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The analgesic NSAID lornoxicam inhibits cyclooxygenase (COX)-1/-2, inducible nitric oxide synthase (iNOS), and the formation of interleukin (IL)-6 in vitro

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Abstract. *Objective*: To investigate anti-inflammatory effects of lornoxicam in vitro on COX-1/COX-2, on NO formation from iNOS and on the formation of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8.
Materials and Methods: COX-1 inhibition in intact cells was assessed employing two systems: measurement of aggregation in human washed platelets and assessment of TXB_2 formation in HEL cells. COX-2 inhibition was assessed by measuring 6-keto-$PGF_{1\alpha}$ in supernatants of intact cells of LPS-stimulated J774.2 cells (murine) and of Mono Mac 6 cells (human). In whole blood inhibition of COX-1 was performed by measuring TXB_2 formation after clotting, and COX-2 inhibition was examined in LPS-stimulated whole blood cultures. The reduction of NO levels as a measure of the inhibition of cellular NO formation was assayed in supernatants of LPS-stimulated RAW 264.7 cells using the Griess reaction. Compound influence on the formation of TNF-α, IL-1β, IL-6, and IL-8 was examined using LPS-stimulated monocytic cells (THP-1) and measurement of cytokine concentrations by specific ELISAs.
Results: In intact human cells, lornoxicam showed a balanced inhibition of COX-1/-2 exhibiting the lowest IC_{50} (0.005 µM/0.008 µM) of the large panel of NSAIDs tested. Similar results were obtained in the whole blood for COX-1/-2. NO formation was dose-dependently inhibited by lornoxicam (IC_{50} of 65 µM) whereas piroxicam, diclofenac, ibuprofen, ketorolac and naproxen inhibited the NO formation markedly less. Indomethacin was approximately equipotent with lornoxicam. In stimulated monocytic cells (THP-1), lornoxicam showed a marked inhibition of IL-6 formation (IC_{50} 54 µM) while the formation of TNF-α, IL-1β and IL-8 was only moderately affected.
Conclusions: Of the panel of NSAIDs tested, lornoxicam was found to be the most potent balanced inhibitor of human COX-1/-2. The equipotent COX-isoenzyme inhibition by lornoxicam is complemented by a marked inhibition of IL-6 production and of iNOS-derived NO formation. The in vitro activities described support the marked anti-inflammatory and analgesic activities of lornoxicam found in animal models as well as in clinical studies.

Key words: Lornoxicam – COX-1 – COX-2 – Prostaglandin – IL-6 – iNOS – Cytokines

Introduction

Lornoxicam (chlortenoxicam) is a novel non-steroidal anti-inflammatory drug (NSAID) of the oxicam class of NSAIDs. Lornoxicam is distinguished from established oxicams by its short plasma half life (3–5 h) [1] and good gastrointestinal tolerability [2]. As with other NSAIDs the principle mechanism of action of lornoxicam relates to the inhibition of cyclooxygenase (COX), the key enzyme of the arachidonic acid pathway, resulting in the inhibition of prostaglandin (PG) synthesis [3–5]. Thus, in rat polymorphonuclear leukocytes lornoxicam potently inhibited the formation of PGD_2 in vitro (IC_{50} 20 nM) [1]. In vivo lornoxicam exhibited anti-inflammatory activities in acute (carrageenan-induced paw oedema) as well as in chronic inflammation (adjuvant-induced arthritis) that were more potently than the ones of other oxicams. In addition lornoxicam showed effective anti-nociception in the acetylcholine induced writhing test in mice [1].

The in vivo anti-inflammatory and anti-nociceptive properties of lornoxicam were also observed in clinical trials. Lornoxicam effectively relieved the symptoms of osteoar-

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thritis, rheumatoid arthritis and ankylosing spondylitis (for review see [6]) [7, 8]. Strong analgesic effects of lornoxicam were also observed after dental surgery, hysterectomy, lumbar disk surgery and in controlling lower back pain [9–12].

