Pharmacokinetic and pharmacodynamic study of NO-donating aspirin in F344 rats

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Abstract. Nitric oxide-donating aspirin (NO-ASA) represents class of promising chemopreventive NO-NSAIDs. NO-ASA combines the beneficial effects of ASA and the gut-sparing effect of the NO moiety. There is, however, limited information on its pharmacokinetic and pharmacodynamic effects *in vivo*. Herein, experiments were designed to identify the optimal dose, the effective route of administration, and targeted markers in plasma and colonic tissues of male F344 rats. Seven weeks old male F344 rats were randomized into 9 groups (16/group) and fed the control diet. At eight weeks of age, groups 2-5 were each administered one of four different doses of NO-ASA by gavage (33, 66, 132 and 264 mg/kg) and each of groups 6-9 were fed diets containing NO-ASA (35, 700, 1,400 and 2,800 ppm) for two weeks. Rats were sacrificed 2 and 10 h after completion of the two weeks of treatment with NO-ASA and plasma and colonic mucosa were collected and analyzed for NO-ASA, its metabolites, and PGE_2 and TXB_2 levels. Our results indicate that NO-ASA is rapidly metabolized, predominantly to salicylic acid; no intact NO-ASA was detected in plasma. Compared to diet-fed NO-ASA, gavaging generated much higher salicylic acid levels over a wide range of doses and a relatively broad time period (10 h). Regardless of its route of administration, NO-ASA lowered the levels of PGE_2 in colonic tissues and plasma, as well as TxB_2 in plasma in a dose- and time-dependent manner. These findings may have practical utility for the administration of NO-ASA to humans.

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Introduction

NO-donating aspirin is the best studied member of an emerging class of compounds, the NO-NSAIDs, which hold significant promise as chemopreventive agents against colon and other cancers (reviewed in ref. 1). NO-ASA consists of an aspirin molecule which has been covalently modified to contain a NO-releasing moiety. Extensive preclinical data both *in vitro* and *in vivo* indicate that NO-ASA is a pleiotropic agent that may bring about its chemopreventive effect by modifying multiple signaling pathways in the preneoplastic cell, including inhibition of proliferation, induction of apoptosis and inhibition of cell cycle phase transitions.

Certain aspects of NO-ASA pharmacology and its effects on the eicosanoid cascade remain, however, unclear. It has been previously shown that while NO-ASA induces the expression of COX-2 in colon cell lines, it inhibits the production of eicosanoids by intestinal mucosa in rats (2,3). NO-ASA's effect on PGE_2 and TxA_2 production is of particular importance since PGE_2 is considered a mediator of carcinogenesis (4,5), whereas TxA_2 is critical to vascular tone and platelet aggregation, both significant parameters of vascular physiology (6).

At present, it would appear that NO-ASA traverses the low pH environment of the stomach intact, followed by progressive deacetylation and hydrolytic cleavages at its salicyloyl ester and/or benzyl nitrate ester bonds (7-10). The exact stage of NO release remains unclear, but it seems to be tightly linked to these biotransformation; esterases are likely involved in its hydrolytic cleavage (11).

To date no complete pharmacokinetic and pharmacodynamic study of NO-ASA has been reported. Therefore, we undertook the present study to assess both its pharmacokinetics and its pharmacodynamic effect on the levels of PGE_2 and TxB_2 in tissue and plasma (TxB_2 is the spontaneous degradation product of TxA_2 and reflects the levels of TxA_2 accurately). In our study, NO-ASA was administered either by oral gavage or admixed with their diet, thus assessing two commonly employed methods of drug administration to experimental animals.

Here, we describe the pharmacokinetics of NO-ASA in rats and document its significant suppressive effect on PGE_2 levels in colon tissue and plasma and its suppressive effect on plasma TxB_2 levels.

