Nitric oxide donors: Novel cancer therapeutics (Review)

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Abstract. The development of cancer cell resistance to various cytotoxic stimuli continues to be a major challenge in oncology and novel therapeutic approaches are urgently needed. Nitric oxide (NO) is emerging as a potential anti-oncogenic agent to overcome tumor cell resistance to conventional therapeutic agents. NO is a ubiquitous, water-soluble, free radical gas that exerts a wide range of biological effects. The actions of nitric oxide are highly variable in oncology with reports in the literature on both sides of the spectrum as an anti-neoplastic vs. a pro-neoplastic agent. The final activity of NO in oncology is dependent on its working microenvironment, including the type of cell exposed to the compound, the redox state of the reaction, as well as the final intracellular concentration and the duration of intracellular exposure to nitric oxide. There is, however, no unifying mechanistic explanation for the biphasic role of nitric oxide in oncology. Nitric oxide donors mimic continuous production of NO in a wide range of time intervals (seconds to days). Thus, multiple biological and (pro- vs. anti-) neoplastic responses are elicited from NO donors depending on the half-life and the type of cell exposed to the compound. The large variety of nitric oxide donors may serve as a tool to explore the wide range of oncologic properties of NO in cancer. In the present report, we discuss classic nitric oxide donors and their potential therapeutic roles as cytotoxic agents or chemo-radio or -immune-sensitizing compounds in the treatment of drug-resistant cancers.

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1. Introduction

The odorless, colorless and highly reactive simple radical nitric oxide (NO) has steadily gained a significant impetus over the past several decades. NO, an air pollutant produced from fuel burning, became the journal of Science 'molecule of the year' in 1992 (1) as a result of the discovery of its wide array of biological functions, including smooth muscle relaxation, inhibition of platelet aggregation, and neurotransmission. Six years later Drs Furchgott, Ingnarro and Murad shared the Nobel Prize for Physiology and Medicine for their major discoveries surrounding NO (1). In 1997, the Academic Press began the publication of the Journal Nitric Oxide: Biology and Chemistry, which is the official journal of the Nitric Oxide Society. Nitric Oxide has been the sole subject of several books and numerous review articles. Over the past decade, nitric oxide has emerged as a molecule of interest in carcinogenesis and tumor growth suppression. In 2007, the first International Conference of Nitric Oxide and Cancer (NO Cancer), was convened in Paris, France. There have been large accomplishments by a small and radical molecule. It is the role of NO in oncology that has stimulated the development of this review, which will summarize the controversy surrounding the dual role of both NO and nitric oxide synthases (NOSs) as pro- vs. anti-neoplastic agents. The focus of the present review will emphasize the potential therapeutic function of the various nitric oxide donors in oncology.

2. Nitric oxide production and nitric oxide synthases

Nitric oxide is generated by the oxidation of the amino acid L-Arginine under the catalytic activity of the nitric oxide synthases (NOSs). This reaction requires NADPH and O_2 as co-substrates and yields NO and L-citrulline as end products (2). The unpaired electron on the outermost orbital of NO permits it to either donate it, thereby participating in oxidation reactions, or accept electrons from other reactive species leading to its anti-oxidant properties (Fig. 1) (3). NO mediates

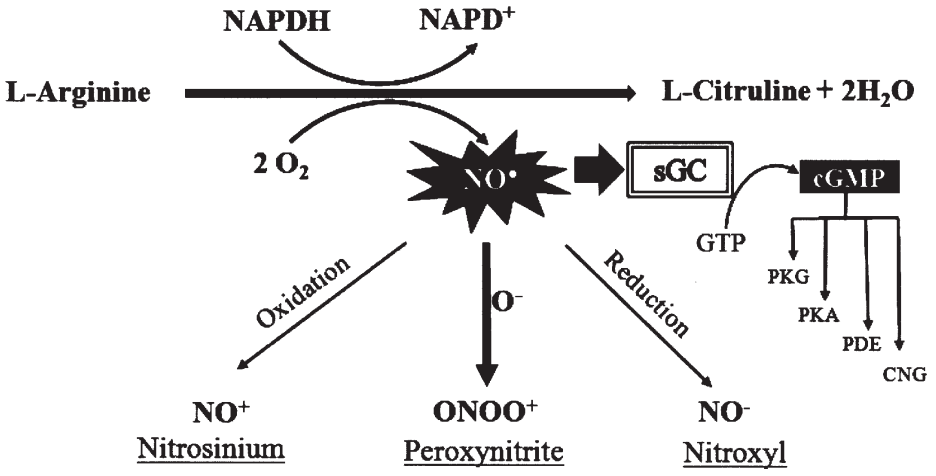

Figure 1. Nitric oxide formation and its redox states. NO's primary function is through its interaction with soluble guanylate cyclase (sGC), which catalyzes GTP to cGMP. cGMP then activates protein kinase G (PKG), protein kinase A (PKA), phosphodiesterases (PDE) and ion gated channels (CNG).

many diverse physiological processes in the body, which are broadly orchestrated by two major mechanisms of action:

i) The cGMP-dependent pathway is the main pathway by which NO exerts most of its biological functions (3). In this pathway, NO binds to the heme moiety of the soluble enzyme guanylate cyclase (sGC) resulting in the production of the second messenger cGMP from GTP. This, in turn, leads to the activation of cGMP-dependent protein kinases, cGMP regulated phosphodiesterases, and cyclic-nucleotide gated ion channels (4), which culminate in the main biological functions of NO, including vasodilation, neurotransmission, inhibition of platelet aggregation and smooth muscle relaxation (3).

ii) The cGMP-independent pathway occurs by the reaction of NO with molecular O_2, superoxide (O_2^-) thiols and transition metals such as zinc. NO can also modify proteins directly without the use of enzymes such as by nitration or nitrosylation. S-nitrosylation of cysteine thiol residues is a reversible modification involved in cell signaling, which regulates the function of many intracellular proteins (3).

There are three major isoforms of nitric oxide synthases, namely, NOS-I [neuronal NOS (nNOS)], NOS-II [inducible NOS (iNOS)] and NOS-III [endothelial (eNOS)]. NO produced from nNOS and eNOS exerts its biological function via the cGMP-mediated pathway. However, NO generated by the catalytic action of iNOS leads to biological and pathological functions, which are largely cGMP-independent (5). Neuronal NOS is constitutively expressed, calcium-calmodulin (Ca^{++}/CaM)-dependent and primarily found in the nervous system where its primary role is in neurotransmission (6,7). The presence of nNOS has also been established in non-neural tissues such as skeletal muscle (8) and epithelial cells of the lung (9). Endothelial NOS is constitutively expressed in myocytes, pyramidal cells of the hippocampus and, as its name implies, endothelial cells. This enzyme is also Ca^{++}/CaM-dependent and it is unique compared to nNOS and iNOS in that it is membrane-bound (10). The wide array of biological functions of eNOS include its participation in vascular tone, stimulation of angiogenesis and inhibition of platelet aggregation (3). Penile erection is mediated by eNOS by its relaxing properties on the corpora cavernosa (11). While nNOS and eNOS are constitutively expressed and Ca^{++}/CaM-dependent, iNOS is transcriptionally regulated and can be induced in cells when stimulated by various inflammatory ligands such as TNF-α, interferon (INF)-λ, interleukin-1 (IL-1), endotoxin, hypoxia and lipopolysaccharides (12). iNOS is expressed in various cell types, including macrophages, hepatocytes, osteoclasts, dendritic cells, astrocytes and epithelial cells (13). The three isoforms of NOS and their main function as well as their typical location are depicted in Table I.

Inducible NOS plays an important role in the immunogenic and cytotoxic response of T-lymphocytes and macrophages as well as in the bacteriostatic activity of the reticuloendothelial cells (14). NO exerts negative feedback on iNOS expression by inhibiting the post-translational modification of IκB, and thus preventing NF-κB activation (15). Of the several biological activities of iNOS, its role in the immunogenic and cytotoxic T-lymphocyte responses as well as its bacteriostatic activity on reticuloendothelial cells have rapidly evolved in the study of NO in oncology. The timing of disease onset and iNOS expression is fundamental in understanding the role of NO in oncology. The signaling effects of NO mediated by nNOS and eNOS are only seconds to hours (16) and are generated in low concentrations (in the nanomolar range) (17), while the effects of NO produced by iNOS occurs for much longer periods of time ranging from hours to days (16). iNOS increases the concentration of NO by a 40-fold (from 0.1 to 4 μM) or higher depending on the stimulus (18). It is the NO produced from iNOS that is largely responsible for the anti-proliferative effects exerted by activated rodent macrophages (19). The regulated pulses between eNOS and nNOS compared to the continuous upregulated NO synthesis of iNOS differentiate between the messenger and cell-death properties of NO (16). The precise half-life of the biological actions of NO are impossible to predict as NO reacts with other compounds, which have various degrees of stability (i.e., *S*-nitrosothiols). These data suggest that NO produced from iNOS and the level of iNOS expression in tissues play an important role in cancer biology.

Table I. Nitric oxide synthases and their main biological functions as well as primary sites.[a]

Nitric oxide synthase	Features	Primary site	Main function
NOS-I (nNOS)	Constitutive Ca^{++}/CaM-dependent	1. Neuronal tissues 2. Skeletal muscle 3. Lung epithelium	1. Neurotransmission 2. Relaxation of vascular and non-vascular smooth muscle
NOS-II (iNOS)	Inducible Ca^{++}/CaM-independent	1. Astrocytes 2. Chondrocytes 3. Dendritic cells 4. Epithelial cells 5. Fibroblasts **6. Macrophages** 7. Osteoclasts 8. Various cancer cells	1. Cytotoxicity 2. Host defense
NOS-III (eNOS)	Constitutive Ca^{++}/CaM-dependent Membrane associated	1. Endothelial cells 2. Cardiac myocytes 3. Hippocampal pyramidal cells	1. Platelet aggregation suppression 2. Vascular tone maintenance 3. Angiogenesis 4. Corpora cavernosa relaxation 5. Smooth muscle proliferation control

[a]Both nNOS and eNOS are constitutively expressed. These enzymes are calcium calmodulin (Ca^{++}/CaM)-dependent NOSs.

