NO-donating NSAIDs and Cancer: An overview with a note on whether NO is required for their action

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Abstract

Nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) consist of a conventional NSAID to which an NO-releasing moiety is attached covalently, often via a spacer molecule. NO-NSAIDs represent an emerging class of compounds with chemopreventive properties against a variety of cancers, demonstrated in preclinical models including cell culture systems and animal tumor models; their potential efficacy in humans has not been assessed. Their mechanism of action appears complex and involves the generation of reactive oxygen species, suppression of microsatellite instability in mismatch repair-deficient cells, and modulation of several signaling cascades that culminate in inhibited cell renewal and enhanced apoptosis. NO, long appreciated to be able to protect from and also promote cancer, is released form NO-NSAIDs and constitutes their defining property. Existing data are consistent with the notion that NO may mediate their anticancer effect. In addition there is evidence that long term administration of NO-donating compounds is not associated with increased incidence of colon cancer. Whether NO release is required for the anticancer effect of NO-NSAIDs has being questioned by recent data indicating that, at least in the case of NO-aspirin, the NO-releasing moiety may serve as a leaving group while the spacer actually being the moiety responsible for its pharmacological action. Regardless of mechanistic issues, these compounds promise to contribute to the control of cancer.

Keywords

Introduction

Cancer represents perhaps the defining medical challenge of our times. The search for pharmacological agents that can control cancer, either as chemotherapeutic or as chemopreventive agents is intense and to date has yielded significant results. Nevertheless, as the continuing morbidity and mortality form cancer indicate, there is a pressing need to identify new agents. Rational design of pharmacological agents includes, among others, modification of known agents in order to optimize their pharmacological properties, mainly their safety and efficacy. Nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) represent a case in point (reviewed in [1]). This emerging class of compounds was designed based on the known properties of NSAIDs and those of the then-newly discovered NO, a molecule that

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plays an important role not only in the cardiovascular system but also in much of human physiology. The expectation was that these molecules would harness the properties of both achieving an enhanced, if not synergistic, effect.

The concept of modifying known pharmacological compounds to make them capable of releasing NO is broader than NSAIDs and encompasses many others, such as steroids, statins, prostaglandin $F_{2\alpha}$ analogs, and antihypertensive agents. Besides cancer, NO-donating compounds hold promise for the control of other diseases, including cardiovascular diseases [2], asthma [3], hypoxic–ischemic brain injury [4], glaucoma [5], and Alzheimer's disease [6]. To date, an extensive body of work including studies in preclinical models and relevant clinical trials underscores their therapeutic potential.

The ability of NO-NSAIDs to release NO is their defining (and name-giving) property. Here, we summarize the current status of NO-NSAIDs as anticancer agents and examine the intriguing question of whether NO is indeed required for their action in cancer.

NO-NSAIDs: Rationale and Structure

The rationale for the initial synthesis of NO-NSAIDs was simple, brilliant and probably inaccurate. NSAIDs damage the gastroduodenal mucosa by inhibiting the synthesis of cytoprotective prostaglandins [7]. Since the action of NO on this mucosa is similar to that of prostaglandins, it was reasoned that if an NSAID could provide locally NO, its mucosal damaging effect would be averted. Numerous animal and some human studies of NO-NSAIDs have clearly demonstrated that NO-NSAIDs indeed protected the gastric mucosa from the damage that the parent NSAIDs were expected to inflict [8,9]. As discussed later, however, there is serious doubt as to whether this notion is correct.

NO-NSAIDs consist of a conventional NSAID to which an NO-releasing moiety is attached covalently, often via a spacer molecule. The first NO-NSAIDs were synthesized by del Soldato and his colleagues (reviewed in [7]); NO-ASA and NO-indomethacin are depicted in Fig. 1. Various molecules, both aliphatic and aromatic, have been used as spacer molecules in NO-NSAIDs

Additional versions of NO-donating molecules, including NO-NSAIDs have been synthesized by various teams attempting to capitalize either on the pharmacological properties of NO per se or on the enhanced efficacy of NO-releasing compounds.

