The influence of hydralazine on the vasculature, blood perfusion and chemosensitivity of MAC tumours

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Summary We have studied the influence of the peripheral vasodilator hydralazine (HDZ) on the vasculature and blood perfusion of two members of a series of subcutaneous murine adenocarcinomata of the colon (MAC tumours), and the influence of HDZ on the efficacy and/or toxicity of TCNU and melphalan. The fluorescent DNA stain Hoechst 33342, showed that HDZ caused a shutdown of tumour vasculature, related in magnitude to both dose and tumour differentiation state; 10 mg kg⁻¹ caused an 80% vascular shutdown of well differentiated MAC 26 tumours, but only a 50% shutdown of the poorly differentiated MAC 15A tumours. 2.5 mg kg⁻¹ was ineffective. The blood perfusion marker ⁹⁹mTechnetium-HMPAO showed that the normal perfusion of MAC tumours was consistently markedly less than that of lung, liver or kidneys (4–5% of lung perfusion). HDZ (10 mg kg⁻¹) decreased MAC 26 perfusion by 63%, and that of MAC 15A by 20%. Again, 2.5 mg kg⁻¹ was ineffective. Use of in vitro to in vitro clonogenic assays showed that HDZ (10 mg kg⁻¹) potentiated the efficacy of melphalan (1–10 mg kg⁻¹ i.p.) by a factor of 2.1, and increased the efficacy of TCNU (1–10 mg kg⁻¹ i.v., factor = 1.7) when given 10 or 15 min respectively after dosing. However, the addition of HDZ increased the acute bone marrow toxicity of melphalan, but not that of TCNU. The clinical relevance of these results is discussed.

An important factor influencing the delivery and therefore effectiveness of antitumour agents is the relative perfusion of tumour and other tissues with blood. It has been known for more than 40 years (Aligire & Legallais, 1951) that perfusion of experimental tumours can be manipulated by the use of vasoactive agents, and a recent paper (Hirst & Wood, 1989) cites more than 25 compounds with the demonstrated ability to alter experimental tumour blood flow, the majority of these causing a decrease.

Typical of these is the antihypertensive hydralazine (HDZ), which has been shown to decrease the blood flow through experimental tumours when administered at doses ranging from 0.5–10 mg kg⁻¹ either intraperitoneally (i.p.) (Brown 1987; Babbs et al., 1982) or intravenously (i.v.) (Chan et al., 1984; Voorhees & Babbs, 1982). The results presented for the influence of lower doses of HDZ appear contradictory: Kalmus et al. (1990) showed that 0.25 mg kg⁻¹ HDZ slightly increased the blood flow through FASII tumours when administered i.p., whereas Brown (1987), showed that, at this dose, HDZ caused a slight decrease in the blood flow through SCCVII tumours. The influence of HDZ on tumour blood flow is thought to be mainly via two mechanisms: firstly the blood vessels within most experimental tumours cannot be dilated because they are poorly formed, lacking smooth muscle and innervation (Denekamp, 1986; Chaplin, 1987), and maximally dilated already (Chaplin, 1987), and secondly, if the level of the arterial blood pressure drops below that of the intratumoural interstitial pressure (reported to be abnormally high (Wiig et al., 1982)), the tumour vasculature will collapse. Also contributing are secondary effects such as the induction of red blood cell rigidity at low pH and homeostatic mechanisms diverting blood flow to critical organs and away from the extremities (Kalmus et al., 1990).

Overall, the effect of HDZ will be to cause both an absolute and relative decrease in tumour perfusion (Chan et al., 1984; Jirtle, 1988).

HDZ has also been shown to enhance the effectiveness of 'bioreductive' agents such as the nitroimidazole RSU 1069 (Chaplin & Acker, 1987) and the benzotriazine di-N-oxide SR 4233 (Brown, 1987), and to increase the efficacy and therapeutic index of the bifunctional nitrogen mustard, L-phenylalanine mustard (melphalan) (Stratford et al., 1988). In the case of melphalan, it was suggested that HDZ decreased the rate of tumour clearance, improving melphalan efficacy by increasing the exposure of tumour cells (Stratford et al., 1987, 1988).