The surprisingly strong analgesia found initiated further pre-clinical studies. One recent study in a model of carrageenan-induced inflammatory nociception demonstrated reduction of the number of activated neurones at that level of the spinal cord that related to the inflamed extremity [13]. The type of neurones affected was suggestive of a predominantly peripheral site of analgesic activity of lornoxicam in this model.

As mentioned above one principal mechanism of action of NSAIDs relies on the inhibition of COX which is the rate limiting enzyme in the arachidonic acid pathway [14]. COX exists in two isoforms, COX-1 and COX-2 (for reviews see [15, 16]). While COX-1 is thought to account for homeostatic amounts of eicosanoids, COX-2 is induced during inflammation leading to the formation of pathologic amounts of prostaglandins. The inhibition of prostaglandin synthesis by NSAIDs has been demonstrated to effectively reduce inflammatory symptoms such as oedema and pain [16–18]. However, this does not satisfactorily explain all the NSAIDs' analgesic effects. Other mediators of inflammation such as reactive oxygen products and cytokines have also been shown to considerably contribute to inflammation and inflammatory pain [19, 20]. So, concurrently to the transcriptional induction of the COX-2 gene, the expression of the gene encoding inducible nitric oxide synthase (iNOS) is induced, leading to increased levels of nitric oxide (NO) in inflamed tissues [21]. In these, NO has been shown to contribute to oedema formation, hyperalgesia and pain [20, 22]. Pro-inflammatory cytokines such as cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8 are interlinked with the formation of small molecular mediators of inflammation and thus contribute to the range of mediators that critically control inflammation [23, 24]. The effects of those pro-inflammatory cytokines have been demonstrated by the marked clinical improvements in recipients of anti-cytokine therapies with monoclonal antibodies directed to TNF-α [25], with soluble IL-1 receptor [26], anti-IL-6 monoclonal antibodies [27], or in in vivo experiments with monoclonal antibodies to IL-8 [24].

To gain further insights into the mechanism(s) of action of lornoxicam, we investigated in vitro effects of lornoxicam on human COX-1 and human COX-2, on iNOS, and on the formation of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8. In order to arrive at in vitro conditions that closely resemble the in vivo situation, intact cell assays or whole blood assays were employed throughout the investigations.

Materials and methods

Compounds

Test compounds were obtained from the following sources: lornoxicam, tenidap and tenoxicam (Nycomed Pharma, Linz, Austria); aspirin, diclofenac, ibuprofen, indomethacin, naproxen, piroxicam (Sigma, Munich, Germany); ketorolac (Cayman, Ann Arbor, MI, USA). Meloxicam was a kind gift from Dr. D. Binder, University of Vienna, Vienna, Austria.

The compounds were dissolved in DMSO, serially diluted in DMSO and added to the assays described below. The final concentration of DMSO was kept constant at 1%, which did not affect the formation of any of the mediators measured. Controls treated with vehicle only were always run in parallel.

Cell culture

HEL 92.1.7 cells, RAW 264.7 cells, THP-1 cells (ATCC, Rockville MD, USA) and J774.2 cells (ECACC, Salisbury, UK) were grown in RPMI 1640 medium (GIBCO-BRL, Vienna, Austria) supplemented with 10% foetal calf serum (FCS), sodium pyruvate (1 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml).

The Mono Mac 6 cell line was provided by Dr. H.W.L. Ziegler-Heitbrock, University of Munich [28]. These cells were initially subcloned and the clone with the highest COX-2 protein expression (Mono Mac 6.X2) used as source for COX-2 [29]. The cells were grown in 1 ml cultures using 24 well plates (Greiner, Kremsmünster, Austria) in RPMI 1640 medium supplemented with 10% FCS tested for low endotoxin content, sodium pyruvate (1 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), oxalacetic acid (1 mM), non-essential amino acids (2X), and bovine insulin (9 µg/ml).

