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Characterization of Eltenac and novel COX-2 selective thiopheneacetic acid analogues in vitro and in vivo

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ABSTRACT

We assessed the effect of novel selective thiopheneacetic acids on cyclooxygenase isoenzymes in vitro and in vivo. Thiopheneacetic acid Eltenac and derivatives were investigated in this study. In human whole blood experiments these derivatives were potent inhibitors of COX-2 (IC_{50} = 0.02–0.4 μM) with less pronounced effect on COX-1 (IC_{50} = 0.15–5.6 μM). With COX-1/COX-2 ratios between 7.5- and 16-fold they are in the range of Celecoxib (13-fold). The parent drug Eltenac demonstrated no selectivity for COX-2.

In a rat paw edema model, these compounds showed reduction of edema volume in the range of 36–45% at 10 mg/kg (Eltenac 52%, Diclofenac 51%). However, the compounds were superior to Diclofenac and Eltenac with respect to their ulcerogenic and gastrointestinal properties. Introduction of a nitrate-ester moiety to either Eltenac or a derivative did neither improve selectivity or potency in vitro, nor ulcerogenicity in vivo.

Molecular modeling of selective thiopheneacetic acid derivatives to the active site of human COX-2 suggested similar binding properties as Lumiracoxib and Diclofenac.

In summary, modification of Eltenac generates moderately selective COX-2 drugs in the range of Celecoxib with respect to potency and selectivity. The drugs showed potent anti-inflammatory properties and significant improvement of animal survival in a sub-chronical experimental set up. Thiopheneacetic derivatives are characterized by low pK_a values, short microsomal half-lives and binding mode to COX-2 similar to Diclofenac and Lumiracoxib. These properties may also have an impact on the transient inhibition of COX-2-dependent prostacyclin, thereby being less associated with vascular complications.

1. Introduction

Ten years after the first publications of cyclooxygenase-2-(COX-2-) selective inhibitors (“COXibs”) [1–3], there is increasing concern that improved gastrointestinal safety comes at the expense of cardiovascular complications such as myocardial infarction, systemic hypertension and thromboembolic effects. The observation that these side effects are increased (compared to placebo) with structurally different chemical classes (e.g. Rofecoxib and Celecoxib) [4,5] may indicate a class-effect. This appears plausible given that COXibs reduce systemic synthesis of prostacyclin

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Abbreviations: NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; COXib, COX-2 selective inhibitor.

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(PGI_2) [6] acting as a vasodilator and anti-aggregatory prostanoid without inhibiting pro-aggregatory thromboxane. Consequently, the shift in prostaglandin formation towards unopposed thromboxane action may be one factor contributing to the increased incidence of cardiovascular side effects with COXibs. Recently Lumiracoxib, another COX-2-selective drug, was introduced to the market. This most selective COX-2 inhibitor available is structurally related to Diclofenac and characterized by an excellent gastrointestinal safety [7,8]. Lumiracoxib is also characterized by a shorter half life in vivo compared to COXibs with methylsulfyl or sulphonamide structures [9]. As a consequence transient inhibition of COX-2-dependent prostacyclin was expected. However, reduction of cardiovascular risk could not convincingly be demonstrated [10].

Based on this and also given the evidence that reduced COX-2 selectivity is believed to minimize cardiovascular risk, we developed a series of novel COX-2 inhibitors of the thiopheneacetic acid class. The structural scaffold of the parent compound Eltenac [11] (used in phase 2 studies as topical (gel) NSAID for osteoarthritis), was employed to identify potent and moderately selective COX-2 inhibitors, with good gastro-intestinal tolerability and having also short half-lives and low pK_a values to minimize permanent inhibition of COX-2.

2. Material and methods

2.1. Reagents

Lumiracoxib, Celecoxib, Eltenac, NO-Eltenac, BYK123, BYK124, BYK127, NO-BYK123 were synthesized in the laboratories of Nycomed, Konstanz. Li-Heparin Liquemin 25000 was from Roche (Roche Diagnostics GmbH, Sandhoferstrasse 116, 68305 Mannheim, Germany). All other drugs and compounds were from Sigma (Grünwalderweg 30, 82041 Deisenhofen, Germany). PGE_2 and TxB_2 EIA were purchased from R&D systems GmbH (Borsigstrasse 7, 65205 Wiesbaden-Nordenstadt).

3. Experimental protocols

Rats: Male Sprague-Dawley rats weighing 155 ± 5 g provided by Charles River (Sandhoferweg 7, 97633 Sulzfeld) were used. Space allocation for animals was 45 cm × 23 cm × 21 cm for three rats. The animals were housed in APECR cages. All animals were maintained in a hygienic environment under controlled temperature (22–24 °C) and humidity (60–80%) with 12 h light dark cycles for at least 1 week in MDS Pharma Services-Taiwan laboratory (158 Li-Teh Road, Peitou Taipei, Taiwan 112 ROC) or Nycomed laboratory prior to use. Unless animals were fasted for special purpose, free access to standard lab chow for rats [MF-18 (Oriental Yeast Co., Ltd., 3-15-15 Azuma, Tsukuda City 305–0031 Japan)] and reverse osmosis water was granted. All aspects of this work including housing, experimentation and disposal of animals were performed in general accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996).

