Selective Exclusion by the Polyamine Transporter as a Mechanism for Differential Radioprotection of Amifostine Derivatives

Herson I. Quiñones, Alan F. List, and Eugene W. Gerner

The University of Arizona, Arizona Cancer Center, Department of Radiation Oncology/Cancer Biology Section [H. I. Q., E. W. G.], and Departments of Biochemistry and Molecular Biophysics [E. W. G.], and Medicine [A. F. L.], Tucson Arizona 85724

ABSTRACT

Amifostine metabolites WR-1065 and the disulfide WR-33278 are thiol-containing polyamine analogues with potent radio- and chemoprotective properties. Some studies suggest that amifostine exerts differential cytprotection in normal versus neoplastic tissues, but this finding remains controversial. To assess the role of the polyamine transport system in radioprotection by amifostine derivatives, human DU-145 prostate cancer cells were transfected with a cDNA that encodes antizyme (OAZ), a polyamine-inducible protein that suppresses polyamine transport under control of a minimal heat shock promoter. Selected clones expressing OAZ displayed heat shock-dependent suppression of polyamine uptake. When added to culture medium under non-reducing conditions, both WR-1065 and WR-33278 were detected as the disulfide form. Each derivative protected both parental and OAZ-transfected DU-145 cells from X-ray-induced cell killing at 37°C. When cultures were heat shocked at 42°C, both derivatives protected parental, but not OAZ-transfected cells from radiation-induced cell killing. Treatment of DU-145 cells with difluoromethylornithine (DFMO) suppressed intracellular putrescine and spermidine content, but increased the uptake of WR-33278-derived aminothiols. The concentration-dependent radioprotection of DU-145 cells by WR-33278 was enhanced by DFMO. Addition of exogenous putrescine reduced WR-33278-mediated radioprotection in both DFMO-treated and untreated DU-145 cells. These data demonstrate that negative regulation of the polyamine transporter, mediated by polyamines or antizyme, suppresses the uptake and radioprotective activity of amifostine derivatives. Selective exclusion of amifostine derivatives by the polyamine transporter could account for differential radio- or chemoprotection in normal versus neoplastic tissues in specific situations.

INTRODUCTION

Amifostine (WR-2721), a phosphoaminothioate, is capable of providing marked protection from both radiation- and selected chemotherapy-induced damage for a wide variety of tissues in both rodents and humans (1–6). In addition to preventing cytotoxicity, amifostine derivatives display anticarcinogenic (7, 8) and antimutagenic (9) properties in rodent model systems as well as hematopoiesis-promoting effects (10, 11).

Amifostine is a prodrug that is dephosphorylated by alkaline phosphatase to its active component (12). The dephosphorylated product, WR-1065, enters cells via passive diffusion (13, 14). Under nonreducing conditions, such as those occurring in tissues in vivo, the thiol form is oxidized to form the disulfide, WR-33278. This amifostine derivative is structurally similar to the polyamine spermine (Table 1) and is actively transported into cells via the OAZ-dependent polyamine transporter (13, 14).

Amifostine has been reported to selectively protect normal tissues in multiple organ systems against the toxic effects of radiation and various cytotoxic drugs while preserving the antitumor effects of these therapies (15). For example, one clinical trial showed that pretreatment with amifostine produced a significant reduction in late radiation toxicities to pelvic organs with no reduction in antitumor efficacy (16). The apparent selective concentrations of amifostine derivatives in normal versus neoplastic tissues have been attributed to several factors. These include low levels of alkaline phosphatase expression in solid tumors; poor neoplastic vascularization leading to either low levels of alkaline phosphatase or low pH, which could affect the efficiency of either alkaline phosphatase enzyme activity; or some other aspect of amifostine derivative uptake (see Ref. 15 for review). However, these factors are not ubiquitous neoplastic features. For example, alkaline phosphatase activity increases with cancer progression in carcinogen-treated rats (17) and in human gastric cancers (18). Furthermore, human tumors are notoriously heterogeneous in terms of vascularization and interstitial pH (19).

We investigated the possibility that selective radioprotection by amifostine derivatives in normal versus neoplastic tissue may be attributable to selective exclusion of amifostine derivatives as a consequence of negative feedback regulation of the polyamine transporter. The activity of this transporter is suppressed by OAZ(3) (13), a polyamine-inducible protein also in-

Received 4/5/01; revised 10/23/01; accepted 1/19/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This investigation was supported in part by Grants CA-23074 and CA-72008 from the National Cancer Institute, and contracts from the Arizona Disease Control Research Commission (1516) and Varian Biosynergy, Inc.