Thatcher's team has also synthesized chimeras able to release NO [10–12]. GT 1061, an NO mimetic compound that contains an ancillary, synergistic pharmacophore, is being investigated as neuroprotective in patients with Alzheimer's disease [6]. In addition, this group has synthesized the NO chimera GT-094. This compound, a novel nitrate containing an NSAID and disulfide pharmacophores, exhibits antiproliferative activity and exerts a G_2/M cell cycle block in cultured colon cancer cells [13]. Finally, the so-called NONO-NSAIDs have been synthesized and studied for their anti-inflammatory and anticancer properties [14–16]. It should not be forgotten that the organic nitrates are also NO-donating compounds. Nitroglycerin, the prototypical pharmacological agent of this category, has been used successfully for cardiac indications for over a century, long before its mechanism of action was unraveled following the discovery of NO as a vasodilating agent [12].

The straightforward mechanism outlined above that involves the release from NO-NSAIDs of cytoprotective NO within the stomach may not be accurate. The strongest evidence against this mechanism comes form the pharmacokinetic studies of NO-aspirin (NO-ASA). Carini *et al* have shown that NO-ASA traverses the stomach intact, thus ruling out this mechanism, at least in the case of NO-ASA [17,18]. The alternative explanation that NO, released beyond the upper

GI tract, can reach the gastric mucosa via the circulation remains unproven; elevated NO levels in the circulation following the administration of NO-NSAIDs have been repeatedly demonstrated (e.g., [18]).

Evidence for the Anticancer Effect of NO-NSAIDs

During the last several years we and other groups have studied NO-NSAIDs as potential agents for the control of cancer. There is ample evidence of their efficacy in preclinical models of cancer. Williams *et al* provided the first evidence that NO-NSAIDs possess chemopreventive properties by showing that NO-ASA, NO-sulindac, and NO-ibuprofen reduce the growth of cultured HT-29 human colon adenocarcinoma cells much more potently than their corresponding NSAIDs [19]. This observation was later extended to additional NO-NSAIDs [20] and additional cell lines [21–23].

Several animal studies, all congruent, have generated results consistent with the conclusions derived from the cell culture studies. For colon cancer, three animal tumor models documented the chemopreventive effect of NO-NSAIDs. Tumor incidence and multiplicity were reduced in *Min* mice and in an azoxymethane model of colon cancer, whereas the size of tumor xenografts was significantly reduced in response to NO-ASA [24–27]. In pancreatic cancer *meta* NO-ASA displayed an astonishing effect, reducing the incidence of pancreatic cancer by 89.5%, while conventional ASA, studied in parallel, was totally ineffective [28].

The mechanism of action of NO-NSAIDs against cancer (mainly of the colon and pancreas) has been pursued from several angles: their cell kinetic effect, effects on cell signaling pathways, and effects on detoxifying enzymes. To a first approximation, NO-NSAIDs have a strong cell growth inhibitory effect, which is the end result of three individual effects: inhibition of cell proliferation, induction of apoptosis and deceleration of cell cycle phase transitions [19,29–31]. The most detailed study on the induction of apoptosis was conducted by Gao *et al* [32], whose work on NO-ASA highlighted three important aspects of this effect: a) the first recognizable effect was the induction of oxidative stress; b) this was followed by activation of the intrinsic apoptosis pathway; and c) inhibition of Wnt signaling was a major component of the proapoptotic effect of NO-ASA. Animal studies confirmed the importance of the induction of apoptosis for the chemopreventive effect of NO-ASA [24,28].

NO-NSAIDs, in particular NO-ASA, the compound that has attracted the greatest number of mechanistic studies, modulate a large array of molecular targets. Their action includes effects on mitogen-activated protein kinase (MAPK) signaling [33]; inhibition of inducible nitric oxide synthase (NOS2) [34]; and inhibition of NF-κB activation [35]. The effect of NO-NSAIDs on COX-2 is still unclear, since some in vitro studies indicate its induction by NO-NSAIDs and some suggest that the opposite effect is likely [25,36]. Finally, NO-ASA modulates drug metabolizing enzymes, including induction of the activity and expression of NAD(P) H:quinone oxireductase (NQO) and glutathione S-transferase (GST) and translocation of Nrf2 into the nucleus, likely by binding to Keap1, the protein that anchors Nrf2 in the cytoplasm [37]. Finally, it was recently shown that NO-ASA isomers were more effective at suppressing microsatellite instability in mismatch repair-deficient cell lines than conventional ASA, raising the possibility that NO-ASA could be an effective chemopreventive agent for hereditary nonpolyposis colorectal cancer (HNPCC) carriers [38].

