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Mini Review

Applications for nitric oxide in halting proliferation of tumor cells

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ABSTRACT

Tumor resistance to cytotoxic therapeutics coupled with dose-limiting toxicity is a serious hurdle in the field of medical oncology. In the face of this obstacle, nitric oxide has emerged as a powerful adjuvant for the hypersensitization of tumors to more traditional chemo- and radio-therapeutics. Furthermore, emerging evidence indicates that nitric oxide donors have the potential to function independently in the clinical management of cancer. Herein, we discuss the role of nitric oxide in cancer and the potential for nitric oxide donors to support conventional therapeutics.

1. Introduction

From its minute relative size and lipophilicity to its highly reactive nature as a free radical, nitric oxide (NO) is an iconic molecule of signal transduction [1,2]. NO plays a central role in cell regulatory pathways [3,4] and is essential in the mediation of leukocytic cytotoxicity via superoxide [5–8]. The physiological processes which depend upon NO vary in the concentrations of NO that they require. For example, while NO-induced cytotoxicity requires high, precipitous concentrations [8], regulation of platelet accretion is governed by specific localization of exacting concentrations of nitric oxide [9,10]. Consequently, several isoforms of NO synthase (NOS) which vary in their expression patterns and rates of productivity are responsible for the synthesis of nitric oxide under varying conditions. These include endothelial NOS (eNOS), a calcium dependent form which functions in cell signaling outside the central nervous system (CNS); neuronal NOS (nNOS), another calcium dependent variant involved in signaling within the CNS; and inducible NOS (iNOS). This inducible form is involved in immunological responses and has the capacity to rapidly generate high concentrations of NO [1,11].

Several studies have highlighted the roles of NO in the regulation of tumor-targeting immunological processes [12–14]. In a similar capacity, NO signaling has been implicated in the initiation of tumor cell apoptosis through its impacts on mitochondrial membrane permeability and the consequent discharge of cytochrome c oxidase [15,16]. The potential therapeutic application for NO donors has been highlighted in hundreds of papers and patent claims [17–20]. A comprehensive investigation of the immunological impacts of NO and the conditions necessary to induce those impacts, along with the identification of specific NO donors with the capacity to release NO at the right concentration and in a temporally regulated, targeted manner will likely lead to a powerful new line of tumor therapeutics.

2. *In vivo* nitric oxide synthesis and activity

In vivo, NO is synthesized by NOS isoforms via oxidation of the amino acid, L-arginine [1,11]. The three principal isoforms of NOS catalyze a two-step reaction that employs NADPH and O_2 as co-substrates to oxidize L-arginine into L-citrulline, releasing NO as a byproduct [21]. An unpaired electron in the outer orbital of NO may either be released conferring an oxidative capacity or it may serve in an antioxidant capacity by accepting and, thereby, stabilizing an electron donor [20,22].

The broad range of physiological processes mediated by NO generally fall under two mechanistic routes, being either cGMP-

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dependent or -independent. The primary path through which NO imparts a host of physiological outcomes is cGMP-dependent whereby NO interaction with guanylate cyclase ultimately facilitates activation of cyclic nucleotide gated ion channels and cGMP-regulated kinases and phosphodiesterases [23]. These, in turn, impact neurotransmission, vasodilation, inhibition of platelet aggregation, and relaxation of smooth muscle [23]. NO products of nNOS and eNOS act through such cGMP-dependent pathways [20,23]. A second cGMP-independent pathway includes NO reactivity with molecular O_2 and metals or direct protein modifications such as nitration and nitrosylation. For example, *S*-nitrosylation of cysteine residues is a key form of cell signaling involving the regulation of many enzymes [24]. NO produced by iNOS functions in this cGMP-independent manner [25] and also has a critical role in T-lymphocyte cytotoxicity and other immunological responses [26].

The role of NO in cytotoxic pathways has broadened research into potential applications of NO donors in the treatment of cancer. The disparity among physiological outcomes induced by NO derived from each NOS highlights the importance of timing, duration, and intensity of NO release in the ultimate determination of biological outcomes. Thus, in deciding upon potential NO donors for antitumor therapeutics, an awareness of disparities among these factors, as induced by NO derived from nNOS, eNOS, and iNOS has been essential. The biological impacts of nNOS and eNOS are produced by relatively minute concentrations of NO generated at short-lived intervals [2]. These are most often observed in the range of nanomolar concentrations for seconds to hours in duration [27,28]. With iNOS, NO is generated at μM concentrations for periods often lasting days [27–29]. Such disparity among the duration and intensity of NO appears to distinguish events related to cell signaling (nNOS; eNOS) from those which impact cell growth and survival (iNOS). Therefore, the evaluation of NO donors as potential anticancer therapeutics should be designed to select for those which most closely model the NO release attributes of iNOS [30,31].

