Review Article

Nitric oxide-mediated sensitization of resistant tumor cells to apoptosis by chemo-immunotherapeutics ☆

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ABSTRACT

The generation of NO by the various NO synthases in normal and malignant tissues is manifested by various biological effects that are involved in the regulation of cell survival, differentiation and cell death. The role of NO in the cytotoxic immune response was first revealed by demonstrating the induction of iNOS in target cells by immune cytokines (e.g. IFN-γ, IL-1, TNF-α, etc.) and resulting in the sensitization of resistant tumor cells to death ligands-induced apoptosis. Endogenous/exogenous NO mediated its immune sensitizing effect by inhibiting NF-κB activity and downstream, inactivating the repressor transcription factor YY1, which inhibited both Fas and DR5 expressions. In addition, NO-mediated inhibition of NF-κB activity and inhibition downstream of its anti-apoptotic gene targets sensitized the tumor cells to apoptosis by chemotherapeutic drugs. We have identified in tumor cells a dysregulated pro-survival/anti-apoptotic loop consisting of NF-κB/Snail/YY1/RKIP/PTEN and its modification by NO was responsible, in large, for the reversal of chemo and immune resistance and sensitization to apoptotic mechanisms by cytotoxic agents. Moreover, tumor cells treated with exogenous NO donors resulted in the inhibition of NF-κB activity via S-nitrosylation of p50 and p65, inhibition of Snail (NF-κB target gene), inhibition of transcription repression by S-nitrosylation of YY1 and subsequent inhibition of epithelial–mesenchymal transition (EMT), induction of RKIP (inhibition of the transcription repressor Snail), and induction of PTEN (inhibition of the repressors Snail and YY1). Further, each gene product modified by NO in the loop was involved in chemo-immunosensitization. These above findings demonstrated that NO donors interference in the regulatory circuitry result in chemo-immunosensitization and inhibition of EMT. Overall, these observations suggest the potential anti-tumor therapeutic effect of NO donors in combination with subtoxic chemo-immuno drugs. This combination acts on multiple facets including reversal of chemo-immune resistance, and inhibition of both EMT and metastasis.

Contents

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1. Introduction

Since the early introduction of various nonspecific chemotherapeutic drugs and, subsequently, radiotherapeutics, hormonal drugs and immunotherapies, there have been significant clinical and objective responses in patients with various malignant diseases. While these therapeutic strategies continue to improve and are still currently in use in oncology, there remains a major problem in that a subset of cancer patients does not initially respond and another responding subset develops resistance to further treatments and both subsets succumb to the disease. Clearly, the non-responding patients underlie the rapid and successful development of new classes of targeted therapeutics based on the various molecular mechanisms that govern resistance. Noteworthy, it is recognized to date that many of the cytotoxic agents (drugs, hormones, immunotherapies) used in cancer therapy mediate their effects by inducing programmed cell death, or apoptosis, in the responding sensitive tumor cells. Hence, a major, but not the only mechanism of resistance, is the mechanism in acquisition of tumor cells of several molecular and genetic means to evade cell death by apoptosis.

The inhibition of pro-apoptotic pathways in cancer cells is the result of tumor cells exhibiting constitutively hyperactivated survival and proliferative signal pathways that counteract the pro-apoptotic pathways. A good example is the constitutively hyperactivated NF-κB pathway in the majority of cancers that was shown to play a pivotal role in tumorigenesis, angiogenesis, resistance, inflammation, immunomodulation, and metastasis. Further, other survival/proliferative/anti-apoptotic pathways are also upregulated in cancer cells such as the PI3K/AKT and MAPK pathways. These various pathways also crosstalk and synergize in their anti-apoptotic effects [1,2].

There have been several strategies to therapeutically inhibit anti-apoptotic pathways in cancer. Among these, many earlier and recent studies suggested the potential cytotoxic and sensitizing effects of NO in examined various cancer cell models, *in vitro* and *in vivo* [3–10]. Recently, Reynolds el al. [11] reviewed the use of NO in the inhibition of tumor cell proliferation and its consideration as a novel therapeutic.

2. Nitric oxide properties and applications

Nitric Oxide is a versatile and pleiotropic molecule. It has been reported to be involved in cell signaling and it regulates a variety of physiological functions in mammals [12,13]. NO is a lipophilic free radical gas that can readily diffuse through cell membranes and chemically reacts with metal containing proteins and other effector molecules. NO is synthesized from arginine by the nitric oxide synthases (NOS). Three types of NOS have been discovered, namely, eNOS (endothelial nitric oxide synthase or type III NOS), nNOS (neuronal nitric oxide synthase or type I NOS), and iNOS (inducible nitric oxide synthase or type II) [14]. Zinc finger proteins have been reported to be important targets of NO [15] and NO can also mediate nitrosation and nitrosylation of various proteins and affects their functions and activities [16–19].

