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Cardioprotection of Rat Heart Myocytes with Amifostine (Ethyol®) and its Free Thiol, WR-1065, *In Vitro*

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Cultured neonatal rat heart myocytes form a synchronously-contracting cell syncytium from one to two days after isolation, plating on plastic and incubation at 37°C. On day 3 after plating, myocytes were exposed to the anthracycline doxorubicin, 0.1–10 μg/ml, for 1 h with or without a 15-min pretreatment with the thiophosphate compound amifostine (WR-2721, Ethyol®) or its dephosphorylated metabolite, WR-1065. The concentration of each WR-compound was limited to 2 μg/ml or 10% of the maximal achievable plasma concentration of amifostine after an intravenous dose of 740 mg/m^2. Both amifostine and the free thiol significantly reduced doxorubicin-induced heart-cell toxicity, measured by adenosine triphosphate content normalised to total cellular protein. A concurrent 1-h exposure to these compounds and doxorubicin was also cardioprotective, but neither compound was effective when administered after doxorubicin. Although both amifostine and WR-1065 were approximately equipotent in preventing doxorubicin-induced cardiotoxicity, only amifostine significantly increased glutathione levels in the myocytes. These results complement prior *in vitro* and *in vivo* studies in rodents showing cardioprotectant activity for amifostine and its free thiol, WR-1065, when administered prior to doxurubicin.

Key words: anthracyclinase, amifostine, cardiotoxicity, chemoprotection, doxorubicin, sulfhydryls
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INTRODUCTION

THE ANTHRACYCLINE-BASED antitumour agents doxorubicin (adriamycin®), daunomycin (cerubidine®) and idarubicin (idamycin®) are extensively used in numerous combination regimens for the treatment of haematological malignancies such as leukaemia and lymphoma and for solid tumours such as breast cancer, lung cancer and sarcoma [1]. Mechanistically, these agents are known to intercalate between DNA base pairs to impair macromolecular synthesis [2] as well as inhibit the strand-passing and religation activity of DNA topoisomerase II enzymes [3]. This leads to the production of protein-linked DNA double strand breaks and an arrest of cell-cycle progression in late S-phase and early G_2-phase [4]. In addition, the quinone moieties in these anthracyclines can bind iron and may be reduced by mammalian flavoenzymes, using nicotinamide-adenine dinucleotide phosphate to yield unstable hydroquinones. These reduced metabolites are capable of transferring electrons to molecular oxygen. The anthracyclines can then recycle back to the quinone form [5]. This redox cycling is believed to be facilitated by iron-bound anthracycline intermediates because these forms are more readily reducible by cytochrome reductases under normal physiological conditions [6]. As a consequence of the postulated reduction/oxidation cycling of the anthracyclines, highly labile oxygen free radicals are produced, leading to cardiac toxicity [7, 8]. Anthracycline-induced oxygen radicals, if not scavenged, can produce diverse oxidative damage to critical cellular components and, in particular, to the membrane lipids in cellular organelles, such as mitochondria and plasma membranes [9]. Normal cellular defences against such oxidative damage include superoxide dismutase (SOD), which catalyses the conversion of superoxide to less reactive hydrogen peroxide; catalase, which scavenges hydrogen peroxide; and glutathione (GSH), the principal intracellular reduced thiol, which maintains the active form of glutathione peroxidase to handle (scavenge) numerous potentially toxic organic peroxides [10].

The acute clinical toxicities of the anthracycline class of agents include myelosuppression (principally neutropenia), nausea, vomiting, alopecia and local skin necrosis if inadvertently extravasated [11]. Cardiac damage is a unique toxicity of chronic doxorubicin therapy when cumulative doses total $>$ 400–500 mg/m^2 [12]. More recent follow-up studies of children who received even lower cumulative anthracycline doses as part of curative treatment for leukaemia suggest that heart damage can be manifested long after dosing has ended

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[13]. Although epidemiological studies suggest that cardiotoxicity is somewhat lessened with daunomycin [14] and idarubicin [15], it is clear that all the quinone-containing agents in this group produce total-dose-limiting myocardial damage, presumably by the oxidative mechanisms described above. Indeed, heart tissues appear to be particularly sensitive to oxidative stress because this organ has relatively low endogenous enzymatic defenses (SOD catalase) and relatively low GSH stores [14]. This contrasts sharply with the liver, an organ that has very high antioxidant enzymes and in which anthracyclines accumulate to high levels, but rarely if ever expresses any degree of serious toxic sequelae following repeated anthracycline dosing [15].