3.1. Gastric irritation

Compounds were tested in groups of 4–6 overnight-fasted Sprague-Dawley male rats weighing 155 ± 5 g. Test compounds were administered by oral gavage. Animals were sacrificed 8 h later and gastric irritation/ulceration was scored for degree of hemorrhage and severity of ulcerative lesions. Gastric irritation/ulceration was scored according to an arbitrary system: 0 = no lesions; 1 = hyperemia; 2 = one or two slight lesions; 3 = more than 2 slight lesions or severe lesions; and 4 = very severe lesions [12]. Test substance-induced mucosal lesions of 50% or more relative to the lesions caused by positive control Aspirin at 150 mg/kg p.o. (as 100% lesion) were considered significant.

In another set up of experiments to check GI safety animals were treated daily for 7 days in groups of 10–30 with a single dose of drug (10 mg/kg for Eltenac and Diclofenac; 30 mg/kg for the thiopheneacetic acid derivatives). Animals (10–30) were observed for additional 14 days and survival was monitored.

3.2. Rat paw edema

Female Sprague-Dawley rats (10 animals/group), weighing 150–190 g and fasted overnight were used. Induction of paw edema was performed by subplantar injection of 0.05 ml/animal of a 1% aqueous carrageenin (type XM, Kraft foods, 7300 S Kedzie Ave, Chicago, IL 60629, USA) suspension in the right hind paw. Paw volume was determined plethysmometrically before and at hourly intervals up to 6 h after injection of carrageenin. Compounds were administered p.o. 1 h before provocation of edema.

3.3. Human COX-1 and COX-2 whole blood experiments

Fresh blood (0.1% Li-Heparin Liquemin 25000, Roche, Cat. No. 47195) was taken from human volunteers (female) with no pharmacological therapy in the last 2 weeks preceding sampling. Blood (480 μl) was mixed by gentle shaking with 1 μl of compounds/controls (in DMSO) on deep-well-plates. For measurement of COX-2 induction, LPS (Salmonella abortus equi, Sigma, L-1887, 1 μg/ml end-concentration/well, total volume of 20 μl PBS) stimulation of blood samples was performed and PGE_2 levels measured. Controls were adjusted to the same volume with PBS and mixed for 1 min on an orbital shaker at 37 °C in humidified atmosphere. After 24 h incubation, blood samples were centrifuged (2250 × *g*, 10 min, 4 °C) and plasma supernatant was harvested on ice. PGE_2 levels were analyzed by EIA (R&D Systems Immunoassay, CtNo. DE0100). For the analysis of COX-1 activity blood samples were allowed to clot for 1 h (37 °C, humidified atmosphere) following TxB_2 release and the assay was stopped by adding 2 μl Diclofenac (25 mM, equivalent to 100 μM end-concentration/well) and mixing for 1 min on an orbital shaker. Plates were centrifuged (2250 × *g*, 10 min, 4 °C). Following centrifugation serum supernatant was harvested on ice and analyzed with a

TxB_2 EIA (R&D Systems Immunoassay, CtNo. DE0700). COX-1 and COX-2-selectivity of each compound were assessed in parallel with every blood sample. Mean values were calculated from at least four independent female donors.

3.4. Isolation and culture of human chondrocytes

Chondrocytes were isolated from cartilage specimen from osteoarthritis patients undergoing total knee or hip replacement. Cells were isolated as recently described by Mais et al. [13] following pronase digestion and suspension in 1.2% sodium alginate (Sigma–Aldrich, Germany) in 150 mM NaCl. The formed alginate beads were cultured in DMEM/F12 supplemented with 20% FBS, 1% L-cystein, 25 μg/ml ascorbate and 50 μg/ml gentamycin (Fluka Biochemicals, Switzerland). To determine prostanoid production chondrocytes were plated in 24-well dishes and cells were stimulated 24 h with 0.5 nM human recombinant interleukin 1β in the presence or absence of inhibitors. PGE_2 was determined in the supernatant of chondrocytes using specific gas chromatography triple stage quadrupole mass spectrometry (GC/MS/MS).

3.5. Molecular modeling

All work of molecular modeling was done using the software package MOE 2006.08 from the Chemical Computing Group Inc. (1010 Sherbrooke St. W, Suite 910 Montreal, Quebec, Canada H3A 2R7). The calculations were performed using the forcefield MMFF94x implemented in MOE 2006.08.

The coordinates of the X-ray structures have been taken from the Protein Data Bank (PDB) [14]. The complex structures with the following accession codes were used: 1pxx (Diclofenac – COX-2) [15], 1cx2 (SC-558 – COX-2) [16]. The inhibitor SC-558 was used as a template for Celecoxib. For preparation of the PDB structures, hydrogen atoms were added, the protonation states of the amino acids were assigned and partial charges were calculated for all atoms. Furthermore, the positions of the added hydrogen atoms were optimized through minimization while keeping the positions of all other atoms fixed. For comparison of the ligand binding modes, the protein structures 1pxx and 1cx2 were superposed using only the Cα atoms of the protein chains. The root mean square distance (RMSD) of the superposed structures is 0.439 Å. The preparation of the inhibitor structures followed the general procedure described for the protein structures. The minimization of each inhibitor in the binding pocket of PDB structure 1pxx was done while all protein atoms were fixed. For comparison Diclofenac of PDB structure 1pxx was also minimized resulting in a very low RMSD of 0.292 Å between the original X-ray coordinates and the minimized structure of Diclofenac.

