Chapter 13
Nitric Oxide–Induced Immunosensitization to Apoptosis by Fas-L and TRAIL

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1 Introduction

The incidence of cancer is only second to heart disease. Treatment of cancer remains one of the biggest challenges, particularly in view of increase in average life span, at least in developed countries; cancer is largely a disease of older individuals. Significant progress has been made in the development of novel therapeutics and the treatment of a large number of cancers. Hence, current therapeutics (chemotherapy, radiation, hormonal, and immunotherapy) have resulted in significant clinical responses and prolongation of life, albeit with little complete remission. One of the major problems in the eradication of cancer is the acquisition/development of resistance and refractoriness to conventional therapeutics. Cross-resistance develops since most cytotoxic therapeutics exerts their antitumor effect by inducing cell death by apoptosis and tumor cells develop mechanisms to resist apoptosis.

The failure to eradicate resistant tumors with current standard therapeutics calls for the use of alternative and less toxic novel therapies. For instance, a detailed understanding of the underlying molecular mechanisms of tumor drug resistance is critical for the development and design of new strategies to overcome the problem of resistance, thus improving the therapeutic outcome. The mechanisms of drug resistance are complex, and include among others poor vascular access and little drug penetration into the tumor mass, acquisition of multi-drug resistance phenotype in which the efflux of the drug is rapid, metabolic inactivation of the drugs, detoxification of accumulated toxic metabolites, enhanced DNA repair mechanism, and amplification of drug target genes [1]. The failure to cure chemoresistant tumors with conventional chemotherapeutic approaches has led to the introduction of immunotherapy. Immunotherapeutic strategies under investigation consider chemoresistant tumors to be sensitive to immunotherapy, as it has been assumed that cytotoxic immune cells attack tumor cells by different mechanisms of action and may not be subjected to the mechanisms of drug resistance. Despite the proposed advantages of immunotherapy over chemotherapy, immunotherapy today still fails to deliver a significant curative rate. It is unclear if drug-resistant tumors are actually sensitive to killing mediated by immune cytotoxic cells and whether cross-resistance is established. Tumor chemoresistance may actually reflect the general tumor resistance mechanism underlying a common cytotoxic pathway mediated by various cytotoxic stimuli, namely, programmed cell death or apoptosis. Such a resistance scheme to a central cytotoxic pathway may also lead to the cellular resistance to other cytotoxic mechanisms, including immunotherapy. With the premise that chemoresistant tumors develop general mechanisms of resistance to apoptosis-mediated stimuli, our hypothesis proposes ways to use immunosensitizing agents that

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can modify the apoptosis regulatory proteins to facilitate the apoptotic signaling cascade induced by the cytotoxic cells/ligands.

Host cytotoxic cells (NK, CTL, macrophages, etc.) mediate their cytotoxic killing by various mechanisms. These include the perforin/granzymes pathway and the death ligand family members (TNF-α, FasL, TRAIL), leading to necrosis/apoptosis. Thus, failure of tumor cells to respond to cytotoxic immunotherapy may be due to the development of resistance to death-induced stimuli by the cytotoxic cells/ligands. Hence, sensitizing agents that can modify the antiapoptotic regulatory mechanisms in tumor cells may be successfully used in combination with cytotoxic immunotherapy in the treatment of immune-resistant tumor cells.

2 Biological Activity of Nitric Oxide

Nitric oxide (NO) is a highly reactive free radical capable of mediating a multitude of reactions [2]. The free radical, NO, is an uncharged molecule containing an unpaired electron in its outermost orbital, allowing it to undergo several reactions functioning either as a weak oxidant (electron donor) or an antioxidant (electron acceptor). NO is able to react with other inorganic molecules (i.e., oxygen, superoxide, or transition metals), structures in DNA (pyrimidine bases), prosthetic groups (i.e., heme) or with proteins (leading to *S*-nitrosylation of thiol groups, nitration of tyrosine residues or disruption of metal-sulfide clusters such as zinc-finger domains or iron-sulfide complexes) [3]. In addition, NO can function as an antioxidant against reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) by diffusing and concentrating into the hydrophobic core of low-density lipoprotein (LDL) [4]. It can react with several ROS, such as superoxide to form peroxynitrite ($ONOO^-$), a highly oxidizing and nitrating reactive nitrogen species (RNS) responsible for mediating protein oxidation reactions under physiologic conditions [5]. Another mechanism of NO-related reactivity is through the addition of an NO group to the thiol side-chain of cysteine residues within proteins and peptides, termed *S*-nitrosylation, which plays a significant role in the ubiquitous influence of NO on cellular signal transduction [6]. NO or NO^+ ion is capable of forming *S*-nitrosothiols (RSNO; product of *S* nitrosylation), which function as potent platelet aggregation inhibitors and vasorelaxant compounds [7]. Other biological effects by NO have been recently reviewed [8].