Previous attempts at blocking or preventing experimental anthracycline-induced cardiotoxicity include the administration of sulphydryls such as *N*-acetylcysteine [16], antioxidant vitamins such as α-tocopherol [17] and the intracellular iron-chelating agent dexrazoxane (ICRF-187) [18–20]. Of these, only dexrazoxane has shown unequivocal evidence of significant cardioprotection in randomised clinical trials [21, 22]. This agent was recently approved for marketing in the United States for breast cancer patients receiving high cumulative doses of doxurubicin as part of the cyclophosphamide, doxorubicin and 5-fluorouracil regimen [21]. Clearly, other approaches to anthracycline cardioprotection are desirable. In this regard, several preclinical studies have suggested that the phosphorothiol, amifostine, and its dephosphorylated active metabolite, WR-1065, may be useful. In mice, WR-1065 prevented a doxorubicin-induced increase in myocardial damage without impairing experimental therapeutic efficacy against Sarcoma-180 tumours [23]. Similar results have been reported in nude mice bearing human MDA-MB-435 breast cancer tumours [24]. *In vitro* studies in heart mitochondria also showed that amifostine and WR-1065 scavenged hydroxy radical spin-trap signals. In addition, WR-1065 was able to scavenge superoxide anions [25]. Overall, these findings suggested that amifostine and WR-1065 might reduce doxorubicin-induced cardiotoxicity. This was further investigated in a rat heart myocyte model to determine the dose–response and time course for such protective activities.

MATERIALS AND METHODS

Chemicals

Doxorubicin was obtained from Pharmacia (Columbus, Ohio, U.S.A.) as a 10-mg lyophilised powder. It was reconstituted in 100 ml 0.9% sodium chloride for injection, USP, and stored at −80°C in plastic vials before use. Amifostine [S-2-(3-aminopropylamino)ethylphosphorothioic acid, Ethyol,® or WR-2721] and the dephosphorylated, reduced thiol congener, WR-1065 or actifostine, were obtained as white powders from U.S. Bioscience (West Conshohocken, Philadelphia, U.S.A.). The drugs were initially dissolved in double-distilled sterile water, filtered through 0.2-μm nitrocellulose, frozen and subsequently diluted into sterile M-3 culture medium immediately prior to use. Radiolabelled WR-1065 (ethyl-1,2-^{14}C) was synthesised at 96% purity for U.S. Bioscience at Chemsyn Science Laboratories (Lenexa, Kansas, U.S.A.). The specific activity was 35.6 mCi/mmol and it was supplied as a lyophilised powder containing 500 μCi/2.91 mg. All other chemicals were of analytical grade.

Heart cell culture

The isolation and culture of neonatal rat heart cells was performed as described in detail previously [26]. Hearts were obtained by aseptic surgical excision from one to two day-old mixed-gender Sprague–Dawley rat pups (Harlan Laboratories, Philadelphia, U.S.A.). The hearts were minced into fragments and dissociated with seven serial 15-min exposures to 0.2% trypsin (Difco Laboratories, Detroit, Michigan, U.S.A.) dissolved in calcium and Mg^2-free Hank's balanced salt solution (Irvine Scientific, Santa Ana, California, U.S.A.). The supernatants from the last five digestions were collected, pooled and washed twice in Liebovitz's M-3 culture medium [27]. The myocytes were initially plated at 3–4 × 10^7 cells/150 cm^2 at 37°C for 2 h on plastic plates to allow for fibroblast attachment. Subsequently, the myocyte-enriched supernatant was counted and seeded on to 24-well Costar® plastic plates at a density of approximately 4 × 10^3 myocytes/mm^3. The cultures were placed in an incubator with 5% CO_2/95% filtered room air for seven days.