3.6. Statistical analysis

Values presented are means ± S.E.M. Statistical differences were determined using unpaired Student *t*-test (GraphPad Prism 4, GraphPad Software Inc., 215 San Diego, USA). Differences in mean values were considered statistically significant at $p < 0.05$.

4. Results

4.1. Effects and selectivity of thiopheneacetic acids on cyclooxygenases in vitro

Table 1 describes the chemical structures and similarities between Diclofenac, Lumiracoxib, Eltenac and its derivatives. The Eltenac molecule can be characterized as thienyl-analogue of Diclofenac. Inhibition of COX activity was measured in human whole blood to assess the selectivity of compounds with COX-1 and COX-2 inhibitory activity. Table 2 summarizes the effect of thiopheneacetic acids in comparison to reference inhibitors. The most potent inhibitors of COX-2 activity (measured as inhibition of PGE_2 synthesis) were BYK127 followed by Eltenac, Diclofenac and BYK124 (IC_{50} = 20–50 nM). Lumiracoxib, Celecoxib and BYK123 inhibited COX-2 in the upper nanomolar range (IC_{50} = 200–400 nM).

Nitroxyalkylesters of either Eltenac (NO-Eltenac) or BYK123 (NO-BYK123) revealed a trend for reduced activity on COX-2 (IC_{50} of 80 nM for NO-Eltenac versus 30 nM for Eltenac; IC_{50} of 1.4 μM for NO-BYK123 versus 400 nM for BYK123). This observation is most likely due to limited or impaired metabolism of the nitroxyalkylesters to the active acids in human blood in vitro.

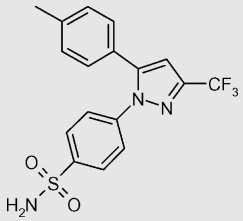
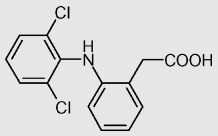
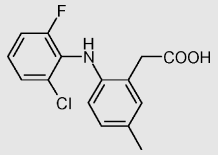
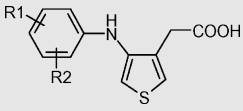
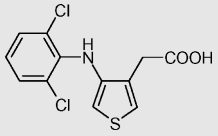
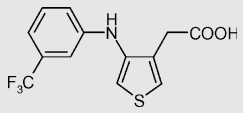
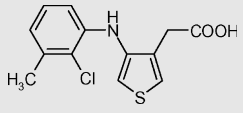
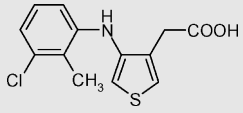
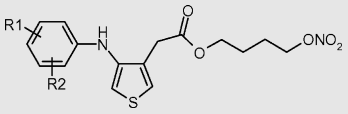
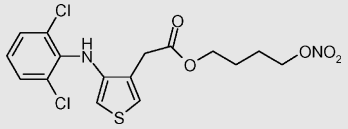
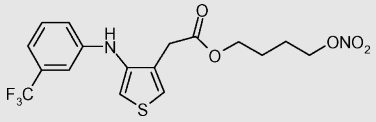
The most COX-2 selective drug in our hands was Lumiracoxib (>500-fold) followed by BYK124, BYK123 and Celecoxib (all 13–15-fold). Eltenac and Diclofenac demonstrated the lowest selectivity. The aforementioned nitrate-ester modification of Eltenac and BYK123 only slightly altered the selectivity of these drugs.

Chondrocytes are known to be the major source of COX-2-dependent PGE_2 synthesis responsible for inflammation and pain and thus represent a natural target for NSAID therapy. Therefore, we investigated the effect of selective Eltenac derivatives on COX-2 activity in primary human chondrocytes. Similar to naive chrondrocytes these cells are embedded in enriched extracellular matrix proteins, mimicking the natural penetration barrier. The ranked order of potency on COX-2 within this group of selective inhibitors was also reflected in their suppression of IL-1 evoked PGE_2 synthesis in chondrocytes with IC_{50} values of 1.2 nM and 2 nM for BYK127 and BYK124, respectively. BYK123 had an approximately 25 times lower potency (IC_{50} of 50 nM, Table 3).

4.2. Assessment of COX-2 inhibitors on inflammation and gastric injury in vivo

In vivo potency of thiopheneacetic acids was evaluated in the rat paw edema model, a standard model of acute inflammation. Injection of carrageenin into the footpad resulted in a marked increase of paw volume over 5 h (not shown). With administration 1 h prior to carrageenin, Eltenac and Diclofenac (10 mg/kg) efficaciously reduced the increase in paw volume by 52% and 51%, respectively (the maximal suppression obtained with NSAIDs was around 50–60% in this experimental set-up). The selective compounds BYK123, 124 and 127 were less potent with values of 36%, 45% and 39% in the 10 mg/kg dose, respectively (Table 4). Furthermore, ED_{30} values were determined for Eltenac and Diclofenac. Eltenac (ED_{30} = 1.9 mg/kg) was approximately 4-fold more potent that

Table 1 – Chemical structures of thiopheneacetic acids and tool compounds

Structure	Compound			pK_a
	Celecoxib			9.7
	Diclofenac			4.18
	Lumiracoxib			4.7
	Thiophenacetic acid derivatives	R1	R2	
	Eltenac	2-Cl	6-Cl	4.13
	BYK123	3-CF_3	H	4.16
	BYK124	2-Cl	3-Me	4.15
	BYK127	2-Me	3-Cl	4.16
	Thiopheneacetic acid nitrate-esters			
	NO-Eltenac	2-Cl	6-Cl	0.16
	NO-BYK123	3-CF_3	H	2.78

Summaries of chemical structures and pK_a values of the thiopheneacetic acids, nitroxyalkylesters and reference compounds.

