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A comparison of the cyclooxygenase inhibitor-NO donors (CINOD), NMI-1182 and AZD3582, using in vitro biochemical and pharmacological methods

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Abstract

Cyclooxygenase (COX, EC 1.14.99.1) inhibitor-nitric oxide (NO) donor (CINOD) hybrid compounds represent an attractive alternative to NSAID and coxib therapy. This report compares two CINODs, NMI-1182 (naproxen–glyceryl dinitrate) and AZD3582 (naproxen–*n*-butyl nitrate), for their ability to inhibit COX-1 and -2, deliver bioavailable nitric oxide, and release naproxen, using in vitro biochemical and pharmacological methods. In human whole blood, both CINODs showed inhibition, comparable to naproxen, of both COX isozymes and slowly released naproxen. Both CINODs donated bioavailable NO, as detected by cGMP induction in the pig kidney transformed cell line, LLC-PK1, but NMI-1182 was more potent by 30–100 times than AZD3582, GTN, GDN, and ISDN and considerably faster in inducing cGMP synthesis than AZD3582. The nitrate groups of GTN, NMI-1182, and AZD3582 appeared to be bioactivated via a common pathway, since each compound desensitized LLC-PK1 cells to subsequent challenge with the other compounds. Similar cGMP induction also occurred in normal, untransformed cells (human renal proximal tubule epithelial cells and hepatocytes from man, rat, and monkey); again, NMI-1182 was superior to AZD3582. NMI-1182 was also the more metabolically labile compound, releasing more absolute nitrate and nitrite (total NO_x) in human stomach (in which NO is salutary) and liver S9 homogenates. Naproxen was also more rapidly freed from NMI-1182 than AZD3582 in human stomach, although liver S9 hydrolyzed both CINODs with similar rates. These in vitro tests revealed that NMI-1182 may be a better CINOD than AZD3582 because of its superior NO donating and naproxen liberating properties.
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Keywords: Nitric oxide (NO); Nitric oxide donor; Cyclooxygenase (COX) inhibitor; CINOD; Coxib therapy; Naproxen

1. Introduction

Cyclooxygenases (Prostaglandin G/H Synthase, COX, EC 1.14.99.1) are enzymes responsible for the synthesis of the first member of the prostaglandin pathway from arachidonic acid. The discovery that there are two isoforms of cyclooxygenase, COX-1 and -2, and the realization that COX-1 has salutary properties in stomach, while COX-2 is induced in inflammation led to the creation of COX-2 selective inhibitors, also termed coxibs, for the treatment of chronic inflammation and pain [1,2]. Because coxibs spared COX-1, it was thought they would avoid the major problem of gastric ulceration/bleeding associated with the non-selective COX inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs). The coxibs, celecoxib (Celebrex®)[3], rofecoxib (Vioxx®)[4], valdecoxib (Bextra®)[5], and etoricoxib (Arcoxia®)[6] have been introduced for therapeutic use. However, a recent clinical

Abbreviations: cGMP, cyclic guanosine 3′,5′-monophosphate; CINOD, COX inhibitor NO donor; COX, cyclooxygenase; GI, gastrointestinal; GDN, glyceryl dinitrate; GTN, glyceryl trinitrate; HPLC, high pressure liquid chromatography; ISDN, isosorbide dinitrate; LPS, lipopolysaccharide; NO, nitric oxide; NO_x, nitrate and nitrite; NSAID, non-steroidal anti-inflammatory drug; RPTEC, renal proximal tubule epithelial cells; SOD, super oxide dismutase

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trial with rofecoxib has exposed the potential cardiovascular risk of COX-2 selective inhibitors [7–9] and led to its withdrawal. It is postulated that very selective COX-2 inhibitors may totally shut down prostacyclin synthesis (due to COX-2) in the vascular endothelium with no accompanying inhibition of thromboxane synthesis (by COX-1) in platelets, resulting in a pro-thrombotic environment [1].

Accordingly, an alternative to coxib therapy is warranted. This new drug therapy should be as effective as NSAIDs in alleviating inflammation and pain with the same, proven, safe, cardiovascular profile, but without the complication of gastric ulceration. One possible approach is the use of cyclooxygenase-inhibitor-nitric oxide donor (CINOD) hybrid compounds ([10,11] and Fig. 1), in which a NSAID is tethered through its carboxyl group in an ester or amide linkage to a nitric oxide (NO) donating moiety. Such NO donating compounds would be GI safe, because of the gastroprotective properties of NO (enhanced mucous production and increased blood flow secondary to vasodilation [11–15]), as well as cardiovascular-protective [16,17], due to the anti-thrombotic [18–20], anti-platelet [21,22], anti-hypertensive [16], and anti-inflammatory [23] action of NO. Furthermore, hydrolysis of the tether linkage would liberate the NSAID, allowing it to act as a COX inhibitor, but unlike a coxib, with inhibition of both COX-1 and -2. Such dual inhibition outside of the GI tract has been accepted over many years of therapeutic usage to be anti-inflammatory and analgesic without added cardiovascular risk. For example, the NSAID, naproxen, has been shown to have relatively low cardiovascular risk [24,25].

Recently, a CINOD, AZD3582 [15,26], in which naproxen is linked via a *n*-butyl group to a mononitrate (Fig. 1), has undergone clinical trials. This report compares AZD3582 with a novel CINOD, NMI-1182 (Fig. 1), in which naproxen is tethered to glyceryl dinitrate (GDN), for their ability to release the NO moiety with donation of bioavailable NO and to liberate naproxen in in vitro human tissue extracts and relevant human and animal cells.