3 Sensitization of Tumor Cells to Fas-L–Induced Apoptosis by IFN-γ: Pivotal Role of NO

Immunosensitization is the process by which cells are made sensitive to immune-mediated cytotoxicity (Fig. 13.1). Molecular mechanisms of immunosensitization such as transcriptional upregulation of proapoptotic proteins and downregulation of antiapoptotic proteins have been proposed to facilitate apoptosis by immunocytotoxic stimuli. Interestingly, NO has been found to be involved in the sensitization of tumor cells to various apoptotic stimuli, such as FasL (APO-1/CD95), TRAIL, and TNF-α. One mechanism responsible for the eradication of tumor cells by cytotoxic immune lymphocytes is Fas-mediated apoptosis, and Fukuo et al. [9] found that NO caused an increased expression of the Fas receptor in aortic vascular smooth muscle cells and increased sensitivity to FasL-mediated apoptosis. IFN-γ, together with many other proinflammatory cytokines (TNF-α, IL-1, LPS, etc.), can stimulate the induction of NOS II and the subsequent generation of NO. Through treatment with IFN-γ and the NO donor SNAP (alone or in combination), we have shown that human ovarian carcinoma cell lines (A2780 and AD10) were sensitized to FasL-mediated apoptosis by IFN-γ, partly due to NOS II induction and the consequent upregulation of Fas gene expression by RNS [10, 11]. These findings demonstrated that NO and RNS can regulate the sensitivity of tumor cells to FasL-mediated cytotoxic immune lymphocytes. A similar study by Park et al. [12], using ionizing radiation (IR) in combination with SNAP, showed sensitization to FasL-induced apoptotic cell death of HeLa human cervical cancer cells parallel to our findings with regard to the role of NO as an immunosensitizer. We have also previously reported, using a Fas promoter–driven luciferase reporter system, that the transcription factor Yin Yang 1