The present study aimed to use both the vital bis-benzo-imide fluorescent dye Hoechst 33342 (H33342) and the blood flow marker technetium-99m labelled hexamethylpropyleneamine oxime (⁹⁹mTechnetium-HMPAO) to characterise the vasculature and perfusion of two members of a panel of transplantable adenocarcinomata of the mouse colon (MAC tumours). This panel, which was derived from a series of primary tumours induced in NMRI mice by prolonged administration of 1,2-dimethylenehydrazine (Double et al., 1975) has been shown to be a good model for clinical large bowel cancer in terms of range of histology and chemosensitivity (Double & Ball, 1975). These two agents were used to investigate the influence of HDZ on the vasculature and perfusion of these MAC tumours. We investigated by the use of both in vivo to in vitro clonogenic assays and in vivo tumour growth inhibition assays the influence of HDZ on the efficacy of both melphalan and taumustine (TCNU) against tumours. TCNU (1-(2-chloroethyl) -3-[2-(dimethyl-amino-sulphonyl) ethyl]-1-nitrosourea) is a novel nitrosourea shown to have good activity both clinically (Smyth et al., 1987; Gundersen et al., 1989) and against the MAC tumours (Bibby et al., 1988), (also reviewed in Workman, 1987), whose dose limiting toxicity in patients is myelosuppression (Smyth et al., 1987; Gundersen et al., 1989). A positive correlation has been demonstrated between the in vivo and in vitro sensitivity of the MAC tumours to TCNU (Phillips et al., 1988). Finally, we investigated the influence of HDZ on the acute bone marrow toxicity of TCNU and melphalan in mice by the use of the spleen colony unit assay of Till & McCulloch (1961).

Materials and methods

Animals

Pure strain NMRI male mice, aged 6–8 weeks, from our inbred colony were used. The mice received CRM Diet (Labsure, Croydon, England) and water ad libitum. All animal procedures were carried out under a project license issued by the Home Office, London, and UKCCCR guidelines (Workman et al., 1988) for the use of animals in experiments were adhered to throughout.
Tumour system

Two different tumours have been used in the present study: MAC 15A – a rapidly growing, poorly differentiated tumour induced by the subcutaneous (s.c.) injection of a suspension of 1 x 10^6 ascites tumour cells, and MAC 26 – a slow growing, well differentiated cystic tumour induced by s.c. implantation of solid tumour fragments. Mice bearing MAC 15A tumours were treated after 7 days, and MAC 26 tumour bearing animals were treated after 16 days. At the time of treatment, the mean tumour volume of the MAC 15A tumours were 624 mm^3 (range = 162 - 1300), and that of the MAC 26 tumours was 109 mm^3 (range = 30 - 360). These sizes were chosen in view of data obtained in preliminary investigation, which showed that, whereas MAC 26 tumours had an established vasculature from the time when they were just palpable, MAC 15A tumours needed to be well established before they had a consistent, measurable vasculature. Mice were allocated to groups so that variation in group mean tumour sizes was within 10%.

Test compounds

H33342, TCNU and HDZ were dissolved in sterile physiological saline. Melphanal was dissolved in 2% acetic acid and then sonicated in 10% propylene glycol buffered physiological saline and HDZ (administered intravenously (i.v.) via the tail vein) and melphanal (intraperitoneally (i.p.)) were administered in 1 ml per 100 g bodyweight, and H33342 was administered i.v. in 0.5 ml per 100 g bodyweight. The HDZ and H33342 were obtained from Sigma, TCNU and melphanal were gifts from Pharmacia Leo and Burroughs Wellcome & Co. respectively. All drugs were prepared immediately before use.

