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ORIGINAL ARTICLE

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In vitro studies of the hyperthermic enhancement of activated ifosfamide (4-hydroperoxy-ifosfamide) and glucose isophosphoramide mustard

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Abstract *Purpose*: To study the effect of hyperthermia on the cytotoxicity of glucose isophosphoramide mustard (D-19575), a derivative of ifosfamide, which does not require activation and preclinically demonstrates less nephrotoxicity and myelosuppression than ifosfamide. *Methods*: In vitro studies (using a crystal violet cell survival assay) of the interaction of hyperthermia with D-19575, as well as the activated form of ifosfamide (4-hydroperoxy-ifosfamide, D-18851), were performed using L929 and OVCAR-3 cell lines held at various temperatures (i.e. 37 °C (control), 40.5 °C, 41.8 °C, 42.5 °C, and 43 °C) for 65 min. *Results*: The following thermal enhancement ratios (TER) were demonstrated: D-19575 in L929 1.2, 2.0 and 2.3 at 40.5, 41.8 and 42.5 °C, respectively; for D-18851 in L929 1.7 at 41.8 °C; for D-19575 in OVCAR-3 2.1, 3.2 and 3.3 at 40.5, 41.8 and 42.5 °C, respectively; for D-18851 in OVCAR-3 4.6 at 41.8 °C. *Conclusion*: The significant observed increase in cytotoxicity of D-19575 caused by hyperthermia taken together with its known preclinical toxicity profile, encourage its further preclinical and ultimately clinical testing, including its use with whole body and regional hyperthermia.

Key words Whole body hyperthermia · Chemotherapy · Ifosfamide

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Introduction

The cytotoxic effects of alkylating agents appear to be significantly enhanced by hyperthermia. In this regard, Wiedemann et al. have demonstrated the ability of hyperthermia to increase the cytotoxicity of ifosfamide in a series of murine experiments involving tumor transplantation [9]. This preclinical study led to the investigation of ifosfamide in a series of clinical whole body hyperthermia (WBH) trials involving patients with refractory cancer [10–13]. As ifosfamide is a prodrug requiring hepatic metabolism, its use in the setting of clinical WBH requires its administration at prehyperthermic temperatures to allow for proper activation. Although the clinical use of ifosfamide in combination with other agents and WBH has resulted in dramatic results in phase I and phase II trials, its use is compromised by its tendency to cause renal toxicity and myelosuppression [10–13]. The need to administer this agent prior to reaching hyperthermic temperatures [9] leads to a further potential compromise of its therapeutic efficacy [5, 14]. An ifosfamide analogue not requiring metabolic activation (coupled to a reduced toxicity profile discussed below) should provide further enhancement of the therapeutic index (over its parent compound) in the clinical setting of 41.8 °C WBH.

Some of the major toxicities of ifosfamide (e.g. hemorrhagic cystitis) are attributable to its metabolites. In this regard, ifosfamide is initially metabolized by the hepatic microsomal system to 4-hydroxy-ifosfamide, which through additional spontaneous equilibration reactions forms ifosfamide mustard (the active alkylating agent) and the urothelial toxin acrolein. Thus, delivery of an ifosfamide mustard analogue should provide activity without the toxicity of acrolein. Relevant to this, glucose isophosphoramide mustard (D-19575), a sugar-linked isophosphoramide derivative (scheduled to enter

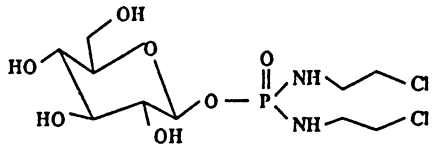

Fig. 1 Chemical structure of D-19575. Molecular weight 383.1 kDa

clinical trials in the spring of 1997; J. Pohl, personal communication) appears to encompass all of these desired pharmacological properties. D-19575 is an ifosfamide mustard in which glucose has an ester-like link to the phosphoric acid part of the molecule (Fig. 1). This agent is directly cytotoxic in vitro, lacks renal toxicity, and its toxicity to white blood cells, colony forming units, and spleen colony forming units is considerably diminished as compared to ifosfamide [4]. Furthermore, the activity of D-19575 in vitro is comparable to isophosphoramide mustard, the final active metabolite of ifosfamide. A comparison of the antitumor activity of equimolar amounts of D-19575, ifosfamide, and cyclophosphamide has shown that D-19575 has the highest antineoplastic activity of these agents in transplanted murine Ehrlich ascites carcinoma [4].