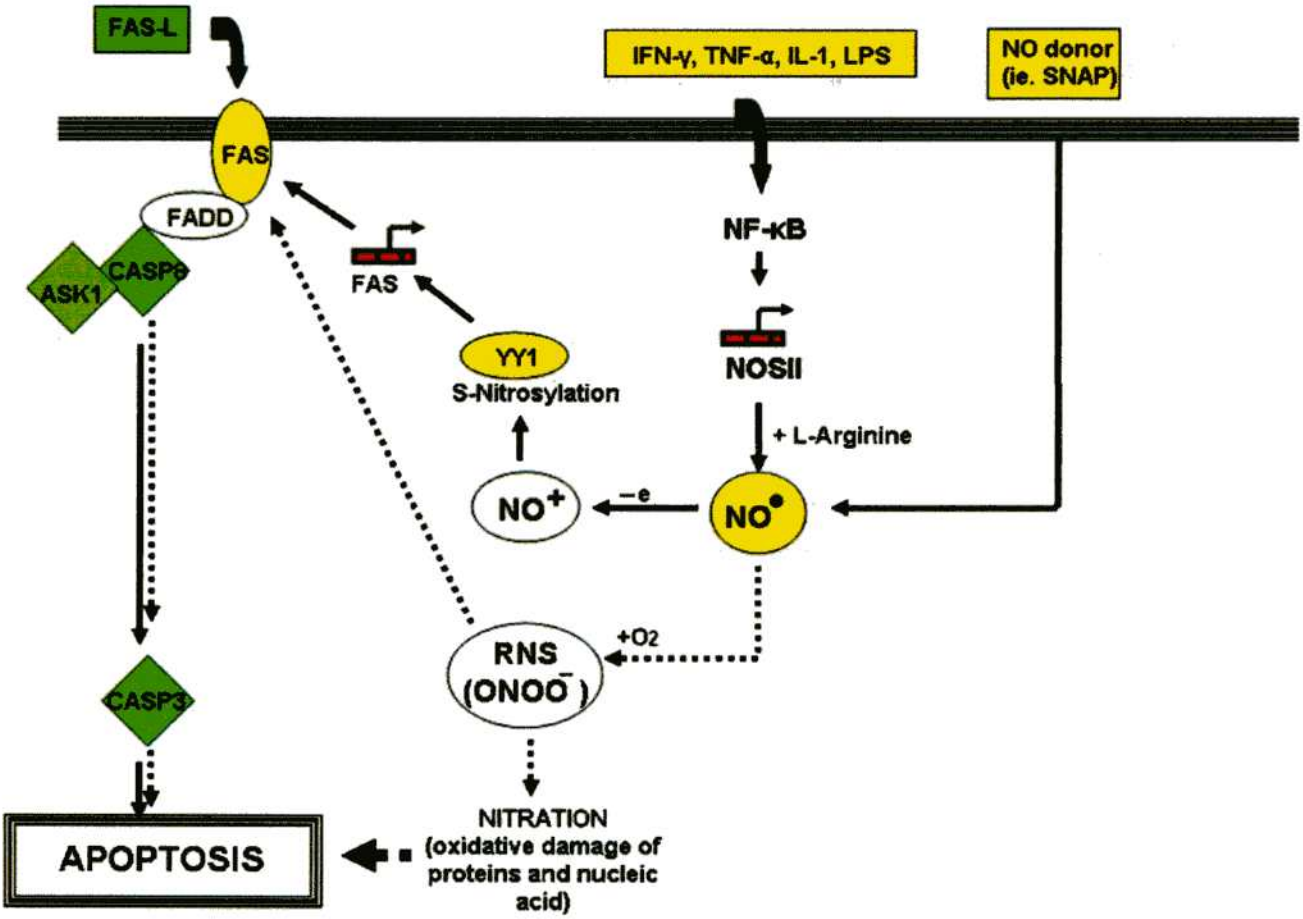

FIGURE 13.1. Mechanism of tumor cell sensitization to Fas-L–induced apoptosis by IFN-γ: Pivotal role of NO. IFN-γ or other agents, such as TNF-a, IL-1, or LPS, upregulate NF-kB, which in turn regulates positively the transcription of NOSII. NOSII catalyses the biosynthesis of NO by L-arginine. NO can also be released in the cytosol by treatment of cells with an NO donor such as SNAP or DETANONOate. Free nitric oxide may react with O_2 (discontinuous line), resulting in the formation of reactive nitrogen species (RNS) such as ($ONOO^-$), which upregulate Fas and cause oxidative damage in protein and nucleic acids leading to apoptosis. Alternatively (continuous line), NO or NO^+ ion is capable of forming S-nitrosothiols resulting in S-nitrosylation of several proteins, including YY1, which acts as a repressor of Fas transcription. Thus, inducible levels of Fas by NO are able to overcome tumor resistance to Fas-L and sensitize them to Fas-L–mediated apoptosis (*See Color Plates*)

(YY1) (which normally represses Fas expression by binding to a *cis*-element clustered at the silencer region of the Fas promoter) negatively regulates Fas expression through its interaction with the silencer region of the Fas promoter [13]. YY1 is a 414 amino acid Kruppel-related zinc transcription factor that binds to the CG (A/CC) CATNTT consensus DNA element located in promoters and enhancers of many cellular and virus genes [14]. YY1 physically interacts with and recruits histone-acetyl-transferase, histone-deacetylase and histone-methyl-transferase enzymes to the chromatin and may thus direct histone-acetylation, deacetylation and methylation at YY1 activated or repressed promoters [14]. NO-mediated inhibition of YY1 resulted in upregulation of Fas expression and sensitization of ovarian carcinoma cells to FasL-induced apoptosis [13]. Recently, we have found that the treatment of the B non-Hodgkin's lymphoma cell line (B-NHL), Ramos, with rituximab (chimeric anti-CD20 Ab) or with specific NF-κB inhibitors (e.g., Bay 11-7085 and DHMEQ) and/or inhibition of YY1(through the use of the NO donor, DETA/NONOate), resulted in sensitization

to FasL-induced apoptosis [15]. Noteworthy, the NO-mediated inhibition of YY1 activity (in the absence of rituximab) resulted in significant upregulation of surface Fas expression and sensitized Ramos cells to CH-11 (Fas agonist mAb)-induced apoptosis. Until now, the mechanism of YY1 inhibition by NO was unclear. However, Hongo et al. [16] demonstrated that treatment of prostate cancer (PC-3) cells with DETA/NONOate resulted in the *S*-nitrosylation of YY1, thereby upregulating Fas expression and sensitizing tumor cells to FasL-induced apoptosis through a direct NO-mediated mechanism.