For stimulation experiments LPS from *E. coli*, serotype 026:B6 (Sigma) was used throughout the study.

Assessment of COX-1 activity in human platelets

Informed consent was obtained from both male and female healthy volunteers (10 individuals, age of 25 to 35) that had not taken any drugs within the previous 2 weeks. Blood was drawn into vacutainer tubes (9 volumes (vol.) blood and 1 vol. 0.129 M citrate) and centrifuged at 450 g for 4 min at room temperature (RT) to obtain platelet rich plasma (PRP). About 4 vol, PRP was mixed with 1 vol. 0.129 M citrate (pH 6.5) and centrifuged at 250 g for 10 min. Then, the platelets were resuspended in HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES and 0.05 U/ml hirudin; pH 7.4) and adjusted to about 3×10^8/ml. Platelets (200 µl) were incubated in a dual channel aggregometer cuvette for 4 min at 37 °C. Fibrinogen, 25 µl, (1 mg/ml final conc.) and 2.5 µl compound were added and incubated with stirring at 1000 rpm for 2 min. Then, 25 µl arachidonic acid (10 µM final conc.) was added and aggregation was monitored for 4 min. Maximum aggregation obtained during monitoring was taken as 100%.

Assessment of COX-2 activity in J774.2 cells

The inhibition of COX-2 activity in the murine monocytic J774.2 cells was assessed as previously described with minor modifications [30]. Briefly, J774.2 cells were stimulated with LPS (10 µg/ml) for 16 h. After exchange of the culture medium, compounds were added for 30 min at 37 °C. Then, arachidonic acid (Sigma) was added to a final concentration of 30 µM for a further 15 min at 37 °C. Thereafter, 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) was assessed in cell free culture supernatants by ELISA (Cayman) according to the manufacturer's instructions.

Assessment of human COX-1 activity in intact cells

The inhibition of COX-1 activity in intact cells was assessed as previously described [31]. Briefly, cells of the human erythroleukemic cell line HEL were harvested, suspended in fresh medium (10^6/ml) and incubated with compound for 30 min at 37 °C. Then, arachidonic acid (30 µM; Sigma) was added for 15 min at 37 °C. Thereafter, thromboxane B_2 (TXB_2) was assessed in cell free culture supernatants by ELISA according to the manufacturer's instructions.

Assessment of human COX-2 activity in intact cells

Inhibition of COX-2 was performed as previously described [31]. Mono Mac 6 cells (10^6/ml) were cultured in 24-well plates for 6 h in the presence of 100 ng/ml LPS. The culture medium was renewed and compound was added for 30 min at 37 °C. Then, arachidonic acid (30 µM; Sigma) was added for 15 min at 37 °C. Thereafter, $PGF_{1\alpha}$ was assessed in cell free culture supernatants by ELISA according to the manufacturer's instructions.

Assessment of COX-1 activity in whole blood

Whole blood was drawn from healthy donors (same panel of volunteers as above) by veinipuncture after informed consent had been obtained. The donors had not taken any drugs the previous two weeks. The blood (100 µl) was immediately mixed with graded amounts of compound or vehicle and incubated for 1 h at 37 °C as described by Patrigniani and colleagues [32]. Thereafter, serum was obtained by centrifugation at 1200 g, 4 °C for 10 min and assayed for TXB_2 by ELISA according to the manufacturer's instructions.

Assessment of COX-2 activity in whole blood

Heparinized blood was collected from the same volunteers as for COX-1 assessment. About 100 µl were incubated with LPS (10 µg/ml), acetylsalicylic acid (10 µg/ml) (Sigma) and compound for 24 h at 37 °C as described [32]. Then, plasma was obtained by centrifugation at 1200 g, 4 °C for 10 min and assayed for PGE_2 by ELISA according to the manufacturer's instructions.

