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ORIGINAL ARTICLE

Consequences of increased vascular permeability induced by treatment of mice with 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and thalidomide

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Abstract
Purpose 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) (AS1404), a small-molecule vascular disrupting agent currently in clinical trial, increases vascular permeability and decreases blood flow in both murine and human tumours. DMXAA induces tumour necrosis factor (TNF) in mice and the effects on vascular permeability are hypothesised to result from both direct (DMXAA) and indirect (TNF) effects. Skin temperature decreases in mice treated with high doses of DMXAA, raising the question of whether host toxicity is mediated by the induction of increased vascular permeability in normal tissue. Thalidomide is an anti-inflammatory agent that potentiates the anti-tumour activity of DMXAA but decreases induction of TNF in plasma. We wished to determine how it potentiated the effects of DMXAA.
Methods Vascular permeability was measured in Colon 38 tumour and liver tissue by uptake of Evans Blue dye. Blood haematocrit and body temperature were also measured.
Results Tumour vascular permeability was increased following administration of DMXAA (25 mg/kg i.p.), minimally affected following thalidomide (100 mg/kg i.p.) but strongly increased following co-administration of both drugs. In contrast, dye uptake into liver tissue was decreased following administration of DMXAA, thalidomide or both drugs. Administration of DMXAA at a potentially toxic dose (35 mg/kg i.p. or 50 mg/kg orally) was found to decrease body temperature and to increase the blood haematocrit, while administration of thalidomide alone (100 mg/kg i.p.) had no effect. Co-administration of thalidomide potentiated the effects of DMXAA on both body temperature and haematocrit but surprisingly did not increase toxicity.
Conclusions The results are consistent with the hypothesis that the host toxicity of high-dose DMXAA is mediated by effects on host vasculature. Co-administration of thalidomide increases the effective dose of DMXAA by reducing clearance but also, by inhibiting production of circulating TNF, reduces the host toxicity of DMXAA.

Keywords Vascular disrupting agents · Vascular permeability · Thalidomide · TNF

Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA; AS1404) is a low molecular weight vascular disrupting agent [16] that has completed three Phase-I trials and has undergone four Phase II clinical trials in combination studies with carboplatin and taxanes [13, 14]. In mice, DMXAA induces tumour endothelial cell apoptosis [5], increases tumour vascular permeability [19], decreases tumour blood flow [20] and increases the production of cytokines including tumour necrosis factor (TNF) [3]. Evidence has been obtained in Phase-I trials for DMXAA-induced decreases in tumour blood flow and/or increases in vascular permeability [9], suggesting a similar action in humans. DMXAA is thought to have both a direct effect on tumour vascular permeability and an indirect effect mediated by TNF, other cytokines and possibly other vasoactive mediators such as serotonin and nitric oxide. The resulting loss of plasma from blood vessels

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and consequent decreased blood vessel diameter and increased blood viscosity lead to decreased tumour blood flow and in some cases to catastrophic failure [1].

The mechanism for the dose-limiting host toxicity of DMXAA in mice has not been extensively investigated. Treated mice show a pronounced drop in skin temperature for several hours after treatment, suggesting that decreased blood flow to peripheral tissues could be involved in toxicity. Such a change could be a result of increased vascular permeability of normal blood vessels, which would be expected to lead to leakage of plasma from these vessels, with a corresponding increase in haematocrit and decrease in blood flow. On the other hand, the reduced susceptibility of TNF knockout [3] and of TNF receptor knockout mice to DMXAA toxicity [17] suggests an involvement of TNF in toxicity. Thalidomide, an anti-inflammatory agent with antiangiogenic properties, reduces plasma concentrations of TNF induced by DMXAA but paradoxically potentiates the activity of DMXAA in the murine Colon 38 carcinoma with minimal increase in host toxicity [4]. Thalidomide might therefore be useful in helping to separate host toxicity mediated directly by DMXAA from that mediated by TNF.

In this communication, we have investigated the hypothesis that thalidomide potentiates the action of DMXAA by potentiating its induction of tumour vascular permeability. We have used Evans Blue, which after administration binds tightly to albumin, to track the tissue distribution of albumin with time [12]. We have also measured two parameters that may be related to normal tissue vascular permeability, body temperature and blood haematocrit, to determine whether thalidomide potentiates the effect of DMXAA. The results help to explain how thalidomide interacts with DMXAA and also helps to explain the toxicity of DMXAA in mice.

