Novel agents for cancer prevention based on nitric oxide

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Abstract
NO (nitric oxide) biology has provided the impetus for the development of anticancer agents based on their ability to release NO. NO-NSAIDs (NO-donating non-steroidal anti-inflammatory drugs), consisting of a conventional NSAID to which an NO-releasing moiety is covalently attached, are promising chemopreventive agents against cancer. Compared with their parent compounds, NO-NSAIDs are up to several hundred times more potent in inhibiting the growth of cancer cell lines and prevent colon and pancreatic cancer in animal models. Their chemopreventive effect is due to inhibition of proliferation, induction of cell death and inhibition of cell-cycle-phase transitions. NO-ASA (NO-aspirin), the best-studied NO-NSAID, induces oxidative stress in target cells. Major downstream signalling effects involve the Wnt, NOS2 (nitric oxide synthase 2), MAPK (mitogen-activated protein kinase), NF-$\kappa$B (nuclear factor $\kappa$B) and Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) pathways. NO-NSAIDs, particularly NO-ASA, appear to be safe compounds, as suggested by many animal and early human studies. An ongoing clinical trial is designed to determine whether NO-ASA can inhibit early stages of colon carcinogenesis in subjects at risk for colon cancer. It is clinical trials that will ultimately determine the role of NO-NSAIDs in cancer prevention and perhaps treatment.

Introduction
Cancer represents one of the major medical challenges of our time. Two dreadful statistical facts [1] underscore the enormity of the problem: first, cancer is a leading cause of death. In 2004, 27.2% of deaths in U.S.A. were due to heart diseases, while 23.1% were caused by cancer. For 2007, the American Cancer Society predicts that 289550 American males and 270100 females will die of cancer. The second and even more sober statistic concerns the lack of any significant progress in solving 'the cancer problem'. For example, in U.S.A., the death rate from cancer (expressed per 10^3) has remained essentially unchanged between 1950 and 2004, being 192.9 and 185.8 respectively. In contrast, over the same period of time, the death rate from heart diseases has been reduced by more than 60% (from 586.8 to 217.0).

The great progress in diminishing the death toll of heart diseases is attributed, to a significant degree, to prevention measures, including the control of such contributors to vascular damage as hypertension and dyslipidaemias and the use of aspirin as a preventive agent. The stark paradigm of cardiology has compelled the examination of other-than-conventional-treatment approaches to the control of cancer. Prevention has emerged as a promising option for cancer for three reasons: it commands an inherent logic; the alternative, as outlined above, is not appealing; and, as of late, it appears feasible. In the present paper, I summarize one of the several current pharmacological approaches to cancer prevention, the use of novel agents based on NO (nitric oxide).

Cancer prevention as a promising approach
Cancer prevention is defined as active measures to decrease the incidence of cancer. Much of its promise comes from epidemiological studies demonstrating the association between specific cancers and modifiable factors (life style and environmental exposures, including those to infectious agents). Interventional studies have proven the validity of the concept.

Cancer prevention may be categorized as primary, secondary or tertiary [2]. Primary prevention modifies genetic, environmental and biological factors aetiological to a given tumour to diminish their effects on tumorigenesis. Elimination of chemicals, for example, constitutes primary prevention. Secondary prevention screens for pre-malignant and early neoplastic lesions, which it treats expeditiously. Endoscopic screening for colorectal cancer, an excellent example of secondary prevention, reduces the attendant mortality by 15–33% [3]. Tertiary prevention or chemoprevention utilizes specific pharmacological agents or nutrients to prevent, delay or retard the development or recurrence of cancer.

Leads for the development of cancer prevention strategies and chemopreventive agents are provided by epidemiological studies and by laboratory research. NSAIDs (non-steroidal anti-inflammatory drugs), perhaps the prototypical...
chemopreventive agents, are a case in point. A rapid transition from epidemiological, basic science and animal studies culminated in interventional studies that formally proved their chemoprevention efficacy [4,5]. Although their effect appears modest [6], nevertheless, collectively these studies constitute a landmark achievement, as they established the concept of chemoprevention.

**NO and cancer biology**

An unexpected lead to cancer prevention has come from the discovery of NO, one of the simplest biological molecules in Nature, initially identified as a signalling molecule in the cardiovascular system [7]. Produced through the oxidation of l-arginine by any of three NOSs (nitric oxide synthases), NO is a free radical, a property that renders it very reactive and unstable. Besides regulating blood flow and thrombosis, it is now appreciated that NO regulates virtually every critical cellular function [8]. NO reacts readily with another radical, superoxide anion (O$_2^-$), forming peroxynitrite, which interacts with lipids, DNA and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms. Chronic inflammatory diseases and cancer are among the many pathogenetic mechanisms mediated by peroxynitrite.

