

The mechanism of action of nitric oxide-donating aspirin

Khosrow Kashfi ^{a,*}, Basil Rigas ^{b,*}

^a Department of Physiology and Pharmacology, City University of New York Medical School, 138th Street & Convent Avenue, New York, NY 10031, USA

^b Division of Cancer Prevention, Department of Medicine, Life Sciences Building 06, SUNY at Stony Brook, Stony Brook, NY 11794-5200, USA

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Abstract

NO-donating aspirin (NO-ASA) is a promising anticancer drug. We studied the contribution of NO-ASA's components (*ASA*, *NO-releasing moiety*, and *spacer* linking them) to its effect. The *ASA* and *NO-releasing moieties* play no biological role: *ASA* inhibits the growth of colon cancer cells >100-fold less potently than NO-ASA; and denitrated NO-ASA plus the *NO-donor SNAP* releasing the same amount of *NO* as NO-ASA, inhibit the growth of cancer cells >50-fold less potently than NO-ASA. The biologically active moiety of NO-ASA is the *spacer*: it is chemically reactive (studies with NO-ASA radiolabeled at the *spacer* demonstrated that it binds to proteins); and compounds in which the *ASA* or the *NO-releasing groups* are replaced inhibit cell growth similar to NO-ASA. We propose a mechanism of action of NO-ASA involving formation of quinone methide from its *para* and *ortho* isomers and of a carbocation from the *meta*, with the *NO-releasing group* functioning as a leaving group.

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Nitric oxide-donating compounds represent a novel emerging class of pharmaceutical agents that capitalize on the biological properties of *NO*, one of the smallest yet most powerful biological molecules [1]. NO-donating aspirin (NO-ASA) and other NO-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) were amongst the first to be synthesized. They consist of a conventional NSAID to which the *NO* releasing moiety $-\text{ONO}_2$ is attached covalently through a *spacer* (Fig. 1). Their design was based on the expectation that by releasing *NO* they would render the stomach harmless from the inhibition of cyclooxygenase brought about by the NSAID, an effect generally thought to account for their gastroduodenal toxicity. It is unlikely that this is an operational mechanism, especially in view of the finding that NO-ASA passes intact through the upper gastrointestinal tract of rats that is spared the well-known damaging effects of *ASA* [2].

For the past 7 years we have been exploring the mechanism of action of NO-NSAIDs with emphasis on their application to cancer [3,4]. It is clear that amongst the various NO-NSAIDs, NO-ASA is the most effective as judged by its IC_{50} for cell growth inhibition when used in various cancer cell culture systems [5] and its ability to inhibit the neoplastic process in animal models [6,7]. Although the effect of NO-ASA on a wide variety of signaling and other cellular mechanisms has been deciphered by us and others, several key questions remain unanswered. One of them concerns the contribution to the overall biological effect of each of the three structural components of NO-ASA. Thus, we conducted a series of studies designed to assess the roles of the *ASA*, the *spacer*, and the *NO-releasing moiety*.

In this paper, we report our efforts to understand the mechanism of action of NO-ASA and our unexpected conclusion that the *NO-releasing moiety*, although it appears defining (and name-giving), is not required for the observed biological effects. Rather, it is the *spacer* that is responsible for the biological actions of NO-ASA, with the *NO-releasing moiety* restricted to the role of a leaving group that

* Corresponding authors. Fax: +1 212 650 7692 (K. Kashfi), +1 631 632 1992 (B. Rigas).

E-mail addresses: Kashfi@med.cuny.edu (K. Kashfi), basil.rigas@stonybrook.edu (B. Rigas).