Materials and methods

Thalidomide, a kind gift from Dr. George Muller (Celgene Corporation, Summit, NJ, USA), was dissolved in DMSO at 100 mg/kg and injected i.p. in a volume of 2.5 μl/g body weight of mouse. DMXAA, synthesised in this laboratory, was dissolved in saline at 25 mg/kg and administered i.p. in a volume of 10 μl/g body weight of animal. Evans blue dye and sodium benzalkonium chloride were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals and solvents were of analytical grade.

Mice and tumours

C57Bl/6 mice between 8 and 12 weeks of age obtained from the Animal Resources Unit, Faculty of Medical and Health Sciences, University of Auckland. All experiments were approved by the Animal Ethics Committee and conformed to the Guidelines for Animal Welfare set out by the United Kingdom Co-ordinating Committee on Cancer Research. Murine Colon 38 tumours were implanted as previously described [6]. Mouse body temperature was measured by insertion of a small temperature probe into the rectum of mice. Blood samples were obtained from the ocular sinus of mice anaesthetised with halothane and placed in heparinised tubes on ice. Haematocrits were measured in duplicate in haematocrit capillaries, with centrifugation for 5 min, and the average was recorded.

Permeability assay

Evans blue dye (1 mg/ml in saline) was administered i.v. (10 μl/g body weight). The concentration of the dye in liver and tumour tissues was determined by a published method [2]. The tissues were blotted dry then digested for 15 h at room temperature with 2 ml concentrated HCl. Sodium benzalkonium chloride (2 ml; 10% w/v in water) was added and after 1 h incubation at room temperature, chloroform (1 ml) was added and the mixture was allowed to stand for 1 h at room temperature. The chloroform layer was removed and the concentration of Evans blue dye was determined spectrophotometrically at 630 nm, using a calibration curve (0.5–40 μg/ml) and the best-fit straight line derived from linear least-square regression analysis. Plasma concentrations of Evans blue in plasma was also determined spectrophotometrically against the standard curves over the range 6.25–200 μg/ml. Statistical difference between treatment groups was tested using one-way ANOVA.

Results

Evans blue dye concentration-time profiles in plasma, liver and tumour tissues

Evans blue (10 mg/kg) was administered i.v. in non-tumour bearing mice and blood was removed after 15 min, 2, 4, 6, 8 and 12 h. The concentration of the dye in plasma showed an exponential decrease with time, consistent with elimination from the circulation (Fig. 1a). Evans blue concentrations were also determined in tumour and liver tissue of Colon 38 tumour-bearing mice. Maximal concentrations were found after 4 h in tumour (Fig. 1b) and 6 h in liver tissue (Fig. 1c). The 4 h time point was selected to investigate the effect of administration of thalidomide, and since thalidomide was dissolved in DMSO, an initial experiment was carried out to check the effect of the solvent. Administration of DMSO alone (2.5 μl/g body weight) did not affect uptake of Evans blue dye in tumour and liver tissues.

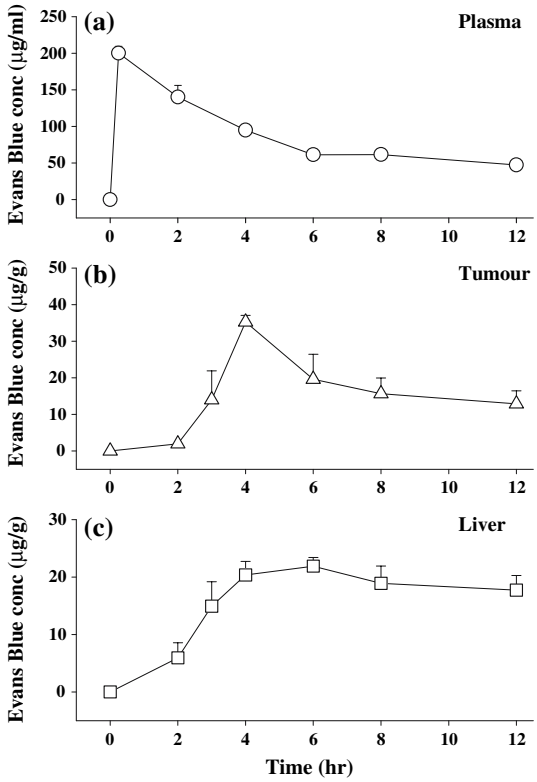

Fig. 1 Concentration of Evans blue dye in plasma (**a**), tumour (**b**) and liver (**c**) tissues following i.v. injection of the dye into the tail vein. Each point represents the mean ± SD from 3 to 5 mice

