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Gastric-sparing nitric oxide-releasable 'true' prodrugs of aspirin and naproxen

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ABSTRACT

Nitric oxide-releasing non-steroidal anti-inflammatory drugs (NO-NSAIDs) are gaining attention as potentially gastric-sparing NSAIDs. Herein, we report a novel class of '1-(nitrooxy)ethyl ester' group-containing NSAIDS as efficient NO releasing 'true' prodrugs of aspirin and naproxen. While an aspirin prodrug exhibited comparable oral bioavailability and antiplatelet activity (i.e., TXB_2 inhibition) to those of aspirin, a naproxen prodrug exhibited better bioavailability than naproxen. These promising NO-NSAIDs protected experimental rats from gastric damage. We therefore believe that these promising NO-NSAIDs could represent a new class of potentially 'Safe NSAIDs' for the treatment of arthritic pain, inflammation and cardiovascular disorders in the case of NO-aspirin.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and naproxen are widely used to reduce pain, arthritis and inflammation. However, NSAIDs can cause undesirable gastrointestinal (GI) toxic effects such as bleeding, dyspepsia and peptic ulcers. The NSAID-induced GI toxicities could be of either systemic or local origin.[1,2] While the systemic effects are thought to be due to the inhibition of COX-1 isozyme, the local effects are largely due to the presence of free carboxylic acid group, which can cause local irritation upon oral administration. A widely used approach to reduce local GI effects is to temporarily mask the free carboxyl group in the form of an ester prodrug.[3–7] It was for the first time, Bayer introduced aspirin (acetylsalicylic acid) as a less gastric irritating derivative of the NSAID sodium salicylate in 1899. Based on known definitions of prodrugs, aspirin can be considered as a prodrug as it is hydrolyzed by plasma esterases to release its parent drug salicylate in vivo. However, aspirin also exhibits intrinsic pharmacological activity such as inhibition of COX isozymes.[8]

Aspirin (*O*-acetylsalicylic acid) is the most versatile pharmaceutical agent known with antipyretic, analgesic, anti-inflammatory, and antiplatelet properties. Aspirin can inhibit platelet cyclooxygenase, thromboxane production and protect against arterial thrombosis at low doses of 75–100 mg per a day.[9] However, similar to other NSAIDs, chronic treatment with aspirin even at smaller doses can increase the risk of major bleeding complications. Unfortunately, the ester prodrug strategy cannot be applied easily to aspirin to reduce the local GI toxicity by temporarily masking of its free carboxyl acid group. This is due to the fact that aspirin exits in carboxylate form at biological pH and the carboxylate anion seems to stabilize the adjacent *O*-acetyl group from hydrolysis. However, esterification of carboxyl group in aspirin, which amounts to neutralizing the negative group of the aspirin, renders the adjacent *O*-acetyl group extremely susceptible to enzymatic cleavage.[10] In order to act as a 'true' prodrug of aspirin, its carboxyl masking group must be cleaved faster than its *O*-acetyl group. It is therefore a challenging task to design prodrugs of aspirin. Hence, there are only a few reported examples of true prodrugs of aspirin in the literature.[11–13,10,14–19]

Another potentially promising approach to reduce NSAID-induced gastrotoxicity is to attach a nitric oxide (NO)-releasing moiety to standard NSAIDs to yield a new class of compounds called NO-releasing NSAIDs (NO-NSAIDs), which are also sometimes known as cyclooxygenase inhibitory NO-donors (CINODs). The rationale behind this strategy is to harness the beneficial effects of NO, which is recognized as a critical mediator of GI mucosal defense, influencing factors such as mucus secretion, mucosal blood

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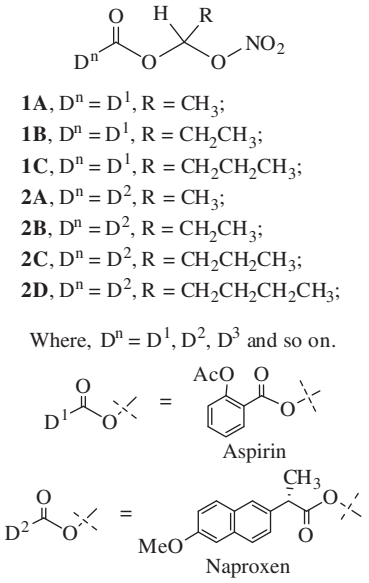


Figure 2. Structures of NO-aspirin prodrugs (**1A–C**) and NO-naproxen prodrugs (**2A–D**).

flow, ulcer repair, down-regulation of inflammatory responses, scavenging of various free radical species and protection of the GI mucosa from injury induced by many topical irritants.[20–22] As expected, NO-NSAIDs have been shown to cause reduced GI damage, while exhibiting comparable analgesic and anti-inflammatory activity to those of their respective parent NSAIDs.[23] Except NO-aspirin compounds, all other reported NO-NSAIDs acted as true prodrugs by releasing nitric oxide and their respective parent NSAIDs in vivo or in plasma. Even a widely studied NO-aspirin compound such as NCX-4016 did not act as true prodrug of aspirin and released only NO and salicylic acid in vivo or in plasma.[24] Here again, it is a very challenging task to design a true NO releasing prodrug of aspirin. Hence, there are only a few examples of NO-aspirin prodrugs which can release NO and some amount (9–71%) of aspirin along with salicylic acid in human plasma or serum. Structures of known NO-releasing true prodrugs of aspirin are shown in Figure 1 (see Supplementary material).[25–29]

