

Amifostine Aminothiols and Protection of Keratinocyte Apoptosis and DNA Damage

To the Editor:

Amifostine (S-2-3-aminopropylaminoethyl phosphorothioic acid), also known as WR-2721, is an aminothiol developed during the 1950s by the U.S. Army as a radio-protective agent (Murray, 1998). Amifostine is dephosphorylated *in vivo* to the active metabolite WR-1065, which enters cells by passive diffusion (Mitchell *et al*, 1995) and acts as a potent scavenger of free radicals and inhibitor of DNA damage (Peters and van der Vijgh, 1995). Amifostine protection of human leukemia cells against apoptosis induced by ionizing radiation (IR) relates to its ability to prevent DNA strand breaks (Warters *et al*, 1997). It effectively protects normal tissues against the acute effects of IR and chemotherapy, and the drug is widely used in cancer patients to increase the therapeutic benefit of radiotherapy and chemotherapy (Wasserman, 1999). Amifostine has been particularly useful in reducing nephrotoxicity associated with cisplatin administration (Markman, 1998) and radiation-induced xerostomia in patients with squamous cell carcinoma of the head and neck (Brizel *et al*, 2000). In addition, amifostine in some cases has demonstrated anti-proliferative effects (Rubin *et al*, 1996) and anti-tumor effects (Grdina *et al* 2002), suggesting its potential as a chemopreventive as well as chemoprotective agent.

To the best of our knowledge, there are no studies examining direct effects of these aminothiols on keratinocytes and whether they can block keratinocyte apoptosis induced by IR. In addition, their potential as protective agents against ultraviolet (UV) B radiation, the primary carcinogen in sunlight and etiologic agent for nonmelanoma skin cancers, has not been formally addressed.

We thus examined the activity of both WR-1065 and amifostine on the model keratinocyte cell line HaCat, which undergoes apoptosis in response to both IR and UVB (Petit-Frere *et al* 2000). First, HaCat cells (Grossman *et al*, 1999a) were preincubated for 15 min in Falcon 353001 dishes in the presence or absence of freshly prepared WR-1065 (kindly provided by Dr Robert Schultz, Drug Synthesis and Chemistry Branch, NCI, Bethesda, MD) or amifostine (Ethyol, Alza Pharmaceuticals, Palo Alto, CA), and then exposed to IR (7.5 Gy per min, J.L. Shepherd ¹³⁷Cs source) as described previously (Wilmore *et al* 2001). After replacing the medium and culturing for 72 h, apoptosis was assessed by propidium iodide staining of cellular DNA and flow cytometry as described previously (Grossman *et al*, 1999b). As shown in **Fig 1(A)**, IR induced a considerable level of apoptosis that was significantly reduced ($p = 0.004, 0.01$) in the presence of WR-1065. The amifostine prodrug had a milder protective effect (not shown), likely due to its decreased cell permeability compared with WR-1065 (Calabro-Jones *et al*, 1985). Concentrations of either drug in excess of 5 mM resulted in variable cytotoxicity, and concentrations of 1 mM were less protective (not shown).

Next, we examined the ability of these aminothiols to protect against UVB radiation. HaCat cells were preincubated in the absence or presence of WR1065 or amifostine as above, and

then exposed to UVB (2 J per m² per s, FS20T12-UVB bulbs, National Biological Corporation, Twinsburg, OH) as described previously (Grossman *et al*, 2001). After replacing the medium and culturing for 24 h, apoptosis was assessed as above. UVB induced a high level of apoptosis that was unaffected by WR-1065 (**Fig 1B**) or amifostine (not shown). Failure to protect against UVB-induced keratinocyte apoptosis suggested that these aminothiols, in contrast to their ability to block IR-induced DNA strand breaks (Warters *et al*, 1997), are unable to block UVB-induced DNA damage, i.e., formation of UV photoproducts (Livneh *et al*, 1993). To confirm this, HaCat cells were preincubated in the presence or absence of amifostine and WR1065 and then exposed to UVB as above. Cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidone (6-4) photoproducts were detected using TDM-2 and 64M-2 monoclonal antibodies (Mori *et al*, 1991), respectively (kindly provided by Dr Toshio Mori, Nara Medical University, Nara, Japan). Genomic DNA was prepared as described previously (Grossman *et al*, 2001) and spotted on Biotrace PVDF membrane (ISC BioExpress, Kaysville, UT) for immunodot-blot assay as described elsewhere (Adimoolam *et al*, 2001). As shown in **Fig 2**, both CPD and 6-4 photoproducts were readily detected immediately following higher levels of UVB exposure, whereas none were seen in unirradiated cells. In addition, UV photoproducts were not detected in cells exposed to 10 or 20 Gy IR (not shown). Consistent with the apoptosis responses above, in aminothiol-treated HaCat cells there was no reduction in levels of either UV photoproduct compared with cells not exposed to the drugs at the time points examined (**Fig 2**).