Table 2 – Effect of thiopheneacetic acids and reference compounds on COX-1 and COX-2 in human whole blood

	IC_{50} Cox-1 (μM)	IC_{50} COX-2 (μM)	Ratio
Diclofenac	0.06	0.04	1.5
Lumiracoxib	>100	0.2	>500
Eltenac	0.03	0.03	1
BYK 123	5.6	0.4	14
BYK 124	0.8	0.05	16
BYK 127	0.15	0.02	7.5
NO-Eltenac	0.15	0.08	1.9
NO-BYK123	14	1.4	10
Celecoxib	2.6	0.2	13

Effects of thiopheneacetic acids, nitroxyalkylesters and reference compounds on human COX-1 and COX-2 isoenzymes. IC_{50} represent means calculated from concentration response curves of the respective NSAIDS of at least four independent donors. Selectivity ratios are calculated by IC_{50} COX-1 [μM] divided by IC_{50} COX-2 [μM].

Table 3 – Inhibition of PGE_2 synthesis by thiophenacetic acid derivatives from IL-1ß induced human chondrocytes

	BYK123	BYK124	BYK127
IC_{50}	50 nM	2 nM	1.2 nM
Slope	1.2	1.8	2.1

PGE_2 synthesis in human chondrocytes was induced approximately 65-fold (ranging from 5 ng/1.5 × 10^5 cells (basal level) to 324 ng/1.5 × 10^5 cells) after stimulation with IL-1β (0.5 nM) for 24 h. Compounds were co-incubated over this period in different concentrations. PGE_2 accumulation in the supernatant was determined via GC/MS/MS. IC_{50} values were calculated from two independent concentrations response curves from different donors via GraphPad Prism 4 and are given as means.

Diclofenac (ED_{30} = 7.4 mg/kg). Nitroxyalkylesters of BYK123 and Eltenac were not tested in this approach, because neither their anti-inflammatory activity in vitro (Table 2), nor gastric injury in vivo (see below and Fig. 2) was improved.

Table 4 – Anti-inflammatory features and chronic GI toxicity of thiopheneacetic acid derivatives

	Rat paw edema Inhibition%	Chronic GI toxicity Survival%
BYK123	36% $p < 0.01$ ($n = 10$)	100% (30 mg/kg/d) ($n = 10$)
BYK124	45% $p < 0.01$ ($n = 10$)	100% (30 mg/kg/d) ($n = 10$)
BYK127	39% $p < 0.01$ ($n = 10$)	100% (30 mg/kg/d) ($n = 10$)
Eltenac	52% $p < 0.001$ ($n = 30$)	30% (10 mg/kg/d) ($n = 30$)
Diclofenac	51% $p < 0.001$ ($n = 30$)	13% (10 mg/kg/d) ($n = 30$)

Anti-inflammatory effects of thiopheneacetic acids and Diclofenac were determined in single dosage and are given in % reduction of edema to controls. Chronic GI toxicity was determined following a 7 days daily treatment period with the indicated drugs. After an observation period of 14 days survival was monitored. Values are given as percentage to untreated controls for the respective dose. Student *t*-test. Numbers of animals were 10 for BYK123, 124, 127 and 30 for Eltenac and Diclofenac.

All thiopheneacetic acid derivatives are characterized by short half-lives in rat microsomes ($t_{1/2}$ for BYK123, 124, 127 and Eltenac were 101 min, 126 min, 105 min and 76 min, respectively). The $t_{1/2}$ for Eltenac in vivo in the rat was with 5 h comparable to that of Lumiracoxib [24] (for comparison $t_{1/2}$ of Lumiracoxib in our microsomal in vitro experiments was 176 min). Thus, the pharmacokinetic characteristics of thiopheneacetic acids discriminate them from classical COXibs and meet an essential prerequisite for transient prostacyclin suppression. However, we decided to pursue with BYK123 because its selectivity was improved over BYK127, and BYK124 showed massive microsomal (human and rat) degradation (data not shown). In addition, fluorine substitution has been utilized to block metabolism of aromatic methoxy groups and resulted in improved pharmacokinetic properties [17].

In an acute model of GI toxicity the reference compound Diclofenac caused ulcers in rats with an UD_{50} of 6 mg/kg 8 h following drug administration. Eltenac was slightly superior in this regard causing ulcer induction with UD_{50} value of 12 mg/kg. However, this was obtained following short time exposure with the drugs. In contrast, damage scores were significantly lower both in high and low doses for the selective compound BYK123 (Fig. 1).

In a sub-chronical experimental set up (7 days daily treatment, 14 days additional observation) survival was dramatically reduced to 13% and 30% in Diclofenac and Eltenac treated animals (10 mg/kg dose). In contrast, the improved gastro-intestinal safety of BYK123 (and also for BYK124 and BYK127) was reflected by a 100% survival, even in the higher dose of 30 mg/kg/d (Table 4).

Several investigators noticed improved GI safety following introduction of NO donating nitroxy butlyester moieties to unselective NSAIDs [18]. We were, however, unable to minimize ulcer formation in rat stomach despite introducing nitrate-esters to unselective Eltenac or BYK123 (Fig. 2).