Tumour vasculature and blood flow

H33342 The use of fluorescent dye, H33342, to visualise and quantify tumour functional vasculature when frozen sections are viewed under ultraviolet light has been described (Smith et al., 1988). H33342 was administered at 40 mg kg^-1, and tumours removed 1 min after dosing. This dose was chosen in view of preliminary investigations, where 40 mg kg^-1 was shown to give the clearest, most consistent visualisation of the tumour blood vessels. It has been established that H33342 is vasoactive at doses above 10 mg kg^-1 (Trotter et al., 1990). However, this would not alter the validity of these experiments, as only the absolute values of tumour vasculature obtained might be altered, not their relative values, and all results for treated tumours were compared to those obtained for controls. Tumours were snap frozen in liquid nitrogen, and stored at -20°C until sectioning at 8-10µm using a Bright cryostat. Sections were air dried and then examined under ultraviolet illumination using a Vickers microscope fitted with an epiphysiological source (magnification × 250). Adjacent sections to those used for the vascular quantification were retained, air dried for at least 24 h and then stained using Haematoxylin and Eosin (H & E). Vascular quantification was by use of a point scoring system similar to that described by Chalkley (1943). Briefly, a graticule with a grid of 400 points was focused on five different areas of each of 10-20 sections per tumour, and a count made of the number of points falling within the fluorescent values of H33342 labelled cells. The percentage functional vasculature was calculated for each tumour from the equation:

\[
\% \text{ vasculature} = \left(\frac{\text{No. of positive points}}{\text{total points}}\right) \times 100
\]

This technique was used to investigate the influence of HDZ (2.5 - 10 mg kg^-1) on the functional vasculature of both tumours and that of TCNU (30 mg kg^-1) on the vasculature of MAC 26 tumours. In the case of the HDZ, H33342 was administered 5 min after dosing, and in the case of the TCNU, 10 min after.

\(^{99m}\text{Tc-HMPAO}\) The use of \(^{99m}\text{Tc-HMPAO}\) to measure clinical (Holmes et al., 1985; Ell et al., 1985; Rowell et al., 1990), and experimental (Hammersley et al., 1987) blood flow has been described. This technique relies on the principle, first described by Sapirstein (1956) that, for a given time after its intravenous administration, the fractional distribution of an indicator among the organs will correspond to the fractional distribution of the cardiac output amongst them. Work-up experiments using non-tumour bearing NMRI mice showed that relative tissue levels of \(^{99m}\text{Tc-HMPAO}\) remained constant between 10 min and 1 h after administration, and that the radiochemical purity of the \(^{99m}\text{Tc-HMPAO}\) was not significantly reduced for at least an hour after the addition of the \(^{99m}\text{Tc}\).

Non-anaesthetised mice were killed by cervical dislocation 10 min after i.v. administration of 37 kBq of \(^{99m}\text{Tc-HMPAO}\) (0.1 ml). Lung, liver, kidney, tumour and tail tissues were removed. The lungs were washed by brief immersion in isoton to remove any surface blood contamination and then blotted. All tissues were weighed, and the radioactivity present in each tissue/organ measured by use of a Wallac 1282 Compugamma Gamma Counter. The radioactivity/g tissue was then calculated for each sample and results normalised for radioactivity remaining in the tails, and for radioactive decay during both the experiment and sample assay. From this distribution figure, the relative distribution of blood was deduced.

The time course of the effects of HDZ on relative tissue/organ perfusion was studied by injecting either non-tumour bearing mice, or those bearing MAC 26 or MAC 15A tumours with \(^{99m}\text{Tc-HMPAO}\) 5 min, 1, 2 or 4 h after administration of 10 mg kg^-1 HDZ, and then proceeding as above. For inter-experiment comparisons, results were expressed as percentages of control values, which were obtained from animals which had received only \(^{99m}\text{Tc-HMPAO}\). Examination of the data obtained showed that the absolute values of counts/g tissue varied between experiments. This was due to expected alterations in the radioactivity of different batches of \(^{99m}\text{Tc-HMPAO}\). However, the lungs consistently had the highest counts/g tissue. Therefore, for intra-experimental comparisons, the lungs were arbitrarily selected as a reference tissue against which to compare the other tissues. All results were compared to a control experiment, where non-tumour bearing animals were injected with saline (1 ml per 100 g bodyweight), and then \(^{99m}\text{Tc-HMPAO}\) according to the time schedule described above.

The relationship between HDZ dose and perfusion was investigated for both tumours. \(^{99m}\text{Tc-HMPAO}\) was administered 5 min after HDZ (2.5 or 10 mg kg^-1), and the assessment of perfusion made as above.

