Int. J. Radiation Oncology Biol. Phys., Vol. 42, No. 4, pp. 849–853, 1998

0360-3016/98/$–see front matter

ELSEVIER

PII S0360-3016(98)00303-4

● *Modulation of Tumor Oxygenation*

THE COMPARATIVE EFFECTS OF THE NOS INHIBITOR, N^{ω}-NITRO-L-ARGININE, AND THE HAEMOXYGENASE INHIBITOR, ZINC PROTOPORPHYRIN IX, ON TUMOUR BLOOD FLOW

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Purpose: To determine the relative effects of inhibiting nitric oxide synthase (NOS) and haemoxygenase (HO) on blood flow to the rat P22 carcinosarcoma.

Methods and Materials: HO is the enzyme responsible for *in vivo* production of carbon monoxide (CO). The vascular effects of zinc protoporphyrin IX (ZnPP), a competitive inhibitor of HO, were compared with those of copper protoporphyrin IX (CuPP), a poor inhibitor of HO, in isolated *ex vivo* perfusions of the P22 tumour and in intact tumour-bearing rats. In *ex vivo* perfusions, tumour vascular resistance was calculated from measurements of perfusion pressure at a known flow rate. In intact animals, blood flow to tumour and normal tissues was calculated using a radiotracer uptake method. The effects of ZnPP were compared with those of the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NNA), and the combination of the two drugs.

Results: HO activity in the P22 tumour was reduced by 50% following administration of either ZnPP or CuPP directly to *ex vivo* perfused tumours, suggesting an indirect effect on the enzyme. Enzyme inhibition was not associated with any significant vasoactive effect. Neither ZnPP nor CuPP, at a dose of 45 μmol · kg^{-1} administered i.p., inhibited tumour HO *in vivo*. However, they did significantly decrease tumour blood flow to 60–70% of control, with similar effects in skin and brain. Skeletal muscle blood flow was increased to 150% of control. L-NNA decreased both tumour and skeletal muscle blood flow to around 40% of control. These differences suggest that the nonspecific effects of ZnPP and CuPP were not mediated by NOS inhibition. The combination of ZnPP and L-NNA improved the selective reduction in tumour blood flow achieved with either agent alone.

Conclusion: This suggests that the HO/CO pathway does not play a major vasodilatory role in this tumour. However, ZnPP and CuPP could be useful for inducing a relatively selective decrease in tumour blood flow *via* mechanisms unrelated to HO inhibition, especially when combined with NOS inhibition. © 1998 Elsevier Science Inc.

Tumour blood flow, Carbon monoxide, Haemoxygenase, Nitric oxide.

INTRODUCTION

Tumours are characterised by a vasodilatory tone, the precise nature of which is unknown. A prime candidate for a vasodilatory mediator in tumours is the free radical nitric oxide (NO), generated by the activity of the enzyme nitric oxide synthase (NOS). Both the constitutive and inducible isoforms of NOS have been identified in experimental and human tumours, suggesting that NO levels in tumours are high [see 1 for review]. Systemic, competitive inhibition of NOS, using analogues of L-arginine, results in a decrease in blood flow to experimental tumours as a result of vasoconstriction (2). This approach, therefore, has potential for enhancing a wide variety of cancer treatments. Indeed, NOS inhibition has been found to increase tumour sensitivity to the cytotoxic effect of the bioreductive drug RB6145 in some tumour lines, suggesting an influence on tumour oxygenation and/or pharmacokinetics (3–5). Systemic administration of the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NNA), to BD9 rats bearing the P22 carcinosarcoma, was found to produce a relatively selective decrease in tumour blood flow over a wide dosage range (2). The reduction in tumour blood flow levelled out at high doses due to a dose-dependent increase in mean arterial blood pressure (MABP). Methods for improving both the extent and selectivity of tumour blood flow reduction are currently being sought.

Presented at the 10th International Conference on Chemical Modifiers of Cancer Treatment, Clearwater, FL, Jan 28–31, 1998.