4.3. *Molecular modeling*

The overlay of the inhibitory modes of SC-558 (template for Celecoxib, white carbons, PDB code 1cx2) and Diclofenac (magenta carbons, PDB code 1pxx) to COX-2 shows their different binding behavior to the protein (Fig. 3A). The COX-2 selectivity of Celecoxib is structurally related to the binding of its sulfon amide group to COX-2. Recently, the crystal structure of murine COX-2 was resolved and Lumiracoxib was described to bind in an orientation similar to Diclofenac [15]. As shown in Fig. 3B, the binding modes of BYK123 (cyan carbons), Diclofenac (magenta carbons) and Lumiracoxib (orange carbons) to COX-2 are similar. The COX-2 selectivity of Lumiracoxib is structurally related to the methyl group attached to the central phenyl ring. BYK123 comprises structural elements of Celecoxib and also of the arylacetic acids Diclofenac and Lumiracoxib with its trifluormethyl moiety.

5. Discussion

Full control of osteoarthritis and rheumatoid arthritis pain and inflammation without causing significant side effects on either

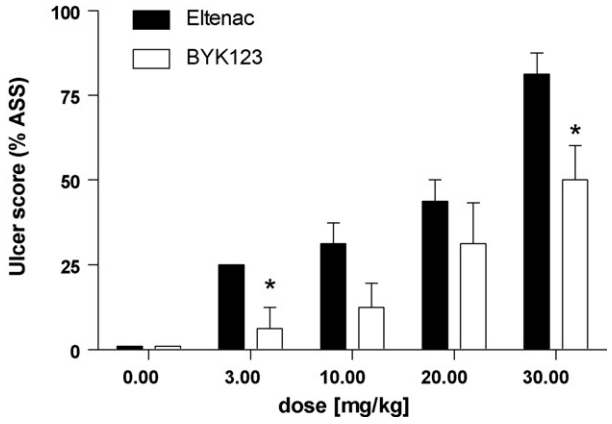


Fig. 1 – Ulcerogenic properties of Eltenac versus BYK123. Gastric damage following oral administration of BYK123 in comparison to Eltenac in rats is shown. Ulcer score is given as percentage to Aspirin provoked lesions (100%, 150 mg/kg). Bars are means ± S.E.M. of six rats per group. (*) Significant $p < 0.05$, unpaired t-test.

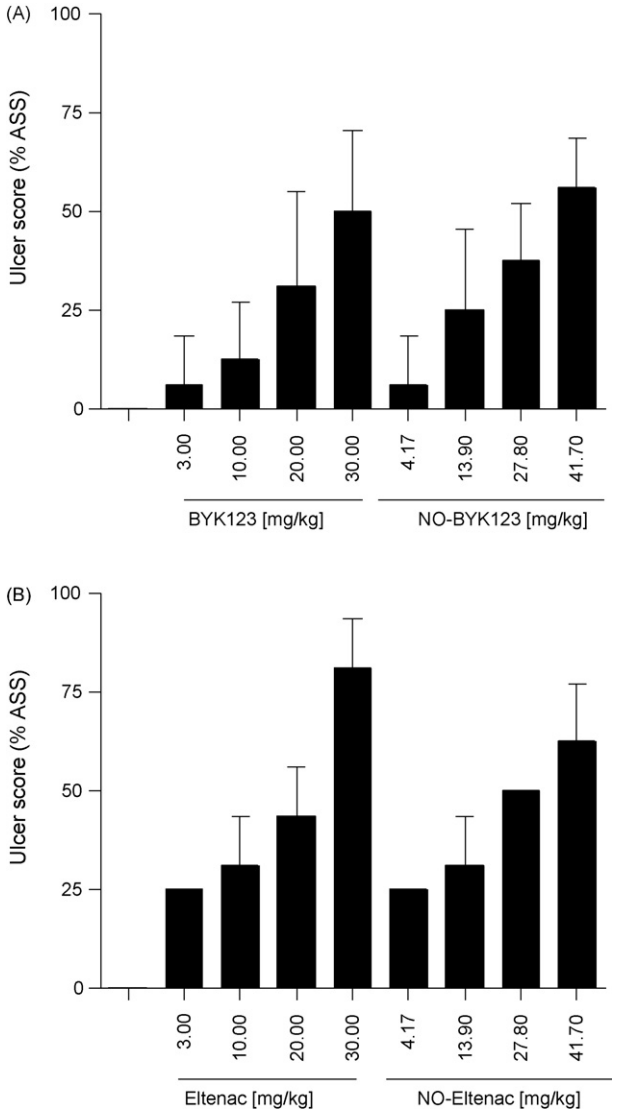


Fig. 2 – Comparison NO-linked drugs to parent compounds BYK123 and Eltenac. Severity of gastric damage in rat following oral administration of BYK123 and NO-BYK123 (upper panel) and Eltenac and NO-Eltenac (lower panel). The doses of NO-BYK123 and NO-Eltenac represent equipotent doses to that of BYK123 and Eltenac. Bars are means ± S.E.M. of six rats per group.

