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ORIGINAL ARTICLE

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Mechanisms of enhancement of the antitumour activity of melphalan by the tumour-blood-flow inhibitor 5,6-dimethylxanthenone-4-acetic acid

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Abstract Several studies show that the antitumour activity of melphalan (MEL) and other alkylating agents can be enhanced by the selective inhibition of tumour blood flow, although the mechanism(s) underlying these interactions are unclear. 5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a new anticancer agent currently in phase I clinical trial, inhibits blood flow in murine tumours. DMXAA increased the activity of MEL against the MDAH-MCa-4 mouse mammary tumour maximally when MEL was given about 2 h after DMXAA, without compromising the maximal dose of the alkylating agent that could be given. The plasma pharmacokinetics of MEL were unchanged by DMXAA pretreatment, but the area under the concentration-time curve (AUC) for the tumour increased by 33% as a result of decreasing clearance (consistent with falling tumour blood flow). However, inhibition of tumour blood flow also leads to microenvironmental changes (e.g. acidosis and hypoxia) that might influence sensitivity to MEL. The sensitivity of KHT cells (freshly isolated from tumours) to MEL in vitro was increased by lowering of either pH or oxygen concentration (pO_2), with an overall dose-modifying factor of 15 being recorded for aerobic cells at pH 7.4 versus hypoxic cells at pH 6.5. The cellular uptake of MEL by KHT cells was increased by 74% under hypoxia. Thus, DMXAA appears to augment the antitumour activity of MEL through two different mechanisms, increased exposure (via decreased tumour clearance of MEL) and increased sensitivity resulting from changes to the tumour microenvironment, both of which result from inhibition of tumour blood flow.

Key words 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) · Melphalan · Hypoxia · Acidosis · Tumour blood flow

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Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a potent analogue of flavone acetic acid (FAA), is currently in phase I clinical trial as an antitumour agent. FAA selectively inhibits blood flow [9, 11, 28], induces hypoxia [7], and enhances the activity of hypoxia-selective cytotoxins [8, 25] in murine tumours. DMXAA also inhibits tumour blood flow selectively [6, 29], probably through induction of tumour necrosis factor-alpha (TNFα) [4, 5, 15] and differs from FAA in its ability to induce TNFα production by human as well as murine macrophages [4]. We have previously reported that DMXAA also enhances the antitumour effects of hypoxia-selective cytotoxins and suggested this interaction to be due to induction of additional hypoxia by DMXAA, leading to increased bioreductive drug activation [6]. In subsequent investigations, reported herein, we noted a similar enhancement by DMXAA of the antitumour activity of melphalan (MEL), which does not require reductive activation. The antitumour effects of MEL are also known to be enhanced by several other agents that inhibit tumour blood flow [1, 3, 16, 19, 23].

The mechanism by which tumour-blood-flow inhibitors enhance the activity of MEL is not clear, but three possibilities have been suggested. Firstly, altered systemic pharmacokinetics of MEL could lead to

increased exposure of the tumour cells to the alkylating agent [3, 14]. Secondly, falling tumour blood flow could lead to entrapment of MEL in tumours with a consequent increase in tumour cell exposure [1, 19]. Thirdly, both increased hypoxia and lowered pH, two of the physiological consequences of reduced tumour blood flow, independently increase the cytotoxic effects of MEL in vitro [3, 13, 20, 22] and could be responsible for the enhanced antitumour effect in vivo [3, 19]. The first two mechanisms appear not to have been tested directly by investigation of the effects of tumour-blood-flow inhibitors on the plasma and tumour pharmacokinetics of MEL.

The present study investigated the mechanism of therapeutic interaction between DMXAA and MEL against MDAH-MCa-4 mouse mammary tumours. The effects of DMXAA on the plasma and tumour pharmacokinetics of MEL were investigated, and the implications of environmental changes resulting from tumour-blood-flow inhibition (i.e. lowering of extracellular pH and oxygen concentration) on MEL sensitivity were examined in vitro using KHT cells obtained from freshly excised tumours.