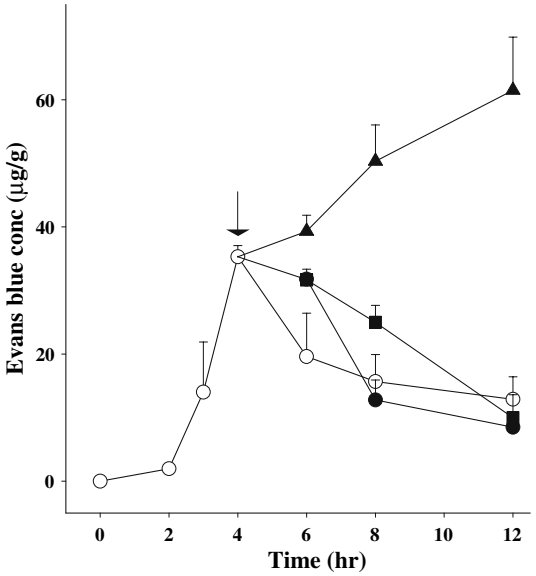

Fig. 2 Concentration of Evans blue in tumour following treatment with no drug treatment (*open circle*), following thalidomide (100 mg/kg) (*filled circle*) following DMXAA (25 mg/kg) (*filled square*) or following thalidomide and DMXAA (*filled triangle*). Drugs were administered i.p. 4 h (*arrow*) after Evans blue. Mean ± SD from 3 to 5 mice per group

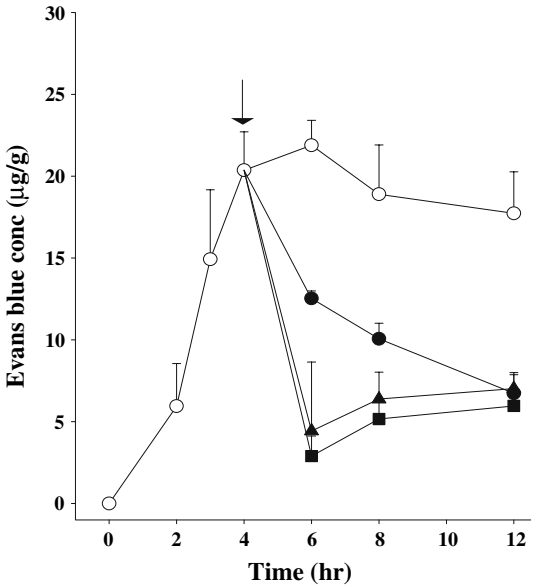

Fig. 3 Concentration of Evans blue in liver with no drug treatment (*open circle*), following thalidomide (100 mg/kg) (*filled circle*) following DMXAA (25 mg/kg) (*filled square*) or following thalidomide and DMXAA (*filled triangle*). Drugs were administered i.p. 4 h (*arrow*) after Evans blue. Mean ± SD from 3 to 5 mice per group

Effects of DMXAA and thalidomide on tumour uptake of Evans blue dye

Mice were treated with Evans blue as above after 4 h, DMXAA (25 mg/kg i.p.), thalidomide (100 mg/kg i.p.) or with a combination of both drugs were administered. Tumour and liver concentrations of dye were measured 2, 4 and 8 h later. Evans blue dye concentrations tumour tissue in DMXAA-treated mice were found to be significantly higher ($P < 0.05$) than those of control mice at the 2 and 4 h time points and thalidomide-treated mice were significantly higher only at the 2 h time point (Fig. 2). However, dye concentrations in mice co-administered DMXAA and thalidomide increased relative to those at the time of drug administration, with highly significant and the differences at all time points ($P < 0.001$).

Effects of DMXAA and thalidomide on liver uptake of Evans blue dye

In contrast to the results obtained in tumour tissue, Evans blue dye concentrations in liver tissue of DMXAA- and thalidomide-treated mice were found to be significantly lower ($P < 0.01$) than those of control mice at all three time points measured (Fig. 3). The effect of DMXAA was greater than that of thalidomide and the effect of combined DMXAA and thalidomide treatment was similar to that of DMXAA alone.

Effect of DMXAA and thalidomide on plasma concentrations of Evans blue dye

To check that the observed changes in tissue dye uptake caused by thalidomide and DMXAA did not reflect drug-induced changes in plasma concentrations of Evans blue dye, plasma concentrations of dye were determined in non-tumour-bearing mice. Evans blue dye plasma concentrations in mice co-administered thalidomide and DMXAA were not significantly different to those from mice that received no treatment (Table 1).