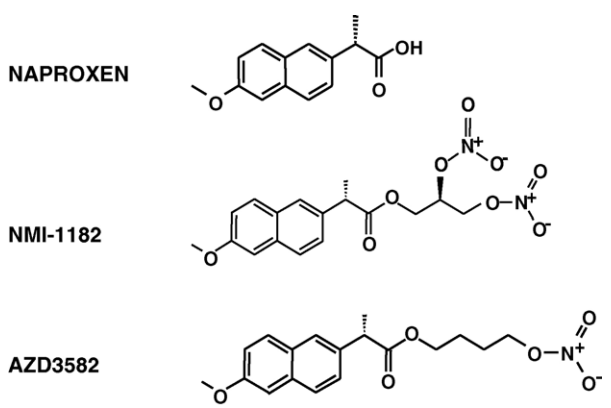

Fig. 1. Chemical structures of naproxen, NMI-1182, and AZD3582.

2. Materials and methods

2.1. Materials

Human whole blood was collected in sodium heparin from NSAID-free (for 2 weeks) staff members at a local hospital. Human stomach mucosal lining S9 fractions were prepared from quick-frozen, whole stomach autopsy specimens from NDRI (Philadelphia, PA) by scraping the mucosal lining and Dounce homogenization in 100 mM Tris–HCl buffer, pH 7.4, 2 mM EDTA, 0.3 mM dithiothreitol (DTT) supplemented with a Protease Inhibitor Cocktail (Roche Diagnostics, Hague Road, IN). The homogenate was centrifuged at 9000 × *g* for 30 min at 4 °C; the supernatant was dialyzed against 10 mM Tris–HCl buffer, pH 7.4, 0.2 mM EDTA, 0.03 mM DTT and stored frozen at −80 °C. Human liver S9 fractions, pooled from 15 individual livers, was purchased from In Vitro Technologies (Baltimore, MD). NMI-1182 ((2*R*)-2,3-bis(nitrooxy)propyl (2*S*)-2-(6-methoxy(2-naphthyl)propanoate), AZD3582 (4-(nitrooxy)butyl (2*S*)-2-(6-methoxy(2-naphthyl))propanoate), and naproxen were synthesized at NitroMed (Lexington, MA). LLC-PK1 (CRL-1392, grown in DME/F12 with 10% heat-inactivated fetal bovine serum or FBS) and human renal proximal tubule epithelial cells (RPTEC, grown in REGM medium without hydrocortisone) were acquired from ATCC (Rockville, MD) and CAMBREX (Walkersville, MD), respectively. Plated human hepatocytes (maintained in hepatocyte incubation medium, HIM) were provided by In Vitro Technologies, CAMBREX, and CellzDirect (Tucson, AZ). DME/F12-FBS medium, REGM, and HIM were the products of Invitrogen Life Technologies (Rockville, MD), CAMBREX, and In Vitro Technologies, respectively. Lipopolysaccharide (LPS, *E. coli* serotype 026:B6), A23187 (calcium ionophore), isobutylmethylxanthine (IBMX), zaprinast, superoxide dismutase (SOD), glutathione, β-NADP, β-D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (Type VII ammonium sulfate suspension) were bought from Sigma (St. Louis, MO). Sildenafil HCl was prepared at NitroMed from Viagra® tablets (Pfizer, Groton, CT). Glyceryl trinitrate (GTN), dissolved at 5 mg/ml in 30% ethanol, 30% propane-1,2-diol, and 40% water, was the commercial product (Nitroject®) of Omega (Montreal, CN).

2.2. Five hour human whole blood COX-1, -2 inhibition assay

This assay for the inhibition of COX-1 and -2 enzyme activity in human whole blood has been previously described [27,28]. Briefly, human whole blood from donors who had not received any NSAIDs for 2 weeks was collected in sodium heparin (20 units/ml). Test compounds (dissolved in DMSO; final concentration of

DMSO 0.1%) at various concentrations were added in duplicate to 1 ml aliquots of blood in 24 well plates. After 15 min incubation in a 37 °C, CO_2 incubator, LPS at 10 μg/ml was added to select wells to induce COX-2. At 4.5 h after test compound addition, A23187 was added at 25 μM to select wells to activate COX-1. (Vehicle control wells received equal volumes of DMSO.) At 30 min after A23187 addition, all reactions were terminated by placing the 24 well plates on ice and adding 2 mM EGTA. Plasma was collected and extracted with methanol overnight at −20 °C. After evaporation thromboxane B_2 (TXB_2) in each sample was measured in duplicate via EIA (Thromboxane B_2 EIA Kit, Cayman Chemical, Ann Arbor, MI). The results were normalized against the vehicle control values and expressed as % control.

2.3. Cellular cGMP induction

Hepatocytes, LLC-PK1, or RPTEC cells (~1.5–3.0 × 10^5 cells per well of 24 well plates) were pre-treated for 30 min with phosphodiesterase (PDE) inhibitors by replacement of their media with fresh media containing the appropriate inhibitors (1 mM IBMX/200 μM sildenafil HCl for LLC-PK1 cells and 1 mM IBMX/1 mM zaprinast for hepatocytes and RPTEC cells). SOD (200 units/ml) was added for the last 5 min of the pre-treatment. Test compounds were dissolved and diluted in DMSO (final DMSO concentration, 0.1%), except for GTN which was diluted in its own solvent (see Section 2.1 above). Reactions were initiated with the addition of the test compounds, including DMSO as vehicle control, and incubated at 37 °C in a CO_2 incubator for 15 min (except for Fig. 5). Reactions were terminated by replacement of the media with 0.3 ml of 0.1N HCl and, after extraction with HCl at room temperature for 1 h, the plates with the samples were stored frozen at −80 °C. For assay, the thawed plates were centrifuged and the samples collected and assayed for cGMP by competitive EIA (Format A cGMP EIA Kit, BIOMOL, Plymouth Meeting, PA). The vehicle control values, which represented basal cGMP levels, were subtracted from all results.

