Further Evidence That the Radioprotective Aminothiol, WR-1065, Catalytically Inactivates Mammalian Topoisomerase II

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Abstract

It has recently been proposed that the thiol form of the cytoprotective drug amifostine that is designated WR-1065 [2-((aminopropyl)amino)ethanethiol] exerts its cytoprotective effects in part via a catalytic inhibition of DNA topoisomerase II (topo II) α. This in turn leads to the subsequent accumulation of cells in G2 phase and a prolongation of the cell cycle. We have used a Chinese hamster V79 cell-based micronucleus assay to further evaluate this hypothesis. It is demonstrated that WR-1065 strongly inhibits the clastogenesis of the topo II poisons etoposide and cinafloxacin at clinically attained exposure levels while having no effect on clastogenesis induced by topo II-noninteractive chemicals. These findings are consistent with the hypothesis that WR-1065 is a catalytic inhibitor of topo II in mammalian cells. These studies also suggest that WR-1065 might be expected to reduce the toxicity and clastogenicity in clinical applications of etoposide or quinolone antibiotics in dose-limiting normal tissues.

Introduction

The aminothiol amifostine, on dephosphorylation to its active thiol form WR-1065, has shown remarkable radio- and chemoprotective effects in vitro and in vivo. It is currently approved for clinical use as a protective agent against renal toxicity induced by cisplatin in patients being treated for ovarian cancer and against xerostomia induced by ionizing radiation in patients with head and neck cancer (1). The underlying mechanisms of action usually attributed to this drug are its ability to scavenge free radicals, to participate in direct chemical repair through the donation of hydrogen atoms, and to induce intracellular hypoxia as a result of undergoing auto-oxidation (2). Each of these mechanisms requires that WR-1065 must be present at the time of radiation or drug treatment (3). However, the ability of WR-1065 to protect against mutation induction even when administered up to 3 h after insult (3) has been difficult to explain. Rather than acting directly to reduce initial damage, the postaddition effectiveness of WR-1065 in preventing mutagenesis can be attributed to its effects on endogenous enzyme systems that are implicated in damage processing and/or repair. An important parameter in the cellular response to DNA-damaging agents is the ability of the cell to accurately repair damage to its DNA before it becomes fixed at cell division. Agents that can prolong the duration of the cell cycle to allow for more time to complete repair would presumably enhance the fidelity of repair as evidenced by a reduction in clastogenic (4) and mutagenic damage (3). Lending support to this hypothesis is the ability of WR-1065 to perturb cell cycling by causing an accumulation of cells in G2 (5, 6). The mechanism of this G2 delay remains an open question, but evidence has accumulated suggesting that WR-1065 interferes with the normal activity of DNA topo II (5, 7). Specifically, it was suggested that WR-1065 inhibits the normal phosphorylation of topo II, rendering topo II catalytically inactive.

We have recently reported a modification of the in vitro micronucleus assay in which catalytic inhibitors of topo II are used to probe the mechanism of clastogenic activities of test articles (8). With this technique, it was demonstrated that the clastogenicity (micronucleus induction) of topo II-active poisons such as etoposide and cinafloxacin was nearly completely antagonized by catalytic inhibition of topo II by the known catalytic topo II inhibitors ethiidium bromide, chloroquine, and sodium azide. Catalytic inhibition renders topo II incapable of forming “cleavable complex,” which is thought to be the primary cause of clastogenic lesions after treatment with topo II poisons. The present studies evaluated the ability of WR-1065 to inhibit the clastogenic activity of topo II poisons in this V79 cell-based system.

Materials and Methods

Chemicals. The aminothiol WR-1065 was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Cinafloxacin was synthesized according to published literature. All other compounds used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO). [3H]Thymidine (25 Ci/mmol) was obtained from Amersham Life Sciences (Arlington Heights, IL).

Cells and in Vitro Micronucleus Assay. Chinese hamster lung V79 cells were grown and maintained in Eagles’ minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum. All studies were conducted by seeding 400,000 cells/well in a 6-well tissue culture dish and growing the cells for 24 h before any drug treatments. Conduct of the in vitro micronucleus assay was essentially as reported previously (9). Briefly, drug treatments were for 30 min in medium, followed by removal of the drug and the addition of fresh medium containing 3 μg/ml cytochalasin B for approximately 16 h. Cells were then harvested with trypsin-EDTA, hypotonically swollen, affixed to glass slides, fixed, stained, and coverslipped. Slides were examined for relative numbers of binucleate and mononucleate cells to assess the antiproliferative effects of the treatment. Between 300 and 500 binucleate cells were also examined for the presence of micronuclei.