Regarding normal tissue toxicity, the LD_{50} of D-19575 is three times that of ifosfamide (on a molar basis) in the rat and 1.5 times that of ifosfamide in the mouse [4]. Pohl et al. have postulated that this new alkylating agent has increased antitumor selectivity secondary to an active transmembrane transport mechanism [4]. Interestingly, relatively high doses of D-19575 appear to be required for short in vitro exposure to produce cytotoxicity, presumable because of the rate-limiting action of active transport [4, 7, 8].

Since D-19575 has significant indications for clinical use in the setting of 41.8 °C WBH, a series of preclinical investigations were undertaken to study the effect of hyperthermia on its cytotoxicity. The effect of hyperthermia on D-19575 has not previously been studied in vitro. In this report, the relative dose enhancement effects (in terms of the thermal enhancement ratio, TER) of hyperthermia on D-19575, as well as 4-hydroperoxy-ifosfamide (D-18851) the activated form of ifosfamide, are described.

Material and methods

Cell lines, cell culture, and reagents

The human ovarian adenocarcinoma cell line OVCAR-3 (ATCC no. HTB161) and the murine fibrosarcoma cell line L929 (ATCC no. CCL1) were purchased from the American Type Culture Collection, Rockville, Md. Cells were grown at 37 °C under an atmosphere containing 5% CO_2 in RPMI-1640 with 10% fetal calf serum, 10 μg/ml insulin, and antibiotic/antimycotic (OVCAR-3) or in DMEM with 5% fetal calf serum and antibiotic/antimycotic (L929). Both cell lines were maintained by serial passage of the monolayer and were regularly found to be free of *Mycoplasma* contamination (GEN PROBE, San Diego, Calif.). D-19575 (MW 383.1 kDa) and D-18851 (MW 293 kDa) were provided by ASTA Medica, Frankfurt, Germany.

Drug/heat-mediated cytotoxicity assays

Survival assays were performed over a 7-day period. On day 0, tumor cells were planted in 100 μl medium/well on a Corning 96-well plate at a concentration of 1×10^3 cells/well. On day 1, serial dilutions of either D-19575 or D-18851 were applied to exponentially growing cells which were then exposed to one of five temperatures (37 °C, 40.5 °C, 41.8 °C, 42.5 °C, or 43 °C). Plates were heated as previously described [2, 3] by placing them into sealable plastic bags (Hamilton Beach, Washington, N.C.) and filled with air containing 5% CO_2. The plates were then submerged into gently shaking water baths maintained at 37 °C, 40.5 °C, 41.8 °C, 42.5 °C, or 43 °C $\pm <$ 0.05 °C for 65 min. (It required ~5 min for plates to equilibrate. The 60-min incubation time was chosen as a standard condition as it is optimal for clinical WBH and should provide an adequate area under the curve to allow for WBH enhancement.) After treatment, the medium was removed from all 96 wells and the cells were washed with sterile medium containing no additives. After washing, the cells were replenished with fresh medium and returned to the incubator at 37 °C until day 7.

On day 7, cells were stained and fixed with 0.5% crystal violet in methanol for 45 min. The dye was gently removed with distilled water after staining and the plates were dried. Absorbed dye was then dissolved and solubilized in 33% acetic acid, and the plates were read with a microtiter plate photometer (SLT Laboratories, Salzburg, Austria) at 550 nm.