2.4. Desensitization of LLC-PK1 cells by pre-treatment with nitrates

LLC-PK1 cells in 24 well plates were exposed to vehicle, 10 μM GTN, 30 μM NMI-1182, or 30 μM AZD3582 for 5 h. The media were then washed out with unsupplemented medium and replaced with IBMX/sildenafil/SOD medium. Incubation of the cells continued with vehicle, 30 μM GTN, NMI-1182, or AZD3582 for 15 min. After termination of the incubations by withdrawal of the media and addition of 0.1N HCl, the cGMP was measured as above.

2.5. Tissue S9 homogenate incubations for naproxen and nitrite/nitrate (total NO_x) release

Test compounds (at 70 μM for NO_x formation or 35 μM for naproxen release) or DMSO (0.1%, as vehicle control) were incubated in reaction mixtures (total volume of 300 μl) consisting of tissue (stomach, liver) S9 homogenates (10 mg protein/ml) in 50 mM Tris–HCl, pH 7.4 buffer supplemented with reduced glutathione (1 mM) and a NADPH regenerating system (NRS), comprised of 425 μg/ml of β-NADP, sodium salt, 1.95 mg/ml of β-D-glucose-6-phosphate, sodium salt, and 375 munits/ml of glucose-6-phosphate dehydrogenase in 0.5% $NaHCO_3$. The NRS formulation was taken from the In Vitro Technologies (supplier of microsomes and S9) protocol ''Instructions for Using Microsomes and S9 Fractions'' (November 27, 2001). The reactions in open microfuge tubes were incubated in a 37 °C, ambient atmosphere incubator on a gently shaking platform shaker for the indicated times. To terminate the NO_x formation reactions, the reaction tubes were capped, placed in boiling water baths for 5–10 min, and stored frozen at −80 °C. Prior to assay, the tubes were centrifuged at 15,000 × *g* for 10 min at 4 °C and the supernatants filtered through Millipore (Bedford, MA) 10 K mw cutoff centrifuge filter units. The filtrates were assayed for nitrite/nitrate by the Greiss reaction (see below). The naproxen release reactions were terminated by adding 300 μl acetonitrile (ACN) to each reaction mixture, vortexing, centrifugation at 15,000 × *g* for 10 min at 22 °C, and filtering twice through membrane filters. The filtrates were stored at −80 °C prior to assay by HPLC.

2.6. Human whole blood incubations for naproxen and nitrite/nitrate (total NO_x) release

Aliquots of human whole blood were incubated at 37 °C in a CO_2 incubator with the test compounds (at 100 μM) and the vehicle, DMSO (0.1%), for the indicated times and placed on ice to stop the reaction. Plasma was prepared at 4 °C from each aliquot and stored frozen at −80 °C. The plasma samples were ultrafiltered through Millipore 10 K cutoff centrifuge filter units and the filtrates measured for total NO_x by the Greiss reaction.

2.7. Greiss reaction for nitrite/nitrate (total NO_x)

Samples processed as above were assayed for nitrite alone and nitrate separately (by reduction of nitrate to nitrite with a bacterial nitrate reductase) to yield total NO_x (nitrite/nitrate) via the Greiss Reaction using the Nitrate/Nitrite Colorimetric Assay Kit supplied by Cayman Chemical Co. (Ann Arbor, MI). Results were expressed after subtracting the vehicle control (0.1% DMSO) values, which represented basal nitrate levels.

2.8. HPLC analysis of naproxen, NMI-1182, and AZD3582

Naproxen, NMI-1182, and AZD3582 from the ACN-treated samples were analyzed by HPLC (Shimadzu 10Avp) in a water-ACN (0.05% TFA) gradient, using a Supelcosil® LC-F column (25 cm × 4.6 mm × 5 μm). The metabolites were detected by absorbance at 232 nm.

2.9. Statistical analysis

Most experiments were the averages of three experiments ±S.E.M., except where noted otherwise. (The values taken from each individual experiment were also the means of three or more determinations.) Significant differences were evaluated by one- or two-way ANOVA, followed by the Newman–Keuls test or the Bonferroni post test, as indicated. The calculated *p*-values are shown in the legends.

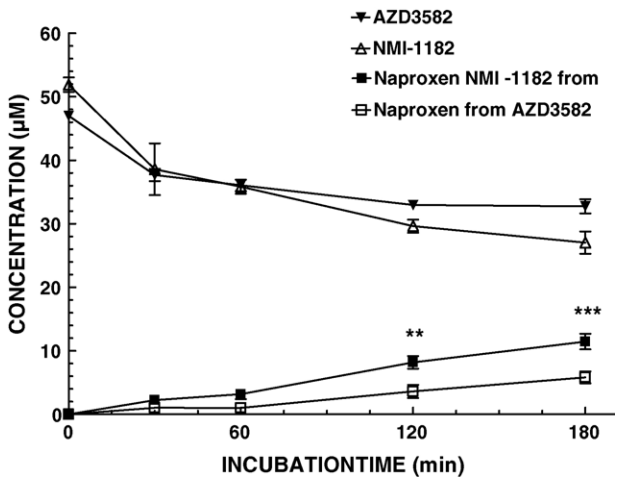

Fig. 3. Naproxen was slowly released from NMI-1182 and AZD3582 in human whole blood. NMI-1182 and AZD3582 were incubated at 35 μM in human whole blood for the indicated times. Aliquots of blood were extracted with an equal volume of ACN. After centrifugation, the supernatants were filtered and analyzed for the original compound or naproxen by HPLC. The results were the averages ±S.E.M. of three experiments; $^{**}p < 0.01$ and $^{***}p < 0.001$ between naproxen from NMI-1182 and naproxen from AZD3582 by two-way ANOVA, Bonferroni post test.