What is both remarkable and perplexing is that in theory each of these effects could prevent cancer. It remains, however, unclear whether only one, more than one or all of these effects are required for the drug's overall pharmacological effect. Resolving this dilemma, formulated as mechanistic *dominance* (one effect) versus *redundancy* (multiple effects) [39], will require further work.

Regardless of preclinical effects and mechanistic studies, only clinical trials will definitively assess the role of NO-NSAIDs in cancer control. Unfortunately, a clinical trial of NO-ASA for the prevention of colon cancer was recently terminated prematurely due to concerns about its potential genotoxicity [40]. The expectation is that resumption of this trial should follow the successful resolution of this issue.

NO Release from NO-NSAIDs and their Anticancer Effect

NO is long appreciated to have a dichotomous effect on cancer, being able to protect from and also promote cancer [41]. The outcome depends on a host of factors, including mainly the concentration of NO and the location where it is released. Consequently, the presence of the NO-releasing moiety on the various permutations of NO-donating compounds intended to be used as anticancer agents engenders three questions:

a. Does the NO that NO-NSAIDs ultimately release have an anticancer effect or simply a cytoprotective effect in the stomach as initially envisioned?

b. Could the NO derived form NO-NSAIDs instead of protecting form cancer *promote* carcinogenesis, especially after their long-term administration? And, finally,

c. Is the NO actually *required* for the anticancer effect of NO-NSAIDs?

NO as the mediator of the action of NO-NSAIDs

There is clear evidence that NO-NSAIDs do release their NO. First, NO levels (measured as NOx) are increased in the circulation following NO-NSAID administration [17,42]. Second, no intact NO-NSAID has been identified in the circulation or target tissues [17,18] and our own unpublished data], suggesting that the breakdown of the NO-NSAID molecule occurs either prior to or rapidly after it reaches the circulation. Third, S-nitrosylation, an unambiguous marker of NO action, following exposure to NO-NSAIDs has been repeatedly identified [43]. Finally, Govoni *et al* using electron paramagnetic resonance demonstrated that NO-flurbiprofen generated NO in erythrocytes, with hemoglobin mediating this biotransformation [44]. Their work and that of others suggests that the NO-releasing moiety ($-ONO_2$) undergoes $1\ e^-$ reduction to NO_2^-, which is then either converted to NO or oxidized to NO_3^-. The issue then appears to be whether the NO released from the NO-NSAIDs is the moiety that brings about their cytoprotective effect and/or their anticancer effect.

It is fair to assume that those who conceived the idea of NO-NSAIDs expected them to release NO in the upper GI tract, protecting it from the mucosal damage brought about by conventional NSAIDs [45][46]. As mentioned earlier, NO-ASA traverses the stomach intact [17,18], thus ruling out such a mechanism. Therefore, the only remaining possibility, that could link gastric cytoprotection to the NO that is released from NO-NSAIDs, is that NO reaches the mucosa via the circulation. This is entirely possible as elevated circulating NO levels have been documented following NO-NSAID administration [18,42]. Whether, however, such a mechanism is operative alone or as part of a complex series of events remains uncertain; NO is indeed an important mediator of mucosal defense in the stomach [47].

The issue whether NO actually mediates the anticancer effect of NO-NSAIDs is still unresolved. When it was shown that NO-NSAIDs were more potent than their parent NSAIDs, the intuitive interpretation of the data was that NO release accounted for this effect. Similar to gastroprotection, such an effect would also require, to a first approximation, one of two general mechanisms. The first mechanism requires that the NO-NSAIDs have somehow a tropism to the target tissue where they travel intact and deliver their NO in situ, perhaps on account of a property of the neoplastic tissue. The second mechanism will require that NO released from NO-NSAIDs reaches the neoplastic cells and kills them. There is very little support for the first

mechanism, as already discussed above. In fact, the most important obstacle in the field of NO donor drugs is represented by the difficulty in targeting NO release, and thereby its effects, to a particular tissue [48].