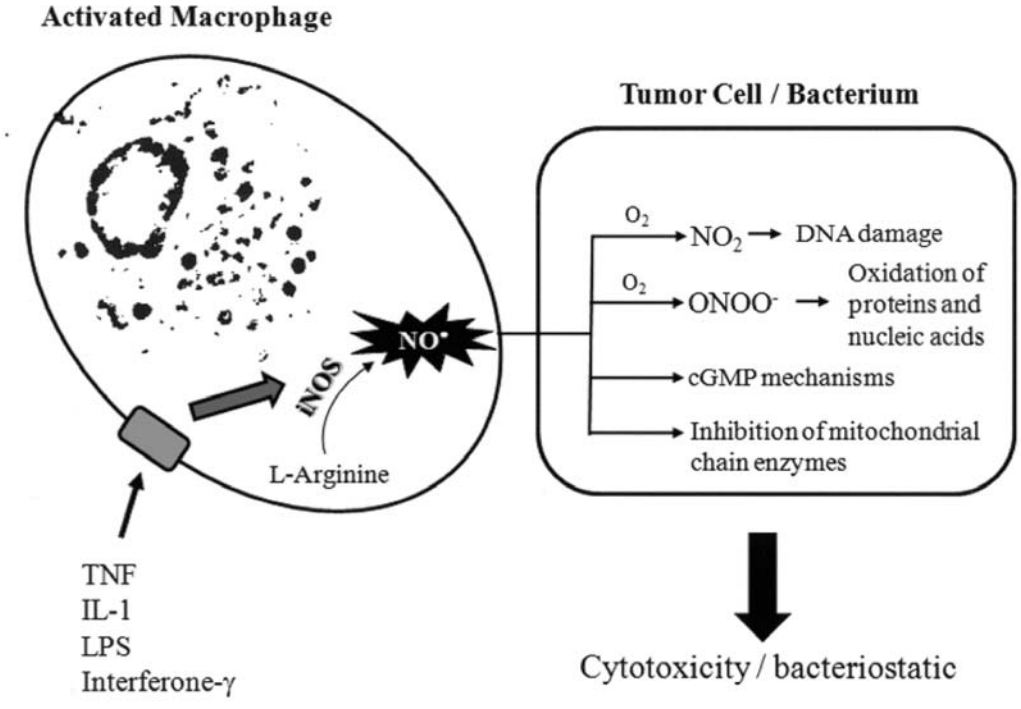

Figure 2. The initial observations of NO in cancer biology emanate from the observation that activated macrophages liberation of NO resulted in cytostatic and cytotoxic activity of target cells.

3. Nitric oxide in cancer

The role in tumor growth arrest by NO was initially suggested by the observation that murine activated macrophages synthesized nitrite and nitrate (20) leading to cytotoxicity of tumor cells and bacteria (21,22) (Fig. 2). This anti-tumor activity of NO first opened the door to identifying a function of NO as a potential oncologic agent. Indeed, this initial

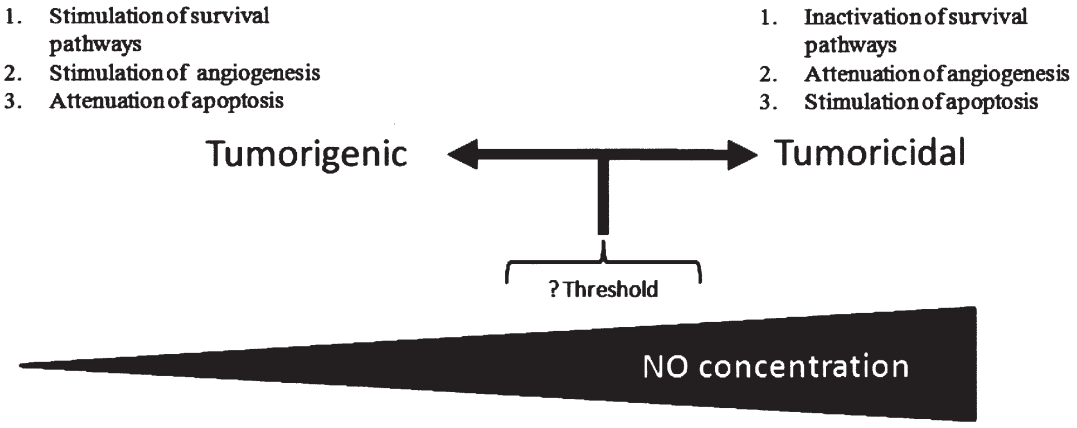

Figure 3. The concentration of NO (as represented by the width of the arrow) has been suggested to be responsible for its dual nature. The concentration at which this switch occurs is not clear.

observation has stimulated a number of reviews regarding the role of NO in oncology (16,23-25). However, whether NO or iNOS function as pro-neoplastic vs. anti-neoplastic effectors is still the center of much controversy. It is this fundamental difference where the nitric oxide donors can provide a great deal of insight.

While not inclusive, the following examples are representative excerpts from the literature that divide this controversy in half. In general, it has been suggested that at high concentrations NO may have an anti-neoplastic function whereas at low levels it can be pro-angiogenic and pro-tumor formation (26) (Fig. 3). NO at high concentrations causes programmed cell death and at low levels protects the cell from apoptosis, which has been suggested to be the result of a dual role of *p53* (17). In this phenomenon, low concentrations of NO may induce *p53* alterations or mutations, which cause tumor cell resistance; however, at high concentrations, the DNA damage induced by NO increases wild-type *p53* leading to programmed cell death. This hypothesis is supported by the findings that human DLD-1 cells engineered to produce NO from murine iNOS by transfection manipulations resulted in inhibition of cell growth *in vitro* compared to non-transfected DLD-1 cells. However, iNOS-transfected-DLD-1 xenografts grew faster than wild-type xenografts. The NOS activity in this model was 1-2 orders of magnitude lower than the NOS activity required to cause cytotoxicity and apoptosis (27). Similarly, iNOS-produced NO inhibits metastasis at high levels, but at low levels permits tumor growth (28). Nitric oxide mediated-cell death occurs by both necrosis and apoptosis (16) and a balance between apoptosis and necrosis by S-nitrosylation has also been suggested (29). Sustained NO production leads to caspase-mediated apoptosis, whereas at low physiological doses, it has the opposite effect on programmed cell death (30). To shed some light into the biphasic role of NO in oncology (i.e., pro-neoplastic vs. anti-neoplastic) excerpts from the literature are presented separately.

Pro-neoplastic activity of NO

Mechanisms of action that may lead to NO tumor formation include the following:

i) Apoptosis inhibition by: a) S-nitrosylation-inactivation of caspases-1, 2, 4, 8 and 3, 6, 7 (31); b) Inhibition of apoptosis by disruption of the Apaf-1/caspase-9 complex (32); c) Induction of heat-shock protein 70 (Hsp 70) (3); d) Mutation of *p53* (33-35); and e) Activation of cyclo-oxygenase-2 (36).

ii) Cell proliferation by activation of oncogenes (18).

iii) Angiogenesis simulation (37-39).

iv) Multifactorial by: a) Direct DNA damage by reactive nitrogen and oxygen species (40,41); b) Genotoxicity caused by deamination of guanine, cytosine, and adenine DNA bases (42,43); c) Nitrosation of biological amines leading to inhibition of DNA repair enzymes (44,45); and d) Release of toxic substances and a result of protein structure loss (46).

These above mechanisms of action have been demonstrated in models of carcinogenesis *in vivo*, *in vitro* and *ex vivo*. For instance, *in vitro* tumor cell production of NO may inhibit T-cell proliferation and induce them to undergo apoptosis (12). Low output of NO may promote angiogenesis and increase tumor blood flow (27). Additionally, multiple *in vivo* studies have shown a pro-neoplastic role of iNOS as depicted in Table IIA (47-53). In *ex vivo* models, increased iNOS expression and activity have been documented in pre-malignant and malignant conditions, including breast cancers (54), colonic neoplasms (55), esophageal Barrett's (56), gynecological malignancies (57), head and neck tumors (58,59), lung neoplasms (60), malignancies of the central nervous system (61), melanomas (62), prostate cancers (63,64), and gastric malignancies (65).

Anti-neoplastic activity of NO. Mechanisms of action that lead to the anti-tumorigenic properties of NO include the following:

i) Apoptosis stimulation by: a) *p53* up-regulation (34,66,67); b) Proteosomal degradation of anti-apoptotic mediators (68); c) Induction of Smac release (69); d) Increase in mitochondrial permeability changes leading to cytochrome *c* release (70); and e) Formation of $ONOO^-$ leading to increased in *p53* levels (24).

ii) Proliferation inhibition by: a) High NO production by macrophages can induce tumor cell cytostasis and cytotoxicity (12); b) Cell cycle arrest (require high NO concentrations) (17); c) Cell death by necrosis (71).

iii) Angiogenesis attenuation (72-74).

iv) Protection against tumor metastasis by NO production in microvessels adjacent to the tumor (75).

v) Anti-oxidant terminating cell damaging radical propagation and thus effectively function in cytoprotection (16).

Table II. Studies on iNOS and the putative role in oncology.[a]

A, Studies where the putative role of iNOS shows a pro-neoplastic phenotype.