Assessment of inducible nitric oxide synthase mediated NO accumulation

RAW 264.7 cells were suspended in culture medium without phenol red (Gibco-BRL, Vienna, Austria) and adjusted to 10^6/ml. Then, the cells were incubated with graded amounts of compound and with or without LPS (2 µg/ml) for 24 h at 37 °C as described previously [33]. Thereafter, culture supernatants were removed and accumulated nitric oxide, as a measure of NO synthase activity was assessed in cell-free culture supernatants by the Griess-reaction [34]. To 100 µl culture supernatant 100 µl Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) was added and colour development was assessed at λ 550 nm with a microplate reader (*v-max* spectrophotometer, Molecular Devices, Munich, Germany). Standard curves were generated with a serial dilution of sodium nitrite dissolved in culture medium. The sensitivity of the assay was 0.5 µM.

Assessment of the formation of TNF-α, IL-1β, IL-6, and IL-8

THP-1 cells (0.8×10^5/ml) were incubated with compound for 10 min at room temperature. Then, cells were cultured for 15h at 37 °C with or without LPS (5 µg/ml). Thereafter, TNF-α, IL-1β, IL-6 and IL-8 were assessed in cell free culture supernatants by capture ELISAs specific for the respected human cytokines as previously described [29]. Briefly, 96 well plates (Nunc, Vienna, Austria) were coated with mouse monoclonal antibody directed to the respective cytokine (TNF-α, IL-1β, IL-6 and IL-8 (R&D Systems, Oxon, UK) and blocked with BSA. Cell-free culture supernatants serially diluted in PBS containing 0.1% BSA, 0.05% Tween 20 and 0.02% NaN_3 were added to the plates and incubated overnight at 4 °C. Thereafter, the plates were washed four times with PBST (PBS with 0.05% Tween 20 and 0.1% NaN_3). Goat antibodies directed to the respective cytokines (R&D Systems, Oxon, UK) were added and after extensive washing followed by alkaline phosphatase-conjugated donkey $F(ab')_2$ to goat immunoglobulin (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). After another cycle of four washes p-nitrophenylphosphate (1 mg/ml) (Sigma) dissolved in substrate buffer (9.7% (v/v) diethanolamine (Sigma) in bi-distilled H_2O, 4 mM $MgCl_2$, pH 9.8) was added and colour development was assessed with a microplate reader (*v-max* spectrophotometer, Molecular Devices) at λ 405 nM. Reference measurements were performed at λ 650 nm. The concentrations of cytokines were calculated using standard curves for each cytokine that were generated with purified human recombinant cytokines (TNF-α, IL-1β, and IL-6 from R&D Systems, Oxon, UK; IL-8 from Genzyme, Cambridge, MA, USA). The sensitivity for each ELISA was 10 pg/ml.

Assessment of IL-6 steady-state mRNA levels

We used RT-PCR to quantify IL-6 steady-state mRNA levels. RNA was isolated [35] and amounts were determined by measuring the absorbency at λ 260 nm. RT-PCR was performed using the GeneAmp RNA PCR Kit (Perkin-Elmer Corporation, Vaterstetten, Germany) applying 1 µg RNA to synthesise oligo$(dT)_{16}$-primed cDNA. The cDNA was normalised using β-actin specific primers (5′-ATC TGG CAC CAC ACC TTG TAC AAT GAG CTG CG-3′ and 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′) and PCR-mimics (PCR-MIMIC-kit and RT-PCR amplimer set for IL-6 Clontech, Palo Alto, CA, USA) according to the manufacturer's instruction. Then, β-actin and IL-6 specific PCR was performed applying equal amounts of the normalised cDNA. The sequences of the IL-6 specific oligonucleotide primers were 5′-ATG AAC TCC TTC TCC ACA AGC GC-3′ and 5′-GAA GAG CCC TCA GGC TGG ACT G-3′. The 50 µl PCR mixtures comprised 0.4 µM Primer (Clontech, Palo Alto, CA, USA) 0.2 µM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 8.3, 50 mM KCl, 2 U AmpliTaq DNA polymerase. After an initial step of 94 °C for 5 min, amplification was performed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min followed by a final extension of 7 min at 72 °C. The PCR products (8 µl/lane) were resolved on 1.5% agarose gels and visualised by staining with ethidium bromide (Sigma, Munich, Germany). The β-actin and IL-6 specific PCR generated fragments of 838 bp and 628 bp, respectively.