3. Nitric oxide and tumors

The role of NO in tumor biology has been the source of much debate. Early studies presented a promising portrait of NO as a potent inducer of macrophage-mediated cytotoxicity [32,33]. Since that time, there have been dozens of studies investigating the potential of NO donors as cancer therapeutics [20,34]. However, the results have been frustratingly inconsistent. In many cases, NO actually appears to augment tumor cell proliferation [35]. A common idea emerging in recent literature related to NO and cancer is that the capacity of NO to enhance or inhibit tumor proliferation is dependent upon the concentration of NO. Higher levels of NO are correlated with induction of tumor cell apoptosis while lower levels have been correlated with enhanced tumor survival [35,36]. A popular model has since emerged in which high concentrations of NO have been linked to high levels of wild-type p53, ultimately leading to apoptosis. Conversely, it is suggested that lower concentrations of NO lead to p53 mutations and, consequently, cell survival [37–39].

Beyond concentration of NO, accumulating evidence suggests that cell type and the respective pattern of protein expression significantly affect the impact of NO on cell survival and proliferation [40–42]. Such dichotomous outcomes have even been observed within variations of the same cell type as illustrated with subtypes of estrogen receptor positive (ER+) versus ER negative (ER−) breast tumors where prognosis associated with some subtypes of ER− tumors fare worse in the presence of high levels of NO [43]. Our own studies have supported these claims as we have observed dramatic inhibition of ER+ breast tumor lines in response to NO donors (Fig. 1A). In a similar portrait of dichotomy, we have also observed these same donors actually augment growth and survival of several prostate tumor lines (Fig. 1B). Our observations in breast versus prostate tumors are consistent with other recent findings [44,45]. Thus, although NO holds promise as a potent supplement to the current anticancer arsenal, its applications will likely be tumor specific. To facilitate discrimination of NO tumor applications, we are currently documenting a library of tumor lines which are NO-sensitive versus those for which growth, survival, and aggressiveness are augmented by NO.

4. Nitric oxide donors as cancer therapeutics

A number of NO-donors have been investigated as potential cancer therapeutics, as recently reviewed by Huerta et al. [20] and Bonavida et al. [46,47]. Examples of these include organic nitrites glyceryl trinitrite, (GTN), metal-nitrosyl complexes sodium nitroprusside, (SNP), *S*-nitrosothiols (*S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione (GSNO)), Sydnonimines 3-morpholinosydnonimine, (SIN-1), and diazeniumdiolates [20,46,47]. A synopsis of the anticancer potential for each of these NO donors follows.

Studies have shown that GTN induces apoptosis in colon cancer cells through sensitization to Fas-mediated cell death by increasing the expression of Fas and decreasing the expression of several endogenous inhibitors of apoptosis [48]. Low concentrations of NO-mimetic GTN have also been found to inhibit the hypoxia-mediated metastatic potential of B16F10 murine melanoma cells [49] and increase the chemosensitivity of human prostate tumor xenografts [50]. NO-released from SNP has been shown to inhibit invasion of human cancer cells (PC-3M and T24) via inhibition of hypoxia inducible factor 1 (HIF-1) as well as through impairment of mitochondria [51]. The NO donor, SNAP, results in an increase in apoptosis and cell death in human neuroblastoma SH-SY5Y cells caused by enhanced expression of p53 and consequent nucleosomal DNA fragmentation [52,53]. Moreover, SNAP has also been effective in the radiosensitization of hypoxic EMT-6 tumor cells [54]. GSNO has been shown to induce apoptosis in colon tumor lines, HCT116 (p53 wild-type) and SW620 (p53-deficient) [55]. Interestingly, the p53-deficient SW620 cells were found to be more sensitive to NO-induced apoptosis than the HCT116 cells. In higher concentrations GSNO also exhibited inhibition of cell growth and induced apoptosis in HCA7, HT29, and HCT116 colon cancer cell lines, regardless of their Cyclooxygenase-2 (COX-2) expression and activities [56]. SIN-1, a peroxynitrite generator, is highly effective in inducing cancer cell apoptosis through oxidative DNA damage with activation of caspase 3-like proteases [57]. SIN-1 also inhibits the activity of endogenous arylamine *N*-acetyltranferase isoform 1 (NAT1) in MCF7 breast cancer cells [58] and induces apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells [59].

