Anti-tumor actions of ruthenium (III)-based nitric oxide scavengers and nitric oxide synthase inhibitors

by

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Abbreviations:
AG, 1-amino-2-hydroxyguanidine
AMD6221, [Ru(H3DTPA)Cl]
AMD6245, [Ru(HEDTA)H2O]
AMD3689, [Ru(H2DTPA)NO]
DAB, diaminobenzadine tetrahydrochloride
DTPA, diethylenetriaminepentaacetic acid
EDTA, ethylenediaminetetraacetic acid
L-NAME, N⁶-nitro-L-arginine methyl ester
L-NNA, N⁶-nitro-L-arginine
MVD, microvascular density
NO, nitric oxide
NOS, nitric oxide synthase
PBS, phosphate-buffered saline
PECAM, platelet endothelial cell adhesion molecule
RNS, reactive nitrogen species
1400W, N-[3-(aminomethyl)benzyl]acetamidine hydrochloride
ABSTRACT

The role of endogenous NO in the growth and vascularization of a rat carcinosarcoma (P22) has been investigated. Tumor-bearing animals were treated with (a) nitric oxide synthase (NOS) inhibitors, administered via the drinking water, including L-NAME, a non-isoform-selective inhibitor, and two others that target the inducible (NOS-II) enzyme preferentially, namely AG or 1400W; or (b) daily injections (i.p.) of two Ru (III)-polyaminocarboxylates, AMD6221 and AMD6245, both of which are effective NO scavengers. L-NAME, AMD6221 and AMD6245 reduced tumor growth, by ~ 60-75% of control rates. Tumor sections stained with abs to CD-31/PECAM-1 or NOS III showed that this was associated with a marked reduction (60-77%) of tumor MVDs. 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 re-establishing conditions that allow tumors to begin growing again.
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 L-arginine (1) by three 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\(^{2+}\)], generating localized NO 'transients' in the pM-nM range. NOS II is controlled transcriptionally and provides a ‘high-output’ pathway for NO, creating sustained [NO]s in the μM range independently of changes in intracellular [Ca\(^{2+}\)] (2,3).

NOS isoforms have been identified in malignant tumors where their expression levels correlate with tumor grade. NOS II and NOS III are co-expressed 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 L-arginine that competitively inhibit all three isoforms (11), together with inhibitors with much greater selectivity for NOS II, such as AG (12,13) and 1400W (14).

NO can inhibit or enhance tumor growth (15), depending on its concentration. Low [NO]s 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 [NO]s 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 isoenzyme 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).
Ruthenium(III) polyaminocarboxylates of EDTA or DTPA function as pentadentate ligands that satisfy these criteria.

Several chemically-related Ru(III) complexes, including K[Ru(H$_3$EDTA)Cl] (AMD1226), [Ru(HEDTA)H$_2$O] (AMD6245), and [Ru(H$_3$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 demonstrated 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 L-NAME, a non-selective 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 Figure 1.
MATERIALS AND METHODS

The procedures used throughout complied with the requirements of the Animals (Scientific Procedures) Act 1986 and with the prevailing UKCCCR 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. 1mm³) 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 performed 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 p.o., C-Vet) or buprenorphine (0.5mg.kg⁻¹ s.c., Schering) were administered for 24-48 hours to alleviate post-operative 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, \( V = \frac{1}{6} \pi (X^2 \times Y) \). 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 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 (0.2M, 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/PECAM-1 (Serotec) mab, followed by secondary anti-mouse pre-absorbed labelled-streptavidin-biotin immunoperoxidase antibody. Diaminobenzadine tetrahydrochloride (DAB) 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 as above, overlaid with abs to NOS II (Santa Cruz Biotech) or NOS III (Transduction Labs) for 45 min and then rinsed in phosphate-buffered saline (PBS). Primary abs were detected using fluorescein- or rhodamine-labelled mouse secondary antibodies (Santa Cruz Biotech). Low power photomicrographs were taken on a Zeiss Ultraphot fluorescence microscope.

**Tumor microvascularization**

Two methods were used to quantify tumor vascularization from immunostained
sections. First, MVDs 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 (x 40) to identify regions showing the greatest vascularization (39). Three 'hotspots' were selected and examined at higher magnification (x 250; field of view = 0.126mm$^2$). 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 J 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 (31).

**Statistical analyses**

Power analysis was used to estimate the sample size (n) required to reliably detect a change in tumor growth rate of ~ 25%. The probabilities of incorrectly rejecting the null hypothesis (= $\alpha$) and of accepting a false null hypothesis (= $\beta$) 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 while others were allowed to recover. Unequal samples of $n_1 = 16$ and $n_2 = 10$ would allow an effect size of $\pm 25\%$ 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 differences in Chalkley scores. P values of $<0.05$ were considered significant.
RESULTS

Tumor growth curves

All growth curves show mean tumor volumes (+/- SEM) versus time, where n = (number of tumors per rat) x (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 vs control growth rates, expressed throughout as mm$^3$/day.