Each plate contained control rows which received no drug but were submitted to all other treatments. All experiments were performed at least three times.

Data analysis

Dose enhancement ratios for drug killing (also termed TER if heat is the independent variable) were calculated from the slope of the linear portion of the survival curves. Briefly, dose enhancement ratios were calculated by taking the ratio of the survival curve slopes (e.g. the slope of the survival curve using D-19575 or D-18851 and hyperthermia divided by the slope of the curve using drug alone) as previously reported [2, 6]. Standard errors for dose enhancement ratios were computed by the method of propagation of errors. All *P*-values are two-sided. Test statistics were computed by comparing the difference in estimated slopes with the estimated standard error of the difference. Using this convention, additive interactions produce parallel curve slopes (e.g. the slope for D-19575 at 41.8 °C equaled the slope for D-19575 at 37 °C). An increase in slope (yielding a positive dose enhancement ratio) indicates supraadditive cytotoxicity (e.g. the slope of D-19575 at 41.8 °C exceeded the slope for D-19575 at 37 °C with the *P*-value indicating the statistical significance of this difference).

Results

Figures 2a and 3a show survival of L929 and OVCAR-3 cells, respectively, following simultaneous exposure to D-19575 and heat. Similarly, Fig. 2b and 3b show survival of L929 and OVCAR-3 cells following simultaneous treatment with D-18851 and heat. Figures 4 and 5 show survival of L929 and OVCAR-3 cells, respectively, following simultaneous exposure to D-19575 at 37 °C, 40.5 °C, 41.8 °C, 42.5 °C, and 43 °C. (The data shown are not corrected for heat killing; the ordinate represents killing by heat alone.) In all experiments cells were incubated at various concentrations of drug for 1 h at the indicated temperatures. Each graph represents the mean results from three experiments. Survival decreased exponentially as a function of drug concentration at all

