Chapter 17
Nitric Oxide, Coagulation and Cancer

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Abstract Nitric Oxide (NO) is a well-known potent and rapid vasodilator and inhibitor of coagulation. Synthesized from an L-arginine precursor, NO is produced via the Nitric Oxide Synthase enzyme which is expressed constitutively in endothelial cells. Nitric oxide has a wide range of biological properties that maintain vascular homeostasis and protection of the vessel from injurious consequences. The decreased production of NO in pathological states causes deleterious effects, creating an endothelial dysfunction state with a wide variety of subsequent diverse biological effects. There is now evidence of the link between hypoxia and/or reduction of NO availability and coagulopathies. NO is also a modulator of various cancer-related events and has anti-tumor properties. Cancer is a known hypercoagulable state and hypoxia is a typical feature of the tumor micro-environment. Cancer patients—particularly those with advanced or metastatic states—are at higher risk of developing venous and arterial thromboembolic events. The dichotomous nature of nitric oxide with regard to its tumorigenic and tumoricidal properties are at present under intense investigation. The transcendent field of nanotechnology has moved into the realm of NO donor therapy, though currently there are no commercially available carriers of NO. While nanotechnology is not quite at the translational research stage, it poses the greatest potential for storage and site-specific delivery of high concentrations of NO to tumors. In this chapter, we review the effects of NO on various hemostatic elements, the pro-tumoral and anti-tumoral effects of NO and finally shed some light on the link between NO, cancer and coagulopathies.

Keywords Cancer · Coagulation · Fibrinolysis · Nitric oxide · PAI-1 · Platelets

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Abbreviations

NO	nitric oxide
NOS	nitric oxide synthase
aPTT	activated partial thromboplastin time
vWF	von Willebrand factor
LPS	lipoproteinpolysaccharides
TEG	thromboelastography
TF	tissue factor
MPs	microparticles
PCa	prostate cancer
GTN	glyceryl trinitrate
tPA	tissue plasminogen activator
uPA	urokinase type plasminogen activator
PAI	plasminogen activator inhibitors
L-NMMA	L-NG-monomethylarginine
nNOS	neuro-isoform of NOS
eNOS	endothelial isoform of NOS
iNOS	inducible NOS
PGE2	prostaglandin E2
VEGF	vascular endothelial growth factor
COX-2	cyclooxygenase-2
EMT	epithelial-mesenchymal transition
MMP	matrix metalloproteinases
NODD	NO-donating drugs
NSAID	non-steroidal anti-inflammatory drug

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Introduction

It is well known that nitric oxide (NO) is a potent and rapid vasodilator and inhibitor of coagulation. Synthesized from an L-arginine precursor, NO is produced via nitric oxide synthase (NOS), a calcium-calmodulin-dependent enzyme which is expressed constitutively in endothelial cells. The synthase enzyme is also known to be activated in the presence of inflammatory mediators as well as lipopolysaccharide (LPS) indicating the critical relationship between NO and inflammation. Studies have indicated that inducing NOS activity such as after an endotoxic shock challenge increases NO output detectable in 3–12 h [1–3].

NO has a wide range of biological properties that maintain vascular homeostasis, including modulation of vascular dilator tone, regulation of local cell growth, and protection of the vessel from injuries from activated platelets and white cells in the

blood, thereby playing a crucial role in the normal endothelial function. Hypertension, hypercholesterolemia, smoking, diabetes mellitus, heart failure and cancer are associated with diminished release of NO into the arterial wall either because of impaired synthesis or excessive oxidative degradation. NO is a modulator of various cancer-related events and has anti-tumor properties. The decreased production of NO in some pathological states causes deleterious effects in the endothelial equilibrium, resulting in endothelial dysfunction and a wide variety of subsequent diverse biological effects [4]. Evidence exists from pathological conditions such as obstructive sleep apnea [5] and placental alterations in rat models [6] of the relationship between hypoxia and coagulopathies.