Experimental model	Putative role of iNOS	Study findings	Authors/Ref.
iNOS in Apc^{min} mice	Promotes tumorigenesis	Apc^{min} express iNOS in normal mucosa and adenomas. L-arginine-deficient diets and iNOS inhibition decreased adenoma development. Apc^{min}-iNOS knock-out mice developed less adenomas vs. Apc^{min}-iNOS wild-type mice	Ahn and Ohshima (47)
Melanoma B16-F1 xenografts in iNOS knockout mice	Promotes tumorigenesis and decreased VEGF transcription	iNOS knockout mice had decreased B16-F1 melanoma tumor growth and decrease levels of VGEF mRNA	Konopka *et al* (51)
Rodent C-6 models of glioma tumorigenesis	Promotes tumorigenesis	iNOS C-6 knockout cells had decreased tumor mass when implanted in mice compared to control-implanted implanted cells. *In vitro* proliferation was not affected	Yamaguchi *et al* (53)
Polyomavirus middle T antigen targeted to murine mammary gland bred into iNOS knockout mice	Promotes early tumorigenesis, but not metastasis	Carcinogenic virus induced-mammary gland targeted murine models had delayed progression of mammary tumors in the iNOS knockout combined bred vs. iNOS wild-type combined bred. iNOS knockout mice retained similar metastatic potential vs. iNOS wild-type	Ellies *et al* (48)
iNOS in murine models of gastric cancer	Promotes tumorigenesis	The incidence of gastric cancer was less in iNOS knock-out mice vs. iNOS wild-type mice whose gastric cancer was induced either by carcinogens or with *H. pylori* infection	Nam *et al* (52)
iNOS in murine models of lung adenocarcinoma	Promotes tumorigenesis and decreased VEGF content	iNOS deficient mice developed less tumors and had less VEGF expression compared to iNOS-wild type mice	Kisley at al (50)
Irradiated-induced mammary tumors in rats	Promotes tumorigenesis	iNOS inhibition either p.o. or i.p. decreased mammary tumors in rats previously treated with gamma X-ray irradiation	Inano and Onoda (49)

B, Studies where the putative role of iNOS demonstrates anti-neoplastic properties.

Experimental model	Putative role of iNOS	Study findings	Authors/Ref.
B16 melanoma and Lewis lung cancer implanted cells in mice	Inhibits metastasis	The iNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) increased the metastatic potential of melanoma and lung cancer cell xenografts	Yamamoto *et al* (83)
Retroviral iNOS transfection into highly metastatic human renal cancer cells	Inhibits tumor growth and metastasis	iNOS transfected cells produced smaller tumors in the infected kidneys and also produced less lung metastasis compared to sham-injected mice	Juang *et al* (78)
Rat model of colon cancer	Inhibits tumor formation	iNOS inhibition by L-NAME increased formation of pre-neoplastic changes in a rat model of colon cancer	Schleiffer *et al* (80)

Table IIB. Continued.

Experimental model	Putative role of iNOS	Study findings	Authors/Ref.
Knockout iNOS/ Apc^{min} mice	Inhibits tumor formation	Apc^{min}-iNOS knockout mice developed more adenomas vs. Apc^{min}-iNOS wild-type mice	Scott *et al* (81)
iNOS knockout fibrosarcoma cells in iNOS knockout mice	Inhibits tumor growth and metastasis	iNOS knockout fibrosarcoma cells injected subcutaneously grew faster and in $iNOS^{-/-}$ mice and when these cells were injected i.v. there were more lung metastasis in the $iNOS^{-/-}$ mice vs. iNOS wild-type	Wei *et al* (82)
Lymphoma and sarcoma xenografts on *p53* and iNOS knockout mice	Inhibits tumor early development on a *p53* knockout background	Lymphomas and sarcomas developed at a faster rate in $p53^{-/-}$ $iNOS^{-/-}$ mice vs. either $p53^{-/-}$ $iNOS^{+/-}$ or $p53^{-/-}$ $iNOS^{+/+}$ mice	Hussain *et al* (77)
Transfection of iNOS knockout vectors and vectors with iNOS mutations in multiple cancer cell lines	Inhibits tumor growth and metastasis	NO-producing cells derived from various human tumors formed no tumors and resulted in an inability of these cells to metastasize in ectopic of orthotopic xenografts. Vectors harboring iNOS mutations resulted in a wide range of NO production in transfected cancer cells. The level of NO produced correlated with antitumor activity both *in vitro* and *in vivo*	Le *et al* (79)

[a]A dichotomy in the role of iNOS is observed with the number of reports divided in half by portraying iNOS as an anti-neoplastic marker vs. studies showing a potential pro-neoplastic phenotype dictated by tissue expression of iNOS.

These above mechanisms of action have been established in models of carcinogenesis *in vitro* and *in vivo*. For instance, *in vitro*, nitric oxide derived from macrophages, Kupffer, natural killer, and endothelial cells exhibits cytotoxic and cytostatic activities on target cells (2,20,76). Several studies *in vivo* have established an anti-neoplastic role for iNOS. These studies are depicted in Table IIB (77-83). In *ex vivo* models, increased expression of NOS activity in tumor vs. normal tissue does not occur in all human cancers (54). For instance, high iNOS activity was observed in only 25% of cervical cancers studied. Similar to previous observations, the level of NOS activity in cancer tissues was low compared to the level of activity required to cause cytotoxicity and apoptosis, which supports the finding that low levels of NO are pro-neoplastic and high levels are anti-neoplastic. These studies have suggested stromal macrophages and endothelial cells as the main sources of NO (54).

The dual role of nitric oxide in tumor formation continues to be divided in the literature. It is the microenvironment, the cellular background, amount and duration of exposure of NO to the targets that may explain such a biphasic nature of this radical. It is by the development of the instrumentation of NO donors that this dissection may be performed to yield a unifying mechanistic role of NO in oncology.

4. NO donors

Because of the highly reactive nature of NO, it is difficult to predict its biological effects on a given system from single doses of this agent even if provided by the longer action of iNOS. Thus, NO donors capable of producing a sustained released with a wide range of half-lives, and with a predictable estimated dose have become useful tools to study the biological properties of NO in cells and *in vivo* models of carcinogenesis. For an in depth review of the chemistry of the various classes of NO donors and their preparation, the reader is referred to reports by Wang *et al* (84) and Feelisch and Stamler (85).

The specific advantages of NO-donors in oncology include: i) varied half-life depending on the compound; ii) spontaneous release of NO depending on the compound; iii) NO release can be provided at a controlled rate; iv) multiple chemical reactions based on the parent compound; v) safety for clinical application by some compounds previously used in the cardiovascular system (i.e., glyceryltrinitrate); and vi) multiple mechanisms of NO release (Fig. 4) such as: a) spontaneous release of NO; b) chemical reaction with acid, alkali, metals and thiol; and c) enzymatic oxidation.

The classification of nitric oxide donors may take several forms because all nitrogen oxygen-bound compounds rapidly undergo oxidation and reduction reactions. A system suggested by Wang *et al* (84) takes into account the similarity in structure of the NO donor and its form of NO generation. This classification is summarized in Fig. 5.

The following are important parameters to consider when selecting the appropriate NO donor in a particular system: i) the byproducts released during the decomposition of the NO donor as they can have a significant effect on the outcome

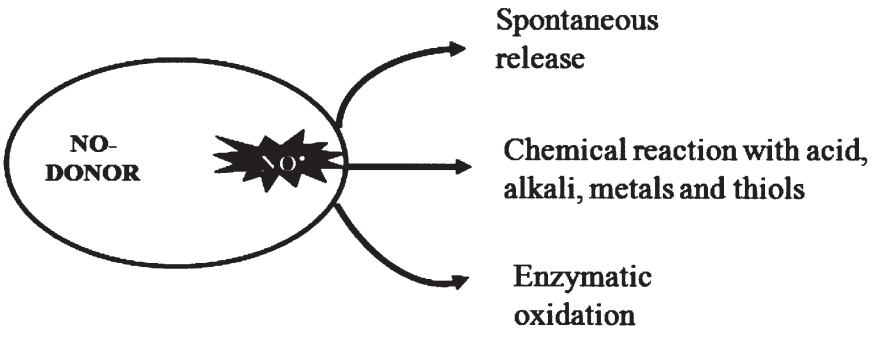

Figure 4. Mechanisms by which NO is released from NO donors (84).