Measurement of tumour chemosensitivity

Clonogenic assays

This method was used to investigate the influence of HDZ on the efficacy of TCNU (1-20 mg kg^-1) and melphanal (1-10 mg kg^-1) against MAC 15A tumours. HDZ was administered 10 min after the TCNU, which is the time of peak tumour concentration in NMRI mice (Double et al., 1988), and 15 min after the melphanal, which is the time of peak plasma concentration in C3H mice (Lee & Workman 1986). Tumours were removed 24 h after dosing, and each tumour assayed individually. Each tumour was made into a single cell suspension by mincing with a scalpel and gently pressing through a 625 holes cm^-2 sterilised wire mesh. These cell suspensions were washed in two changes of Hanks Balanced Salt Solution (HBSS), centrifuged and the resulting cell pellet resuspended in RPMI 1640 supplemented with foetal calf serum (10%), sodium pyruvate (1 mM), penicillin-streptomycin (50 IU ml^-1) and buffered by HEPES (25 mM); complete media. From each tumour cell suspension, 10⁴ viable cells (as determined by trypsin blue exclusion) were plated out in duplicate in complete media. This cell inoculum was
chosen from preliminary experiments as giving an appropriate, reproducible number of colonies. Colonies comprising 50 cells or more were counted 4–5 days later with the aid of a 10×10 mm eyepiece grid lattice on an inverted microscope with a ×4 objective lens. Ten grid area counts were made in each culture well, and from this, the mean counts/well calculated by multiplying the mean counts/grid by the (area of the well/the area of the grid). Cytotoxict effects for each treatment were assessed by comparison of the plating efficiencies obtained for dosed tumours with those for control tumours at the same time. In this study, the average yield of viable cells from untreated MAC 15A tumours was 1.6 × 10^5 cells per g tumour tissue. This cell yield was not affected by any of the treatments used. The plating efficiency of control tumours was 10.8–25%.

In vivo assay

Due to differences in the morphology and growth characteristics of MAC 26 and MAC 15A tumours, different protocols were necessary to assess their sensitivity to TCNU and melphalan: the method for the assessment of the chemosensitivity of the slow growing MAC 26 tumours has been described (Bibby et al., 1988). Tumour volumes were assessed over a period of 2–3 weeks by twice-weekly caliper measurements of tumour diameter. Volumes were calculated from the equation: Volume = (a²b)/2, where a is the smaller diameter and b the larger diameter of the tumour (Geran et al., 1972). Volume measurements for each tumour were normalised to their initial values, and a calculation made of the time taken for the tumours to reach a relative tumour volume of 2 (RTV2). The chemosensitivity of the rapidly growing MAC 15A tumours was assessed in terms of both tumour weight inhibition at 7 days after treatment (Bibby et al., 1989b) and time to reach RTV2.

Bone marrow toxicity

In vivo colony forming units-spleen (CFU-S) assay

An adaptation of the method of Tall & McCulloch (1961) was used to assess in vivo the influence of HDZ on the acute bone marrow toxicity of TCNU (2.5–30 mg kg\(^{-1}\)) or melphalan (2.5 or 5 mg kg\(^{-1}\)) in mice bearing MAC 15A tumours (two per group). HDZ (10 mg kg\(^{-1}\)) was administered 10 min after the TCNU or 15 min after the melphalan, and bone marrow toxicity assessed 24 h after the last dose. Each experiment involved investigating one drug concentration with or without HDZ.

Removal of bone marrow

Bone marrow was aspirated from both femora of each pair of mice by flushing with 1–2 ml HBSS. These individual suspensions were pooled to give one per group. An additional control suspension was obtained from two untreated mice for each drug level tested. These marrow suspensions were made up to 10 ml and stored on ice until injection, which was always within 1 h of removal from the animal. The number of cells in each suspension was counted using a Neubauer haemocytometer and dilutions made to give approximately 10^5 cells ml\(^{-1}\). These diluted cell suspensions were then injected (0.2 ml mouse\(^{-1}\) i.v.) into groups of 5–6 mice which had previously been anaesthetised (Saffan 36 mg kg\(^{-1}\) or ether) and exposed to supralethal X-irradiation (11.7 Gy mouse\(^{-1}\)). Seven days later these mice were killed, their spleens removed, fixed in Bouin’s fluid and the number of surface colonies counted. The surviving fraction was calculated as the number of colonies observed in the treated groups compared with those in the control.

