Nitric Oxide Synthase Inhibition Enhances the Tumor Vascular-Damaging Effects of Combretastatin A-4 3-O-Phosphate at Clinically Relevant Doses

Gillian M. Tozer,1 Vivien E. Prise,2 Gemma Lewis,2 Shaoping Xie,3 Ian Wilson,2 and Sally A. Hill4

Abstract Purpose: The therapeutic potential of combining the prototype tumor vascular-disrupting agent combretastatin A-4 3-O-phosphate (CA-4-P) with systemic nitric oxide synthase (NOS) inhibition was investigated preclinically.

Experimental Design: Vascular response (uptake of 125I-labeled iodoantipyrine; laser Doppler flowmetry) and tumor response (histologic necrosis; cytotoxicity and growth delay) were determined.

Results: Inducible NOS selective inhibitors had no effect on blood flow in the P22 rat sarcoma. In contrast, the non–isoform-specific NOS inhibitor Nω-nitro-L-arginine (L-NNA; 1 and 10 mg/kg i.v. or chronic 0.1 or 0.3 mg/mL in drinking water) decreased the P22 blood flow rate selectively down to 36% of control at 1 hour but did not induce tumor necrosis at 24 hours. CA-4-P, at clinically relevant doses, decreased the P22 blood flow rate down to 6% of control at 1 hour for 3 mg/kg but with no necrosis induction. However, L-NNA administration enhanced both CA-4-P–induced tumor vascular resistance at 1 hour (chronic L-NNA administration) and necrosis at 24 hours, with 45% or 80% necrosis for 3 and 10 mg/kg CA-4-P, respectively. Bolus L-NNA given 3 hours after CA-4-P was the most effective cytotoxic schedule in the CaNT mouse mammary carcinoma, implicating a particular enhancement by L-NNA of the downstream consequences of CA-4-P treatment. Repeated dosing of L-NNA with CA-4-P produced enhanced growth delay over either treatment alone in P22, CaNT, and spontaneous T138 mouse mammary tumors, which represented a true therapeutic enhancement.

Conclusions: The combination of NOS inhibition with CA-4-P is a promising approach for targeting tumor vasculature, with relevance for similar vascular-disrupting agents in development.

Combretastatin A-4 3-O-phosphate (CA-4-P) is the lead compound of a group of tubulin-binding/microtubule-depolymerizing agents, which cause extensive vascular damage to solid tumors. CA-4-P is currently in clinical trial as a novel anticancer vascular-disrupting agent (VDA), and its pharmacokinetics in man are known (1). At equivalent drug exposures to those found in man at the maximum tolerated dose, CA-4-P (10 mg/kg) caused a rapid shutdown in blood flow rate in the P22 rat sarcoma model, with few effects in normal tissues (2). However, by 24 hours after treatment, the tumor blood flow had recovered substantially (2). In the clinical trials of CA-4-P, tumor and normal tissue vascular response was assessed by the uptake of a gadolinium chelate, gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA), with the use of dynamic contrast-enhanced magnetic resonance imaging. CA-4-P effects resembled those found in the rat model, typically with significant recovery in the tumor uptake of the marker by 24 hours after treatment (3). Extensive vascular damage in solid tumors leads to induction of hemorrhagic necrosis, and this is the most obvious manifestation of tumor cell cytotoxicity in animal tumors after treatment with CA-4-P (4). Unfortunately, the consequence of rapid blood flow recovery is much reduced necrosis induction and, although different tumors are variably susceptible to CA-4-P, a sustained reduction in blood flow is clearly an essential component of vascular-disrupting therapy. Therefore, further advances need to be made to improve...
on the therapeutic efficacy currently achievable with CA-4-P and similar agents.