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Acknowledgments—Supported by the Cancer Research Campaign.
Accepted for publication 6 July 1998.

Carbon monoxide (CO) is a gaseous signalling molecule that shares some of NO's properties: it activates guanylate cyclase and relaxes vascular smooth muscle cells, causing vasodilation (6, 7). High levels of NO can activate haemoxygenase (HO), the enzyme responsible for the majority of CO production *in vivo* (8). A constitutive and an inducible isoform of HO (HO-2 and HO-1, respectively) have been identified, and HO-1 expression has been reported in human brain tumours (9) and in tumour spheroids (10). These properties suggest that CO may contribute to the vasodilatory tone in tumours, but the effect of HO inhibition on tumour vasculature is currently unknown. Competitive inhibition of HO can be achieved using various metalloporphyrins, including zinc protoporphyrin IX (11, 12). The aim of this study was to determine the effect of HO inhibition relative to NOS inhibition on tumour and healthy tissue blood flow.

METHODS

Tumours

The P22 carcinosarcoma, transplanted into male BD9 rats, was used (13). Subcutaneous tumours in the left flank were used for blood flow measurements in the intact rat when tumours weighed 1.48 ± 0.05 g (mean ± 1 SE). Tumours were transplanted into the right inguinal fat pad, to produce tumours with a single vascular supply (14). These were used for isolated, *ex vivo* tumour perfusions, when they weighed 1.21 ± 0.14 g.

Blood flow

Tissue blood flow was measured, in anaesthetised rats, using uptake of ^{125}I-labelled iodoantipyrine (^{125}I-IAP) (Institute of Cancer Research, Sutton, UK), as described previously (2). Mean arterial blood pressure (MABP) was monitored *via* a tail artery catheter.

Drugs were obtained from Sigma Chemical Co. Ltd. (Poole, UK), except for copper protoporphyrin IX (CuPP) and some of the zinc protoporphyrin IX (ZnPP), which were obtained from Alexis Corporation (UK) Ltd. (Nottingham, UK). Care was taken to shield protoporphyrins from visible light to prevent their inactivation (12).

The effects of the HO inhibitor ZnPP were compared with those of CuPP, which has no direct inhibitory effect on HO *in vivo* (15) and is a poor inhibitor *in vitro* (16). The effects of ZnPP were also compared with those of L-NNA and the combination of ZnPP and L-NNA. Protoporphyrins were administered i.p. in a volume of 3 ml · kg^{-1} and L-NNA was administered i.v. in a volume of 1.7 ml · kg^{-1}.

At the appropriate time, 0.3 MBq (8 μCi) ^{125}I-IAP was infused i.v. with simultaneous collection of timed arterial blood samples (2). Tumour and selected normal tissues were excised, weighed, and gamma-counted. Tissue blood flow rates were calculated from tissue counts, the equilibrium partition coefficient of IAP in the different tissues, and the arterial input function derived from gamma counts in the blood samples (17). Tissue perfusion pressure was assumed to change in direct proportion to MABP for calculating tissue vascular resistance from MABP divided by blood flow rate. Results were generally expressed as a fraction of control values obtained from animals given only the drug vehicle.

Ex vivo perfusions

The method for *ex vivo* perfusion of tissue-isolated tumours has been described previously (14, 17). Briefly, rats were anaesthetised and catheterised for connection to the perfusion apparatus. Tumours were perfused with a modified Krebs–Henseleit (KH) buffer gassed with 5% carbon dioxide/95% oxygen, and were left *in situ* for the duration of the experiment. Perfusate flow rate was kept constant and perfusion pressure was continuously monitored. Vascular resistance was calculated from perfusion pressure divided by perfusate flow rate per gram of tumour.

Escalating doses of ZnPP or CuPP from 1 to 100 μM (30 min per dose) were administered to the tumour tissue as constant infusions. Control tumours received drug vehicle only for the same total length of time. Results for drug-treated tumours were expressed as vascular resistance at the end of each drug dose, as a percentage of the baseline vascular resistance prior to drug administration.