Materials and methods

Mice and tumours

Specific pathogen-free C_3H/HeN mice weighing 20–25 g were used for all experiments. They were bred and maintained under constant temperature and humidity, with sterile bedding, food and water being provided according to institutional guidelines. All animal procedures were approved by the Animals Ethics Committee of The University of Auckland. MDAH-MCa-4 mouse mammary tumours (6th transplant generation) were grown in female mice in the gastrocnemius muscle to a leg plus tumour diameter of 10 mm (ca. 0.5 g). Tumour growth delay was assessed from the time required for growth to a diameter of 13 mm (ca. 1.4 g). KHT tumours were grown s.c. in the inguinal region to 0.5–1.0 g.

Drugs

DMXAA was provided by Prof. W.A. Denny, Cancer Society Research Laboratory, The University of Auckland. MEL was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Drug solutions were injected i.p. (0.01 ml/g body weight). DMXAA was formulated in phosphate-buffered saline. MEL (15.5 m*M*) was dissolved in 60% propylene glycol with 40% sodium citrate and diluted in water to 3.4 m*M*. Controls received vehicle only. For the in vitro experiments, MEL was dissolved in acidified ethanol (0.5 *N* HCl) and diluted with 9 vols. propylene glycol to a concentration of 1.6 m*M* and was subsequently diluted at least 100-fold into the cell cultures. The pH of the cell suspensions was unchanged after the addition of MEL.

Toxicity

Male non-tumour-bearing mice were used for the assessment of drug toxicity by determination of the weight loss measured on the 5th day after drug administration and of the number of mice surviving at 28 days after drug treatment (six mice per group). The drug dose was increased by 1.33-fold increments and the maximum tolerated dose (MTD) was determined as the highest dose that did not cause deaths.

Measurement of MEL in plasma and tumour

The MEL extraction and assay methods were based on a published method [14]. Plasma was prepared after collection of blood from the retroorbital sinus, and MeCN containing 1% (v/v) concentrated HCl (4 vols.) was added to precipitate proteins. After storage at –80 °C, samples were thawed and centrifuged. The supernatants were evaporated to dryness, resuspended in mobile phase and centrifuged briefly, and 100-µl samples were analysed by high-performance liquid chromatography (HPLC) using a refrigerated autosampler and a Waters µBondapak C_{18} column. The mobile phase consisted of a linear gradient of MeCN in ammonium formate (0.45 *M*, pH 4.5). Detection was carried out by diode-array absorbance at a wavelength of 264 nm. Whole tumours (ca. 0.5 g) were removed immediately after the bleeding of mice and were homogenised in 2 vols. of ice-cold water using a Polytron homogeniser. The homogenates were deproteinised with 4 vols. of acidified MeCN and prepared for HPLC as described for plasma samples except that 165-µl samples were analysed. The precision of detection of MEL was determined by analysis of 5-nmol samples taken from a freshly prepared standard solution, resulting in an inter-experiment coefficient of variation (CV) of 4.1% and an intra-experiment CV of 3.5%. The extraction efficiency determined for MEL by spiking of control plasma and tumour samples with MEL at a concentration of 5 µ*M* was 66 ± 3% and 90 ± 7% (mean ± SE, $n = 4$), respectively. The sensitivity limits for quantitation of MEL were about 0.15 µmol/l and 0.1 µmol/kg in plasma and tumour, respectively.

Pharmacokinetic model

Plasma and tumour pharmacokinetics of MEL were fitted using the extended least-squares modeling system MKMODEL [12]. Plasma pharmacokinetics were fitted by a first-order-absorption, one-compartment, first-order elimination model. Tumour pharmacokinetics assumed first-order transfer from plasma to tumour with a one-compartment distribution model. Clearance from tumour was assumed to be first order in the absence of DMXAA. After pretreatment with DMXAA, tumour clearance was assumed to decrease as an exponential function of time starting at some time, T*lag*, after DMXAA administration. The half-time of the decrease, T*eqx*, and the asymptotic extent of inhibition, I*max*, were estimated as well as T*lag*. The models were expressed as a system of differential equations and were integrated using a relative error tolerance of 1×10^{-6}.