In our quest for finding a potentially gastric-sparing 'Safe NSAID', we have earlier designed, synthesized and evaluated a number of novel NO-NSAIDs containing 'SS-nitrates' as NO-releasing moieties and we were successful in identifying a few promising NO-NSAIDs such as NO-aspirin (P1539), NO-diclofenac (P2026) and NO-naproxen (P1853), which showed significant oral bioavailability, anti-inflammatory activity and protected experimental rats from NSAID-induced gastric damage, which could be attributable to the physiological actions of NO released from these promising prodrugs.[30–32] Unfortunately, like NCX-4016, P1539 also failed to act as a true prodrug of aspirin (i.e., it released only salicylic acid in human plasma). We now report in this preliminary communication, our design, synthesis and evaluation of a novel class of 1-(nitrooxy)alkyl esters of NSAIDs as efficient NO-releasing 'true' prodrugs of aspirin and naproxen (Fig. 2). To our knowledge, this is the first report of such unique NO-releasing true prodrugs of aspirin and naproxen. We also believe that it is the first report of a prodrug of naproxen which exhibits significantly superior oral bioavailability than its parent drug naproxen.

Design of NO-NSAIDs

A salient feature in our design of these novel NO-NSAIDs is the introduction of a unique '1-(nitrooxy)alkyl ester–acetal linkage' represented by '—C(=O)—O—C(H)(R)—O—NO_2', which can undergo facile hydrolysis (either by acid-catalyzed or esterase-catalyzed) to release the constituent NSAID as shown by plausible mechanisms in Figure 3. In fact, these prodrugs can be structurally considered as double esters of an organic carboxylic acid such as aspirin or naproxen and an inorganic acid such as nitric acid. However, these prodrugs also possess a unique acid-labile acetal function, which can force these unique prodrugs to degrade only in a particular way to release nitrate species under acidic aqueous conditions. Thus, under acidic aqueous conditions such as in simulated gastric fluid (SGF, pH 1.2), initial protonation on the ester/acetal oxygen atom of either inorganic acid (i.e., Pathway A) or carboxylic acid (i.e., Pathway B) would trigger cleavage of the prodrug to release free carboxylic acid (i.e., aspirin or naproxen), the corresponding aldehyde (i.e., RCHO) and nitrate group (NO_3^-) as shown in Figure 3. In simulated intestinal fluid (SIF, pH 6.8) or human plasma, esterases can initiate cleavage of carboxyl ester group, which can trigger further cleavage to release the same species (i.e., aspirin or naproxen, the corresponding aldehyde and nitrate) as shown in Figure 3. Thus, as per the proposed mechanisms of cleavage of these prodrugs, an aldehyde (RCHO) and nitrate (NO_3^-) species

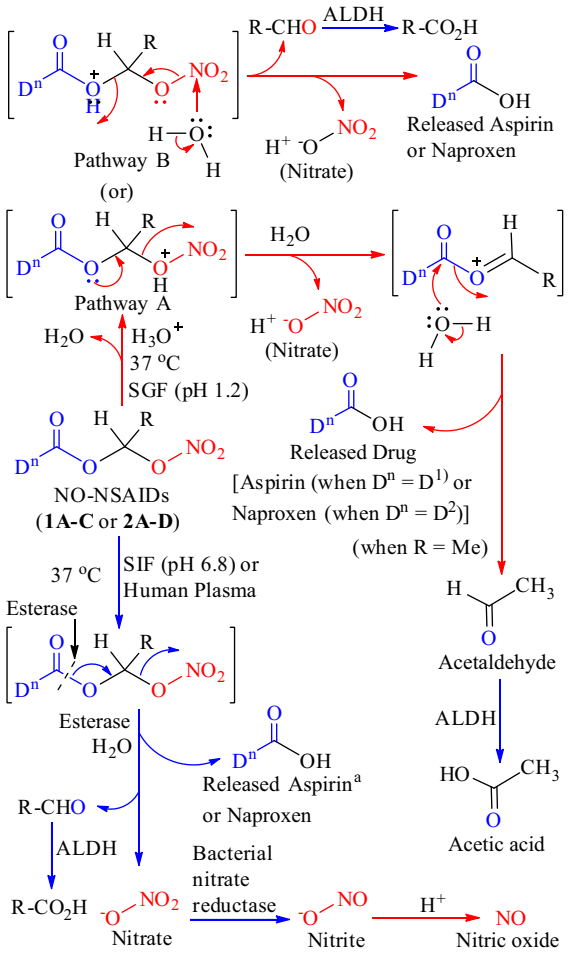
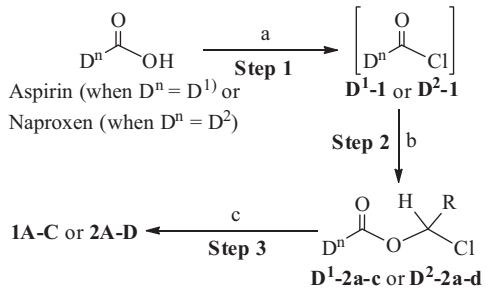


Figure 3. Proposed plausible mechanisms of drug release from the reported NO-NSAIDs either by acid-catalyzed (shown by red arrows) or by enzyme-catalyzed (shown by blue arrows) hydrolysis. [a]In human plasma, NO-aspirin prodrug **1A** released only 8–9% of aspirin and the rest as salicylic acid. Where R = CH_3, CH_3CH_2, $CH_3CH_2CH_2$ or $CH_3CH_2CH_2CH_2$; ALDH = aldehyde dehydrogenase; SGF = simulated gastric fluid at pH 1.2; SIF = simulated intestinal fluid, pH 6.8.