4 Sensitization of Tumor Cells to TRAIL-Induced Apoptosis by NO: Roles of NF-kB and Bcl-xL

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a cytotoxic molecule that has been shown to exert, selectively, antitumor cytotoxic effects both in vitro and in vivo with minimal toxicity to normal tissues [17, 18]. TRAIL has been considered a new therapeutic and preclinical studies demonstrate its antitumor activity alone or in combination with drugs [17, 19–21]. However, many tumor cells have been shown to be resistant to TRAIL [22–25]. We and others have reported that various sensitizing agents like chemotherapeutic drugs [1, 22, 26], cytokines [27], and inhibitors [28], are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis. Further, we [29] and others [30, 31] reported that (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1, 2-diolate (DETANONOate) can also sensitize tumor cells to TRAIL-mediated apoptosis.

The mechanism underlying the NO-mediated sensitization to TRAIL is not known. We hypothesized that NO-mediated sensitization of tumor cells to apoptosis may be due to NO-induced inhibition of constitutive NF-kB activity and this, in turn, results in the downregulation of the antiapoptotic resistant factor, Bcl-xL. Hence, downregulation of the antiapoptotic gene product Bcl-xL results in the sensitization of CaP cells to TRAIL-mediated apoptosis. The mechanism by which DETANONOate mediated the sensitization was examined. DETANONOate inhibited the constitutive NF-kB activity as assessed by EMSA. Also, p50 was S-nitrosylated by DETANONOate, resulting in inhibition of NF-kB. Inhibition of NF-kB activity by the chemical inhibitor Bay 11-7085, like DETANONOate, sensitized tumor cells to TRAIL-induced apoptosis. In addition, DETANONOate downregulated the expression of Bcl-2-related gene (Bcl-xL), which is under the transcriptional regulation of NF-kB. The regulation of NF-kB and Bcl-xL by DETANONOate was corroborated by the use of Bcl-xL and Bcl-xL kB reporter systems. DETANONOate inhibited luciferase activity in the wild-type and had no effect on the mutant cells. Inhibition of NF-kB resulted in downregulation of Bcl-xL expression and sensitized tumor cells to TRAIL-induced apoptosis. The role of Bcl-xL in the regulation of TRAIL apoptosis was corroborated by inhibiting Bcl-xL function by the chemical inhibitor 2-methoxyantimycin A3, and this resulted in sensitization of the cells to TRAIL apoptosis. Signaling by DETANONOate and TRAIL for apoptosis was also examined. DETANONOate altered the mitochondria by inducing membrane depolarization and releasing modest amounts of cytochrome c and Smac/DIABLO in the absence of downstream activation of caspases-9 and -3. However, the combination of DETANONOate and TRAIL resulted in activation of the mitochondrial pathway and activation of caspases-9 and -3, and induction of apoptosis [29]. These findings demonstrate that DETANONOate-mediated sensitization of tumor cells to TRAIL-induced apoptosis is via inhibition of constitutive NF-kB activity and Bcl-xL expression.

5 Sensitization of Tumor Cells to TRAIL-Induced Apoptosis by NO: Roles of YY1 and DR5

Several reports have revealed that treatment with certain sensitizing agents, such as chemotherapeutic drugs, upregulate DR4 and/or DR5 expression, and upregulation of these receptors correlated with sensitivity to TRAIL-induced apoptosis (Fig. 13.2) [32–34]. However, the molecular mechanisms by which these receptors are upregulated by sensitizing agents are not known. The transcriptional regulation of DR5 expression has been