The guanylyl cyclase (sGC) α-1 and α-2 heterodimers are found in many tissues and are the receptors for NO [20,21]. The generation of NO by nitroglycerine or nitroprusside activates soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) that, in turn, activates Protein kinase C (PKC) to phosphorylate proteins [22]. The recent findings by Bian and Murad [23] established that the NO/sGC/cGMP axis is involved in the regulation of proliferation and differentiation. They also reported that undifferentiated tumor cells and stem cells have low levels of sGC. Thus, by elevating endogenous cGMP it resulted in the inverse correlation with the proliferation and colony formation of glioma cells.

The various NOS isoforms' functions are interconnected and operate as a pivotal part of Red-OX-based molecular signaling systems that result in major impacts on the cell's physiology and behavior and include S-nitrothiols (SNOs), which are generally produced by S-nitrosation of cysteine thiols by NO. These result in the disruption or dysregulation of normal cell signaling and in the impairment of cellular functions [19,24]. Both eNOS and nNOS are calcium-dependent, but iNOs is calcium independent. Further, eNOS and nNOS release lower levels of NO whereas iNOS releases high levels of NO [25]. Of relevance, NO signaling has been reported to initiate apoptosis through its activity on the mitochondrial membrane permeability and the consequent release of cytochrome c oxidase [6, 26].

NO-mediated apoptosis consists also in the endogenous formation of reactive nitric oxide species (RNOS). These RNOS include superoxide (O_2^-) that reacts with NO to form peroxynitrite ($ONOO^-$) that strongly induces apoptosis. Peroxynitrite acts as a DNA oxidant and induces single strand breaks in DNA [27,28]. Also, NO induces changes in the mitochondrial permeability transition pore (MPTP) and its opening results in the release of cytochrome c into the cytoplasm and induces apoptosis via binding Apaf-1 that activates caspase-9 leading to the activation of caspases 7 and 3 and downstream apoptosis [29]. The release of cytochrome c also results in the significant release of intracellular calcium resulting in cell death [30].

The consideration of the potential therapeutic application of NO donors has been the subject of many reports and reviews [7–9,11,13,31–33]. In this review, we will briefly discuss (1) the roles of NO-mediated regulation/interference of gene products that form a loop, namely, the NF-κB /Snail/YY1/RKIP/PTEN loop, that

has been shown to be dysregulated in cancerous cells and that is also involved in drug/immune resistance (2) the roles of NO-mediated chemo- and immunosensitizations of resistant cancer cells to apoptosis by chemo-immuno cytotoxic agents (3) the role of NO donors in the inhibition *in vivo* in mice bearing tumor xenografts when used in combination with chemotherapy and (4) the clinical response in cancer patients treated with NO-mediated therapeutics.

3. NO-mediated interference in the dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop responsible for chemo- and immune-resistance in cancer cells

Based on reported studies by us and others, we have established the presence of a dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in cancer cell model systems that regulates resistance and metastasis [34–38]. Each gene product of the loop was pivotal in the regulation of tumor cell sensitivity to various cytotoxic apoptotic stimuli (e.g., chemotherapeutic drugs, cytotoxic cells, cytotoxic ligands including FasL, TRAIL, and TNF-α). Treatment of tumor cells with high levels of NO donors (e.g., greater than 500 mM DETA-NONOate) resulted in the inhibition of the upregulated gene products in the loop, namely, NF-κB, Snail, and YY1 and the derepression of down regulated gene products, namely, RKIP and PTEN. These NO-mediated effects were both direct and indirect and resulting in tumor cells' inhibition of cell proliferation and their sensitization to apoptotic stimuli.

Briefly, we present below the findings of NO-mediated effects on each of the gene products of the above loop.

3.1. NO-mediated inhibition of NF-κB

The transcription factor NF-κB is constitutively hyper-activated in many cancers and regulates, in large part, survival, proliferation, anti-apoptotic activity, angiogenesis, and metastatic properties of cancer cells [39]. The role of NF-κB in resistance to apoptotic stimuli is the result of the activation downstream of anti-apoptotic gene products such as XIAP, Bcl-xl, Bcl-2, and YY1 [40–43]. Hence, inhibition of NF-κB may reverse the above tumorigenic properties and reverse drug resistance. The inhibition of NF-κB by various agents, including drugs, small molecule inhibitors, siRNA, etc., results in the reversal of tumor cell resistance to apoptotic stimuli and sensitization to apoptosis by chemo-immunotherapeutic drugs [44–48].