[3H]Thymidine Pulse Studies. Catalytic topo II inhibitors and topo II poisons were evaluated for their effects on the DNA replicative process during the course of cell treatments by thymidine pulse experiments. Cells in 6-well plates were exposed to 1 μCi of [3H]thymidine for 30 min in the presence or absence of test articles. Cells were rapidly washed with three changes of ice-cold PBS, harvested by trypsinization, and placed onto Whatman 3M filter paper. Trichloroacetic acid-insoluble incorporation was estimated from triplicate determinations.

Results and Discussion

The clastogenicity of DNA topo II poisons is believed to form the basis for their antitumor activity (e.g., etoposide) or their antibacterial activity (e.g., quinolones such as ciprofloxacin and
clinafloxacin). This clastogenicity is due to the formation of the so-called “cleavable complex,” protein-associated DNA double-strand breaks that arise when the normal nicking/closing activity of topo II is inhibited midstream. Failure to resolve this lesion before DNA replication results in chromosome breaks that may be visualized using standard metaphase or micronucleus analysis. We have previously demonstrated that a variety of chemical agents capable of catalytically inhibiting DNA topo II dramatically antagonize the ability of topo II poisons to produce cleavable complex (8). These catalytic inhibitors have different mechanisms of action ranging from prevention of binding of topo II to DNA to interference with topo II-dependent ATP binding/hydrolysis. We have proposed that this approach might have utility in determining whether clastogenesis induced by novel experimental compounds might be caused by topo II poisoning (8).

It has been proposed that WR-1065 may catalytically inactivate topo II by reducing its phosphorylation state (5, 7). If this is the case, one would expect that WR-1065 would protect against micronucleus formation by topo II poisons in the same way as described for other classes of catalytic topo II inhibitors.

Fig. 1A demonstrates that a 30-min treatment with 2 μg/ml etoposide induces approximately 8% micronucleated cells (untreated control value is approximately 1%). Concomitant treatment of cells with 4 mM or 4 μM WR-1065 resulted in a highly statistically significant reduction in micronuclear formation. Treatment with 0.4 μM WR-1065 had no such protective effect. Concentrations of WR-1065 ranging from 0.4 μM to 4 μM, when tested alone, had no effect on the background levels of micronucleated cells (data not shown).

Fig. 1B demonstrates a similar protection by WR-1065 against micronuclear formation by the quinolone antibiotic clinafloxacin. It is also shown that the ribonucleotide reductase inhibitor HU has no effect on clinafloxacin clastogenesis. This suggests that reduction in topo II poison-dependent micronuclear formation is not simply a function of reduction in DNA synthesis and the subsequent reduced requirement for topo II. Table 1 shows that, as expected, HU markedly reduces DNA synthesis during the 30-min treatment, whereas WR-1065 has only marginal effects on DNA synthesis at 4 mM and has no effects on DNA synthesis at the equally protective concentration of 4 μM.

The selectivity of the protective response of WR-1065 for topo II-active clastogens is shown in Fig. 1C. Micronucleus formation by the topoisoisomerase I poison camptothecin, the intercalating alkylating agent mitomycin C, and the bisintercalating agent echinomycin was not affected by WR-1065 treatment.

These findings are consistent with the hypothesis that exposure of cells to WR-1065 results in catalytic inactivation of topo II. From a chemopreventive and chemoprotective standpoint, this topo II inactivation would have the effect of slowing cell cycling, thus providing more time for DNA repair to occur. This could result in protection of normal tissues against the clastogenic effects of antitumor and antibiotoxic therapies. Such differential protection by WR-1065 is well documented in the case of γ-irradiation.

Catalytic inhibition of topo II occurs at WR-1065 concentrations as low as 4 μM. This is well below the concentration of WR-1065 (100 μM) known to exhibit cytoprotection after γ-irradiation (10), indicating that concentrations affording protection against mutagenesis are unlikely to antagonize the antineoplastic activity of irradiation and certain drug therapies. However, because both the clastogenic (mutagenic) and cytotoxic activities of topo II poisons likely arise from the same event, stabilization of the cleavable complex, such differential protection of normal tissues by WR-1065 after treatment with topoisoisomerase-active drugs remains to be demonstrated.

Table 1 Effects of WR-1065 and HU on DNA synthesis in V79 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of Control</th>
<th>% HU incorporation</th>
<th>% Control proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 μM WR-1065</td>
<td>102 ± 5</td>
<td>96 ± 5</td>
<td>96 ± 8</td>
</tr>
<tr>
<td>4 μM WR-1065</td>
<td>91 ± 5</td>
<td>104 ± 2</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>4 mM WR-1065</td>
<td>79 ± 12</td>
<td>90 ± 11</td>
<td>90 ± 11</td>
</tr>
<tr>
<td>4 mM HU</td>
<td>12 ± 9</td>
<td>91 ± 13</td>
<td>91 ± 13</td>
</tr>
</tbody>
</table>

* Values as measured by the proportion of binucleated cells at 16 h relative to untreated controls.

References


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