytotoxicity and mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase locus in V79 Chinese hamster cells was examined. With a standard exposure time of 30 min for both agents, WR1065, at a final working concentration of 4 mM, was added to cells either prior to, during, or immediately following treatment with selected doses of cis-DDP. With respect to cell survival, dose modification factors of 2.9, 1.4, and 1.4 were obtained for cells treated under each of these conditions, respectively. The induction of mutants under all conditions was linear as a function of cis-DDP concentration. Mutation frequencies per μg of cis-DDP were 25 × 10^-7, 1 × 10^-7, 5 × 10^-7, and 11 × 10^-7 for protocols involving no protector present or WR1065 added before, during, or after cis-DDP treatment, respectively. No WR1065-mediated cytotoxicity to cells derived from either wild-type or mutant colonies was observed. These data demonstrate that WR1065, the free thiol of S-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR2721) which is currently being evaluated in clinical trials, affords substantial protection against the cytotoxic and mutagenic effects of cis-DDP, with the most effective protection occurring when the protector is administered prior to cis-DDP treatment. Due to their ability to better protect normal as compared to tumor tissue against acute effects, these protectors have generated considerable interest for use in improving the therapeutic gain of radiation therapy and chemotherapy. The ability of these compounds to also protect against the mutagenic effects of therapy agents may be an additional important benefit for consideration in their use in the treatment of human neoplasia.

INTRODUCTION

cis-DDP is currently one of the most valuable antineoplastic agents used in the treatment of human cancers (1). It is, however, a highly toxic agent to normal cells and has been shown to be clastogenic (2), mutagenic (3), and capable of inducing sister chromatid exchanges and morphological transformation (4). These deleterious effects appear to be due to the ability of cis-DDP to produce both inter- and intrastrand DNA cross-links (5).

While these lesions are thought to be irreversible, it has been reported that SH-containing compounds such as thiols can protect against cis-DDP-induced cytotoxicity and mutagenicity (6-9). When administered either concomitantly or up to 4 h following the removal of cis-DDP, cysteamine was observed to be protective against cell toxicity (7). Likewise, thiourea was found to be effective in protecting against cis-DDP-induced cytotoxicity and mutagenicity (8). With respect to molecular damage, thiourea has been characterized as being effective in reversing cis-DDP-induced interstrand cross-links without the concomitant breakdown of target DNA molecules (9).

The radioprotective effect of aminothiols such as WR2721 has been demonstrated in a variety of biological systems. Clinical interest in WR2721 resulted from the observation that normal, as compared to neoplastic, tissues were, for the most part, differentially protected from radiation damage (10, 11). In addition, Phases I and II clinical trials have been performed which suggest that WR2721 provides protection against cis-DDP-induced nephrotoxicity (12) without concomitant tumor protection (13). Thus, efforts have been directed at applying WR2721 to radiation therapy protocols in the attempt to extend a therapeutic advantage by increasing the effective dose to solid tumors as compared to surrounding, but dose-limiting, normal tissues (14). Recently, it has been reported, using a rodent system, that WR2721 can also exhibit an anticarcinogenic activity (15). Our recent work suggests that radiation-induced mutagenesis (16), as well as transformation in vitro, can be effectively altered and interfered with using the free thiol form of WR2721, e.g., WR1065. Thus, it was of interest to us to investigate the effects of WR1065 on cis-DDP activity. In particular, we have extended our studies to include the evaluation of WR1065 as an agent capable of modulating the processes of cis-DDP-induced cytotoxicity and mutagenicity using a V79 Chinese hamster lung cell system.

MATERIALS AND METHODS

Cells and Culture Conditions. V79-B310H Chinese hamster cells were maintained as stock cultures in α-minimal essential medium (Gibco) with 10% fetal calf serum (Biologos) in a humified atmosphere containing 5% CO₂ and 95% air at 37°C. Cells for use in mutation studies were grown for 24 h under the previously described conditions in α-minimal essential medium containing hypoxanthine (10 μg/ml), thymidine (5 μg/ml), and aminopterine (0.4 μg/ml) (HAT medium), to reduce the number of preexisting mutants at the HGPRT locus (18). Upon removal from HAT medium the cells were grown in normal growth medium for at least two passages prior to use. Doubling time of cells under these conditions was 11 h. To ensure that the spontaneous background mutation rate would remain at acceptable levels, cell populations treated with HAT medium were passaged no more than 30 times for use in these experiments.

Drug Treatment. WR1065 used in these studies was supplied by the
Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, and was made up in PBS (8.1 mM Na2HPO4-1.5 mM KH2PO4-0.14 mM NaCl-2.6 mM KCl) buffer at a 1 mM concentration and sterilized by filtration immediately before use. Under these conditions, the protector was stable throughout the duration of the experiment (16). cis-DDP (Sigma Chemical Co., St. Louis, MO) was prepared as a stock solution of 1 mg/ml in PBS buffer prior to each experiment. The desired concentration of drug was then added to each V79 cell test culture.

