Nitric Oxide/Nucleophile Complexes Inhibit the in Vitro Proliferation of A375 Melanoma Cells via Nitric Oxide Release

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ABSTRACT

Cell-mediated antitumor effects have, in part, been attributed to the production of NO. Compounds which generate NO might, therefore, be useful in attenuating the growth of tumor cells. Six nitric oxide/nucleophile adducts that release NO spontaneously in solution were tested for their effectiveness in inhibiting DNA synthesis in A375 human melanoma cells. The complexes of NO with spermine, 3-(n-propylamino)propylamine (PAPA/NO), and diethylamine reduced thymidine incorporation by 50% at concentrations of 24, 44, and 128 μM, respectively. The degree of inhibition was, in general, related to the rate and extent of NO release in solution. A melanoma cell clone sensitive to interleukin 1-mediated cytostasis (A375-C6) was no more sensitive to PAPA/NO than a clone resistant to interleukin 1 (A375-C5), suggesting that the differing inhibitory effects of interleukin 1 in the two A375 cell clones are not due to a differential sensitivity to nitric oxide. Oxymyoglobin (125 μM), a known scavenger of NO, restored the ability of A375-C6 cells to incorporate thymidine in the presence of up to 200 μM PAPA/NO. When PAPA/NO was added to a solution of oxymyoglobin, nitrosomyoglobin was formed, indicating that the protective effect of myoglobin was due to scavenging of NO. The results are consistent with a nitric oxide-mediated mechanism for NO/nucleophile cytostasis and suggest that such compounds may be useful as tools for investigating the role of reactive nitrogen intermediates in cytostasis and cytotoxicity.

INTRODUCTION

Cytotoxic activated macrophages, important in the immune response to foreign agents, act in part through the generation of reactive nitrogen intermediates, including NO (1–8). Normal hepatocytes (9) and tumor cells (3–6) are susceptible to NO cytostasis. There are several mechanisms of NO action, including inhibition of DNA synthesis (5, 10, 11), inhibition of mitochondrial respiration (1, 6), and interference with iron-sulfur proteins (5, 12, 13). Recently NO has been shown to inhibit cytochrome P-450 (14). An inverse relationship between the generation of NO by tumor cells and their metastatic potential has been proposed (15).

The cytostatic effects of nitric oxide imply that compounds which release NO might be useful in attenuating tumor cell growth. Glycerol trinitrate and SNP, which yield NO after metabolic activation, are two clinically useful nitrovasodilators (16). Both glyceryl trinitrate and SNP inhibited the DNA synthesis of RACS-1 vascular smooth muscle cells but not of Chinese hamster fibroblast V79 cells (17). SNP has been reported variously to have a role in killing primary rat neuronal cells (18) and attenuation of glutamate-induced cell death (19). SNP also induced iron release from ferritin and catalyzed lipid peroxidation (12). Experiments in which SNP was used as a means of delivering NO should be interpreted carefully, due to the potential for biological activity from the cyanoferriate portion of the molecule (19). A third agent, S-nitroso-N-acetylpenicillamine, also releases NO in solution and was effective at inhibiting DNA synthesis and proliferation of BALB/c 3T3 fibroblasts lacking soluble guanylate cyclase activity (20).

Recently the vascular effects of a group of compounds which do not require metabolism or reduction to release NO were described (21). These nitric oxide/nucleophile complexes are of the general structure [XN(O)NO]−, where X is a nucleophile residue and can, in principle, liberate 2 molecules of NO/molecule of starting material. In practice, the extent of NO release, as well as the rate, can be modified by changing the structure of the nucleophile residue (21). The present investigations were designed to determine if the NO/nucleophile compounds would be cytostatic (or cytotoxic) and, if so, the extent to which the effect could be attributed to nitric oxide. The results bear on the selection and design of NO/nucleophile complexes for studies of potential tumoricidal activity.