Measurement of tumour concentration does not directly allow the parameters of clearance and volume of distribution for tumour tissue to be identified. We assume that the tumour is 2% of body weight and that the partition coefficient for MEL between plasma and tumour is unity. With these assumptions the pharmacokinetic parameters of MEL in the presence and absence of DMXAA may be estimated to describe the effect of DMXAA. Residual error was fitted assuming a constant proportional residual error. The optimal model was selected by visual assessment of the predicted curves as supported by the Schwarz criterion [18]. The area under the tumour concentration-time curve (AUC) was computed using the trapezoidal rule and was extrapolated to infinity by addition of the value C_t/Z, where C_t is the concentration measured at the last time point and Z is the terminal slope determined by linear regression.

In vitro cytotoxicity

Suspensions of KHT tumour cells (10^6 cells/ml) were obtained by enzymatic dissociation (40 min at 37 °C) of tumours in a cocktail of pronase (0.5 mg/ml), collagenase (0.2 mg/ml) and DNAase (0.2 mg/ml) in αMEM containing 5% fetal calf serum, penicillin and streptomycin. Cells were exposed to MEL in αMEM containing 5% fetal calf serum with a gas phase of 95% N_2/5% CO_2 (hypoxia) or 95% air/5% CO_2 (air) at pH 6.5 or 7.4 as described elsewhere [21]. The pH was checked with an electrode at the end of the 1-h exposure period. Cells were then washed and plated in αMEM containing 10% fetal calf serum, 0.25% agar, penicillin and streptomycin in 24-well plates to determine clonogenicity as previously described [10]. Cytotoxic potency was expressed as the concentration of MEL that reduced cell survival to 10% of the control value (C_{10}).

Uptake of MEL

Suspensions of KHT tumour cells (5×10^7 cells/10 ml) were prepared and incubated (1 h) with MEL (16 μ*M*) as described above. Cell viability was then determined with trypan blue. Cells were separated from the medium by a double-spin technique: cells were centrifuged (ca. 200 g for 10 min) and the medium was aspirated, which was followed by another brief step of centrifugation (2 min) and aspiration to remove any residual medium adhering to the tube wall. Cells (ca. 50 μl) were then lysed in 200 μl water and precipitated with 1000 μl MeCN containing 1% (v/v) HCl. Intra- and extracellular concentrations of MEL were measured by HPLC as described above.

Results

In Fig. 1 the time course of the interaction between MEL (at 80% of its MTD) and DMXAA (at 80% of its MTD) against MDAH-MCa-4 tumours is shown. The effect was greater than that expected from addition of the growth delays induced by the individual drugs and appeared to be maximal when MEL was given after DMXAA. The MTD of MEL was 42 μmol/kg i.p. and was not changed by combination with DMXAA (given at 80 μmol/kg 2 h before MEL); however, a significant weight loss of 14.3 ± 3.1% (mean ± SD) was observed at day 5 after administration of both drugs at these doses, whereas weight losses were not significant for MEL or DMXAA alone (7.0 ± 5.2% and 2.3 ± 1.9%, respectively).

The plasma and tumour concentrations of MEL measured in untreated and DMXAA-pretreated mice are shown in Fig. 2. The model for plasma pharmacokinetics indicated that MEL was rapidly absorbed from the i.p. injection site (half-time 6.2 min). Plasma concentrations reached a peak at about 15 min after administration and then declined mono-exponentially with a half-time of 21 min. Peak levels of MEL in tumour tissue were reached at slightly later times. The apparent volumes of distribution were 0.55 and 0.70 l/kg for the plasma and tumour compartments, respectively (Table 1), indicating low-level tissue binding for MEL. DMXAA had no effect on the plasma pharmacokinetics of MEL or on the exchange of MEL

