Nitrosative stress in cancer therapy

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TABLE OF CONTENTS
1. Abstract
2. Introduction
   2.1. Free radicals in cancer biology
   2.2. Synthesis of (NO)$^\cdot$ in biological systems
   2.3. Biological reactions involving NO$^\cdot$ and generation of RNS
3. NO$^\cdot$ and cancer therapy
   3.1. Inhibition of RNS generation.
   3.2. Enhancing nitrosative stress for therapeutic benefit
   3.3. Enhancing conventional therapies
   3.4. Enhancing novel therapies
4. Perspective
5. References

1. ABSTRACT

Reactive nitrogen species play important roles in cell signalling, but when present at high concentrations they can subject cells to nitrosative stress, which may lead to cell death. Nitric oxide (NO) is now recognized as playing important roles in cancer aetiology and progression and it can influence the outcome of cancer treatment. It is synthesised by the action of nitric oxide synthases (NOSs) on the amino acid arginine. Although NO$^\cdot$ is not highly reactive with biological molecules, it reacts readily with other oxygen radicals to generate highly damaging reactive nitrogen species such as peroxynitrite, nitrogen dioxide and dinitrogen trioxide. These are potent inducers of apoptosis and necrosis. They may also inhibit DNA repair mechanisms, leading to mutation and carcinogenesis. Both inhibition and over-production of NO have been investigated as strategies for cancer therapy. There is clear evidence that administration of competitive inhibitors of NOS can significantly slow the growth of solid tumors in rodent models, probably by reducing blood flow, and this creates a hypoxic environment that is conducive to the activation of bioreductive anticancer agents. Alternatively, generation of NO concentrations in the high micromolar range by NO donor drugs or gene therapy with inducible NOS is directly cytotoxic to cells and has been shown to inhibit tumor growth. At these high concentrations NO is also an excellent sensitizer to radiation and to some chemotherapeutic agents, particularly cisplatin. Thus, manipulation of NO levels in tumors offers exciting opportunities to improve the effectiveness of cancer treatment.

2. INTRODUCTION

2.1. Free radicals in cancer biology

The role of free radicals as key players in the biology and therapy of cancer is well-established (1). By far the most biologically important of these are reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can be generated both by endogenous metal-catalysed reactions and by exposure of cells to certain anticancer agents such as bleomycin (2) and tirapazamine (3) or to ionizing or UV radiation (4). ROS are known to activate numerous signalling cascades. The most significant of these involve nuclear transcription factors and protein kinases, which in turn control cell cycle checkpoints, stress responses and differentiation and are upstream regulators of numerous cytokines (1). Reactions involving ROS are also seen as potential targets for cancer therapy (5) and have been widely studied.

The most important source of nitrogen for the generation of reactive nitrogen species (RNS) in vivo is nitric oxide (NO). It is well known to be a product of the catalytic action of the nitric oxide synthase enzyme family on L-arginine (6). However, recent evidence suggests that it can also be formed by reduction of nitrite, which can arise in the body by ingestion or from bacterial metabolism (7). Low concentrations of NO exert subtle effects in cells, some of which are the key homeostatic regulators in several tissues. Nitrosative stress on the other hand arises when the rate of generation of RNS exceeds the capacity of a cell to neutralise them and damaging interactions with macromolecules (lipids, proteins and nucleic acids) compromise cellular function (1). It can originate from the
Nitrosative stress

endogenous generation or exogenous addition of nitric oxide (NO), which in turn interacts with ROS to generate numerous nitrogen oxides of widely differing reactivity (8).

The past five years have seen an explosion of interest in the impact of NO on the aetiology, progression, treatment and prognosis of cancer. In 2003 Wink and Mitchell, two major contributors to the field, summarised the available data on the subject in a concise review (9), concluding that “In tumor biology, NO continues to confuse and confound us”. Since then over 3000 articles have been published containing evidence of relevance to this field; some of these have helped clarify the role of NO, while others have added additional complexity to the picture. This review will attempt to summarise our current understanding of the importance of RNS in cancer biology and their potential roles in cancer therapy.