We have thus shown that the aminothiol amifostine, and particularly its active metabolite WR1065, can protect keratinocytes from apoptosis induced by IR. Reduced keratinocyte apoptosis is the likely basis for the tissue-sparing effect seen in patients with head and neck squamous cell carcinoma receiving amifostine prior to radiotherapy. Our data suggest, however, that these aminothiols are limited in their protective capacity for keratinocytes to IR-induced DNA damage, and thus are likely not to be useful as UV-protective agents in skin.

Miyoung J. Diedrich,‡ Raymond L. Warters,† and Douglas Grossman*‡Departments of *Dermatology, †Radiation Oncology, and ‡the Huntsman Cancer Institute, University of Utah Health Sciences Center, Salt Lake City, UT, U.S.A.

D.G. is supported by NIH grant KO8AR48618, the Huntsman Cancer Foundation, and a Fellowship-to-Faculty Transition Award from the University of Utah funded in part by the Howard Hughes Medical Institute. A pilot project award from grant P30CA42014 also funded this work. R.L.W. is supported by DOE grant DEFGH ER63240. We thank Robert Schultz for providing WR-1065, Toshio Mori for antibodies against UV-photoproducts, and Sancy Leachman for helpful discussions in the early phase of these studies.

Manuscript received 2002; revised 2002; accepted for publication 2002
Corresponding author is Dr Grossman; Email doug.grossman@hci.utah.edu. Reprints not available from authors.

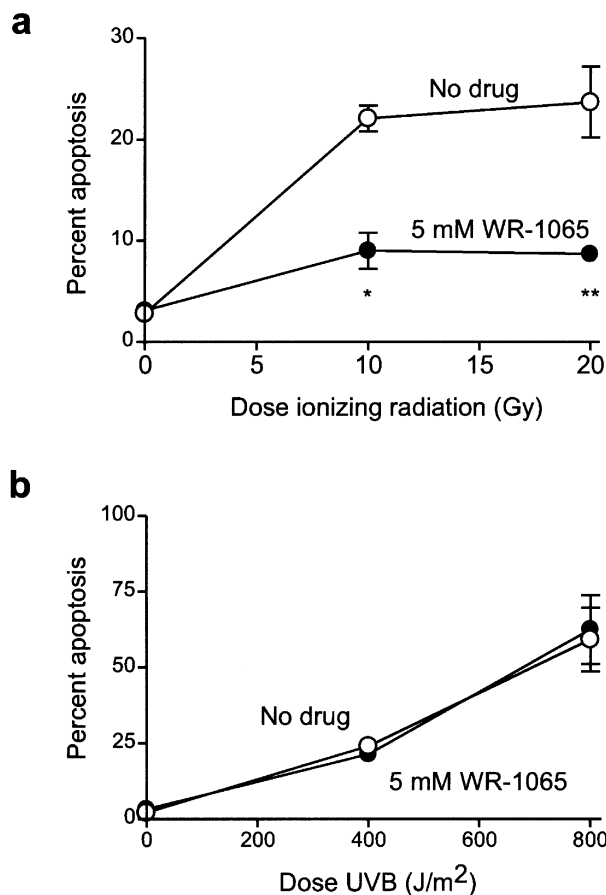


Figure 1. Apoptotic responses of HaCat keratinocytes and the effect of WR-1065 preincubation. HaCat cells were either untreated (○) or preincubated (●) with 5 mM WR-1065 for 15 min prior to exposure to the indicated doses of (a) IR or (b) UVB. After 72 h (a) or 24 h (b), all cells were recovered and percent apoptosis was assessed by propidium iodide staining and flow cytometry. Apoptotic cells were identified as the sub-G₁ (subdiploid) population and quantitated as a percentage of the total cell population. Error bars indicate SEM from four independent experiments performed. p-values determined by unpaired t tests using Prism (Graphpad software, San Diego, CA): *p = 0.004, **p = 0.01. p-values for unirradiated cells and UVB-irradiated cells were not significant (p > 0.05).