the gastric mucosa or the cardiovascular system has not yet been obtained in clinical practice. Although the moderately selective NSAIDs such as Celecoxib show significantly improved gastrointestinal safety [19], these drugs show an inherent complication on cardiovascular safety, most likely due to constant suppression of cardioprotective prostacyclin. Despite belonging to structurally diverse compound classes (diarylheterocycles like Celecoxib, Rofecoxib, Valdecoxib, and Etoricoxib or sulfanilides like Flosulide and Nimesulide) all marketed COXibs, with the exception of Lumiracoxib, are chemically characterized by 4-methylsulfonyl or 4-sulfonamido groups. This residue is responsible for the selectivity versus COX-2 due to being time-dependent inhibitors, while COX-1 is inhibited only competitively [20–22]. The aim of this study was to develop new NSAIDs with firstly, moderate COX-2 selectivity, secondly, short half-lives, thirdly, low pK_a for accumulation in joints and finally, a binding mode different from classical COXibs. All of these characteristics should contribute to only a transient inhibition of cardio-protective prostacyclin. Our study describes the in vitro and in vivo effects of novel carboxyl, non-4-methylsulfonyl or 4-sulfonamido containing drugs with potent anti-inflammatory properties. The biochemical potency was tested in the state of the art assay of human whole blood. The rank order of potency of the reference compounds Celecoxib, Diclofenac and Lumiracoxib was in accordance with recently published work employing similar assays [23,24]. The lead compound Eltenac, the structural basis on which we optimized selectivity, was unselective versus COX-1 and COX-2. The in vitro potency was in the range of Diclofenac and superior to Lumiracoxib. However, the in vivo anti-inflammatory potency of the drug was approximately four times higher than Diclofenac and equipotent to Lumiracoxib (based on ED_{30} values) [24]. In addition, the ulcerogenicity of Eltenac was superior to Diclofenac with respect to acute ulcerogenicity (UD_{50} = 12 mg/kg vs 6 mg/kg), and also superior regarding 21 days survival. In other words, despite being unselective for COX-2, Eltenac demonstrates an improved therapeutic window compared to Diclofenac. The reason for this observation is hitherto unexplained. Exchanging the 2,6 dichloro substitution pattern in the phenylaminogroup of Eltenac to 3-chloro-2-methyl or 2-chloro-3-methyl further increased in vitro selectivity 7.5- to 16-fold.

The biochemical selectivity for COX-2 inhibition by optimized thiopheneacetic acid derivates was reflected by their enhanced acute gastric tolerability and was most prominent on survival parameters in the tested dose. Survival clearly discriminates unselective versus selective thiophenacetic acids in our hands.

To further minimize the potential of gastric damage we introduced NO-releasing residues (nitroxybutyl moiety) to