Myocyte viability was assessed by quantitation of cellular adenosine triphosphate (ATP), using luminometry [28], normalised to total protein (as an estimate of cell number) measured by the Bradford method (BioRad, Richmond, California, U.S.A.) [29]. The resultant ATP: protein ratio was compared with that of the untreated controls (assumed 100% viability) to yield a per cent of control viability end point. Luminescence from the luciferin–luciferase reaction (Sigma Chemical, St Louis, Missouri, U.S.A.) was read on a model 1251 luminometer (LKB Wallac, Turku, Finland). The myocytes were also visually observed for synchronous contractions that begin spontaneously at a rate of 40–50 per min one–two days after plating.

Doxorubicin exposures for 1 h at 37°C in M-3 medium were performed on day 3, when contraction rates and total protein concentrations are maximal [26]. Amifostine or WR-1065 was added to the myocytes by three exposure sequences: (1) for 15 min and then removed immediately by washing prior to doxorubicin, (2) for 1 h after doxorubicin, or (3) simultaneously for 1 h with doxorubicin. The *in vitro* concentrations of amifostine and WR-1065 were limited to 2.0 μg/ml, which is one tenth of the peak plasma concentration achievable after standard amifostine doses of 740 mg/m^2 are administered to humans [30]. The use of one tenth of the peak concentration is based on correlative clinical pharmacokinetic studies with anticancer agents tested in the human tumour colony-forming assay [31]. These studies showed good predictions of anticancer efficacy when *in vitro* drug exposures were limited to one tenth of the peak achievable plasma level in patients. Following these drug exposures, the cells were washed twice, placed in fresh M-3 medium and returned to the 37°C incubator for three days to allow for maximal, time-dependent loss of viability following the 1-h anthracycline exposure [26, 32].

To evaluate aminothiol uptake and retention characteristics, 20 μg/ml radiolabelled WR-1065 was added to untreated myocyte cultures for 15, 30, 45 and 60 min, followed by two washes in ice-cold medium to remove unbound drug. Efflux of the radiolabel WR-1065 was followed for 15 min after a 15-min exposure. Per cent uptake was calculated as the fraction of cell-associated radioactivity (by scintillation) divided by the total radioactivity in the exposure concentration.

The effect of doxorubicin and/or amifostine and of WR-1065 on myocyte glutathione levels was measured using the spectrophotometric/enzymatic recycling method of Tietze [33]. This method simultaneously quantitates both oxidised and reduced (GSH) forms of cellular glutathione.

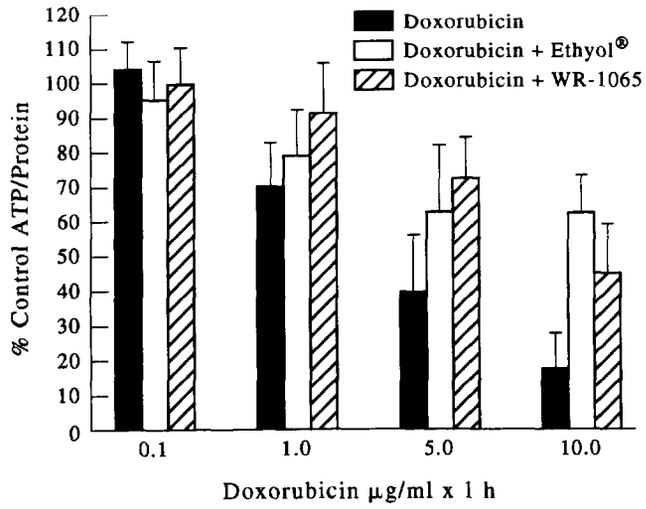

Figure 1. A comparison of myocyte viability following doxorubicin alone ■ for 1 h or with a 15-min pretreatment with 2.0 μg/ml of either WR-2721 ▨ or WR-1065 □. Each bar represents the mean (± S.E.M.) of 12 determinations (two experiments involving six wells per experiment). ATP, adenosine triphosphate.