Materials and methods

Animals, diets, NO-ASA. Weanling male F344 rats were from Charles River Breeding Laboratories (Kingston, NY). NO-ASA (NCX-4016; meta positional isomer) was provided by the NCI's DCP repository. The purity of NO-ASA, ascertained by HPLC analysis, was ≥98%. All ingredients of the semi-purified diet (Bioserv, Frenchtown, NJ) were stored at 4˚C prior to its preparation. The composition of the modified AIN-76A semi-purified diet was as follows: casein, 20%; DL-methionine, 0.3%; corn starch, 52%; dextrose, 13.2%; alphacel, 5.0%; corn oil, 5.0%; mineral mix, 3.5%; vitamin mix, 1.0%; and choline bitartrate, 0.20%. NO-ASA was incorporated into the diet with a Hobart mixer; its uniform distribution in the diet was monitored as described (12). Control and NO-ASA-containing diets were prepared weekly in our laboratory and stored at 4˚C. NO-ASA was stable in the diet under our experimental conditions, as determined periodically in multiple samples by HPLC; recoveries from the diet were >96%.

Experimental design. At seven weeks of age, 144 male F344 rats were randomized into 9 groups [16 rats/group (Table I)] and fed the AIN-76 modified control diet. At eight weeks of age groups 2-5 were administered four different doses of NO-ASA (33, 66, 132 and 264 mg/kg) by gavage for two weeks. NO-ASA was dissolved in corn oil every day prior to its administration to rats by gavage. At eight weeks of age, groups 6-9 were fed for 2 weeks diets containing 350, 700, 1,400 and 2,800 ppm NO-ASA. The control group (Group 1) received no NO-ASA and was fed only AIN-76 modified control diet. Body weights were recorded twice weekly. All animals were examined daily for signs of toxicity. Rats were sacrificed 2 and 10 h after completion of the two weeks of treatment with NO-ASA either by gavage or through their diet. At each of these two time-points rats were bled and plasma was harvested after centrifugation. All rats were necropsied and the colonic mucosa was harvested by scrapping the mucosal epithelial layer with a glass slide at 4˚C. Plasma and mucosal samples were stored at -80˚C until analyzed.

Reagents. Phosphoric acid, aspirin (ASA), salicylic acid (SA) and acetonitrile were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All general solvents and reagents were of HPLC-grade or of the highest grade commercially available.

HPLC analysis. A Waters 2690 series HPLC System with a HP (G1314A) UV absorbance detector and a LiChrospher C8 reverse-phase column (250x4.6 mm; particle size 5, Alltech Inc., Beerfied, IL) with a manual sample injector was used to analyze metabolites of NO-ASA. The column was maintained at room temperature. Buffer A consisted of water-acetonitrile-phosphoric acid (9:1:0.01, v/v/v); Buffer B consisted of acetonitrile. The flow rate was 1 ml/min. We applied gradient elution from 100% buffer A to 60% buffer B from 0 to 30 min; it was maintained at 60% buffer B until 43 min, becoming 100% buffer B at 50 min. The linearity and recovery of the method regarding salicylic acid were assessed, salicylic acid was the major metabolite of NO-ASA detectable in plasma. The response was linear between salicylic acid concentrations of 19.3 and 483.1 μg/ml ($R^2 = 0.9996$); the recovery (defined as: Recovery = Detected Salicylic Acid ÷ Actual Salicylic Acid x 100)% was complete.

Table I. Experimental design to determine the optimal and effective dose of NO-ASA.

Group no.	NO-ASA dose	Route of administration
1	0 ppm	Diet
2	33 mg/kg BW	Gavage
3	66 mg/kg BW	Gavage
4	132 mg/kg BW	Gavage
5	264 mg/kg BW	Gavage
6	350 ppm	Diet
7	700 ppm	Diet
8	1,400 ppm	Diet
9	2,800 ppm	Diet

16 F344 rats/group; all treated for 2 weeks; BW, body weight.

Eicosanoid assays. 0.7-0.9 g of tissue samples were sonicated in homogenization buffer (0.1 M phosphate, pH 7.4, containing 1 mM EDTA and 10 μM indomethacin) and 500 μl of plasma samples were used for the analysis. Several samples were spiked with tritium-labeled PGE_2 and all samples were purified in accordance with the manufacturer's protocol (Prostaglandin E_2 EIA Kit Monoclonal/Thromboxane B_2 EIA Kit; Cayman Chemicals, Ann Arbor, MI). All purified samples were stored at -80˚C until assayed. TxB_2 and PGE_2 concentrations were measured directly by the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's directions. Sample and standard dilutions were made with experimental medium.