Data analysis

Results of 3 or more independent experiments each performed in triplicates were calculated as mean ± SD of percent eicosanoid formation, NO and cytokine levels relative to control (DMSO vehicle). The IC_{50} values were calculated either by sigmoidal curve fitting [36], or by logarithmic-linear regression analysis using values in the range of 20% to 80%.

Results

Effects of lornoxicam on cyclooxygenase-1/-2 activity in intact cells

To assess the effects of lornoxicam on both COX isoenzymes separately intact cell assays specific for COX-1 and COX-2 were used.

In a first set of experiments, lornoxicam's inhibitory potency on COX-1 and COX-2 was assessed using arachidonic acid-induced platelet aggregation for COX-1. For COX-2 assessment, we used the LPS-stimulated mouse macrophage cells J774.2 which have been shown to display only COX-2 [30]. From these cells, the production of $PGF_{1\alpha}$ was measured. As presented in Table 1, lornoxicam effectively inhibited the aggregation of human platelets as well as the formation of $PGF_{1\alpha}$ in that COX-1 was nine-fold more

Table 1. Inhibition of arachidonic acid-induced platelet aggregation (COX-1) and $PGF_{1\alpha}$ formation in LPS-stimulated J774.2 cells (COX-2) by lornoxicam and other NSAIDs.

Compound	IC50 for COX-1 [µM] ± SD (n)	p-Value[a]	IC50 for COX-2 [µM] ± SD (n)	p-Value[a]	Ratio COX-1/COX-2	p-Value[b]
Lornoxicam	0.005 ± 0.0015 (6)	–	0.045 ± 0.019 (6)	–	0.11	n.s.
Tenoxicam	0.16 ± 0.0 (3)	< 0.001	2.0 ± 0.5 (3)	< 0.05	0.08	< 0.05
Piroxicam	0.16 ± 0.0007 (3)	< 0.001	1.4 ± 0.8 (3)	< 0.05	0.11	n.s.
Aspirin	16.0 ± 0.07 (3)	< 0.001	157 ± 73 (3)	<0.05	0.10	< 0.05
Diclofenac	0.014 ± 0.0002 (3)	< 0.01	0.046 ± 0.01 (3)	n.s.	0.30	< 0.05
Indomethacin	0.015 ± 0.0005 (3)	< 0.01	0.22 ± 0.06 (3)	< 0.05	0.07	< 0.05
Tenidap	0.16 ± 0.0 (3)	< 0.001	3.2 ± 1.8 (3)	< 0.05	0.05	n.s.

n.s. = not significant.
[a] p-value by t-test comparing lornoxicam with each of the other NSAIDs.
[b] p-value by t-test, COX-1 < COX-2.
n = number of independent experiments.

potently inhibited than COX-2. The other NSAIDs tested for comparison, showed similar preferential COX-1 inhibition as lornoxicam with the exception of indomethacin and tenidap which inhibited COX-1 about 15-fold and 20-fold more potently than COX-2. Lornoxicam's inhibitory activity on COX-1 and COX-2 was about 30-fold greater than of the other oxicams piroxicam and tenoxicam. Of the NSAIDs tested, lornoxicam showed the lowest IC_{50} value on both isoenzymes (Table 1).

In a second set of experiments lornoxicam's inhibitory potency was examined in two almost identical assays using different human cell types specific for COX-1 or COX-2. Lornoxicam dose-dependently inhibited TXB_2 formation in HEL cells and $PGF_{1\alpha}$-formation in LPS-stimulated Mono Mac 6 cells (Fig. 1). Both COX isoenzymes were inhibited almost equipotently, with IC_{50} values of 0.003 µM for COX-1 and of 0.008 µM for COX-2. The maximum inhibition of COX-1 was observed at 10 µM, resulting in 98% inhibition of TXB_2 formation. For COX-2, maximum inhibition was obtained at 1 µM yielding 94% reduction of the $PGF_{1\alpha}$ production. Thus lornoxicam appears to be an NSAID exhibiting strong and equipotent COX-1/-2 inhibition.