2 To whom requests for reprints should be addressed, at The University of Arizona, Arizona Cancer Center, Room 3999, 1515 N. Campbell Avenue, Tucson, Arizona 85724-0001. Phone: (520) 626-2197; Fax: (520) 626-4480; E-mail: egerner@azcc.arizona.edu.

3 The abbreviations used are: OAZ, ornithine decarboxylase antizyme; ODC, ornithine decarboxylase; FBS, fetal bovine serum; HSP70B, heat shock protein 70B; HPLC, high-pressure liquid chromatography; DFMO, D,L-α-difluoromethylornithine.
Selective Exclusion of Amifostine by Antizyme

Table 1 Comparison of polyamine and aminothiol structures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td>(\text{NH}_2(\text{CH}_2)_2\text{NH}(\text{CH}<em>2)</em>\text{NH}_2)</td>
</tr>
<tr>
<td>WR-2721</td>
<td>(\text{NH}_2(\text{CH}<em>2)</em>\text{NH}(\text{CH}<em>2)</em>\text{S-PO}_4)</td>
</tr>
<tr>
<td>WR-1065</td>
<td>(\text{NH}_2(\text{CH}<em>2)</em>\text{NH}(\text{CH}<em>2)</em>\text{S-H})</td>
</tr>
<tr>
<td>WR-33278</td>
<td>(\text{NH}_2(\text{CH}<em>2)</em>\text{NH}(\text{CH}_2)_2\text{S-(CH}_2)_3\text{NH})</td>
</tr>
<tr>
<td>Spermine</td>
<td>(\text{NH}_2(\text{CH}<em>2)</em>\text{NH}(\text{CH}<em>2)</em>\text{NH}(\text{CH}<em>2)</em>\text{NH}_2)</td>
</tr>
</tbody>
</table>

Involved in the regulated degradation of ODC, the first enzyme in polyamine synthesis (20). ODC activity and polyamine contents are elevated in several epithelial cancers in both experimental animals and humans (see Ref. 21 for a review). ODC is a transcriptional target of the c-myc oncogene (22, 23), and ODC activity is elevated in several human cancers compared with relevant normal tissues (24). ODC is also induced in the Min mouse model of intestinal carcinogenesis as a consequence of inactivation of the APC tumor suppressor gene (25). Thus, the selective suppression of the polyamine transporter as a result of activation of ODC and elevation of polyamines could be a mechanism for the selective exclusion of extracellular polyamines and their analogues, including the amifostine derivative WR-33278, in specific cancers compared with corresponding normal tissues.

MATERIALS AND METHODS

Cell Lines and Culture Techniques. The human prostatic cancer cell line DU-145 (doubling time, 16–20 h) was routinely maintained in DMEM supplemented with 10% FBS plus 1% penicillin-streptomycin solution. Cultures were maintained at 37°C in a humidified 5% CO_2 -95% air atmosphere.

Plasmids and Transfections. Vector M5 was constructed by inserting a 451-bp BamHI-HindIII element of the HSP70B promoter (StressGen) into pcDNA3 (Invitrogen) upstream of a multiple cloning site. The responsiveness of this vector to heat shock has been described elsewhere (26). A 954-bp fragment containing rat OAZ clone Z1 cDNA, kindly provided by Dr. S. Hayashi (The Jikei University School of Medicine, Tokyo, Japan) (27), was isolated by EcoRI digestion of plasmid pUC118Z1 and ligated into the EcoRI site of vector M5. DU-145 cells were transiently transfected with the HSP70B promoter-driven OAZ construct by use of a calcium phosphate transfection protocol (28).

Cell Survival Analysis. Survival of cells exposed to various treatments was assessed by colony-forming assays, as described previously (29). Cells were irradiated with 60Co γ-rays at a dose rate of 50 cGy/min. Cultures were then harvested, counted, diluted, and seeded in 100-mm dishes at numbers estimated to give ~100 colonies/dish. A minimum of three replicate dishes were plated and counted for each exposure point, and the experiments were repeated at least twice. After an incubation period of 2–3 weeks, the plates were rinsed with PBS, fixed in ethanol, and stained with crystal violet. Colonies per dish were counted manually on a dissecting microscope.

Polyamine and Aminothiol Determination. After treatments and cell harvest, as described above, cultures were washed three times in PBS and sonicated in 0.1 N HCl to disrupt cell membranes. Proteins and other large molecules were precipitated by addition of HClO_4 (final concentration, 0.2 N). Polyamines and aminothiols in the acid-soluble fraction were detected by HPLC methods, described elsewhere (25), that used postcolumn derivatization techniques to detect free primary amine groups. Measured values of polyamines and aminothiols were normalized to protein content in the acid-insoluble fraction of samples.

