Summary

The radioprotector S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) was evaluated for its ability to protect against cytoxan-induced mutagenesis at the hypoxanthine-guanine phosphoribosyl transferase (hppt) locus in mouse splenocytes under conditions that would not interfere with the therapeutic effectiveness of cytoxan in the treatment of fibrosarcoma lung tumors. Mutations at the hppt locus increase in frequency as a function of the dose of cytoxan used. With a spontaneous mutation frequency in C3H mice of $1.5 \times 10^{-6}$, mutation frequencies increased from $6.2 \times 10^{-6}$ to $2.0 \times 10^{-5}$ as the dose of cytoxan increased from 50 to 200 mg/kg. C3H male mice were injected in their tail veins with $3.5 \times 10^5$ viable fibrosarcoma (FSa) cells. This protocol gave rise to an average of 68 tumor colonies per mouse. Four days following injection animals were treated with cytoxan at a dose of 100 mg/kg, which gave rise to significant tumor cell killing and a reduction in tumor colony number to less than an average of one per animal. WR-2721 at a concentration of 100 mg/kg did not affect on cytoxan’s therapeutic effectiveness. However, a 100 mg/kg dose of WR-2721 was effective in reducing the cytoxan induced hppt mutation frequency in mice from 160 to 35 per $10^5$ viable cells regardless of whether it was administered 30 min before or 2 h following cytoxan treatment.
Introduction

Chemoprevention is the prevention of the carcinogenic process through the use of inhibiting chemical agents (Meyskens, 1991). While the first step of carcinogenesis is initiation, the carcinogenic process proceeds via a number of mutagenic events which can occur involving both suppressor genes and oncogenes (Vogelstein et al., 1988). WR-2721 has been demonstrated in mice to be both an effective antimutagen (Kataoka et al., 1992; Grdina et al., 1992) and an anticarcinogen (Milas et al., 1984; Grdina et al., 1991). Using the hprt as a reporter gene for mutagenic damage, it was demonstrated that WR-2721 was uniformly effective in protecting against mutation induction by fission-spectrum neutrons over a concentration range of 50 to 400 mg/kg and when administered either 30 min before or 3 h following irradiation (Grdina et al., 1992).

As therapies for cancer treatment have become more effective, the risk of therapy-induced secondary tumors has risen (Dorr and Coltman, 1985; Swendlow et al., 1992). It is the inherent mutagenicity of certain anti-cancer drugs that leads to this increased secondary cancer risk (Sugimura et al., 1978). An example of this type of chemotherapeutic agent is cytoxan (cyclophosphamid), which was demonstrated as early as 1973 to increase cancer incidence in mice (Walker and Bole, 1983).

In this report we describe the use of WR-2721 as a possible chemopreventive agent for use in cancer treatment. Using a C3H mouse system, we have evaluated the effectiveness of a nonradioprotective and relatively low dose of WR-2721 (i.e., 100 mg/kg) in reducing the frequency of cytoxan-induced mutagenesis at the hprt locus in splenocytes while not diminishing its therapeutic effectiveness in the treatment of FSa lung tumors.

Materials and Methods

WR-2721 was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, National Institutes of Health. It was weighed and diluted with physiological saline solution immediately before use (Grdina et al., 1992). WR-2721 was injected i.v. Cytoxan was supplied by Mead Johnson, Evansville, IN, USA, and was made up in sterile water just before use. Cytoxan was injected i.p.
All studies were performed using C3H/Sed mice obtained from Edwin L. Steele Laboratory for Radiation Biology, Massachusetts General Hospital, Boston, MA, USA. Male mice were at least 100 days of age at the initiation of experiments. Mice were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. All animal procedures used in these studies complied with the guidelines listed in the Guide for the Care and Use of Laboratory Animals (U.S. Public Health Service) and were approved by an Animal Care and Use Committee (Animal Welfare Assurance No. A-3089-01).