2.2. Synthesis of (NO) in biological systems

The cellular generation of NO occurs by an oxidation reaction involving L-arginine as the substrate and tetrahydrobiopterin (10) nicotine adenine dinucleotide, flavin adenine dinucleotide, flavin mononucleotide, and protoporphyrin IX (a source of haem) as cofactors (11,12). This reaction, is catalysed by three main isoforms of the nitric oxide synthase (NOS) enzyme family (13), although NOS activity has also been demonstrated in mitochondria and this can probably be attributed to a fourth distinct isoform (14). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are expressed constitutively and regulated post translationally by association with other proteins including caveolins (15). Their expression is largely, but not exclusively, restricted to the vasculature (16) and nervous tissues in the absence of pathology (22). Thus, the presence of specific sequences in the reductase domains of the enzymes (19).

The inducible isoform of NOS (iNOS) is not constitutively expressed in normal tissues and its concentration is regulated mainly at the transcriptional and translational levels (20), though some post translational regulation is now recognised (21). Once generated in the cell, iNOS is capable of generating very high concentrations (high micromolar) of NO over a prolonged period (20). An increasing number of cytokines and other signalling molecules are now known to mediate iNOS expression (22); in addition, iNOS expression is sensitive to environmental stress factors such as hypoxia (23). While all three isoforms of NOS have been detected in tumors (24), given the conditions prevailing in the tumor microenvironment, such as hypoxia and increased superoxide production, it is not surprising that iNOS is the isoform most usually associated preferentially with malignant tissue and that it is rarely detected in normal tissues in the absence of pathology (22). Thus, the conditions existing in the microenvironment of tumors are conducive to the generation of high concentrations of NO and there is compelling evidence that this influences their biology, progression and response to therapy. There is also strong evidence that NO generation plays a key role in the aetiology of many cancers (25) and may be a major mediator of carcinogenesis associated with inflammation (22, 26, 27) particularly in the colon (28) and gastro-oesophageal junction (29).

2.3. Biological reactions involving NO and generation of RNS

A broad spectrum of biologically reactive molecules is now known to be generated by the interaction of NO with O$_2$ and reactive oxygen species under physiological conditions (8). These in turn react with thiols, proteins and lipids with profound consequences for the integrity of the cell. However, the most significant reaction of NO in the absence of pathology is its binding to the prosthetic haem group of soluble guanylate cyclase, which massively increases the activity of the enzyme (30). This leads to rapid synthesis of cyclic GMP, which in turn mediates relaxation of vascular smooth muscle (31). This is the most important mechanism maintaining steady state control of vascular tone.

Two key reactions of NO with either O$_2$ or the O$_2^-$ radical initiate the generation of a wide spectrum of RNS (8). Some of these species are highly reactive with cellular macromolecules, causing, for example, lipid peroxidation and nitrating tyrosine-containing proteins (32), impairing or modifying their function. Perhaps the best characterised reaction of NO with reactive oxygen species is its very rapid interaction with superoxide (O$_2^-$). This leads to generation of peroxynitrite (OONO$^-$), a potent oxidising agent (33, 34) and mediator of cellular damage that triggers apoptosis in many cell types via a variety of mechanisms (35-38). Common features are lipid peroxidation (39, 40) and the activation of caspase-3 (41). There is also compelling evidence that NOSs can generate O$_2^-$ in addition to NO (42-45) creating the ideal circumstances for OONO$^-$ formation. Clearly, however, this does not happen with high efficiency in healthy tissue, largely because O$_2^-$ generation from NOS occurs only under conditions where its L-arginine substrate is depleted (46) and/or tetrahydrobiopterin is absent (43, 44) leading to uncoupling of the enzyme. In addition, superoxide dismutases, an enzyme family present as three isoforms in mammalian cells, act as highly efficient antioxidants, rapidly catalysing the conversion of O$_2^-$ to much less reactive species such as H$_2$O$_2$ and molecular oxygen (47, 1). There is also compelling evidence that one isoform in particular, Mn-SOD, affects tumor growth and progression (48, 1). While detailed consideration of these mechanisms lies beyond the scope if this review, the presence of O$_2^-$ appears to be a crucial determinant of whether or not exposure to NO will lead to apoptosis in a given cell type; furthermore, this may form the basis of high levels of apoptosis induction by NO in transformed cells (49-50) particularly compared to normal cells (51). Generally, then, peroxynitrite formation from NO and O$_2^-$ is a key reaction in determining if apoptosis will be triggered. In the absence of O$_2^-$, lipid peroxidation will not occur and indeed NO has been shown
Nitrosative stress

to be protective against peroxidation and other damaging radical reactions (52).