Temperature and haematocrit changes following DMXAA and thalidomide

Body temperatures were first measured hourly for 4 h following administration of DMXAA (25 mg/kg i.p.) or thalidomide (100 mg/kg i.p.) and found not to be significantly different to those of untreated mice (results not shown). Experiments were therefore carried out using higher doses of DMXAA (35 mg/kg i.p. and 50 mg/kg orally). Such doses are known eventually to lead to toxicity, with deaths generally occurring between 12 and 24 h after drug administration, but previous experiments showed that mice did not show signs of toxicity at earlier times and measurements were carried out at 4 h. A significant decrease in body temperature was noted at this time and the skin was cold to touch, suggesting that the peripheral circulation was being affected.

One possible explanation for the effects on body temperature is that the blood haematocrit is increased because of leakage of plasma from capillaries, raising peripheral blood viscosity and reducing blood flow. In a further experiment, both haematocrit and body temperature were measured in mice receiving no treatment, thalidomide alone, DMXAA alone (35 mg/kg i.p. and 50 mg/kg orally) or DMXAA and thalidomide. The results are shown in Fig. 4. DMXAA (35 mg/kg i.p.) and DMXAA plus thalidomide both provided significant reductions of body temperature ($P < 0.05$ and <0.0001, respectively). Similarly, DMXAA (35 mg/kg i.p.) and DMXAA plus thalidomide both provided significant increases in haematocrit ($P < 0.05$ and <0.001, respectively). For the combined data, haematocrits correlated significantly with body temperature ($r = 0.84$ and $P < 0.0001$).

Table 1 Effect of treatment with thalidomide plus DMXAA on Evans blue concentrations in plasma

Time (h)[a]	Evans blue dye concentration (μg/ml)[b]	
	No drug treatment	DMXAA and thalidomide
2	61.4 ± 2.6	57.0 ± 7.6
4	61.6 ± 4.2	58.9 ± 6.2
8	48.0 ± 8.3	46.7 ± 3.5

[a] Evans blue dye was injected i.v., and after 4 h thalidomide (100 mg/kg) and DMXAA (25 mg/kg) were injected i.p. Evans blue concentrations in plasma were measured at the indicated times after drug treatment

[b] Mean ± SD from three mice per group

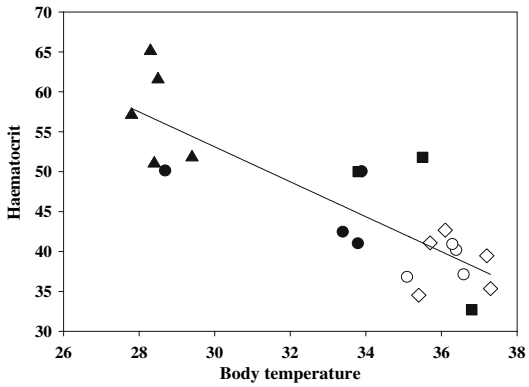

Fig. 4 Haematocrit and temperature readings in tumour-bearing mice. Each point represents readings from a single mouse taken 4 h after treatment. Values are shown for control mice (*open circle*), mice treated i.p. with DMXAA (35 mg/kg) (*filled circle*), mice treated orally with DMXAA (50 mg/kg) (*filled square*), mice treated i.p. with thalidomide (100 mg/kg) (*open diamond*) and mice co-administered DMXAA (50 mg/kg orally) and thalidomide (100 mg/kg i.p.) (*filled triangle*)

Discussion

Since Evans blue dye is tightly bound to albumin, concentrations of dye extracted from tumour tissue, liver tissue and plasma (Fig. 1) provide estimates of albumin concentration [12]. In the current experiments, injected Evans blue dye had a plasma half-life of ~3 h, presumably as a result of elimination of labelled albumin through the liver. The increased concentration of Evans blue dye in tumour tissue relative to that in plasma is a result of extravasation of albumin into the extravascular compartment of this tissue. The amount of dye in tumour tissue increased to a maximum by 4 h (Fig. 2) and decreased thereafter with a half-life of several hours, consistent with the gradual elimination of the dye from the body. Following co-administration of DMXAA, the amount of dye in tumour tissue decreased with time but at a rate slower than that for plasma. Thus, the results are consistent with increased extravasation of albumin into the tumour extravascular compartment in response to DMXAA (Fig. 2). This result suggests that DMXAA increases vascular permeability of Colon 38 tissue, confirming previous data obtained using a slightly different experimental protocol [18].