NO is a core molecule in endothelial and blood vessel functions. The endothelial cell surface in an adult human is composed of approximately $1–6 \times 10^{13}$ cells, and spans a surface area of approximately 350 m^2 with a mass of about 110 g [7, 8]. The endothelium contains growth factors, coagulant and anticoagulant proteins, lipid transporting particles, metabolites and hormones; it also expresses proteins and receptors that control cell-cell and cell-matrix interactions [9]. Stimulation of the endothelium in conditions of shear stress or inflammation induces a prothrombotic and antifibrinolytic microenvironment. Given the wide distribution and heterogeneity of the endothelium, every organ system including blood vessels within these organs can be affected as result of endothelial damage or insult. Moreover, endothelial cells from diverse tissues are heterogeneous with respect to their surface phenotype and protein manufacturing, release and expression. This heterogeneity manifests itself differently in pathological states including thrombosis [10] and cancer [11–14]. In this chapter, we will review NO's specific effects on hemostatic parameters including its important role in physiological endothelium, platelets, coagulation factors, and other procoagulant and fibrinolytic elements (Fig. 17.1). We will also discuss tumoral and antitumoral effects of NO and shed some light on cancer therapeutics.

NO and Pro-Coagulant Factors

There have been no reports showing significant change in prothrombin time upon exposure to NO [15]. Inhaled NO in healthy males did not alter hemostasis significantly, as measured by the activated partial thromboplastin time (aPTT) and the bleeding time [16]. However, inhalation of NO in animals resulted in prolongation of the bleeding time [17], and the inhibition of NO synthesis shortened the prolonged bleeding time in uremic rats [18]. The reduction of NO did not significantly increase levels of factors II, V, or VII, but NOS inhibitors were associated with moderate increases in the plasma von Willebrand factor (vWF) [19], endothelial thrombogenicity, and vascular platelet thrombi [15, 20]. These effects indicate that this vascular damage can be causally linked to the increased vWF.

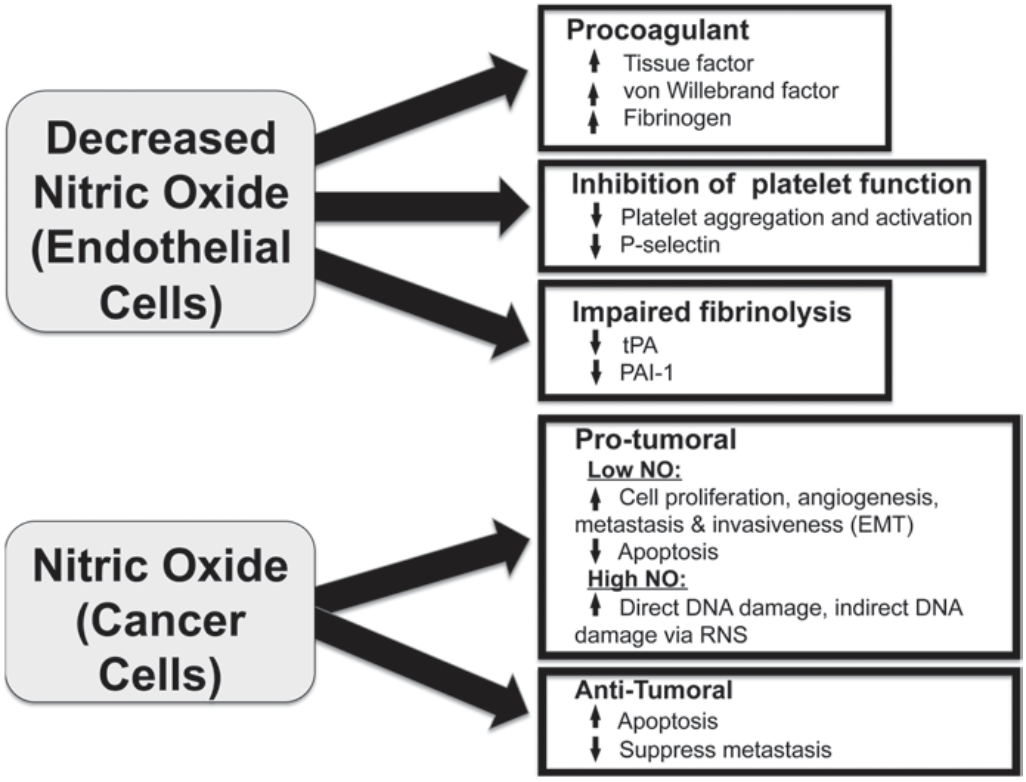

Fig. 17.1 NO is protective to endothelial cells; decreased NO results in a prothrombotic phenotype. In cancer cells, NO is both carcinogenic (pro-tumoral) and anti-tumoral. TF = tissue factor;TF-MPs = tissue factor bearing microparticles; TEG = thromboelastography; EMT = epithelial-mesenchymal transition; RNS = reactive nitrogen species

In a porcine model of endotoxin septic shock, a NOS inhibitor administration resulted in coagulation changes consistent with disseminated intravascular coagulation. In response to bacterial membrane lipoproteinpolysaccharides (LPS), there was a fourteen-fold elevation in thrombin and antithrombin complexes over the baseline; this increased further to twenty-seven-fold the baseline with the use of NOS inhibitors [15]. Moreover, prolonged inhibition of NOS significantly elevated fibrinogen levels in a dose-dependent manner in murine models [21].