The current study investigates the potential for improving the efficacy of CA-4-P by combination with systemic nitric oxide synthase (NOS) inhibition. All NOS isoforms catalyze the production of nitric oxide (NO) from l-arginine, NADPH, and oxygen. NO is a gaseous signaling molecule and a highly reactive free radical. It exerts various vascular effects via binding to the haem group of soluble guanylyl cyclase, which increases cyclic guanosine 3′,5′-monophosphate (cGMP), and also via S-nitrosylation of cysteine thiol protein residues (5, 6). NOS expression and NO production are commonly up-regulated in various cellular compartments of tumors, and the exact role of NO in tumors is thought to be determined both by its cellular source of production and concentration (7). NO production by constitutive NOS in tumor endothelial cells (ceNOS) has been found to promote tumor progression via acting downstream of vascular endothelial growth factor in a proangiogenic role, promoting vascular maturation, inducing vascular hyperpermeability, and reducing leukocyte–endothelial cell interactions (8). Constitutive neuronal NOS produced by glioma cells and inducible NOS (iNOS) were found to contribute to the disorganization of glioma vasculature (9). Therefore, the inhibition of NOS in tumors has therapeutic potential. In addition, in preliminary studies, we showed that the combination of chronic high-dose administration of the nonselective NOS inhibitor Nω-nitro-l-arginine methyl ester (L-NNAME), the prodrug for Nω-nitro-l-arginine (L-NNA), with CA-4-P significantly increased tumor blood flow reduction induced by either agent alone in the P22 rat tumor model (10). L-NNA itself, when given i.v., caused the constriction of tumor-supplying arteries in the P22 tumor growing in rat dorsal skin fold ‘window chambers’, which induced an ~50% reduction in red cell velocity in the tumor microcirculation but no vascular disruption when given alone (11). However, in the same study, the addition of L-NNA administration to relatively high-dose treatment with CA-4-P (30 mg/kg) did increase the vascular disruption obtained by CA-4-P alone. L-NNA was also the most effective NOS inhibitor of a range of commercially available agents, for increasing CA-4-P-induced reduction in the perfused vascular volume and induction of necrosis in CaNT and SaSa mouse tumors (12).

Based on these previous findings, the first aim of the current study was to compare the effects of the nonselective NOS inhibitor, L-NNA, with those of various inhibitors that have selectivity for the iNOS isoform on both tumor and normal tissue vasculature. L-NNA was chosen, in preference to L-NAME, because we found a delayed appearance of L-NNA in the blood of L-NAME–treated rats, which complicated the comparison of the effects of acute versus chronic drug administration.6 The second aim was to test the hypothesis that chronic inhibition of NO (via L-NNA in the drinking water) before CA-4-P treatment on tumor cytotoxicity and therapeutic response in a broader range of preclinical tumor models, including a spontaneous mouse mammary tumor.

#### Materials and Methods

**Animals and tumors.** All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and with local ethical committee approval.

Male 7-to-9-wk-old BDIX rats were used for quantitative blood flow and blood pressure measurements. The rat sarcoma P22 (13) was implanted s.c. into the right flank, and the animals were treated when the mean tumor diameter reached 12 to 16 mm 2 to 3 wk after implantation. The murine mammary carcinoma CaNT (14), grown s.c. on the back of 10-to-14-wk-old female syngeneic CBA/Gy F1 mice, was used for tumor cytotoxicity assays at 5 to 6.5 mm mean diameter 3 to 4 wk after implantation. P22 and CaNT tumors, at the same sizes as described above, and a spontaneous murine mammary carcinoma growing in female T138 mice more than the age of 5 mo (15) at 5 to 6.5 mm were used for growth experiments.

**Drug treatments.** The NOS inhibitor L-NNA (Sigma-Aldrich Co. Ltd.) was dissolved in acidic water and given as a bolus i.v. injection into a catheterized rat tail vein at various doses, at 1.7 mL/kg, and, on one occasion, with an additional maintenance dose via i.v. infusion of 1.7 mL/kg/h for 15 min before blood flow determination. Alternatively, chronic NOS inhibition was achieved by administration of L-NNA in the drinking water; 20 or 60 mg L-NNA was dissolved in a few drops of 1N HCl and then made up to 200 mL in 1% dextrose water to give final concentrations of 0.1 or 0.3 mg/mL of the drug. Aminoguanidine (5 mg/kg) and aminoethyl isothiourea (1 mg/kg; Sigma-Aldrich Co. Ltd.), which preferentially inhibit iNOS over constitutive NOS (16, 17), were dissolved in water (acidified for aminoguanidine) and injected i.v. with a maintenance dose as described for L-NNA. Doses were chosen for their in vivo efficacy as published (18, 19). A third iNOS inhibitor, 1400W [N-(3-aminomethyl)benzyl] acetamidine (20), was dissolved in saline and injected i.v. at 1.6 mg/kg or 16.0 mg/kg in a volume of 1.7 mL/kg as described above. In the mouse, L-NNA was injected i.p. in a volume of 10 mL/kg acidified saline at various doses.

CA-4-P (provided by Prof. George R. Pettit, University of Arizona, Tempe, AZ) was dissolved in 0.9% saline with a few drops of 5% Na2CO3 and given i.p. in a volume of 3 mL/kg (rats) or 10 mL/kg (mice).