3. Results

3.1. NMI-1182, AZD3582, and naproxen as comparable COX inhibitors: inhibition of COX-1 and -2 activity in human whole blood

Naproxen, NMI-1182, and AZD3582 dose dependently inhibited COX-1 and -2 with comparable efficacy (Fig. 2). There was no statistically significant difference between the dose response curves for all three compounds. The ester bonds linking the nitrate tethers to naproxen in both NMI-1182 and AZD3582 were hydrolyzed by esterases in whole blood, albeit at a slow rate (Fig. 3). Concurrent with the appearance of naproxen was the disappearance of the parent NMI-1182 and AZD3582 molecules (Fig. 3). The slow liberation of naproxen suggests that some, but,

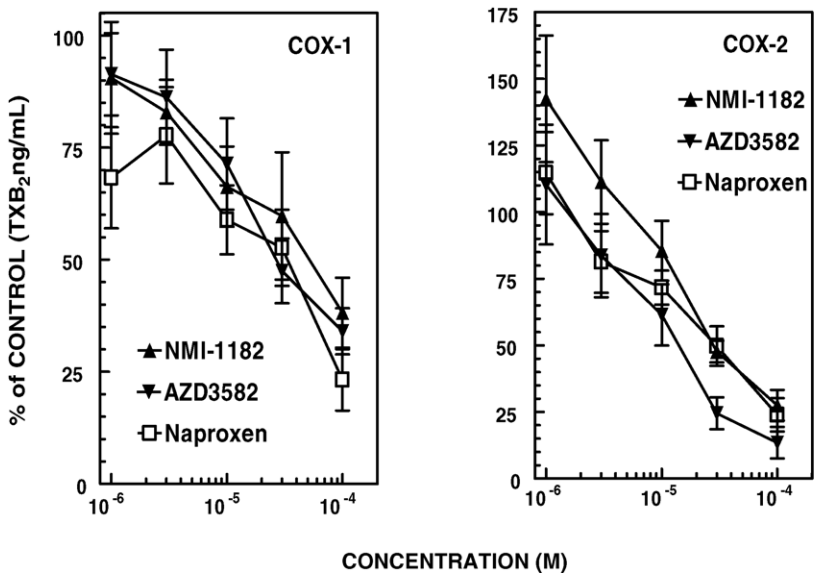

Fig. 2. NMI-1182, AZD3582, and naproxen showed comparable inhibition of COX-1 and -2 in human whole blood. COX-1 and -2 were activated or induced in human whole blood in the presence of varying concentrations of these three compounds in the 5 h human whole blood COX-1, -2 inhibition assay described in Section 2. Thromboxane B_2 (TXB_2) at ng/ml was measured by EIA and all values were normalized with respect to control (% of control). The absolute control values varied between human blood donors used in separate assays, but the averages were 297 ± 62 (S.E.M.) and 30 ± 6.4 (S.E.M.) for COX-1 and -2, respectively. The % of control values were the means ± S.E.M. ($n = 6$). There was no statistical difference between any of the concentration curves for either COX-1 or -2 inhibition, as determined by one-way ANOVA, Newman–Keuls multiple comparison test.

perhaps not all, of the inhibition of COX-1 and -2 by NMI-1182 and AZD3582 may be attributable to free naproxen.

3.2. NMI-1182 and AZD3582 as NO donors

To qualify as a NO donor, these CINODs must demonstrate the capability of releasing nitric oxide. This can be routinely done by the activation of soluble guanylate cyclase, the iron-heme group of which is considered the primary receptor of nitric oxide. On activation by NO, soluble guanylate cyclase synthesizes cGMP, which is then taken as an indirect measure of nitric oxide. The demonstration that these CINODs donate bioavailable NO is complicated by the requirement of organic nitrates for enzymatic bioactivation to NO [29]. Fortunately, this can be accomplished using the transformed pig kidney epithelial cell line, LLC-PK1, which has the two required enzymatic systems: organic nitrate reductases for bioactivation of organic nitrates to release NO and soluble guanylate cyclase for the detection of the generated NO by cGMP induction [26,30]. When NMI-1182 and AZD3582 were tested for their ability to donate NO in LLC-PK1 cells, it was found that NMI-1182 was a more potent NO donor than not only AZD3582, but also the classic organic nitrates, GTN, 1,2-GDN, and ISDN, with detectable cGMP induction occurring in the presence of as little as 3 nM NMI-1182 (Fig. 4). NMI-1182 was 30–100 times more potent than the other organic nitrate NO donors. The rate of induction of cGMP by NMI-1182 was also much faster than that of AZD3582, although it was similar to the classic organic nitrates tested (Fig. 5).