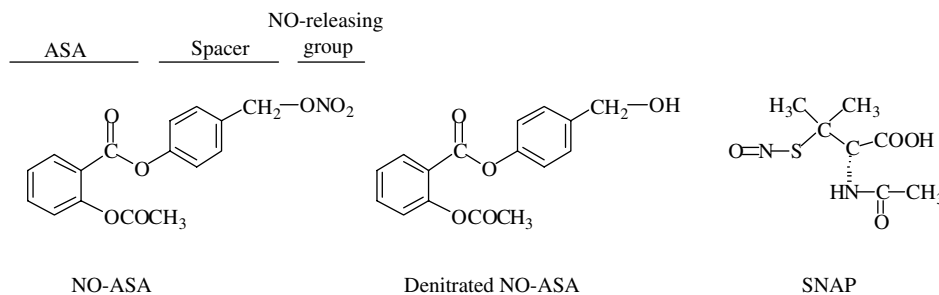


Fig. 1. NO-ASA, its denitrated derivative and SNAP. The three key structural components of NO-ASA, the conventional ASA moiety, the spacer, and the NO-releasing group are shown. The denitrated derivative of NO-ASA, missing the $-\text{ONO}_2$ moiety, is incapable of releasing NO. The structure of SNAP, a NO donor used in our studies, is also shown.

facilitates the release and activation of the spacer, while the ASA component apparently makes very limited or no biological contribution.

Materials and methods

Reagents. Stock (100 mM) solutions of NO-ASA (2-(acetyloxy)benzoic acid 4-(nitrooxy methyl)phenyl ester), its denitrated form and ASA were prepared in DMSO (Fisher Scientific, Fair Lawn, NJ); the final DMSO concentration was adjusted in all media to 1%. S-nitroso-N-acetylpenicillamine (SNAP) and other reagents were from Sigma, St. Louis, MO, USA. The benzene ring of the spacer was [^3H]-labeled by an exchange reaction following standard protocols. NO-ASA and its derivatives were synthesized by us as described [8].

Cell culture. HT-29 human colon adenocarcinoma cells were from American Type Tissue Collection, Rockville, MD. Cells were grown in 5% CO_2 at 37 °C in McCoy 5A medium supplemented with 10% fetal calf serum, 10,000 IU/ml penicillin, and 10 mg/ml streptomycin. Cells were counted using a hemacytometer.

NO assay. NO levels were determined in cell culture media using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI) and following the instructions of the manufacturer. This assay is a modification of the classical Griess method, which relies on a diazotization reaction.

The reaction of [^3H]-NO-ASA with albumin. Bovine serum albumin (BSA, Sigma) was dissolved in phosphate buffered saline (PBS, pH 7.4) at 0.5%. It was reacted with [^3H]-NO-ASA 200 μM plus either solvent (DMSO) or unlabeled NO-ASA 500 μM . In a third reaction, BSA was incubated with NEN 10 μM for 1 h prior to addition of [^3H]-NO-ASA 200 μM . All reaction volumes were 40 μL . Reaction mixtures were incubated at 37 °C for 24 h. At that time we added to each reaction 160 μL ethanol, the mixture was centrifuged at 15,000 rpm for 15 min and the pellet was washed twice with 200 μL methanol and resuspended in 400 μL PBS. Aliquots of the resuspended protein pellet and the supernatant were counted by scintillation counting. Protein concentration was determined using the biuret protein assay.

Statistical Analysis. Data are presented as mean \pm SEM for at least three to five different sets of treatment groups or experiments. $P < 0.05$ was considered significant using Student's *t* test.

Results

The effect of NO-donating group of NO-ASA on cell growth

Initially, we determined the contribution of the ASA moiety and the NO-releasing moiety ($-\text{ONO}_2$) to the biological effect of NO-ASA. HT-29 colon cancer cells were treated with ASA, NO-ASA, its denitrated derivative or

the exogenous NO donor SNAP for up to 72 h (Fig. 1). We also determined the amount of NO released in the culture medium at 24, 48, and 72 h. The concentration of SNAP was adjusted to generate essentially identical amounts of NO released by NO-ASA at those three time points. IC_{50} s for cell growth inhibition were determined at 24, 48, and 72 h.