Colon cancer represents an instructive case for the involvement of NO in its pathogenesis [reviewed in [9]]. Increased expression of iNOS (inducible NOS) has been observed in colon (and several other) tumours. In the colon, it seems to be particularly pronounced in the transition from adenoma to carcinoma. Overexpressed iNOS, common in chronic inflammation, generates sustainable amounts of NO, which through critical signalling effects have profound consequences for carcinogenesis. Encouraging prevention results with selective inhibitors of iNOS in both chemical and transgenic models of colon cancer underscore the importance of NO in colon carcinogenesis.

Inflammation and DNA damage engendered by NO provide general links to cancer. Recent work is unravelling the involvement of NO in discrete steps of the complicated process that leads from normalcy to malignancy [9]. For example, NO affects tumour angiogenesis, metastasis, blood flow and immune surveillance [10]. Even eNOS (endothelial NOS) can modulate cancer-related events, such as angiogenesis, apoptosis, cell cycle, invasion and metastasis [11]. NO has also the potential to enhance both radio- and chemo-therapy, but such strategies depend on achieving appropriate levels of NO [10].

NO displays a biphasic nature in cancer biology [12]. This unusual feature arises from its concentration-dependent ability to regulate tumour growth, migration, invasion, survival, angiogenesis and metastasis. NO flux, the chemical redox environment and the duration of NO exposure determine the final outcome of such processes. These considerations are very important in the design of NO-based strategies for the control of cancer.

**NO-based approaches to cancer control**

Given the properties of NO and its potential role in carcinogenesis, it is not at all surprising that NO-based approaches have been pursued over the last several years. They include attempts to revive or modify older agents; nitroglycerine, for example, has been in use for decades before its ability to release NO was known. NO-related therapeutic agents span the range from prodrugs that elevate NO levels, to scavengers of NO, inhibitors of endogenous NO synthesis and substrates for NOS such as $N$-hydroxarginine derivatives (reviewed in [13]). Reflecting the duality of NO function in cancer, both anti-NO and NO-based anticancer strategies appear effective in several preclinical models (reviewed in [14]). As already alluded to, the major challenge of NO-centred anticancer strategies is to align NO signalling activated by a given agent with the desired pharmacological outcome.

Examples of NO-generating strategies are the NO chimaeras developed by Thatcher’s group [15], diphenyldiazole synthesized as COX-2 (cyclo-oxygenase-2) inhibitors/NO donors [16], and the NO-donating NSAIDs [17], the main subject of this paper.

**NO-donating NSAIDs and cancer prevention: the case of cancers of the colon and pancreas**

NO-NSAIDs consist of a conventional NSAID to which the NO-releasing moiety –ONO$_2$ has been attached via a chemical linker [18] (Figure 1). The spacer can vary in its chemical structure, providing a great number of derivatives. NO-ASA (NO-aspirin) is the best-studied NO-NSAID. There are three positional isomers of the NO-ASA molecule, ortho, meta and para, generated by varying the position of the –CH$_2$ONO$_2$ group with respect to the ester bond linking the two benzenes [19].

Preclinical results to date have established that NO-NSAIDs possess properties consistent with a chemopreventive effect. They can be grouped into those establishing an *in vitro* favourable cytokinetic effect and those demonstrating efficacy in animal models of cancer. Several NO-NSAIDs, including NO-ASA, NO-sulindac and NO-ibuprofen, NO-salicylic acid, NO-indomethacin and NO-flurbiprofen, have greater potency in inhibiting, much more potently than the corresponding parent compounds, the growth of cancer cell lines, e.g. colon, prostate, lung, pancreas, tonsil, breast cancer and leukaemia [20–23]. In the case of colon cancer cell lines, for example, the IC$_{50}$ values of NO-NSAIDs were enhanced between 1.7- and 1083-fold. The growth-inhibitory effect of NO-NSAIDs is due to a profound cytokinetic effect, consisting of reduced cell proliferation, enhanced cell death and inhibition of cell-cycle-phase transitions. Remarkably, this effect was independent of COX inhibition. Beyond classical apoptosis, there was another form of cell death induced by NO-ASA that was termed atypical cell death [19]. Atypical cell death, initially described *in vitro*, may actually occur *in vivo*. Ouyang et al. [24] recently described a sequence
Figure 1 | The structure of representative NO-NSAIDs

NO-NSAIDs consist of a conventional NSAID, the spacer molecule and the NO-releasing moiety (–ONO₂). The conventional NSAID is in the boxed area.

NO-Aspirin

NO-Indomethacin

NO-Ibuprofen

NO-Sulindac

of morphological changes in intestinal tumours of Min mice treated with NO-ASA that strongly suggest its existence.