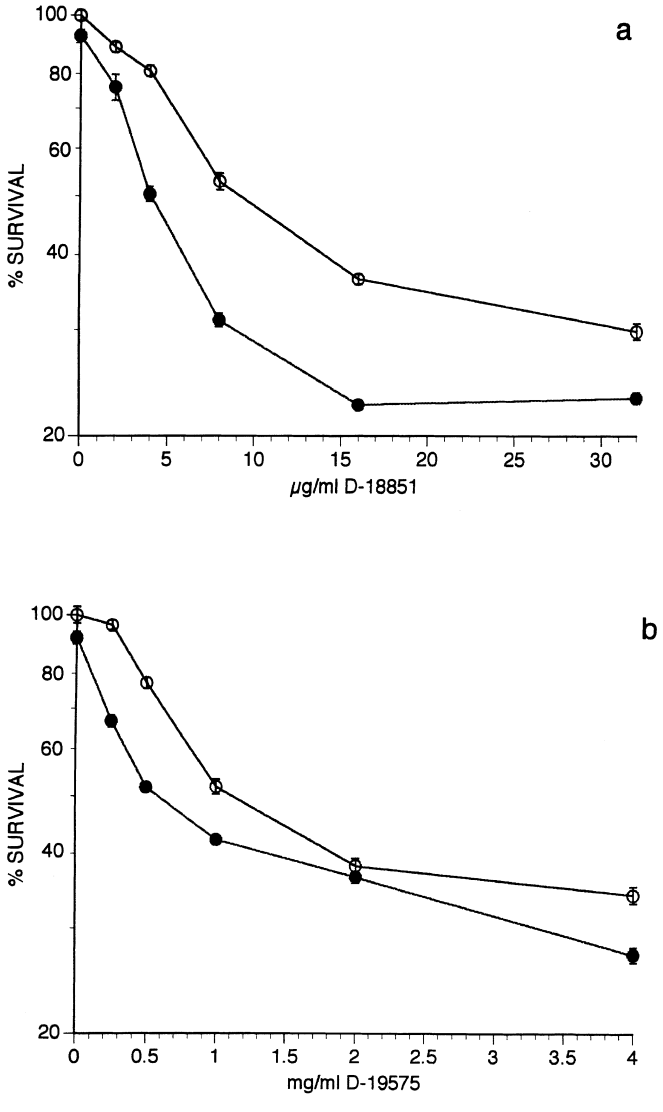

Fig. 2a,b Survival of L929 at 37 °C (○) or 41.8 °C (●) with D-18851 (**a**) and with D-19575 (**b**). Cells were simultaneously heated and exposed to varying concentrations of D-18851 or D-19575 for 65 min. Survival was calculated relative to that of untreated controls

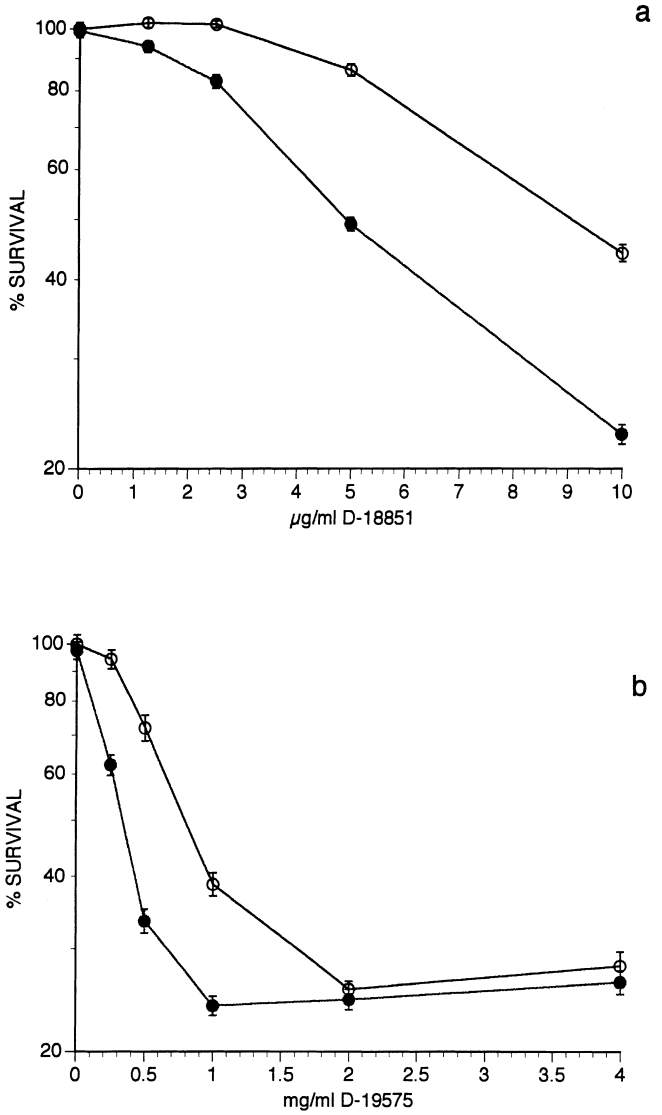

Fig. 3a,b Survival of OVCAR-3 at 37 °C (○) or 41.8 °C (●) with D-18851 (**a**) and with D-19575 (**b**). Cells were simultaneously heated and exposed to varying concentrations of D-18851 or D-19575 for 65 min. Survival was calculated relative to that of untreated controls

temperatures except 43 °C in both cell lines. This allowed for valid calculation of the TER at each temperature except 43 °C.

The TERs are shown in Table 1. In both cell lines the TERs at 41.8 °C and 42.5 °C were significantly increased in comparison with the 37 °C control for D-19575, as well as for D-18851.The significant cell killing by heat alone (particularly of L929 cells) prevented valid calculation of the TER at 43 °C.