Survival Studies. Exponentially growing cultures of V79 cells were treated with various concentrations of cis-DDP for 30 min. In the case of the radioprotector WR1065, the protective efficiency against radiation-induced cell killing was maximal but essentially indistinguishable between WR1065 concentrations of 4 and 10 mM (19). Thus, to conserve the amount of the radioprotector used in these studies, experiments were routinely performed using a concentration of 4 mM. An exposure time of 30 min was chosen for exposure to the radioprotector, because this exposure time was observed to ensure significant protection against radiation-induced cell killing (16). Four treatment protocols were followed: (a) cells were exposed to cis-DDP only; (b) cells were exposed to WR1065 for 30 min, washed free, and then exposed to cis-DDP; (c) cells were exposed simultaneously to cis-DDP and WR1065; and (d) cells were exposed to cis-DDP, washed free, and then exposed to WR1065 for 30 min. After treatment all cultures were washed twice with PBS and trypsinized. Cell survival was determined by plating appropriate numbers of cells to give between 80 and 200 colonies per dish, 6 dishes per experimental point, after 7 days of incubation.

Mutation Assay for HGPRT Mutants. Cells were plated into 150-cm² T-flasks (Corning) at a cell density of 2 × 10⁶ cells per flask. After 24 h of growth, cells were treated with cis-DDP and WR1065 according to the protocols described earlier. Cells were then washed, trypsinized, counted, and plated. Mutation induction was assayed by seeding at least 10⁶ surviving cells per experimental point. Cells were grown in nonselective medium for 6 days to allow mutations to be expressed. At this time the plating efficiency of each experimental group was determined by seeding 200 cells per plate in standard growth medium. Mutation induction for each group was determined by seeding 8 × 10⁶ cells into each of 20 plates containing standard growth medium and 6-TG (Sigma) at a concentration of 5 µg/ml. Following 7 days of growth, resulting mutant colonies were stained with 0.5% methylene blue and counted. Mutation frequencies were expressed as the number of 6-TG-resistant cells per 10⁶ surviving cells. Spontaneous mutation frequencies under these conditions were about 2 × 10⁻⁶. 6-TG-resistant V79 clones were obtained from representative mutation assay plates. Cells were harvested from isolated individual colonies and were maintained separately in continuous cultures in the absence of 6-TG. We have tested clones that were resistant to 5 µg of 6-thioguanine per ml for stability by assaying their ability to survive in varying concentrations of 6-thioguanine. Clones which have been passaged for up to 3 months, some of which were thawed from frozen stock, can grow in concentrations of 6-thioguanine up to 10 µg/ml before significant killing is observed. In our hands normal V79 stocks are reduced to a survival level of 2 to 3 × 10⁻⁸ by 6-thioguanine at concentrations above 1 µg/ml.

RESULTS

Data presented in Fig. 1 demonstrate the protective effects of WR1065 on cis-DDP-induced cytotoxicity to V79 cells. This protection was evidenced by a shift of the resulting survival curves to the right following WR1065 treatment. The amount of cis-DDP required to reduce cell survival to the 1% level was 52, 84, 92, and 134 µg/ml for cis-DDP only and WR1065 before, during, or after cis-DDP exposure, respectively.

Fig. 2 contains data describing the effects of WR1065 on cis-DDP-induced mutations in V79 cells at the HGPRT locus. The frequencies were expressed as the number of 6-TG-resistant cells per 10⁶ surviving cells per experimental point. Cells were grown in nonselective medium for 6 days to allow mutations to be expressed. At this time the plating efficiency of each experimental group was determined by seeding 200 cells per plate in standard growth medium. Mutation induction for each group was determined by seeding 8 × 10⁶ cells into each of 20 plates containing standard growth medium and 6-TG (Sigma) at a concentration of 5 µg/ml. Following 7 days of growth, resulting mutant colonies were stained with 0.5% methylene blue and counted. Mutation frequencies were expressed as the number of 6-TG-resistant cells per 10⁶ surviving cells. Spontaneous mutation frequencies under these conditions were about 2 × 10⁻⁶. 6-TG-resistant V79 clones were obtained from representative mutation assay plates. Cells were harvested from isolated individual colonies and were maintained separately in continuous cultures in the absence of 6-TG. We have tested clones that were resistant to 5 µg of 6-thioguanine per ml for stability by assaying their ability to survive in varying concentrations of 6-thioguanine. Clones which have been passaged for up to 3 months, some of which were thawed from frozen stock, can grow in concentrations of 6-thioguanine up to 10 µg/ml before significant killing is observed. In our hands normal V79 stocks are reduced to a survival level of 2 to 3 × 10⁻⁸ by 6-thioguanine at concentrations above 1 µg/ml.