Nitric oxide, endogenous or exogenous, mediates contrasting effects on tumor cells, namely, it can either promote or inhibit tumor cell survival and growth. These contrasting effects were found to be dependent on the level of NO; low level of NO is protumorigenic whereas high level is anti-tumorigenic [49]. High level of NO inhibits NF-κB activity by inhibition of both the phosphorylation and degradation of IκBα and, thus, preventing NF-κB nuclear translocation and gene transcription [50]. NO will react with reactive oxygen species such as superoxide resulting in an impaired activation of NF-κB [51]. In addition, NO mediates direct effects by S-nitrosylation of NF-κB p50 and p65 and reduces its DNA-binding activity [52,53].

3.2. NO-mediated inhibition of Snail

Snail is a member of the Snail superfamily of zinc finger transcription factors. It plays important roles in embryonic development, neural differentiation, cell division, survival, invasion, EMT and metastasis [54,55]. Snail transcription is regulated, in part, by both NF-κB [56] and YY1 [57]. Snail represses the transcription of the metastasis suppressor E-cadherin [58] and RKIP [59].

Treatment of tumor cells with the NO donor, DETA-NONOate, inhibited Snail expression via NO-mediated inhibition of NF-κB and its target genes *Snail* and *YY1*[37,47]. Also, inhibition of Snail resulted in the derepression of E-cadherin and the induction of RKIP.

3.3. NO-mediated inhibition of YY1

The transcription factor YY1 is a DNA-binding factor that is multifunctional; it activates, represses or initiates transcription through either direct binding to DNA or indirectly through complexing with other DNA-binding proteins [60]. Our earlier findings demonstrated that YY1 represses the transcription of both Fas and DR5 and, hence, its role in immune resistance [61,62]. We have also reported that the treatment of tumor cells with the NO donor, DETA-NONOate, resulted in the S-nitrosylation of YY1 and further inhibition of its DNA-binding activity [63].

3.4. NO-mediated induction of RKIP

The Raf kinase inhibitory protein (RKIP) is a member of the phosphatidylethanolamine-binding protein family (PEBP) and it has been reported to play a role in lipid metabolism and phospholipid membrane biogenesis [64]. Yeung and colleagues were the first to clone RKIP and demonstrate its inhibitory effects on both the Raf/MEK/ERK and NF-κB pathways [65,66]. It has also been reported that RKIP negatively regulates the activation of G-protein coupled receptors (GPCRs) [67]. The metastatic inhibitor RKIP is significantly depressed in many tumors and almost absent in metastatic tumors [68–72].

Treatment of tumor cells with the NO donor, DETA-NONOate, resulted in significant upregulation of RKIP expression. This upregulation was the result of NO-mediated inhibition of NF-κB and its target the RKIP repressor Snail. The upregulation of RKIP subsequently results in the inhibition of both the NF-κB and the MAPK pathways and, thus, amplifying the inhibition of NF-κB, Snail and YY1 and upregulation of PTEN and RKIP.

3.5. NO-mediated induction of PTEN

PTEN is involved in the inhibition of the PI3K/AKT pathway [73]. It has been reported that both Snail and YY1 negatively regulate PTEN expression. In tumor cells whereby the NF-κB, Snail and YY1 are activated, they result in in the inactivation of PTEN and, thus, promoting the activation of the survival and anti-apoptotic PI3K/AKT pathway. The inhibition of NF-κB, Snail and YY1 by NO results in the derepression of PTEN and its upregulation and, thus, resulting in PTEN-mediated inhibition of the PI3K/AKT pathway and downstream target genes involved in both proliferation and anti-apoptosis [38].

4. NO-mediated immune-sensitization to apoptosis

Cytotoxic lymphocytes, primarily CTL and NK cells, mediate their cytotoxic activities by both necrosis and apoptosis. The induction of apoptosis is the result of CTL or NK activation following recognition and binding to the corresponding receptors on the target cell. The activation of the cytotoxic cell results in the utilization of the perforin/granzyme mechanism as well as the extracellular activation of the Fas-L, TRAIL and TNF-α- and their interactions and activation of their corresponding receptors [74].