REFERENCES

- Adimoolam S, Lin CX, Ford JM: The p53-regulated cyclin-dependent kinase inhibitor, p21 (cip1, waf1, sd1), is not required for global genomic and transcription-coupled nucleotide excision repair of UV-induced DNA photoproducts. *J Biol Chem* 276:25813–25822, 2001
- Brizel DM, Wasserman TH, Henke M, et al: Phase III randomized trial of amifostine as a radioprotector in head and neck cancer. *J Clin Oncol* 18:3339–3345, 2000
- Calabro-Jones PM, Fahey RC, Smoluk GD, Ward JF: Alkaline phosphatase promotes radioprotection and accumulation of WR-1065 in V79-171 cells incubated in medium containing WR-2721. *Int J Radiat Biol Relat Stud Phys Chem Med* 47:23–27, 1985

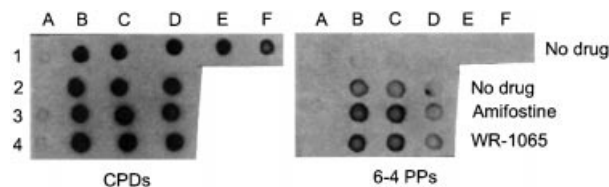


Figure 2. Immunodetection of photoproducts induced by UVB. HaCat cells were either untreated (rows 1 and 2) or preincubated with 5 mM amifostine (row 3) or WR-1065 (row 4) as indicated for 15 min, prior to mock exposure (unirradiated; A1, A3, A4) or exposure to 100 J per m² (B1, C1, D1, E1, F1) or 400 J per m² (B2, B3, B4, C2, C3, C4, D2, D3, D4) UVB. Cells were then harvested immediately (column B) or after incubating for 0.5 h (column C), 2 h (column D), 6 h (column E) or 24 h (columns A, F) for preparation of genomic DNA. No DNA was spotted at A2. For detection of CPD, 0.1 μg DNA was spotted at each point. For detection of 6–4 photoproducts, 0.2 μg DNA was spotted. Shown is a representative experiment of two performed. The decreased level of CPD (F1) and 6–4 photoproducts (D2, D3, D4) after prolonged incubation reflects DNA repair of photoproducts.

- Grdina DJ, Kataoka Y, Murley JS, Hunter N, Weichselbaum RR, Milas L: Inhibition of spontaneous metastases formation by amifostine. *Int J Cancer* 97:135–141, 2002
- Grossman D, McNiff JM, Li F, Altieri DC: Expression of the apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line. *Lab Invest* 79:1121–1126, 1999a
- Grossman D, McNiff JM, Li F, Altieri DC: Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. *J Invest Dermatol* 113:1076–1081, 1999b
- Grossman D, Kim PJ, Blanc-Brude OP, Brash DE, Tognini S, Marchisio PC, Altieri DC: Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. *J Clin Invest* 108:991–999, 2001
- Livneh Z, Cohen-Fix O, Skalter R, Elizur T: Replication of damaged DNA and the molecular mechanism of ultraviolet light mutagenesis. *Crit Rev Biochem Mol Biol* 28:465–513, 1993
- Markman M: Amifostine in reducing cisplatin toxicity. *Semin Oncol* 25:522–524, 1998
- Mitchell JL, Judd GG, Diveley RR Jr, Choe CY, Leyser A: Involvement of the polyamine transport system in cellular uptake of the radioprotectants WR-1065 and WR-33278. *Carcinogenesis* 16:3063–3068, 1995
- Mori T, Nakane M, Hattori T, Matsunaga T, Ihara M, Nikaido O: Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6–4) photoproduct from the same mouse immunized with ultraviolet-irradiated DNA. *Photochem Photobiol* 54:225–232, 1991
- Murray D: Aminothiols. In: Bump E, Malaker K (eds). *Radioprotectors. Chemical, Biological and Clinical Perspectives*. Boca Raton: CRC Press, 1998, pp 53–109
- Peters GJ, van der Vijgh WJ: Protection of normal tissues from the cytotoxic effects of chemotherapy and radiation by amifostine (WR-2721): preclinical aspects. *Eur J Cancer* 31A:S1–S7, 1995
- Petit-Frere C, Capulas E, Lyon DA, et al: Apoptosis and cytokine release induced by ionizing or ultraviolet B radiation in primary and immortalized human keratinocytes. *Carcinogenesis* 21:1087–1095, 2000
- Rubin DB, Drab EA, Kang HJ, Baumann FE, Blazek ER: WR-1065 and radioprotection of vascular endothelial cells. I. Cell proliferation, DNA synthesis and damage. *Radiat Res* 145:210–216, 1996
- Warters RL, Roberts JC, Wilmore BH, Kelley LL: Modulation of radiation-induced apoptosis by thiolamines. *Int J Radiat Biol* 72:439–448, 1997
- Wasserman T: Radioprotective effects of amifostine. *Semin Oncol* 26:89–94, 1999
- Wilmore BH, Cassidy PB, Warters RL, Roberts JC: Thiazolidine prodrugs as protective agents against gamma-radiation-induced toxicity and mutagenesis in V79 cells. *J Med Chem* 44:2661–2666, 2001