RESULTS

Selection of Transfected Cells Conditionally Expressing OAZ. DU-145 cells were transfected with a plasmid, described in “Materials and Methods,” that directed heat-inducible expression of the rat OAZ gene and constitutive expression of the neomycin resistance gene. Transfected cells were selected for growth in G418, and individual drug-resistant clones were isolated by serial dilution methods. Twenty clones were evaluated for OAZ expression, by measuring the effect of heat shock on polyamine uptake because this process is known to be OAZ dependent (20). Fig. 1 shows radiolabeled putrescine uptake in five transfected clones plus the nontransfected DU-145 parental cells 4 h after exposure to either 37°C or 42°C. Putrescine uptake was unaffected by heat shock in the nontransfected DU-145 cells and clones 9, 12, and 15. However, heat shock suppressed putrescine uptake in clones 10 and 13. Clones 10 (DUN4-C10) and 13 (DUN4-C13) were judged to conditionally express OAZ and were used for further study.

Influence of Culture Medium on Aminothiol Forms. Because culture conditions are known to affect the forms of WR-1065 and WR-33278, their concentrations were measured after the addition of 100 μM of either drug to PBS (nonreducing
shown. Results are from a single representative experiment, which was replicated twice. ■, WR-1065; □, WR-33278.

The results described above suggested that OAZ expression suppressed radioprotection by WR-33278 under other conditions that altered the uptake of this drug. DFMO, a potent inhibitor of polyamine synthesis, suppresses expression of endogenous antizyme protein and enhances polyamine uptake (20). Cultures were incubated for 48 h after subculture in full PBS-containing medium with and without 500 μM DFMO. This medium was then removed, and PBS containing WR-33278 (50 μM) was added for 1 h. Cultures were then harvested and levels of aminothiols and polyamines were determined. As shown in Table 2, DFMO suppressed the levels of putrescine and spermidine, but not spermine, in DU-145 cells. DFMO treatment enhanced aminothiol uptake when cells were treated with WR-33278. When added to the culture medium, the disulfide was detected as the free thiol in cells, presumably because of the reducing environment within the cell. These results demonstrate that DFMO treatment increased aminothiol uptake and that the disulfide form was presumably converted to the free sulfide form once taken up by cells.

DU-145 cells, treated with and without DFMO for 48 h in PBS-containing medium, were then exposed to either 10 or 50 μM WR-33278 in PBS for 1 h and then irradiated with a single 5-Gy γ-ray dose. As shown in Fig. 4, radioprotection induced by WR-33278 was concentration dependent. Treatment of cells with DFMO increased radioprotection at both aminothiol concentrations. Addition of putrescine suppressed radioprotection by WR-33278 in cultures treated with and without DFMO. This suppression was presumably attributable to both competition for uptake of extracellular amines/aminothiols and restoration of intracellular amines by exogenous putrescine.

DISCUSSION

The data presented here show that expression of OAZ, which suppressed polyamine uptake, decreased radioprotection mediated by amifostine derivatives. This finding was corroborated by the observation that treatment with DFMO, which suppressed putrescine and spermidine pools and increased aminothiol uptake, increased radioprotection mediated by amifos-
Selective Exclusion of Amifostine by Antizyme

Table 2 Effects of DFMO on polyamine and aminothiol uptake by DU-145 cells Cells previously exposed to 500 μM DFMO for 48 h were treated with 50 μM WR-33278 for 1 h in PBS (exposure to DFMO, or no exposure, continued during this period). Cellular contents of aminothiols and polyamines were evaluated as described in Materials and Methods.11

<table>
<thead>
<tr>
<th>Treatment (nmol/pg protein)</th>
<th>Aminothiola</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFMO</td>
<td>+</td>
<td>NDb</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>ND</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

a WR-33278 added to the culture medium was detected as the free thiol after uptake into cells (see text for discussion).
b ND, not detected, limit of detection 0.05 nmol/mg protein.

Fig. 4 Effect of polyamine suppression by DFMO on radioprotection by WR-33278. DU-145 cells were subcultured in T-25 flasks in medium with (●) or without (○) 500 μM DFMO for 48 h. Putrescine (1 mM) was added to one dish with (□) and without (□) DFMO for the last 18 h of this interval. Medium was then removed and replaced with PBS containing WR-33278 at the concentrations shown for 1 h. Cultures were then irradiated with 5 Gy of 60Co γ-rays. Shown are means ± SE (when larger than symbols) for surviving fractions from a single representative experiment, which was replicated.

Polyamines are known to regulate their active transport via a mechanism involving OAZ (20). Together, these data suggest that radioprotection mediated by the disulfide WR-33278 in these human prostate tumor-derived cells is influenced by the OAZ-dependent polyamine transporter. This conclusion is consistent with a previous report (13), which implicated the polyamine transporter in uptake of WR-33278 in a rat hepatoma cell line but did not evaluate the consequences of this transporter on radioprotection.