There have been many approaches undertaken to reverse tumor cell resistance to cytotoxic stimuli. Among these are the use of nonspecific as well as specific sensitizing agents that modify the anti-apoptotic threshold of resistance and, thus, rendering the modified cells more susceptible to respond and be killed by the

conventional therapeutic agents [75]. We have reported that treatment of drug/immune resistant tumor cells with NO donors sensitizes the cells to apoptosis by chemo-immunotherapeutic agents.

The majority of our studies used the NO donor, DETA-NONOate, a member of the family of diazoniumdiolate (formerly NONOate). DETA-NONOate spontaneously dissociates in a pH-dependent first order process and has a half-life of 20 h and 50 h at 37 °C and 22–25 °C, pH 7.4, respectively. It liberates two moles of NO per mole of the parent compound DETA-NONOate [76,77].

4.1. NO-mediated sensitization to FasL apoptosis

Our initial original findings regarding the role of NO-mediated sensitization of immune resistant cancer cells to apoptosis was the result of investigating the underlying mechanism by which interferon-γ (IFN-γ) sensitized Fas-resistant tumor cell lines to FasL apoptosis. We observed that treatment of the tumor cells with IFN-γ induced the upregulation of Fas expression on the cell surface [78]. It was also known that IFN-γ induces iNOS and NO release and, thus, we hypothesized that NO may be involved in the sensitization to FasL-mediated apoptosis. Hence, when the tumor cells were treated with an NO donor, SNAP, it resulted in the upregulation of Fas and sensitization to FasL-mediated apoptosis, mimicking IFN-γ treatment. The direct role of NO-mediated sensitization by IFN-γ was corroborated by the use of the NO inhibitor, L-NAME, which abrogated IFN-γ-mediated sensitization to FasL [78]. The molecular mechanism of NO-mediated upregulation of Fas was likely the result of either the activation of a transcription factor on the Fas promoter or the inhibition of a Fas transcription repressor. Analysis of the Fas promoter identified a silencer region with consensus binding sites for the transcription factor YY1. YY1 has been reported to act as both an activator and as a repressor [60]. Hence, deletion of the silencer region of the Fas promoter resulted in a significant augmentation of the Fas reporter activity. Also, treatment of tumor cells with DETA-NONOate resulted in the inhibition of YY1-DNA binding activity as assessed by EMSA [61]. In addition, DETA-NONOate treatment of the tumor cells resulted in the S-nitrosylation of YY1 and inhibition of its DNA-binding activity [41]. Overall, the findings revealed that treatment of tumor cells with DETA-NONOate resulted in the significant release of NO and consequent inhibition of the Fas YY1 repressor, the induction of Fas and sensitization to FasL-mediated apoptosis. The sensitization was the result of activation of the apoptotic pathway triggered by Fas and by NO and involved both type I and type II apoptotic pathways.

4.2. NO-mediated sensitization to TNF-alpha apoptosis

Several lines of evidence supported the role of the constitutively activated NF-κB pathway in cancer cells as pivotal of tumor cells resistance to TNF-α apoptosis [79,80]. TNF-α interacts with two distinct cell surface receptors, TNF-R1 and TNF-R2, and most cytotoxic effects of TNF-α are mediated by interaction with TNF-R2 [81]. TNF-α triggers the induction of various reactive oxygen species (ROS) [82], hence, the use of antioxidants inhibited TNF-α-mediated effects [83].

The ROS generated by TNF-α results from enzymatic partial reduction of oxygen yielding superoxide anion (O_2^-), which is immediately reduced by superoxide dismutase to H_2O_2 or reacts with NO to generate $ONOO^-$ [84].

Treatment of tumor cells with IFN-γ sensitized the cells to TNF-α apoptosis and induction of iNOS. Treatment of tumor cells with the NOS inhibitor, L-NMA, inhibited IFN-γ-mediated sensitization to TNF-α. In contrast, treatment with an NO donor (e.g., S-nitroso-N-acetylpencillamine) sensitized tumor cells to TNF-α apoptosis

[51]. This finding shows that NO interferes with TNF-α-induced NF-κB activity by reacting with O_2^- and reducing the TNF-α H_2O_2-mediated activation of NF-κB.

