Antitumor Actions of Ruthenium(III)-Based Nitric Oxide Scavengers and Nitric Oxide Synthase Inhibitors

Frederick W. Flitney, Rebecca J. Pritchard, Gordon D. Kennovin, Stuart K. Bisland, David G. Hirst, and Simon P. Fricker

Abstract

The role of endogenous nitric oxide (NO) in the growth and vascularization of a rat carcinosarcoma (P22) has been investigated. Tumor-bearing animals were treated with (i) nitric oxide synthase (NOS) inhibitors, administered via the drinking water, including N\textsuperscript{G}-nitro-\textsuperscript{1}-arginine methyl ester (L-NAME), a nonisoenzyme-selective inhibitor, and 2 others that target the inducible (NOS II) enzyme preferentially, namely 1-amino-2-hydroxyguanidine or N-[3(aminomethyl)benzyl]acetamidine hydrochloride; or (ii) daily injections (intraperitoneally) of 2 Ru(III) polyaminocarboxylates, AMD6221 and AMD6245, both of which are effective NO scavengers. L-NAME, AMD6221, and AMD6245 reduced tumor growth by approximately 60% to 75% of control rates. Tumor sections stained with abs to CD-31/platelet endothelial cell adhesion molecule-1 or NOS III showed that this was associated with a marked reduction (60%–77%) of tumor microvascular density (MVD). Tumors resumed growing promptly when treatment was discontinued, accompanied by partial or complete restoration of MVDs. In contrast, NOS-II selective inhibitors had no effect on tumor growth or vascularization, indicating that both responses require complete blockade of NO production. The results corroborate the view that endogenous NO facilitates tumor development. We suggest that NO deprivation causes tumor feeder vessels to constrict, reducing tumor blood flow. The delivery of oxygen and essential nutrients to the developing tumor is impaired as a consequence, hampering further growth. Normalizing NO levels by withholding treatment causes tumor feeder vessels to dilate, increasing tumor perfusion and reestablishing conditions that allow tumors to begin growing again.

Mol Cancer Ther; 10(9); 1–10. ©2011 AACR.

Introduction

Nitric oxide (NO) is a potent vascular smooth muscle relaxant that plays a pivotal role in regulating blood flow and pressure throughout the cardiovascular system. NO is synthesized from \textsuperscript{1}-arginine (1) by 3 isoenzymes known as nitric oxide synthases (NOS): NOS I (neuronal or brain NOS), NOS II (inducible NOS), and NOS III (endothelial NOS). NOS I and NOS III are expressed constitutively and are regulated by fluctuations in intracellular Ca\textsuperscript{2+}, generating localized NO transients in the pM-nM range. NOS II is controlled transcriptionally and provides a high-output pathway for NO, creating sustained NOs in the \textmu mol/L range independently of changes in intracellular Ca\textsuperscript{2+} (2, 3).

NOS isoforms have been identified in malignant tumors where their expression levels correlate with tumor grade. NOS II and NOS III are coexpressed in some human (4–8) and rodent tumors (9). Other tumors reportedly express either NOS III (6, 8) or NOS II exclusively (10). The relative contributions of the different isoforms to the growth of a tumor can be assessed in principle, using synthetic analogues of \textsuperscript{1}-arginine that competitively inhibit all 3 isoforms (11), together with inhibitors with much greater selectivity for NOS II, such as 1-amino-2-hydroxyguanidine (AG; refs. 12, 13) and N-[3(aminomethyl)benzyl]acetamidine hydrochloride (1400W; ref. 14).

NO can inhibit or enhance tumor growth (15), depending on its concentration. Low NOs facilitate blood flow, ensuring the efficient delivery of nutrients and oxygen to the tumor (16–19). They also enhance vascular permeability (10, 20) and stimulate angiogenesis, facilitating the development of the tumor neovasculature (21, 22). These effects promote tumor growth and assist metastatic spread. However, other studies report that NO can suppress tumor growth (23), typically following induction of...
the NOS II isoform. The high NOs generated by this enzyme react with oxygen-derived radicals to generate highly reactive nitrogen species (RNS), a condition called nitrosative stress. RNS react with cellular macromolecules (proteins, lipids, nucleic acids), generally with adverse outcomes that can result in cell death, for example, by initiating apoptosis or cell necrosis (24).