Statistical analysis. Experimental values were expressed as mean ± SEM. Differences between mean values were evaluated using Student's t-test and the association between variables was determined using regression analysis; $p<0.05$ was considered statistically significant.

Results

Pharmacokinetic results. In agreement with previous findings (10), the most prominent and consistently detectable peak in the HPLC chromatograms was that corresponding to salicylic acid. Shown in Table II and Fig. 1 are the concentration of salicylic acid in plasma at 2 and 10 h post-dosing. At either time-point, no intact NO-ASA was detected in the plasma, which is in line with the findings of Carini *et al* (8,9).

Table II. The effect of NO-ASA on rat plasma salicylic acid levels.

Salicylic acid, μg/ml, mean ± SEM Gavage, mg/kg BW			
33	66	132	264
2 h			
73.46±4.00	132.70±7.46[a]	189.55±8.70[b]	275.30±21.64[c]
N=8	N=8	N=8	N=8
10 h			
58.48±1.27	99.82±1.48	172.06±5.52[d]	237.88±7.46[e]
N=8	N=8	N=8	N=8
Diet, ppm			
350	700	1,400	2,800
2 h			
35.64±1.52	100.00±17.57[f]	122.44±10.25	175.54±7.74
N=8	N=8	N=8	N=8
10 h			
21.18±3.39	44.21±7.32	77.28±13.29	105.19±13.79
N=8	N=8	N=8	N=8

Salicylic acid was not detected in vehicle control animals. The differences in salicylic acid levels between the gavage and diet groups were statistically significant for all doses and at both time-points except for [a] vs. [f]; [b] vs. [d]; [c] vs. [e].

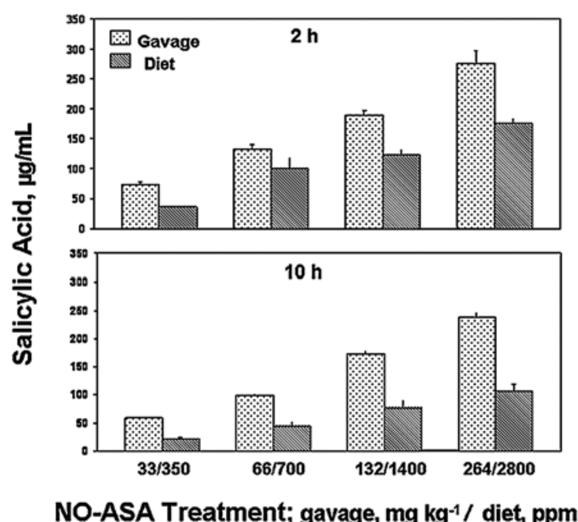

Figure 1. Salicylic acid plasma levels in rats treated with NO-ASA. F344 rats were treated with NO-ASA administered by oral gavage or added into their diet. Four increasing NO-ASA doses were used as described. Plasma salicylic acids levels, 2 and 10 h post-dosing, were determined by HPLC as in Materials and methods. Values are mean ± SEM.

The higher the dose of NO-ASA, whether by gavage or through diet, the higher the salicylic acid levels; this was true for both time-points. The ratio of the salicylic acid level between the highest and the lowest drug intake ranged between 3.74 and 4.93. Thus, at every level of NO-ASA intake, gavage produced consistently higher plasma salicylic acid levels at both time-points. The ratios of plasma salicylate levels between gavage and diet for each corresponding level of NO-ASA were always >1 (range 1.33-2.76).