4.3. NO-mediated sensitization to TRAIL apoptosis

Reported studies using chemotherapeutic drugs and NF-κB inhibitors showed that their use with TRAIL-resistant cancer cells resulted in their sensitization to TRAIL apoptosis concurrently with upregulation of DR-4 or DR-5 expression [34,85–87]. The finding that NO inhibits NF-κB led us to hypothesize that NO can also sensitize TRAIL-resistant tumor cells to apoptosis by TRAIL. Treatment of tumor cells with DETA-NONOate sensitized the tumor cells to TRAIL apoptosis concomitantly with upregulation of DR-5 [34]. Similar to our findings with Fas-induced upregulation by NO, there were YY1 consensus binding sites on the DR-5 promoter. Treatment with DETA-NONOate augmented DR-5 reporter activity and transcription of cells with DR-5 constructs lacking YY1 binding sites or mutation of the YY1 sites, all of which resulted in enhancement of reporter activity. The direct role of YY1 on the suppression of DR-5 transcript was corroborated by the use of siRNA for YY1. The sensitization by DETA-NONOate of TRAIL-induced apoptosis was mediated by the activation of type-I and type-II apoptotic pathways. The combination treatment activated both caspases 9 and 3 and PARP cleavage [34].

Overall, the above findings demonstrated that NO modulates the dysregulated NF-κB/Snail/YY1/RKIP loop and results in the inhibition of NF-κB, Snail and YY1 and the induction of RKIP. These findings suggested that each of the gene products in the loop contributes to the resistance and that each gene product alone is directly involved in the sensitization of tumor cells to immunotherapeutic agents.

5. NO-mediated chemosensitization to apoptosis

Several mechanisms have been postulated regarding drug-resistance of tumor cells. Drug-resistance is also a consequence of the anti-apoptotic machinery in cancer cells since many chemotherapeutic drugs mediate their cytotoxic activity via apoptosis. Inhibitors of survival pathways have been reported to reverse drug resistance. For instance, inhibitors of the constitutively activated NF-κB pathway have reversed Cis-Diammine-Dichloro-Platinum (CDDP)-resistant cancer cell lines to respond to CDDP treatments [88]. The finding that NO treatment inhibits NF-κB activity led us to postulate it will also sensitize tumor cells to apoptosis by chemotherapeutic drugs. Indeed, treatment of human prostate cancer cell lines with DETA-NONOate and CDDP resulted in significant synergistic apoptosis. Treatment with DETA-NONOate inhibited anti-apoptotic gene products, particularly, Bcl_{xl} and XIAP in these lines. The combination treatment activated the type-II apoptotic mitochondrial pathway [89]. Similar findings have also been reported with CDDP-resistant metastatic colon cancer cell lines [90]. These findings corroborated an earlier and a first report by Wink's Group, whereby, and under conditions of high concentrations of NO, tumor cells were sensitized to various chemotherapeutic drugs [91].

The NO-mediated chemosensitization in prostate cancer cell lines revealed that both YY1 and Bcl_{xl} were inhibited and played pivotal roles in chemosensitization. Treatment of tumor cells with either siRNA YY1 or siRNA Bcl_{xl} sensitized tumor cells to CDDP apoptosis. The in vitro findings of the synergistic apoptotic activity by the combination treatment were corroborated in vivo in mice bearing PC3 tumor xenografts. Treatment with the combination of CDDP and DETA-NONOate in vivo, but not a single agent alone, resulted in synergistic inhibition of tumor

growth [89].

6. NO-mediated interference in the NF-κB/Snail/YY1/RKIP loop and the role of each gene product in chemo-immunosensitization

6.1. Role of NO-mediated inhibition of NF-kB in chemo-immunosensitization

The NF-κB activity is inhibited by NO and resulting in the inhibition of the anti-apoptotic target genes as well as the derepression of pro-apoptotic gene products [34,92,93]. Various NO donors have been reported which inhibited NF-κB activity and sensitized drug-resistant tumor cells to cytotoxicity [94,95]. The implication of NF-κB -induced inhibition by NO in chemo-immunosensitization was corroborated by the use of NF-κB inhibitors, for example DHMEQ, which mimicked NO in both chemo and immunosensitizations of tumor cells to CDDP and TRAIL apoptosis, respectively [34,96].

6.2. Role of NO-mediated inhibition of YY1 in chemo-immunosensitization

The transcription factor YY1 is under the transcriptional control of NF-κB [42]. Hence, inhibition of NF-κB will also result in the inhibition of YY1. In addition, NO directly nitrosylates YY1 and inhibits its DNA-binding activity [63]. The direct role of YY1 in chemo-sensitization was corroborated by the use of siRNA YY1. Treatment of tumor cells with siRNA YY1 sensitized tumor cells to CDDP apoptosis [89].