NOS II has been highlighted as the predominant iso-enzyme that facilitates tumor progression (25), prompting the search for novel therapies based on highly selective NOS-II inhibitors (26). However, this strategy can offer only limited therapeutic benefit where constitutive isoforms are the major, or perhaps the only, source of NO (27). An alternative approach is to scavenge NO before it can exert its effects. Scavengers will react with NO from all sources and their efficacy will depend upon the rate of reaction with the ligand and the tissue distribution (compartmentalization) of different NOS isoforms. Assuming second-order reaction kinetics, scavenging will be most effective in regions where the NO concentration is greatest, reducing NO preferentially in regions of inappropriate overproduction, while having less effect on essential basal NO levels.

NO is a ligand for transition metals, especially ruthenium, where the formation of nitrosyl complexes is a notable feature of its chemistry (28). Ru(III) readily reacts with NO to form Ru(II) mononitrosyls containing a linear Ru-N-O bond. The Ru-N-O bond is very stable; consequently the nitrosyl moiety is not readily displaced (29). Ru can be chelated with a suitable ligand to confer water solubility and ensure rapid clearance in vivo, while providing an accessible binding site for NO (30). Ru(III) polyaminocarboxylates of EDTA or diethylenetriaminepentaacetic acid (DTPA) function as pentadentate ligands that satisfy these criteria.

Several chemically related Ru(III) complexes, including K[Ru(H$_2$EDTA)Cl] (AMDI226), [Ru(HEDTA)H$_2$O] (AMD6245), and [Ru(H$_2$DTPA)Cl] (AMD6221), are able to scavenge NO in different biological systems. For example, nitrite production by activated RAW-264 macrophages in culture was reduced by AMD6245 and AMD6221, but not by AMD3689 (31), the nitrosyl adduct of AMD6221. NO scavenging was also shown in several animal disease models. AMD6245 and AMD6221 reversed the hypotension in rodent (32) and porcine models of endotoxic shock (33). AMD3689 was identified in the supernatant of activated RAW264 cells (31) and also in the plasma of rats treated with AMD6221 in a model of cardiac allograft rejection (34), providing clear evidence for NO scavenging as the underlying mechanism of action. AMD6221 also reduced ocular inflammation in a rabbit model of lipopolysaccharide-induced uveitis. Significantly, one study (31) showed that AMD6221 did not alter either the level of expression or catalytic activity of NOS II.

Here, we report on the effects of AMD6245 and AMD6221 on the growth and vascularization of a rat carcinosarcoma P22 (35). We have compared the results with those obtained using NG-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, and with AG and 1400W, both of which display greater selectivity for NOS II than for NOS III (13, 14). The molecular structures of these compounds are shown in Fig. 1.

**Materials and Methods**

The procedures used throughout complied with the requirements of the Animals (Scientific Procedures) Act 1986 and with the prevailing UKCCR guidelines.

**Tumor implants**

Male BD-IX rats (10–12 weeks; 300–380g) were used. Animals were age-matched to minimize differences in both the angiogenic response (36) and vascular NOS III expression (37).

The mixed P22 carcinosarcoma was used for all experiments (35). Small fragments (ca. 1 mm$^3$) from the cortex of a freshly passaged tumor were implanted subcutaneously on to the dorsum of syngeneic male rats, close to the mid line (either 2 or 4 per animal; see below). Alternatively, tumor slurries were prepared and injected s.c. (0.05 mL). Surgery was done under general anesthesia (90 mg.kg$^{-1}$ ketamine, Willows Francis Ltd., and 10 mg.kg$^{-1}$ xylazine, Bayer Ltd.). Lidocaine (Astra Pharmaceuticals Ltd.) was used as an analgesic on wound sites immediately after surgery. Carprofen...
(10 mg·kg⁻¹ orally, C-Vet) or buprenorphine (0.5 mg·kg⁻¹ s.c., Schering) were administered for 24 to 48 hours to alleviate postoperative pain.

**Tumor growth curves**

The long (Y) and short (X) axes of each tumor were measured daily or on alternate days using skin-fold calipers. Tumor volumes (V) were estimated using the hemi-ellipsoid formula, \( V = \frac{1}{2}X^3 \times Y \times \pi \). Total tumor burden was not permitted to exceed 10% body weight, in line with the prevailing UK Home Office (Project License) requirements (1997).