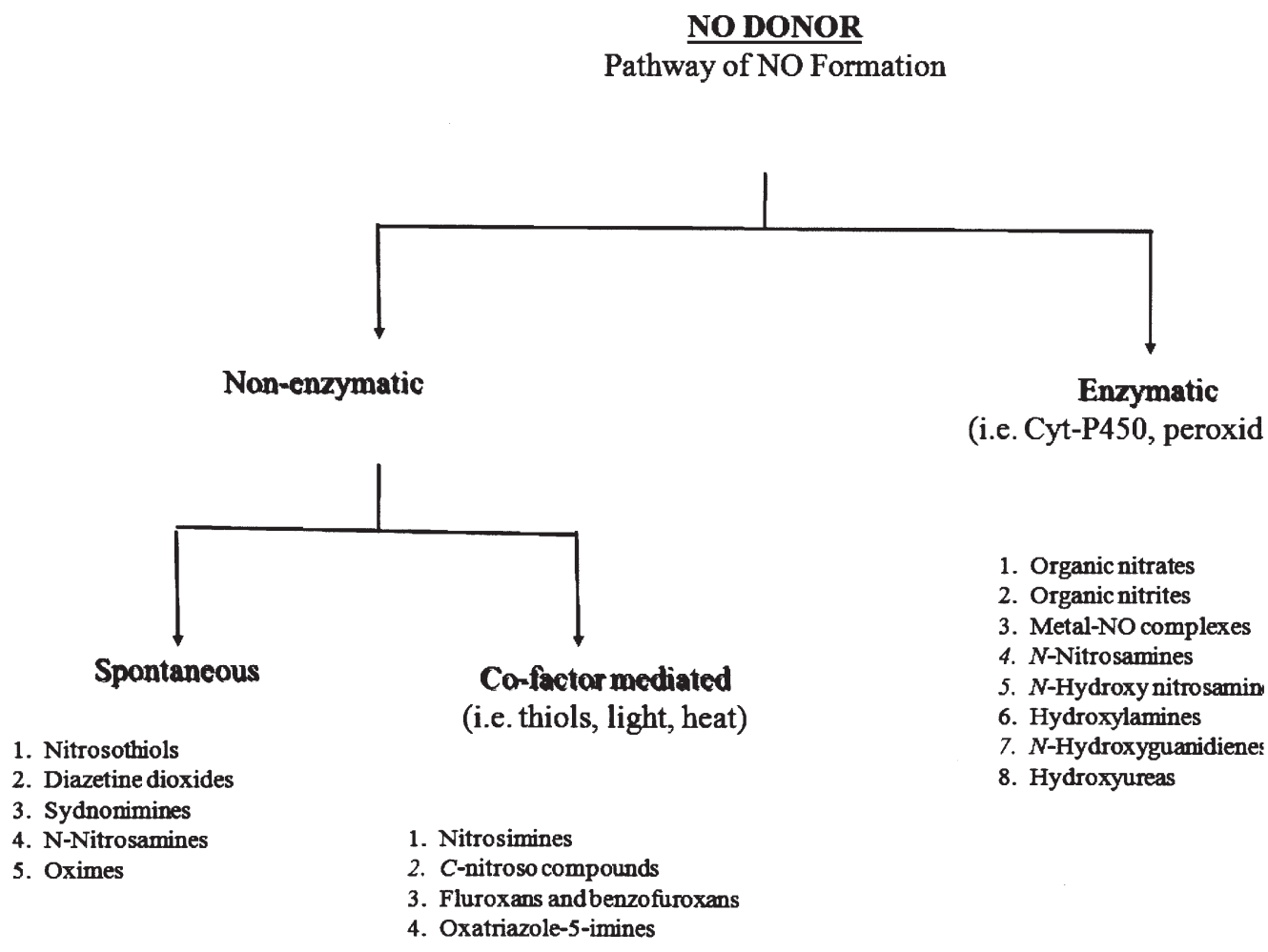

Figure 5. Classification of NO-donors based on their pathway of NO generation. Several compounds share non-enzymatic and enzymatic pathways for NO generation.

such as sodium nitroprusside, which generates cyanide as its byproduct; ii) the microenvironment of the reaction such as pH and temperature which could affect the half-life and the release of the NO donor; iii) the half-lives of NO donors (a wide range of NO production from 1.8 sec to 56 h can be accomplished depending of the NO donor as well as the microenvironment of the reaction); and iv) the mechanism of NO release as some NO donors may require enzymatic activation for the generation of NO (Fig. 4).

5. NO donors in cancer

Several NO donors have been used to study the role of nitric oxide in tumor biology. Of these donors, the group belonging to the class of the *N*-Nitroso compounds (Fig. 5) has gained substantial interest in the area of oncology. One of the first of such compounds prepared was dimethylnitroamine, which was tumorigenic as described over half a century ago by Magee and Barnes (86). This ignited the synthesis of multiple com-

pounds to test their oncologic properties (87,88). The *N*-Nitroso compounds can be divided broadly into nitrosamines, hydroxy-*N*-nitrosoamines, nitrosoimines and diazeniumdiolates.

The therapeutic application of NO donors has been limited by potential systemic effects exerted *in vivo*. These adverse effects include vasodilation leading to pronounced hypotension and accumulation of toxic metabolites such as cyanide (89). Search is currently underway for the ideal NO donor with maximal anti-proliferative properties and minimal side effects. The development of NO-drug hybrids, whereby an NO moiety is attached to currently known anti-cancer agent (i.e., NO-NSAID), provides additive anti-tumor effects by each compound while minimizing their respective side effects (i.e., NSAID induced peptic ulcer disease). NO-NSAIDs demonstrate promise as anti-cancer agents and are currently in clinical trials by an NCI sponsored phase I randomized studies [i.e., NO-aspirin in high-risk patients with colorectal cancers (90)].

In the present discussion, we outline the important classes of NO donors that are being examined in cancer research and their outcomes in various studies. The major classes of NO donors in cancer include: i) Organic nitrates; ii) Metal-NO complexes [sodium nitroprusside (SNP)]; iii) *S*-nitrosothiols; iv) Sydnonimines; v) Diazeniumdiolates (NONOates); and vi) NO-drug hybrids.

i) Organic nitrates. Organic nitrates are the oldest class of NO donors in clinical use (84). These compounds are nitric acid esters of mono- and polyhydric alcohols with the general formula ($RONO_2$). Commonly used organic nitrates include glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN). Glyceryl trinitrate (Fig. 6A) has been used for over a century as a therapeutic agent for relaxation of the coronary vessels leading to improvement of chest pain (91). GTN is a prodrug, which requires denitration for NO generation. Multiple mechanisms have been proposed to account for denitration reactions and these are still the center of a disputed controversy. These mechanisms include denitration by reactions with sulfhydryl groups, enzymatic activation by: glutathione S-transferase, cytochrome P450, and/or xanthine oxidoreductase and catalytic activity mediated by mitochondrial aldehyde dehydrogenase (91). GTN has a biological half-life of 1-4 sec, but its metabolites (1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate) have a half-life of up to 40 min (91).

The clinical application of GTN for over ten decades in the cardiovascular system has proven it to be a safe agent with a relatively short side effect profile, including hypotension and headaches. One of the major limitations of nitrates is the development of tolerance following continued administration (92). However, its current clinical administration makes GTN an attractive candidate to determine its properties in the area of oncology.

In cancer, the hypoxia-mediated metastatic potential of murine melanoma B16F10 cells was inhibited by GTN. Established tumor xenografts with these cells decreased the ability of murine melanoma cells to metastasize and form lung nodules *in vivo* if the nude mice had received GTN (93). GTN sensitized colon cancer cells to Fas-mediated apoptosis and resulted in activation of caspases-1 and -10 (94). GTN also demonstrated a down-regulation of the ß-catenin/TGF signaling pathway as a result of ß-catenin-proteasome-dependent degradation in colon cancer cells (95). GTN, at a low dose (0.1 nM), chemosensitized prostate cancer TRAMP-C2 and PC-3 cells to doxorubicin (12.5 μM) under hypoxic conditions (0.5% O_2) (96).

GTN transdermal patches have been used in the United States for the management of angina since the 1980s (91). However, the 24-h sustained release of plasma GTN limits its therapeutic applicability in the cardiovascular system as a result of the rapid development of tolerance (97). In cancer, the development of tolerance in the cardiovascular system is desirable as this would limit the systemic side effects of the continuous release of NO while taking advantage of the anti-neoplastic or chemosensitizing properties of GTN patches. Fredericksen *et al* (96) demonstrated the potential role of GTN as a sensitizing agent *in vivo* by transdermal application of this compound in a murine model of prostate cancer. GTN patches were applied to mice with prostate cancer xenografts and treated with doxorubicin. These mice showed a decrease in tumor growth compared to the xenografts treated with doxorubicin alone (98). More recently, nitroglycerine patches have been shown to have a therapeutic effect on small cell lung cancer patients treated with chemotherapeutic drugs, with minimal side effects (99,100).

ii) Metal-NO complexes [sodium nitroprusside (SNP)]. Nitric oxide has great affinity for metals. In fact, NO has higher affinity to bind metals than to bind CO and O_2 (84). Under bioregulatory conditions, iron is a primary target for NO binding (101,102). Thus, metal nitrosyl compounds (M-NO) may be nitric oxide donors. Of the metal nitrosyl compounds, the most widely used agent is SNP [$Na_2Fe(CN)_5NO$], which liberates 1 mol of NO per M-NO. Owning to the formation of NO (103), SNP is an excellent therapeutic agent for the management of hypertensive emergencies and has been in clinical use for over seven decades (84). While the generation of NO from SNP is not well understood, NO release requires irradiation or thiol-mediated reduction reactions (84). *In vivo*, NO production from SNP may be either enzymatic or non-enzymatic. Release of NO in tissues requires reduction-mediated reactions and leads to the generation of cyanide as well (104). Thus, the use of large doses of SNP may lead to cellular toxicity, not only as a result of cyanide formation but also from peroxinitrite (105) and H_2O_2 production (106). Fortunately, the vasodilator properties of SNP do not require large doses, which produce no substantial cyanide toxicity (84).

In cancer, studies *in vitro* showed that SNP suppressed *de novo* synthesis of TGF-ß1 mRNA, which is upregulated in advanced prostate cancer (107). PC-3 and DU145 prostate cancer cells demonstrated down-regulation of TGF-ß1 mRNA by more than a 2-fold in a dose-dependent manner following treatment with SNP (0.25 nM to 1.0 nM) (107). This inhibition was attenuated by co-incubation with NO scavengers and the iNOS inhibitor N-methyl-arginine (NMA) (107). NO from SNP suppressed invasion of prostate PC-3 and bladder T24 cancer cells *in vitro* without causing cytotoxicity (108). Matrigel-coated invasion-chamber invasion of PC-3 and T24 cells was associated with down-regulation of hypoxia-inducing factor-1α (HIF-1α) and caused attenuation