Furthermore, the expression of tissue factor, the key trigger of coagulation, was inhibited in association with NO release in response to statin derivatives [22]. This inhibition was also associated with inhibition of collagen-induced platelet-aggregation, collagen-induced platelet P-selectin expression, platelet adhesion to collagen-coated coverslips under high shear stress indicating strong antithrombotic effects for these drugs, likely mediated through NO release.

NO and Global Hemostasis

Inhalation of NO has no major impact on hemostasis in healthy subjects, because no significant changes in platelet counts and levels of coagulation activation markers have been found in venous blood after drug administration [23]. However, in neonates with persistent pulmonary hypertension, inhalation of NO inhibited coagulation as evidenced by the more sensitive global hemostatic test thromboelastography (TEG) [24]. This was also supported by another study that showed an inhibitory effect for NO on all TEG parameters in platelet rich plasma and whole blood: it caused a longer reaction time (R), decreased angle, and reduced maximum amplitude (MA) in a dose-dependent manner [25].

Bradykinin is a potent stimulator of NO formation and prostacyclin release from the endothelium. Bradykinin binds to B1 and B2 receptors on endothelial cells, opens endothelial calcium channels, and activates NOS [26]. A study with bradykinin B receptor knockout mice showed reduction of thrombosis risk; this was shown to be mediated by an overriding mechanism involving angiotensin II which induced an elevation in NO and prostacyclin [27].

NO and Pro-Coagulant Microparticles

Microparticles (MPs) are membrane vesicles with procoagulant and proinflammatory properties released during cell activation or apoptosis. MPs can be released from all types of cells and carry phenotypic markers of these cells. Tissue factor (TF)-bearing MPs can serve as novel signaling elements and trigger hemostasis. Studies have shown that LPS administration leads to an increase in the numbers of MPs released from platelets, monocytes, and endothelium but inhalation of NO did not influence them [28].

However, patients with prostate cancer (PCa) have shown increased plasma procoagulant MPs. Moreover, hypoxia was recently shown to induce the release of TF-MPs by human PCa cell lines *in vitro,* which was reduced by the NO mimetic nitroglycerin (glyceryl trinitrate, GTN) [29]. In a pregnancy rat model of abnormal inflammation, inflammation-induced systemic coagulopathies were associated with placental hemostatic alterations and impaired placental hemodynamics [6]. In a closely similar model, GTN prevented inflammation-associated coagulopathies and fetal death, indicating a role for NO in triggering inflammation-induced coagulopathy [30]. These data add to the evidence of the link between hypoxia, nitric oxide, and coagulation.

NO and Natural Coagulation Inhibitors

Little is known about the relationship between of NO and natural coagulation inhibitors. Antithrombin III pretreatment was shown to reduce NO levels and improve survival in a rat model of heat stress-induced acute inflammation [31]. Activated protein C administration in rat model of experimental septic shock improved hemodynamics and myocardial efficiency by downregulating the inducible nitric oxide synthase pathway and reducing myocardial oxidative stress [32]. These data indicate once again the close link of hypoxia, inflammation and coagulation.

NO and the Fibrinolytic System

The plasminogen-plasmin system consists of plasminogen, a precursor of the active protease plasmin, which is then converted to plasmin by plasminogen activators, tissue plasminogen activator (tPA) and urokinase type plasminogen activator (uPA). The process is inhibited by several plasminogen activator inhibitors (PAI), of which PAI-1 has been shown to play an important role in both physiologic and pathologic conditions [33, 34]. NO is involved in modulating the fibrinolytic activity in blood. Sodium nitroprusside given to rabbits results in increase in tPA [35], believed to be due to the inhibition of clearance of tPA by PAI-1. This was confirmed by findings in human volunteers when after administration of a NO donor, mobsidomine, PAI-1 was reduced concomitantly with an increase in tPA [36] Conversely, the inhibition of NOS by L-N^G-monomethylarginine (L-NMMA) can block the increase in tPA [37].