**Effect of NOS inhibitors on tumor growth**

Male BD-IX rats were maintained on a normal laboratory diet, supplemented by the addition of L-NAME (1–10 mg/mL), AG (1 mg/mL) or 1400W (1 mg/mL) to the drinking water. Chronic oral administration of these compounds at these doses has been shown to be effective at inhibiting NOS isoforms in several different models (18; and Discussion). The drinking water also contained sucrose (10 mg/mL) to mask the taste of NOS inhibitors. The time at which treatment with NOS inhibitors commenced, relative to the time of implantation, and the duration of each treatment, varied in different experiments. Animals were either treated continuously until sacrificed, or treatment was discontinued and animals were maintained for a further 7 days before sacrifice. Some effects of L-NAME were reversed in the latter experiments and for this reason we refer to this interval as the recovery period.

Control groups of animals were allowed access to water containing sucrose only.

**Effect of Ru NO scavengers on tumor growth**

Animals used in this study (24 BD-IX rats) received 2 implants. After surgery they were randomly placed into 3 groups of 8 animals (\( n = 16 \) tumors/group). Group 1 contained control animals that received intraperitoneally (i.p.) injections of sterile saline only. Animals in groups 2 and 3 were given daily i.p. injections of AMD6245 or AMD6221 dissolved in sterile phosphate buffer (0.2 mol/L, pH 7.4, 50 mg/mL), for 18 days commencing on day +10. The dose used was 50 mg/kg body weight, based on our experience of their efficacy in previous whole animal studies (32, 34). Three animals in each group were sacrificed on day +28 and the remaining 5 were maintained for a further 7 days without treatment (recovery period) before sacrifice.

**Immunohistochemistry**

Cryostat sections (7 µm) of snap-frozen tumors were mounted on poly-l-lysine-coated slides, air dried and fixed in acetone (10 min). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 70% methanol (30 min). Sections were then overlaid with mouse anti-rat CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1; Serotec) mab, followed by secondary anti-mouse preabsorbed labelled-streptavidin-biotin immunoperoxidase antibody. Diaminobenzidine tetrahydrochloride was the substrate (LSAB-IP kit, DAKO).

The distribution of NOS II and NOS III isoforms was also studied, using double immunofluorescence. Tumor sections were fixed in acetone (10 min). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 70% methanol (30 min). Sections were then overlaid with mouse anti-rat CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1; Serotec) mab, followed by secondary anti-mouse preabsorbed labelled-streptavidin-biotin immunoperoxidase antibody. Diaminobenzidine tetrahydrochloride was the substrate (LSAB-IP kit, DAKO).

The distribution of NOS II and NOS III isoforms was also studied, using double immunofluorescence. Tumor sections were fixed in acetone (10 min). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 70% methanol (30 min). Sections were then overlaid with mouse anti-rat CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1; Serotec) mab, followed by secondary anti-mouse preabsorbed labelled-streptavidin-biotin immunoperoxidase antibody. Diaminobenzidine tetrahydrochloride was the substrate (LSAB-IP kit, DAKO).

The distribution of NOS II and NOS III isoforms was also studied, using double immunofluorescence. Tumor sections were fixed in acetone (10 min). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 70% methanol (30 min). Sections were then overlaid with mouse anti-rat CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1; Serotec) mab, followed by secondary anti-mouse preabsorbed labelled-streptavidin-biotin immunoperoxidase antibody. Diaminobenzidine tetrahydrochloride was the substrate (LSAB-IP kit, DAKO).

The distribution of NOS II and NOS III isoforms was also studied, using double immunofluorescence. Tumor sections were fixed in acetone (10 min). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in 70% methanol (30 min). Sections were then overlaid with mouse anti-rat CD31/platelet endothelial cell adhesion molecule 1 (PECAM-1; Serotec) mab, followed by secondary anti-mouse preabsorbed labelled-streptavidin-biotin immunoperoxidase antibody. Diaminobenzidine tetrahydrochloride was the substrate (LSAB-IP kit, DAKO).