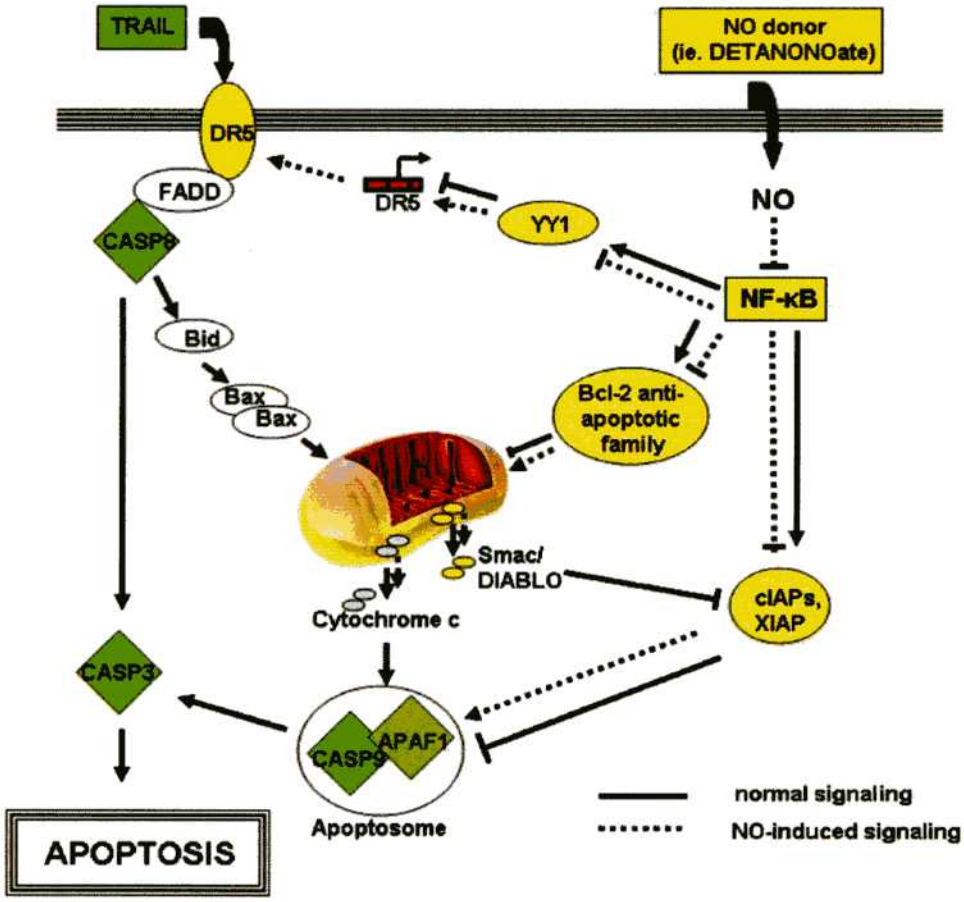

FIGURE 13.2. Mechanism of tumor cell sensitization to TRAIL-induced apoptosis by NO. Treatment of several tumor cell lines with NO donors such as DETANONOate and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent partially activates the apoptotic pathway and the combination results in apoptosis. The signal provided by NO partially inhibits NF-kB activity, and this leads to downregulation of antiapoptotic proteins of the Bcl-2 family such as Bcl-xL, and inhibition of cIAP family members (i.e., XIAP, cIAP-1, cIAP-2). In addition DETANONOate also partially activates the mitochondria and release of modest amounts of cytochrome C and Smac/DIABLO into the cytosol in the absence of caspase-9 activation. The NO-induced NF-kB suppression also inhibits the negative transcriptional regulator of DR5, YY1, resulting in DR5 upregulation. Thus, the combination treatment with TRAIL and DETANONOate results in significant activation of the mitochondria and release of high levels of cytochrome C and Smac/DIABLO, activation of caspases-9 and -3, promoting apoptosis. The role of Bcl-xL in the regulation of TRAIL apoptosis has been corroborated by the use of the chemical inhibitor 2MAM-A3 in several cell lines, which also sensitized the cells to apoptosis (*See Color Plates*)

investigated by using a pDR5-reported system and demonstrated that the transcription factor Sp-1 is a major factor that regulates DR5 expression [35]. In a recent study, we have identified the transcription factor YY1 as a transcription repressor on the Fas promoter [10, 13]. Thus, we reasoned that upregulation of DR5 expression by sensitizing agents may be due to inactivation of a transcription repressor, such as YY1 on the DR5 promoter. Examination of the DR5 promoter revealed the presence of a putative DNA-binding site for YY1. Preliminary findings demonstrated that the nitric oxide donor DETANONOate sensitized TRAIL-resistant tumor cells to TRAIL-induced apoptosis concomitant with DR5 upregulation. Thus, we hypothesized that the upregulation of DR5 expression by DETANONOate may be due to the inhibition of the YY1 repressor activity.