As shown in Table 1, conventional ASA failed to significantly inhibit cell growth at concentrations exceeding 1 mM, in agreement with previous reports [9]. NO-ASA and its denitrated derivative showed the expected results [10], with the former being inhibitory and the latter much less so. SNAP inhibited HT-29 cell growth, but its IC_{50} s were markedly different from those of NO-ASA (870 \pm 47, 545 \pm 32, and 327 \pm 27 μM at 24, 48, and 72 h, respectively compared to 7 \pm 2, 4 \pm 2 and 3 \pm 1 μM for NO-ASA at comparable time points). The interesting observation here is that the NO concentrations in the culture media of cells treated with either NO-ASA or SNAP were virtually identical. For example, treatment of the cells with 50 μM NO-ASA gave 65 \pm 5, 75 \pm 3, and 67 \pm 4 μM NO at 24, 48, and 72 h and with 90 μM SNAP, NO levels were 75 \pm 6, 80 \pm 2, and 75 \pm 4 μM at the same time points. Similarly, treatment of the cells with 500 μM NO-ASA gave 250 \pm 7, 375 \pm 8, and 490 \pm 15 μM NO at 24, 48, and 72 h and with 900 μM SNAP, NO levels were 275 \pm 8, 400 \pm 4, and 450 \pm 9 μM at the same time points (Table 2).

Table 1
 IC_{50} values for cell growth inhibition in HT-29 cells

Treatment	IC_{50} (μM)		
	24 h	48 h	72 h
ASA	>1000	>1000	>1000
NO-ASA	7 \pm 2*	4 \pm 2*	3 \pm 1*
Denitrated NO-ASA	>800	350 \pm 27	283 \pm 32
SNAP	870 \pm 47	545 \pm 32	327 \pm 27
Denitrated NO-ASA + SNAP	637 \pm 32	265 \pm 15	155 \pm 25

Cells were treated with various concentrations of the test agents shown above as described in Materials and methods. Cell numbers were determined at 24, 48, and 72 h, from which IC_{50} values were calculated. Results are mean \pm SEM of three to five different experiments done in duplicate.

* $P < 0.001$ compared to all other treatment groups.

Table 2
Nitric oxide released

Treatment (μM)		[NO] released (μM)		
		24 h	48 h	72 h
ASA	1000	10 \pm 3	15 \pm 2	15 \pm 3
NO-ASA	50	65 \pm 5	75 \pm 3	67 \pm 4
	500	250 \pm 7	375 \pm 8	490 \pm 15
Denitrated NO-ASA	50	15 \pm 3	15 \pm 2	13 \pm 3
	500	15 \pm 2	13 \pm 2	15 \pm 3
SNAP	90	75 \pm 6	80 \pm 2	75 \pm 4
	900	275 \pm 8	400 \pm 4	450 \pm 9
Denitrated NO-ASA + SNAP	50 + 90	57 \pm 6	75 \pm 4	67 \pm 8
	500 + 900	280 \pm 7	420 \pm 7	460 \pm 5

Cells were treated with various concentrations of the test agents shown above and at different time points NO concentrations were determined as described in Materials and methods. Background concentrations of NO, i.e. without treatment with any test agent, varied between 10 \pm 3 and 15 \pm 3 μM . Results are mean \pm SEM of three to five different experiments done in duplicate.

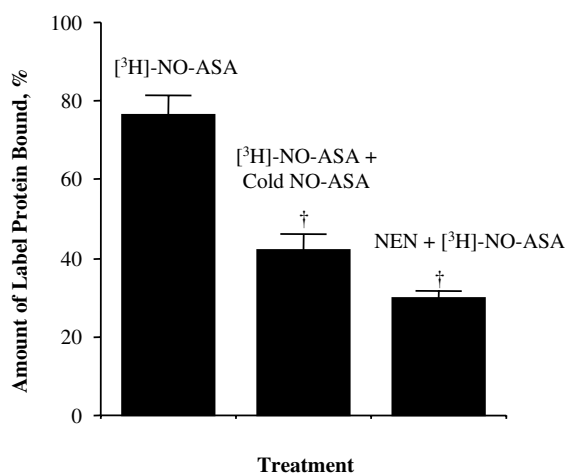
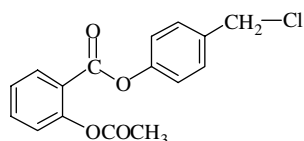
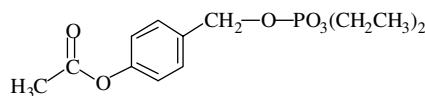


Fig. 2. The chemical reactivity of the spacer of NO-ASA. NO-ASA labeled with ³H in the benzyl ring of its spacer was reacted *in vitro* with albumin as described in Material and methods. The labeled spacer binds to albumin (*first bar*), the unlabeled spacer of simultaneously added cold (unlabeled) NO-ASA competed with the labeled spacer of [³H]-NO-ASA for binding to albumin (*middle bar*), whereas pretreatment with maleimide that blocks thiol groups, reduced the binding of the spacer to albumin even more (*last bar*). Results are mean \pm SEM of three different experiments done in duplicate. [†]*P* < 0.01 compared to [³H]-NO-ASA].