Scheme 1. Synthetic method for NO-aspirin prodrugs (**1A–C**) and NO-naproxen prodrugs (**2A–D**). Reagents and conditions: (a) oxalyl chloride, DMF (1 or 2 drops), DCM, rt, 3 h or thionyl chloride, rt or reflux; (b) $ZnCl_2$, DCM, −15 °C to rt, R-CHO (**Aa-d**), 23% (**D¹-2a**), 30% (**D¹-2b**), 43% (**D¹-2c**), 80% (**D²-2a**), 30% (**D²-2b**), 65% (**D²-2c**), 23% (**D²-2d**); (c) $AgNO_3$, acetonitrile, rt or reflux, 81% (**1A**), 65% (**1B**), 47% (**1C**), 74% (**2A**), 31% (**2B**), 57% (**2C**), 35% (**2D**).

are also generated along with the free Aspirin or Naproxen. Under normal physiological conditions, the aldehyde species (RCHO, possibly toxic) would be quickly oxidized further by the enzyme aldehyde dehydrogenase (ALDH) (EC 1.2.1.3) to their respective acids that are likely to be less toxic and can be excreted from the body easily. However, it is preferable to select methyl as R group so that the liberated aldehyde would be a less toxic acetaldehyde, which is the usual metabolite formed in our body via oxidation of ethyl alcohol by the enzyme alcohol dehydrogenase (ADH) (EC 1.1.1.1) and the acetaldehyde thus generated can be oxidized further to an innocuous acetic acid by the enzyme ALDH. The liberated nitrate group can be reduced by oral bacterial nitrate reductase to nitrite ion, which in turn can get reduced to NO in the acidic conditions of the stomach.[33,34] Thus, after oral administration, these prodrugs are expected to be hydrolyzed quickly in the acidic stomach region to release NO, acetaldehyde (i.e., when R = CH_3) and the free NSAID. Notably, prodrugs derived from aspirin acted as true prodrugs of aspirin by releasing quantitative amounts of aspirin in biologically relevant fluids. In fact, this platform prodrug technology can be applied to any therapeutic agent containing at least one derivatizable carboxylic acid group.

Synthesis of NO-NSAIDs

Synthesis of NO-aspirin prodrugs (**1A–C**) and NO-naproxen prodrugs (**2A–D**) was performed in 3 steps as shown in Scheme 1. Thus, in the first step, aspirin (D^1-C(=O)OH) or naproxen (D^2-C(=O)OH) was converted to their respective acid chlorides (**D^1-1** or **D^2-1**) by a known procedure such as treating with oxalyl chloride–DMF or thionyl chloride. In the second step, the acid chloride was reacted with an appropriate aldehyde [i.e., acetaldehyde (**Aa**) or propionaldehyde (**Ab**) or butyraldehyde (**Ac**) or pentanaldehyde (**Ad**)] in presence of zinc chloride to yield the respective α-chloroalkyl ester (**D^1-2a–c** or **D^2-2a–d**). In the third step, the resulting α-chloroalkyl ester (**D^1-2a** or **D^1-2b** or **D^1-2c** or **D^2-2a** or **D^2-2b** or **D^2-2c** or **D^2-2d**) was treated with silver nitrate in acetonitrile[35] to afford the respective NO-aspirin prodrugs **1A–C** and NO-naproxen prodrugs **2A–D**.

Metabolic studies in biologically relevant fluids

The NO-aspirin prodrug **1A** and the NO-naproxen prodrugs **2A–D** were incubated in biologically relevant simulated gastric fluid (SGF at pH 1.2) at 37 °C, which is close to acidic stomach environment, and estimated their half-lives ($t_{1/2}$) and the amount (%) of drug release over 1–3 h (Table 1). The aspirin prodrug **1A** was also incubated in simulated intestinal fluid (SIF) and 100% human plasma and estimated its drug release profile (Table 1).

Thus, all the prodrugs released their respective parent drug [either aspirin (D^1-C(=O)OH) or naproxen (D^2-C(=O)OH)], but the rate of drug release was found to be dependent on the nature of their R group. Thus, both the NO-aspirin prodrug **1A** (R is methyl) and the NO-naproxen prodrug **2A** (R is methyl) decomposed in SGF with half-lives of <10 min and <30 min, respectively, and released quantitative amounts (i.e., 99–100%) of their parent drugs aspirin and naproxen, respectively, within 60 min of incubation in SGF. However, the NO-naproxen prodrugs **2B–D** exhibited half-lives ($t_{1/2}$) of ~60 min (when R is ethyl), <3 h (when R is *n*-propyl) and >3 h (when R is *n*-butyl) and released 93%, 61% and 29% of naproxen, respectively, after 3 h of incubation is SGF. This is expected because the amount of drug release from these prodrugs is proportional to their hydrophobicity, which is expected to increase proportionally with increase in the size of R group. In SIF, the aspirin prodrug **1A** decomposed with a half-life of <20 min and released 100% of aspirin in about 2 h of incubation. However, in 100% human plasma, this aspirin prodrug **1A** decomposed with a half-life of <5 min. It seems that this prodrug has acted more like a prodrug of salicylic acid by releasing 79% of salicylic acid and only 8–9% of aspirin within 5 min of incubation is human plasma (Table 1).