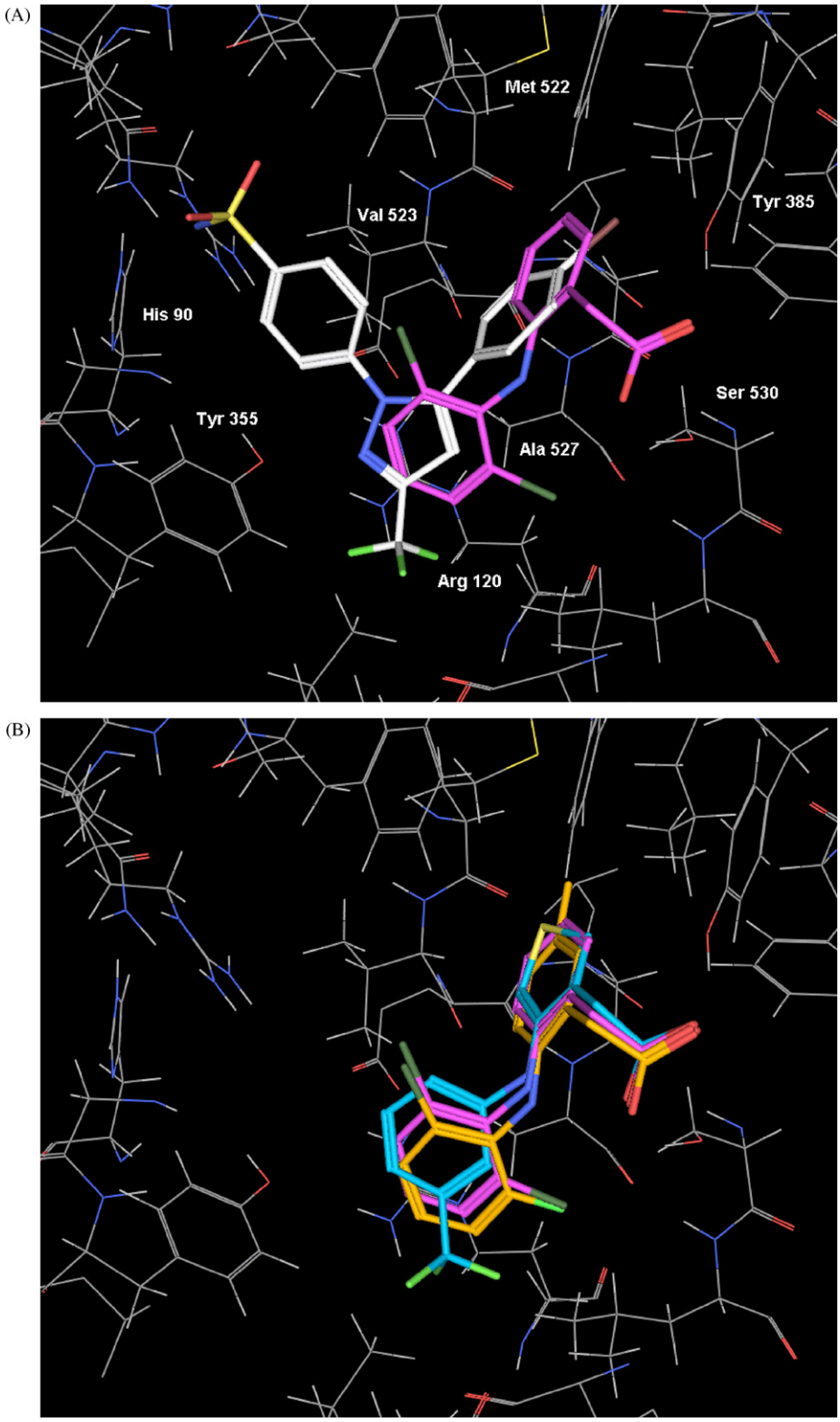


Fig. 3 – Structural comparison of BYK123, Diclofenac, Lumiracoxib and Celecoxib bound to COX-2. (A) The overlay of the inhibitory modes of SC-558 (template for Celecoxib, white carbons, PDB code 1cx2) and Diclofenac (magenta carbons, PDB code 1pxx) to COX-2 shows their differential binding to the protein. The COX-2 selectivity of SC-558/Celecoxib is structurally related to the binding of its sulfon amide group to COX-2. The protein atoms of the binding pocket are shown. The residues are labelled regarding PDB file 1pxx. (B) The binding modes of BYK123 (cyan carbons), Diclofenac (magenta carbons) and Lumiracoxib (orange carbons) to COX-2 are similar. The COX-2 selectivity of Lumiracoxib is structurally related to the methyl group attached to the central phenyl ring [30]. All three inhibitors were minimized in the binding pocket of the COX-2 structure (PDB code 1pxx) as described. The protein atoms of the binding pocket are shown.

either Eltenac or BYK123. The rationale of the coupling of an NO-releasing moiety to standard NSAIDs was, that released NO would exert beneficial effects on the gastric mucosa by enhancing the mucosal defensive ability and mucosal blood flow, thus accelerating repair mechanisms [25]. Our biochemical data demonstrated a 2–5-fold decrease in potency on cyclooxygenases, whereas the degree of selectivity was maintained. The impaired potency is most likely due to limited or lack of specific ester hydrolysis in whole blood [26]. In vivo, where proper hydrolysis of esters should be given due to hepatic clearance, we could not observe differences between nitroxyalkylesters and the parent compounds with regard to mucosal damage. This observation is inconsistent with other findings for NO-NSAIDs [27,28]. However, we cannot exclude that in long-term models of chronically induced ulcers and models comprising intestinal toxicity a protective effect would be seen. Furthermore, NO-NSAIDs are metabolized differently than their parent NSAIDs [29] and we did not check pharmacokinetics in vivo or concentrations of NO oxidation products (nitrite/nitrate) in plasma.

The thiopheneacetic acid compounds we developed display a low degree of ionization (pK_a 4.1–4.6) and consequently demonstrate preferential distribution into inflamed tissue (e.g. joints). As further summarized by Brune et al. the volume of distribution of the relatively lipophilic sulphonamides and methlysulphones is 5–30-fold higher than that of the arylacetic acids [9].

Our molecular modeling approach suggested that thiopheneacetic acids (e.g. BYK123) bind to COX-2 in an inverted conformation with its carboxylate group hydrogen-bound to Tyr-385 and Ser-530, identical to Diclofenac and Lumiracoxib [15]. The observed improved selectivity of the compounds can be possibly explained by the spatial position of the trifluoromethyl group, which is similar to that described for the trifluoromethyl group of Celecoxib [16]. Several distinct binding sites contribute to tight inhibitor binding to the COX active site. One is the aforementioned Tyr-385 and Ser-530 at the top of the active site. Another major anchor point for binding is the site pocket defined by the amino acids Tyr-355, Val-523, His-90, Gln-192 and Arg-513. All COXibs, except Lumiracoxib utilize the pocket by interaction with their methlysulfyl or sulphonamide groups, resulting in potency and selectivity. BYK123 and related thiopheneacetic acids avoid interaction with this domain and thus might differ in the tightness of the inhibitor-COX-2 complex.

Taken together, the pharmacology and the physicochemical properties of moderately selective thiopheneacetic acids make this drug class favorable for long-term use in osteoarthritis and rheumatoid arthritis. Low pK_a values, short half-lives and a different binding mode discriminate them from sulphonamide/methlysulfyl containing COXibs, moderate selectivity discriminates them from latest generation compounds like Lumiracoxib. It is intriguing to speculate that all these characteristics of thiopheneacetic acids and not at least the structural similarities to old and well established drug classes, may lead to a potent anti-inflammatory drug with good gastrointestinal safety combined with an improved cardiovascular profile, due to only transient inhibition of prostacyclin.