Haemoxygenase activity

Tumour haemoxygenase activity was assayed by spectrophotometric detection of bilirubin extracted from microsomes isolated from frozen tumour and liver samples. This method has been published previously (18).

Statistics

A standard ANOVA followed by the Tukey–Kramer honest significant difference test was used to test the significance of differences between groups used in the ^{125}I-IAP uptake studies. The Wilcoxon rank sum test for nonparametric data was used for groups that were not normally distributed. JMP statistics for the Apple Macintosh (SAS Institute Inc., Cary, NC) was used throughout.

RESULTS

Tumour HO activity *in vivo* was found to be high, at around 63% of that in the liver of tumour-bearing rats (Table 1). Control HO activity in tumours at the end of several hours *ex vivo* perfusion was approximately half that of the tumours *in vivo* (0.6 ± 0.1 vs. 1.5 ± 0.1 nmoles bilirubin per mg protein per h, respectively). Neither ZnPP nor CuPP had any effect on HO activity of tumours *in vivo* whereas, under *ex vivo* conditions, both drugs decreased tumour HO activity to around 60% of the activity in vehicle-perfused tumours (Table 1). Because CuPP is a poor inhibitor of HO, this suggests that the effect is indirect. Different drug doses and exposure times probably account for the different effects of ZnPP/CuPP *in vivo* compared to *ex vivo*. In the isolated, *ex vivo* perfusions, a moderate increase in tumour vascular resistance was observed toward the end of the perfusion time for tumours perfused with ZnPP or CuPP. However, this was not significantly different from the in-

Table 1. Haemoxygenase (HO) activity in frozen tissue samples from rats bearing s.c. P22 tumours and from isolated tumour perfusions

	Tumour HO activity (nmoles bilirubin per mg protein per h)*	
	In vivo†	*ex vivo*‡
Control	1.5 ± 0.1 (4)	0.6 ± 0.1 (3)
ZnPP	1.4 ± 0.2 (4)	0.3 ± 0.1 (3)§
CuPP	1.2 ± 0.1 (5)	0.4 ± 0.1 (3)§

Equivalent activity in liver of untreated, tumour-bearing BD9 rats was 2.4 ± 0.3 (9).

* Values are means ± 1 SE for the number of samples shown in brackets. A significant difference between treated and control values is denoted by §$p < 0.01$.

† Rats were administered 28 mg · kg^{-1} ZnPP i.p., 24 mg · kg^{-1} CuPP i.p., or the vehicle for the drugs (control group) and sacrificed 15 min later for tumour excision.

‡ Isolated perfused tumours were administered escalating doses of ZnPP or CuPP up to 100 μM, as shown in Fig. 1, or drug vehicle only (control group).

crease in vascular resistance observed for the control tumours receiving vehicle only (Fig. 1).

In the intact rat, there were some significant blood flow changes induced by ZnPP and CuPP, despite the lack of effect on HO activity. The blood flow changes induced by ZnPP were very similar to those induced by CuPP (Fig. 2a). Tumour blood flow was significantly reduced to 60–70% of control by both drugs, with similar effects in skin and brain. Conversely, blood flow to skeletal muscle was increased to 150% of control. Because tumour HO activity, under these conditions, was not modified by either ZnPP or CuPP, the blood flow responses must have been due to nonspecific effects of the drugs. No effects were found for the other normal tissues, and blood pressure was unaffected. Heart rate was significantly raised to 120% of control by each drug.