Statistical analysis

Differences between control and treated groups were quantified by use of a two tailed un-paired students t-test.

For the clonogenic assay results, the per cent survival/drug dose curves were fitted by least squares linear regression on the log transformed data. Ninety five per cent confidence limits were calculated for the slopes of all curves (Clarke, 1980).

Results

Tumour vascularisation and blood flow

Control tumours

Comparison of Figures 1a and 1b shows that, in this well differentiated MAC 26 tumour, the functional blood vessels are associated with the tumour stroma, with this stroma supporting a thin layer of viable tumour cells. The results presented here show that MAC 26 tumours also have a high per cent functional vasculature, which is consistent with the fact that these tumours can grow very large (greater than 10 × initial volume) without becoming necrotic. MAC 15A tumours, by contrast, are poorly differentiated (Figure 1c) and poorly vascularised (Figure 1d).

Figure 1c shows the formation of ‘cords’ of viable tumour cells, with necrosis at distances greater than ~150 μm from the central, functional blood vessel.

The results obtained by the use of \(^{99m}\)Tc-HMPOA showed that the perfusion of the normal tissues studied was consistently in the rank order: lungs > kidneys > liver, with the tumour tissues being considerably less well perfused than the liver tissue, (Figure 2). There was no difference between the non-tumour animals, and those bearing MAC 26 or 15A tumours in terms of the perfusion of the normal tissues studied.

Influence of HDZ on tumour vascularisation and blood flow

In this study, all doses of HDZ were well tolerated, with no morbidity or mortality observed.

HDZ caused both a decrease in tumour perfusion and a shutdown of tumour vasculature. Previous work in this laboratory (N. Patel, unpublished data) using H33342 has shown that this vascular shutdown was maximal by 5 min. We have shown here (Figure 3) that the magnitude of the vascular shutdown was related to both dose and tumour differentiation state: 10 mg kg\(^{-1}\) caused a 80% shutdown of MAC 26 vasculature, from 5.4% to 1.1% (P < 0.001), but a 50% shutdown of MAC 15A vasculature, from 1.9% to 0.95% (P < 0.1). No shutdown was seen with 2.5 mg kg\(^{-1}\) HDZ, or with 30 mg kg\(^{-1}\) TCNU. The influence of 5 mg kg\(^{-1}\) HDZ on the vasculature of MAC 26 tumour was intermediate between that of 2.5 mg kg\(^{-1}\) and that of 10 mg kg\(^{-1}\) (Figure 3).

The use of \(^{99m}\)Tc-HMPOA showed that the HDZ-induced decrease in tumour perfusion was also related to both dose and differentiation state: 10 mg kg\(^{-1}\) caused a 63% decrease for MAC 26 (P < 0.1), but only a 20% decrease for MAC 15A (NS). Perfusion of both tumours was returning to approximately control levels by 4 h after dosing (Figure 4).

Again, 2.5 mg kg\(^{-1}\) was ineffective. Saline did not affect the perfusion of any of the tissues studied (data not shown).

As a dose of 10 mg kg\(^{-1}\) was found to have the greatest influence on vasculature/perfusion, this was the dose used to investigate the influence of HDZ on the efficacy of melphalan or TCNU.

Chemosensitivity

HDZ (10 mg kg\(^{-1}\)) enhanced the activity of melphalan (1–10 mg kg\(^{-1}\)) against MAC 15A tumours (enhancement ratio = 2.1), but the effect on the activity of TCNU (1–20 mg kg\(^{-1}\)) was less marked (enhancement ratio = 1.7). These enhancement ratios were calculated as the ratio of the slopes of the regression lines shown in Figures 5a and 5b. The gradients of these lines (to 95% confidence limits) were: melphalan alone = \(-0.315 \pm 0.062\), melphalan with HDZ = \(-0.674 \pm 0.057\); TCNU alone = \(-0.111 \pm 0.021\), TCNU with HDZ = \(-0.189\).
± 0.043. It was found that 20 mg kg⁻¹ TCNU gave a per cent survival of 0 when administered without HDZ. Therefore the data presented in Figure 5b is for 1–10 mg kg⁻¹.