Diazeniumdiolates (NONOates) have been the most extensively evaluated class of NO-donors for the treatment of cancer [60]. Simeone et al. evaluated the use of diethylamine NONOate/AM (NONO-AM, synthesized from DEA/NO) as an effective chemo preventive agent against bone metastatic breast cancer [61]. Their studies showed that NONO-AM induced cell apoptosis in a dose-dependent manner in both MDA-MB-231 and F10 bone metastatic breast cancer cell lines. However the F10 cells were found to be more sensitive to NONO-AM than the MDA-MB-231 cells. NO released from PAPA/NO was found to be effective in radiosensitization of hypoxic EMT-6 tumor cells [62] and caused apoptosis in HT29 human colon cancer cells [63]. NO generated from SPER/NO provided a rapid enhancement of heme oxygenase-1 (HO-1)

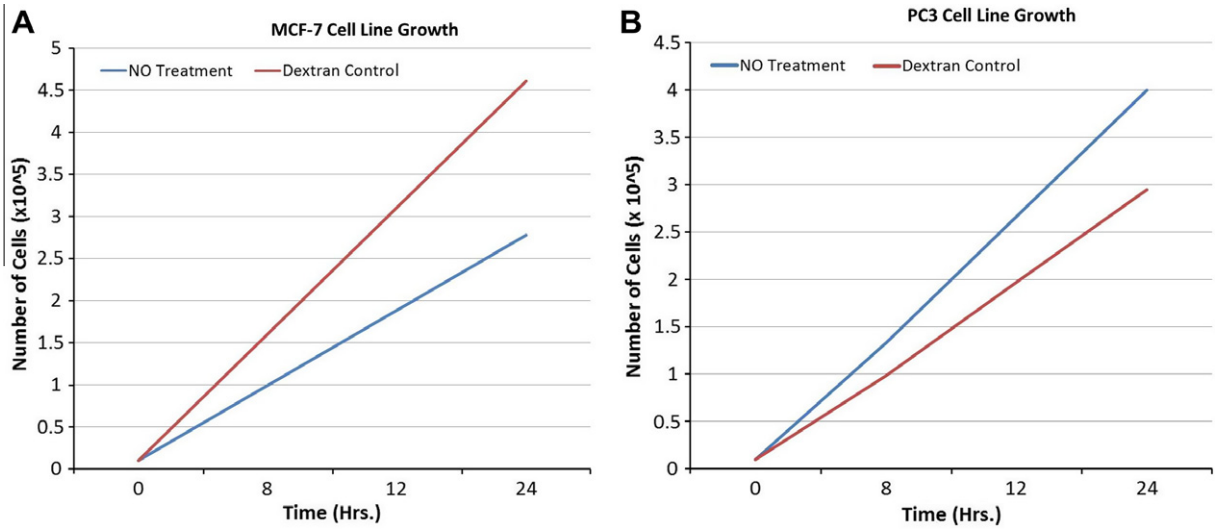

Fig. 1. Effect of NO on proliferation rate of cancer cells. (A) Proliferation of NO-treated MCF-7 human breast cancer cells (blue) is significantly inhibited relative to untreated cells (red). (B) NO treatment enhances proliferation of PC3 prostate cancer cells (blue) relative to untreated cells (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression and resulted in cytoprotective outcomes in C6 rat glioma cells against cadmium induced cytotoxicity [64] and in COH-BR1 cells against light-induced chain peroxidation and necrosis [65]. Lastly, DETA/NO induced cytostasis and cell-cycle arrest in human breast cancer cell lines MDA-MB-231 [45] and MDA-MB-468 [66] through dephosphorylation of extracellular signal-regulated kinase ERK1/2 [67].

5. Controlling NO delivery

Unfortunately, the majority of NO-donors lack target specification, extended half-lives, and controlled release kinetics. In order to achieve the required therapeutic effect, NO-hybrid pro-drugs which can deliver NO site-specifically and in a controlled manner are gaining interest. To that end, protected NO-donors as well as NO-donors incorporated into macromolecules have been evaluated as stable pro-drugs for controlled NO delivery. JS-K (O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate) is a diazeniumdiolate based NO-releasing pro-drug evaluated as a potential cytotoxic agent against human non-small-cell lung cancer (NSCLC) cell lines and induced apoptosis in human multiple myeloma cells *in vitro* and *in vivo* [68,69]. In addition, site specific tumor cell-targeting glyco-*S*-nitrosothiols (sugar-SNAP) have been prepared and evaluated by Hou et al. [70] against DU145 human prostate cancer cells. Those studies illustrated that an increased uptake of this NO-donor observed in the cancer cells was due to the enhanced transport of saccharides in mammalian cells, thereby resulting in a greater cytotoxicity than SNAP, alone.