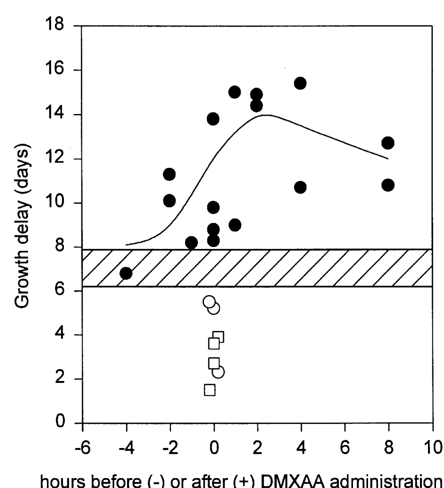

Fig. 1 Time dependence of administration of MEL (34 μmol/kg) relative to DMXAA (80 μmol/kg) on the growth of MDAH-MCa-4 tumours (○ MEL alone, □ DMXAA alone, ● MEL + DMXAA). Each symbol represents a group of 5–7 mice. SEM values were typically 10–15% of the mean and have been omitted for clarity. The expected additive response is shown as a *hashed box* (range for three experiments in which both DMXAA and MEL were tested individually)

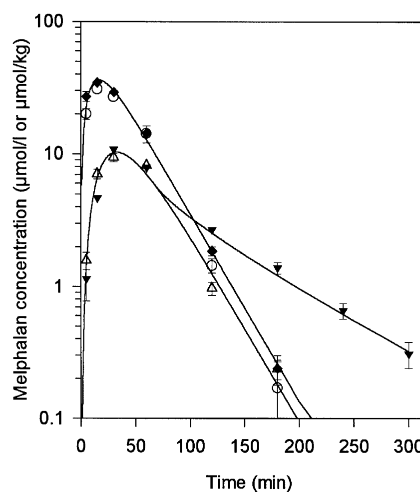

Fig. 2 Time course of MEL injected i.p. at 34 μmol/kg in plasma (○,◆) and MDAH-MCa-4 tumours (△,▼) as determined in the presence (*black symbols*) and absence (*white symbols*) of DMXAA given at 80 μmol/kg 2 h before MEL. Data represent mean values ± SE for 4–7 mice. The *lines* are fits to the pharmacokinetic model described in Materials and methods in which MEL clearance from the tumour falls exponentially to 15% of the value recorded for non-DMXAA-treated mice, with a half-time of 36 min, starting at 186 min after DMXAA treatment

from plasma into the tumour. MEL concentrations in tumours were significantly increased, but only at late time points (≥ 2 h) after DMXAA pretreatment (Fig. 2). Calculation of the AUC using the trapezoidal rule with extrapolation to infinity indicated a 33% increase in tumour AUC after DMXAA treatment.

Assumption of a fixed fractional change in tumour clearance and/or volume of distribution after pretreatment with DMXAA did not give a good prediction of tumour concentrations at times later than 2 h after dosing with MEL (data not shown). The model predicting an exponential decrease in clearance after a lag period (using the parameter values shown in Table 2) described the observations better (Fig. 2). Inhibition of tumour clearance was estimated to start at 186 min (T*lag*) after dosing with DMXAA. The half-time recorded for increase of inhibition (T*eqx*) was 36 min with an estimated maximal extent of inhibition (I*max*) of 85%. This model predicted an increase of 27% in the AUC for tumour.

In a separate experiment, MDAH-MCa-4 tumours were clamped for 15 min starting at 1 h after MEL administration (34 μmol/kg i.p.) and MEL levels in the tumours were assessed without release of the clamp prior to excision. The MEL concentration detected after clamping (4.29 ± 0.46 μmol/kg; mean ± SE, $n = 4$) was not significantly different from the preclamp value (3.48 ± 0.74 μmol/kg; $n = 4$).

The effects of acidification and hypoxia on the cytotoxicity of MEL in vitro were assessed using KHT cells freshly isolated from tumours (Fig. 3). Lowering of the pH to 6.5 enhanced the toxicity of MEL under either aerobic or hypoxic conditions; MEL was also more toxic under hypoxic than under aerobic conditions either at pH 6.5 or at pH 7.4. The C_{10} values were 3.1 (air, pH 7.4), 0.8 (hypoxia, pH 7.4), 1.5 (air, pH 6.5) and 0.2 μ*M* (hypoxia, pH 6.5).