Previous studies indicated that the free thiol WR-1065 is transported into cells via a passive diffusion mechanism (13, 14). Pharmacokinetic studies indicate that after administration in animals and humans, the free thiol form rapidly disappears in blood and that disulfides, especially mixed disulfides with cysteine, are formed (31). The relevance of the mechanism described here for exclusion of amifostine derivatives in humans is unclear. Whether the polyamine transporter influences the uptake of WR-1065 cysteine disulfides is not known. However, the data presented indicate that if the free thiol exists in a nonreducing environment outside cells, it will form the disulfide WR-33278 (Fig. 2). This disulfide is then transported into DU-145 human prostate cancer cells via a polyamine- and OAZ-dependent mechanism. A definitive test of the relevance of this mechanism in vivo could be conducted in polyamine transporter knockout mice. Unfortunately, the OAZ-dependent polyamine transporter in mammals has not yet been defined in biochemical or genetic terms.

The studies presented here show that treatment of DU-145 cells with WR-1065 or WR-33278 at concentrations of 50–100 μM in a nonreducing medium (PBS; see Fig. 2) is sufficient to protect cells by factors of 4–6 (Figs. 3 and 4). When Chinese hamster ovary cells were treated with WR-1065 in full medium with serum, a condition shown here to promote formation of the free thiol, concentrations in excess of 1 mM were required to obtain similar levels of protection (32). These could be cell line-specific differences. More likely, they reflect differences in the mode of aminothiol transport. Uptake of exogenous polyamines by the polyamine transporter results in concentrations of intracellular amines until feedback regulation by endogenous amines suppresses the transporter. For example, when cells are treated with DFMO to deplete polyamine and, consequently, OAZ levels, addition of micromolar concentrations of polyamines to the medium causes intracellular levels to reach almost millimolar concentrations (33). Uptake by passive diffusion mechanisms does not result in intracellular concentration of drugs.

Polyamine contents are often increased in epithelial cancers (see Ref. 21 for a review). One mechanism of increased polyamine contents is attributable to the adenomatous polyposis coli-dependent expression of ODC in experimental models of intestinal carcinogenesis (25). OAZ is known to be induced via a novel polyamine-dependent mechanism (34). These results predict that tissues with normal polyamine homeostasis that express OAZ would exclude molecules such as WR-33278, which are transported by the OAZ-dependent polyamine transporter. The data presented here show for the first time that pharmacological suppression of intracellular polyamines can enhance radioprotection by WR-33278 and that OAZ-mediated suppression of WR-33278 uptake can reduce radioprotection by this agent.

Together, these results demonstrate the plausibility of a mechanism by which the amifostine metabolite WR-33278 would be selectively excluded by some cancers. Extracellular dephosphorylated amifostine could exist in the disulfide form under nonreducing conditions in vivo. Transport into cells could occur predominantly via the OAZ-dependent active transport mechanism described by others (13, 14). Tumors expressing high levels of polyamines would suppress this uptake process, whereas uptake would be unrestrained in normal tissues. Along with other proposed mechanisms discussed earlier, this selective exclusion mechanism could account for long-standing observa-
tions suggesting that amifostine accumulates to lower levels in some normal tissues compared with tumor tissue (15).

Another consequence of this proposed mechanism is that some tumors that express a polyamine transporter may be protected by amifostine. There is experimental evidence for tissue-specific normal tissue radioprotection and site-specific tumor radioprotection by amifostine in transplanted rodent models (4, 35) and radioprotection of spontaneously arising sarcomas in dogs (5). Others have noted that clinical evidence against radio- or chemoprotection of tumors is sparse (36). Randomized clinical trials to document protection of specific normal but not tumor tissues, such as the study by Brizel et al. (37), are warranted.

The relevance of this mechanism in human cancer therapy is testable by measuring the levels of OAZ protein expression and amifostine accumulation in tumor and normal tissues. Amifostine has already been shown to protect normal rectal tissue from postsurgical radiotherapy-induced toxicity (16). In this setting, patients could receive amifostine before surgery to assess levels of amifostine and OAZ in both the tumor and adjacent normal tissue.

ACKNOWLEDGMENTS

Aminothiols were the generous gifts of US Biosciences (Gaithersburg, MD) and Dr. David Grdina (University of Chicago, Chicago, IL).

REFERENCES


Selective Exclusion by the Polyamine Transporter as a Mechanism for Differential Radioprotection of Amifostine Derivatives

Herson I. Quiñones, Alan F. List and Eugene W. Gerner


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/5/1295

Cited articles
This article cites 35 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/5/1295.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/8/5/1295.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.