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WR-1065, the Active Metabolite of Amifostine (Ethyol®), does not Inhibit the Cytotoxic Effects of a Broad Range of Standard Anticancer Drugs Against Human Ovarian and Breast Cancer Cells

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Amifostine (WR-2721, Ethyol®), a phosphorylated thiol, demonstrates the unique ability to protect normal but not tumour tissue from cytotoxic damage induced by radiation therapy and chemotherapy. This study tested the effect of amifostine's active metabolite, the free thiol, WR-1065, on the cytotoxicity of standard anticancer drugs against human A2780 ovarian and MCF7 breast cancer cell lines *in vitro*, using the well-characterised sulphorhodamine B assay. 50% inhibitory concentration (IC_{50}) values were determined for each of 16 different anticancer drugs in the presence and absence of the highest nontoxic dose of WR-1065 from concentration–response curves constructed in triplicate and based on 18 replicate cell culture plates for each tested drug concentration. Pretreatment with WR-1065 had no statistically significant effect on the IC_{50} value of any of the 16 drugs tested against either the A2780 or MCF7 human tumour cells. These data expand upon previous reports showing that amifostine does not protect tumours from the cytotoxic effects of anticancer agents. The ability of amifostine to protect against dose-limiting toxicity to a variety of normal tissues without protection of tumour should enhance the efficacy ratio of a wide range of standard anticancer drugs. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

AMIFOSTINE (WR-2721, Ethyol®) is a phosphorylated thiol that was selected for development from 4400 potential radioprotective compounds screened by the United States Army as having the most effective radioprotective properties. Amifostine demonstrates the unique ability to protect selectively a broad range of normal tissues, including those of bone marrow, kidney and the nervous system, but not tumour tissues from damage induced by radiotherapy or chemotherapy [1–5]. This selective protection is based on differential dephosphorylation by alkaline phosphatase at the tissue site and the preferential uptake of the active thiol metabolite, WR-1065, by cells in normal tissue. Of these two processes, it appears that the selective cytoprotective effect of amifostine is more closely related to the faster rate of its dephosphorylation than to an increased rate of membrane transport of the dephosphorylated thiol [6].

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An extensive literature exists concerning the cytoprotective effects of amifostine on normal tissues without its inhibiting the cytotoxic effects of a variety of anticancer drugs in *in vivo* animal model systems. Most of these studies have evaluated the cytoprotective effects of amifostine against alkylating agents and platinum compounds [7, 8]. Additionally, there is no clinical evidence of a reduction in antitumour activity when amifostine is administered prior to various chemotherapeutic agents [9, 10]. The present study was undertaken to expand the preclinical experience of amifostine combined with a much broader variety of standard anticancer drugs by testing the effect of its active metabolite, WR-1065, on the cytotoxicity of both cell-cycle dependent and independent agents against human A2780 ovarian and human MCF7 breast cancer cell lines *in vitro*.

MATERIALS AND METHODS

A2780 ovarian and MCF7 breast cancer tumour cell lines

The A2780 cell line was obtained from the laboratory of Robert F. Ozols, Fox Chase Cancer Center, Philadelphia,

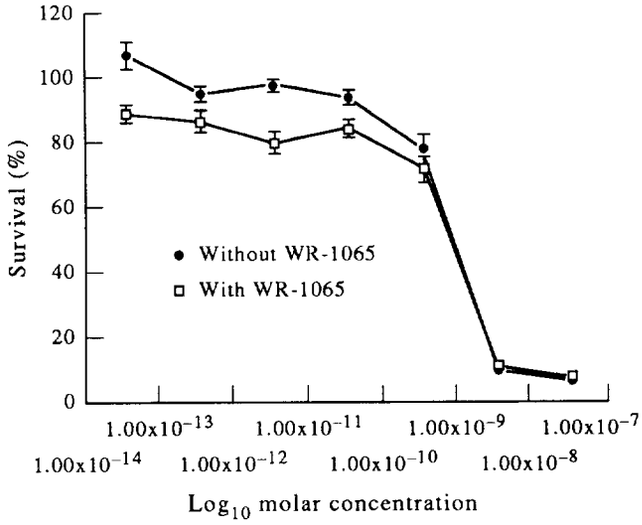

Figure 1. *In vitro* concentrations of paclitaxel with and without WR-1065, the active metabolite of amifostine (Ethyol®), versus per cent survival of human MCF7 breast cancer cells. Bars indicate the mean standard errors at each drug concentration point.