Tumor microvascularization

Two methods were used to quantify tumor vascularization from immunostained sections. First, microvascular density (MVD) were measured by Chalkley point counting (38). A graticule containing 25 randomly positioned points was inserted into the microscope eyepiece (Leitz Laborlux 12) and sections were scanned (×40) to identify regions showing the greatest vascularization (39). Three hotspots were selected and examined at higher magnification (×250; field of view = 0.126 mm²). Any endothelial cell, or cluster of endothelial cells was taken to represent a vessel and a recognizable lumen was not necessary. Second, digitized images of immunostained material were obtained using an edge-detection algorithm, from which the fractional areas occupied by endothelial cells were calculated (NIH Image) Software.

**Reagents and sources**

BD-IX rats were supplied by the Gray Laboratory, Mount Vernon Hospital. L-NAME and AG were purchased from Sigma Ltd., 1400W was synthesized by Dr. Malcolm Stewart (University of St Andrews) and AMD compounds were synthesized by Dr. Beth Cameron (AnorMED Inc., British Columbia) as in reference (31).

**Statistical analyses**

Power analysis was used to estimate the sample size (\( n \)) required to reliably detect a change in tumor growth rate of approximately 25%. The probabilities of incorrectly rejecting the null hypothesis (\( H_0 \)) and of accepting a false null hypothesis (\( H_1 \)) were assigned values of 0.05 and 0.20, respectively. The coefficient of variation (SD/mean) was assumed to be 0.25 for treated and untreated groups. Calculation showed that a sample size of \( n = 11 \) for control and treated groups (\( n_1 = n_2 \)) would detect an effect size of 25% with a statistical power of 0.8 (1 – \( \beta \)). Some experiments called for unequal sample sizes, namely when comparing tumor growth rates for control groups of animals with treated groups in which some animals were sacrificed whereas others were allowed to recover. Unequal samples of \( n_1 = 16 \) and \( n_2 = 10 \) would allow an effect size of ± 25% to be detected with a statistical power of 0.85.

Data sets were analyzed (GraphPad Prism software) by 2-factor ANOVA, controlled for multiple comparisons, to determine whether differences between growth curves (treated vs. control animals) were significant. Student’s unpaired \( t \) test was used to determine the significance of

---

Nitric Oxide and Tumor Growth
differences in Chalkley scores. *P* values of less than 0.05 were considered significant.

**Results**

**Tumor growth curves**

All growth curves show mean tumor volumes (± SEM) versus time, where *n* = (number of tumors per rat) × (number of rats in each group). Tumor volumes increased in a near linear fashion while animals were being treated and also afterwards during the recovery period. The slopes of the regression lines are used to compare treated versus control growth rates, expressed throughout as mm$^3$ per day.

The nonselective NOS inhibitor L-NAME impairs tumor growth

Tumors grew more slowly on animals treated with L-NAME when compared with control (untreated) animals. This effect was reversed when treatment was withdrawn and animals were given water only. Figure 2 shows results from an experiment in which 18 rats were divided into 3 groups of 6 (4 = 24 tumors, 6 rats per group): group 1 served as controls (curve 1); group 2 received L-NAME (3 mg/mL) from day +15 to day +35 (curve 2); and group 3 received L-NAME from day +10 until day +28, at which time treatment ceased and animals were maintained for a further 7 days before being sacrificed (curve 3).

The growth of tumors on the animals of group 2 decreased during treatment with L-NAME, from 378 ± 19 mm$^3$/d ($r^2 = 0.97$; curve 1) to 114 ± 7 mm$^3$/d ($r^2 = 0.95$; curve 2; Fig. 2A), a reduction of 70% (2-factor ANOVA, *P* < 0.001). A similar reduction was seen in the animals of group 3, where the rate during treatment decreased by 67% to 126 ± 6.0 mm$^3$/d ($r^2 = 0.98$; curve 3; Fig. 2B; 2-factor ANOVA, *P* < 0.001). The subsequent removal of L-NAME from the drinking water in this group on day +28 allowed tumors to resume growing again. Data from a similar, independent experiment,