6.3. Role of NO-mediated inhibition of Snail in chemo-immunosensitization

The transcription regulation of the zinc transcription factor Snail is under the control of both NF-κB [56] and YY1 [57]. It has also been reported that Snail represses E-cadherin [58] and RKIP [59].

Treatment of tumor cells with the NO donor DETA-NONOate inhibited Snail mRNA and protein expressions. In addition, Snail was S-nitrosylated that inhibited its DNA-binding activity. Further, Snail was also involved in the reversal of chemo-immune resistance. The direct role of Snail was demonstrated by treating the resistant cells with Snail siRNA and the tumor cells were sensitized to apoptosis by both chemotherapeutics and immunotherapeutics [37,97]. Treatment with Snail siRNA resulted in modification of the regulatory loop by inhibiting NF-κB, YY1 and Snail and the induction of RKIP.

6.4. Role of RKIP-mediated upregulation by NO in chemo-immunosensitization

The first demonstration that RKIP plays a role in the regulation of drug resistance was reported by Chatterjee et al. [98], whereby they reported that the overexpression of RKIP in prostate cancer cell lines sensitized the cells to apoptosis by Camptothecin (CPT-1). Also, we have reported that overexpression of RKIP sensitizes cells to TRAIL apoptosis [45].

Since treatment of tumor cells with DETA-NONOate resulted in the upregulation of RKIP, we postulated that the induction of RKIP concomitantly with inhibition of the resistant factors NF-κB, Snail and YY1 will be involved in chemo-immunosensitization of resistant tumor cells. Treatment of tumor cells with DETA-NONOate resulted in the upregulation of RKIP mRNA and protein expressions in tumor cell lines and the cells were sensitized to apoptosis

by cytotoxic drugs. The direct role of RKIP in chemo-immunosensitization was corroborated by the use of cells treated with siRNA RKIP, which resulted in the reversal of tumor cell sensitivity to TRAIL apoptosis [45].

Clearly, all of the above findings point out that each gene product of the loop plays a direct role in chemo-immunosensitization to apoptosis by both chemo and immunotherapeutic drugs. However, the direct role of Snail, YY1 and RKIP in chemo-immunosensitization is not totally clear since they are all interregulated. To approach the direct roles, one has to investigate tumor cells that are, for example, inhibited for NF-κB activity and then were transduced to overexpress Snail, YY1 or RKIP and examine their sensitization to drugs.

## 7. Comparison between NO donors and other chemo-immunosensitizing agents

There have been many reports of using various agents that reverse tumor cell resistance and sensitized the cells to apoptosis by chemo-immunotherapeutics [99]. Various agents have targeted the NF-κB, Snail, YY1, RKIP loop and the results showed similar findings to those mediated by treatment with NO donors. For example, we have reported that treatment of tumor cells with subtoxic concentrations of chemotherapeutic drugs sensitized the cells to both FasL and TRAIL apoptosis [100–102]. These findings were subsequently corroborated by treatment with NO donors [103]. Also, treatment with subtoxic concentrations of drugs sensitized the tumor cells to TRAIL apoptosis [104].

Treatment with chemotherapeutic drugs resulted in the upregulation of DR4 or DR5, concomitantly with sensitization to TRAIL apoptosis [105,106]. However, the molecular underlying mechanisms were not known. In our prior findings [96] we reported that treatment with an NO donor upregulated DR5 concomitantly with sensitization to TRAIL apoptosis. The mechanism of DR5 upregulation was the result of NO-mediated inhibition of the DR5 repressor YY1. We have also reported that treatment of B-NHL tumor cells with rituximab sensitized the cells to both chemo and immunotherapeutics. Sensitization was the result of inhibition of the NF-κB, Raf-1, and PI3K pathways [36,107–109]. These findings were reproduced with NO donors and showed that rituximab and the NO donor behaved similarly. Inhibitors for NF-κB also mimicked NO donors in tumor cell sensitization. Hence, treatment of tumor cells with NF-κB inhibitors sensitized the tumor cells to apoptosis by cytotoxic drugs. One example is the use of the NF-κB inhibitor, DHMEQ, which resulted in sensitization to FasL and TRAIL and chemotherapeutic drugs [44]. Likewise, we have shown that the NO donor, which inhibited NF-κB, also resulted in the reversal of resistance and, thus, mimicking the NF-κB inhibitor. In addition, treatment with proteasome inhibitors resulted in tumor cell sensitization mimicking NO donors. For example, treatment with bortezomib was shown to sensitize tumor cells to TRAIL apoptosis. Bortezomib inhibits NF-κB activity like NO donors [45]. Like NO donors, treatment with another proteasome inhibitor, NPI0052, resulted in the inhibition of NF-κB, Snail, and YY1 and induction of RKIP.