Pennsylvania, U.S.A. The doubling time of this cell line is 24 h. The MCF7 cell line was obtained from American Type Tissue Culture, Rockville, Maryland, U.S.A. The doubling time of this cell line is 30 h. Cells were maintained in monolayer culture in RPMI 1640 media supplemented with 5% foetal bovine serum (Gemini Bioproducts, Calabases, California, U.S.A.), 2 mM L-glutamine, 50 IU/ml of penicillin and 50 μg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell lines were monitored for the absence of mycoplasmas using the Mycoplasma Detection Kit (Boehringer Mannheim Corporation, Indianapolis, Indiana, U.S.A.). Subculturing was carried out at subconfluent densities. The cells were dispersed with a phosphate buffered saline solution of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid.

Sulphorhodamine B in vitro *drug assay*

The A2780 ovarian and MCF7 breast tumour cells were plated at 4000 cells per well into 96-well plates in 180 μl of media on day 0 for use in the three-day sulphorhodamine B assay. The cell densities for the A2780 and MCF7 tumour cells in the seven-day assay were 250 and 1000 cells per well, respectively. On day 1, 20 μl of appropriate drug concentrations with vehicle was added to the wells. Cells were preincubated for 15 min with WR-1065, as follows:

Three-day assay (cell-cycle independent drugs): A2780 cells, 0.5 μg/ml; MCF7, 50 μg/ml,

Seven-day assay (cell-cycle dependent drugs): A2780 cells, 0.05 μg/ml; MCF7, 5 μg/ml.

The medium containing WR-1065 was removed and fresh medium with the appropriate anticancer drugs added. On day 3 or 7, the plates were fixed with cold trichloroacetic acid for 1 h at 4°C, washed four times with distilled water and stained with 0.4% sulphorhodamine B for 10 min. Excess stain was removed by washing four times with 1% acetic acid. Dye was solubilised with 50 mM unbuffered TRIS and plates were read on an automatic plate reader at wavelength 540 nm.

Per cent survival of tumour cells was calculated on the basis of the following equation:

% survival = [mean optical density of treated wells/mean optical density of control wells] × 100.

Chemicals and assay conditions

WR-1065 was obtained for *in vitro* use from U.S. Bioscience (West Conshohocken, Pennsylvania, U.S.A.). Thirteen of the drugs were obtained for *in vitro* use only from Sigma (St Louis, Missouri, U.S.A.). Idarubicin was obtained from Adria Laboratories (Dublin, Ohio, U.S.A.). Mitoxantrone was obtained from American Cyanamid Company (Lederle Laboratories, Pearl River, New York, U.S.A.). Docetaxel was obtained from Rhone–Poulenc Rorer (Collegeville, Pennsylvania, U.S.A.). For each of the 16 standard anticancer drugs, a dose–response curve was constructed over a seven-log concentration range to establish a 50% inhibitory concentration (IC_{50}) with and without a 15-min preincubation with WR-1065. Each drug concentration was plated in sextuplet and each drug comparison was performed in triplicate.

Statistical analyses

IC_{50} values were estimated in the following automated manner for each of the triplicate experiments for each anticancer drug with and without preincubation with WR-1065. Regression lines were fitted to tumour cell survival versus log concentration by successively omitting the data from the lowest concentration until the implied IC_{50} value and the per cent of explained variation were maximised. Because the survival curves tended to be flat initially, this had the effect of selecting the portion of the curve over which the dose–response was linear. The estimated IC_{50} was then taken to be the log dose for which the fitted regression predicted 50% survival. As a check, all curves were plotted and visual estimates of IC_{50} were compared with the regression results. In 2 cases, the regression was found to be artifactual, so the doses in the linear part of the curve were selected manually. Comparison of IC_{50} values between experiments was carried out using the Aspin–Welch version of the two-sample *t*-test (which does not assume equal variances) to compare the mean IC_{50}s on the log scale. Two-sided *P*-values below 0.05 were taken to indicate statistical significance.