Table 1
Stability of NO-aspirin prodrug (**1A**) and NO-naproxen prodrugs (**2A–D**) in SGF, SIF and human plasma and percentage (%) of drug {aspirin [D1-C(=O)OH]} or {naproxen [D^2-C(=O)OH]} or salicylic acid (**SA**) release

Time (min)	SGF (pH 1.2)										SIF (pH 6.8)		100% HP	
	1A		**2A**		**2B**		**2C**		**2D**		**1A**		**1A**	
	1A[a]	**Asp**[b]	**2A**[a]	**Np**[b]	**2B**[a]	**Np**[b]	**2C**[a]	**Np**[b]	**2D**[a]	**Np**[b]	**1A**[a]	**Asp**[b]	**Asp**[b]	**SA**[c]
0	100	0	100	0	100	0	100	0	100	0	100	0	0	0
2	78	22	–	–	–	–	–	–	–	–	91	9	–	–
5	65	35	85	15	94	6	96	4	98	2	82	18	8	79
10	45	55	75	25	90	10	94	6	96	4	52	48	9	86
15	–	–	65	35	74	26	83	17	87	13	–	–	–	–
20	14	86	–	–	–	–	–	–	–	–	32	68	8	90
30	–	–	33	67	62	38	83	17	87	13	–	–	–	–
40	12	88	–	–	–	–	–	–	–	–	–	–	6	90
60	0	99	0	100	51	49	75	25	87	13	4	96	0	95
120	–	–	–	–	39	61	58	42	80	20	0	100	0	100
180	–	–	–	–	7	93	39	61	71	29	–	–	–	–
Half-life ($t_{1/2}$)	<10 min		<30 min		~60 min		<3 h		>3 h		<15 min		<5 min	

SGF = simulated gastric fluid; SIF = simulated intestinal fluid; HP = human plasma; – = not determined.
[a] Percentage (%) of NO-NSAID (i.e., **1A** or **2A** or **2B** or **2C** or **2D**) remaining.
[b] Percentage (%) of Asp = aspirin [D^1-C(=O)OH] or Np = naproxen [D^2-C(=O)OH] released.
[c] Percentage (%) of **SA** released.

Biological evaluation

The NO-aspirin prodrugs **1A–C** *and* NO-naproxen prodrugs **2A–D** were evaluated in vivo to establish their bioavailability. Based on their promising bioavailability data, the NO-aspirin prodrug **1A** and the NO-naproxen prodrug **2A** were selected and evaluated further for their nitric oxide release capabilities and their gastric-sparing/damaging effects in comparison to those of their respective parent drugs. The NO-aspirin prodrug **1A** was also evaluated for its ability to inhibit thromboxane B_2 (TXB_2) and compared the result with that of aspirin at equimolar dose.

Bioavailability studies

All the reported NO-NSAIDs **1A–C** and **2A–D** were subjected to pharmacokinetics studies using rat as animal model and determined their bioavailabilities. For NO-naproxen prodrugs **2A–D** and naproxen, the presented bioavailability [area under curve (AUC)] data correspond to the plasma concentration of the released parent drug naproxen. However, in the case of NO-aspirin prodrugs **1A–C**, the reported AUC data corresponds to plasma concentration of the released salicylic acid, since aspirin and NO-aspirin rapidly undergo enzymatic hydrolysis in vivo to generate salicylic acid. Among the NO-aspirin series, as shown in Figure 4 (see Supplementary material), the prodrug **1A** showed nearly comparable bioavailability to that of aspirin (i.e., AUCs: 91.13 ± 12.2 vs 89.78 ± 10.2 μg*h/ml). The other two prodrugs **1B** and **1C** exhibited lower bioavailabilities when compared to that of aspirin. Thus, the prodrug **1A** (with methyl as R group) is the best among three NO-aspirin prodrugs. When R group is ethyl, the corresponding prodrug **1B** showed significantly less bioavailability (AUC: 53.56 ± 15.6 μg*h/ml). When R group is *n*-propyl, the corresponding prodrug **1C** showed slightly better bioavailability (AUC: 73.54 ± 4.9 μg*h/ml) than that of **1B**. Although the AUC values are nearly comparable for aspirin and NO-aspirin prodrug **1A**, they exhibited significantly different T_{max} values (15 min and 2 h, respectively) and C_{max} values (26.12 ± 2.11 μg/ml and 18.57 ± 1.60 μg/ml, respectively).

In order to assess the species-specific differences in oral bioavailability of these prodrugs, we have carried out PK studies on the most promising NO-aspirin prodrug **1A** and aspirin in Wistar rats and the corresponding results are presented in Figure 5 (see Supplementary material). Interestingly, both aspirin and its prodrug **1A** have shown comparable bioavailability (AUCs: 436.8 ± 26.2 μg*h/mL vs 397.6 ± 28.0 μg*h/mL) in Wistar rats also. However, both aspirin and its prodrug **1A** have shown strikingly improved oral absorption in Wistar rats as compared to that in Sprague–Dawley (SD) rats (AUCs for Aspirin: 436.8 ± 26.2 vs 91.13 ± 12.20 at 30 mg/kg equimolar dose; AUCs for prodrug **1A**: 397.6 ± 28.0 μg*h/mL vs 89.78 ± 10.20 μg*h/mL at 44.83 mg/kg, which is equimolar to 30 mg/kg dose of aspirin). Based on the above bioavailability data and on the in vitro drug release profile (Table 1), we have selected the best NO-aspirin prodrug **1A** for further evaluation.