The final fate of peroxynitrite is dependent on the conditions. In most tissues, where CO₂ is present at significant concentrations, CO₂⁻ and NO₂⁻ are generated and these species are capable of undergoing damaging reactions with other cellular constituents (53). In the absence of CO₂, peroxynitrite undergoes slow decomposition to NO₂⁻ (see below) and OH. While these are also potentially very damaging radical species their rate of generation by this reaction is very slow and in any event is unlikely to occur in metabolising tissues with mM concentrations of CO₂. An additional basis for tumor specificity may be the dependence of one mechanism of NO-induced apoptosis on hypoxia, though this appears to be independent of peroxynitrite (54).

If the cellular concentration of NO⁻ is high enough (55) it can be oxidised by physiological concentrations of O₂ to generate, via several intermediates including NO₂⁻, the potent nitrosating agent dinitrogen trioxide (N₂O₃) (8). This is the principal nitrosating agent derived from NO and is capable of interacting with many biologically significant molecules (53). Of particular interest is the interaction with thiols to generate S-nitrosothiols, which in turn can react with other intracellular thiols or thiol-containing proteins (56). In some systems this may convey regulatory signals (57-58) and in the case of cancer cells there is evidence that apoptosis is regulated by S-nitrosoglutathione (via bax signalling) (59) and S-nitrosylation of procaspase-9 (53). Additionally, the nitrosation of glutathione by N₂O₃ and possibly also NO₂⁻ occurs most efficiently at an oxygen tension of 3% (60) typical of the level likely to exist in many viable tumor cells in vivo (61).

The nitrogen dioxide radical (NO₂) can be formed in several different reactions though some are too slow to be of any consequence in vivo (8). The most biologically significant are the reaction of NO at high concentrations with molecular oxygen (which also yields N₂O) and the decomposition of peroxynitrite in the presence of CO₂ to yield NO₂⁻ as well as CO₂⁻ (62). The latter reaction is of particular interest because the cogeneration of NO₂ and CO₂⁻ shows selectivity for important biological molecules and, acting together, they are highly effective and selective in nitrating proteins (63,8), which can contribute to numerous disease processes (64) including cancer (65).

3. NO AND CANCER THERAPY

We have recently reviewed the importance of NO in cancer therapy (25) and there is now an extensive literature available (22, 66-69). As a general rule, malignant tumors express significant levels of NOS enzymes and often at higher concentrations than their normal tissue counterpart. This has been clearly demonstrated in a wide range of tumors including those of the bladder (70), brain (71, 72), breast (73-75), cervix (76), colon (77), endometrium (78), lung (79, 80), ovary (81), pancreas (82), prostate (83), and in melanoma (84). This implies that tumors develop within an environment with high levels of nitrosative stress and are exposed to a range of RNS (see section 2.3). Potential therapies could therefore be designed to reverse this condition, which as we shall see, is responsible for maintaining the malignant phenotype of many cancers; alternatively, the damaging effects of RNS could be specifically enhanced. Strategies employing these two approaches will now be reviewed.

3.1. Inhibition of RNS generation.

We will focus mainly on methods of intervention to modify the generation of RNS in a manner that could be used to treat established solid tumors. However, it is worthy of note that over-expression of iNOS has been associated with generation of excessive levels of RNS during chronic inflammation and may play an important role in carcinogenesis by causing DNA damage (26, 85-89); there may, therefore, be a role for RNS inhibition in the chemoprevention of cancer. The generation of RNS in tissues including tumors occurs almost exclusively via the catalytic action of the NOS enzyme family; consequently, this offers a discrete target through which generation of NO can be inhibited. Furthermore the availability of a wide variety of inhibitors with selectivity for the NOS family of enzymes, or its specific isoforms, has allowed this concept to be tested both in vitro and in vivo (90-92).