(A) "No NO NO-ASA"



(B) "No NO No ASA NO-ASA"

Fig. 3. Two novel compounds based on the spacer. In the "No NO NO-ASA" compound the NO-releasing moiety has been replaced by -Cl, whereas in the "No NO, No ASA NO-ASA" both the ASA and the NO-releasing moiety have been replaced by the acetyl and diethyl phosphate moieties, respectively.

When we did a reconstitution experiment in which cells were treated with both the denitrated NO-ASA and SNAP at concentrations that provided virtually identical concentrations of "NO-ASA" and NO in the media, the IC₅₀s of the intact NO-ASA molecule were not obtained. Although there was a synergistic effect in terms of cell growth inhibition, the combination of these two molecules failed to reconstitute the potency of NO-ASA. In fact, the respective IC₅₀s were far higher (52–91 fold) than those of NO-ASA (Table 1).

These findings indicate that neither the ASA nor the NO-releasing group can account for the biological activity of NO-ASA, suggesting the spacer as the likely candidate.

The chemical reactivity of the spacer

To determine whether the spacer of NO-ASA is chemically reactive, we radiolabeled NO-ASA by introducing ³H into its benzyl unit. A solution of bovine serum albumin (BSA) was reacted with [³H]-NO-ASA for 24 h and the fraction of the label incorporated into BSA was determined. The fraction of the total label bound to the protein was 76.6%. When BSA was incubated with both [³H]-NO-ASA and cold NO-ASA (the later being in 2.5-fold molar excess), only 42.1% of the label was bound to the protein. Finally, when BSA was preincubated for 1 h, with *N*-ethyl maleimide (NEM), the fraction of label incorporated into the protein dropped to 29.9% of the total. These results are shown in Fig. 2. Similar results were obtained when cancer cells were treated with [³H]-NO-ASA (to be published elsewhere).

These findings establish that the spacer part of NO-ASA is chemically reactive and, in addition to forming a conjugate with glutathione [11,12], it reacts with proteins as well, in particular with their -SH groups.

The effects of "No NO NO-ASA" and "No NO, No ASA NO-ASA" on cell growth

Since the preceding data suggest that the NO-donating moiety of NO-ASA is not crucial for its biological activity and that the spacer is chemically reactive, we synthesized the two compounds shown in Fig. 3. The first represents the "No NO NO-ASA" where the -ONO₂ leaving group

has been replaced by $-Cl$ and the latter the “*No NO, No ASA NO-ASA*” where the ASA moiety has been replaced with an acetate and the $-ONO_2$ leaving group has been replaced by a substituted phosphate group. We then determined their ability to inhibit the growth of cultured HT-29 human colon cancer cells. Both were effective with IC_{50} s of $18.2 \pm 3.1 \mu M$ and $4.8 \pm 1.2 \mu M$ at 24 h, which were comparable to those of NO-ASA done in parallel experiments ($10.5 \pm 2.4 \mu M$).

Discussion

Our findings document that this class of NO-ASA (molecules containing an aromatic spacer) exerts its biological effects through the action of its spacer. This is documented by the demonstration that (a) when reconstituting the structural components of NO-ASA, the effect of the parts does not equal the effect of the whole molecule; (b) the spacer part of the molecule is chemically active; and (c) molecules in which either the ASA or the $-ONO_2$ group have been replaced (the latter by a good leaving group) display biological activity similar to that of NO-ASA.