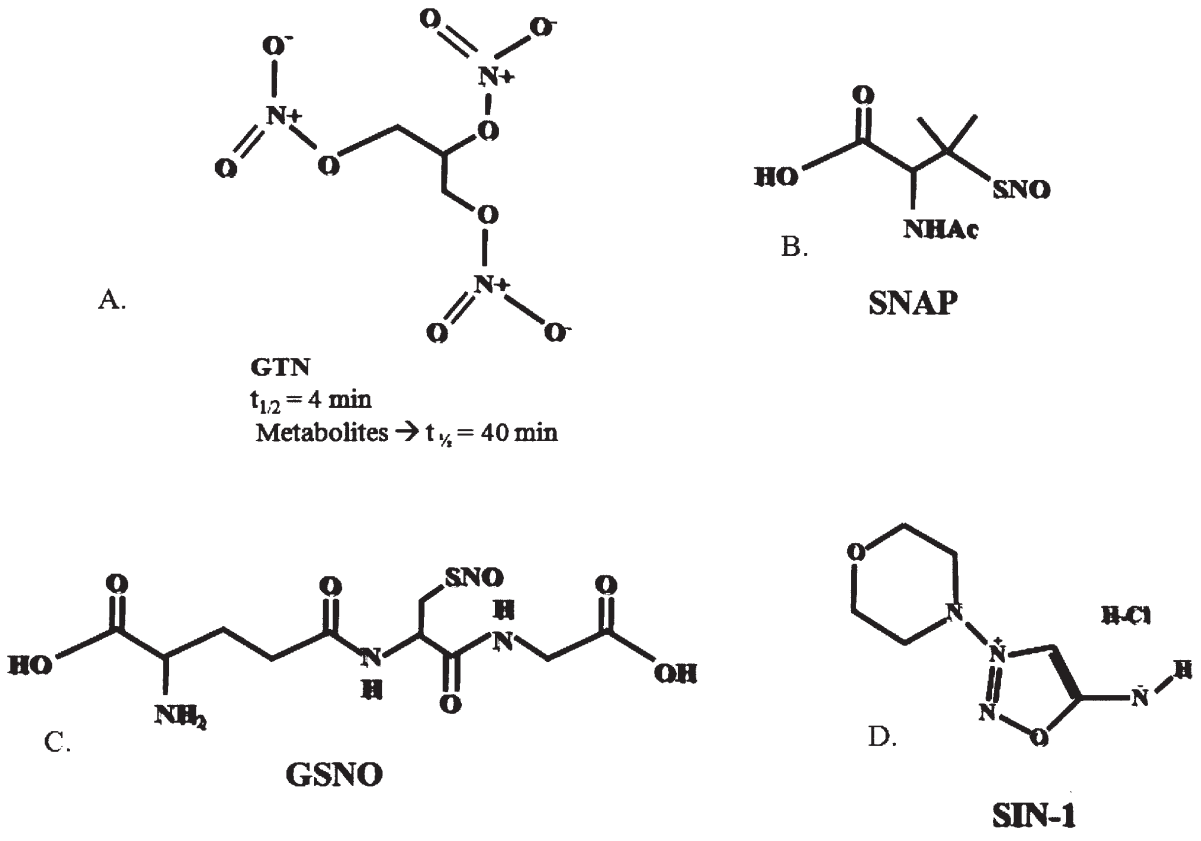

Figure 6. Organic nitrate (GNT), the RSNO compounds (SANP and GSNO) and a classic sydnonimine (SIN-1).

of mitochondrial respiration when the cells were incubated with SNP (1.0 nM) (108). Similarly, SNP decreased the speed of migration of gastric epithelial cells by 18% (at 0.5 nM) and 33% (at 1.0 nM) (109). This effect was associated with an increase of caspase-3 activity in gastric epithelial cells incubated with 0.5 nM of SNP (109). In cervical cancer HeLa cells, NO from SNP caused an increase in the protective protein against oxidative cellular stress (caused by heme oxygenase-1 transcriptional activity) in a concentration-dependent manner via activation of mitogen protein kinases ERK and p38 (110). SNP (0.1 nM) radiosensitized pancreatic cells as a result of enhancement of the formation of single-strand DNA breaks (111). SNP also radiosensitized glioma cells, but the mechanism was not clear (112). Apoptosis stimulation occurred in T cell lymphoma (HuT-78) cells incubated with SNP (1.0 mM) as a result of inhibition of constitutive NF-κB leading to a decrease of Bcl-2 (113). In contrast, SNP (0.5 nM) inhibited Bcl-2 ubiquitination resulting in an increase in its activity and inhibition of apoptosis in human lung carcinoma H-460 cells, which caused a cisplatin-resistant phenotype (114). In C-6 glioma cells, the iron of SNP (0.1-1.0 nM) rather than NO protected them from chemical-induced hypoxic cell death by activation of ion channels culminating in calcium and sodium efflux (115).

The results from the studies of SNP in cancer point to the biphasic nature on nitric oxide on cell death vs. survival; and also to the importance of the microenvironment (i.e., cell type). These observations also illustrate the importance of the NO donor used (i.e., an M-NO donor), where the parent compound may be responsible to the biological activity of the NO donor compound.

iii) S-Nitrosothiols. Nitrosothiol compounds have the general formula 'RSNO' and are typically unstable. However, two relatively stable compounds in this class include: a) S-nitroso-N-acetylpenicillamine (SNAP); and b) S-nitrosoglutathione (GSNO) (Fig. 6B and C) (84). These compounds may serve for the storage, transfer and delivery of NO in many reactions (84). Decomposition of RSNO compounds yields NO, NO^+ and NO^-. The S-NO bond can be disrupted by heat, UV light, and some metal ions, superoxide, and seleno compounds (84). Metal ions (Cu^+, Fe^{++}, Hg^{++} and Ag^+), especially copper, serve as important catalysts for the decomposition of RSNOs (84,88).

a) SNAP. SNAP (Fig. 6B) is a relatively stable tertiary S-nitrosothiol, which functions as an NO donor with a potent vasodilator activity. Its stability in solution varies from seconds to hours depending on temperature, buffer composition and metal content (116-118). At a pH of 6.0-8.0 and a temperature of 37˚C, the half-life of SNAP is approximately six hours in the presence of transition-metal ion chelators (119).

Evidence for the oncogenic properties of SNAP stems, in part, by its role in apoptosis; but more substantially by its function in radiosensitization in various tumors. For instance, SNAP caused an increase in apoptosis and cell death in neuroblastoma cells as a result of an elevation in *p53* levels. However, apoptosis occurred in a Bcl-2/BAX-independent fashion (120). In human neuroblastoma SH-SY5Y cells, SNP (1.0 mM) and SNAP (1.0 nM) caused cell death as a result of

GAPDH and poly-(ADP-ribose) polymerase-1 (PARP-1) ADP ribosylation (121).

As a radiosensitizing agent, SNP caused upregulation of Fas and associated increase in CH-11-mediated apoptosis in human cervical cancer (HeLa) cells following treatment with ionizing radiation (122). Glioma cells pre-treated with SNAP (0.1 nM) led to a sensitizer enhancement ratio of 1.4-1.8 (112). Mitchell *et al* demonstrated that the release of NO under hypoxic conditions and the radiosensitizing capabilities of NO donors were superior when using the RSNO donors SNAP and GSNO (1.0 mM) compared to SNP and 3-morpholinosydnonimine in Chinese hamster V79 lung cells (123,124). Janssen *et al* showed that it was the bioreductive release of NO from SNAP (1.0 mM), which caused hypoxic cell radiosensitization in murine mammary adenocarcinoma EMT-6 cells (125). Thus, the radiosensitizing potential of NO donors varies with the type of NO donor used, especially those requiring bioreduction.

In contrast, SNAP has also been shown to have pro-neoplastic role in various malignancies (58,59,126). In head and neck cancer cells, SNAP (0.05-1.0 nM) caused an upregulation of iNOS and COX-2, resulting in a carcinogenic phenotype (58,126).

b) GSNO (S-nitrosoglutathione). GSNO (S-nitroso-L-glutathione) (Fig. 6C) is the second of the RSNO compounds with relative stability, which serves as a good source of NO based on the cleavage of the S-NO bond (116). GSNO is a potent smooth muscle relaxant and inhibitor of platelet aggregation (127). Its stability in solution varies from seconds to hours depending on temperature, buffer composition, and metal content.

In colon cancer SW620 (*p53*-deficient) and HCT116 (*p53*-wild-type) cells, GSNO induced apoptosis in both cell lines (128). Apoptosis, however, was more prominent in the SW620 suggesting that NO-mediated apoptosis occurred in a *p53*-independent fashion (128). Furthermore, GSNO caused G_0/G_1 cell cycle arrest in SW620, but not in HCT116 cells. Cell cycle arrest was accompanied by GSNO induction of the ERK1/2 and p38 kinase pathway in *p53*-deficient SW620 colon cancer cells (128). In human HT29 colon cancer cells, liberation of NO from GSNO (0.5 mM) was significantly facilitated by the catalytic activity of Cu^{++} and Ni^{++}, which enhanced GSNO-mediated apoptosis (129). Enhanced apoptosis in these cells was accompanied by a 4-fold increase in BAX and Bad with a concomitant decrease in the levels of Bcl-2, which were modulated by Cu^{++} (129). GSNO (10-500 μM) induced the same rate of apoptosis in HCA7 colon cancer cells (expressing relatively high levels of COX-2) and in HCT116 colon cancer cells (which demonstrate no COX-2 expression at base line), suggesting the cell death and GSNO-induced apoptosis in these colon cancer cells were independent of the COX-2 pathway (130). Similar to SNP, GSNO sensitized hypoxic Chinese hamster V79 lung cells to ionizing radiation (123,124).

The pro-neoplastic role of GSNO has been suggested by several studies. In melanoma cells, GSNO resulted in an increase in proliferation as a result in the upregulation of IL-8 (131). In C-6 glioma cells, GSNO conferred chemoresistance against BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea], which is the mainstay chemotherapy in glioblastoma multiforme (132,133). The effects of such chemoresistance were the result of increase levels of iNOS, COX-2, and HIF-1 in C-6 glioma cells (132,133).

iv) Sydnonimines. Sydnonimines are in the general class of NO-releasing heterocycles within the subclass of mesoionic heterocycles (134). The most extensively studied compound in this class is 3-morpholinosydnonimine (SIN-1) (Fig. 6D) (84). SIN-1 is generated from the pro-drug molsidomine by esterase catalysis in the liver and has a half-life of 1-2 h in plasma (84). Sydnonimines release NO in an alkaline pH and its release is facilitated by oxygen and irradiation from visible light (135,136). In the presence of oxygen, sydnonimines produce peroxynitrite from superoxide generated in their decomposition and are, therefore, considered classic donors of peroxynitrite (84). An advantage of these agents is that they do not induce tolerance or the cross-tolerance that occurs with nitrates as the NO releasing mechanism is spontaneous and independent of thiols (84). However, the production of toxic levels of peroxynitrite also limits the therapeutic application of these compounds. The short duration of action requiring frequent dosing of these agents also limits their therapeutic use.