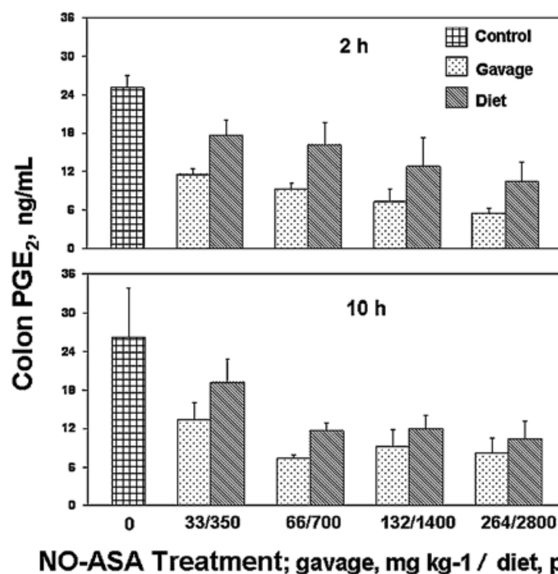

Figure 2. Prostaglandin E_2 colon tissue levels in rats treated with NO-ASA. F344 rats were treated with NO-ASA administered by oral gavage or added into their diet. Four increasing NO-ASA doses were used as described. Colon tissue PGE_2 levels, 2 and 10 h post-dosing, were determined by ELISA as in Materials and methods. Values are mean ± SEM.

In all instances, the highest salicylic acid levels occurred at 2 h. By 10 h salicylic acid levels were substantially lower than those at 2 h. For gavaged animals, the 10 h levels were on average 83% (range 75.22-90.77) of those at 2 h. For animals receiving NO-ASA admixed with their diet the corresponding value was 56.67% (range 44.21-63.12). In all instances the levels of the diet groups were lower than those of the gavage groups. Specifically, the 10 h salicylic acid levels of the diet group were on average 68.27% (range 58.77-74.65) of those of the gavage group.

The effect of NO-ASA on colon tissue and plasma levels of PGE_2 and TxB_2. As summarized in Table III and Fig. 2, NO-ASA inhibited strongly the levels of PGE_2 in colonic tissue in a manner that was: a) dose-dependent, b) time-dependent, and c) administration route-dependent.

Compared to controls, the colon tissue levels of PGE_2 at 2 h in the gavage groups were decreased progressively from 53.78 at the lowest NO-ASA dose to 77.89% at the highest (average 66.49%). At 10 h the corresponding values were 48.74 and 68.75% (average 63.57%). The diet groups displayed a similar pattern wherein the PGE_2 colon tissue levels at 2 h decreased between 29.52% for the lowest NO-ASA dose and 58.45% at the highest (average 43.18%) and at 10 h between 26.63% and 60.18% (average 49.18%). In all instances the values for the gavage group were significantly lower than the corresponding values for the diet group. On average, at 2 h the PGE_2 levels of the gavage groups were lower by 35% compared to diet groups and 27% at 10 h. The

Table III. The effect of NO-ASA on rat PGE_2 colon tissue levels.

PGE_2, ng/ml, mean ± SEM				
Gavage, mg/kg BW				
0	33	66	132	264
2 h				
25.10±1.93[a]	11.60±0.82[b]	9.23±1.02[c]	7.26±2.04[d]	5.55±0.70[e]
N=4	N=8	N=8	N=8	N=6
10 h				
26.14±7.62[f]	13.40±2.59[g]	7.36±0.52[h]	9.16±2.67[i]	8.17±2.34[j]
N=3	N=8	N=6	N=5	N=8
Diet, ppm				
0	350	700	1,400	2,800
2 h				
25.10±1.93[k]	17.69±2.40[l]	16.16±3.56[m]	12.77±4.43[n]	10.43±3.02[o]
N=4	N=7	N=5	N=6	N=7
10 h				
26.14±7.62[p]	19.18±3.70[q]	11.65±1.17[r]	11.90±2.12[s]	10.41±2.66[t]
N=3	N=4	N=6	N=4	N=7

Statistical comparisons were made between controls and PGE_2 levels of individual NO-ASA doses; between 2 and 10 h for each NO-ASA level in the gavage and diet groups; and between gavage and diet groups at each dose and time-point. All differences were statistically significant except for the following: [d] vs. [n]; [e] vs. [o]; [g] vs. [q]; [i] vs. [s]; [j] vs. [t]; [k] vs. [m]; [f] vs. [g]; [p] vs. [q]; [p] vs. [s]; [d] vs. [i]; [e] vs. [j]; [l] vs. [q]; [n] vs. [s]; [o] vs. [t].

Table IV. The effect of NO-ASA on rat plasma PGE_2 levels.