The in vivo studies used animal models of cancer as well as xenotransplants of human cancer cell lines into appropriate murine hosts. For colon cancer, the results from the various models are congruent and demonstrate a clear-cut chemopreventive effect. In Min mice, 3 weeks of treatment with NO-ASA decreased the number of tumours by 55% [25]. In F344 rats treated with the carcinogen azoxymethane, NO-indomethacin and meta NO-ASA significantly suppressed both tumour incidence and multiplicity (NO-indomethacin was more effective than NO-ASA). Of the two NO-ASA isomers, the para is more efficacious than the meta in Min mice [19]. When combined with 5-fluorouracil or oxaliplatin, para NO-ASA showed additive effects [26]. Sequential NO-ASA and oxaliplatin treatment caused higher reduction in tumour growth than single-drug treatments, perhaps by sensitizing colon cancer cells to the effect of anti-tumour drugs.

Studies using a hamster model of pancreatic cancer generated some impressive results [27]. Compared with the control group, NO-ASA reduced the incidence and multiplicity of pancreatic cancer by 88.9 and 94% respectively, whereas conventional ASA had no significant effect. NO-ASA arrested the transition from PanIN2 to PanIN3 and carcinoma by largely reversing the abnormal cytokinetic profile characterizing pancreatic cancer and its precancerous stages.

A consistent finding in numerous animal studies, including those mentioned above, is the safety of NO-NSAIDs, which appears superior to that of their parent NSAIDs. Some clinical studies underscore the same, but they are limited in scope [28,29]. Ongoing clinical trials are expected to address this important issue. The vexing concern that NO-NSAIDs may actually promote cancer by releasing NO has been addressed by Muscat et al. [30], who used the results of the Framingham Heart and Offspring Study to evaluate the effects of nitrovasodilators on the risk of colorectal cancer. There was no increase in colorectal cancer over a sufficiently long period of observation. That NO, released from a pharmacological agent not too dissimilar from NO-NSAIDs, did not change the risk of colorectal cancer offers some level of comfort.

NO-ASA: the mechanistic prototype

An intriguing aspect of NO-NSAIDs is their at times extraordinarily enhanced potency. We and others have attempted to understand this through studies assessing their effects on potentially informative pathways [31]. Most of the studies concern NO-ASA, the most promising agent and the only one currently approved for cancer clinical trials by the FDA (Food and Drug Administration). NO-ASA inhibited important signalling cascades, including NF-κB (nuclear factor-κB), Wnt, MAPK (mitogen-activated protein kinase) and NOS. Results regarding the COX pathway have been mixed: it was consistently induced by NO-ASA in vitro, whereas in animals its activity and the levels of various prostaglandins were inhibited [32,33]. When a comparison was made between the IC₅₀ values for cell growth inhibition and signalling inhibition in response to NO-ASA, the most important event was the inhibition of Wnt (β-catenin) signalling that occurred at concentrations much below those required for inhibition of cell growth. Finally, NO-ASA modulates both in vitro and in vivo drug-metabolizing enzymes such as NQO [NAD(P)H:quinone oxyreductase] and GST (glutathione S-transferase) [34]. Interestingly, NO-ASA modulated in parallel the Keap1 (Kelch-like enoyl-CoA hydratase-associated protein 1)–Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) pathway.
**Figure 2 | Oxidative stress and the action of NO-ASA**

The induction of oxidative stress, probably the result of a complex mechanism, represents a very early and critical event in the action of NO-ASA. The sequence of depicted events is as follows: NO-ASA generates oxidative stress, which activates the intrinsic apoptosis pathway (activation of caspase 9) and/or affects several signalling pathways, all ROS (reactive oxygen species)-responsive, which, alone or in combination, ultimately inhibit cell proliferation and promote cell death. Arrows, positive effect; T-shaped arrows, negative effect.

**Concluding remarks**

A major need in chemoprevention is the development of effective and safe agents. Unfortunately, conventional NSAIDs do not seem to meet these two non-negotiable criteria (discussed in [31]). Based on their pharmacological properties, NO-NSAIDs represent a promising alternative to conventional NSAIDs. This optimistic assessment is based on three considerations. First, the rationale for their development is sound. Secondly, the preclinical evidence suggesting that they are both efficacious and safe has been consistently positive. Thirdly, their putative mechanism of action, involving induction of oxidative stress and downstream modulation of several signalling pathways critical to carcinogenesis, is consistent with a strong effect encompassing many types of cancer. However, as is always the case, the litmus test for the clinical value of NO-NSAIDs will be their performance in chemoprevention clinical trials.

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**References**


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