It has long been recognized by us and others that ASA per se is far less potent in inhibiting the growth of cancer

cells (reviewed in [3]). This was confirmed by the present set of experiments. Furthermore, there is no apparent reason to suspect that ASA undergoes a modification that would enhance its biological activity. Detailed studies of its metabolism have all along supported this notion [11,12]. The main transformation of the ASA moiety of NO-ASA is to salicylic acid, in agreement with the known metabolism of conventional ASA [13].

The first strong evidence that the NO-releasing moiety of NO-ASA and by extension NO, the molecule it releases, are not important for its biological activity is provided by the experiments assessing NO levels in cultured cells. The critical finding is the inability of a “reconstituted” NO-ASA to achieve an effect on cell growth comparable to that of intact NO-ASA; in fact, the potency of NO-ASA is 52- to 91-fold higher than that of the “reconstituted” NO-ASA. The “reconstitution” of NO-ASA using its denitrated analog plus SNAP that releases equimolar amounts of NO into the medium represents a fairly close approximation to NO-ASA. Thus these findings strongly suggest that it is the spacer that accounts for most, if not all, of the biological effect of NO-ASA. An alternative, although unlikely interpretation of these data is that the NO levels in the culture medium do not reflect biologically relevant

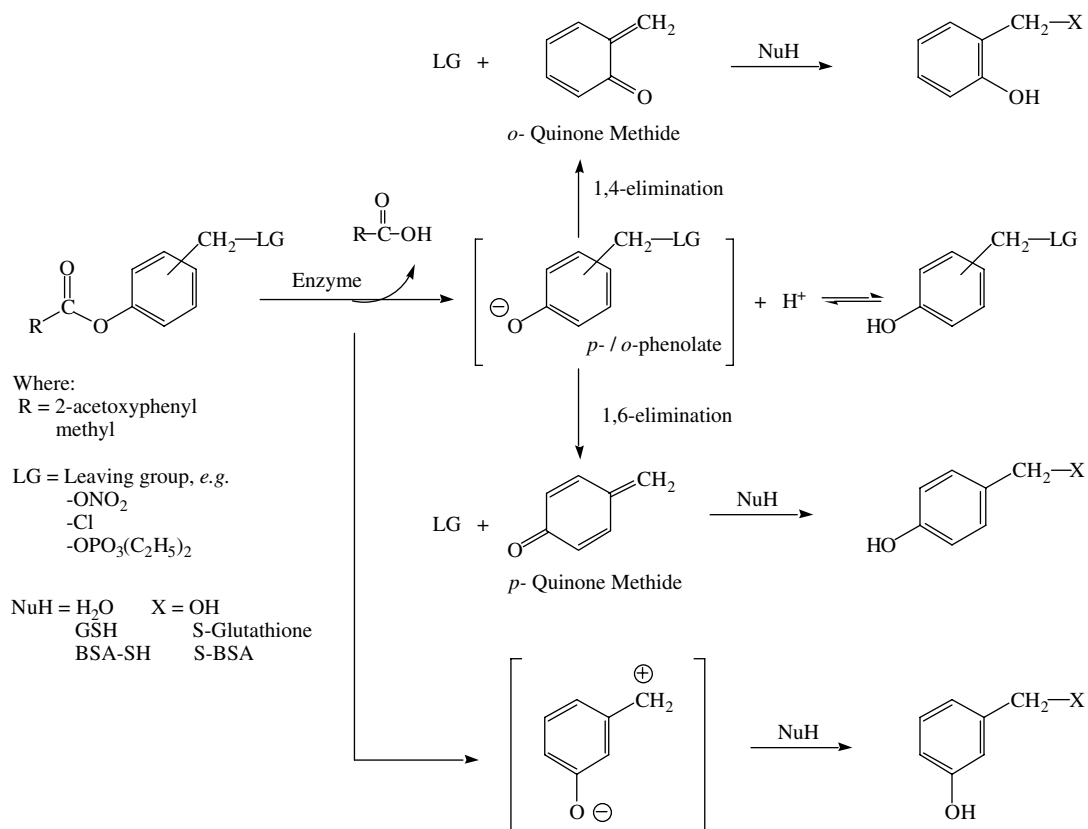


Fig. 4. Proposed mechanism of action of NO-ASA. For the *p*- and *o*-NO-ASA, hydrolysis of the carboxylic ester group of NO-ASA (e.g., by an esterase) produces a phenolate intermediate, which at physiological pH is protonated or undergoes an 1,6- or 1,4-elimination reaction producing the highly reactive electrophile *p*- or *o*-quinone methide, respectively. This, in turn, can react with various cellular nucleophiles; for example, with water it produces the corresponding hydroxy benzylalcohol and with glutathione yields a GSH adduct. For *m*-NO-ASA, ester hydrolysis is followed by formation of a carbocation, which is then converted to a *m*-hydroxy benzylalcohol and other cellular conjugates.