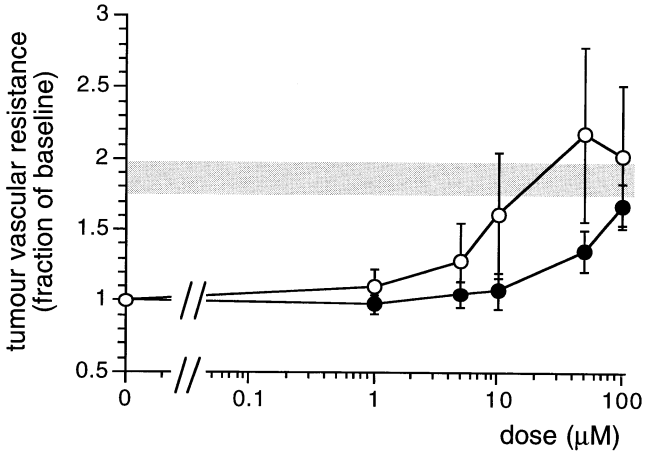

Fig. 1. The effect of ZnPP (closed symbols) and CuPP (open symbols) on vascular resistance in the rat P22 carcinosarcoma perfused *ex vivo*. Points are means ± 1 SE for $n = 6$ (ZnPP); $n = 5$ (CuPP). The shaded area represents vascular resistance (mean ± 1 SE) at the end of the perfusion, for a group of tumours administered vehicle only, $n = 3$.

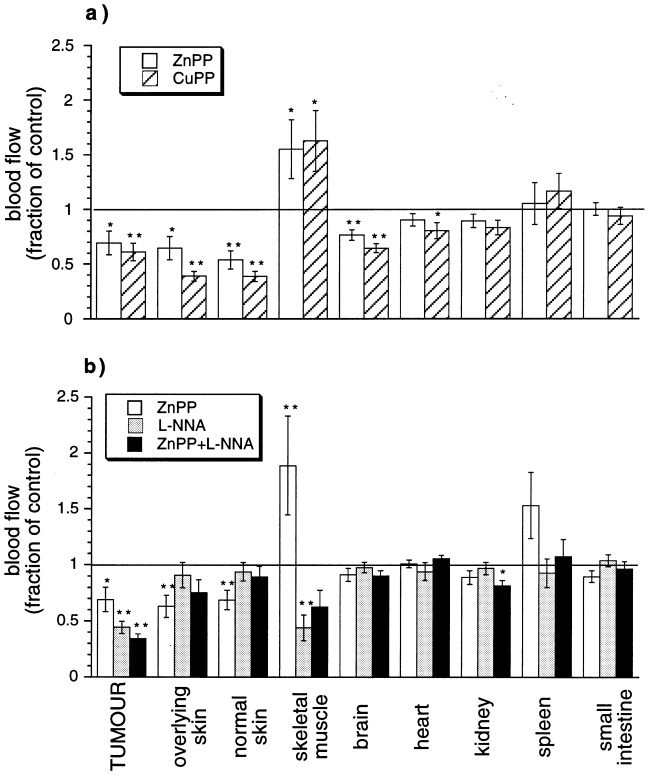

Fig. 2. (a) The effect of 45 μmol · kg^{-1} ZnPP (28 mg · kg^{-1}, unhatched bars) and 45 μmol · kg^{-1} CuPP (24 mg · kg^{-1}, hatched bars) on blood flow to the P22 tumour and a range of normal tissues plotted as a fraction of control blood flow calculated from rats treated with vehicle only. Rats were sacrificed for blood flow calculation at 15 min after drug or vehicle administration. Results are means ± 1 SE for $n = 8$ (ZnPP); $n = 8$ (CuPP) and $n = 7$ (control). (b) The effect of 30 mg · kg^{-1} ZnPP (unshaded bars), 3 mg · kg^{-1} L-NNA (shaded bars) and the combination of ZnPP and L-NNA (solid bars) on blood flow to the P22 tumour and a range of normal tissues. Results are plotted as a fraction of control blood flow calculated from rats treated with vehicle only. Rats were sacrificed for blood flow calculation at 20 min after drug or vehicle administration. Results are means ± 1 SE for $n = 12$ (ZnPP); $n = 11$ (L-NNA); $n = 11$ (ZnPP + L-NNA) and $n = 11$ (control). A significant difference between treated and control values is denoted by *$p < 0.05$; **$p < 0.01$.