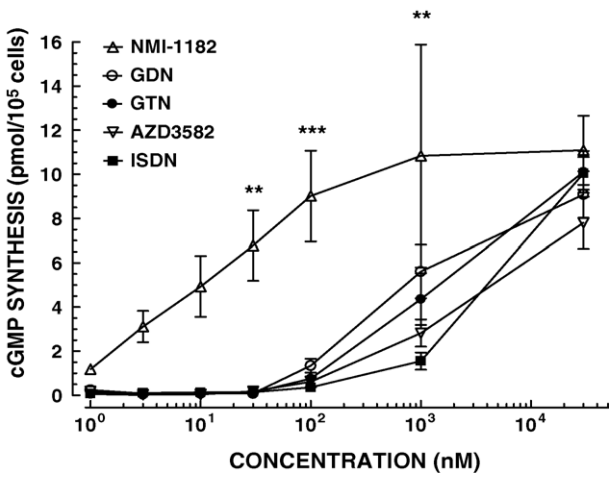

Fig. 4. NMI-1182 was more potent than AZD3582 and the classic nitrates, GTN, 1,2-GDN, and ISDN in stimulating cGMP synthesis in LLC-PK1 cells. Induction and synthesis of cGMP in LLC-PK1 cells by these compounds were measured as described in detail in Section 2. The results were the averages ±S.E.M. of three experiments (each performed in triplicate); $^{**}p < 0.01$ and $^{***}p < 0.001$ between the indicated concentrations of NMI-1182 and AZD3582 by two-way ANOVA, Bonferroni post test.

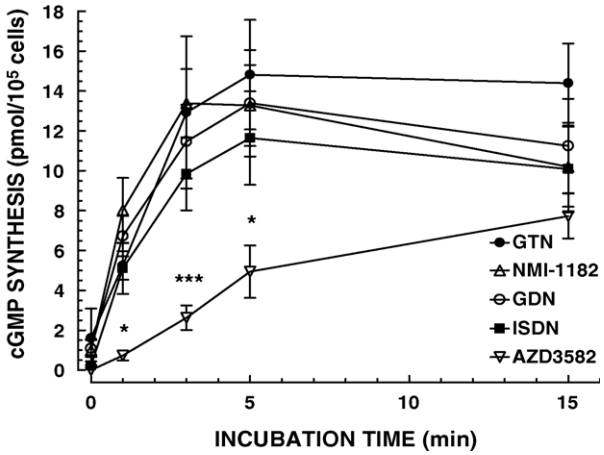

Fig. 5. NMI-1182 stimulated cGMP synthesis in LLC-PK1 cells faster than AZD3582. The kinetics of cGMP synthesis in LLC-PK1 cells were measured as in Fig. 4 by terminating the reactions at the indicated times. The results were the averages ±S.E.M. of three experiments (each performed in triplicate); $^{*}p < 0.05$ and $^{***}p < 0.001$ between NMI-1182 and AZD3582 at the indicated times, by two-way ANOVA, Bonferroni post test.

It has been proposed by Schröder and co-workers [26] that the bioactivation of AZD3582 in LLC-PK1 cells occurs via the same pathway that bioactivates GTN. This was revealed through the mutual desensitization of the putative bioactivation pathway by pre-treatment with either GTN or AZD3582. In the present study, NMI-1182 also showed similar desensitization with GTN and AZD3582 (Fig. 6), suggesting a common bioactivation pathway. In this case, pre-treatment with GTN at 10 μM (a higher concentration than used by Schröder), NMI-1182 or AZD3582 (both at 30 μM) greatly reduced subsequent cGMP induction by each of the three compounds at 30 μM. AZD3582 was the most suppressed, while NMI-1182 the

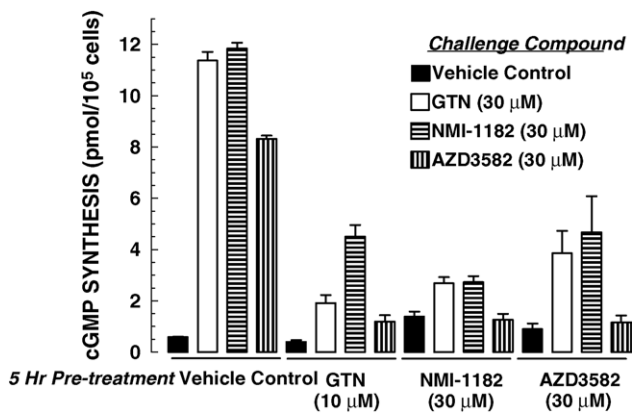

Fig. 6. GTN, NMI-1182, and AZD3582 desensitized LLC-PK1 cells to further stimulation of cGMP induction by the other NO donors. The cells were pre-treated for 5 h with vehicle (0.1% DMSO), 10 μM GTN, 30 μM NMI-1182, or 30 μM AZD3582 (*x*-axis reagents), then after replacement of the media with fresh media with IBMX/sildenafil/SOD, challenged with DMSO (0.1%), GTN, NMI-1182, or AZD3582, all three at 30 μM, for 15 min. cGMP was measured as described in detail in Section 2. The results were the averages ±S.E.M. of three experiments (each performed in triplicate).