The non-selective 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 three groups of 6 (n = 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$^3$/day ($R^2 = 0.97$; curve 1) to 114 +/- 7 mm$^3$/day ($R^2 = 0.95$; curve 2; Figure 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 $126 \pm 6.0 \text{ mm}^3/\text{day}$ ($R^2 = 0.98$; curve 3; Figure 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, where treatment with L-NAME (1 mg/ml) commenced on day +12 and ceased on day +24 (arrows), are presented in Figure 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), while the other two 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 (Figure 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 (1mg/ml), administered from day +17-35 (2-factor ANOVA; p = 0.90; n = 12 tumors, 3 rats). Similar results (not shown) were obtained when treatment with AG began (a) before the tumors were implanted (day -7; 2-factor ANOVA; p = 0.59; n = 16 tumors, 4 rats); or (b) 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 (Figure 4A) or AMD6221 (Figure 4B) from day +10 (downward arrows) until day +28 (upward arrows), at which time three 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 17-28 was 433 +/- 26 mm³/day (R² = 0.97) compared with (a) 118 +/- 6 mm³/day (R² = 0.98) for AMD6245-treated animals (A), a reduction of 73% (2-factor ANOVA, p < 0.001; n = 16 tumors, 8 rats); and (b) 173 +/- 12 mm³/day (R² = 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; Figure 2).

**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 (A-C), or type III (endothelial) NOS (G and H). Chalkley counts (Figure 6) revealed a large reduction in MVD (~ 77%; unpaired t-test, p< 0.01) in tumors from animals treated with AMD6245 (B; n = 9), while a similar though smaller decrease (~ 60%) was observed in tumors from AMD6221-treated animals (C; unpaired t-test p<0.01; n = 6). Treatment with L-NAME (H) was also associated with a large decrease in MVD (~ 68%) when compared with tumors from untreated animals (G; 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 (panels D-F in Figure 5). The results revealed a significant reduction (66%) when compared with control tumors. The individual values obtained ranged from 0.14-0.25, with a mean (+/-
SEM) value of 0.21 +/- 0.01 (n = 11) for control tumors, compared with (a) 0.06-0.26 with a mean of 0.14 +/- 0.03 for AMD6221-treated tumors (n = 10; unpaired t-test p< 0.01); and (b) 0.06-0.20, with a mean of 0.14 +/- 0.02 for AMD6245-treated tumors (n = 9; unpaired t-test, p< 0.01). Figure 5 I, J shows profiles of control and treated sections immunostained with NOS-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 (Figure 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 mm$^3$/day during treatment to 500 +/- 75 mm$^3$/day ($R^2 = 0.92$), a 4.4-fold increase (Figure 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 mm$^3$/day to 275 +/- 15 mm$^3$/day ($R^2 = 0.98$), and 3.4-fold after AMD6221 treatment, from 173 +/- 12 mm$^3$/day to 589 +/- 32 mm$^3$/day ($R^2 = 0.98$).

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
mm$^3$/day; $R^2 = 0.92$) respectively. The data for L-NAME shown in Figure 2B cannot be analysed 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 Figure 2D, tumor growth during recovery increased 4.2-fold, from 74 +/- 3 mm$^3$/day ($R^2 = 0.97$) during treatment to 310 +/- 25 mm$^3$/day ($R^2 = 0.99$) during the recovery period. The latter amounted to 64% of the control value (484 +/- 75 mm$^3$/day; $R^2 = 0.95$), 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 three 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 ~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 Figure 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). This view would be corroborated if alternative means of depleting NO also curbed tumor growth. We therefore studied the effects of two 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 two NOS II-selective inhibitors, AG and 1400W.

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

Treatment with L-NAME (Figure 2) or with NO scavengers (Figure 4) retarded tumor growth and decreased MVDs (Figure 5,6). The effects of AMD6245 (Figure 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 (Figure 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 (>10³ fold) than for AMD6221, and with pharmacological measurements showing that the clearance rate for AMD6221 is ~ 20x 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 (Figure 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 (Figure 3) or vascularization (Figure 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 anti-vascular 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, while the remaining two (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. Since 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, re-opening 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. L-NMMA reduced blood flow in a rat mammary carcinoma (43) while 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 > 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 ‘anti-vascular’ mechanism outlined above arises from our observation that growth retardation is invariably associated with reduced MVDs. This does not preclude an anti-angiogenic effect however, because this would present a similar histological 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 anti-angiogenic 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 (Figure 2C). Second, it is unlikely that a well-differentiated, fully-functional neovasculature could assemble within the short time
taken to establish new steady-state rates of growth (ca 1 day) after discontinuing treatment. However, re-inflating a pre-existing 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 constricting 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.