![Figure 2. Effects of L-NAME on tumor growth rates. A, curve 1, growth curve for control tumors. Addition of L-NAME (3 mg/mL) to the drinking water on day +15 to animals of group 2 (curve 2) resulted in a marked decrease (~70%) in tumor growth rates. *n* = 24 tumors, 6 rats for curves 1 and 2. B, a similar reduction in growth rate (~67%) was seen in animals of group 3 when treatment commenced on day +10 and was halted on day +28. Tumor growth increased on removing L-NAME from the drinking water (day +28; curve 3). C, effect of L-NAME showed a small dependence on concentration administered: filled squares, 1 mg/mL; empty diamonds, 6 mg/mL (n = 24 tumors, 6 rats). Addition of L-NAME (10 mg/mL) on day +20 (curve 4, filled triangles) halted tumor growth altogether (n = 16 tumors, 4 rats). D, results from an experiment in which L-NAME treatment (1 mg/mL) began on day +12 (left arrow) and was withdrawn on day +24 (right arrow), also showing resumption of tumor growth during the 7 day recovery period. Filled triangles, L-NAME treated; empty circles, control tumors. Means ± SEM shown (n = 12 tumors, 3 rats). The results in A and B were published previously in preliminary form. Reproduced with permission from Kennovin, G.D., Hirst, D.G., Stratford, M.R.L. and Flitney, F.W. [S. Moncada, M. Feelisch, R. Busse & E.A. Higgs, (eds.) (1994) Inducible nitric oxide synthase is expressed in tumourassociated vasculature: inhibition retards tumour growth, The Biology of Nitric Oxide, 259–263. © the Biochemical Society.]
where treatment with L-NAME (1 mg/mL) commenced on day +12 and ceased on day +24 (arrows), are presented in Fig. 2D. Again, growth was retarded during treatment (2-factor ANOVA, \( P < 0.05; n = 12 \) tumors, 3 rats) and resumed promptly after treatment ceased.

The efficacy of L-NAME depended upon the time at which treatment commenced relative to the time of implantation (day 0). In an independent experiment (not illustrated), one group of animals served as controls \((n = 16 \) tumors, 4 rats), whereas the other 2 were treated with L-NAME (1 mg/mL) commencing on day –7 or on day +12. Again, the growth of tumors on animals treated from day +12 was impaired relative to that of controls (2-factor ANOVA; \( P < 0.05; n = 12 \) tumors, 3 rats). However, tumors on animals treated with L-NAME from day –7 grew at a rate that was not significantly different from that of control tumors (2-factor ANOVA; \( P = 0.61; n = 16 \) tumors, 4 rats). In contrast, the ability of L-NAME to impede tumor growth was enhanced when treatment commenced at even later times. Thus, treatment that began on day +20 halted tumor growth completely (Fig. 2C; curve 4; \( n = 16 \) tumors, 4 rats).

Finally, the decrease in tumor growth rates showed only a small dependence on the concentration of L-NAME used. Figure 2C shows data for animals treated with 1 or 6 mg/mL (filled squares; empty diamonds, respectively), commencing on day 10 (\( n = 24 \) tumors, 6 rats). Growth retardation is near maximal at the lowest dose (i.e., 1 mg/mL).

**NOS II selective inhibitors have no effect on tumor growth**

The results obtained with AG and 1400W differ markedly from those obtained with L-NAME. Figure 3A shows that tumor growth was unaffected by AG (1 mg/mL), administered from day +17 to 35 (2-factor ANOVA; \( P = 0.90; n = 12 \) tumors, 3 rats). Similar results (not shown) were obtained when treatment with AG began (i) before the tumors were implanted (day –7; 2-factor ANOVA; \( P = 0.59; n = 16 \) tumors, 4 rats); or (ii) on day +12 following implantation, instead of on day +17 (2-factor ANOVA; \( P = 0.38; n = 20 \) tumors, 5 rats). Figure 3B shows that tumor growth was also unaffected when animals were treated with 1400W (2-factor ANOVA, \( P = 0.72; n = 12 \) tumors, 3 rats).

**Effect of Ru(III) polyaminocarboxylate scavengers on tumor growth**

Daily injections (i.p) of AMD6245 (Fig. 4A) or AMD6221 (Fig. 4B) from day +10 (downward arrows) until day +28 (upward arrows), at which time 3 animals were sacrificed and the remaining 5 were maintained for 7 days without further treatment. The growth rates of control tumors and of tumors during and after treatment were measured over the same time intervals to validate direct comparisons (viz: day 17–28 during treatment and day 29–35 during recovery).