Table 1 Thermal enhancement ratios of D-19575 and D-18551 in L929 and OVCAR-3 cell lines at different temperatures

	37 °C	40.5 °C	41.8 °C	42.5 °C
L929				
D-19575	1.0*	1.2	2.0*	2.3*
D-18551	1.0*		1.7*	
OVCAR-3				
D-19575	1.0**	2.1	3.2**	3.3**
D-18551	1.0**		4.6**	

*$P \leq 0.05$, **$P \leq 0.05$

Discussion

The data presented in Figs 2–5 are not corrected for heat killing. The ordinate on each curve represents heat killing, as this point defines cell survival in the absence of drug. These cell lines are relatively heat resistant below 43 °C. Thus, the obvious supraadditive effect of hyperthermia on D-19575 and D-18851 cells is graphically illustrated. It is noteworthy that in the two cell lines studied there was a dramatic increase in TER up to

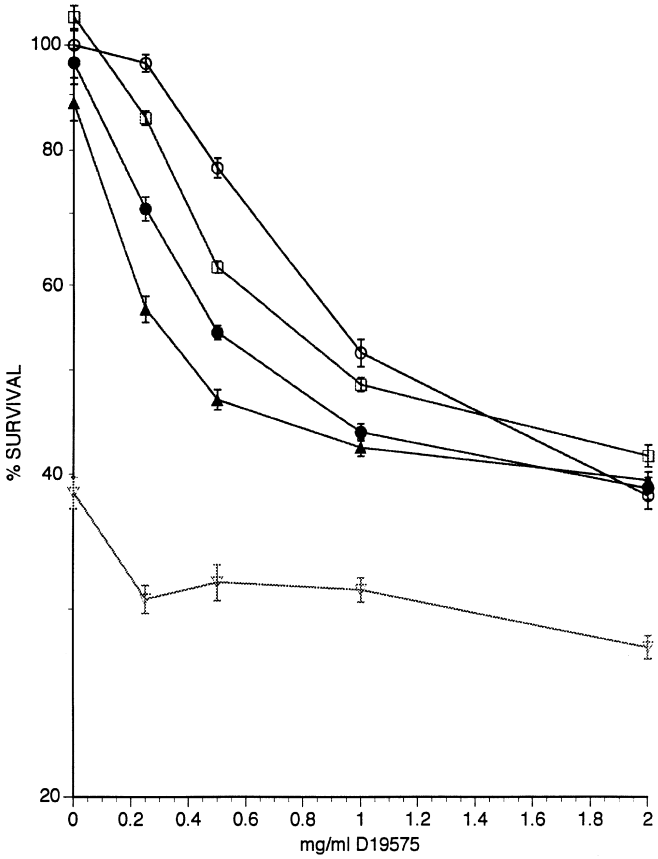

Fig. 4 Temperature/concentration profile of L929 with D-19575 at increasing temperatures. Cells were simultaneously heated at 37 °C (○), 40.5 °C (□), 41.8 °C (●), 42.5 °C (▲), or 43 °C (▽) with varying concentrations of D-19575 for 65 min. The TERs were: 1.2, 2.0 and 2.3 at 40.5, 41.8 and 42.5 °C, respectively (The overriding effect of heat cytotoxicity prevented meaningful estimation of $TER_{43\ °C}$)