In cancer biology, SIN-1 has been mainly used as a peroxynitrite generator to study the cytotoxic effects of peroxynitrate ($OONO^-$) (137). Peroxynitrate induces cellular damage by causing single stranded DNA breaks, induction of protein nitration and by inhibition of mitochondrial respiration (138-140). In rodent cortical cell cultures, the neurotoxicity elicited by SIN-1 is exclusively the result of the formation of peroxynitrite (141). SIN-1 (2.5-25 μM) impaired the mitotic activity of glioma C-6 cells and this effect was reversed by co-treatment of cells with LPS (100 ng/ml) (142). In human MCF7 breast cancer cells, peroxynitrite generated from SIN-1 (0.5-1.0 mM) inactivated enzymes that play a crucial role in the elimination of several carcinogens [arylamine-N-acetyltransferases (NATs)] (143). This effect was suggested to contribute to the carcinogenesis and tumor progression in MCF7 cells by peroxynitrate (143). Trackey *et al* showed that SIN-1-induced neuronal cell death depending on the antioxidant status of the cell (141). SIN-1 was less potent of a mutagen in lymphoblastoid TK6 cells, which are *p53* wild-type compared to WTK cells containing *p53* mutations (69). SIN-1 also resulted in a more substantial increase in apoptosis in the *p53* wild-type cells compared to WTK cells (69). In human esophageal adenocarcinoma cells, the genotoxic effects of the carcinogen (myosmine) were substantially augmented in the presence of SIN-1, as a result of its contribution in nitrosative stress (144).

In contrast to the pro-neoplastic effects of SIN-1 from its nitrosative stress, SIN-1 has been shown to have anti-neoplastic properties. For instance, SIN-1 has anti-tumor activity in Hep3B, Neuro2A and HeLa cells as a result of NO-mediated decrease in HIF-1α (145). Similarly, the effects of the hypoxia mimicking agent, cobalt chloride, were antagonized by co-incubation of human acute myeloid leukemic NB4, U937, and Jasumi-1 cells with SIN-1, and this was also the result of decreased levels of HIF-1α (146). Additionally, SIN-1 increased apoptosis in human neuroblastoma cells by activating the p38 MAPK pathway and inducing caspase-3 like proteases activation (147,148).

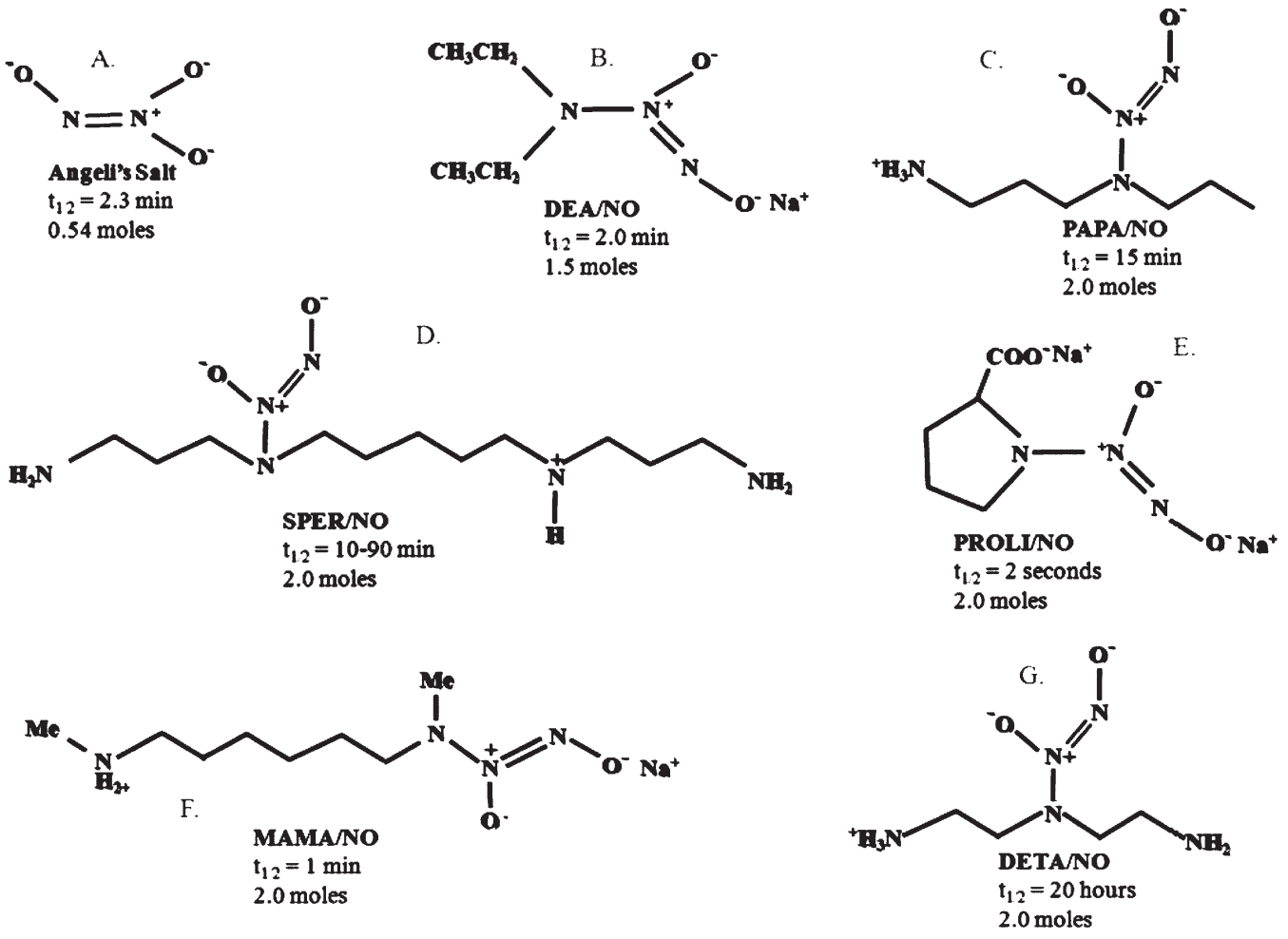

Figure 7. *N*-diazeniumdiolates (NONOates) and their respective half-lives ($t_{1/2}$) at a pH of 7.4 and temperature of 37˚C. The number of moles depicted below the half-lives of each compound refers to the number of NO moles released per parent compound.

v) Diazeniumdiolates (NONOates). The diazeniumdiolates are a group of compounds also known as 'NONOates'. These compounds have the basic structure: **X-[N(O)NO]**$^-$, in which 'X' is typically a secondary amine. In Angeli's salt, 'X' is 'O-' (149). At a pH 7.4 and a temperature of 37˚C, most of these compounds generate up to 2 mol of NO per [N(O)NO]$^-$. These compounds have a wide range of half-lives from 2 sec to 20 h (149). The first compound of this family was synthesized by Drago *et al* in 1960 (150) and since then several others have been synthesized by Keefer's group at the NCI (151). These compounds are attractive because they are stable as solids, but can be triggered to release NO at controlled rates by simple chemical reactions such as hydrolysis. The amount of NO generated can be calculated as most of them generate 2 mol of NO per NO donor. These features are useful properties for the study of NO in oncology (151).

The main compounds in this group are depicted in Fig. 7. Their accepted nick names rather than the chemical formal name will be consistently used for simplicity. The compounds in this group include: a) Sodium α-oxyponitrite (Angeli's salt); b) DEA/NO; c) PAPA/NO; d) SPER/NO; e) PROLI/NO; f) MAHMA/NO; and g) DETA/NO.

a) Sodium α-oxyhyponitrite (Angeli's salt). Angeli's salt (Fig. 7A) is regarded as a classical nitroxyl (NO$^-$) donor. It spontaneously dissociates in a pH-dependent, first-order process with a half-life of 2 to 3 min at 37˚C (pH 7.4) to liberate 0.54 mol of NO per mole of parent compound. Hydrolysis of Angeli's salt generates the hydroxyl radical (•OH) and hydrolysis of this radical forms nitroxyl (HNO) (152). Angeli's salt is a typical donor of nitroxyl (HNO), which is the 1-electron and protonated form of NO (153). HNO, from Angeli's salt, suppressed the growth of estrogen-dependent and estrogen-independent breast cancer cells and its established xenografts in nude mice in a dose-dependent manner (154). Inhibition of proliferation and reduction of tumor growth correlated with a decrease of tumor vessel formation *in vivo* and a low level of serum VEGF as well as a decrease in hypoxia-inducible factor *in vitro* (154).

HNO, from Angeli's salt, demonstrated high toxicity in neuroblastoma cells *in vitro* at a low pH (6.0), which was a 10-fold higher than at a pH 7.0. Because tumor cells have a lower pH (6.0-7.0) compared to normal tissue (7.4), these data emphasize the importance of the microenvironment in the generation and biological action of NO-donors. These findings are supported by the observation that established neuroblastoma xenografts (a model of pheochromocytoma) in nude mice were inhibited by Angeli's salt at doses that were not toxic in normal tissues (152). Nitric oxide generated

from Angeli's salt, in combination with electron acceptors (i.e., ferricyanide or tempol) radiosensitized V79 Chinese hamster lung fibroblast, which was the result of tumor-induced hypoxia (155). NO derived from Angeli's salt without such electron acceptors failed to produce radiosensitizing effects in these cells. These findings further support the importance of the microenvironment in the response elicited by NO.