PGE_2, ng/ml, mean ± SEM				
Gavage, mg/kg BW				
0	33	66	132	264
2 h				
0.50±0.09[a]	0.41±0.05[b]	0.25±0.05[c]	0.21±0.02[d]	0.16±0.00[e]
N=6	N=7	N=4	N=3	N=4
10 h				
0.50±0.09[f]	0.45±0.07[g]	0.21±0.04[h]	0.15±0.04[i]	0.11±0.02[j]
N=6	N=3	N=6	N=7	N=4
Diet, ppm				
0	350	700	1,400	2,800
2 h				
0.50±0.09[k]	0.35±0.02[l]	0.34±0.05[m]	0.09±0.05[n]	0.08±0.02[o]
N=6	N=3	N=4	N=3	N=3
10 h				
0.50±0.09[p]	0.46±0.07[q]	0.40±0.08[r]	0.10±0.00[s]	0.10±0.01[t]
N=6	N=5	N=4	N=3	N=4

Statistical comparisons were made between controls and PGE_2 levels of individual NO-ASA doses; between 2 and 10 h for each NO-ASA level in the gavage and diet groups; and between gavage and diet groups at each dose and time-point. The following differences were statistically significant: [g] vs. [q]; [h] vs. [r]; [k] vs. [n]; [k] vs. [o]; [f] vs. [h]; [f] vs. [i]; [f] vs. [j]; [p] vs. [s]; [p] vs. [t].

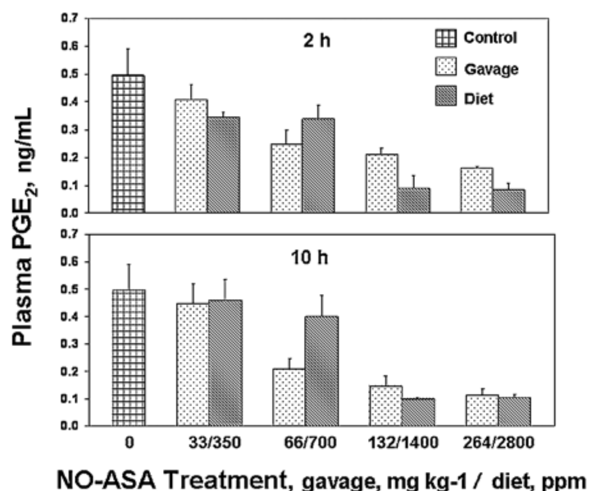

Figure 3. Prostaglandin E_2 plasma levels in rats treated with NO-ASA. F344 rats were treated with NO-ASA administered by oral gavage or added into their diet. Four increasing NO-ASA doses were used as described. Plasma PGE_2 levels, 2 and 10 h post-dosing, were determined by ELISA as in Materials and methods. Values are mean ± SEM.

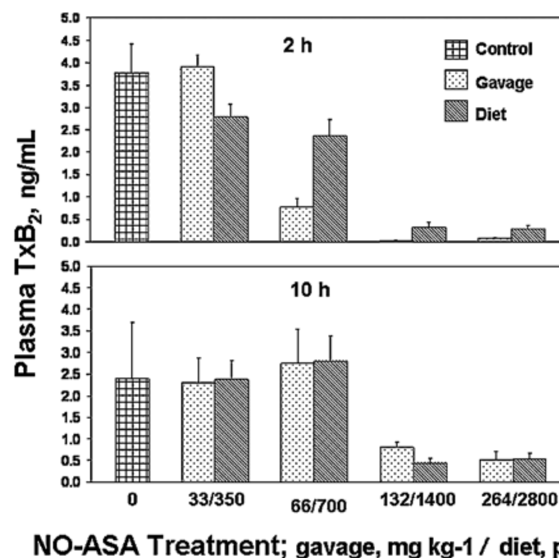

Figure 5. Thromboxane B_2 plasma levels in rats treated with NO-ASA. F344 rats were treated with NO-ASA administered by oral gavage or admixed with their diet. Four increasing NO-ASA doses were used as described. Plasma TxB_2 levels, 2 and 10 h post-dosing, were determined by ELISA as in Materials and methods. Values are mean ± SEM.