Among the NO-naproxen series also, as shown in Figure 6 (see Supplementary material), the prodrug **2A**, which contains methyl as R group, exhibited superior and statistically significant increase in bioavailability (AUC: 272.60 ± 8.50 μg*h/mL, $^{**}p$ <0.01) over that of naproxen (AUC: 207.80 ± 18.20 μg*h/mL) in SD rats. However, it is interesting to see their important PK parameters: while their T_{max} values are different (i.e., 15 min for naproxen vs 1 h for the prodrug **2A**), their C_{max} values are not significantly different (i.e., 54.97 ± 2.42 μg/ml and 49.37 ± 5.61 μg/ml, respectively). It is also interesting to see that the plasma drug concentration in prodrug treated animals was found to be between 30 and 35 μg/mL during the period from 0.5 h to 6.0 h (between 40 and 55 μg/mL during the period between 1 h and 4 h). However, the plasma drug concentration in naproxen treated animals, although showed a C_{max} of above 55 μg/mL at 15 min, quickly reached to just above 30 μg/ml in 2 h and to just above 20 μg/mL in a period of 4 h and it further dropped to below 15 μg/ml in a period of 8 h. So, the prodrug **2A** has exhibited controlled release of higher amounts of naproxen over a longer period of time (over 30 μg/mL*** up to 6 h duration) when compared to naproxen at equimolar doses. This prodrug is therefore expected to offer better pain relief for a longer period of time than the parent drug naproxen although the parent drug is expected to offer quicker relief from pain than its prodrug due to its faster absorption within 15 min of administration of the drug. The remaining prodrugs in the naproxen series (i.e., **2B**, **2C** and **2D**) exhibited either comparable (**2B** with an AUC value of 182.70 ± 8.10 μg*h/mL) or slightly less (i.e., **2C** and **2D** with AUC values of 178.60 ± 8.10 μg*h/mL and 177.40 ± 4.10 μg*h/mL, respectively) bioavailability when compared to that of naproxen with an AUC value of 207.80 ± 18.20 μg*h/mL and also showed some decreasing trend, although not significant, in bioavailability with increasing chain length of 'R' group. Based on the above bioavailability data and on the in vitro drug release profile (Table 1), we have selected the best NO-naproxen prodrug **2A** for further evaluation. To our knowledge, this is the first report of a naproxen prodrug, which has shown significantly superior oral bioavailability than its parent drug naproxen.

Anti-inflammatory efficacy

It is known that the anti-inflammatory activity of an NSAID is directly proportional to the plasma concentration of the drug.[30,31] Thus, based on their comparable oral bioavailability data, the promising NO-aspirin prodrug **1A** is expected to show comparable anti-inflammatory activity to that of the parent drug aspirin. Similarly, based on its superior and improved bioavailability, the NO-naproxen prodrug **2A** is expected to show superior or at least comparable anti-inflammatory activity to that of naproxen in the carrageenan-induced rat paw edema model. We have chosen to defer these in vivo experiments to save experimental animals and resources but the anti-inflammatory activity of these promising prodrugs can be readily assessed in carrageenan-induced rat paw edema model according to the reported procedure.[36]

Estimation of NO release from the promising NO-NSAIDs

We have evaluated the NO releasing capability of the promising NO-NSAIDs **1A** and **2A** in SD rats. The NO release profile in the blood plasma which is an indirect measure of the NO (i.e., nitrate/nitrite) released in the blood was estimated by using Griess method[37] and the data obtained from these experiments is presented in Figure 7 (see Supplementary material) and Table 2. As shown in Table 2, the NO-aspirin prodrug **1A** released more NO than the NO-naproxen prodrug **2A** (AUCs: 1481.00 μM*h vs 686.80 μM*h). However, their NO release profiles were different in that the NO-aspirin prodrug **1A** treated rats showed plasma NOx concentration of ~180 μM over a period of 8 h, where as the

Table 2
Estimation of NO release (i.e., plasma nitrate/nitrite concentration) from the most promising NO-NSAIDs **1A** and **2A** in SD rats

Prodrug[a]	Plasma nitrate/nitrite AUC (μM*h)
Vehicle	371.10
1A[b]	1481.00
2A[c]	686.80

[a] All the compounds were administered orally.
[b] At a dose equimolar to 10 mg/kg dose of aspirin.
[c] At a dose equimolar to 30 mg/kg dose of naproxen.

NO-naproxen prodrug **2A** treated rats have shown ~150 μM plasma NOx concentration during first 1 h, which fell to ~120 μM by 2 h and to ~60 μM by 4 h, which finally reached to basal level by 6 h.