Both NO scavengers retarded tumor growth. Figure 4 shows that the rate of growth of control tumors (\( n = 16 \) tumors, 8 rats) from day 17 to 28 was 433 ± 26 mm\(^3\)/d (\( r^2 = 0.97 \)) compared with (i) 118 ± 6 mm\(^3\)/d (\( r^2 = 0.98 \)) for AMD6245-treated animals (Fig. 4A), a reduction of 73% (2-factor ANOVA, \( P < 0.001; n = 16 \) tumors, 8 rats); and (b) 173 ± 12 mm\(^3\)/d (\( r^2 = 0.95 \)) for AMD6221-treated animals (B), a reduction of 61% (2-factor ANOVA, \( P < 0.001; n = 16 \) tumors, 8 rats). As seen during the recovery from L-NAME, tumor growth quickly resumed when treatment was discontinued (upward arrows; Fig. 4).

**The growth-retarding effects of L-NAME and Ru scavengers were accompanied by a reduction in MVD**

Figure 5 shows tumor sections immunostained with mAbs to CD31/PECAM-1, a specific marker for endothelial cells (Fig. 5A–C), or type III (endothelial) NOS (Fig. 5G and H). Chalkley counts (Fig. 6) revealed a large reduction in MVD (~77%; unpaired t test, \( P < 0.01 \)) in tumors from animals treated with AMD6245 (Fig. 5B; \( n = 9 \),

---

Figure 3. Tumor growth rates were not impaired by NOS-II selective inhibitors. Tumor growth rates were unaffected by either AG (A) or 1400W (B), both of which show greater selectivity toward NOS II. Open circles, control tumors (\( n = 12 \) tumors, 3 rats). Filled circles, treated tumors (A, \( n = 12 \) tumors, 3 rats; B, \( n = 12 \) tumors, 3 rats). Means ± SEM shown. Dose administered: 1 mg/mL for both AG and 1400W.
while a similar though smaller decrease (~60%) was observed in tumors from AMD6221-treated animals (Fig. 5C; unpaired \( t \) test \( P < 0.01; n = 6 \)). Treatment with L-NAME (Fig. 5H) was also associated with a large decrease in MVD (~68%) when compared with tumors from untreated animals (Fig. 5G; unpaired \( t \) test \( P < 0.01; n = 9 \)).

Microvascular profiles of CD31-stained sections were used to estimate the fractional area occupied by endothelial cells in tumors treated with NO scavengers (Fig. 5D–F). The results revealed a significant reduction (66%) when compared with control tumors. The individual values obtained ranged from 0.14 to 0.25, with a mean (± SEM) value of 0.21 ± 0.01 (n = 11) for control tumors, compared with (i) 0.06 to 0.26 with a mean of 0.14 ± 0.03 for AMD6221-treated tumors (n = 10; unpaired \( t \) test \( P < 0.01 \)); and (ii) 0.06 to 0.20, with a mean of 0.14 ± 0.02 for AMD6245-treated tumors (n = 9; unpaired \( t \) test, \( P < 0.01 \)). Figure 5I and J shows profiles of control and treated sections immunostained with NO III ab, respectively.

In striking contrast to the results obtained with L-NAME and both NO scavengers, treatment with either AG or 1400W had no effect on tumor MVDs (Fig. 6).

### Tumor recovery following treatment with L-NAME or Ru scavenger

Tumors resumed growing when treatments with L-NAME or Ru scavengers were discontinued. The mean rate of growth during recovery from L-NAME increased from 114 ± 7 mm³/d during treatment to 500 ± 75 mm³/d \( (r^2 = 0.92) \), a 4.4-fold increase (Fig. 2B). The corresponding values obtained after treating animals with Ru scavengers were less. Figure 4 shows that growth during recovery from AMD6245 increased 2.3-fold, from 118 ± 6 mm³/d to 275 ± 15 mm³/d \( (r^2 = 0.98) \), and 3.4-fold after AMD6221 treatment, from 173 ± 12 mm³/d to 589 ± 32 mm³/d \( (r^2 = 0.98) \).