The above findings demonstrated that treatment with NO donors mimicked many sensitizing agents, some of which are clinically used (Bortezomib) and suggest the use of NO donors *in vivo* in patients in combination with subtoxic cytotoxic therapies. Further, it is not clear whether the development of tumor sensitizing agents can be also applied for novel sensitizing NO donors.
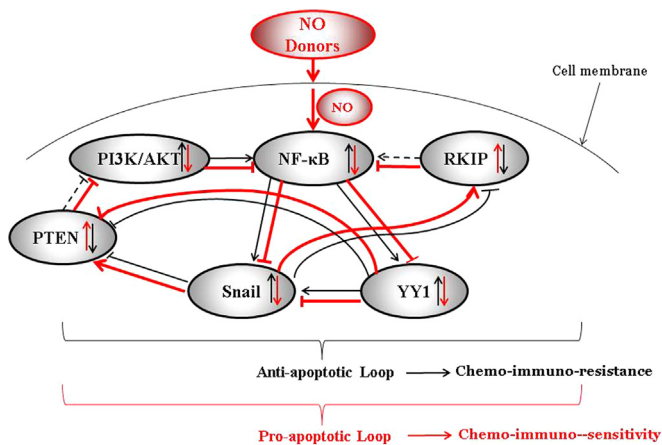
Fig. 1. NO donors convert the anti-apoptotic NF-κB/Snail/YY1/RKIP/PTEN resistant loop into a sensitive pro-apoptotic loop. The schematic diagram represents tumor cells that have a dysregulated NF-κB/Snail/ YY1/RKIP/PTEN resistant loop involved in the maintenance of the tumor cell viability, proliferation, resistance, EMT, and metastasis. Hence, in tumor cells, the expression and the activities of NF-κB, Snail, YY1 are upregulated while those of RKIP and PTEN are downregulated. The loop is the result of NF-κB-mediated activation of its target Snail and YY1 and, in turn, Snail represses RKIP and YY1 represses PTEN. In addition, YY1 activates Snail. In the presence of NO donors, the activities of NF-κB, Snail and YY1 are inhibited and resulting in the de-repression of RKIP and PTEN. In turn, RKIP potentiates its inhibitory activity on NF-κB and its targets and, likewise, PTEN inhibits the PI3K/Akt pathway which controls NF-κB. Overall, the NO treatment inhibits tumor cell viability, proliferation and sensitizes the cells to chemo-immunotherapies-induced apoptosis.
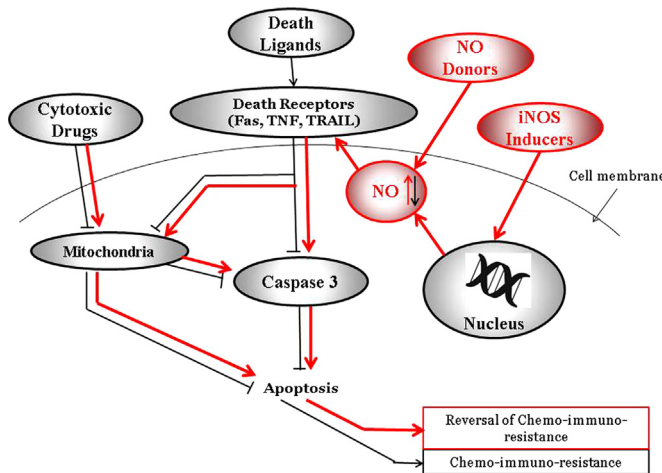
Fig. 2. Mechanism of NO-mediated chemo-immunosensitization of resistant tumor cells to apoptosis. The schematic diagram represents the NO-mediated effects that results in the sensitization of resistant tumor cells to drug-immune-induced apoptosis. Both NO donors and iNOS induction will result in the upregulation of NO, resulting in the upregulation of death receptors and sensitization to death ligands (FasL, TRAIL, TNF-α) and activation of the effector caspase 3 and downstream apoptosis. Also, NO will inhibit anti-apoptotic gene products such as Bcl-2 and allowing cytotoxic drugs to act on the mitochondrial membrane and resulting on the activation of caspase 3 or induction of AIF, all of which result in apoptosis.