The second mechanism appears, however, plausible. Various NO donor molecules that are structurally dissimilar to NO-NSAIDs have anticancer properties. For example, S-nitrosoglutathione inhibits the growth of various cancer cells [49] [50]. In addition, organic nitrates [51] diazeniumdiolates [52], syndominides [53], S-nitrosothiols [54] and even metal-NO complexes [55], all display anticancer properties in preclinical models of cancer, mostly cell lines. Since they differ structurally so broadly and their "common denominator" is that they are NO donors, it is fair to conclude that their anticancer properties are due or related to the NO that they release. One can then extrapolate this conclusion to NO-NSAIDs and, in the context of the mechanistic assumptions mentioned above, attribute at least part of their effect to NO. It should be emphasized that stating that NO contributes to the anticancer effect of NO-NSAIDs is not equivalent to stating that the release of NO is *required* for this effect. Since doubts about such a requirement have been voiced in recent literature, we will examine this question in a later section of this review.

NO-NSAIDs as potential promoters of carcinogenesis

Carcinogenesis is in general a lengthy process, in some cases estimated to require several years until a full blown cancer develops. If the NO released form NO-NSAIDs is to behave as a carcinogen or promoter of carcinogenesis, this would require either a prolonged exposure to NO or some crucial effect on genes whose mutation may be contribute to carcinogenesis. A rather compelling case has been described by Ambs *et al* [56] who studied the mutations of p53 in the vicinity of NOS and demonstrated that the NO produced through the catalytic activity of this enzyme was responsible for critical mutagenesis.

It was in the face of such considerations that we were prompted to examine the possibility that the NO released by NO-NSAIDs may promote carcinogenesis, even in a manner akin to second tumors developing following anticancer treatment with some chemotherapeutic agents. The immediate problem was the absence of human trials with NO-NSAIDs that had a sufficient lag time between treatment and follow up for cancer development. Our approach was to examine cohorts of patients treated with nitrovasodilators and for whom there were long-term follow up observations. Therefore, we evaluated data from the Framingham study, an on-going population-based cohort study initiated in 1948 which now includes the original participants, and a second cohort ("The Framingham Offspring Study") of the children of the original participants and their spouses. We analyzed the latter cohort. This database included exam information that included questions on medicinal nitrate use, which started in 1983/1984 through November 1999. There were 195 newly diagnosed incident cases of colon cancer during this period. Three controls were selected for each case, matched by study cohort, age (within five years) and sex. Analysis of the effects of nitrovasodilators on the risk of colorectal cancer showed that the odds ratio for colorectal cancer associated with nitrovasodilator use was 1.2 (95% confidence interval 0.6, 2.2), indicating that NO does not change the risk of colorectal cancer. Aspirin use, used as an internal control, had the expected preventive effect. Perhaps remarkably, nitrovasodilators showed no protection from cancer. These results offer a level of comfort, even as indirect as it is, that medicinal NO may not be carcinogenic in the colon.

The NO requirement for the anticancer effect of NO-NSAIDs

Recently, our group [57]and Hulsman *et al* [58] raised the possibility that, at least in the case of NO-ASA, the NO releasing moiety is not required for its anti-cancer effect. Both sets of data demonstrate that carboxylic ester hydrolysis of NO-ASA leads to the formation of quinone

methide which reacts at least with cellular GSH. It is assumed that the formation of quinone methide is the key biological event in this mechanism and that the $-ONO_2$ moiety serves simply as a good leaving group, simply facilitating the release of quinone methide (Fig. 2)

The strongest support for such a mechanism comes from the demonstration that analogues of NO-ASA deprived of the aspirin or the $-ONO_2$ moiety are as potent as the classic NO-ASA against the growth of cancer cells. Very recently, we actually showed that "phosphoaspirin" (a *meta* isomer of NO-ASA in which $-ONO_2$ was replaced by diethylphosphate) was very effective in inhibiting the growth of colon cancer xenografts in nude mice [59]. Thus, at least in the case of NO-ASA, NO is not required for its anticancer effect.

Concluding Remarks

NO-NSAIDs represent a highly promising development in the area of cancer prevention and treatment. These compounds are part of a larger family of agents sharing their ability to release NO, a molecule which appears to exert a very important, although not always predictable (or even desirable) effect on cancer. The key questions concerning the pharmacology of NO-NSAIDs are three:

1. Are these compounds effective against cancer in humans?

2. What is the mechanism underlying their higher potency and limited gastrointestinal toxicity, two practically important pharmacological properties? And

3. Is the NO-releasing moiety required for their anticancer effect?

All evidence to date, essentially all of it preclinical, suggests that they could prove effective against colon and a host of other cancers. There has been enough background preclinical work to justify evaluating them in suitable groups of patients and it is only through such trials that the eagerly awaited answers will be obtained.