Administration of thalidomide alone at 4 h appeared to have an effect on the uptake of albumin by Colon 38 tissue only at only one time point, suggesting a minimal effect. On the other hand, co-administration of thalidomide in combination with DMXAA provided a dramatic increase in dye concentration over the next 8 h (Fig. 2), reaching a value that approached that expected for saturation of the extracellular compartment. A potential reason for this effect is a pharmacokinetic interaction between thalidomide and DMXAA. We have previously shown that co-administration of thalidomide strongly reduces clearance of DMXAA from plasma, increasing its plasma half-life from 1.7 to 3.2 h and increasing the area under the time-concentration curve by 1.9-fold, but only slightly increasing its maximal plasma concentration [10]. However, this does not explain why such potentiation is not accompanied by a corresponding increase in toxicity.

Induction of increased vascular permeability in normal tissues, by analogy with the situation in tumour tissue, would be expected to lead to leakage of plasma from blood vessels with a consequent increased blood haematocrit and viscosity, decreased blood vessel diameter, decreased pressure differences along vessels and consequent cessation of blood flow [1]. The permeability of skin to DMXAA did not increase in response to a therapeutic dose of DMXAA (25 mg/kg) [18]. The results of this study show that while this dose of DMXAA had no effect on body temperature, larger i.p. and oral doses of DMXAA (35 and 50 mg/kg, respectively) caused a decrease in body temperature and a corresponding increase blood haematocrit, consistent with a vascular effect on normal tissues. Surprisingly, while administration of thalidomide alone did not induce changes to body temperature and blood haematocrit, thalidomide clearly potentiated the DMXAA effect (Fig. 4).

The ability of thalidomide to potentiate the effects of DMXAA on body temperature and blood haematocrit appears to conflict with previous observations that thalidomide, when co-administered with therapeutic doses of DMXAA, caused minimal change in host toxicity [4]. However, this can be understood in view of evidence that the overall effect of DMXAA on tumour vasculature appears to be a combination of direct and indirect actions [1]. DMXAA can act directly to induce endothelial apoptosis and permeability changes [5, 18] and indirectly by the induction of TNF, which binds to TNF receptors on endothelial cells and increases vessel permeability [15]. It is possible that DMXAA at a potentially toxic dose exerts both direct and indirect effects in normal tissues. Co-administration of thalidomide with DMXAA might then increase the direct effect of DMXAA on normal vascular permeability but decrease the indirect effect mediated by TNF production, leading to the observed minimal change in overall toxicity.

On the basis of the results found for tumour tissue permeability, it might be expected that DMXAA might increase vascular permeability in liver tissue and that thalidomide might potentiate the effect. However, administration of DMXAA and/or thalidomide appeared to decrease uptake of Evans blue into liver (Fig. 3). The liver has a fenestrated endothelium whose permeability is physiologically controlled by the size of the fenestrations [7]. One of the main functions of the liver is the uptake and processing of lipoproteins and other plasma components that are generated from the diet. Various types of stresses can temporarily halt this function by closure of the fenestrations [7]. It is possible that DMXAA and thalidomide are able to induce such stress, leading to reduced protein uptake and consequent decreased dye accumulation.

The results described here support a mechanism of action whereby DMXAA acts on the vasculature through both direct and indirect (TNF-mediated) effects. At a therapeutic dose, these two actions are selective for tumour vasculature, while at a higher dose they affect normal vasculature. In mice treated with DMXAA (25 mg/kg), co-administration of thalidomide (100 mg/kg) leads to a 1.9-fold increase in AUC but also to a decrease in circulating (but not in tumour tissue-associated) TNF. These two opposing effects appear to result in a substantially unchanged maximum tolerated dose (MTD) for DMXAA. It is known that knockout mice lacking TNF or the TNFR1 receptors are resistant to DMXAA-induced toxicity, such that the MTD rises from 27.5 mg/kg to more than 100 mg/kg [3, 17]. Colon 38 tumours still respond to DMXAA at the higher dose, indicating that TNF is not essential for activity. Interestingly, DMXAA does not increase circulating TNF in rats or humans and the corresponding MTDs for DMXAA in rats (300 mg/kg) [11] and in cancer patients (~120 mg/kg) [8] are higher than those in mice. In rats, DMXAA clearly has anti-tumour activity and also increases TNF in tumour tissue [11]. A study of the effects of DMXAA on TNF production in human tumour tissue would be of great interest.

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