Mutation assays were performed 56 days following exposure of animals to cytoxan according to methods described in detail elsewhere (Kataoka et al., 1992; Grdina et al., 1992). Briefly, 56 days following exposure to cytoxan animals were sacrificed and spleens were removed and made into single cell suspensions (i.e., three spleens per suspension). Mononuclear cells were isolated in Histopaque (Sigma, St. Louis, MO, USA), counted, and then plated at known numbers in 96-microwell plates in medium containing 12% fetal bovine serum, concanavalin-A, human recombinant interleukin-2, and mercaptoethanol (Kataoka et al., 1992). Mutant cells were selected for by growth in medium containing 6-thioguanine (Sigma, St. Louis, MO, USA) at a concentration of 2.5 µg/ml. Each of 384 microwells per group contained $5 \times 10^4$ viable mononuclear cells along with $5 \times 10^4$ heavily irradiated mononuclear feeder cells. Cultures were grown for 12–16 days. Positive wells were those that contained colonies. Cloning efficiencies were calculated using Poisson statistics (Albertini, Castle, and Barcherding, 1982).

Mice were injected with $3.5 \times 10^5$ viable tumor cells from a cell suspension prepared according to the method described in detail elsewhere (Milas et al., 1982). Mice not treated with cytoxan were sacrificed 14 days later. The lungs were removed, fixed in Bouin’s solution, and the resulting tumor colonies were identified as white round nodules on the surface of the yellowish lungs and were visually counted. Mice exposed to cytoxan were treated in the same manner with the exception that they were sacrificed 20 days after injection with tumor cells. Different groups of mice were evaluated for mutagenesis as compared to tumor responses.

Results

The mutagenic effectiveness of cytoxan was evaluated by administering i.p. doses of 50 to
200 mg/kg body weight to experimental groups, each containing six mice. The results of three separate experiments are presented in Figure 1. Mutation frequency increased as a function of cytoxan dose.

A cytoxan dose of 100 mg/kg was chosen for further studies because it not only was significantly mutagenic, but it was also reported to be effectively cytotoxic to FSa tumor cells growing in the lungs of C3H mice (Milas et al., 1982).

Animals treated with 100 mg/kg cytoxan were also injected i.v. with WR-2721 at a concentration of 100 mg/kg. This dose of WR-2721 was chosen because it is significantly below the optimum protective dose of 400 mg/kg, which had been demonstrated previously as being effective in protecting FSa microlung colonies against the cytotoxic activity of cytoxan (Milas et al., 1982). Presented in Figure 2 are data describing the antimutagenic effects of WR-2721. WR-2721 was equally effective in reducing the mutation induction regardless of whether it was administered 30 min before or 2 h following cytoxan treatment.

A comparison of the anticytotoxic and antimutagenic effects of WR-2721 on cytoxan-treated animals is presented in Table I. A 100 mg/kg dose of cytoxan was cytotoxic to FSa tumor cells, as indicated by the resulting reduction of tumor colony number from an average of 68 to less than one per treated animal regardless of whether WR-2721 was administered or not. While not affecting the cytotoxic effects of cytoxan, WR-2721 was effective in preventing the induction of hprt mutations in splenocytes by cytoxan.

Discussion

Clinical interest in the phosphorothioate WR-2721 as a radiation protector developed as a result of early reports that it was preferentially taken up by normal as compared to tumor tissues (Yuhas, 1980). However, concern for its possible protection of tumor cells has persisted as a result of early reports using rodent tumor systems in which WR-2721 was found to protect small tumors by factors ranging from 1.5 to 2.5 (Milas et al., 1982; Penhaligon, 1984; and Stewart, Rojas, and Denekamp, 1983). Also complicating its use as a radio- and chemoprotector has been the requirements of relatively large doses for protection and that it must be present at the time of treatment (Giambarresi and Jacobs, 1987).
These factors do not affect the use of WR-2721 as a chemopreventive agent. In contrast to the requirements for radio and chemoprotection, WR-2721 can be administered at relatively low concentrations which do not affect cell toxicity but do inhibit the mutagenic process(es) (Grdina et al., 1992; Grdina et al., 1995). WR-2721 is equally effective whether it is administered 30 min before or up to 3 h following either exposure to highly mutagenic fission-spectrum neutrons (Grdina et al., 1992) or, as described in this report, to cytoxan (see Fig. 2). It has also been demonstrated in this report that cytoxan is both highly mutagenic at the hprt locus (see Fig. 1) as well as highly toxic to fibrosarcoma cells growing in the lungs of mice (see Table I). These data support the continued evaluation of WR-2721 as a chemopreventive agent for use in the clinic to reduce the mutagenic risk of radiation and chemotherapeutic agents such as cytoxan in the treatment of cancer. Treatment protocols of WR-2721 for use as a chemopreventive agent as compared to a radio- or chemoprotective agent are shown in Table II. When administered at low nonprotective doses prior to exposure to therapeutic agents or at either low or high doses afterwards, WR-2721 does not affect the cytotoxic response of cells. Under these conditions it is a potent antimutagen, which makes it a strong candidate drug for chemoprevention.