Regardless of the underlying mechanism, the evidence from preclinical studies using NOS inhibitors in rodent tumor models in vivo clearly demonstrates inhibition of tumor growth. This was first shown over ten years ago when administration of L-NAME (94) or a similar arginine analogue N⁶-guanyl-L-arginine (NNLA) (95). A more recent study in a human pancreatic cancer model in nude mice showed that the addition of 1 mg/ml NNLA to the drinking water was shown to reduce tumor growth rates by a factor of ~2, in a fully and rapidly reversible manner, such that tumor growth rates returned to normal within 24 h of drug withdrawal (93). Several other investigators have since demonstrated very similar results using L-NAME (94) or a similar arginine analogue N⁶-guanyl-L-arginine (NNLA) (95). More recently, several additional tumor parameters to elucidate the mechanisms behind the growth inhibition. Blood vessel diameters were reduced by 50% as was vessel perfusion (measured by Hoechst 33342) in the NNLA-treated tumors; there was also a small increase in apoptosis. A reduction in tumor perfusion in response to NOS inhibitors has previously been reported by others in a variety of experimental systems. Window chamber models have been used to demonstrate reductions in diameter and perfusion of rat tumor isografts (97) and human tumor xenografts (98). Similar results have also been obtained in orthoptic glioma (99, 100) and tissue isolated rat tumor models (101,102). Because the NOS inhibitors used in all of the above studies will inhibit all NOS isoforms, and all isoforms are known to be expressed in tumors (24) these data do not allow a more detailed analysis of the isoform's responsible for enhancing tumor perfusion. This information would be important for planning a clinical strategy because the inhibition of nNOS and particularly
eNOS has been shown to induce significant cardiovascular pathology (103-106), specifically after chronic administration of L-NAME, as would be required to maintain tumor growth inhibition (93). One study, however, clearly demonstrates the importance of iNOS in the tumor growth response to NOS inhibitors (107). Treatment of mouse tumor isografts and human tumor xenografts with the specific iNOS inhibitor 1400W by continuous infusion, reduced tumor growth rates to the same degree (50%) as that seen in most studies with the non-specific NOS inhibitors. Tumors that did not express iNOS showed no response to 1400W. This suggests that iNOS is the main contributor to NO generation in tumors, at least in the models tested and that its inhibition could be the basis for a clinical strategy to inhibit tumor growth. Few human studies with 1400W have been carried out, but there is evidence that it can be used safely in man (108). In summary, it is reasonable to conclude that tumors are exposed to high levels of nitrosative stress, predominantly as a consequence of iNOS expression; this in turn leads to a highly dilated vasculature that shows marked constriction in response to inhibition of the source of NO production.

NOS inhibitors have also been shown to enhance the effectiveness of other therapies that depend on reduced blood flow or hypoxic conditions for their effectiveness. The toxicity of the bioreductive cytotoxin, RB6145, was significantly enhanced by administration of the non-specific NOS inhibitor L-nitro arginine in mouse tumors (KHT and SCCVII), while the inhibitor had no effect on RB6145 toxicity in bone marrow (109). Inhibition of NO synthesis with L-NAME was found to increase heat-induced growth delay of FSaII tumors in mice, probably as a result of a reduction in the cooling effect of blood flow (110).

Another well established action of NO at constitutive levels is to stimulate angiogenesis, both in tumors and normal tissues. Numerous investigators have studied the role of NO (mainly with the use of non-isofrom specific NOS inhibitors) on different aspects of angiogenesis (111-115) and have observed that inhibition of NO synthesis results in potent inhibition in vitro and in vivo. A potent anti-angiogenic effect was also demonstrated for aminoguanidine, an inhibitor with specificity for iNOS (116). One in vivo study also showed that NO specifically activated angiogenesis by longitudinal splitting of capillaries and had no effect on sprout formation (117). Several studies have attempted to identify downstream mediators of the angiogenic action of NO. One likely candidate is vascular endothelial growth factor (VEGF); it has been known for some time that NO can upregulate VEGF in liver and brain tumor cells via a guanylate cyclase-dependent mechanism that is dependent on de novo protein synthesis and leads to stabilisation of VEGF RNA (118). Increased expression of VEGF in response to administration of the NO donor S-nitroso-glutathione was demonstrated in normal cells (119, 120). The association between NO and VEGF has also been established in human primary astrocytomas and their expression correlated directly with grade of disease (71).

One particularly valuable study directly demonstrated the biparat character of NO in angiogenesis (121). Exposure of endothelial cells to 50-200 µM concentrations of the NO donor S-nitro-N-acetyl penicillamine (SNAP) caused a dose-dependent increase in several angiogenic end points and in the phosphorylation of PKC, ERK and c-Jun, and AP-1 activation. At higher concentrations (up to 4 mM) these endpoints were markedly inhibited by SNAP in a dose-dependent manner.

IL-8 is another cytokine with a well-established role as a mediator of angiogenesis (122, 123) and there is now considerable evidence that NO upregulates IL-8 (together with VEGF) expression (probably at the transcriptional level) in colon cancer (124) and melanoma (125) cells.