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FIGURE LEGENDS
Figure 1. Structural formulae of NOS inhibitors and Ru scavengers. A. $N^G$-nitro-L-arginine methyl ester (L-NAME); B. 1-amino-2-hydroxyguanidine (AG); C. N-[3-(aminomethyl)benzyl]acetamidine hydrochloride (1400W); D. [Ru(H$_3$DTPA)Cl] (=AMD6221). E. [Ru(HEDTA)H$_2$O] (=AMD6245).

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). N = 24 tumors, 6 rats for 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 and are presented here with permission).

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 towards 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.

**Figure 4. Effects of Ru scavengers on tumor growth.** Daily injections (50mg/kg) of AMD6245 (A) and AMD6221 (B) impaired tumor growth. The effect was partially reversible: tumor growth resumed rapidly when treatment ceased on day + 28 (upward arrow). Open circles, control tumors (n = 16 tumors, 8 rats); filled circles, treated tumors (n = 16 tumors, 8 rats for AMD6245 and AMD6221). Means +/- SEM shown. Arrows indicate period of treatment. Three animals were sacrificed on day +28 and remaining 5 were allowed to recover for 7 days.

**Figure 5. Effects of L-NAME and Ru scavengers on tumor vascularization.** Tumor sections were stained with mab to CD31/PECAM-1 (A-C) or type III NOS (G, H). A, G: control tumors; B, AMD6245 treated; C, AMD6221 treated; H, L-NAME treated. D-F and I and J: microvessel profiles obtained using an edge-detection algorithm (NIH Image J software) showing reduced occurrence of vessel lumena in treated tumors as compared to corresponding (above) control vessels. Scale bar = 500 μm.

**Figure 6. Effects of NOS inhibitors and NO scavengers on MVD.** Treatment with L-NAME, but not aminoguanidine or 1400W, significantly reduced MVDs as compared to control animals. MVDs were also reduced in tumors from animals receiving daily injections of AMD 6245 or AMD 6221. The effects of Ru compounds were partially (AMD6245) or fully (AMD6221) reversed (open columns) when treatment ceased. Means +/- SEM shown. * = P<0. 05; ** = P<0. 01 (all test vs controls).
Figure 7. Tumor growth resumed rapidly when treatment with L-NAME or Ru scavengers was terminated. A, Data from 3 independent experiments in which animals received either 1mg/ml (open circles and open triangles) or 3 mg/ml (filled circles) L-NAME for 14-15 days and were then allowed to recover. B, Regrowth of tumors on animals previously treated (day 10-28) with AMD6245 (filled circles) or AMD6221 (empty circles) during the 7 day recovery period. All results are normalized to tumor volumes measured at time of discontinuing treatment (day 0). Mean values only shown.
Figure 1

A

\[ \text{H}_2\text{N} - \text{NNO}_2 \]

\[ \text{H}_2\text{N} - \text{CO}_2\text{CH}_3 \]

B

\[ \text{NH}_2 \]

\[ \text{HN} \]

\[ \text{N} - \text{NH}_2 \]

\[ \text{H} \]

C

\[ \text{HN} \]

\[ \text{N} - \text{N} \]

\[ \text{CH}_3 \]

\[ \text{NH}_2 \]

D

\[ \text{O} \]

\[ \text{C} \]

\[ \text{N} - \text{Ru} - \text{Cl} \]

\[ \text{O} \]

\[ \text{O} \]

E

\[ \text{N} - \text{Ru} - \text{OH}_2 \]

\[ \text{O} \]

\[ \text{N} - \text{Ru} - \text{OH}_2 \]

\[ \text{O} \]
Figure 2

![Graph showing tumor volume over days post implantation for different groups.]

A: Tumor volume (mm³) vs. Days (post implantation) with groups 1 and 2.
B: Tumor volume (mm³) vs. Days (post implantation) with groups 1 and 3.
C: Tumor volume (mm³) vs. Days (post implantation) with groups 1 and 4.
D: Tumor volume (mm³) vs. Days (post implantation) with groups 3 and 4.
Figure 3
Figure 4
Figure 5
Figure 6

Chalkley score (% of control)

- L-NAME
- AG
- 1400W
- 6245
- 6221
- 6345
- 6221

NSD NSD NSD NSD NSD NSD NSD

* **
Figure 7

Relative tumor volume vs. time from halting treatment (days)

(A) Panel showing the relationship between relative tumor volume and time.

(B) Panel showing a similar relationship with an arrow indicating a specific time point.

Time from halting treatment (days)
Molecular Cancer Therapeutics

Anti-tumor actions of ruthenium (III)-based nitric oxide scavengers and nitric oxide synthase inhibitors

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

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