**Blood flow, tissue vascular resistance, and systemic blood pressure.** Absolute blood flow rate to the P22 tumor and to normal tissues of the rat

---

6 Unpublished results.
was measured by the uptake of the readily diffusible tracer iodoantipyrine (IAP), labeled with $^{125}$I, as described previously (13). Briefly, the animals were anesthetized with a mixture of fentanyl-fluanisone (Hypnorm, Janssen Animal Health) and midazolam (Hypnovel, Roche Products Ltd.), body temperature was maintained via a thermostatically controlled heating pad, and two tail veins and one tail artery were cannulated to allow for i.v. administration of drugs and tracer, and to monitor mean arterial blood pressure (MABP) via a physiologic pressure

Fig. 1. Blood flow rate in the P22 rat sarcoma (A and B) and normal skeletal muscle (C and D), and MABP (E and F) 15 min after i.v. treatment of tumor-bearing BDIX rats with various NOS inhibitors. Data in A, C, and E (control untreated; 1.0 mg/kg + 1 mg/kg/h L-NNA; 5.0 mg/kg + 5.0 mg/kg/h aminoguanidine; 1.0 mg/kg + 1.0 mg/kg/h aminoethyl isothiourea) were acquired from a separate group of animals from data in B, D, and F (control untreated; 1.6 mg/kg (low) 1400 W and 16.0 mg/kg (high) 1400 W). Points, means ± SE for 5 to 6 animals per group. ***, significant difference from the value in the control untreated group (Tukey-Kramer HSD test; $P < 0.01$). HSD, honest significance difference.
transducer. The animals were heparinized, and free-flowing arterial blood was collected into preweighed vials at 1-s intervals during a 30-s i.v. infusion of 0.3 MBq (8 μCi) 125I-IAP. At 30 s, rats were killed by i.v. injection of euthatal (Rhône Mérieux Ltd.), and the tumor and various normal tissues (skin, skeletal muscle, spleen, kidney, small intestine, heart, and brain) were excised immediately. Blood and tissue samples were then weighed and counted on a Wallac autogamma counter.

Blood flow rate in milliliter per gram tissue per minute was calculated from the tissue counts, the equilibrium partition coefficient for IAP in each tissue, and the arterial input function derived from the arterial blood counts (21). Perfusion pressure was presumed to change in direct proportion to MABP, and changes in vascular resistance for tumor and normal tissues were calculated from the MABP divided by the blood flow rate.

Laser Doppler flowmetry. Relative changes in microvascular perfusion were measured in the P22 tumor with the use of the Oxford Array multichannel laser Doppler system (Oxford Optronics; ref. 22). Rats were anesthetized as described above, and up to 6 probes of ∼300 μm diameter were randomly inserted into the tumor to a depth of at least 1 mm. Baseline erythrocyte flux was recorded for at least 10 min, followed by injection of l-NNA or vehicle i.v. via a cannulated tail vein and a further 60 min of recordings. Further readings were made after a lethal dose of euthatal to obtain the biological zero erythrocyte flux for each probe. Readings, with biological zero readings subtracted, were time averaged and plotted relative to readings at time 0 (injection of drug or vehicle).

Necrosis induction. P22 tumors were excised 24 h after CA-4-P or vehicle injection and fixed immediately in 10% neutral buffered formalin, followed by standard paraffin wax processing. Sections (4 μm) were cut from the center of each tumor and stained with H&E. Necrosis was identified by reduced cellular density, pale cytoplasm, and pyknotic nuclei or completely disrupted cells, with or without red blood cell infiltration. Small regions of stroma (primarily located at the tumor periphery) were excluded from the analysis. With the use of a grid eyepiece graticule marked in 100 squares, each corresponding to a tissue region of 100 × 100 μm, tumor sections were assessed by scoring each square as undamaged tumor or necrosis, moving systematically over the whole section. A percentage necrosis was then calculated for each tumor.

Cytotoxicity assay. The level of tumor cell kill produced by CA-4-P, either alone or in combination with l-NNA, was measured in the CaNT tumor by an in vivo to in vitro colony-forming cell survival assay as described previously (23). Tumors were excised 18 to 24 h after drug injection, weighed, disaggregated, and enzyme digested. Known numbers of viable cells were added to a feeder layer of heavily irradiated V79-379A Chinese hamster cells and incubated for 7 to 10 d until macroscopic colonies were visible to count. Cell survival was calculated from the product of the colony-forming cell fraction and cell yield per gram of tumor in treated relative to untreated tumors. By incorporating cell yield per gram of tumor into the calculation, tumor cells that are killed rapidly by the treatment, before tumor excision, are included in the calculated cell survival parameter.

Tumor growth delay. Tumors were measured with calipers, in three orthogonal diameters, at intervals after drug treatment, and mean growth curves were produced for each group of animals.