In addition to upregulating pro-angiogenic cytokines, NO has been shown to down-regulate inhibitors of angiogenesis (such as angiostatin) in heart muscle (126). To what extent, then, does abrogation of this process by NOS inhibition contribute to the reduced growth rate of tumors in vivo? Few data are available, but one recent study (96) showed that vessel density determined histologically in a pancreatic tumor model was reduced modestly (~20%) when the host animals were given the NOS inhibitor NNLA, but the most dramatic effect was a 40% reduction in the cross sectional area of the tumor vasculature. Given that the resistance of a blood vessel to flow is inversely proportional to the 4th power of the radius, blood flow reduction is likely to be the predominant mechanism contributing to the 50% reduction in tumor growth rate seen in that study.

3.2. Enhancing nitrosative stress for therapeutic benefit

As we have seen (2.3), NO is not highly reactive with cellular macromolecules, but is capable of undergoing interactions with molecular oxygen to generate dinitrogen trioxide and with O2 to yield ONOO•, which, on decomposition in the presence of CO2 yields NO2. All of these RNS are capable of damaging reactions with lipids, proteins and nucleic acids. A common consequence is the induction of apoptosis following recognition of the damage by the cell, though death by necrosis also occurs (127). However, there is also clear evidence that NO can inhibit apoptosis in some cells, while promoting it in others (128) and that this probably occurs via inhibition of mitochondrial respiration and inhibition of caspases by S-nitrosylation (129). In attempting to generalise the cellular response to NO, it is clear that, concentration is important. This is a notoriously difficult parameter to control in vitro let alone in vivo, which may account for enormous variation in responses between studies. One study, however, used a membrane delivery system to maintain tight control of NO concentration in lymphoblastoid cells and was able to demonstrate that thresholds for steady state concentration (~0.5 µM) and total cumulative dose (~150 µM.min) must both be exceeded if cell death was to occur (130). Interestingly, a lymphoblastoid line lacking wild type p53 required a higher cumulative dose (~300 µM) to kill the cells (131). Variations in NO production are not the only confounding factors determining response in a given cell system: interaction with thiols, metal ions,
Nitrosative stress

Table 1. Enhancement of the effects of chemotherapeutic drugs by NO

<table>
<thead>
<tr>
<th>Drug</th>
<th>Experimental model</th>
<th>Sensitizer enhancement ratio</th>
<th>Cell/animal survival ratio</th>
<th>Source of NO</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>Fibroblasts in vitro</td>
<td>NA</td>
<td>~60</td>
<td>NO gas in solution</td>
<td>155</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Human leiomyoma</td>
<td>NA</td>
<td>800-3000</td>
<td>NO donor drugs</td>
<td>155</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Hamster lung fibroblasts in vivo</td>
<td>NA</td>
<td>2.8 at 50% survival</td>
<td>NO donor drug</td>
<td>156</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Mouse leukemia in vivo</td>
<td>NA</td>
<td>2.6 (animal survival ratio at 60 d)</td>
<td>NO donor drug</td>
<td>159</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Rat liver epithelial cells in vitro</td>
<td>NA</td>
<td>3.7-5.8 at 50% survival</td>
<td>NO donor drugs</td>
<td>157</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Human breast cancer cells in vitro</td>
<td>NA</td>
<td>3</td>
<td>NO donor drug</td>
<td>158</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Colon cancer in vitro</td>
<td>NA</td>
<td>3.7-4.0</td>
<td>NO donor drug</td>
<td>146</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>Colon ovarian cancer cells in vitro</td>
<td>NA</td>
<td>2.7 (apoptotic ratio; 60% reduction in growth rate)</td>
<td>NO donor drug</td>
<td>146</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Human breast cancer in vitro</td>
<td>NA</td>
<td>8</td>
<td>NO gas in solution</td>
<td>163</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Human breast cancer in vitro</td>
<td>NA</td>
<td>-1.6</td>
<td>NO donor drug</td>
<td>160</td>
</tr>
<tr>
<td>Taxol</td>
<td>Prostate cancer in vitro</td>
<td>NA</td>
<td>5-12</td>
<td>NO donor drug</td>
<td>164</td>
</tr>
<tr>
<td>Taxol</td>
<td>Neuroblastoma in vitro</td>
<td>NA</td>
<td>1-3</td>
<td>NO donor drug</td>
<td>164</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Mouse melanoma in vitro</td>
<td>NA</td>
<td>1.6 (inhibition of metastasis)</td>
<td>NO donor drug</td>
<td>159</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Mouse leukemia</td>
<td>NA</td>
<td>100% versus 0% animal survival</td>
<td>NO donor drug</td>
<td>159</td>
</tr>
</tbody>
</table>

NA= not available

proteins and interaction with ROS will all differ (128).