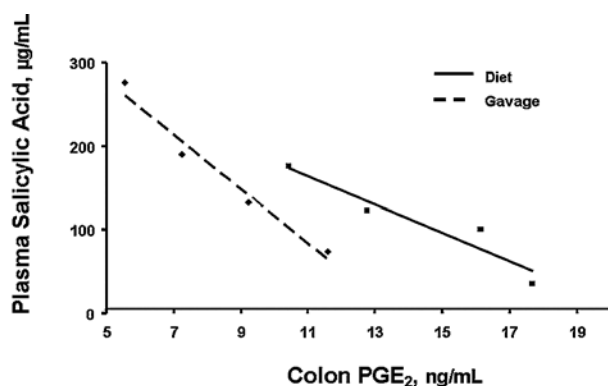

Figure 4. Association between plasma salicylic acid levels and colon PGE_2 levels in NO-ASA treated rats. The 2 h salicylic acid levels in rats treated with NO-ASA administered to rats either by gavage or added to their diet are significantly correlated (P<0.05 for both) with the corresponding PGE_2 levels in colon tissue. Both parameters were determined as in Materials and methods.

apparent asso-ciation between NO-ASA dose and the suppression of colon tissue PGE_2 levels is statistically significant (p<0.001-0.05 for all except for gavage at 10 h where it is significant for trend).

As shown in Table IV and Fig. 3, the inhibitory effect of NO-ASA on tissue levels of PGE_2 was also observed in plasma PGE_2 levels. Gavaged NO-ASA reduced plasma PGE_2 levels dose-dependently. This reduction at 2 h was 17.1% at the lowest dose increasing to 97.61% at the highest (average 48.39%) and correspondingly at 10 h, 9.66 increasing to 77.26% (average 53.87%). PGE_2 plasma levels were also inhibited in those groups receiving NO-ASA through the diet. At 2 h this reduction went from 30.5% for the lowest dose to 83.3% for the highest (average 56.94%) and at 10 h from 70.4% to 79.28% (average 46.38%). In all cases, the degree of association between the dose of the drug and the levels of PGE_2 was statistically significant (p<0.001-0.05).

The levels of salicylic acid correlated with the levels of PGE_2 in colon tissue, the target tissue for its chemopreventive effect. As shown in Fig. 4, there is a statistically significant inverse association (p<0.05) between salicylic acid levels and PGE_2 levels at both 2 and 10 h, suggesting that it was indeed the NO-ASA treatment that brought about the suppression of the PGE_2 synthesis in the colon.

NO-ASA also had a significant inhibitory effect on plasma levels of TxB_2 (Table V and Fig. 5). As was the case for PGE_2, suppression of TxB_2 plasma levels was both dose- and time-dependent. NO-ASA decreased TxB_2 levels in the gavage group on average by 68.3% at 2 h and by 33.4% at 10 h. The diet group displayed similar responses: 61.8% average reduction at 2 h and 35.8% at 10 h. It is of interest that the levels of TxB_2 were nearly completely suppressed at the two highest NO-ASA doses at 2 h (below our analytical methodology). In general, NO-ASA administered through the diet was less effective in suppressing plasma TxB_2 levels than when given by gavage.

Discussion

The findings presented here demonstrate that in rats NO-ASA undergoes metabolic transformations similar to those previously described in rats (8), humans (9), rat liver (7) and in cultured cells (10), regardless of its route of administration. The results also demonstrate that NO-ASA has a significant and unambiguous *in vivo* inhibitory effect on the metabolic transformations of arachidonic acid via the cyclooxygenase pathway.

There are three notable findings from our pharmaco-kinetic study. First, no intact NO-ASA was detected in plasma examined at 2 and 10 h post last dosing. Second, NO-ASA is rapidly metabolized, producing predominantly salicylic acid. Both findings are in close agreement with

Table V. The effect of NO-ASA on rat plasma TxB_2.