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REFERENCES

[1] Klein T, Nusing RM, Pfeilschifter J, Ullrich V. Selective inhibition of cyclooxygenase 2. Biochem Pharmacol 1994;48:1605–10.
[2] Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 1994;47:55–9.
[3] Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, et al. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci USA 1994;91:3228–32.
[4] Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, et al. Cardiovascular events associated with Rofecoxib in a colorectal adenoma chemoprevention trial. N Engl J Med 2005;352:1092–102.
[5] Solomon SD, McMurray JJ, Pfeffer MA, Wittes J, Fowler R, Finn P, et al. Cardiovascular risk associated with Celecoxib in a clinical trial for colorectal adenoma prevention. N Engl J Med 2005;352:1071–80.
[6] Cullen L, Kelly L, Connor SO, Fitzgerald DJ. Selective cyclooxygenase-2 inhibition by nimesulide in man. J Pharmacol Exp Ther 1998;287:578–82.
[7] Farkouh ME, Kirshner H, Harrington RA, Ruland S, Verheugt FW, Schnitzer TJ, et al. Comparison of Lumiracoxib with naproxen and ibuprofen in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET), cardiovascular outcomes: randomised controlled trial. The Lancet 2004;364:675–84.
[8] Schnitzer TJ, Burmester GR, Mysler E, Hochberg MC, Doherty M, Ehrsam E, et al. Comparison of Lumiracoxib with naproxen and ibuprofen in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET), reduction in ulcer complications: randomised controlled trial. The Lancet 2004;364:665–74.
[9] Brune K, Hinz B. Selective cyclooxygenase-2 inhibitors: similarities and differences. Scand J Rheumatol 2004;33:1–6.
[10] Topol EJ, Falk GW. A coxib a day won't keep the doctor away. The Lancet 2004;364:639–40.
[11] Sandelin J, Harilainen A, Crone H, Hamberg P, Forsskahl B, Tamelander G. Local NSAID gel (Eltenac) in the treatment of osteoarthritis of the knee. A double blind study comparing Eltenac with oral Diclofenac and placebo gel. Scand J Rheumatol 1997;26:287–92.
[12] Cashin CH, Dawson W, Kitchen EA. The pharmacology of benoxaprofen (2-[4-chlorophenyl]-alpha-methyl-5-benzoxazole acetic acid), LRCL 3794, a new compound with antiinflammatory activity apparently unrelated to inhibition of prostaglandin synthesis. J Pharm Pharmacol 1977;29:330–6.
[13] Mais A, Klein T, Ullrich V, Schudt C, Lauer G. Prostanoid pattern and iNOS expression during chondrogenic differentiation of human mesenchymal stem cells. J Cell Biochem 2006;98:798–809.
[14] Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. Nucleic Acids Res 2000;28:235–42.

[15] Rowlinson SW, Kiefer JR, Prusakiewicz JJ, Pawlitz JL, Kozak KR, Kalgutkar AS, et al. A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385. J Biol Chem 2003;278:45763–9.
[16] Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, et al. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. Nature 1996;19–26(384):644–8.
[17] Chauret N, Guay D, Li C, Day S, Silva J, Blouin M, et al. Improving metabolic stability of phosphodiesterase-4 inhibitors containing a substituted catechol: prevention of reactive intermediate formation and covalent binding. Bioorg Med Chem Lett 2002;12:2149–52.
[18] Wallace JL, Cirino G, McKnight GW, Elliott SN. Reduction of gastrointestinal injury in acute endotoxic shock by flurbiprofen nitroxybutylester. Eur J Pharmacol 1995;280:63–8.
[19] Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, et al. Gastrointestinal toxicity with Celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. J Am Med Assoc 2000;284:1247–55.
[20] Walker MC, Kurumbail RG, Kiefer JR, Moreland KT, Koboldt CM, Isakson PC, et al. A three-step kinetic mechanism for selective inhibition of cyclo-oxygenase-2 by diarylheterocyclic inhibitors. Biochem J 2001;357:709–18.
[21] Klein T, Nusing RM. Mechanism of cyclooxygenase-2 inhibition by indanone derivatives. Adv Exp Med Biol 1997;407:503–7.
[22] Selinsky BS, Gupta K, Sharkey CT, Loll PJ. Structural analysis of NSAID binding by prostaglandin H2 synthase: time-dependent and time-independent inhibitors elicit identical enzyme conformations. Biochemistry 2001;40:5172–80.
[23] Chan CC, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, et al. Rofecoxib [Vioxx, MK-0966; 4-(4′-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. J Pharmacol Exp Ther 1999;290:551–60.
[24] Esser R, Berry C, Du Z, Dawson J, Fox A, Fujimoto RA, et al. Preclinical pharmacology of Lumiracoxib: a novel selective inhibitor of cyclooxygenase-2. Br J Pharmacol 2005;144:538–50.
[25] Burgaud JL, Riffaud JP, Del Soldato P. Nitric-oxide releasing molecules: a new class of drugs with several major indications. Curr Pharm Des 2002;8:201–13.
[26] Keeble JE, Moore PK. Pharmacology and potential therapeutic applications of nitric oxide-releasing non-steroidal anti-inflammatory and related nitric oxide-donating drugs. Br J Pharmacol 2002;137:295–310.
[27] Davies NM, Roseth AG, Appleyard CB, McKnight W, Del Soldato P, Calignano A, et al. NO-naproxen vs. naproxen: ulcerogenic, analgesic and anti-inflammatory effects. Aliment Pharmacol Ther 1997;11:69–79.
[28] Wallace JL, Reuter B, Cicala C, McKnight W, Grisham M, Cirino G. A Diclofenac derivative without ulcerogenic properties. Eur J Pharmacol 1994;257:249–55.
[29] Fiorucci S, Antonelli E, Burgaud JL, Morelli A. Nitric oxide-releasing NSAIDs: a review of their current status. Drug Saf 2001;24:801–11.
[30] Blobaum AL, Marnett LJ. Molecular determinants for the selective inhibition of cyclooxygenase-2 by Lumiracoxib. J Biol Chem 2007;282:16379–90.