These new rates of growth were compared with those of the corresponding control tumors measured over the same time interval. The values obtained for tumors recovering after AMD6245 and AMD6221 were 31% and 66% of the control rate (894 ± 34 mm³/d; \( r^2 = 0.92 \)) respectively. The data for L-NAME shown in Fig. 2B cannot be analyzed in the same way because control animals were sacrificed before recovery commenced, to comply with restrictions on tumor loading. However, in the experiment with L-NAME (1 mg/mL) illustrated in Fig. 2D, tumor growth during recovery increased 4.2-fold, from 74 ± 3 mm³/d \( (r^2 = 0.97) \) during treatment to 310 ± 25 mm³/d \( (r^2 = 0.99) \) during the recovery period. The latter amounted to 64% of the control value (484 ± 75 mm³/d; \( r^2 = 0.95 \)), similar to that seen after AMD6221 treatment but greater than during recovery from AMD6245.

The delay between discontinuing treatment with L-NAME or Ru scavengers and establishing these new rates of growth was remarkably short. Figure 7 illustrates results from 3 independent studies in which animals were allowed to recover after L-NAME (A), or after treatment with AMD6245 (B, filled circles) or AMD6221 (B, empty circles). Tumor volumes are here represented as a multiple of the mean value recorded on the day that treatment was suspended (time 0). These normalized data show that new steady-state rates of growth were attained very rapidly, probably within approximately 1 day of discontinuing treatment (vertical arrows).

Immunostaining of tumors from animals treated with NO scavengers revealed that tumor vascularization improved dramatically during the recovery period, in parallel with the rapid increase in tumor growth. The results of Fig. 6 (empty columns) show that Chalkley counts for tumors previously treated with AMD6221 recovered fully (unpaired \( t \) test, \( P > 0.1 \)). MVDs also increased when AMD6245 treatment was discontinued, but the recovery was incomplete and the mean value remained significantly lower than for control tumors (unpaired \( t \) test, \( P < 0.05 \)).

### Discussion

The ability of L-NAME to suppress tumor growth implies that NO facilitates tumor development (18).
view would be corroborated if alternative means of depleting NO also curbed tumor growth. We therefore studied the effects of 2 Ru-based NO scavengers, AMD6221 and AMD6245, on the growth and vascular morphology of the rat P22 carcinosarcoma, comparing their efficacies with those of L-NAME and 2 NOS II-selective inhibitors, AG and 1400W.

**Effects of L-NAME and NO scavengers on tumor growth and vascularization**

Treatment with L-NAME (Fig. 2) or with NO scavengers (Fig. 4) retarded tumor growth and decreased MVDs (Fig. 5, Fig. 6). The effects of AMD6245 (Fig. 4A) resembled those seen with L-NAME most closely: tumor growth rates were reduced by the same amount (~70%) and both treatments were associated with large decreases in tumor MVDs (Fig. 6). Tumors quickly began to grow again when treatment with L-NAME or NO scavengers was suspended. The experiments with AMD6221 and AMD6245 showed that renewed growth during the recovery period was accompanied by an increase in tumor MVDs.

Quantitatively, AMD6245 was more effective than AMD6221 at retarding tumor growth and decreasing MVDs. There were other notable differences too, in the way tumors responded after withdrawing treatment. Specifically, MVDs were fully restored during recovery from AMD6221 but only partially so after AMD6245; and the rate of tumor regrowth was greater for AMD6221 than for AMD6245.

These observations accord with kinetic studies, showing that the rate constant for the reaction of NO with AMD6245 is much greater (>10^3-fold) than for AMD6221, and with pharmacologic measurements showing that the clearance rate for AMD6221 is approximately 20-fold greater than for AMD6245 (29, 30). The latter result may help...
explain why MVDs were reinstated fully during recovery from AMD6221, but only in part after withholding AMD6245 (Fig. 6). The recovery period (7 days) was time enough for MVDs to recover fully from AMD6221, but tumors treated with AMD6245 may have required longer. The effect of prolonging the recovery period was not tested and therefore we cannot exclude the possibility that AMD6245 has other adverse effects on tumor growth that are not shared by AMD6221.