NO-NSAIDs appear safer than their parent compounds, especially with respect to the all-important gastrointestinal toxicity. The question of the potential genotoxicity of NO-ASA is at present just that, a question that needs to be addressed. Regardless of the outcome of such re-evaluation, it should be noted that such genotoxicity may be of concern, if at all, only for chemoprevention applications and not for chemotherapy applications. In the latter case, concerns about genotoxicity are viewed differently because of the underlying disease (full-blown cancer), the duration of treatment (very brief compared to chemoprevention) and their reference point (many currently used chemotherapeutic agents are genotoxic).

Although their precise mechanism of action may not be resolved in sufficient detail for quite some time, it is clear that the ability of NO-NSAIDs to generate ROS and to suppress microsatellite instability may be key proximal events. Delineating the mechanism of action of NO-NSAIDs may guide efforts to develop effective drug combinations or guidelines for chemoprevention.

The most recent development concerns the requirement of NO-NSAIDs to have the NO-releasing moiety. Although at first glance this question is totally iconoclastic, it needs not be perceived as such. The evidence is strong that NO-NSAIDs do release NO and that NO does have an effect against cancer. On the other hand, at least in the case of NO-ASA and perhaps of other such derivatives, the NO-releasing moiety can be replaced by moieties that do not release NO without any drastic change in anti-cancer efficacy in preclinical models. At the very least, this development appears to lead to new classes of compounds and their synthesis may be a useful outcome of the mechanistic studies.

In conclusion, NO-NSAIDs are poised to become a new class of anti-cancer agents and to generate perhaps equally promising and certainly equally puzzling "offspring", the "non-NO NO-NSAIDs." Their future seems full of promise, excitement – and more work!