RESULTS

Dose–response curves were constructed for 16 standard anticancer drugs with and without a 15-min WR-1065 preincubation in A2780 ovarian and MCF7 breast cancer cell lines. The curves were generated based on 18 replicates of each drug concentration. WR-1065 had no statistically significant effect on the cytotoxicites of any of the anticancer agents tested in either cell line.

As seen in Figure 1, the dose–response curve for MCF7 cells incubated with WR-1065 prior to paclitaxel (Taxol®) tends to fall below that of paclitaxel alone. Figure 2 illustrates the overlapping dose–response curves generated in A2780 ovarian cancer cells for cisplatin with and without WR-1065. These two graphs are representative of the results for each anticancer drug tested. In no instance did WR-1065 protect the A2780 ovarian or MCF7 breast cancer cells against the cytotoxicites of either cell-cycle-dependent or -independent agents.

Shown in Tables 1 and 2 are the *in vitro* growth-inhibitory effects of the 16 standard anticancer drugs with and without WR-1065 pre-exposure against the human MCF7 breast and human A2780 ovarian cancer cell lines, respectively. The IC_{50} values are expressed in molar concentrations. WR-1065 pre-exposure had no statistically significant effect on the IC_{50} concentration for any of the standard anticancer drugs against either the MCF7 breast or A2780 ovarian cancer cells. Only one

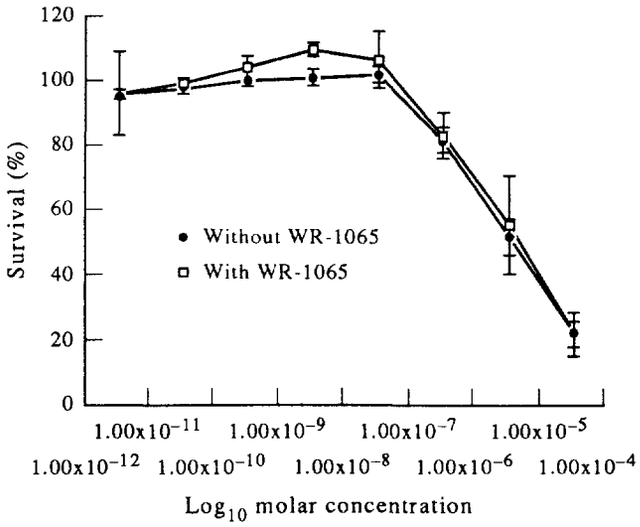

Figure 2. *In vitro* concentrations of cisplatin with and without WR-1065, the active metabolite of amifostine (Ethyol®), versus per cent survival of human A2780 ovarian cancer cells. Bars indicate the mean standard errors at each drug concentration point.

of the *P* values was less than 0.1 (Table 1), and in this instance the addition of WR-1065 appeared to be associated with a trend toward greater cytarabine cytotoxicity.

DISCUSSION

Both preclinical and clinical studies of various alkylating agents and the platinum compounds, cisplatin and carboplatin, have documented that amifostine can prevent drug-induced severe myelotoxicity and nephrotoxicity while preserving and possibly enhancing cytotoxicity to tumours. Our comprehensive *in vitro* screening data suggest that amifostine will not inhibit the cytotoxicity of a wide variety of anticancer drugs, regardless of whether the expression of cytotoxicity is cell-cycle dependent or independent.

The fact that WR-1065 was used in the present *in vitro* studies instead of the prodrug, amifostine, strengthens the results, showing no protection of anticancer drug-induced cytotoxicities against tumour cell lines. A large body of research has proven that the selective cytoprotection of amifostine is based on its differential dephosphorylation by alkaline phosphatase at the tissue site and the preferential uptake of its active thiol metabolite, WR-1065, by normal tissue cells. While the precise mechanism of this selective uptake remains to be fully elucidated, the fact remains that amifostine is concentrated rapidly in normal tissues and the temporal aspects of this selective metabolite accumulation forms a basis for the protective effects. The limited dephosphorylation of amifostine to WR-1065 at the tumour tissue site provides the first major barrier to drug uptake and subsequent potential tumour protection. However, in the present study, despite direct tumour cell exposure to the active amifostine metabolite, there was no evidence of a reduction in anticancer-induced cytotoxicity.