Statistics. Statistical analysis was carried out with the use of JMP Statistics version 5.1 for the Apple Macintosh (SAS Institute Inc.). ANOVA followed by the Tukey-Kramer honest significance difference test was used for group comparisons or Dunnett’s test for comparison of groups with a control group. In all cases, differences between groups were described as significant if the probability corresponding to the appropriate statistic was <0.05.

Results

iNOS-selective NOS inhibitors had no effect on tissue blood flow rate. The effects of three NOS inhibitors with selectivity for iNOS: amino guanidine, aminoethyl isothiourea, and 1400 W, were compared with l-NNA, a nonselective NOS inhibitor, for their effects on P22 tumor and normal tissue blood flow and MABP in the BDIX rat (Fig. 1). Low-dose l-NNA (1 mg/kg, with a maintenance dose of 1 mg/kg/h for a further 15 minutes) significantly reduced tumor blood flow to ∼70% of control at the 15-minute time point (Fig. 1A), confirming previous data (24–28). However, none of the iNOS-selective inhibitors had any effect on tumor blood flow rate (Fig. 1A and B). None of the inhibitors had any significant effect on normal tissue blood flow (skin, spleen, kidney, small intestine, heart, and brain; data not shown). There was a tendency towards blood flow reduction in skeletal muscle for l-NNA, but this did not reach statistical significance, and there was no effect for the other inhibitors in this tissue (Fig. 1C). There was a mild hypertensive effect of l-NNA (Fig. 1E), which is well known (29), but no effect of the iNOS-selective NOS inhibitors on MABP (Fig. 1E and F), consistent with the role of eNOS in blood pressure regulation (30). In consideration of these results, l-NNA was chosen for further investigation.
L-NNA caused a sustained reduction in tumor blood flow. Laser Doppler flowmetry showed a sustained dose effect of bolus L-NNA (1 and 10 mg/kg) in the P22 tumor (Fig. 2A), indicating that the additional maintenance dose of L-NNA used above was unnecessary. Uptake of $^{125}$I-IAP confirmed the sustained effect in the tumor for 10 mg/kg and additionally showed that the blood flow rate in skeletal muscle was similarly affected. The blood flow changes were accompanied by a significant hypertensive effect at 15 minutes after drug treatment, which resolved by 75 minutes after drug treatment (Fig. 2B). As for 1 mg/kg L-NNA, 10 mg/kg L-NNA had no significant effects on blood flow rate in any other normal tissue investigated (data not shown).

L-NNA pretreatment increased the tumor vascular-damaging effects of CA-4-P. Having established the sustained effect of L-NNA on tumor blood flow rate, the effect of combining L-NNA with a very low dose of CA-4-P (3 mg/kg i.p.) was investigated. To determine whether an extended period of NOS inhibition had a different influence on response to CA-4-P than NOS inhibition just before CA-4-P treatment, both bolus dose (15 minutes before CA-4-P administration) and chronic administration (for 24 hours in the drinking water before CA-4-P administration) of L-NNA were investigated. On its own, CA-4-P reduced P22 tumor blood flow rate to ~6% of control by 1 hour after administration (Fig. 3A). L-NNA (10 mg/kg i.v.) alone reduced tumor blood flow to ~36% at 1 hour (Fig. 3A). Pretreatment with 10 mg/kg i.v. L-NNA did not significantly increase the tumor vascular effects of CA-4-P alone at 1 hour, either in terms of blood flow rate (Fig. 3A) or tumor vascular resistance (Fig. 3C). However, pretreatment with p.o. given L-NNA (0.3 mg/mL) for 24 hours before CA-4-P had a substantially greater influence on CA-4-P-induced increase in tumor vascular resistance than i.v. administration of L-NNA (Fig. 3D). Taking the data in Figs. 2 and 3B together, tumor blood flow was approximately the same at the time of CA-4-P administration in all the L-NNA–pretreated groups irrespective of whether L-NNA was given acutely as a bolus i.v. injection or chronically in the drinking water at two different doses (Fig. 2B, closed symbols, and Fig. 3B, open bars), indicating that blood flow level at the time of CA-4-P administration was not the key factor in determining the strength of interaction.