Gastric tolerance study

Since the reported prodrugs release their parent NSAIDs quantitatively in the stomach region following their oral administration, it is important see whether the released NSAIDs could cause the usual NSAID-induced gastric bleeding, lesions and ulcers in experimental animals. We have therefore evaluated the most promising aspirin prodrug **1A** and the naproxen prodrug **2A**, after acute oral dosing of rats with 298.8 mg/kg of **1A**, which is equivalent to 200 mg/kg of aspirin and 138.67 mg/kg of **2A**, which is equivalent to 100 mg/kg of naproxen, to determine their gastric tolerance capability compared to gastric ulcer-causing potential of their respective parent drugs, aspirin and naproxen (both at doses of 100 mg/kg). The results associated with these experiments are presented graphically in Figures 8 and 9, respectively. The actual stomach images from these experiments are presented in Figures S1 and S2, respectively, (see Supplementary material). These results clearly establish that none of the animals treated with the prodrugs **1A** and **2A** showed any significant development of gastric lesions or ulcers. However, severe hemorrhagic lesions and ulcers were developed in rats administered with parent drugs, aspirin and naproxen (both at doses of 100 mg/kg). We believe that the observed gastric-sparing effects of these promising prodrugs **1A** and **2A** could be partly attributable to the physiological functions of NO released from these novel prodrugs. However, it is possible that the masking of carboxylic acid groups of NSAIDs as ester prodrugs might have partly contributed to the gastric-sparing effects of these prodrugs.[38–40]

TXB_2 inhibition assay

Aspirin shows its antiplatelet activity via inhibition of platelet cyclooxygenase (COX), which is responsible for generation of a potent platelet activator thromboxane A_2 (TXA_2), which in turn indirectly inhibits the formation of its stable metabolite serum TXB_2.[41] It is therefore possible to achieve complete suppression of platelet TXA_2 (and the TXB_2) formation via chronic administration of aspirin at a dose of 30 mg/daily.[42] The antiplatelet activity of aspirin (30 mg/kg) and its prodrug **1A** (at a dose equimolar to 30 mg/kg dose of aspirin) was evaluated in SD rats through estimation of serum TXB_2 levels.[43] As expected, aspirin (30 mg/kg, p.o., o.d., 7 days) and its prodrug **1A** (44.82 mg/kg, equivalent to 30 mg/kg of aspirin, p.o., o.d., 7 days) exhibited nearly comparable inhibition of platelet TXB_2 formation (75.97% vs 72.59%) at equimolar doses as shown in Figure 10. This result unequivocally establishes that the compound **1A**, which exhibits significant antiplatelet activity (a unique property of the wonder drug aspirin), is indeed a true prodrug of aspirin.

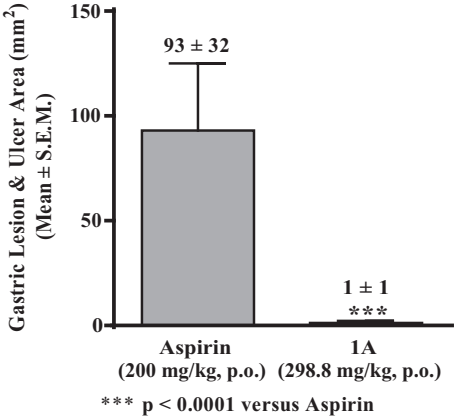


Figure 8. Gastric lesion & ulcer area (mm^2) of rat stomachs after acute oral dosing of rats with aspirin (100 mg/kg) and its prodrug **1A** (298.85 mg/kg, which is a dose equimolar to 200 mg/kg of aspirin).

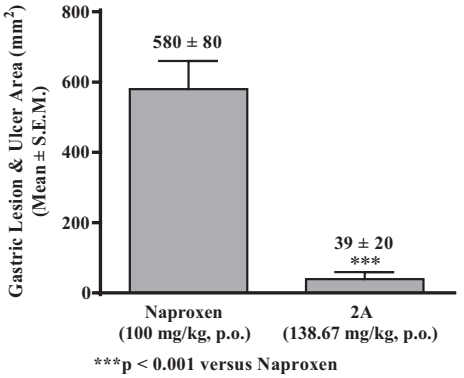


Figure 9. Gastric lesion area (mm^2) of rat stomachs after acute oral dosing of rats with naproxen sodium (109.52 mg/kg, which is a dose equimolar to 100 mg/kg dose of naproxen) and its prodrug **2A** (138.67 mg/kg, which is a dose equimolar to 100 mg/kg of naproxen).

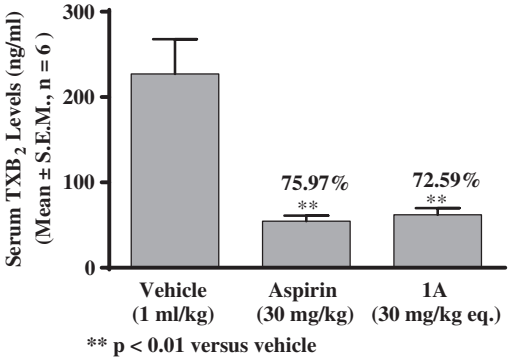


Figure 10. In vivo inhibition of TXB_2 (i.e., indicated by the reduction in serum TXB_2 levels) after oral dosing of rats with aspirin (30 mg/kg) and its promising prodrug **1A** (44.82 mg/kg, which is equimolar to 30 mg/kg dose of aspirin).

Stability studies

An investigational drug must show sufficient stability at room temperature (rt) so that it can have an acceptable shelf life when it is developed as a drug. This stability issue is even more critical for aspirin prodrugs as they tend to be less stable by design. We have therefore tested stability of the promising NO-aspirin prodrug **1A** and the NO-naproxen prodrug **2A** at room temperature and at 50 °C over a period of 25–30 days and the results are presented in Table S1 (see Supplementary material).