MATERIALS AND METHODS

Chemicals. DEA/NO (Chemical Abstracts registry number 92382-74-6), IPA/NO (89603-57-6), OX1/NO (37035-81-7), SPER/NO (136587-13-8), and SULFI/NO (66375-30-2) were synthesized according to procedures in the literature (21, 22). Isopropylamine, 3-(n-propylamino)propylamine, and diethylenetriamine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Spermine and N-nitrosodiethylenetriamine were purchased from Sigma Chemical Co. (St. Louis, MO). Nitric oxide gas was purchased from Matheson Gas Products (East Rutherford, NJ). Sodium nitrite was obtained from Fisher Scientific Chemicals (Springfield, NJ). RPMI 1640 and PBS were obtained from Advanced Biotechnologies, Inc. (Columbia, MD). Fetal bovine serum was purchased from HyClone (Logan, UT). All remaining reagents were of analytical grade or better and were purchased from major suppliers.

Preparation of PAPA/NO. A solution of 3-(n-propylamino)propylamine (10.0 g, 86 mmol) in 150 ml of anhydrous ethyl ether was placed in a standard grade or better and were purchased from major suppliers.

Preparation of PAPA/NO. A solution of 3-(n-propylamino)propylamine (10.0 g, 86 mmol) in 150 ml of anhydrous ethyl ether was placed in a standard glass Parr hydrogenation bottle (Parr Instrument Company, Moline, IL) and deoxygenated with nitrogen. The solution was maintained at about 22°C and placed under NO gas at a pressure of 5 atmospheres. The reaction was contained in a modified Parr model 3911 hydrogenation apparatus equipped with a magnetic stir bar and constructed of stainless steel to resist corrosion. The mixture was stirred, and NO was added as required to maintain a pressure of 70–75 psig. After 3 days the excess pressure was vented, and the mixture was flushed with nitrogen for 2 min. The voluminous white precipitate was filtered, washed with ether, and dried in vacuo for 2 h to afford 3.03 g of PAPA/NO (89%). The white solid was recrystallized twice from 10 ml of methanol to afford 1.7 g of white PAPA/NO (89%). The yields were found to be 89% and 91% after two recrystallizations, respectively. The purity of the product was confirmed by spectral analysis.

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3 The abbreviations used are: SNP, sodium nitroprusside; IPA/NO, the isopropylamine/NO adduct sodium salt; DEA/NO, the diethylenetriamine/NO adduct sodium salt; OX1/NO, sodium trioxodinitratotelurite (Angel’s salt); SPER/NO, the spermine/NO adduct monohydrate; SULFI/NO, the bis(ammonium) salt of N-nitrosohydroxylationamine-N′-sulfonate; PAPA/NO, the NO adduct of 3-(n-propylamino)propylamine; PBS, phosphate-buffered saline; t½, half-life of first-order decomposition; Eg50, the number of moles of NO spontaneously liberated per mole of starting material decomposed; MB02, oxymyoglobin; [1H]dThd, tritiated thymidine; IC50, concentration required to inhibit [1H]dThd incorporation by 50%.

564

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the rates and extents of nitric oxide release for DEA/NO, IPA/NO, SPER/NO, OXII/NO, and SULFI/NO (21). In the same manner the rate of NO release from PAPA/NO was determined spectrophotometrically by following the disappearance of the chromophore at 252 nm ($E_{\max } = 8.1 \pm 0.2 \text{ mM}^{-1} \text{ cm}^{-1}$) in 37°C PBS, pH 7.2. The half-life was calculated from the slope of a plot of ln (absorbance – absorbance) versus time. $E_{\max }$ for PAPA/NO was determined at pH 7.2 and 37°C using a chemiluminescence method, in which released NO is reacted with ozone (21, 23).

Oxymyoglobin. MbO2 was prepared by reduction of ferric myoglobin from horse skeletal muscle (Sigma Chemical Co., St. Louis, MO). Myoglobin (350 mg) was dissolved in 5 ml of distilled water and dialyzed against 500 ml of 50 mM ascorbate in PBS at 4°C. After 12 h the solution was dialyzed twice against 500 ml of PBS at 4°C and filtered. A portion was diluted, and the MbO2 concentration was determined from the absorbance at 579 nm ($\epsilon = 14.4 \text{ mM}^{-1} \text{ cm}^{-1}$) (24) using a Hewlett Packard 8451A diode array spectrophotometer. The remainder was diluted to yield a stock MbO2 solution of 2.5 mM, 0.05 ml of which was added per ml of incubate (final MbO2 concentration, 125 $\mu$M).