RESULTS

Amifostine and the free thiol, WR-1065, were nontoxic to the myocytes at concentrations of up to 10μg/ml for a 72-h exposure. This allowed subsequent testing of each agent at a concentration of 2 μg/ml for 15 min prior to a 1-h doxorubicin exposure. Doxorubicin produced a concentration-dependent loss of myocyte viability over the range of 1-h drug concentrations of 0.1 μg/ml (100% viability) to 10 μg/ml (17.3% viability). A 15-min pre-treatment with 2 μg/ml of either amifostine or WR-1065 followed by the same 1-h doxorubicin exposure resulted in a significant degree of protection (Figure 1). Of interest, pre-treatment with either amifostine or WR-1065 appeared to be equally efficacious at preventing doxorubicin-induced losses of myocyte viability. Time course experiments with these compounds and doxorubicin showed that pretreatment or concurrent treatment with WR-1065 is effective at blocking doxorubicin-induced cardiotoxicity. In contrast, a 1-h post-treatment with WR-1065 resulted in no significant protection from doxorubicin-induced cardiotoxicity *in vitro*. These results are summarised in Table 1.

The results of the ^{14}C-WR-1065 uptake studies showed that maximal uptake by myocytes at 37°C was achieved by a 30-min exposure to a concentration of 20 /ml. This resulted in a cellular WR-1065 concentration of 0.41 μg/ml or roughly 2.1% of the extracellular exposure concentration. Uptake was approximately one-half maximal at 5 min: 0.17 μg/ml or 0.86% of the extracellular exposure concentration. When the myocytes were extensively washed in ice-cold phosphate-buffered saline after a 15-min exposure at 37°C and then allowed to incubate in drug-free medium for 15 min at 37°C, ^{14}C-WR-1065 concentrations decreased by only 14%, to 0.15 μg/ml or 0.74% of the exposure concentration. This indicates that the limited uptake of WR-1065 is rapid and that retention is avid even after a brief exposure at 37°C.

A 15-min exposure to amifostine also raised myocyte glutathione levels significantly. Following a 15-min exposure to 1 μg/ml, glutathione levels in three-day-old myocyte cultures increased from a control value of 111 ± 9.0 nmol/mg of protein to 159 nmol/mg (a 43% increase [P = 0.001 by t-test]). Following the same 15-min exposure to WR-1065, myocyte GSH levels were unchanged from controls at 107 ± 18.6 nmol/mg of protein.

CONCLUSIONS

Amifostine is a unique organic thiophosphate that has been shown to protect against normal tissue toxicity induced by ionising radiation [34, 35], electrophilic DNA binding agents (including alkylating agents [36, 37] and platinum-containing compounds [38, 39]). Mechanistically, amifostine is believed to be selective for normal (nontumorous) tissue because of physiological differences between normal cells and cancer cells. These differences include increased drug distribution into normal tissues because of relatively poorer tumour vascularity [40] and greater activation by enzymatic dephosphorylation of amifostine to WR-1065 in normal tissues. The latter is believed to result from a higher relative pH and thereby greater alkaline phosphatase activity in normal tissues compared with solid tumour masses, which may have more acidic pH values [41]. Greater drug distribution into normal tissues compared with tumours has been experimentally documented in rats given amifostine, wherein heart and liver tissues took up a 6–10-fold greater amount of drug than did 3M2N tumour tissues [40]. Studies in BALB/c mice show that a similar 4–6-fold concentration gradient in heart tissues compared with tumours is maintained for 1 h after intravenous (i.v.) dosing with WR-1065 [42]. In concert with the current cellular studies, the uptake of WR-1065 into heart tissues of mice was shown to be rapid, peaking at 0.65 μmol/g within 15 min after an i.v. injection of 500 mg/kg of body weight [42]. Of interest, this prior study reported no increase in tissue GSH levels following WR-2721 dosing. This contrasts with the current finding that myocyte GSH levels did increase significantly following a brief exposure to amifostine. However, our *in vitro* studies showed that both amifostine and WR-1065 (which did not increase GSH levels)

Table 1. *Time dependence of the cardioprotective effect of the amifostine free thiol,* WR-1065, *with doxorubicin (DOX) in rat heart myocytes* in vitro

		WR-1065		
DOX (μg/ml × 1 h)	No WR-1065 compounds	15 min before DOX	1 h simultaneous	1 h after DOX
1.0	69 (12.7)	94.0 (11.7)*	76.0 (17.6)	71.5 (12.9)
10	38.5 (6.9)	78.0 (13.4)*	81.9 (11.8)*	37.1 (10.4)
50	17.3 (10.1)	61.7 (19.1)*	59.4 (13.4)*	11.9 (13.1)

* $P < 0.05$ by t-test compared with DOX control (without WR-1065).
ATP, adenosine triphosphate; S.E.M., standard error of the mean.

were approximately equally effective cardioprotectants for doxorubicin. This suggests that GSH elevation is not directly involved in the cardioprotectant mechanism of amifostine and WR-1065. This finding is also compatible with prior *in vitro* myocyte studies showing no enhancement of doxorubicin cardiotoxicity with concomitant GSH depletion by the inhibitor of GSH synthesis, L-buthionine sulphoximine [43].