Many investigators have studied the effect of delivering high concentrations of NO and its reactive products. Delivery methods include nitric oxide donor drugs and iNOS gene transfer that can achieve concentrations of NO in the micromolar range. This lead to apoptosis in a wide variety of human cancer cells including bladder (132-134), breast (135), colon (136, 137), pancreas (138, 139), and prostate (139). There is also some evidence that NO mediated apoptosis may show some selectivity for transformed compared with normal cells (fibroblasts) and that this can be attributed to increased superoxide production in transformed cells leading, on interaction with NO, to peroxynitrite production (51). Further evidence suggests that solid tumors containing hypoxic regions may be specifically vulnerable to peroxynitrite-induced apoptosis because of enhanced generation of ROS (including O2-) under hypoxic conditions (140). Is there any in vivo evidence? The first study used direct injection of a plasmid containing the iNOS gene into an experimental thyroid cancer model in rats and showed significant inhibition of tumor growth even though only 1% of the cells in the tumors had been transfected (141). Several studies have since investigated apoptosis induction by NO using iNOS DNA constructs and have seen extensive apoptosis within 24 hours of transfection in rodent tumor (142, 143) or human tumor xenograft (144,145) models. Nitric oxide donor drugs have since been shown to induce similar effects in colon cancer xenografts (146). Thus, expression of NO in tumors at high concentrations has therapeutic potential, but none of the above studies suggests that NO could be effective as a stand alone treatment; it is likely to be combined with other conventional or novel therapies.

3.3. Enhancing conventional therapies

NO or its reaction products have been shown to interact with and damage proteins; in particular, it can nitrosate zinc finger containing enzymes leading to their denaturation (147, 148). This includes many of the DNA repair proteins such as Fpg (149), DNA ligase (150), O6-methylguanine-DNA-methyltransferase (151), poly (ADP-ribose) polymerase (152), and enzymes involved in nucleotide excision repair (153). Compromised repair capacity induced by high concentrations of NO should, therefore, increase the toxicity of most DNA damaging agents; there is now a considerable body of evidence to support this, particularly for cisplatin (154).

Chemosensitization by RNS has been studied most extensively in combination with cisplatin. A variety of techniques have been used to deliver high NO concentrations (high µM) to cells in culture including NO gas saturation, NO donor drugs or iNOS gene transfer. The first study (155) used NO-saturated medium or the NO donors DEA/NO or PAPA/NO to sensitize fibroblasts to cisplatin. While no enhancement ratio can be calculated from their data, surviving fraction after cisplatin was reduced by over 1000 fold by the addition of NO donors. Since then, chemosensitization has also been demonstrated in head and neck carcinoma cells (156), in liver cells (157) and in ovarian cancer cells (158). The latter study also included evidence for increased intracellular retention of cisplatin by a mechanism involving mitogen activated kinases, with obvious implications for multidrug resistance. Whatever the mechanism, enhancement of the antitumor effect of platinum-based drugs can also be achieved in vivo. Significantly prolonged survival was seen in leukaeimia bearing mice treated with a combination of cisplatin and an NO donor compared with animals treated with cisplatin alone (159). A recent study using oxaliplatin in combination with the NO donor NCX 4040 achieved a 60% reduction in tumor growth rate compared with oxaliplatin alone and a 3 fold increase in the number of apoptotic cells (146).

Recently, a study in breast cancer cells grown as spheroids demonstrated that the NO donor DETA/NO was
Nitrosative stress

able to reverse acquired resistance to doxorubicin (160). There is also evidence that inhibition of drug efflux by high concentrations of NO (induced by cytokines) may be another mechanism of enhancement of the cytotoxicity of this drug (161). These authors have also shown that the ability of statins to revert the resistance of colon cancer cells to this agent may be mediated by NO (162). An important insight into the mechanisms of NO induced enhancement of doxorubicin is provided by a recent study (163), which demonstrated the importance of the sequencing of NO delivery in relation to cytotoxic drug exposure. NO delivered 30 min before doxorubicin enhanced its cytotoxicity by eight fold (in terms of cell survival), but when doxorubicin was given 30 min before or simultaneously there was little or no enhancement. These authors proposed that time might be required for NO to modify critical signalling pathways regulating apoptosis. We suggest that inhibition of DNA repair enzymes by NO (153) may also occur during that time. Finally, using AdiNOS gene transfer this group were able to show enhanced doxorubicin toxicity to breast cancer cells, but not to normal cardiac myoblasts under the same conditions. This provides a basis for selectivity of NO therapy.