As anticipated, the aspirin prodrug **1A** was found to be very stable at RT up to 1 month. However, when it was incubated at 50 °C, it degraded slightly (~1%) after 5 days and about 11% after 1 month. After 1 month of incubation at 50 °C, about 2.8% of aspirin and 0.6% of salicylic acid were generated. In the case of naproxen prodrug **2A**, the prodrug remained stable both at rt and at 50 °C for up to 25 days (period of study) and released only negligible amounts (~0.20% at rt and ~0.33% at 50 °C) of naproxen after 25 days.

Although we have disclosed only the NO releasing prodrugs of NSAIDs aspirin and naproxen here, the linker technology is not lim-

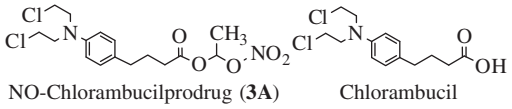


Figure 11. Structures of NO-chlorambucil prodrugs (**3A**) and chlorambucil [i.e., D^3-C(=O)OH].

ited to NSAIDs and it can be extended to any other therapeutic agent containing at least one derivatizable carboxylic acid group. Thus, we have made an example using an anti-cancer drug, chlorambucil and the structure of the corresponding prodrug **3A** is shown in Figure 11.

As anticipated, the NO-chlorambucil prodrug **3A** decomposed in SGF to give 100 % of the parent drug chlorambucil [D^3-C(=O)OH] with a half-life ($t_{1/2}$) of <5 min (data not shown).

In summary, we reported a novel class of '1-(nitrooxy)ethyl ester' group-containing NSAIDs as efficient NO releasing 'true' prodrugs of aspirin and naproxen. The most promising NO-aspirin prodrug **1A** exhibited nearly comparable oral bioavailability and antiplatelet activity to those of aspirin. The most promising NO-naproxen prodrug **2A** also exhibited significantly superior bioavailability to that of naproxen. Both of these promising NO-NSAID prodrugs protected rats from NSAID-induced gastric damage, which could be attributable to the beneficial effects of NO-released from these prodrugs. However, rats treated with equimolar doses of aspirin and naproxen suffered from severe hemorrhagic gastric lesions and ulcers. Based on the above data, we anticipate that these promising NO-NSAIDs could represent a new class of potentially 'Safe NSAIDs' for the treatment of arthritic pain, inflammation and also for the treatment of cardiovascular disorders in the case of NO-aspirin.

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Supplementary data

Supplementary data (Figs. 1, 4–7, S1 and S2, Table S1, experimental details for the synthesis and characterization of all reported compounds and procedures for biological experiments) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.096.