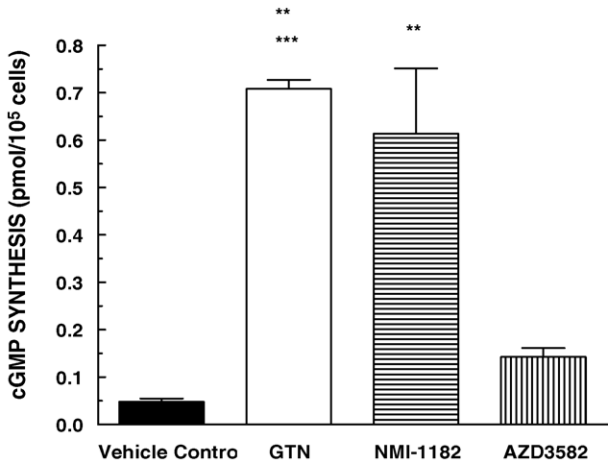

Fig. 7. NMI-1182 was comparable to GTN and superior to AZD3582 in cGMP induction in human renal proximal tubule epithelial cells (RPTEC). The RPTEC cells from In Vitro Technologies were used within eight cumulative population doublings from the frozen cryovial. The results were the averages ±S.E.M. of three experiments (each performed in triplicate); $^{**}p < 0.01$ vs. AZD3582 and $^{***}p < 0.001$ vs. vehicle control (0.1% DMSO) by two-way ANOVA, Bonferroni post test.

least, perhaps, reflecting the greater potency (Fig. 4) of the latter compound.

These CINODs can also be shown to donate bioavailable NO, as detected by cGMP induction, in relevant, normal, untransformed cells. RPTEC cells play a major role in maintaining water and salt balance in the nephron. These cells respond to NO as part of this homeostatic role. In cell culture they synthesized cGMP when exposed to GTN, NMI-1182, and AZD3582 (Fig. 7). Again, the responses of GTN and NMI-1182 were comparable and significantly greater than that of AZD3582.

The liver is considered a major organ for nitrate metabolism. Tissue culture plated, human, cynomolgus monkey, and rat hepatocytes also synthesized cGMP upon treatment with NMI-1182, AZD3582, and GTN and, again, the amounts of cGMP induced by GTN and NMI-1182 were significantly greater than that of AZD3582 (Table 1). It should be noted that there was good agreement between the three species.

3.3. Metabolic fate of the CINODs, NMI-1182, and AZD3582

These CINODs, which consist of nitrate tethers linked to naproxen through an ester bond, have two sites of metabolic instability: the nitrate moiety which can be reduced or bioactivated to NO, which eventually appears as inorganic nitrite and nitrate, and the ester bond which upon hydrolysis liberates naproxen. For a CINOD to be gastroprotective, it must release a significant portion of its NO in the stomach; for it to have the same anti-inflammatory pharmacodynamic properties as its parent NSAID, it must release its NSAID quickly to the circulation. To answer these questions, human tissues (whole blood) or tissue homogenates (S9 of stomach and liver) were obtained and the CINODs were incubated with these tissues or homogenates in vitro. Human stomach S9 homogenates reduced over a third (~50 μM out of a possible 140 μM, as measured by the Greiss reaction) of the nitrate moiety in NMI-1182 by 30 min and over half by 1 h (Fig. 8). AZD3582 released less than half as much NO_x in absolute amounts (although, proportionally, an equivalent amount, given that NMI-1182 is a dinitrate and AZD3582 a mononitrate) over the same time period. On the other hand, human whole blood (Fig. 8) had little organic nitrate reductase activity for these CINODs. Hydrolysis of the ester link in NMI-1182 occurred more readily in human stomach S9, freeing more naproxen than AZD3582 (Fig. 9). Again, as shown in Fig. 3, the appearance of naproxen was matched by the disappearance of the parent NMI-1182.

Table 1
cGMP synthesis (as %GTN) induced by NMI-1182 and AZD3582 in human, monkey, and rat hepatocytes

	Vehicle	NMI-1182	AZD3582
Human hepatocytes	1.7 ± 0.9	64.9 ± 5.7$^{***,\#}$	12.7 ± 1.2*
Cynomolgus monkey hepatocytes	8.0 ± 0.1	73.6 ± 0.3$^{***,\#}$	24.4 ± 0.2***
Rat hepatocytes	14.1 ± 0.1	86.6 ± 0.1$^{***,\#}$	26.3 ± 0.1***

The experimental details are described in Section 2. All test compounds were added at 70 μM. The results were the averages ±S.E.M. of three experiments (each performed in triplicate), except for monkey hepatocytes which were averages of four experiments. The absolute GTN values (fmol/10^5 cells ± S.E.M.) were 382 ± 138, 153 ± 66, and 209 ± 60 for human, monkey, and rat, respectively.
* One-way ANOVA, Newman–Keuls test: $p < 0.05$ vs. vehicle.
*** One-way ANOVA, Newman–Keuls test: $p < 0.001$ vs. vehicle.
$^{\#}$ One-way ANOVA, Newman–Keuls test: $p < 0.001$ vs. AZD3582.

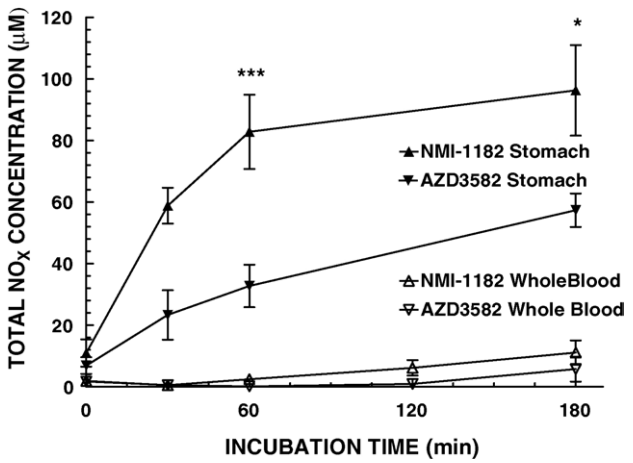

Fig. 8. NMI-1182 generated more nitrate and nitrite (total NO_x) than AZD3582 in human stomach S9 homogenates, but both produced little NO_x in human whole blood. Tissue S9 homogenate and human whole blood incubations of test compounds for NO_x were performed as described in detail in Section 2. The results were the averages ±S.E.M. of three experiments (each performed in triplicate); $^{*}p < 0.05$ and $^{***}p < 0.001$ between stomach total NO_x values for NMI-1182 and AZD3582 at the indicated times, by two-way ANOVA, Bonferroni post test.