Nitric Oxide Solutions. Saturated solutions of NO (Chemical Abstracts registry number 10102-43-9) were prepared by degassing 50 ml of 0.1 M PBS under vacuum (20 millitorr) for 2 h. Nitric oxide was introduced into the degassed solution after passing through concentrated KOH. Samples were immediately inverted in order to minimize exposure of septa to concentrated NO gas, leaving only an inert (glass) surface in contact with the headspace. Saturation was confirmed by checking the chemiluminescence response as described above.

Cell Lines. The human melanoma cell line A375 established by Giard et al. (25) was subjected to cloning by the limiting dilution method, yielding two clones, one sensitive (A375-C6) and one resistant (A375-C5) to interleukin-1-mediated cytoidal effects (26). These clones were maintained in RPMI 1640 supplemented with 5% fetal bovine serum.

DNA Synthesis. Incorporation of [3H]dThd was used as a measure of DNA synthesis. Subconfluent A375 cells were detached by a brief exposure to trypsin, washed twice with PBS, and resuspended in RPMI 1640 containing 5% fetal bovine serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. Cells were diluted to 5.3 $\times$ 10^5 cells/ml. 0.05-$\mu$l aliquots were distributed into microtubes. Samples were immediately inverted in order to minimize exposure of septa to concentrated NO gas, leaving only an inert (glass) surface in contact with the headspace. Saturation was confirmed by checking the chemiluminescence response as described above.

RESULTS

Five of the six nitric oxide/nucleophile complexes we studied inhibited DNA synthesis in A375-C6 human melanoma cells in culture. SPER/NO was the most potent inhibitor of DNA synthesis, with PAPA/NO, DEA/NO, OXII/NO, and, to a lesser extent, IPA/NO also showing activity (Fig. 1). The IC$_{50}$ was obtained with the aid of a computer program (TableCurve; Jandel Scientific, Corte Madera, CA). The data were fit to a dose-response equation of the form

$$\text{relative incorporation} = a + \frac{b}{1 + \left(\frac{\text{dose}}{c}\right)^d}$$

where $a$, $b$, $c$, and $d$ are constants. This equation is equivalent to the sigmoid $Emu^*$ model with baseline correction as described by Holford and Sheiner (27). Values of the IC$_{50}$ obtained from 3–6 separate trials were used to calculate average IC$_{50}$ and error estimates.

Cell Viability. The cytotoxic activity of NO/nucleophile complexes was determined by assessing cell viability by trypan blue exclusion. A375-C6 cells (0.95 ml, 5.3 $\times$ 10^5 cells/ml) were added to individual wells of 24-well tissue culture plates (Costar). Fifty $\mu$l of test agent (or PBS control) were added and allowed to incubate at 37°C in 5% CO$_2$ for 8 h. Trypsin (100 $\mu$l) was added and allowed to incubate for 5 min, after which cells were gently detached with a rubber policeman. Cells were mixed with trypan blue, and the fraction of nonviable cells was determined.