The cellular content of MEL was measured under the same conditions used in the cytotoxicity assays. Hypoxia increased the cellular MEL content of KHT

Table 1 Pharmacokinetic parameter estimates for MEL in plasma and tumour

Parameter	Value	CV%[a]
Distribution volume of total body (l/kg body weight)	0.54	4.6
Total body clearance (l/h/kg body weight)	1.07	3.2
Distribution volume of tumour (l/kg tumour)	0.70	8.5
Tumour clearance (l/h/kg tumour)	2.90	4.1

[a] Calculated from the asymptotic standard error

Table 2 Pharmacokinetic-dynamic parameters describing the inhibitory effect of DMXAA on MEL tumour clearance

Parameter	Value	CV%[a]
T*lag*, lag time after DMXAA dosing before inhibition starts (min)	186	31.6
T*eqx*, half-time for increase of inhibition (min)	36	55.5
I*max*, maximal extent of inhibition (%)	85	8.5

[a] Calculated from the asymptotic standard error

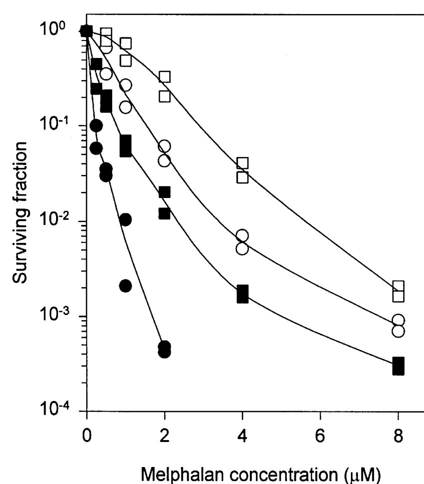

Fig. 3 Survival curves generated for KHT cells incubated with MEL under air pH 7.4 (□), air pH 6.5 (○), hypoxia pH 7.4 (■) and hypoxia pH 6.5 (●). Average control plating efficiencies observed under these conditions were 0.30, 0.29, 0.34 and 0.22, respectively. The results are pooled data from two independent experiments

cells by a factor of approximately 1.7, whereas acidification was without effect (Table 3). As measured with trypan blue, little metabolic cell killing was observed under these conditions (Table 3).

Discussion

The plasma pharmacokinetics of MEL were in close agreement with published data reporting a half-life of 26 min (cf. 21 min in the present study) after i.p. administration to C_3H mice [14] and were unaffected by pretreatment with DMXAA (Fig. 2). Consistent with this, there appeared to be no increase in host mortality due to MEL in DMXAA-pretreated animals, although a small increase in host toxicity was suggested by the weight loss observed when MEL was combined with DMXAA. The observation that the increase in the systemic toxicity of MEL seen in the presence of vasoactive agents is substantially lower than the enhancement of the tumour response has been used as indirect evidence for a tumour-specific action of these vasoactive agents [1, 3, 19, 23].

The increase in tumour AUC noted for MEL after treatment of mice with DMXAA provides direct evidence that entrapment of the alkylating agent as a result of falling tumour blood flow contributes to the enhanced antitumour effect (Fig. 1). The pharmacokinetic model assuming that tumour clearance falls exponentially starting at 3 h after DMXAA treatment fitted the data well (Fig. 2). The parameters of this model are also broadly consistent with direct measurements of blood flow in MDAH-MCa-4 tumours, which show a lag of about 2 h and 70–75% inhibition of flow by 4 h after DMXAA treatment ([6]; C.J. Lash and

Table 3 Effect of hypoxia and acidosis on MEL uptake (initial concentration 16 μ*M*) by KHT cells (5×10^6 cells/ml). Data represent mean values from triplicate measurements ± SEM. Cells incubated without MEL had a viability of 96%

Conditions	Cell viability (%)	Extracellular MEL concentration (μ*M*)	Cellular MEL content (pmol/10^6 viable cells)
Air pH 7.4	92	10.6 ± 0.2	36.0 ± 3.9
Air pH 6.5	98	12.7 ± 0.2	34.2 ± 0.2
Hypoxia pH 7.4	92	11.2 ± 0.4	60.6 ± 1.5
Hypoxia pH 6.5	90	12.5 ± 0.1	62.0 ± 1.3

W.R. Wilson, unpublished results). The apparent lag time of 3 h after DMXAA treatment before MEL clearance decreased is also consistent with the finding that plasma concentrations of TNFα, the probable mediator of the blood flow effect, are maximal at 3 h after DMXAA treatment of BDF_1 mice with colon 38 tumours [15].