References and notes

1. Schoen, R. T.; Vender, R. J. *Am. J. Med.* **1989**, *86*, 449.
2. Wolfe, M. M.; Lichtenstein, D. R.; Singh, G. *N. Eng. J. Med.* **1999**, *340*, 1888.
3. Bundgaard, H. *Adv. Drug Delivery Rev.* **1989**, *3*, 39.
4. Bundgaard, H. In *Delivery Systems for Peptide Drugs*; Davis, S. S., Illum, L., Tomlinson, E., Eds.; Plenum: New York and London, 1986. pp 49.
5. Maag, H. In *Prodrugs*; Stella, V. J., Borchardt, R. T., Hageman, M. J., Oliyai, R., Maag, H., Tilley, J. W., Eds.; Springer: New York, NY, USA, 2007; pp 3–29. Part 2, Chapter 3.1.
6. Halen, P. K. K.; Murumkar, P. R.; Giridhar, R.; Yadav, M. R. *Mini-Rev. Med. Chem.* **2009**, *9*, 124.
7. Koc, E.; Kucukguzel, S. G. *Mini-Rev. Med. Chem.* **2009**, *9*, 611.
8. Vane, J. R.; Botting, R. M. *Thromb. Res.* **2003**, *110*, 255.
9. Patrono, C. *Platelets*, 3rd ed.; Academic Press, 2013; p 1099. Chapter 53.
10. Bundgaard, H.; Nielsen, N. M.; Buur, A. *Int. J. Pharm.* **1988**, *44*, 151.
11. Hussain, A.; Truelove, J.; Kostenbauder, H. *J. Pharm. Sci.* **1979**, *68*, 299.
12. Loftsson, T.; Kaminski, J. J.; Bodor, N. *J. Pharm. Sci.* **1981**, *70*, 743.
13. Loftsson, T.; Bodor, N. *J. Pharm. Sci.* **1981**, *70*, 750.
14. Nielsen, N. M.; Bundgaard, H. *J. Med. Chem.* **1989**, *32*, 727.
15. Falborg, L.; Senning, A. *Acta Chem. Scand.* **1993**, *47*, 514.
16. Bowden, K.; Huntington, A.; Powell, S. *Eur. J. Med. Chem.* **1998**, *32*, 987.
17. Babin, P.; Bennetau, B. *Tetrahedron Lett.* **2001**, *42*, 5231.
18. Moriarty, L. M.; Lally, M. N.; Carolan, C. G.; Jones, M.; Clancy, J. M.; Gilmer, J. F. *J. Med. Chem.* **2008**, *51*, 7991.
19. Pathak, R. K.; Marrache, S.; Choi, J. H.; Berding, T. B.; Dhar, S. *Angew. Chem., Int. Ed.* **2014**, *53*, 1963.
20. Kubes, P.; Wallace, J. L. *Mediators Inflamm.* **1995**, *4*, 397.
21. Elloitt, S. N.; Wallace, J. L. *J. Gastroenterol.* **1998**, *33*, 792.
22. Barrachina, M. D.; Panes, J.; Esplugues, J. V. *Curr. Pharm. Des.* **2001**, *7*, 31.
23. Stefano, F.; Distrutti, E. *Curr. Top. Med. Chem.* **2007**, *7*, 277.
24. Cena, C.; Lolli, M. L.; Lazzarato, L.; Guaita, E.; Morini, G.; Coruzzi, G.; McElroy, S. P.; Megson, I. L.; Fruttero, R.; Gasco, A. *J. Med. Chem.* **2003**, *46*, 747.
25. Gilmer, J. F.; Moriarty, L. M.; Clancy, J. M. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3217.
26. Jones, M.; Inkielewicz, I.; Medina, C.; Santos-Martinez, M. J.; Radomski, A.; Radomski, M. W.; Lally, M. N.; Moriarty, L. M.; Gaynor, J.; Carolan, C. G.; Khan, D.; O'Byrne, P.; Harmon, S.; Holland, V.; Clancy, J. M.; Gilmer, J. F. *J. Med. Chem.* **2009**, *52*, 6588.
27. Lazzarato, L.; Donnola, M.; Rolando, B.; Chegaev, K.; Marini, E.; Cena, C.; Di Stilo, A.; Fruttero, R.; Biondi, S.; Ongini, E.; Gasco, A. *J. Med. Chem.* **2009**, *52*, 5058.
28. Lazzarato, L.; Chegaev, K.; Marini, E.; Rolando, B.; Borretto, E.; Guglielmo, S.; Joseph, S.; Di Stilo, A.; Fruttero, R.; Gasco, A. *J. Med. Chem.* **2011**, *54*, 5478.
29. Rolando, B.; Lazzarato, L.; Donnola, M.; Marini, E.; Joseph, S.; Morini, G.; Pozzoli, C.; Fruttero, R.; Gasco, A. *ChemMedChem* **2013**, *8*, 1199.
30. Nemmani, K. V. S.; Mali, S. V.; Borhade, N.; Pathan, A. R.; Karwa, M.; Pamidiboina, V.; Senthilkumar, S. P.; Gund, M.; Jain, A. K.; Mangu, N. K.; Dubash, N. P.; Desai, D. C.; Sharma, S.; Satyam, A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5297.
31. Pathan, A. R.; Karwa, M.; Pamidiboina, V.; Deshattiwar, J. J.; Deshmukh, N. J.; Gaikwad, P.; Mali, S. V.; Desai, D. C.; Dhiman, M.; Mariappan, T. T.; Sharma, S.; Satyam, A.; Nemmani, K. V. S. *Inflammopharmacology* **2010**, *18*, 157.
32. Borhade, N.; Pathan, A. R.; Halder, S.; Karwa, M.; Dhiman, M.; Pamidiboina, V.; Gund, M.; Deshattiwar, J. J.; Mali, S. V.; Deshmukh, N. J.; Senthilkumar, S. P.; Gaikwad, P.; Tipparam, S. G.; Mudgal, J.; Dutta, M. C.; Burhan, A. U.; Thakre, G.; Sharma, A.; Deshpande, S.; Desai, D. C.; Dubash, N. P.; Jain, A. K.; Sharma, S.; Nemmani, K. V. S.; Satyam, A. *Chem. Pharm. Bull.* **2012**, *60*, 465.
33. Doel, J. J.; Benjamin, N.; Hector, M. P.; Rogers, M.; Allaker, R. P. *Eur. J. Oral Sci.* **2005**, *113*, 14.
34. Lundberg, J. O.; Weitzberg, E.; Gladwin, M. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 156.
35. Ferris, A. F.; McLean, K. W.; Marks, I. G.; Emmons, W. D. *J. Am. Chem. Soc.* **1953**, *75*, 4078.
36. Al-Swayeh, O. A.; Clifford, R. H.; Del Soldato, P.; Moore, P. K. *Br. J. Pharmacol.* **2000**, *129*, 343.
37. Takeuchi, K.; Ukawa, H.; Konaka, A.; Kitamura, M.; Sugawa, Y. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 115.
38. Bandgar, B. P.; Sarangdhar, R. J.; Viswakarma, S.; Ahamed, F. A. *J. Med. Chem.* **2011**, *54*, 1191.
39. Bandgar, B. P.; Sarangdhar, R. J.; Ahamed, F. A.; Viswakarma, S. *J. Med. Chem.* **2011**, *54*, 1202.
40. Huang, Z.; Velázquez, C. A.; Abdellatif, K. R.; Chowdhury, M. A.; Reisz, J. A.; DuMond, J. F.; King, S. B.; Knaus, E. E. *J. Med. Chem.* **2011**, *54*, 1356.
41. Cox, D.; Maree, A. O.; Dooley, M.; Conroy, R.; Byrne, M. F.; Fitzgerald, D. J. *Stroke* **2006**, *37*, 2153.
42. Patrignani, P.; Filabozzi, P.; Patrono, C. *J. Clin. Invest.* **1982**, *69*, 1366.
43. Esser, R.; Berry, C.; Du, Z.; Dawson, J.; Fox, A.; Fujimoto, R. A.; Haston, W.; Kimble, E. F.; Koehler, J.; Peppard, J.; Quadros, E.; Quintavalla, J.; Toscano, K.; Urban, L.; Duzer, J. V.; Zhang, X.; Zhou, S.; Marshall, P. J. *Br. J. Pharmacol.* **2006**, *144*, 538.