In vivo assay

The addition of HDZ (10 mg kg⁻¹) increased the time taken for MAC 26 tumours administered with melphalan (10 mg kg⁻¹) to reach a relative tumour volume of 2 (RTV2) from 8.7 ± 2.1 to 15.5 ± 1.1 days, an increase by a factor of 1.8. HDZ (10 mg kg⁻¹) also caused a dose related enhancement of the efficacy of melphalan against MAC 15A tumours, whether the results are expressed in terms of tumour weight inhibition, or of the time taken for tumours to reach RTV2 (Table I). Melphalan alone caused a 43 ± 13% reduction in tumour weight, and delayed the time to RTV2 from 2.8 ± 0.5 to 4.1 ± 0.6 days. The addition of HDZ (10 mg kg⁻¹) increased the tumour weight inhibition, by a factor of 1.6, to 70 ± 16% (P<0.01), and delayed the time to RTV2, by a factor of 2.5, from 4.1 ± 0.6 to 10.3 ± 5.3 (P<0.01). HDZ (2.5 mg kg⁻¹) was ineffective.

HDZ (10 mg kg⁻¹) did not significantly alter the efficacy of TCNU (30 mg kg⁻¹) against either MAC 26 tumours (data not shown), or MAC 15A tumours (Table I). TCNU alone increased the time for MAC 15A tumours to reach RTV2 from 2.5 ± 1.0 days to 6.3 ± 2.1 days. After the addition of HDZ, the time to RTV2 was 6.1 ± 1.9 days.

Bone marrow toxicity assay

HDZ (10 mg kg⁻¹) did not alter the acute toxicity of TCNU (2.5–15 mg kg⁻¹) against murine bone marrow stem cells (data not shown), but did increase that of melphalan (5 mg kg⁻¹) on both occasions that this experiment was carried out (Table II) (P<0.001).

Discussion

We have used a combination of two complimentary techniques; H33342 and ⁹⁹mTc-HMPAO, to investigate the vasculature and perfusion of two members of the panel of MAC tumours. The use of H33342 provides information which cannot be deduced by the use of radioactive isotopes such as ⁹⁹mTc-HMPAO. It allows direct visualisation of the tumour vasculature which is functional at a given moment, and showed both that the well differentiated MAC 26 tumours were well vascularised, with blood vessels running through...
the tumour stroma, and the poorly differentiated MAC 15A tumours were poorly vascularised with a 'corded' structure similar to that shown in many papers, from Thomlinson & Gray (1955) to Hirst et al. (1991). We have shown previously that the vascularisation of five members of the panel of MAC tumours was directly related to their differentiation state (Quinn et al., 1991), with well differentiated tumours being significantly better vascularised than those which were poorly differentiated. The two tumours described here represent the two ends of this spectrum.

The results obtained by the use of $^{99m}$Tc-HMPAO showed that both MAC 26 and MAC 15A tumours were poorly perfused with blood, relative to several normal tissues. These are in agreement with the results of Hammersley et al. (1987) who used $^{99m}$Tc-HMPAO to investigate relative tissue perfusion in Balb c mice bearing either PM 2 sarcoma or PC 6 plasmacytoma. Both the H33342 and the $^{99m}$Tc-HMPAO techniques showed that the vasoactive agent HDZ caused a decrease in functional tumour blood perfusion, the magnitude of which was related to both HDZ dose and tumour differentiation state. The time of onset of the shutdown effect, as shown in our previous experiments using H33342, was similar to that shown by Okunieff et al. (1989). The degree of vascular shutdown of both of the tumours in this study was greater than that shown by Trotter et al. (1989), who demonstrated that 10 mg kg$^{-1}$ HDZ caused the abolition of perfusion in 36% of the vessels within SCCVII tumours. This may reflect the differentiation state of this tumour. There was good agreement between the H33342 and the $^{99m}$Tc-HMPAO methods as to the magnitude of the influence of 10 mg kg$^{-1}$ HDZ.