On the other hand, tPA can also affect NO production. In the central nervous system, NO is an important modulator in mediating neurogenesis [26, 38], synaptic plasticity [35], and neuronal signaling [36]. Under physiologic conditions, tPA reduces NO by dislocating the neuro-isoform of NOS (nNOS) in neuronal cultures and, through the activation of plasminogen to plasmin, by proteolysis of NOS [41]. In stressed conditions with excitotoxicity, NO modulates neurodegeneration [42]. tPA regulates NO production through its proteolytic action on NOS [43, 44]. Much less is known of tPA in the other organs. Notably, hypertension, aortic arteriosclerosis, and coronary perivascular fibrosis developed in experimental animals given long-term treatment with N^{Ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS. These changes are not seen in PAI-1 knock-out animals 45. In these animals, there is upregulation of PAI-1. PAI-1 is known to have a role in the pathogenesis of a number of vascular pathologies, including hypertension and atherosclerosis and in pulmonary fibrosis. It is, thus, hypothesized that this interaction with the endothelial isoform of NOS (eNOS) may account for the role of PAI-1 in the vasculopathy and fibrosis. This concept was verified in experiments when an inhibitor of PAI-1, TM5441, was able to prevent hypertension and vascular changes

in L-NAME-treated animals [46, 47]. Such experimental therapeutic approaches pave the way for further exploration of the NO system for many vasculopathies.

NO and Platelets and Thrombosis

The NO derived from endothelial cells also affects platelet function. It impairs platelet aggregation [48, 49] and activation [50]. It also down-regulates P-selectin, thus preventing platelet-endothelial adhesion [51]. By the same token, inhibition of NO results in platelet adhesion [51–53] and aggregation [53].

Thus, the enhancement of NO may be of potential therapeutic benefit. This has been explored in sepsis, where platelet adhesion to endothelium can impair the microcirculation [54]. In sepsis, there is increased oxidative stress leading to platelet adhesion and microcirculatory blockage. It has been shown that the administration of the antioxidant ascorbic acid inhibits this series of events and that the action of ascorbic acid is dependent on local NO produced by eNOS [54].

Recently, studies showed that NO also participates in the pathology of the antiphospholipid syndrome, in which the presence of high levels of anti-beta2 glycoprotein I antibodies is predictive of thrombotic complications [55]. This antibody inhibits eNOS and attenuates the production of NO on the endothelial surface [56], leading to a prothrombotic endothelial phenotype. A correlation between low plasma NO levels and the titer of antiphospholipid antibodies as well as the number of vascular occlusive lesions had been observed in patients with antiphospholipid antibody syndrome [57].

NO and Cancer

NO is a modulator of various cancer-related events with additional anti-tumor properties [58]. Among the isotypes of NOS, inducible NOS (iNOS) has been found to be activated in various types of tumors, including breast, colon, head and neck, esophagus, lung, prostate, bladder, and pancreatic carcinomas, brain tumors, mesothelioma, Kaposi's sarcoma, and hematologic malignancies [59]. iNOS is induced by cytokines and lipopolysaccharides within the inflammatory milieu [60]. NO production is particularly important in these inflammatory states, as it can form reactive nitrogen species (RNS) nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), which in turn cause DNA and lipid damage via oxidative and nitrosative stress. Moreover, RNS are involved in inducing a tumor specific immune response that ultimately inhibits T-cell penetration and function in the tumor microenvironment that aids in tumor growth [61].

Pro-Tumoral Effects of NO

Under normal physiologic circumstances, NO in low concentrations performs many vital functions including regulating blood flow, iron hemostasis, and neurotransmission. At higher concentrations, it acts as an immune regulator; iNOS generates large amounts of NO in macrophages and neutrophils in order to eliminate a variety of pathogens [60].

Nonetheless, in the setting of inflammation and/or cancer, both low and high NO concentrations can have deleterious pro-tumoral properties. Low NO concentrations can act to increase cell proliferation, angiogenesis, metastasis and invasiveness, and decrease apoptosis. High levels—though it has the effect of increasing apoptosis—cause extensive DNA damage, but more importantly cause oxidative and nitrosative stress. However, the direct modification of DNA and inactivation of DNA repair enzymes by NO alone are simply not enough to lead to carcinogenesis [62].