The model described above assumes that tumour clearance of MEL is dependent on tumour blood flow. The finding that MEL concentrations in MDAH-MCa-4 tumours are raised after DMXAA treatment contrasts with the observation that MEL was below detectable levels in RIF-1 tumours treated with "early" photodynamic therapy [17]. The latter causes rapid and severe inhibition of tumour blood flow, which would prevent replenishment of MEL from the systemic pool, and it was therefore suggested that tumour levels might fall rapidly as a result of metabolism of MEL in the tumour [17]. However, in the present study the MEL concentration measured after a relatively short period of clamping (15 min) was not significantly different from the preclamp value. This result, again, argues that clearance of MEL from tumours is due to blood flow rather than to metabolism of the drug.

The marked increase seen in the sensitivity of KHT cells to MEL under hypoxic or acidic conditions suggests that microenvironmental changes induced by DMXAA in tumours provide a second mechanism that contributes to the observed therapeutic interaction. The lack of a clonogenic assay for MDAH-MCa-4 cells precluded in vitro sensitivity testing of these cells. However, other studies have shown enhancement of MEL cytotoxicity by low pH with MIR cells [13] and RIF-1 cells [24] and by both low pH and hypoxia with V79 spheroids [3], KHT/iv cells [19, 20] and V79-WNRE and SiHa cells [22]. We have also observed enhancement of MEL cytotoxicity by both low pH and hypoxia in AA8 cell cultures (data not shown). It therefore appears that the effects of hypoxia and acidosis are qualitatively similar for all cell lines tested and that this can be expected to contribute to the enhancement of MEL antitumour activity against MDAH-MCa-4 tumours by DMXAA. It is interesting that inhibition of tumour blood flow by hydralazine or by clamping enhanced MEL activity against KHT tumours more than did induction of hypoxia with BW12C, which decreases oxygen availability [23]. This finding is consistent with the view that entrapment and acidosis, not just hypoxia, are required for the enhancement of MEL antitumour activity by DMXAA.

The mechanism underlying the increased cytotoxicity of MEL under hypoxia and acidosis is not known. Low pH might be expected to increase the stability of MEL by reducing its rate of hydrolysis, but between pH 6.5 and pH 7.4 there does not seem to be any difference in the rate of degradation of MEL in phosphate-buffered saline [2, 3]. Studies showing enhancement of melphalan activity by nigericin at low extracellular pH in culture [24] and in vivo [27] suggest that acidification of the intracellular compartment is responsible for the pH effect. It has been reported that the formation of MEL-DNA monoadducts is enhanced to a greater extent than that of DNA interstrand cross-links at acidic pH [13], but the possibility of reduced repair of MEL-induced cross-links at low pH or under hypoxia has not been specifically addressed. MEL is actively transported into mammalian cells via two separate amino acid transport systems [26], and the concentrations measured in KHT cells (approximately 3-fold higher than the extracellular level after 1 h of aerobic exposure, assuming an intracellular water volume of 1 pl/cell) are consistent with this. MEL uptake by KHT cells was increased by a factor of about 2 by hypoxia but not by low pH (Table 2). Similarly, whereas the cytotoxicity of MEL to SiHa cells was increased by either hypoxia or low pH, only hypoxia increased the net drug uptake [22]. As in the present study, the effect of hypoxia on uptake by SiHa or V79-WRNE cells was weaker than its effect on cytotoxicity [22], suggesting that there are other oxygen-dependent processes mediating the latter.

This study shows that the new antitumour drug DMXAA, currently in clinical trial, causes an increase in the antitumour effects of MEL against MDAH-MCa-4 tumours. The mechanism of this interaction does not involve a change in the plasma pharmacokinetics of MEL but appears to be partially due to an increase in tumour exposure as a result of entrapment of the alkylating agent caused by falling tumour blood flow. Furthermore, inhibition of blood flow is also likely to induce changes in the tumour microenvironment that could increase the sensitivity of tumour cells to MEL. This study demonstrates the potential of DMXAA to induce microenvironmental changes in tumours that can be exploited by bioreductive drugs and other agents with selectivity for hypoxic and/or acidic conditions.

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