We have, likewise, evaluated pro-drugs which are polysaccharide based dextran thiomers prepared via a covalent incorporation of an NO-donor [71]. This results in a stabilization of the donors against the rapid and uncontrolled release of NO thereby providing a stable pro-drug to release NO quantitatively under physiological conditions. Moreover the use of polysaccharides provides a better option than monosaccharides for drug delivery applications by improving the microcirculatory flow through decreased viscosity of blood and inhibition of erythrocytic aggregation due to their colloidal nature. The NO-donors used for the proliferation assays depicted Fig. 1A and B were produced in this manner. Representative real-time NO-release profiles and the average total NO-release curves for the *S*-nitrosated dextran-cysteamine and -cysteine derivatives are presented in Fig. 2A and B, respectively.

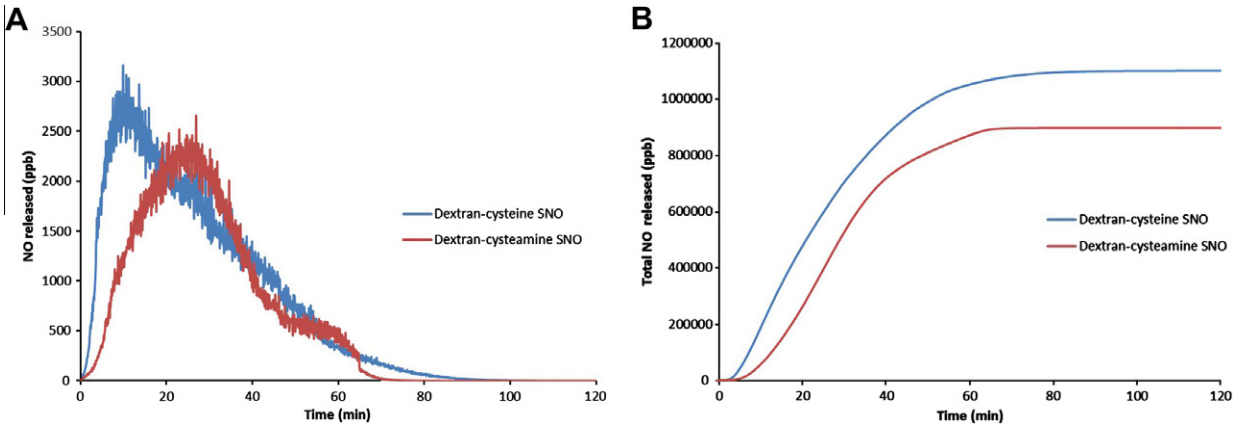

Fig. 2. Release profiles for two polysaccharide based dextran thiomer NO donors. (A) Representative real-time release profiles for *S*-nitrosated dextran derivatives over a 120 min period under cell media conditions. (B) The average total NO released from the *S*-nitrosated dextran derivatives over a 120 min period under cell media conditions.

Table 1
Summary of NO recovery at over 2 h at 37 °C under cell media conditions.

Dextran derivative	Total NO released (mmol/g)	pH of media after NO release
Dextran-cysteamine SNO[a]	0.098 ± 0.019	7.31 ± 0.075
Dextran-cysteine SNO[b]	0.119 ± 0.049	7.35 ± 0.015

Note: average from multiple experiments.
[a] $N = 4$.
[b] $N = 5$.

In both cases, the donor release analyses were conducted in the same media and under the same conditions as those in the proliferation assays indicated in Fig. 1. Nitric oxide release from dextran-cysteamine demonstrated an initial rapid rate of release which reached a maximum at 25 min. In a similar manner, the dextran-cysteine reached a maximum rate of release within 15 min which then gradually decreased until a baseline level was achieved. The dextran-cysteamine derivative released a total of 0.098 ± 0.019 mmol NO/g, and the dextran-cysteine derivative released a total of 0.119 ± 0.049 mmol NO/g in 2 h under cell culture conditions (Table 1). We have found these release profiles to most closely match those which are associated with the previously reported antitumor potential of NO. Thus, we have evaluated the impacts of these NO-donors on numerous cancer cell lines in our ongoing studies to create a library of tumor lines which are NO-sensitive.

6. Discussion

Accumulating evidence demonstrates the potential of NO donors as anticancer therapeutics. However, it is clear that such donors must exhibit a modestly controlled release at relatively high concentrations as exhibited by the polysaccharide based dextran thiomer examples highlighted in this review. Moreover, given the disparity in physiological outcomes of NO treatments in varying cancer cells, additional information is required related to the specific tumor cell types that may be best suited as targets of NO therapy. The absence of any kind of library of data related to the impacts of specific NO donors on a broad array of tumors highlights a serious gap in the current body of NO-related literature. A broad investigation cataloging such information in one manuscript would be most useful to the field of NO therapeutics.

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