The indirect effect of NO—the production of RNS—is the true pathogenic and carcinogenic culprit. Excess NO, particularly in the setting of inflammation, reacts with superoxide anion to form the powerful oxidant peroxynitrite ($ONOO^-$) [58, 60, 63]. Peroxynitrate is involved in several carcinogenic pathways, including genotoxic mechanisms (inducing DNA damage, suppressing DNA repair enzymes such as p53, and modifying post-translational proteins), antiapoptotic effects, promotion of angiogenesis, promotion of metastasis, inhibition of antitumor immune responses, and promotion of the epithelial-mesenchymal transition [58, 62–65].

NO alone can cause direct DNA damage by way of strand breaks, oxidation, and deamination of nucleic acids. The more potent derivative peroxynitrite induces DNA damage by forming 8-nitroguanine, in addition to inducing lipid peroxidation that ultimately creates more reactive species to form DNA adducts [62]. Chronic nitrosative stress is further genotoxic via its post-translational alteration of proteins involved in intracellular signal transduction; this in turn leads to abnormal growth and proliferation of cells [62]. NO can cause further genome infidelity by inhibiting DNA repair processes via nitrosylation of DNA alkyl-transferase, xeroderma pigmentosum-A, and 8-oxoguanine glycosylase-1 [59].

But it is truly the antiapoptotic effects exerted by NO and its derivatives that allow for the aforementioned mutations to escape repair or inactivation. NO has been shown to cause loss-of-function mutations in p53 in HPV [65], inhibit caspase activation via S-nitrosylation [66], activate cyclooxygenase, inhibit release of cytochrome C, and increase bcl-2 expression [67]. All of these actions serve to create a permissive milieu for cancer cells to thrive.

The metabolically active cancer cells need a robust vascular network in order to maintain growth. NO produced by eNOS dilates arterioles that assist in augmenting tumor blood flow, in addition to decreasing leukocyte endothelial adhesion and increasing vascular permeability via increased prostaglandin E2 (PGE2) production [68, 69]. NO is perhaps most influential in angiogenesis by mediating up-regulation of the vascular endothelial growth factor (VEGF) by cGMP pathways [69, 70].

Production of cyclooxygenase-2 (COX-2) by NO also stimulates proangiogenic factors and prostaglandins that lead to neovascularization to increase a tumor's invasiveness and metastatic potential [68, 70]. To achieve effective invasiveness and metastasis, however, the tumor environment must undergo the epithelial-mesenchymal transition (EMT). EMT refers to a complex set of events that are responsible for the rapid changes in the epithelial cell phenotype, during which they assume mesenchymal cellular properties. EMT essentially enables transformed cells to travail through the basement membrane that is encapsulating a tumor, and invade lymphatic or blood vessels so as to gain access to other organs [63, 71, 72]. The effect of NO on EMT is controversial, with evidence supporting its ability to both augment and attenuate the EMT dedifferentiation and hence tumor invasiveness and metastasis. In gastric carcinomas, for example, a significant correlation was found between iNOS expression in tumor cells and loss of differentiation. iNOS expression was most notable in 'EMT-like' dedifferentiation areas with loss of cohesion and an invasive phenotype [73]. Similarly, a study involving colorectal adenocarcinoma cells expressing iNOS (HRT-18 cells) versus those not expressing iNOS (HRT-29 cells) were shown to be nearly three times more invasive; further supporting this result was the increased invasiveness of the HRT-29 cells in the presence of an NO donor and inflammatory cytokines [74]. The mechanism by which NO increases tumor invasiveness is thought to be related to up-regulation of matrix metalloproteinases (MMP-2 and MMP-9) and downregulation of tissue inhibitors of MMPs such as TIMP-2 and TIMP-3 [75].

Anti-Tumoral Effects of NO

The dichotomous nature of NO with regard to its tumorigenic and tumoricidal properties is not to be understated. Low concentrations of NO promote tumor cell survival and angiogenesis, whereas high levels of NO (typically > 500 nM) have a cytotoxic propensity, particularly as it pertains to inducing apoptosis [63, 76]. The concept of a dose threshold for NO is key to understanding NO-induced cytotoxicity.

NO has the potential to induce apoptosis through various mechanisms, including S-nitrosylation of NF-kappa-B, glyceraldehyde-3-phosphate dehydrogenase, Fas receptor and Bcl-2 [77, 78]. Furthermore, NO ignites the caspase cascade that is responsible for releasing mitochondrial cytochrome C into the cytosol that is ultimately responsible for initiating the chain of apoptotic events [67, 79]. NO and its relationship to p53 has proven to be baffling. Wild-type p53 appears to be activated by low-dose NO, which in turn exerts a negative feedback loop to inhibit further NO generation. High NO concentrations appear to inactivate p53 via peroxynitrite tyrosination, causing mutations in the *p53* gene itself that lead to the loss of repressor activity. This, in turn, leads to increased iNOS expression, which feeds a cycle of NO generation, DNA damage, and additional mutations [59].