Fig. 1. DNA synthesis by A375-C6 cells exposed to various NO/nucleophile complexes as measured by [3H]dThd incorporation. Each curve represents 3 experiments, with 3–6 wells/experiment/point (n = 9–18 wells/point). Points, means; bars, 95% confidence intervals. The compounds tested were: SULFI/NO ($\bullet$); IPA/NO ($\circ$); OXII/NO ($\Delta$); DEA/NO ($\triangle$); PAPA/NO ($\triangle$); and SPER/NO ($\circ$). Curves were fit to the data using a dose-response equation as described in the text. The concentrations of each agent required to inhibit [3H]dThd incorporation by 50% are listed in Table 1. Authentic NO, added as 53 $\mu$M of a saturated solution to 950 $\mu$M of cells, decreased thymidine incorporation to 72 ± 17% of the control. SULFI/NO, which releases minuscule amounts of NO, did not inhibit incorporation (110 ± 15% relative incorporation at 500 $\mu$M). In 23 experiments, radioactivity values for the controls ranged from 15,000 to 50,000 (average 32,000) cpm.
test agents in PBS in the absence of medium, and cell viability was assessed before \[^{3}H\]dThd was added to equal numbers of viable cells. This format provided for the separation of cytostatic and cytotoxic effects within a given experiment. Later tests were conducted with the exposure to drugs occurring in the presence of RPMI 1640 with 5% fetal bovine serum for a longer time (8 h versus 30 min) and with a shorter recovery period (8 versus 20–22 h). Use of the shorter recovery period and longer exposure time in the presence of serum resulted in a slightly enhanced cytostatic effect for DEA/NO and PAPA/NO but did not influence the effect of OXI/NO. Using the shorter exposure times, the IC\textsubscript{50} was approximately 200 \mu M for DEA/NO and PAPA/NO, and 100–400 \mu M for OXI/NO. Exposure of cells to drugs in the presence of a complex medium containing serum is more representative of an in vivo situation and was therefore used for the experiments reported here.

Two A375 clones, one sensitive to interleukin 1\alpha (A375-C6) and one resistant to interleukin 1\alpha (A375-C5), did not differ in their cytostatic effects (Table 1). Spermine, the polyamine decomposition product of spermine/NO, inhibited DNA synthesis by 49% at 500 \mu M. This effect of polyamines on melanoma cells has been reported previously (35). However, although it is apparent that a portion of the activity of spermine/NO resulted from spermine released upon decomposition, spermine/NO was still approximately 200-fold more potent than the free amine.

Macrophage-mediated cytotoxicity and cytostasis result, in part, from NO synthesis induced by cytokines. NO inhibits DNA synthesis by inhibiting ribonucleotide reductase, a rate-limiting enzyme in the process (5, 10, 11). NO also inhibits mitochondrial respiration by reacting with aconitase (1, 5). Other proteins containing iron-sulfur clusters may be influenced as well, and NO has been reported to increase iron loss from cells (summarized in Ref. 5) as well as the release of iron from ferritin (12). Finally, NO can inhibit protein synthesis (9) and may have other mechanisms of action as well (33).

Whether NO acts as a cytostatic or cytotoxic agent is likely to depend, in part, upon the cell type. In the present report we describe the finding that a group of NO-releasing compounds which have previously been shown to have vasodilatory (21) and genotoxic (34) properties also inhibit DNA synthesis in melanoma cells in a manner consistent with an NO-mediated mechanism. Cytostasis, the inhibition of cell division, was measured using thymidine incorporation (6) as an index of DNA synthesis, while trypan blue exclusion was used to measure cell viability (18).

Several of these nitric oxide/nucleophile complexes caused substantial inhibition of DNA synthesis in the human melanoma cell line A375. The two most potent, SPER/NO and PAPA/NO, inhibited thymidine incorporation by 50% at concentrations of 24 and 44 \mu M, respectively (Table 1). Spermine, the polyamine decomposition product of SPER/NO, inhibited DNA synthesis by 49% at 500 \mu M. This effect of polyamines on melanoma cells has been reported previously (35). However, although it is apparent that a portion of the activity of SPER/NO resulted from spermine released upon decomposition, SPER/NO was still approximately 200-fold more potent than the free amine.

| Table 1 Inhibition of \[^{3}H\]dThd incorporation by NO/nucleophile complexes |
|-----------------------------|-------|-------|-------|-------|-------|-------|-------|
| Compound | IC\textsubscript{50} (\mu M) | 125 | 250 | 500 | 1000 |
| SPER/NO | 24 ± 6 | 44 ± 12 | 128 ± 6 | 287 ± 51 | 4 ± 2 |
| PAPA/NO | 24 ± 6 | 44 ± 12 | 128 ± 6 | 287 ± 51 | 4 ± 2 |
| DEA/NO | 24 ± 6 | 44 ± 12 | 128 ± 6 | 287 ± 51 | 4 ± 2 |
| OXI/NO | 287 ± 51 | 574 ± 59 | 1148 ± 45 | 2296 ± 89 | 62 ± 2 |
| IPA/NO | >500 | 298 ± 41 | 636 ± 52 | 1272 ± 105 | 62 ± 2 |
| SULF/NO | NR | 24 ± 6 | 44 ± 12 | 128 ± 6 | 287 ± 51 |

\( a \) Inhibited thymidine incorporation by 43% at 500 \mu M.