Limited data exist for other drug combinations. NO delivery using a donor drug increased the cytotoxicity of taxol in prostate cancer cell lines, but had no effect in neuroblastoma cell lines (164). An NO donor has also been used in combination with 5-fluorouracil, but in this case toxicity was only additive (146). Enhancement of the cytotoxicity of cyclophosphamide by an NO donor has also been demonstrated in vivo, leading to an impressive delay in tumor regrowth (158).

NO is also a potent radiation sensitizer. The radiosensitization of hypoxic by NO was first described at about the same time as the oxygen effect in radiation biology was characterised in mammalian systems (165-167). While the evidence existed then that NO has a sensitizing efficiency similar to that of O₂, it did not generate the same level of interest as in the “oxygen effect”. This may have been because NO was considered to be an environmental pollutant and not the fundamental regulator of cellular signalling pathways that we now recognise (168, 169). Within the last ten years, however, extensive studies have been carried out to quantify the radiosensitizing effect of NO using a variety of strategies. While it is possible to deliver authentic NO gas in radiosensitization experiments in vitro (170) most investigators have found it more practical to use a variety of drugs that donate NO in the cellular environment (171-172). Radiosensitization by NO generated via a redox reaction with Agnelli’s salt was also demonstrated (173). In these studies NO gave sensitizer enhancement ratios of 1.6 -2.0 when the donors were added at 0.1 – 1.0 mM concentrations; this is similar to what would be expected for oxygen.

Rather than introducing NO donor drugs, an alternative approach is to exploit the action of iNOS as a potent and long lasting generator of NO from L-arginine. The first study to do this (174) achieved an enhancement ratio of 2.5 in hypoxic EMT-6 tumor cells in culture by stimulating them with interferon gamma, even though the peak NO levels generated by this method were lower than those achieved with an NO donor. This suggests that the duration of NO exposure rather than just the concentration at the time of irradiation is important to the cellular response and in this respect differs from the effect of oxygen (175).

Developments in gene transfer have opened up the possibility of introducing iNOS into cells as a suicide gene therapy to be combined with other modalities, including radiation. In our own studies we have focussed on gene transfer using liposomal delivery of vectors expressing high levels of iNOS in rodent and human tumor cells in vitro and as solid tumors in vivo. Constitutive (CMV) and radiation- inducible (WAF-1) promoters were used to drive expression of the iNOS gene in RIF-1 tumor cells in culture and achieved enhancement of radiosensitivity similar to that seen with oxygen (176). This strategy can also be applied successfully in vivo: we used direct injection of WAFiNOS and CMViNOS constructs in a liposomal vector to transfect RIF1 (mouse) and HT29 (human) tumors and achieved enhancement ratios of 1.6 -2.0 in combination with radiation (142, 144). Adenoviral delivery can also be used successfully with iNOS gene therapy of tumors (177). Very similar enhancement ratios were obtained in human colon cancer xenografts in combination with single dose and fractionated (2 Gy) irradiation. It is perhaps surprising that enhancement ratios close to the full oxygen effect can be achieved in vivo with a technique that expresses iNOS in a very small proportion of the tumors cells (transfection efficiencies ~1%). Therefore, in considering the role of NO in the sensitivity of tumors in vivo it is important to differentiate the direct effects of NO from physiological changes that could alter oxygenation. Two explanations are likely: a) NO generation could lead to enhanced tumor blood flow and oxygen delivery, so sensitizing previously hypoxic cells. b) NO is a highly diffusible molecule with very short half life in the presence of oxygen or haem proteins (178), but in regions of tumors with few blood vessels and very low oxygen tensions its lifetime could be much longer (several seconds) and diffusion distance greater. There is evidence for the former mechanism: intra venous administration of the NO donor SIN-1 within a narrow dose range (0.5-2.0 mg/kg) resulted in increased oxygenation and radiosensitivity, which was attributed to improved blood flow (179), furthermore, iNOS gene therapy using adenoviral delivery (177) caused an increase in tumor vascularity, which could allow non-transfected bystander cells to be sensitized. However, it is reasonable to suppose that diffusion of NO must play an important part in tumor radiosensitization in vivo after iNOS activation or transfection because the catalytic action of NOS on arginine to generate NO requires O₂ as the source of an oxygen atom (13). Further studies are required to distinguish the relative importance of these two mechanisms, which will probably differ between tumors.