b) DEA/NO. DEA/NO (Fig. 7B) is a short acting NO donor and spontaneously dissociates in a pH-dependent, first-order process with a half-life of 2 and 16 min at 37 and 22-25˚C, pH 7.4, respectively, to liberate 1.5 mol of NO per mole of the parent compound (134). The properties of DEA/NO alone and in combination with other NO-donors have been studied in various oncologic systems. The role of DEA/NO as an anti-neoplastic agent was suggested in breast cancer cells and their bone metastases (156). An NO pro-drug that was synthesized using DEA/NO, NONO-AM, induced apoptosis in both parental breast cancer cells and bone metastases of these cells. These effects were accompanied by a decrease in matrix metalloprotease-9 activity (156). In human neuroblastoma NB69 cells, DEA/NO showed that the anti-proliferative effect of NO was mediated through the inhibition of EGFR (epidermal growth factor receptor) tyrosine kinase activity; this was time- and concentration-dependent, fully reversible, and occurred without affecting the cell viability or apoptosis (157). This study also showed that NB69 cells expressed iNOS and nNOS suggesting that NO is an endogenous growth regulator of this cell line. In this study, the potency of the anti-proliferative effect was proportional to the half-life of the NO donor used, i.e., DETA/NO > DEA/NO > SNAP (157). These observations are matched by studies demonstrating a pro-tumor activity by NO derived from DEA/NO. For instance, DEA/NO and DETA/NO as NO donors showed that NO can cause c-GMP-mediated endothelial cell migration into the hypoxic tissue in subnanomolar concentrations in the umbilical vein endothelial cells (158). This mechanism, in addition to stimulation of VEGF by NO, may contribute to the role of nitric oxide in promoting tumor growth and metastasis. Transforming growth factor beta-1 (TGF-ß1) produced by tumor cells suppressed the function of macrophages and inhibited iNOS and production of NO by neighboring cells (159). In human lung cancer cells, DEA/NO caused nitrosative stress and resulted in an augmentation in TGF-ß1 activity, which may contribute to the ability of these cells to escape macrophage killing (160).

c) PAPA/NO. PAPA/NO (Fig. 7C) spontaneously dissociates in a pH-dependent, first-order process with a half-life of 15 and 77 min at 37 and 22-25˚C, respectively, pH 7.4, to liberate 2 mol of NO per mole of parent compound (134). Evidence of the role of PAPA/NO in carcinogenesis was originally suggested by radiosensitization of hypoxic murine mammary adenocarcinoma cells by IFN-γ-induced iNOS upregulation resulting in the increase levels of NO (161). When compared to the spontaneous NO donors PAPA/NO and SNAP, the nitric oxide level induced by IFN-γ was 3-10-fold higher. Yet, PAPA/NO had a similar or higher radiosensitizing effects in EMT-6 cell (161), which suggests that vasodilation and/or other systemic side effects mediated by the spontaneous release of NO from PAPA/NO were responsible for the radiosensitizing effects in these cells (161). In HT29 human colon cancer cells, a dual effect of nitric oxide in apoptosis was noted with PAPA/NO in a time-dependent manner. At 24 h, irrespective of the concentration, NO had a reversible cytostatic effect without any cell death whereas at 48 h most of the cells treated with PAPA/NO (10^{-3} M) underwent apoptosis. Cell-cycle analysis showed a significant accumulation of cells in the G_2/M phase (162). The cytostatic effect was the result of the rapid and reversible inhibition of ribonucleotide reductase, followed by re-entry of the tumor cell into the cell cycle at high concentrations of PAPA/NO, which caused an increase in apoptosis (162). Furthermore, NO-induced cell death was prevented by blocking the re-entry of cells into the cell cycle (162). While the evidence in tumor suppression with the employment of the diazeniumdiolate PAPA/NO is modest, few studies point to the importance of the microenvironment. For instance, determining the secondary effects of the NO donor employed is important in the final result of the cell type under investigation. Establishing the particular stage of the cell on the cell cycle to determine its ultimate fate may lead to variations on the effect NO in carcinogenesis.

d) SPER/NO. SPER/NO (Fig. 7D) spontaneously dissociates in a pH-dependent, first-order process with a half-life of 39 and 230 min at 37 and 22-25˚C, pH 7.4, respectively, to liberate 2 mol of NO per mole of the parent compound (134). The effects of SPER/NO as a potential anti-cancer agent have been studied in various *in vitro* settings. NO generated from SPER/NO (0.1 mM-100 mM) increased hemoxygenase-1 mRNA expression and protected the cells against cadmium-induced cytotoxicity in rat C-6 glioma cells (163). Low flux NO from SPER/NO (0.4 mM) was cytoprotective in COH-BR1 breast cancer cells induced to cell death by photodynamic cell killing (164). SPER/NO (0.1-2.0 mM) radiosensitized hypoxic cells to a similar magnitude as DEA/NO in murine mammary carcinoma SCK cells (165). In this experiment, SPER/NO at the higher doses was cytotoxic in aerobic conditions, but not in anaerobic conditions (165). Similarly, in human neoplastic salivary gland (HSG), NO from SPER/NO had opposing effects depending on the concentration used. At low doses (20 μM), SPER/NO caused HSG cells to escape from the G_2/M phase of the cell cycle leading to cell growth and proliferation; while at higher doses (100-500 μM), it had the opposite effects (166). These observations underscore the importance not only of the microenvironment of the tumor cells, but also the concentration, and the NO donor employed in the experiment.

e) PROLI/NO. PROLI/NO (Fig. 7E) spontaneously dissociates in a pH-dependent, first-order process with a half-life of 2 sec at 37˚C and pH 7.4 to liberate 2 mol of NO per mole of the parent compound (167). Evidence of the anti-neoplastic properties of PROLI/NO has been suggested *in vivo* in smooth muscle of canine endarterectomized arteries (168). In this study, NO from PROLI/NO (100 mM) inhibited smooth muscle proliferation by 43% in injured arteries (168). Because of the short half-life and the local delivery of PROLI/NO, systemic side effects were not observed (168). A 40% increase in survival was observed in rats with C-6 gliomas receiving combination treatment with carboplatin (20 mg/kg) and PROLI/NO (10 nM) compared to carboplatin, PROLI/NO, or vehicle alone (169).

f) MAHMA/NO. MAHMA/NO (Fig. 7F) spontaneously dissociates in a pH-dependent, first-order process with a half-life of 1 min at 37˚C and pH 7.4 to liberate 2 mol of NO per mole of the parent compound (170). In RAW 264.7 macrophages, NO from MAHMA/NO (500 μM to 1.0 mM) enhanced pathological inflammation as suggested by the transcriptional upregulation of interferon-ß and IκB-α when cells were co-incubated with LPS (100 ng/ml) (171). These responses are important in propagation of both the immune system as well as the inflammatory process. In HT-29 colon cancer cells, MAHMA/NO was not as effective in suppressing essential enzymes for proliferation [Ornithine decarboxylase (ODC)] as SNP (172). This effect was suggested to be the result of the effect of the production of different nitrite species of MAHMA/NO compared to SNP, which had different efficiency in OCD activity (172).

g, DETA/NO. Of the several compounds belonging to the diazeniumdiolates, DETA/NO is an attractive compound to be used in cancer owing this to its long half-life as it closely mimics sustained endogenous NO release for 20 h or more depending on the temperature of the reaction. DETA/NO (Fig. 7G) is a stable NO donor with one of the longest half-lives of the NO diazeniumdiolates. DETA/NO spontaneously dissociates in a pH-dependent, first-order process with a half-life of 20 and 56 h at 37 and 22-25˚C, pH 7.4, respectively, to liberate 2 mol of NO per mole of parent compound (170). The role of DETA/NO as an anti-neoplastic agent has been established by several investigators. For instance, NO from DETA/NO (1.0 mM) induced cytostasis and cell-cycle arrest in human breast cancer cells MDA-MB-231 as a result of down-regulation of cyclin D1 (173). Similarly, exposure of breast cancer cell MDA-MB-468, which are nitric oxide synthase deficient, lead to apoptotic cell death (174). In this experiment, upregulation of the phosphatase MKP-1 leading to dephosphorylation and inactivation of the mitogen-activated protein kinase (ERK) was the critical step in committing these breast cancer cells to programmed cell death (174). In spheroid cultures of breast cancer cells, DETA/NO and GTN attenuated the doxorubicin resistance by a c-GMP-dependent mechanism (175). The combination of low concentrations of DETA/NO (25-100 μM) with a farnesyltransferase inhibitor potentiated NO-induced apoptosis selectively in breast cancer cells and reduced cytotoxicity to the normal breast epithelial cells (176).

In addition to a primary role in tumor growth suppression, DETA/NO has been shown to play a role in chemo-immune-sensitization in many forms of cancer. The NO-donors (GTN and DETA/NO) in low doses reversed the hypoxia-mediated resistance to chemotherapeutic agents such as 5-FU and doxorubicin in human breast carcinoma MDA-MB-231 cells and B16F10 mouse melanoma cells (177). *In vivo*, DETA/NO enhanced cisplatin mediated toxicity in Chinese hamster V79 lung fibroblasts as well as head and neck squamous cell carcinoma cells (178,179). *In vivo*, melanoma B16 models of carcinogenesis were subjected to cisplatin (1.0 mg/kg) i.p. with or without DETA/NO (10 μmol/l) i.t. Mice receiving combination treatment had a significant reduction of xenografts and an increased survival compared to mice treated with cisplatin or vehicle alone (180). In prostate cancer, DETA/NO (500-1000 μM) immunosensitized PC-3 cells to TRAIL- and FasL-mediated apoptosis by S-nitrosylation of p50 leading to NF-κB inhibition and Bcl-xL upregulation (181). DETA/NO also led to S-nitrosylation of zinc finger proteins as well as YY-1 (Yin Yang-1, transcription repressor) (182) and inhibited its DNA-binding and repressor activity with upregulation of Fas expression, which effectively sensitized prostate cancer cells to FasL-mediated apoptosis.