Comparison of the effects of ZnPP alone, L-NNA alone, and the combination of the two drugs, on tissue blood flow in the intact rat, is shown in Fig. 2b. Results for ZnPP alone generally confirm those shown in Fig. 2a, except that blood flow to the brain was unaffected. Tumour blood flow was significantly reduced to around 40% of control by L-NNA alone, with a similar effect in skeletal muscle. Blood flow to other normal tissues was unaffected. These results confirm those previously published for L-NNA, except that a significant blood flow reduction in spleen was observed in the earlier publication (2). The combination of ZnPP and L-NNA caused the greatest reduction in tumour blood flow (to around 33% of control), although the difference between the combination and L-NNA alone was not significant. The combination of drugs also induced the most selective reduction in tumour blood flow with, of the normal tissues studied, only a minor reduction in kidney blood flow reaching

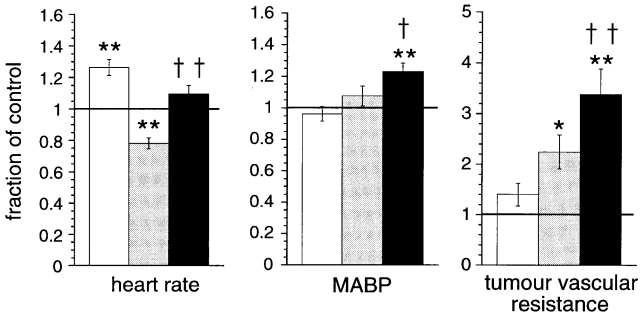

Fig. 3. The effect of 30 mg · kg^{-1} ZnPP (unshaded bars), L-NNA (shaded bars), and the combination of ZnPP and 3 mg · kg^{-1} L-NNA (solid bars) on heart rate, MABP, and tumour vascular resistance. Results are means ± 1 SE for the same group of animals as shown in Fig. 2b. A significant difference between treated and control values is denoted by $*p < 0.05$; $**p < 0.01$. A significant difference between values for the combination and L-NNA alone is denoted by $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$.

statistical significance. Blood flow to skeletal muscle following the combination was not significantly different from either the control group or the L-NNA-alone group.

Figure 3 shows the changes in heart rate, MABP, and tumour vascular resistance for the same group of animals as shown in Fig. 2b. Neither of the drugs alone caused a significant change in MABP, whereas the combination caused a significant increase to around 120% of control. Induced heart rate changes were in the opposite direction for the two drugs. ZnPP increased heart rate to 125% of control and L-NNA decreased it to 80%. Heart rate for the combination was not significantly different from that in control. It was also significantly less than for ZnPP alone and significantly more than for L-NNA alone. In the absence of any change in MABP for ZnPP alone and L-NNA alone, the changes in tumour vascular resistance reflect changes in tumour blood flow shown in Fig. 2b. A tendency for tumour vascular resistance to increase with ZnPP was not significant, whereas L-NNA significantly increased tumour vascular resistance to around 122% of control. The combination of ZnPP and L-NNA increased tumour vascular resistance to around 134% of control, which was significantly greater than that induced by L-NNA alone.

DISCUSSION

These experiments showed that: (a) inhibition of tumour HO, where that was possible (*ex vivo* perfusions), had little or no vasoactive effect; (b) inhibition of tumour HO by ZnPP under *ex vivo* conditions was unlikely to be due to its direct effect as a competitive inhibitor of HO because CuPP had a similar effect; (c) neither ZnPP nor CuPP inhibited tumour HO *in vivo* at the times and doses used; (d) the same doses of ZnPP and CuPP significantly reduced tumour blood flow and increased skeletal muscle blood flow *in vivo*, effects that could not be due to HO inhibition; (e) the blood flow effects of ZnPP and CuPP were different from those of the NOS inhibitor, L-NNA; (f) the combination of ZnPP and L-NNA significantly increased tumour vascular resistance compared with the effects of either agent alone, without further compromising blood flow to normal tissues.