Figure 4 shows the effects of the same dosing regimes for L-NNA alone and in combination with CA-4-P on tumor necrosis measured 24 hours after CA-4-P administration. In this case, two clinically relevant doses of CA-4-P were used, 3 and 10 mg/kg. Untreated P22 tumors, at the size used, have negligible necrosis (Fig. 4A and B). L-NNA alone had no significant effect on tumor necrosis in any of the dosing regimes (Fig. 4A). CA-4-P (3 mg/kg) also had no significant effect on necrosis despite the very severe reduction in tumor blood flow measured at 1 hour after treatment (Fig. 3A). CA-4-P (10 mg/kg) alone produced >40% necrosis in three of five tumors treated, but the overall effect was also not significantly different from controls (Fig. 4A). However, the addition of L-NNA to CA-4-P treatment produced a significant increase in necrosis compared with CA-4-P alone for several of the combinations (Fig. 4A). The increase in necrosis with the addition of L-NNA approximated 9-fold for 3 mg/kg CA-4-P, equating to 45% necrosis in the combination groups, and a 2.5-fold increase for 10 mg/kg CA-4-P, equating to 80% necrosis in the combination groups. Although there was a tendency for tumor necrosis induction to be higher for 0.3 mg/mL chronic p.o. L-NNA combined with 3 mg/kg CA-4-P, compared with the i.v. dosing
regime combination, this did not reach statistical significance with the numbers of animals used. Figure 4B shows examples of the extent of necrosis for the different treatment groups. In the majority of cases, the combination of l-NNA and 10 mg/kg CA-4-P produced the characteristic pattern of necrosis induction normally obtained with much higher doses of CA-4-P used as a single agent (4, 31); that is, extensive central necrosis with a thin rim of viable tumor tissue (Fig. 4B). Twenty-five of 41 tumors treated in these groups were >90% necrotic.

**Combined l-NNA and CA-4-P treatment increased cytotoxicity in the CaNT tumor.** Encouraging results for the combination of l-NNA and CA-4-P in terms of necrosis induction in the rat P22 tumor led to further investigation of the cytotoxic interaction in other tumor lines. First, cytotoxicity was measured in the CaNT mouse mammary adenocarcinoma with the use of a clonogenic assay. These results are shown in Fig. 5, in which various doses of l-NNA and CA-4-P were combined, and scheduling of the two compounds were examined. Figure 5A also shows that a moderate dose of CA-4-P alone (50 mg/kg in the mouse) produced slightly <1 log of cell kill in the CaNT tumor, and 10 mg/kg l-NNA alone was less effective. These results are consistent with the relative effects of the two agents on tumor blood flow and necrosis in the rat P22 tumor (Figs. 3 and 4). Figure 5A also shows that l-NNA had no additional effect on cell killing if CA-4-P was given 6 hours before l-NNA or 0 to 6 hours after l-NNA. If CA-4-P was given up to 3 hours before l-NNA, a greater than additive effect was obtained, with 3 hours before being the most effective regime. This timing schedule was subsequently used to investigate the dose response for the combined treatment, as shown in Fig. 5B. Here, doses of l-NNA alone between 2.5 and 20 mg/kg had very similar cytotoxic effects, with no indication of a dose response. This may be due to an increase in vascular resistance being balanced by an increase in MABP and, consequently, perfusion pressure, over this dose range as reported previously for the P22 tumor (28). In contrast, there was a clear dose response for CA-4-P alone (Fig. 5B), and this is consistent with a dose effect for necrosis induction in the P22 tumor (Fig. 4). The combination of CA-4-P given 3 hours before l-NNA produced a greater than additive effect on CaNT cytotoxicity at all doses tested (Fig. 5B).

The therapeutic effect of CA-4-P was improved by addition of l-NNA in spontaneous as well as transplanted tumors. Tumor response to the combination of l-NNA and CA-4-P was further evaluated in the spontaneous T138 mouse mammary tumor model, the CaNT transplanted mouse mammary tumor model, and the P22 rat sarcoma model with the use of repeated dosing schedules and assayed by tumor growth measurements. Rats are more sensitive than mice to the toxic effects of CA-4-P and related compounds, and so a conservative dosing regime (2 × 10 mg/kg i.p. CA-4-P, 3 days apart) was used in combination with p.o. dosing of l-NNA (0.1 mg/mL in the drinking water). A maximum 5% body weight loss was observed (results not shown). Neither agent alone had any substantial effect on the growth of the P22 tumor. However, the combination treatment substantially retarded tumor growth (Fig. 6A). Interestingly, this effect was only apparent after at least 1 day after the first CA-4-P treatment, suggesting that NOS inhibition was effectively enhancing a delayed effect of CA-4-P, consistent with results from the clonogenic assay of CaNT tumors shown in Fig. 5.