Amifostine has a wide spectrum of cytoprotective effects, including the reduction of anticancer drug-induced myelotoxicity, nephrotoxicity and neurotoxicity. Additionally, it reduces the carcinogenic and mutagenic effects of anticancer drugs [11, 12]. Thus, it undoubtedly will receive increasing use in cancer chemotherapy, both to reduce normal tissue toxicities following standard anticancer drug doses and as a method to facilitate more dose-intensive therapy. The data presented in this study provide further validation that amifostine does not inhibit the tumour cytotoxic effects of a wide variety of anticancer drugs.

Table 1. In vitro *growth-inhibitory effects of standard anticancer drugs with and without pre-exposure of WR*-1065, *the active metabolite of amifostine (Ethyol®) against human MCF7 breast cancer cells*

Anticancer drugs	IC_{50} (−) WR-1065	IC_{50} (+) WR-1065	*P* value
Bleomycin	3.43×10^{-6}	1.59×10^{-6}	0.33
Carboplatin	9.04×10^{-2}	9.52×10^{-2}	0.97
Cisplatin	8.91×10^{-3}	1.07×10^{-3}	0.36
Cytarabine	1.39×10^{-7}	0.70×10^{-7}	0.083
Daunorubicin	7.50×10^{-7}	8.64×10^{-7}	0.74
Doxorubicin	1.05×10^{-6}	0.47×10^{-6}	0.33
Etoposide	6.39×10^{-7}	6.69×10^{-7}	0.86
5-Fluorouracil	1.54×10^{-6}	1.08×10^{-6}	0.51
Idarubicin	1.81×10^{-7}	1.80×10^{-7}	0.995
Melphalan	0.96×10^{-3}	1.73×10^{-3}	0.78
Mitomycin-C	1.55×10^{-5}	1.42×10^{-5}	0.94
Mitoxantrone	0.80×10^{-6}	1.16×10^{-6}	0.37
Paclitaxel	1.15×10^{-9}	1.00×10^{-9}	0.73
Taxotere	1.04×10^{-9}	0.96×10^{-9}	0.74
Vinblastine	1.19×10^{-9}	0.74×10^{-9}	0.36
Vincristine	1.24×10^{-9}	0.81×10^{-9}	0.69

IC_{50}, 50% inhibitory concentration (expressed in molar concentration).

Table 2. In vitro *growth-inhibitory effects of standard anticancer drugs with and without pre-exposure of WR*-1065, *the active metabolite of amifostine (Ethyol®) against human A2780 ovarian cancer cells*

Anticancer drugs	IC_{50} (−) WR-1065	IC_{50} (+) WR-1065	*P* value
Bleomycin	3.4×10^{-9}	5.98×10^{-9}	0.16
Carboplatin	1.31×10^{-4}	1.12×10^{-4}	0.84
Cisplatin	1.1×10^{-5}	3.2×10^{-5}	0.72
Cytarabine	0.91×10^{-8}	1.4×10^{-8}	0.19
Daunorubicin	1.1×10^{-7}	1.08×10^{-7}	0.98
Doxorubicin	1.94×10^{-7}	1.27×10^{-7}	0.33
Etoposide	2.93×10^{-7}	1.65×10^{-7}	0.56
5-Fluorouracil	1.17×10^{-6}	1.18×10^{-6}	0.995
Idarubicin	1.87×10^{-8}	2.23×10^{-8}	0.83
Melphalan	4.44×10^{-5}	3.31×10^{-5}	0.83
Mitomycin-C	1.42×10^{-6}	0.75×10^{-6}	0.48
Mitoxantrone	1.26×10^{-7}	0.55×10^{-7}	0.73
Paclitaxel	5.81×10^{-9}	7.91×10^{-9}	0.89
Taxotere	4.17×10^{-9}	3.68×10^{-9}	0.86
Vinblastine	2.22×10^{-9}	3.01×10^{-9}	0.47
Vincristine	0.73×10^{-8}	1.19×10^{-8}	0.74

IC_{50}, 50% inhibitory concentration (expressed in molar concentration).

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