The *ex vivo* results suggest that the HO/CO pathway contributes little to the vasodilatory tone in the P22 tumour. This contrasts with our previous results on NOS inhibition, which showed a large increase in tumour vascular resistance under the same experimental conditions (19). However, the *in vivo* results suggest that metalloporphyrins such as ZnPP and CuPP may be useful for selective tumour blood flow modifications by mechanisms unrelated to HO inhibition. It is well-recognised that ZnPP has several important vasoactive effects, shared by other metalloporphyrins and unrelated to its role as a competitive inhibitor of HO. In particular, it inhibits guanylate cyclase, the enzyme responsible for synthesis of cGMP (20, 21). In contrast, L-NNA is considered a relatively specific inhibitor of NOS (22, 23). Currently, the mechanisms responsible for the blood flow effects we observed for ZnPP and CuPP are unknown. The fact that no vasoactive effects were found for these agents in the isolated tumours suggests that the mechanism may be mediated *via* effects distant from the tumour. Because neither ZnPP nor CuPP affected MABP, the blood flow changes were most likely due to changes in vascular resistance, rather than perfusion pressure, although local changes in perfusion pressure cannot be discounted.

It is possible that ZnPP displays its selectivity for HO *in vivo* only when HO levels are very high. This may be the case, for instance, in endotoxic shock where there is a dramatic increase in the expression of HO-1 in vascular smooth muscle cells, and ZnPP, administered *in vivo*, abrogates the accompanying hypotension (24). In addition, the fact that HO inhibition was achieved in the isolated tumours suggests that tumour uptake of ZnPP may not have been sufficient during the short exposure time of the *in vivo* experiments.

The vascular response to ZnPP *in vivo* was both qualitatively and quantitatively different from that of L-NNA. This suggests that the nonspecific effects of ZnPP were unrelated to NOS inhibition. In particular, blood flow to skeletal muscle was increased with ZnPP but decreased with L-NNA. Because MABP was unaffected by either agent, this suggests that ZnPP acts as a vasodilator, whereas L-NNA acts as a vasoconstrictor, in this tissue. The heart rate changes were also in the opposite direction for the two drugs and appeared to be associated with homeostatic maintenance of blood pressure. The increase in heart rate observed for ZnPP would tend to counteract a tendency for MABP to decrease as a result of skeletal muscle vasodilation. The opposite scenario would apply to L-NNA, as reported previously (2). Interestingly, the combination of ZnPP and L-NNA caused a moderate increase in MABP and no change in heart rate. The change in skeletal muscle blood flow with the combination was not significantly different from that of L-NNA alone, suggesting that the addition of ZnPP to L-NNA had very little modulatory effect on skeletal muscle vasoconstriction induced by L-NNA, but pre-

vented the reflex bradycardia, resulting in an increase in MABP.

Both ZnPP alone and L-NNA alone produced a relatively selective decrease in tumour blood flow, with no major effects on blood flow to critical organs. However, the blood flow decrease in tumour was larger for L-NNA than for ZnPP. In the absence of any change in MABP for either agent, the results suggest that both L-NNA and ZnPP act as vasoconstrictors in tumour tissue; although, as noted above, ZnPP may act indirectly. In general, the blood flow effects of the combination of ZnPP and L-NNA were closer to those of L-NNA alone than to those of ZnPP alone. However, the combination did tend to blunt the reduction in skeletal muscle blood flow and enhance the reduction in tumour blood flow induced by L-NNA alone, thus increasing the selectivity of effect for tumour tissue. In the tumour, the tendency towards a greater blood flow reduction for the combination than for L-NNA alone did not reach statistical significance. However, the calculated changes in tumour vascular resistance were significantly higher for the combination than for L-NNA alone. This suggests that, if the increase in MABP could be avoided, an even larger selective reduction in tumour blood flow could be achieved with the combination of ZnPP and L-NNA.

In conclusion, our results suggest that, in contrast to NO production *via* NOS, the HO/CO pathway does not have a major vasodilatory role in tumours. However, metalloporphyrins may be useful for inducing a relatively selective decrease in tumour blood flow *via* mechanisms unrelated to HO inhibition, especially when combined with NOS inhibition. Understanding the mechanisms behind these effects could lead to even greater benefits.

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