TxB_2, ng/ml, mean ± SEM				
Gavage, mg/kg BW				
0	33	66	132	264
2 h				
3.77±0.65[a] N=5	3.91±0.27[b] N=5	0.77±0.18[c] N=4	0.02±0.01[d] N=4	0.08±0.02[e] N=3
10 h				
2.39±1.31[f] N=2	2.30±0.56[g] N=4	2.74±0.80[h] N=3	0.81±0.13[i] N=5	0.51±0.19[j] N=3
Diet, ppm				
0	350	700	1,400	2,800
2 h				
3.77±0.65[k] N=5	2.78±0.30[l] N=8	2.37±0.35[m] N=6	0.32±0.11[n] N=3	0.29±0.07[o] N=3
10 h				
2.39±1.31[p] N=2	2.37±0.45[q] N=5	2.80±0.60[r] N=6	0.44±0.12[s] N=3	0.53±0.13[t] N=4

Statistical comparisons were made between controls and TxB_2 levels of individual NO-ASA doses; between 2 and 10 h for each NO-ASA level in the gavage and diet groups; and between gavage and diet groups at each dose and time-point. All differences were statistically significant except for the following: [g] vs. [q]; [h] vs. [r]; [i] vs. [s]; [j] vs. [t]; [a] vs. [b]; [k] vs. [l]; [k] vs. [m]; [f] vs. [g]; [f] vs. [h]; [f] vs. [i]; [f] vs. [j]; [p] vs. [q]; [p] vs. [r]; [p] vs. [s]; [p] vs. [t]; [e] vs. [j]; [l] vs. [q]; [m] vs. [r]; [n] vs. [s]; [o] vs. [t].

studies previously reported by us and others on the metabolic fate of NO-ASA (1-3). Third, the route of administration of NO-ASA has a significant effect on the plasma levels of its main metabolite, salicylic acid. Compared to administering NO-ASA though the diet, gavaging consistently generated much higher salicylic acid levels over a wide range of doses and over a relatively broad time period (10 h). Although the reason for this result is unclear, it is possible that the 'bolus' administration, such as represented by gavage, may allow NO-ASA to overcome a saturable inactivation mechanism, albeit alternative explanations are also plausible. Nevertheless, this finding may have practical implications for the administration of NO-ASA to humans.

The data herein also demonstrate that NO-ASA inhibits the transformation of arachidonic acid. The levels of PGE_2 in colon tissue and in plasma as well as the plasma levels of TxB_2 were all suppressed by NO-ASA in a manner that is clearly dose- and time-dependent. In keeping with the pharmacokinetic findings, the prostanoid levels of gavaged animals were, in general, lower than those of animals receiving NO-ASA in their diet.

These findings are consistent with those reported by Ukawa *et al* (13), who studied the effect of NO-ASA on the ulcerogenic and healing responses of the stomach. Gastric prostaglandin levels in murine stomach were reduced by NO-ASA, including increased prostaglandin generation in the ulcerated mucosa. Consistent with the present finding on the effects of NO-NSAIDs on rat colonic mucosa are results previously published by us (3), showing that NO-indomethacin and NO-ASA significantly inhibited the total COX of colon tumors, including COX-2 activity and formation of PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TxB_2 from arachidonic acid.

The significant correlation between salicylic acid levels and colon PGE_2 levels indicates that this effect is brought about by NO-ASA. It is uncertain, however, whether salicylic acid per se is responsible for the observed effect. Both salicylic acid and NO-salicylic acid inhibit the growth of cancer cell lines, with the latter being more potent than the former (14). While no *in vivo* comparisons are available, NO-ASA is much more potent than either one *in vitro*. It is conceivable that salicylic acid may merely reflect the decomposition of NO-ASA that may generate with similar kinetics other bioactive moieties, such as NO or its spacer or both (7-10). It is noteworthy that here too the suppressive effect of NO-ASA on eicosanoid production extends to two products, PGE_2 and TxB_2, and is not restricted to a single tissue.

In conclusion, our data determined the pharmacokinetics of NO-ASA in rats, established its differential bioavailability depending on its mode of administration and demonstrated a direct effect on arachidonic acid metabolism leading to markedly suppressed levels of PGE_2 and TxA_2. Whether suppression of arachidonic acid metabolism by NO-ASA is responsible for its chemopreventive effect is more difficult to assess, since both COX-dependent and COX-independent

effects have been described for conventional ASA and NO-ASA (15). These findings could prove useful in devising strategies for future human applications of NO-ASA.

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