Acknowledgements

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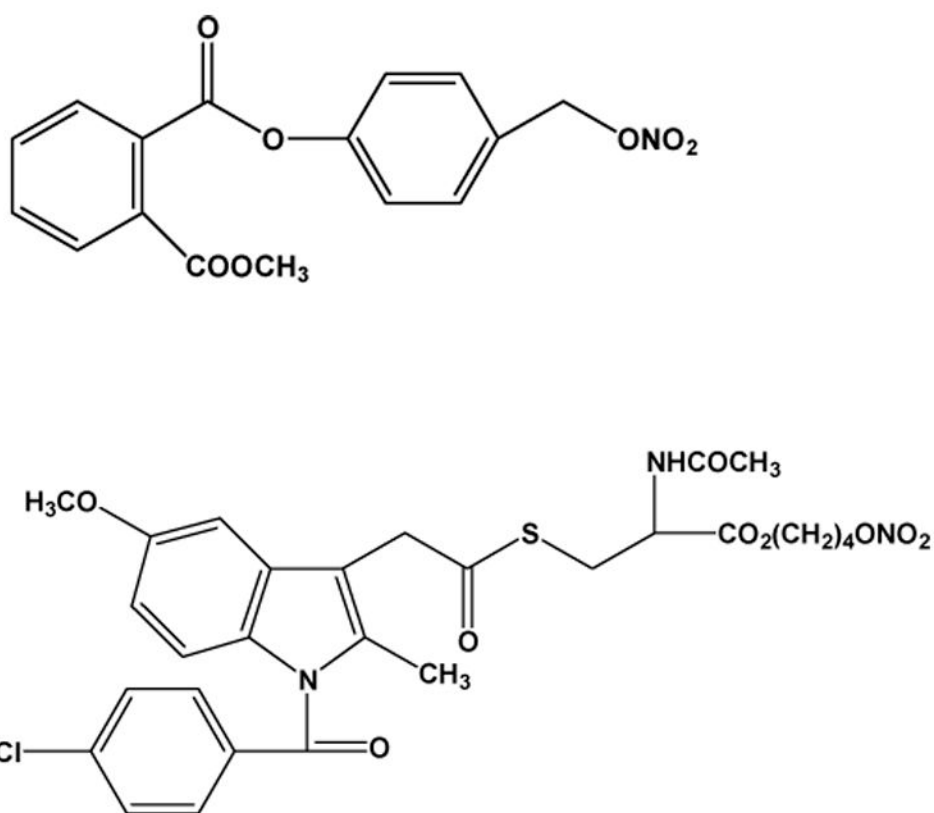
Fig.1. The structures of NO-ASA and NO-indomethacin
The spacer molecules differ between NO-ASA (*top*) and NO-indomethacin (*below*) but the
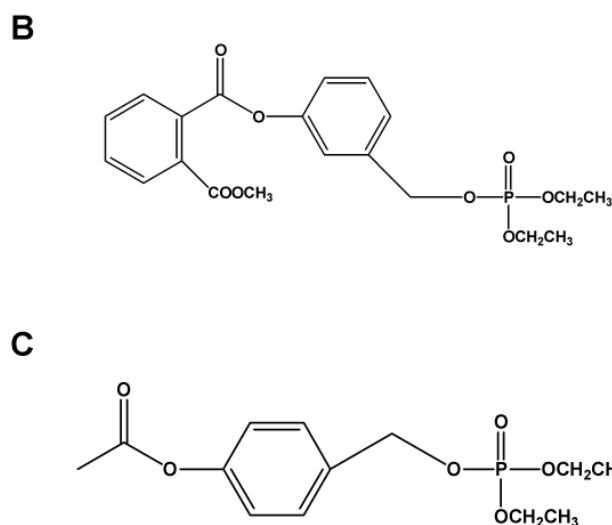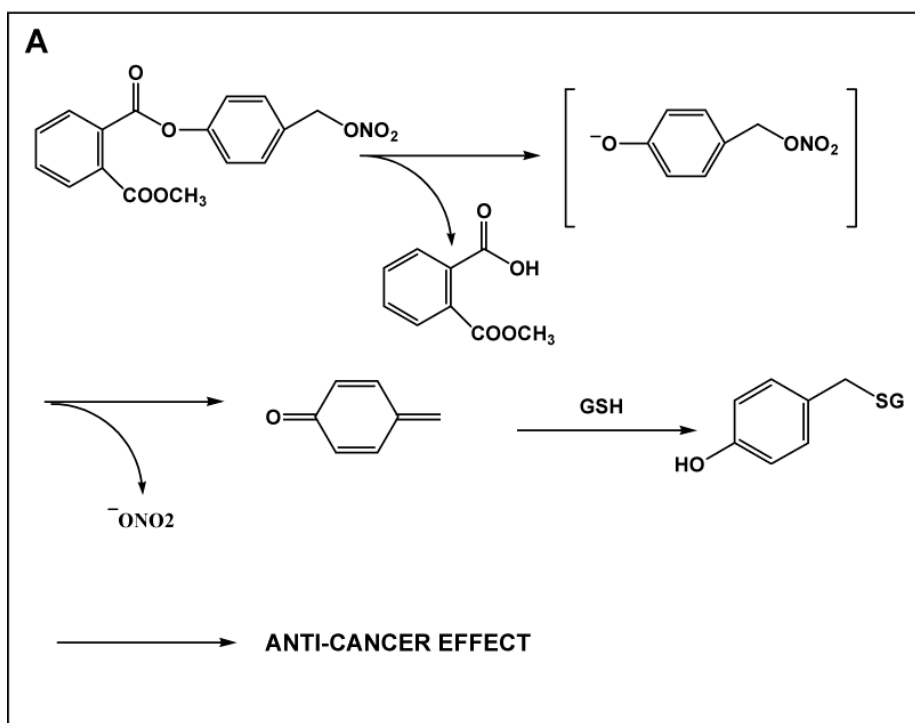NO-releasing moiety is the same ($-ONO_2$).

Fig. 2. Postulated mechanism of action of NO-ASA and the structure of non-NO releasing analogues
A: NO-ASA is hydrolyzed to release quinone methide which can react with glutathione, ultimately leading to cell death. A carbocation-based mechanism has been proposed for its *meta* positional isomer [57]. **B**: This compound has neither aspirin nor the NO-releasing moiety and, similar to **C** (a derivative of aspirin, but without the NO-releasing moiety), it is effective against colon cancer in preclinical models.