Treatment of TRAIL-resistant tumor cells with the nitric oxide donor DETANONOate sensitizes tumor cells to TRAIL-induced apoptosis concomitantly with DR5 upregulation. The mechanism

of DR5 upregulation was examined based on the hypothesis that DETANONOate may inhibit a transcription repressor that negatively regulates DR5 transcription. Treatment of the prostate carcinoma cell line PC-3 cells with DETANONOate inhibited both NF-kappaB and YY1 DNA-binding activity concomitantly with upregulation of DR5 expression and TRAIL-induced apoptosis. The direct role of YY1 in the regulation of TRAIL resistance was demonstrated by transfection of PC-3 cells with YY1 siRNA. The cells exhibited upregulation of DR5 expression and were sensitized to TRAIL-induced apoptosis. The role of YY1 in the transcriptional regulation of DR5 was examined by a DR5 luciferase reporter system (pDR5) and two constructs, namely, the pDR5/-605 construct with a deletion of the putative YY1 DNA-binding region (-1224 to -605) and a construct pDR5-YY1 with a mutation of the YY1 DNA-binding site. Transfection of PC-3 cells with these two constructs resulted in comparable and significant (threefold) augmentation of luciferase activity over baseline transfection with pDR5. The present findings demonstrate that YY1 negatively regulates DR5 transcription and expression and these correlated with resistance to TRAIL-induced apoptosis (Huerta-Yepez et al., unpublished) [1]. Treatment with DETANONOate reverses resistance to TRAIL via inhibition of NF-kappaB and YY1. Inhibitors of YY1 may be used in combination with TRAIL in the treatment of TRAIL-resistant tumor cells.

The in vitro findings with DETANONOate on PC-3 cells, namely, inhibition of YY1 and upregulation of DR5, were examined for validation in an in vivo model of PC-3 tumor xenograft implanted SC into athymic nude mice. Mice were implanted with PC-3 cells and treated intratumorally with DETANONOate as described in Methods. The concentration of DETANONOate used for the in vivo administration was derived from reported studies in rats whereby DETANONOate was used in a noncancer model [36]. Following treatment, tumors were biopsied and analyzed by immunohistochemistry for the expression of YY1 and DR5. The findings demonstrate that treatment with DETANONOate inhibited YY1 expression, as compared with untreated controls, in which there was strong nuclear YY1 expression. The staining for YY1 was specific, as treatment with control IgG did not show any staining. In contrast, DR5 expression was augmented in treated mice as compared with untreated controls. The specificity for DR5 was demonstrated with the use of immunoglobulin control (Huerta-Yepez et al., unpublished). These findings demonstrate that the inhibition of YY1 and upregulation of DR5 following treatment of PC-3 cells with DETANONOate in vitro can also be reproduced in an in vivo model in mice bearing PC-3 tumor xenograft.

6 Concluding Remarks

NO plays several roles in cells and its effects vary depending on its concentration and selective modification of various gene products. Its ultimate manifestation results from a complex set of interactions depending on the type of cells studied. It is also clear from recent findings that NO can play a significant role as a chemopreventive agent in cancer development and cancer therapeutics. The application of NO donors as cancer therapeutics is a new venue that has not been appreciated in the past, as NO was primarily used for the treatment of blood vessel-related diseases and other non-cancer–related applications. The demonstration of NO-mediated cytotoxicity directly on cancer cells and/or indirectly in the tumor microenvironment through its antiproliferative and chemosensitizing roles, presents new challenges for its optimal use in cancer therapy. The data suggest that NO can be used as a chemosensitizing as well as an immunosensitizing agent; thus, one may consider its clinical application using combination treatment of NO donors and chemotherapy or immunotherapy resulting in synergistic activity in the treatment of cancer. It is also conceivable that one might use NO donors complexed with chemotherapeutic drugs or other cytotoxic agents. One may also consider using agents that can activate endogenous NO production via NOS II. Clearly, apart from the direct effects of NO on tumor cells, NO donors would also be functioning as vasodilators and thus have an even enhanced therapeutic potential. Possibly, novel NO donors may be administered orally and thus be more applicable to treatment. For certain tumors, it is also possible to administer NO donors intratumorally, thus reducing the systemic toxic effects that may arise from its route of administration. We expect that the application of NO donors

in cancer therapy will be added to the armamentarium of cancer therapeutics in the near future.

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