The mechanism(s) by which WR-2721 and its metabolites protect against mutagenesis is different from those underlying their protection against cell killing (Grdina et al., 1995). Free radical scavenging, hydrogen atom donation, and auto-oxidation are clearly involved in radiation and chemoprotection (Giambaresi and Jacobs, 1987). The thiol and especially disulfide metabolites of WR-2721 chemically resemble polyamines. Since protection against mutation induction is evident even when WR-2721 or its metabolites are administered up to 3 h following exposure to a mutagen (Grdina et al., 1992; and Grdina et al., 1995), it is reasonable to suggest that protection is mediated via augmentation of endogenous repair systems, presumably via polyamine-related processes. While this can only be considered to be a working hypothesis at this time, it is highly probable that the mechanisms underlying the antimutagenic effects of WR-2721 involve in some manner its interaction with endogenous repair enzymes, DNA synthesis processes, and/or chromation stabilization and structure.

Regardless of the underlying mechanisms of action, the data presented in this report are supportive for the use of WR-2721 as a potential chemopreventive agent for use in radiation and
chemotherapy protocols designed to treat patients having an excellent prognosis for cure but where the associated risk of therapy-induced mutagenesis and carcinogenesis becomes unacceptably elevated.

Acknowledgments

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Figure Legends

Fig. 1 *hprt* mutant frequencies as a function of cytoxan dose for mouse splenocytes isolated from control and cytoxan treated mice. Data represents the average of three separate experiments. Error bars represent 1 S.E.M.

Fig. 2 *hprt* mutant frequencies for mouse splenocytes isolated from control, cytoxan-treated, and cytoxan plus WR-2721 treated mice. WR-2721 was administered at a single dose of 100 mg/kg i.v. either 30 min. before or two hours following a single 100 mg/kg dose of cytoxan administered i.p. Experiments were repeated three times and error bars represent 1 S.E.M.
Cyclophosphamide (mg/kg)
Mutant Frequency

$10^{-6}$  $10^{-5}$  $10^{-4}$

Control  Cytoxan  Cytoxan  Cytoxan
+        +        +
WR-2721  WR-2721
(Before)  (After)
Table I: WR-2721 Effects on Cytoxan-Induced Cytotoxicity and Mutagenesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Fibrosarcoma Lung Colonies</th>
<th>HPRT(^1) Mutant Frequency(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>68 ± 10 (SEM)(^3)</td>
<td>15 ± 3 (SEM)</td>
</tr>
<tr>
<td>Cytoxan Only (100 mg/kg)</td>
<td>0.5 ± 0.3</td>
<td>160 ± 45</td>
</tr>
<tr>
<td>WR-2721 Only (100 mg/kg)</td>
<td>61.3 ± 8.9</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>WR-2721 Administered 30 min Before Cytoxan</td>
<td>0.5 ± 0.3</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>WR-2721 Administered 2 hr After Cytoxan</td>
<td>0.9 ± 0.3</td>
<td>28 ± 4</td>
</tr>
</tbody>
</table>

\(^1\) Hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus
\(^2\) Mutants per 10\(^5\) viable cells
\(^3\) Standard error of the mean
Table II: TIMING OF ADMINISTRATION

<table>
<thead>
<tr>
<th>WR-2721 CONCENTRATION</th>
<th>BEFORE</th>
<th>AFTER</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td>1 RADIO- CHEMO-PROTECTION</td>
<td>2 CHEMO-PREVENTION</td>
</tr>
<tr>
<td>LOW</td>
<td>3 CHEMO-PREVENTION</td>
<td>4 CHEMO-PREVENTION</td>
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