While NO* may radiosensitize by fixation of DNA radicals in a manner similar to oxygen (although this
However, NO⋅ apoptosis via a mitochondria-dependent pathway (187). Sodium nitroprusside enhanced TRAIL-induced bioreductive cytotoxins. Delivery of very high NO⋅ and nutrient deprivation, which can sensitize tumors to inhibition of that constitutive expression leads to reduced blood flow and nutrient deprivation, which can sensitize tumors to bioreductive cytotoxins. Delivery of very high NO concentrations leads to high levels of nitrosative stress, triggering apoptosis. It also sensitizes the tumor cells to other cytotoxic insults. remains a matter of debate) it also exerts more subtle influences by activating a host of signalling pathways within the cell that trigger apoptosis (180). There is also evidence that these death signals can be exported to other cells: microbeam irradiation of individual cells, even if the nucleus was excluded, leads to extensive DNA damage as measured by micronucleus formation in neighbouring unirradiated cells and the effect could be eliminated by the use of NO-specific scavengers (181, 182).

3.4. Enhancing novel therapies
NO may also play an important role in the mechanisms of action of and interaction with novel cancer therapies. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be a selective cytotoxin in some, though not all cancer cells (183-185). There is also clear evidence that NO⋅ can sensitize cancer cells to TRAIL-induced apoptosis; sensitization of prostate cancer cells to TRAIL was also achieved when NO at high concentration was delivered by the donor DETANONate. Inactivation of NF-κB and inhibition of Bel-αL are implicated (186). In another study in human colorectal cancer cells the NO donor sodium nitroprusside enhanced TRAIL-induced apoptosis via a mitochondria-dependent pathway (187). However, NO at lower constitutive concentrations may even protect prostate cancer cells from TRAIL-induced apoptosis (188). This is consistent with the conclusion that NO at low levels in tumors are protective of cell survival, whereas high levels induce death signalling.

4. PERSPECTIVE
The generation of NO⋅ in tumors mainly, though not exclusively by iNOS- plays a key role in maintaining the malignant phenotype. Inhibition of this process consistently reduced the rate of tumor growth, but was unable to prevent growth entirely. In addition, NOS inhibition created a poorly perfused, hypoxic environment (Figure 1). This could be exploited to enhance the cytotoxicity of bioreductive drugs, but would be detrimental to the effectiveness of conventional chemotherapy and radiotherapy. The opposing strategy of targeting very high NO levels to tumors looks more promising in that it can be used to enhance conventional therapies as well as directly causing cell death. While it has clearly been shown in several studies to delay tumor growth by inducing extensive apoptosis, it has also consistently enhanced radiotherapy and chemotherapy given in clinically relevant schedules. Thus, introduction of NO augmentation as an adjuvant therapy should not be problematical. Furthermore, any undesirable systemic effects of NO⋅ on blood pressure can be monitored easily and are readily reversible. These characteristics support the view that introduction of NO augmentation therapy into clinical trials would be a low risk strategy with the potential for considerable patient benefit.

5. REFERENCES
3. Wardman P. Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia. Curr Med Chem 8, 739-761 (2001)
Nitrosative stress

Nitrosative stress

64. Marrogi AJ, WD Travis, JA Welsh, MA Khan, H Rahim, T Tazelaar, P Painrollo, V Trastek, J Jett, NE Caporaso, LA Liotta & CC Harris. Nitric oxide synthase, cyclooxygenase 2, and vascular endothelial growth factor
Nitrosative stress


149. Wink DA & J Laval. The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo. *Carcinogenesis* 15, 2125-2129 (1994)


Nitrosative stress


Abbreviations: AdiNOS: adenovirus/inducible nitric oxide synthase; DETA/NO: diethylenetriamine nitric oxide adduct; DETANONOate: (Z)-1-[2-(2-aminoethyl)-N-(2-aminoethyl) amino]diazen-1-ium-1,2-diolate; CMV: cytomegalovirus; L-NAME: N-nitro-L-arginine; SNAP: N-nitro-L-arginine methyl ester; NNLA: N3-nitro-L-arginine; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; SNAP: S-nitro-N-acetyl penicillamine; VEGF: vascular endothelial growth factor; WAF1: wild-type p53 activated fragment

Key words: Nitrosative Stress, Reactive Nitrogen Species, Cancer, Nitric Oxide, Inos, Enos, Chemotherapy, Radiotherapy, Apoptosis, Review

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