The reason for the roughly comparable degree of cardioprotection with amifostine or WR-1065 in the cardiac myocytes is not known. It suggests that amifostine may have direct protective effects without the need for metabolic conversion to WR-1065. This conclusion is compatible with the low level of alkaline phosphatase activity measured in the myocytes cultured at pH 7.4 [44]. Prior studies showed that the optimum pH for amifostine dephosphorylation in mouse liver homogenates is in the range of 8.6 to 8.8 and, in ascites tumour tissue, the optimum pH was 5.6 [45]. Unfortunately, phosphatase activity was not measured in heart tissues in this trial [45]. However, there was a substantial variation in phosphatase activity among the six different normal tissues studied. This ranged from 935 nmol/mg protein per h in kidney tissues to only 49 nmol/mg per h in lung tissues. Additionally, no phosphatase activity was detected in serum [45]. Furthermore, there was no correlation between the degree of amifostine dephosphorylation and the extent of radioprotection in these different tissues [45]. Thus, the very low value for alkaline phosphatase activity measured in isolated heart cells [44], despite the apparent good cardioprotective action of amifostine and WR-1065, is compatible with the prior findings *in vivo*.

The sequence dependence of the protective effect of amifostine in the myocytes is also compatible with prior studies of cisplatin-induced nephrotoxicity in mice [45]. When administered 30 or 5 min before cisplatin, amifostine significantly reduced kidney toxicity, affording a 2-fold cisplatin dose escalation; however, when administered 30 min after cisplatin, there was no reduction in nephorotoxicity. This requirement for pretreatment has been carried over into all of the clinical trials with amifostine and platinum-containing compounds [35–39]. The similar cardioprotective effects with amifostine, given either beforehand or simultaneously with doxorubicin, in the myocytes suggest that both amifostine and WR-1065 protect critical target sites from anthracycline damage but do not rescue cells already exposed to drug. Pretreatment is also required for cardioprotection with the iron chelator dexrazoxane when used in doxorubicin-based combination chemotherapy regimens in breast cancer patients [21, 22].

The current myocyte findings clearly complement previous preclinical studies with amifostine and doxorubicin. In a defined chemical system, both amifostine and WR-1065 scavenged Fenton's reaction-generated hydroxyl radicals trapped using the spin-trap reagent 5,5-dimethyl-L-pyrroline-*N*-oxide [25]. In addition, WR-1065 scavenged superoxide anions generated by the chemical autoxidation of epinephrine [25]. In nude mice bearing the human breast cancer cell line MDA-MB-435, amifostine protected against lethality from a high-dose regimen of doxorubicin. This regimen produced a significant antitumour effect evidenced by a 45% increase in lifespan. Twice as many amifostine-treated animals survived 90 days after tumour implantation with doxorubicin treatment [24]. Perhaps more relevant to the current *in vitro* myocyte data is the report of reduced doxorubicin cardiotoxicity in BALB/c mice bearing sarcoma-180 ascites tumour [23]. In this trial, amifostine at 50 mg/kg daily for three weeks (throughout the doxorubicin dosing period) prevented a doxorubicin-induced doubling of creatinine phosphokinase (CPK) levels without altering doxorubicin's antitumour effectiveness. The enzymatic results with CPK levels were confirmed by histopathological examinations of the heart. These analyses showed no doxorubicin-induced vacuolisation of myocytes and no swelling or occlusion of capillary lumina in amifostine-treated mice [23].

In summary, amifostine and its dephosphorylated metabolite WR-1065 have been shown to reduce significantly doxorubicin heart toxicity in rat heart myocytes. These results complement prior *in vitro* oxygen radical studies and *in vivo* studies in tumour-bearing rodent models. Because of these findings, the protective effect of amifostine pretreatment on doxorubicin heart toxicity should be evaluated clinically.

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