In contrast to these anti-neoplastic effects, NO from DETA/NO promoted the metastatic potential of breast and papillary thyroid carcinoma cells, by stimulating VEGF (183,184). DETA/NO (50.0 nM) increased tumor cell invasiveness by 3-fold in colorectal HRT-18, which constitutively expresses iNOS compared to the iNOS negative HT-29 adenomarcinoma cells (185). In RKO epithelial colonic cells, DETA/NO (0.25-0.5 mM) induced apoptosis by >50% which was accompanied by cytochrome *c* release and caspase-3 activity (186). These effects were substantially attenuated when transfected RKO cells were induced to overexpress ß-catenin. The same investigators demonstrated that NO-induced apoptosis in RKO colonic epithelial cells was mediated by *p53* through Bcl-xL- and Akt-related pathways (186).

vi) NO-drug hybrids. The specific characteristics of NO in vasodilatation and a potential role as an anti-neoplastic agent on its own and has led to the development of new hybrid drugs with synergistic activities and minimal side effects that may be caused by either drug alone. For instance, a recent study by Chan *et al* showed that the regular use of the non-steroidal anti-inflammatory drug (NASAID), aspirin, reduced the risk of colorectal cancers in tumors that over-expressed COX-2. However, in tumors that showed weak of absent expression of COX-2 the chemopreventive effects were minimal; while the potential side effects of aspirin, including peptic ulcer disease, were retained in both cohorts (187). Owing to the prostacyclin production of nitric oxide, it provides gastric mucosal production while granting potential antiproliferative effects. Since established drugs that inhibit carcinogenesis are COX-2 inhibitors, a logical hybrid is one that combines nitric oxide and the non-steroidal anti-inflammatory drugs (NO-NSAIDs). The NO-aspirin NCX-4016 demonstrated no toxicity when provided to human subjects while maintaining Cox-1 and antiplatelet activity (188). These compounds are emerging as potential agents for chemoprevention and immunochemotherapeutic interventions in many cancers. The NO-NSAIDs have been recently reviewed by Rigas and Kashfi (189). Typical compounds in this category are depicted in Fig. 8.

NO-NSAIDs are composed of typical non-steroidal anti-inflammatory drugs such as aspirin, salicylic acid, indomethacin, ibuprofen or sulindac to which an NO-releasing moiety has been attached via a covalent bond that is cleaved by non-specific esterase activity (190). A wide array of NO-NSAIDs have been synthesized by the Thatcher's group (190). All of these compounds have the characteristic structure: NSAID - Linker - NO-donating compound. It is the bond between the linker and the NSAID that is cleaved by enzymatic activity. Upon cleavage, the NSAID exerts its COX-inhibitory functions and the generated NO acts in synergy to potentiate the actions of both compounds. The compounds designed by Thatcher's group are designated NCX-# (i.e., NCX-4016, NCX-4040, NCX-4215, NCX-976 etc.), several of which

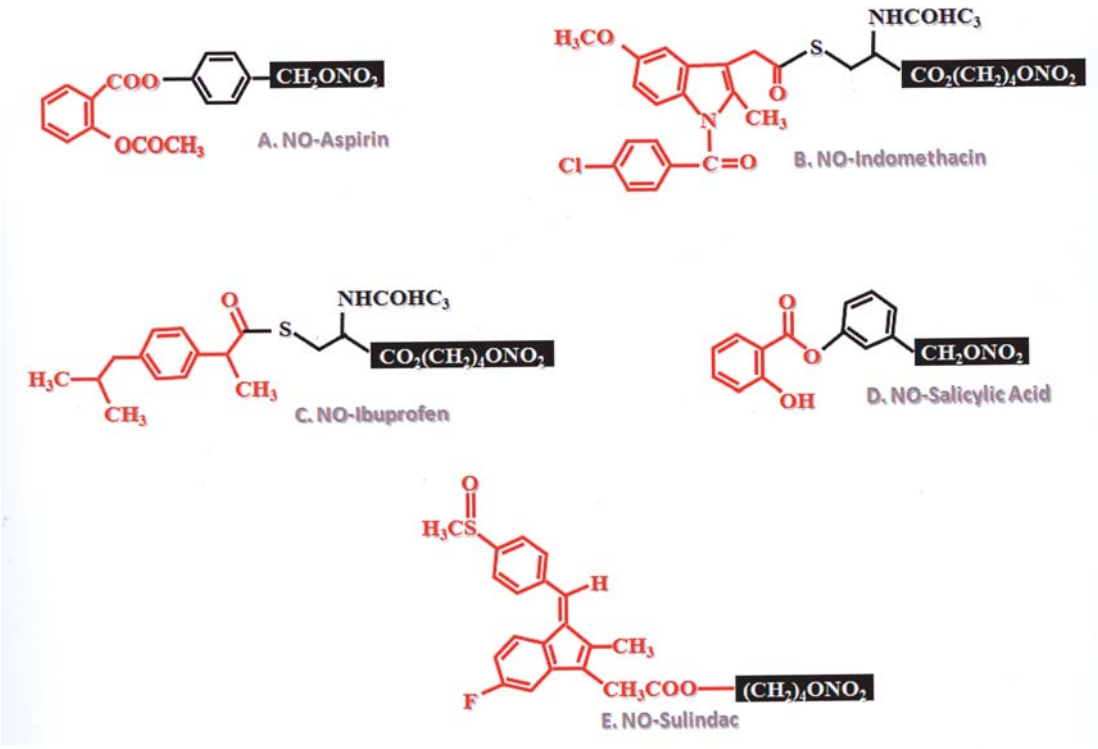

Figure 8. Chemical structure of NO-NSAIDs. The typical NSAID compound is in red. The nitric oxide releasing compound is in the box and the linker molecule is in black.

have been studied in oncology and a substantial number of compounds are on current randomized trials. NCX-4016 and NCX-4215 (NO-aspirins) are compounds that have an aspirin moiety attached to NO. NCX-4016 markedly increased cGMP concentration compared to aspirin alone and when compared to NCX-4215, it is a more potent inhibitor of thromboxane A2 production (190).

In colon cancer, the inhibitory properties of NO-NSAIDs, including: NO-aspirin, NO-salicylic acid, NO-indomethacin, NO-sulindac, NO-ibuprofen, and NO-flurbiprofen, were from 2 to >1000-fold higher compared to their parent compound alone. Because the inhibitory properties of NO-NSAIDs occurred in COX-positive HT-29 and COX-negative HT-15 colon cancer cells, the inhibitory properties of NO-NSAIDs is synergistic and dependent on the structural modifications of the hybrid (191). Similarly, NO-aspirin stimulated phosphorylation of MAP kinases p38 and JNK, which was responsible for the inhibitory properties of this compound in HT-29 colon cancer cells (192). In $APC^{min/+}$ mouse model of early intestinal neoplasia, NO-aspirin (100 mg/kg/day) administered intrarectally for 21 days, reduced polyp formation by 59% compared to sham treated mice (193). *In vitro* models of hereditary non-polyposis colorectal cancer (HNPCC), NO-aspirins were potent suppressors of the microsatellite instability (MSI) phenotype, suggesting their potential role as chemopreventive agents in patients with HNPCC.

Similarly, NO-aspirin (NCX-4060) and NO-ibuprofen (NCX-2111) were potent inhibitors of proliferation of hormone-dependent LNCap and homone-independent PC-3 prostate cancer cells. Both compounds caused caspase-3-dependent apoptosis in these cell lines of prostate cancer (194). In hamster models of pancreatic cancer, NO-aspirin decreased pancreatic carcinogenesis by 88.9% in mice receiving NO-aspirin in the diet (3,000 ppm) compared to mice receiving the same dose of aspirin alone or sham treated mice (195). In human ovarian cancer cells, the NO-aspirin NO-4016 chemosensitized these cells to cisplatin mediated cytotoxicity (196).

A different chimera consisting of nitric oxide bound to a statin compound [i.e., pravastatin (NCX-6550)] inhibited cell proliferation in rat aortic smooth muscle cells and this effect was accompanied by suppression of iNOS and COX-2 (197). While the NO-drug hybrids represent new agents in the armamentarium against carcinogenesis, these initial findings show great potential for their therapeutic use alone or in combination with other cytotoxic drugs.

6. Conclusions

There is substantial dichotomy that splits the literature in halves regarding the role of nitric oxide in oncology. For instance, two studies using knockout mice by two separate groups demonstrated opposite effects in iNOS gene knockouts on intestinal carcinogenesis (47,81). The dual nature is clear in models of carcinogenesis in cells and *in vivo*. The review presented, herein, offers no substantial solution of the dilemma, but suggests that the use of various NO donors may aid to clarify this controversy. Since the literature emphasizes the microenvironment of the experimental conditions such as the concentration of NO, the redox, and pH of the reaction, NO donors provide an excellent approach for their study in oncology in a controlled manner.

There are several NO donors available for *in vitro* and *in vivo* studies on carcinogenesis and therapeutics. The importance to dissociate the dual apposing nature of the

effects of NO in carcinogenesis needs further characterization of the specific cellular pathways that are affected by different concentrations and molecular structures of the NO donors. Only NO donors with various half-lives and redox properties may lead to further delineation of this controversy.

The nitric oxide donors belonging to the class of diazeniumdiolates are promising as they have been shown to be effective chemo- and radio-sensitizing agents along with other attractive properties like long half-lives, target tissue specific delivery, etc. These compounds have also been shown to have anti-cancer properties by themselves and 5 FU/diazeniumdiolate conjugates were more cytotoxic than 5FU in HeLa and prostate cancer cells. Furthermore, the safety of agents such as nitro-glycerine has been established for the management of coronary artery disease, and its role as a chemosensitizing agent as demonstrated by Yasuda *et al* (99,100) not only promises a safe but an affordable alternative for the management of resistant or metastatic tumors.

NO-hybrids are making a unique niche in the armamentarium of anticancer agents. Combining NO to existing drugs affords an advantage of adding or potentiating the effects of NO, to the benefits of drugs like NSAIDs or statins. Development of newer agents, which can selectively deliver NO to specific tissues, should decrease the side effects associated with the systemic delivery of each drug independently. Thus, the future for NO donors in cancer is bright and further studies will help in unraveling the role of NO in tumor biology. Several promising findings strongly support the therapeutic application of NO donors in cancer treatment, used alone or in combination with other subtoxic doses of cytotoxic agents. In addition, the delineation of the molecular and genetic mechanisms that underlie the anti-carcinogenic effect of NO will provide novel prognostic/diagnostic markers and the design of newly targeted therapeutics.

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