In vivo tumour weight inhibition measurement and in vivo to in vitro clonogenic assays, have demonstrated a comparable degree of enhancement of HDZ of the efficacy of melphalan against MAC 15A tumours. This enhancement is similar in magnitude to that demonstrated elsewhere (Stratford et al., 1988; Chaplin et al., 1989). However, Stratford et al. (1988) deduced that the addition of HDZ did not increase
the bone marrow toxicity of melphalan (measured in terms of melphalan LD50 at 90 days). We have demonstrated in this study that HDZ caused a significant increase in the acute bone marrow toxicity of melphalan at a dose where we have shown to be increased by HDZ. Therefore, the increase in activity does not correlate with therapeutic advantage. The enhancement by HDZ of the efficacy of TCNU against MAC 15A tumours, as shown by in vivo to in vitro clonogenic assays, was less marked than the increase shown of melphalan effectiveness, but the acute bone marrow toxicity of TCNU was not increased.

Both Stratford et al. (1988) and Chaplin et al. (1989) demonstrated by the use of clonogenic assays that HDZ could markedly enhance the efficacy of melphalan when the HDZ was administered as early as 2 h before dosing. This result, taken in conjunction with the results presented here, suggests that the potentiation caused by HDZ is due, at least in part, to its influence on drug pharmacokinetics. This seems logical in view of the fact that other chemopotentiators (e.g., misonidazole), known to cause marked selective tumour vascular shutdown (Murray et al. 1987) have also been shown to alter drug pharmacokinetics (Randhawa et al., 1985), and it is to this latter ability that the chemopotentiation has been attributed (Randhawa et al., 1985). The potentiation of CCNU by misonidazole has also been explained on the basis of altered pharmacokinetics (Siemann, 1990). However, additional factors may be the existing tumour hypoxic fraction and the fact that tumour vascular shutdown would alter both intratumoural pH and hypoxia. The importance of these factors would vary with the activity/potentiation of different drugs, depending on their physicochemical properties.

Hydralazine (5 or 10 mg kg\(^{-1}\)) has been shown to cause the mean arterial blood pressure (MABP) of mice to decrease by 41–46% (Bibby et al., 1989a). This is similar to the values presented elsewhere (Horsman 1989; Okunieff et al., 1989). However, the lack of lethality observed in this study and others (Stratford et al., 1988; Okunieff et al., 1989) confirms that the decreased arterial blood pressure is adequately compensated for by the selectively increased rate of perfusion of critical normal tissues. Even so, a >40% decrease in MABP is obviously clinically unacceptable. Decreased MABP is likely to be the explanation for the observation that in NMRI mice, HDZ (10 mg kg\(^{-1}\)) causes decreases in glomerular filtration rate (GFR), as measured by insulin clearance, resulting in decreased TCNU clearance from plasma and tissues (Bibby et al., 1992). Honess & Bleehen (1991) demonstrated similar alterations in GFR by 5 mg kg\(^{-1}\) HDZ in C3H mice. This systemic influence of HDZ not only results in increased drug AUC, but will also have a significant effect on drug metabolism. The degree of potentiation is likely to vary depending on the role of metabolism in the activity of a particular agent, and with the site where metabolism occurs. Nevertheless, on the basis of these observations, in this study the greatest potentiation of antitumour activity should have been seen in those tumours where there was the greatest vascular shutdown. The data for TCNU are contrary to this. It is well known that the sensitivity of cells

![Graph](image-url)  
**Figure 5** a. Influence of HDZ (10 mg kg\(^{-1}\)) on the potency of melphalan (1–10 mg kg\(^{-1}\)) against MAC 15A sc tumours. Each point = mean ± 1 s.d. of 5–6 independent observations. Melphalan, HDZ. b. Influence of HDZ (10 mg kg\(^{-1}\)) on the potency of TCNU (1–10 mg kg\(^{-1}\)) against MAC 15A sc tumours. Each point = mean ± 1 s.d. of 5–12 independent observations. TCNU, HDZ.