NOS II selective inhibitors have no effect on tumor growth or MVDs

AG and 1400W had no effect on tumor growth (Fig. 3) or vascularization (Fig. 6). This was an unexpected result because Western blots (not illustrated) identified NOS II (as well as NOS III) in tumor extracts. It could be explained however if the dose used (1 mg/mL) was insufficient to inhibit NOS II in vivo, or alternatively, if both compounds were degraded en route to the tumor, either in the gut and/or blood stream. AG retains its ability to inhibit NOS II when given orally at doses (0.5–2.0 mg/mL) comparable with the one used here (40–42) and both compounds remain active when administered by i.v. infusion or bolus injection (26), so neither explanation seems likely. Instead, the failure of AG and 1400W to influence tumor growth raises the intriguing possibility that NOS III may be a more important source of NO than NOS II in this particular model.

An antivasculor mechanism for the growth-retarding effects of L-NAME and Ru scavengers

Three of the compounds tested (L-NAME, AMD6221, AMD6245) impaired tumor growth and simultaneously reduced MVDs, whereas the remaining 2 (AG, 1400W) had no effect on either. This correlation implies causality and suggests the following mechanism whereby NO could influence tumor development.

Tumor feeder vessels are much less responsive to vasoconstrictors than normal vessels (18) due to increased NO synthesis following induction of the NOS II (high-output) isoform, therefore they are normally highly dilated, a condition that facilitates tumor perfusion. Treatments that lower NO will restore their sensitivity and cause them to constrict, reducing the amount of blood entering the tumor. Because the patency of vessels within a tumor is dictated by a balance between intra- and extra-vascular fluid pressures, the result would
be to compress the microvasculature and impede the delivery of oxygen and essential nutrients to the cells, hampering further growth. These events would reverse on withdrawing treatment: rising NO levels would cause tumor feeder vessels to dilate and increase blood flow into the tumor, reopening the collapsed vasculature and restoring supplies of oxygen and nutrients needed for renewed growth.

This hypothesis is supported by studies on the effects of NOS inhibitors on tumor perfusion. N\textsuperscript{G}-nitro-L-arginine (L-NNA) reduced blood flow in a rat mammary carcinoma (43), and L-NAME decreased vessel diameter in a human tumor xenograft (9) and a rat glioma (44). L-NNA decreased blood flow in the P22 tumor by as much as 55% and increased vascular resistance greater than 3-fold (19). Interestingly, a recent study (45), also using the P22 model, reported that AG and 1400W had no effect on tumor perfusion. This is an important result, because it suggests that NO must be severely depleted to reduce tumor perfusion sufficiently to check tumor growth and that this can be achieved using L-NAME or NO scavengers, but not by selective blockade of NOS II alone.

The antivasculature mechanism outlined above arises from our observation that growth retardation is invariably associated with reduced MVDs. This does not preclude an antiangiogenic effect, however, because this would present a similar histologic picture. We consider this to be unlikely for the following reasons. First, neither L-NAME nor NO scavengers affected tumor growth when treatment was brought forward (day –7) to encompass the early stages of tumorigenesis, that is, when an antiangiogenic effect would be most disruptive. Instead, treatment was effective only when initiated several days after implantation and was most effective when tumors were well established and of large size (Fig. 2C). Second, it is unlikely that a well-differentiated, fully functional neovascularature could assemble within the short-time taken to establish new steady-state rates of growth (ca. 1 day) after discontinuing treatment. However, reinflat- ing a preexisting, but collapsed vasculature, by dilating tumor feeder vessels, could quickly restore the oxygen and nutrients required for growth to resume.

In conclusion, this study shows that NO deprivation per se suppresses tumor growth, perhaps by constraining tumor feeder vessels and impeding tumor perfusion. It might be possible to exploit this effect when designing new therapies or as an adjunct to established forms of cancer treatment.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Professor Gill Tozer (University of Sheffield) and Professor Ian Megson (University of Highlands and Islands) for valuable discussion and Dr M. Metz, (Genzyme Corp. USA) for assistance in preparing Figure 1.

Grant Support

This work was supported by the Cancer Research Campaign (now Cancer Research UK) and AnorMED Inc. (British Columbia, Canada). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 7, 2010; revised May 31, 2011; accepted June 15, 2011. Published OnlineFirst June 28, 2011.

References


Molecular Cancer Therapeutics

Antitumor Actions of Ruthenium(III)-Based Nitric Oxide Scavengers and Nitric Oxide Synthase Inhibitors

Frederick W. Flitney, Rebecca J. Pritchard, Gordon D. Kennovin, et al.

Mol Cancer Ther Published OnlineFirst June 28, 2011.