8. Efficacy of pre-clinical and clinical applications of NO donors

8.1. Preclinical

An NO donor synthesized by Keefer's Lab at NIH, JS-K, is a diazonium diolate and releases NO enzymatically activated by glutathione and glutathione-S-transferase (GST) [110]. Findings by Weyerbroch et al. [111] reported the inhibition of proliferation and induction of apoptosis in vivo of U87 glioma tumors xenografts treated with JS-K. In a colo-rectal carcinoma model, Huerta et al.

[112] used mice bearing the human metastatic CRC line, SW620, and treated the mice with DETA-NONOate and chemotherapy. This combination treatment resulted in inhibition of tumor cell growth. Pervin et al. [113] reported the treatment of tumor-bearing mice with the combination of DETA-NONOate and a farnesyl transferase inhibitor showed minimal toxicity and slowed the growth of the tumor. Also, the combination of DETA-NONOate and CDDP enhanced the cytotoxic activity of S29 lung fibroblasts [91], and similar results were achieved with head and neck cancers [114]. Huerta-Yepez et al. [89] reported tumor inhibition by treatment with the combination of DETA-NONOate and CDDP in mice bearing human prostate cancer xenografts.

In addition to NO donors, reports utilized gene therapy via inducible NOSII for cancer therapy [115]. Another NO donor, JS-K, was studied in castrated-resistant prostate cancer cells and resulted in the attenuation of functional androgen receptor signaling with inhibition of cell growth [116]. Also, NO-NSAID donors were examined in a variety of tumor cell lines and exhibited inhibition of cell proliferation and induction of apoptosis [117–119].

The anticancer effect of iNOS was first reported by Xie et al. [120]. The in vivo model used mice bearing tumors overexpressing iNOS and resulted in inhibition of tumor growth and additional studies were also reported by Xie and Fiddler [121]. Chung et al. [122] reported significant apoptosis in cells transfected with the iNOS gene and other studies have been reviewed elsewhere [33,115].

The antitumoral properties of NOS-3 overexpression by gene therapy was reported [123,124].

8.2. Clinical

Several reports have delineated the important role of NO in cancer therapy. It may act directly as a cytotoxic agent against cancer cells or sensitizes resistant tumor cells to chemo-immuno-radio-hormonal therapies [125,126].

The initial application of the NO donor, nitroglycerine (GTN), in patients was reported by Yasuda et al. [127,128] whereby they treated patients with lung cancer with the combination of GTN and vinorelbine and CDDP and showed an improved response of 72% and median time to progression compared to patients without GTN. Siemens et al. [129] reported a phase II study using patches releasing GTN in prostate cancer patients who failed primary treatment. This treatment reduced hypoxia-induced progression as measured by a decrease in the PSA doubling time. Siemens et al. [130] used a GTN patch that slowly releases GTN in combination with anti-cancer treatment with drugs that resulted in significant inhibition of PSA in patients with prostate cancer. Arrieta et al. [32] reported the first clinical study that the addition of GTN to induction chemotherapy and concurrent chemo-immunotherapy in patients with local advanced NSCCL resulted in acceptable toxicity profile.

9. Concluding remarks and future directions

It is clear that high levels of NO, either biologically generated by iNOS or by NO donors, exert several anti-tumor activities, namely, inhibition of cell survival and growth, sensitizing the tumor cells to both chemo, immune- and radio-toxicities, and also inhibit EMT and metastasis. The mechanisms mediating these various NO-mediated effects are, in part, under the regulation of the constitutively activated and dysregulated pro-survival/anti-apoptotic of the NF-κB/Snail/RKIP/PTEN loop in cancer cells. NO interferes directly in the loop by inhibiting the activities NF-κB, Snail and YY1 and derepressing the expressions of the anti-survival/pro-apoptotic gene products RKIP and PTEN (see schematic diagrams

Figs. 1 and 2). These NO-mediated manifestations, both in vitro and in vivo in mouse models, support the potential therapeutic application of NO donors in combination with subtoxic therapeutic agents on the reversal of tumor resistance and the inhibition of both EMT and metastasis. Phase I/II clinical studies are under current investigation to support the contention of the anti-tumor-mediated effects of NO [see reviews in this volume]. Clearly, phase III clinical trials will be hopefully investigated in the future and we are eagerly awaiting the findings for the novel therapeutic application of NO in cancer. It must be noted also that while NO donors are not highly tumor specific, their pleiotropic activity in the reversal of resistance and sensitizing activity are paralleled with inhibition of EMT and metastasis. Various laboratories are developing novel NO donors with better efficacies as single agents or complexed with other targeting agents for specificity.

Acknowledgements

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