A more aggressive treatment schedule was used in mice (10 mg/kg i.p. l-NNA followed after 15 minutes by 50 mg/kg i.p. CA-4-P, at daily intervals for 10 days, with a 2-day gap between the 5th and 6th doses). No body weight loss was observed for any

---

**Fig. 4.** Effect of combining l-NNA i.v. (10 mg/kg) or l-NNA given in drinking water (low dose, 0.1 mg/mL; high dose, 0.3 mg/mL) with CA-4-P (3 mg/kg or 10 mg/kg i.p.) on necrosis in the P22 tumor. A, quantitative evaluation as means ± 1 SE for 5 animals per group in untreated and single drug–treated groups, and 12 to 16 animals in combined drug–treated groups. * and **, significant differences between groups (Tukey-Kramer HSD; P < 0.05 and P < 0.01, respectively). B, representative histology (H&E staining) for an untreated tumor (control), a tumor after l-NNA alone (0.3 mg/mL in the drinking water), a tumor after CA-4-P alone (10 mg/kg i.p.), and a tumor after treatment with the combination. l-NNA alone caused little or no necrosis. CA-4-P alone caused some necrosis (bottom left), but most of the tumor was still viable (middle left). The combination caused necrosis in the majority of the tumor volume, shown by pyknotic nuclei and reduced cytoplasmic staining (middle and bottom right). V, viable tumor region; N, necrosis. Bar, 100 μm in all cases.
treatment schedule (results not shown). l-NNA alone had only a minor effect on the growth of the CaNT tumor and no effect on the growth of the T138 tumors (Fig. 6B and C). CA-4-P alone had only a minor effect on the growth of the CaNT tumor but a substantial effect on the growth of T138 tumors as reported previously (15). However, as for the P22 tumor, the combination treatment produced a substantial tumor growth delay in both mouse tumor types, which was greater than additive (Fig. 6B and C).

Discussion

We have shown that, in contrast to several NOS inhibitors that have selectivity for iNOS, the non–isoform-specific NOS inhibitor l-NNA caused a significant and sustained reduction in tumor blood flow rate. Furthermore, pretreatment with l-NNA increased the tumor vascular effects and necrosis induction of a clinically relevant dose of the VDA CA-4-P in a rat sarcoma model. Scheduling studies in the mouse showed that l-NNA was most effective in enhancing CA-4-P–induced tumor cell toxicity when given 3 hours after CA-4-P. Tumor growth delay studies showed that the addition of l-NNA to a repeated dosing schedule of CA-4-P could enhance efficacy in a spontaneous mouse model of breast cancer, as well as in transplanted rat and mouse tumors.

Our data show that it is a constitutive form of NOS, most likely ecNOS, which acts to maintain tumor blood flow, at least in the rat P22 model. This adds to the various roles already elucidated for ecNOS in the tumor microcirculation, most notably its permeability and proangiogenic effects, and its mediating role in mural cell recruitment to angiogenic blood vessels (32–34). Currently, there are no small molecule-selective inhibitors for ecNOS available for preclinical or clinical studies, although a peptide derived from caveolin-1, cavtratin, sequences ecNOS in vivo and is therefore selectively active against ecNOS (33). A bolus dose of l-NNA decreased tumor blood flow for at least 1 hour, which is consistent with sustained plasma levels of l-NNA at these times.7

7 Unpublished data.

Fig. 5. Effect of combining l-NNA with CA-4-P on cytotoxicity in the CaNT tumor. Tumor-surviving fraction was determined by treating tumors in vivo, and measuring cell yield and in vitro clonogenicity of cells from disaggregated solid tumors 18 to 24 h after treatment compared with controls (see text for details). A, effects of l-NNA (10 mg/kg i.p.) and CA-4-P (50 mg/kg i.p.) given alone and in combination at different intervals between the two drugs. Parallel lines, effects of the drugs alone (1 SE each side of the mean value). *, significant difference between the combination group and CA-4-P alone (Dunnett’s test; P < 0.01). B, effects of different doses of l-NNA and CA-4-P, with CA-4-P given 3 h before l-NNA. Open symbols, l-NNA alone; closed symbols, l-NNA combined with CA-4-P. All points in the lower curves were significantly different from l-NNA alone (top curves; Dunnett’s test; P < 0.01). Points, means ± 1 SE for 6 to 8 animals.
A recent phase I clinical study showed that L-NNA, up to a dose of at least 0.9 mg/kg, was well tolerated in cancer patients (35), with only an ~6% increase in blood pressure. Furthermore, computed tomography imaging showed a decrease in tumor perfusion, which is encouraging for further clinical studies of L-NNA or related compounds in tumor vascular-targeting strategies. Previously, we showed that chronic administration of L-NAME, the prodrug of L-NNA, at ~10 times the highest dose used in the current study and for 24 hours before administration of 30 mg/kg CA-4-P, increased the initial blood flow shutdown induced by CA-4-P in the rat P22 tumor (10). In the present study, we focused on L-NNA itself and found that lower doses of both the NOS inhibitor and CA-4-P, in combination, were still effective in increasing the blood flow reduction induced by either agent alone. A dose of 10 mg/kg CA-4-P in BDIX rats is approximately equivalent to the maximum tolerated dose in man in terms of the area under the plasma concentration versus time curve (2). Therefore, the finding in the present study that NOS inhibition combined with 3 or 10 mg/kg CA-4-P effectively enhanced blood flow reduction and, most importantly, that this translated into increased necrosis in the P22 tumor is encouraging for future clinical studies.