NO has also been shown to suppress metastasis by inhibiting the EMT at high concentrations. Indeed, *in vitro* studies have shown that treatment of human metastatic prostate cell lines with NO donors is able to inhibit EMT and reverse the mesenchymal phenotype and cell invasive properties [80]. Given that most studies investigating the anti-tumoral role of NO have been *in vitro*, it is difficult to extrapolate its potential *in vivo* effects. In particular, the question remains whether high enough NO concentrations can be accomplished *in vivo* so as to facilitate apoptosis without up-regulating pro-tumoral pathways.

NO and Cancer Therapeutics

It should follow that NO is a strong candidate and target for anticancer therapeutics, albeit with two drastically different approaches to achieve the same desired effect. Anti-NO cancer therapies have been investigated as a means to modulate the deleterious effects of RNS. NO scavengers have shown promising results, from reducing cancer-related vascular hyperpermeability [81] to inhibiting colon cancer development [82]. NOS enzyme inhibitors, especially iNOS-specific inhibitors, have been shown to decrease the rate of premalignant lesion development in colon cancer [83]. However, NOS inhibitors require long-term administration, as early cessation of therapy can result in tumor regrowth [84].

As it pertains to radiation, NO appears to have both radiosensitizing and radioprotecting properties. NO has the ability to act as a radiosensitizer of hypoxic tumor cells, mimicking the effects of oxygen on fixation of radiation-induced DNA damage [85, 86]. NO can also exert radioprotective effects as it is a free radical that can scavenge other free radicals induced by radiation and inhibit further DNA damage. Moreover, NO can decrease blood flow to bone marrow via the vascular steal phenomenon, which paradoxically causes hypoxia in the marrow and protects those cells from radiation damage [87].

The concept behind NO-based drugs is that its therapeutic consequence is heavily dependent on the concentration and duration of NO delivered. Indeed, effective NO-based drugs must be able to accomplish three tasks: (1) store NO doses for a specific duration, (2) deliver finite amounts of NO over a specific amount of time (i.e. rate), and (3) selectively deliver NO to the tissue of interest given its short half-life [84]. Pro-NO cancer therapies follow this recipe by increasing NO concentrations locally at the tumor site to exert its anti-tumoral (i.e. proapoptotic) effects while sparing healthy cells [88].

Pro-NO strategies include iNOS gene therapy and NO donor drugs. NOS gene therapy was thought to be a workaround to increase NO delivery in the setting of malignancy. However, this strategy has been met with substantial obstacles initially due to the hazard from the viral vectors required to deliver the gene therapy [89], but also secondary to early death of the NOS transfectants. The constitutive expression of NOS can paradoxically lead to the death of the transfectant, thereby decreasing the amount of time that NO can be generated [59].

There are many NO donor therapies (also known as NO-donating drugs, NODD), but the most studied and best understood is NO-NSAID, a NSAID (non-steroidal anti-inflammatory drug) with a NO-donor covalently bound to it [59, 90]. NO appears to enhance the anticancer potential of NSAIDs, though the mechanism by which this occurs is unclear. Some have suggested that NO-NSAIDs accomplish this via direct inhibition of hypoxia-inducible factor-1alpha, a VEGF transcriptional activator [91]. Other evidence suggests that NO-NSAIDs induce apoptosis and modulate the Wnt and NF-kappa-B signaling pathways to achieve its anticancer effect [90]. Regardless, NO-NSAIDs such as NO-acetylsalicylic acid (NO-ASA) exhibit inhibition of colon cancer cell lines *in vitro* and in animal models as well [92]. NO-NSAIDs have also been shown to have proapoptotic and anti-invasive properties in prostate cancer [93, 94]. Other NO-donor drugs have been studied as well, including NONOates DEA/NO and PAPA/NO, SNAP and GSNO, with varying efficacy in numerous cancer cell lines.

The transcendent field of nanotechnology has moved into the realm of NO donor therapy, though there are currently no commercially available carriers of NO. The theory behind nanoparticles is to load high amounts of NO onto a stable material that can be photoactivated. While nanotechnology is not quite at the translational research stage, surely it poses the greatest potential for storage and site-specific delivery of high concentrations of NO to tumors.

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