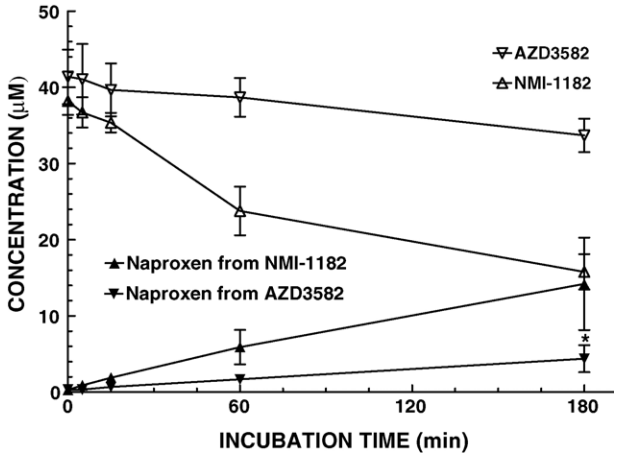

Fig. 9. NMI-1182 was more readily hydrolyzed than AZD3582 to release naproxen during incubation with human stomach S9 homogenates. Incubation of test compounds (added at 35 μM) in human stomach S9 and HPLC analysis for freed naproxen and remaining parent test compounds were performed as described in detail in Section 2. The results were the averages ±S.E.M. of three experiments; $^{*}p < 0.05$ between naproxen values for NMI-1182 and AZD3582 at the indicated time, by two-way ANOVA, Bonferroni post test.

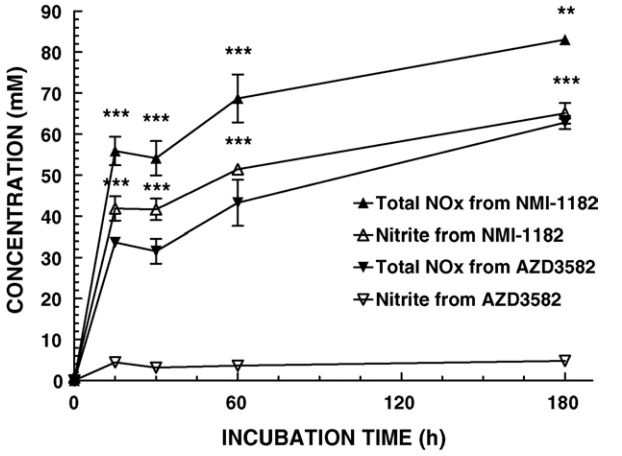

Fig. 11. NMI-1182 produced more absolute total NO_x, much of it as nitrite, than AZD3582; most of the AZD3582 NO_x was in the form of nitrate, not nitrite in human liver S9 homogenates. Liver S9 homogenate incubations of test compounds (added at 70 μM) for NO_x and nitrite were performed as described in detail in Section 2. The results were the averages ±S.E.M. of three experiments (each performed in triplicate); $^{**}p < 0.01$ and $^{***}p < 0.001$ comparing total NO_x from NMI-1182 vs. AZD3582 (closed symbols) or nitrite released by NMI-1182 vs. AZD3582 (open symbols) at the indicated times, by two-way ANOVA, Bonferroni post test.

In keeping with the liver's role as the major metabolizing organ for xenobiotics, human liver S9 homogenates rapidly hydrolyzed the tether esters in both CINODs equally well, releasing all of the naproxen by 30 min (Fig. 10). The nitrate moieties were also rapidly reduced for both CINODs (Fig. 11) at a faster rate than seen in stomach S9 (Fig. 8), releasing about half the available NO_x by 15 min. However, again in absolute terms, NMI-1182 produced about twice as much total NO_x than AZD3582 because of its dinitrate load. It is interesting to note that much of the NO_x released by NMI-1182 was in the form of nitrite, while mostly nitrate and very little nitrite accumulated from AZD3582 (Fig. 11).

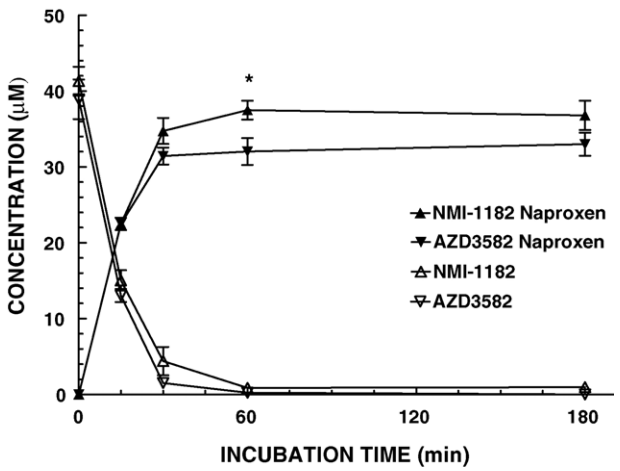

Fig. 10. Both NMI-1182 and AZD3582 were rapidly hydrolyzed to release naproxen in human liver S9 homogenates. Experimental details were as described in Fig. 9. The results were the averages ±S.E.M. of three experiments; $^{*}p < 0.05$ between naproxen values for NMI-1182 and AZD3582 at the indicated time, by two-way ANOVA, Bonferroni post test.