| Treatment | % Tumour Weight Inhibition (± 1 s.d.) | Time to Reach RTV2 (days) (± 1 s.d.) |
|-----------|--------------------------------------|-------------------------------------|
| Control   | 2.8 ± 0.5                            | 2.8 ± 0.5                           |
| Melphalan alone | 43 ± 13                            | 4.1 ± 0.6                           |
| Melphalan plus 10 mg kg\(^{-1}\) HDZ | 70 ± 16\(a\)                      | 10.3 ± 5.3\(\ast\)                 |
| Melphalan plus 2.5 mg kg\(^{-1}\) HDZ | 38 ± 4                              | 3.9 ± 0.3                           |
| Control   | -                                    | 2.5 ± 1.0                           |
| TCNU alone | 63 ± 19                            | 6.3 ± 2.1                           |
| TCNU plus 10 mg kg\(^{-1}\) HDZ | 67 ± 2.4                           | 6.1 ± 1.9                           |

Note: \(\ast P<0.01\)
to nitrosoureas is dependent upon their intracellular concentration of the DNA repair enzyme O⁶-alkylguanine DNA alkyltransferase (AT) (reviewed in D'Incalci et al., 1988). Previous studies in this laboratory have demonstrated MAC 26 tumours to have high levels of AT (Lunn et al., 1989). So, even though MAC 26 tumours are less resistant to TCNU than to other nitrosoureas, a fact which is thought to be due to the increased water solubility of TCNU, it will not be possible to increase the activity of TCNU by the use of HDZ, unless the increase is sufficient to saturate the ability of AT to prevent the formation of DNA-interstrand cross-links. We have some evidence in support of this theory in the form of results obtained from preliminary investigations of the ability of HDZ to increase the activity of TCNU against MAC 13 tumours, which Lunn et al. (1989) demonstrated to have particularly low levels of AT. We found that there was a significant (P < 0.05%) increase in TCNU antitumour activity when measured in terms of tumour weight inhibition at 14 days.

There are several important factors that must be borne in mind when considering the clinical relevance of the results described in this paper. The first is that, in common with most other investigations of the perfusion/vasculature of experimental tumours, all of the tumours used here were grown subcutaneously. While the MAC system of tumours has been shown to be a good model for clinical large bowel cancer in terms of other relevant parameters, it remains to be established if the vascular supply of tumours grown in this superficial site is as representative. Field et al. (1991) showed that HDZ (5 mg kg⁻¹ i.p.) caused a decrease in the blood flow through a group of transplanted malignant fibrous histiocytomas, whereas the primary tumour from which they were derived did not respond. Rowell et al. (1990) showed by the use of SPECT and ⁹⁹ᵐTc-HMPAO that single dose oral HDZ (0.37–2.86 mg kg⁻¹) caused the blood flow through clinical lung tumours to increase rather than decrease. These results highlight the importance of selecting a clinically relevant model for investigation of the influence of vasoactive drugs on antitumour activity.

In conclusion, this study has investigated the vasculature and perfusion of two members of the panel of MAC tumours. We have shown that there are direct relationships between differentiation state and degree of vascularisation and between vascular patterns and tumour architecture in the two tumours described here. We have also demonstrated that the normal perfusion and vascularity of these tumours was consistently markedly less than that of lung, liver or kidneys. Intravenous administration of HDZ caused a shutdown of tumour vasculature and a decrease in tumour perfusion, the magnitude of both of which was related to dose and tumour differentiation state. Use of in vivo to in vitro clonogenic assays showed that HDZ potentiated the efficacy of melphalan, and slightly enhanced the efficacy of TCNU when given 10 or 15 min respectively after dosing. However, the addition of HDZ increased the acute bone marrow toxicity of melphalan, but did not alter that of TCNU. Thus the enhancement of the efficacy of TCNU represents a true therapeutic gain, whereas the increase of melphalan activity does not. Further work is required to investigate the vasculature, perfusion and response to chemotherapy/vasoactives of these tumours at more clinically relevant sites.

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