Fig. 1. The effects of WR1065 on cis-DDP-induced cytotoxicity in V79 cells: O, 30-min treatment with cis-DDP only; •, 30-min treatment with 4 mM WR1065, cells washed, and then 30-min treatment with cis-DDP; 0, 30-min concomitant treatment with WR1065 and cis-DDP; and A, 30-min treatment with cis-DDP, cells washed, and then 30-min treatment with WR1065. Points, mean of 3 experiments; bars, SE. No error bars are included for those data points in which the magnitude of the error bar is less than the size of the symbol.

Fig. 2. The effect of 4 mM WR1065 on the induction of 6-thioguanine-resistant mutants by cis-DDP. Experimental protocols are as described in Fig. 1: O, cis-DDP only; , WR1065 followed by cis-DDP; 0, cis-DDP and WR1065 together; and A, cis-DDP followed by WR1065. Mutation frequencies have been corrected for spontaneous background values of untreated controls. Points, mean of 3 experiments; bars, SE. No error bars are included for those data points in which the magnitude of the error bar is less than the size of the symbol.
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Table 1
6-Thioguanine (5 µg/ml) mutants per 10^8 survivors
Spontaneous mutation frequencies arose in untreated control and corresponding WR1065-exposed (1, 4, or 10 mm for 30 min) V79 cultures.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Control</th>
<th>WR1065</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.6 ± 2.6^a</td>
<td>2.0 ± 1.3 (1)^b</td>
</tr>
<tr>
<td>2</td>
<td>18.26 ± 4.74</td>
<td>7.05 ± 2.94 (4)</td>
</tr>
<tr>
<td>3</td>
<td>8.6 ± 2.6</td>
<td>2.4 ± 1.5 (10)</td>
</tr>
</tbody>
</table>

^a Mean ± SE of 20 plates.
^b Numbers in parentheses, millimolar concentration.

DISCUSSION

Consistent with previous reports concerning the protective effects of selected thiols on cis-DDP toxicity (6–9), the radioprotector WR1065 was observed to be effective in protecting against both drug-induced mutagenesis and cytotoxicity. Maximum protection in the present experiments occurred when the protector was present in the growth medium for 30 min prior to exposure of cells to cis-DDP. While significant protection was observed under all conditions, little if any difference in the magnitude of protection against cell killing was evidenced whether the protector was present either during or immediately following cis-DDP exposure. In contrast, the simultaneous exposure of cells to both the chemotherapeutic agent and WR1065 gave rise to an apparent increased protection against mutagenicity than was observed when the cells were exposed to the protector immediately following cis-DDP treatment.

While the exact mechanism of protection is unclear, it has been suggested that thiols may exert their protective effect by binding to unreacted cis-DDP complexes (6) or otherwise interfere with the formation of interstrand cross-links (9). In particular, thiols may interfere with the binding of cis-DDP to more than one site in DNA so as to prevent the formation of a bifunctional cross-link (8). The protective effects of these thiols and in particular the radioprotectors WR1065 and WR2721, however, extend to other therapeutic but toxic agents, the most notable being ionizing radiation. Protection in the general sense has been proposed to be mediated through a variety of mechanisms including the scavenging of free radicals (20, 21), the donation of hydrogen atoms (22), and the induction of hypoxia in the microenvironment of the cell as a result of cellular metabolism of the protector (23).

Because free radicals have been implicated in the processes of cell killing, mutagenesis, transformation, and carcinogenesis (24) as well as a process involved in the formation of DNA cross-linking (25), it is reasonable to expect that agents capable of scavenging free radicals should play a significant role in modulating these processes. While the scavenging of free radicals may be implicated in the enhanced reduction in the number of background mutants in control populations following exposure to WR1065 (see Fig. 3), it appears that preferential killing of mutant cells by WR1065 is not a contributing factor (see Fig. 3). Within the range of concentrations tested, no differential toxicity was observed for either wild-type cells or cells derived from mutant colonies arising in either protector-treated or untreated controls.

The radioprotector WR1065 and especially its parent compound WR2721 have been extensively studied because of their potential for increasing the therapeutic gain of selected antineoplastic therapies as a consequence of their ability to differentially protect normal as compared to neoplastic tissue. While the focus of their evaluation in clinical trials has been directed at their ability to modulate deleterious acute effects, the observations that these agents can also protect against radiation-induced mutagenesis (16) and carcinogenesis (15) make them even more intriguing for further investigation and application to the clinical situation. By the judicious use of these and similar agents as adjuvants in well-designed therapy protocols, it may be possible to not only improve the therapeutic gain but also to significantly reduce the risk of therapy-induced secondary cancers in patients characterized as having an excellent prognosis for cure and long-term survival.

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