4. Discussion

Coxibs, COX-2 selective inhibitors, once held the hope of being gastro-sparing, highly efficacious, anti-inflammatory pain-killers, but the cardiovascular problems associated with coxib therapy have greatly diminished their promise and created the need for an alternative strategy. Due to their capacity to inhibit both COX-1 and -2 and to release gastroprotective NO in the stomach, CINODs have the potential to provide efficacious medicine that is safe for the gastrointestinal and cardiovascular systems. In a recent study [31], both NMI-1182 and AZD3582 were shown to produce significantly fewer gastric lesions in rats after oral dosing than either naproxen or the des-NO form of NMI-1182. The present report compares a new CINOD, NMI-1182, with AZD3582, using in vitro methodology and human cells and tissues, when available, for their potential as CINODs and provides evidence to suggest that NMI-1182 might prove to be a superior CINOD.

To fulfill effectively its dual functions, a CINOD must inhibit cyclooxygenases either directly or by rapidly releasing its NSAID into the circulation. It must also be a potent donor of bioavailable NO, delivering a substantial proportion of its gastro-protective NO to the stomach, the primary site of the ulceration and bleeding caused by non-selective COX inhibitors. As inhibitors of COX-1 and -2, both CINODs tested here were statistically comparable to naproxen and each other in the in vitro human whole blood COX assay. While both compounds were slowly hydrolyzed in blood, releasing free naproxen, NMI-1182 was the slightly better esterase substrate in blood with slightly faster naproxen release kinetics than AZD3582. On the other hand, the difference in esterase susceptibility was

much more pronounced in human stomach S9 homogenates. Here, NMI-1182 was clearly more rapidly hydrolyzed to liberate free naproxen. These results, coupled with the rapid appearance of naproxen in human liver S9, would suggest that the majority of NMI-1182 would quickly give up its NSAID, naproxen, to the general circulation.

In the matter of NO donation, NMI-1182 is the superior NO donor. Nitric oxide donation from both CINODs was demonstrated by the activation of soluble guanylate cyclase and the resultant synthesis of cGMP in LLC-PK1 and other cell types. Although the enzymatic systems required for the bioactivation of organic nitrates to bioavailable NO have not been definitively identified in LLC-PK1 cells [26] and the general process is still poorly understood [29], both CINODs appeared to be bioactivated to NO by the same pathway as GTN in LLC-PK1 cells. This conclusion was revealed by their mutual desensitization which may involve the down regulation of a common protein(s) [26]. Nevertheless, NMI-1182 was more potent and faster in stimulating cGMP synthesis than AZD3582. As a donor of bioavailable NO, NMI-1182 is more like GTN than AZD3582, a fact further confirmed when these compounds were tested for cGMP stimulation in normal, non-transformed human and animal kidney and liver cells.

Measurements of nitrate and nitrite (total NO_x) released in stomach and liver homogenates revealed that NMI-1182 produced more than twice as much total NO_x as AZD3582. This, of course, can be attributed to the stoichiometric advantage of a dinitrate over a mononitrate, such that, on a proportionate basis, the percentage of total available nitrate released was comparable for both CINODs. However, one should still appreciate the greater absolute amounts of NO_x produced by a dinitrate over a mononitrate on an equal molar dosage basis, and, in the case of NMI-1182, delivered in the stomach, the targetted organ.

An additional advantage that accrues from NMI-1182's dinitrate structure is the potential accumulation of a more metabolically stable mononitrate, either in the form of the naproxen tethered parental molecule, or, more likely, because of hydrolysis of the ester link, as the free glyceryl mononitrate (GMN). This was suggested in the kinetics of NO_x liberation from NMI-1182 seen in Figs. 8 and 11, in which the molar equivalent of one nitrate was released quickly leading to an asymptotic plateau. The second nitrate moiety, possibly now a mononitrate, apparently was more metabolically stable with a longer half-life. This pattern reflects the decades old experience derived from the therapeutic use of multi-and dinitrates, such as GTN and ISDN [32,33]. With these classic, organic multinitrates, the first nitrate is rapidly released, often in the gastrointestinal system and liver, leaving a mono (i.e., ISMN) or dinitrate (GDN) which, having a longer half life, circulates systemically. Often, it is the latter compound which is the actual therapeutic agent. In the case of NMI-1182, the circulating mononitrate may have additional beneficial effects (vasodilatory, anti-thrombotic, anti-inflammatory) on the cardiovascular system. While one might expect AZD3582 to have some circulating mononitrate, especially given its slower NO_x release in stomach, its systemic concentration of mononitrate would, necessarily, be lower than NMI-1182, again, because of the stoichiometric advantage of a dinitrate versus a mononitrate.

Another possibly favorable NO donor property NMI-1182 shares with other multinitrates, such as GTN [29], may be found in the observation that much of the total NO_x released by NMI-1182 is in the form of nitrite and not nitrate (Fig. 11). While nitrate is regarded as an inert end product of NO metabolism, there is considerable interest in the possibility that nitrite may, under appropriate physiological conditions, be converted to NO [34].

In conclusion, the promise of CINODs as gastro-protective, anti-inflammatory, analgesic drugs can be achieved through the design of compounds like NMI-1182. Its ester linkage allows for facile liberation of two already well-characterized and approved drugs, naproxen and GDN. Its dinitrate functionality confers upon it superior properties as a NO donor with abundant NO_x release in the stomach and the survivability of a mononitrate. Such compounds are truly deserving of a thorough evaluation as an alternative to coxib therapy.

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