Some insights into the mechanisms underlying the beneficial effect of NOS inhibition combined with CA-4-P were gained. Interestingly, a single i.v. dose of 10 mg/kg L-NNA 15 minutes before CA-4-P administration did not significantly alter the vascular effects of CA-4-P measured 1 hour after administration, whereas L-NNA given in the drinking water for 24 hours beforehand was effective in enhancing the effects of CA-4-P at this time point (Fig. 3). This difference occurred despite the fact that the different modes of NOS inhibition, when used alone, produced a very similar reduction in tumor blood flow. Therefore, there were no differences in the level of blood flow at the time of CA-4-P administration, which could explain the beneficial effect of chronic versus i.v. bolus NOS inhibition. This suggests that chronic NOS inhibition caused subtle alterations in the tumor vasculature that sensitized it to CA-4-P. NO or related compounds have numerous effects on vascular function in both normal and tumor tissue, in addition to blood flow maintenance. High vascular permeability is considered a predisposing factor for response to CA-4-P (36), and chronic NOS inhibition may impact on vascular permeability by encouraging leukocyte-endothelial interactions (37). Induction of reactive oxygen species by CA-4-P is likely to constitute at least part of the mechanism of action of the drug (38, 39), and leukocyte-endothelial interactions and reduced levels of NO may direct reactive oxygen species induction along a more damaging pathway under chronic NOS inhibition. NOS inhibition has also been shown to increase RhoA activity (40), a pathway activated by CA-4-P, at least in vitro (41). Whatever the precise mechanisms, the combined effect of NOS inhibition and CA-4-P treatment is reminiscent of the increased toxicity of ischemia-reperfusion injury and photodynamic therapy when combined with NOS inhibition, circumstances in which oxidative stress also plays an important role (42, 43).

Clearly, it is important that NOS inhibition not only increased the vascular-damaging effect of CA-4-P but that this translated into an increase in tumor cell toxicity, as shown in Figs. 4 to 6. This effect was more than additive because NOS inhibition on its own had negligible effect on either necrosis.

![Fig. 6. Effect of combining L-NNA with CA-4-P on growth of three rodent tumor lines: P22 (A), CaNT (B), and the spontaneous mammary tumor cell line T138 (C). Points, means ± 1 SE for 5 to 6 animals in A and B, and 10 to 15 animals in C, which reflects a more variable response in T138 tumors. In A: open vertical arrows, dosing time for i.p. CA-4-P; horizontal arrow, period of L-NNA administration in drinking water. Solid vertical arrows in B and C, combined i.p. dosing of L-NNA and CA-4-P. See text for details.](image-url)
in the P22 tumor or cytotoxicity in the CaNT tumor. Presumably, this is due to a lesser effect of l-NNA on tumor blood flow rate compared with CA-4-P (Fig. 3). In the P22 tumor, CA-4-P–induced necrosis was substantially increased when combined with NOS inhibition, amounting to ~80% of the tumor volume at the higher, but still clinically relevant, dose (Fig. 4). In contrast to tumor vascular resistance, measured at 1 hour after CA-4-P administration, chronic administration of l-NNA 24 hours beforehand was no more effective at increasing CA-4-P–induced necrosis than i.v. bolus administration just before CA-4-P administration. This suggests that the net blood flow reduction over the full 24 hours before tumor excision for the necrosis assay was similar for both administration regimes despite the small differences in vascular response detected at 1 hour.