\( b \) No response, i.e., did not inhibit DNA synthesis at 500 \mu M. This compound, a negative control, contains the \( \text{N}^\circ \text{O}^{-} \) functionality but does not release nitric oxide (21).

Because PAPA/NO showed strong cytostatic activity (Fig. 1) without being cytotoxic (Table 2), this agent was chosen for experiments on the blocking effect of myoglobin. MBo2 at a concentration of 125 \mu M prevented the inhibition of DNA synthesis induced by 200 \mu M PAPA/NO (Fig. 2). When 100 \mu M MBo2 was incubated with 260 \mu M PAPA/NO at 37°C and pH 7.2, there was an initial rapid reaction yielding a product with the visible spectrum of metmyoglobin. Formation of the intermediate appeared to peak within 5 min, and over the next hour the nitrosylmyoglobin complex formed (Fig. 3). The spectra in Fig. 3 for MBo2 and nitrosylmyoglobin resemble those reported by Kanner et al. for these compounds (24).

**DISCUSSION**

Nitric oxide has recently been reported to have a number of roles in signal transduction, including vasodilation, inhibition of platelet aggregation, neurotransmission, and nonspecific immunity (29–32).

![Fig. 2. Protective effect of MBo2 on PAPA/NO-induced cytostasis. Data were collected with (C) and without (B) pretreatment with 125 \mu M MBo2 immediately before adding PAPA/NO. MBo2 prevented PAPA/NO-induced inhibition of DNA synthesis. Each curve represents 3 experiments, with 3–6 wells/experiment/point (n = 9–18 wells/point). Points, means; bars, 95% confidence intervals. The IC\textsubscript{50} for PAPA/NO without MBo2 was 69 \mu M.](https://cancerres.aacrjournals.org/article-pdf/53/2/555/2261629/article-pdf-04.png)

**Table 2 Viability of A375-C6 cells exposed to NO/nucleophile complexes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (\mu M)</th>
<th>Percentage nonviable</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4.4 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>SPER/NO</td>
<td>11.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Spermine</td>
<td>4.1 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>PAPA/NO</td>
<td>2.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>DEA/NO</td>
<td>5.3 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>OXI/NO</td>
<td>9.5 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>IPA/NO</td>
<td>5.8 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>SULF/NO</td>
<td>6.6 ± 4.2</td>
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* Percentage of A375-C6 cells killed following an 8-h incubation with the indicated compound. Data represent the mean ± SD of three experiments. Viability was assessed by trypan blue exclusion (n = 8–12 wells).

* PBS, 0.1 M phosphate-buffered saline control (n = 35 wells).
With the exception of spermine, none of the other free amines [diethylamine, isopropylamine, 3-(n-propylamino)propylamine] corresponding to the nucleophiles in the complexes exhibited a cytostatic effect in A375 cells. Potential products of NO/nucleophile decomposition under oxidative conditions include not only the free amines and NO, but N-nitroso derivatives as well. The inactivity of 500 μM N-nitrosodiethylamine (data not shown), a known by-product of DEA/NO degradation, suggests that nitrosamines are not responsible for the cytostatic activity of the NO/nucleophile complexes. Nitrite, an end product of NO oxidation (34), was also inactive at up to 500 μM. Furthermore, SULFI/NO, which contains the N₂O₂⁻ moiety but does not release NO (21), did not inhibit DNA synthesis. These factors suggest that NO, or an oxide of nitrogen derived from it, was the product of NO/nucleophile complex degradation responsible for cytostasis.