NO generated by NO-ASA. For example, NO-ASA may achieve its effects by delivering minute quantities of NO to a critical intracellular location.

This ambiguity is overcome by the two additional sets of experiments. The first set establishes that the spacer is indeed a reactive species, as demonstrated by its ability to react in a quantitatively important way with albumin, a protein that bears several cysteine thiols. The specificity of the observed result is supported by (a) the competition experiment in which cold (unlabeled) NO-ASA reduced the binding of the labeled spacer to albumin by about half, and (b) the blocking experiment in which pretreatment of albumin with maleimide had a similar effect; maleimide readily reacts with the thiol group found on cysteine to form a stable carbon–sulfur bond.

The most compelling evidence is provided by the results obtained with the two novel compounds that have strategically placed structural differences from NO-ASA. Both, the one that does not have an ASA moiety (“*No NO, No ASA NO-ASA*”) and the one that has –Cl instead of –ONO₂ (“*No NO NO-ASA*”), are just as potent as NO-ASA, if not more, in inhibiting the growth of colon cancer cells.

These findings lead us to propose the following mechanism of action of NO-ASA (Fig. 4). Hydrolysis of the carboxylic ester group with an enzyme such as an esterase would produce a phenolate intermediate, which at physiological pH could be protonated or undergo a 1,6-elimination reaction producing the highly reactive electrophile, *para* quinone methide; for the *ortho* compound the corresponding elimination reaction is 1,4-elimination leading to *ortho* quinone methide. This in turn could react with various cellular nucleophiles; for example, with water it would produce *p*-hydroxy benzylalcohol, and with cytoplasmic glutathione (GSH) it would yield a GSH adduct. In the case of *meta* NO-ASA, the initial ester hydrolysis is followed by the formation of a carbocation and its conversion to *m*-hydroxy benzylalcohol and other cellular conjugates. In the studies reported here, using *p*-NO-ASA, which possesses a ³H-labeled spacer, we have shown that the spacer is capable of reacting with thiol groups of albumin.

The proposed mechanism is consistent with several of our prior findings. For example, we have reported that the rate of NO release from the various positional isomers of NO-ASA correlates almost perfectly with their cell growth inhibitory potency [10]. This finding is of course valid but it can now be interpreted as also reflecting the release of the spacer according to the scheme shown in Fig. 4. Consistent with the proposed mechanism is also our previous work with three positional isomers of NO-ASA showing that the metabolism of these compounds produced *para*, *ortho*, and *meta* hydroxy benzylalcohols and *para*, *ortho*, and *meta* hydroxy benzyl conjugates with GSH [11]. The production of these *para* and *ortho* compounds is consistent with the formation of a quinone methide. The production of a *meta* quinone

methide is not thought to be possible. This is why we postulated that the observed products arise from the biotransformation of the *meta* positional isomer of NO-ASA through solvolysis and generation of a carbocation, which then reacts with appropriate nucleophiles. Thus, the *para* and *ortho* compounds generate the appropriate quinone methides as outlined above, whereas the *meta* compound is active through a *less efficient* carbocation mechanism. The proposed mechanism is also consistent with our previous findings in which we reported that the depletion of cellular GSH stores (formation of a GSH-spacer conjugate) induces oxidative stress, which in turn activates the intrinsic apoptotic pathway [14]. Although we have not directly shown the formation of a carbocation or a quinone methide, nevertheless our findings are entirely consistent with such notions.

Our results necessitate that we approach mechanistic studies of NO-ASA keeping in mind this novel mechanism of action and also reinterpret previous reports. These findings may also aid the rational design of newer generations of anti-cancer compounds.

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