The scheduling studies carried out in the CaNT tumor model (Fig. 5) show that NOS inhibition is most effective when given several hours after CA-4-P although, overall, there was still a benefit for l-NNA given for up to several hours before CA-4-P. Together with the growth data for the P22 tumor (Fig. 6), this suggests that NOS inhibition is particularly effective in interacting with the downstream consequences of CA-4-P treatment. Several studies have shown that CA-4-P and related compounds induce increased tumor expression of hypoxia inducible factor-1α and angiogenic growth factors (44–46), as well as mobilizing endothelial progenitor cells from bone marrow (47). Others have shown that the combination of VDAs with antiangiogenic agents are particularly effective (48). NO has been shown to act downstream of important angiogenic growth factors, such as vascular endothelial growth factor (49). Therefore, a potential explanation for the delayed response to l-NNA in combination with CA-4-P is that l-NNA limits the angiogenic response to CA-4-P. The addition of l-NNA to CA-4-P treatment produced more than additive effects on tumor growth delay (Fig. 6) in a spontaneous breast cancer model as well as in transplanted s.c. tumors. Importantly, this was achieved with no apparent toxicity in the mouse, representing a true increase in therapeutic effect. In the rat, similar tumor growth delay was achieved by the addition of l-NNA but with some increase in toxicity. Pancreatic toxicity was associated with l-NAME administration in dogs (50). These results should be borne in mind in the clinical translation of NOS inhibition strategies, although the recent clinical study of l-NNA in cancer patients is encouraging in this regard (35). Repeated dosing of CA-4-P alone had little effect on tumor growth in either of the transplanted tumor lines despite definitive evidence from clonogenic assay (Fig. 4) of ~90% cell kill for a single 50 mg/kg dose of CA-4-P in the CaNT tumor. This result underlines the difficulties associated with extracting cytotoxicity information from tumor growth curves, and the very small effect of CA-4-P is most likely explained by slow clearance of necrotic debris from tumors with vascular insufficiency, as described previously (51). Interestingly, CA-4-P alone was most effective in the T138 spontaneous tumors, which may relate to the slower growth rate of these tumors. The addition of l-NNA prevented the growth of the P22 and T138 tumors for the duration of treatment, and subsequent regrowth occurred at a similar rate to controls. Tumor regression was not achieved in any of the tumor lines, underlining the requirement for vascular-targeted treatment to be combined with conventional chemotherapy or radiotherapy.

In conclusion, we have shown a beneficial therapeutic effect of inhibiting constitutive NOS isoforms in combination with administration of clinically relevant doses of the VDA CA-4-P in a spontaneous preclinical model of breast cancer as well as in transplanted models. Significantly enhanced levels of tumor cell kill were achieved with acceptable toxicity compared with CA-4-P alone, which is promising for the clinical application of this approach. These results may also have relevance for other VDAs in development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Prof. Bob Pettit (Arizona State University, Tempe, AZ) for supplying CA-4-P, and the staff at the Gray Cancer Institute and University of Sheffield for the care of the animals. We also thank Cancer Research UK for grant support.

References

18. Wu C-C, Reutten H, Thiemenmann C. Com- parison of the effects of aminoguanidine and
Nw-nitro-L-arginine methyl ester on the multi-
19. Chatterjee PK, Patel NS, Kvale EO, et al. Inhibi-
40. Wojcik-Stothard B, Torondel B, Tsang LY, et al. The ADMA/DDAH pathway is a critical regu-
41. Kanthou C, Tozer GM. The tumor vascular tar-
geting agent combretastatin A-4-phosphate ind-
tuces reorganization of the actin cytoskeleton and early membrane blebbing in human endo-
42. Parkins CS, Dennis MF, Stratford MRL, Hill SA, Chaplin DJ. Ischemia reperfusion injury in tu-
43. Korbelik M, Parkins CS, Shibuya H, Clecic I, Stratford MF, Chaplin DJ. Nitric oxide produc-
44. Boehle AS, Sipos B, Kliche U, Kohthoff H, Dohr-
45. Sheng Y, Hua J, Pinney KG, et al. Combret-
tastatin family member OXI4503 induces tu-
46. Dachs GU, Steele AJ, Coralli C, et al. Anti-vas-
47. Shaked Y, Ciarrocchi A, Franco M, et al. Ther-
apy-induced acute reversion of circulating endo-
49. Ziche M, Morbidelli L, Choudhri R, et al. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not fibro-
50. Poulsom JM, Dewhirst MW, Gaskin AA, et al. Acute pancreatitis associated with administra-
tion of a nitric oxide synthase inhibitor in tu-
51. Chaplin DJ, Pettit GR, Hill SA. Anti-vascular ap-
Nitric Oxide Synthase Inhibition Enhances the Tumor Vascular-Damaging Effects of Combretastatin A-4 3- O-Phosphate at Clinically Relevant Doses


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/11/3781

Cited articles
This article cites 50 articles, 19 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/11/3781.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/15/11/3781.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.