Cytostatic activity of the NO/nucleophile complexes was, in part, roughly related to the rate and extent of NO release. Compounds which released NO slowly, such as SPER/NO (τₙₛ = 39 min) and PAPA/NO (τₙₛ = 8.0 min), were more potent inhibitors of DNA synthesis than compounds which released NO quickly, such as DEA/NO (τₙₛ = 2.1 min) and IPA/NO and OXI/NO (both with τₙₛ = 2.3 min). Authentic NO gas, which has been reported to have a lifetime of 4 s or less in biological fluids (36), was approximately as cytostatic as OXI/NO and DEA/NO at a dose of 100 μM (Fig. 1). This supports the contention of Stuehr and Nathan (6), who postulated that a sustained exposure to moderate amounts of NO would have a longer-lasting effect than a brief exposure to a greater concentration of NO. In addition to the rate of degradation, the extent of NO release should be an important determinant of activity. Eₓₙₒ, the number of molecules of NO released per molecule of starting material, has a theoretical maximum of 2.0 for the NO/nucleophile complexes. Compounds with substantial values for Eₓₙₒ such as SPER/NO (1.9 ± 0.003) and PAPA/NO (2.1 ± 0.1) were more potent inhibitors of DNA synthesis (Table 1) than compounds with smaller values for Eₓₙₒ such as IPA/NO (0.73 ± 0.04) and OXI/NO (0.54 ± 0.04). DEA/NO, which has an intermediate value for Eₓₙₒ (1.5 ± 0.11), was also intermediate in potency (Table 1). The relationship between Eₓₙₒ and IC₅₀, while suggestive, was not a linear one, in part because the IPA/NO complex released moderate amounts of NO but was cytostatic only at relatively higher doses (Fig. 1). The poor response to IPA/NO was not the result of a short half-life because DEA/NO, with a similar half-life (2.3 versus 2.1 min, respectively), was more active. This indicates that factors in addition to the rate and extent of nitric oxide release influence the activity of the NO/nucleophile complexes. Preliminary experiments, conducted to establish effective conditions for the cytostasis protocol, suggested that the presence of serum and changes in the exposure time and recovery time may influence the activity. Additional factors are likely to include the mechanism by which the compound degrades, the degree of uptake by tumor cells, and the affinity for cellular constituents. On the latter point it may be relevant that spermine binds DNA (37) and that the spermine/NO complex was the most cytostatic.

SPER/NO was also cytotoxic, while the remainder of the NO/nucleophile complexes, including PAPA/NO, DEA/NO, OXI/NO, IPA/NO, and SULFI/NO, did not adversely affect cell viability even at 500 μM (Table 2). The properties of cytostasis and cytotoxicity could be separated from one another, as evidenced by the substantial cytostasis and absence of cytotoxicity induced by PAPA/NO and DEA/NO over this dose range. For OXI/NO, the mechanism of decomposition may not be the same as with the other NO/nucleophile complexes. The products of OXI/NO degradation can include HNO (N₂O) and nitrite (38) as well as NO (39), depending upon the solution conditions. The cytotoxic effects of HNO or other potential intermediates are unknown. Also unknown is whether the NO/nucleophile complexes decreased cell viability at times greater than 8 h, a possibility because acute free radical injury to cells may not be evident during this time frame.

Further support for the role of NO as the mediator of NO/nucleophile cytostasis was provided by the influence of myoglobin on PAPA/NO-induced cytostasis. Myoglobin has previously been shown to inhibit the cytostatic effect of activated macrophages on tumor cells (6), and hemoglobin has been shown to block the cytotoxicity of the NO-generating compound SNP (18). Ferrous and ferric myoglobin form NO derivatives with different spectra over the range 500–600 nm (40). Ferrous myoglobin can bind NO-forming nitrosylmyoglobin, and MbO₂ can react with NO-forming NO₂⁻ and metmyoglobin (40). The result in either case is to lower the free NO concentration and thereby protect against NO-induced cytostasis.

In conclusion, the results presented show that the NO/nucleophile complexes may inhibit DNA synthesis in human melanoma cells and that this inhibition can be attributed to their ability to release nitric oxide. The data suggest that the NO/nucleophile complexes may...
prove useful as research tools for probing the cellular effects of nitric oxide and may provide the basis for the design of new agents for controlling tumor cell proliferation.

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