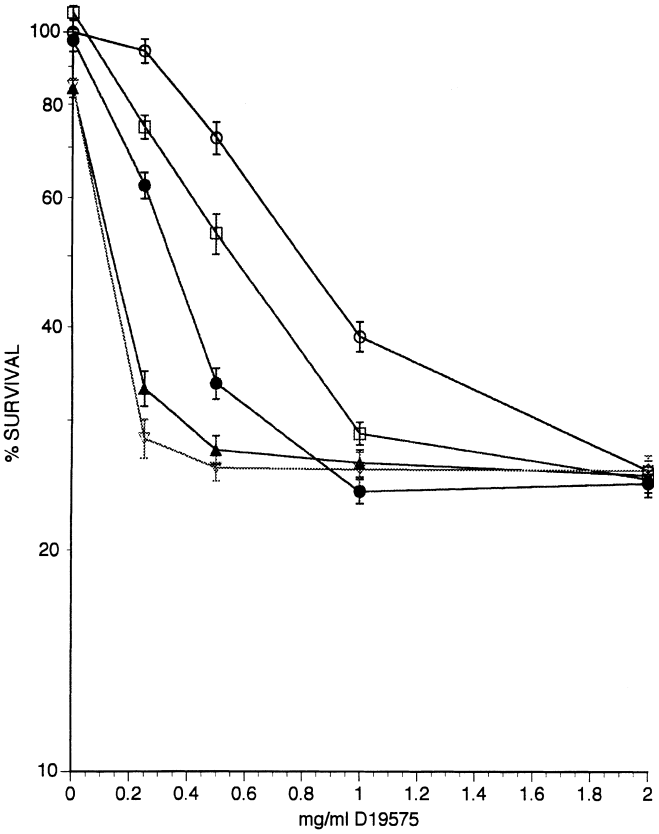

Fig. 5 Temperature/concentration profile of OVCAR-3 with D-19575 at increasing temperatures. Cells were simultaneously heated at 37 °C (○), 40.5 °C (□), 41.8 °C (●), 42.5 °C (▲), or 43 °C (▽) with varying concentrations of D-19575 for 65 min. The TERs were: 2.1, 3.2 and 3.3 at 40.5, 41.8 and 42.5 °C, respectively (The overriding effect of heat cytotoxicity prevented meaningful estimation of $TER_{43\ °C}$)

41.8 °C, and the TER did not increase significantly above the temperatures shown in Figs. 4 and 5. This relationship has been previously observed by Cohen and Robins in studies of carboplatin [1]. Wiedemann et al. also studied D-18851 in an MX-1 cell line which was also heat resistant at 41.8 °C [9]. Although the $TER_{41.8\ °C}$ was not reported for that breast cancer cell line, we reanalyzed the data presented in Fig. 3 of the previous report [9] and found the $TER_{41.8\ °C}$ to be 2.7 ($P < 0.05$); this is quantitatively comparable with our results.

In vitro, significantly higher concentrations of D-19575 than of D-18851 are required for cytotoxicity for short exposures at 37 °C. This phenomenon has been observed by Pohl et al. [4], and is believed to relate to the improved membrane permeability of D-18851 over ifosfamide mustard derivatives (in vitro), which is not evidenced in vivo. In this regard, these workers have shown that an active transport mechanism, i.e. a sodium-dependent glucose transporter, is active in vivo. Drug uptake in vitro is apparently quite different, as discussed in detail by Pohl et al. [4]. This is reflected in animal studies, which have shown that equivalent antineoplastic activity of D-19575 and D-18851 is reached with equimolar doses of each drug. Although the MW of D-19575 is 30% higher than that of D-18551, the intraperitoneal LD_{50} in rats is 10.5 times higher for D-19575 than for D-18551 (personal communication, Dr. Pohl). These observations indicate that D-19575 has a significantly better therapeutic index than D-18551.

It is noteworthy that although these drugs differ in their in vitro cytotoxicity, the dose enhancement effects of hyperthermia were similar. In this regard, the study conditions selected for the experiment shown in Fig. 2 were such that the 37 °C curves for D-18851 and D-19575 are more or less superimposable. Hence, the 41.8 °C curves and there associated TERs are controlled. These observations would suggest a common mechanistic basis for synergy. Clearly, this speculation requires further preclinical investigation.

In summary, the results presented taken together with those of earlier studies encourage further laboratory and clinical investigations of D-19575. In this regard, the potential for D-19575 to be ultimately introduced into a multimodal approach including clinical hyperthermia is promising.

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