For comparison, other NSAIDs were tested in parallel (Table 2). Piroxicam and tenoxicam inhibited COX-1 approximately 100 times less potently than lornoxicam. On COX-2 lornoxicam was about 4 times more potent than tenoxicam and about 70 times more potent than piroxicam. The other NSAIDs, with the exception of diclophenac, were also less potent than lornoxicam on both COX isoenzymes. With the exception of meloxicam, which showed a 14-fold greater selectivity for COX-2, the other NSAIDs were either equipotent COX-1/-2 inhibitors, like lornoxicam, or were more selective for COX-1 (Table 2).

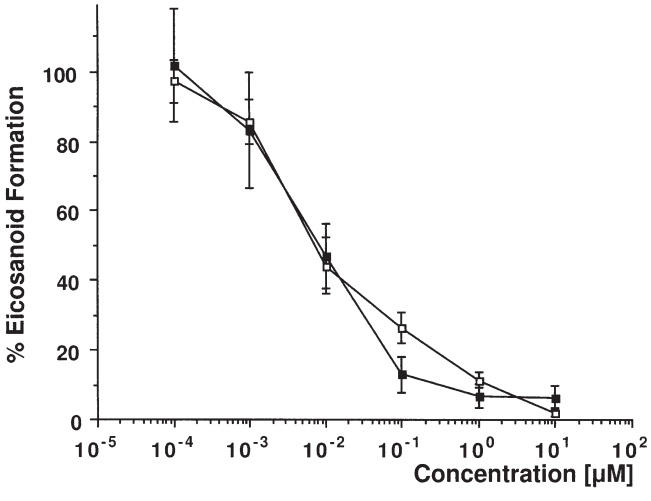

Fig. 1. Inhibition of eicosanoid formation in HEL cells (COX-1) and LPS-stimulated Mono Mac 6 cells (COX-2). Lornoxicam (0.0001 to 10 µM) was incubated with either cell types. After the addition of arachidonic acid COX-1-derived TXB_2 (open symbols) or COX-2-derived $PGF_{1\alpha}$ (closed symbols) were assessed in cell-free supernatants. Values are presented as mean ± SD of the percentage of eicosanoid production of cultures treated with vehicle only.

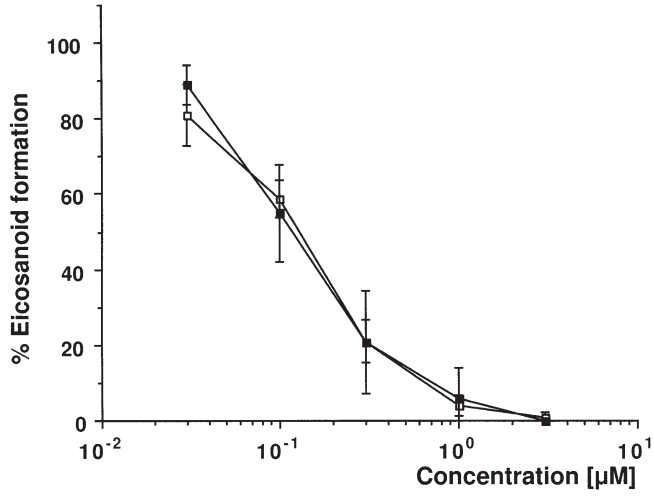

Fig. 2. Inhibition of COX-1-derived TXB_2 from whole blood during clotting and COX-2-derived PGE_2 during LPS-stimulation of whole blood cells. Lornoxicam (0.03 to 3 µM) was added to whole blood and allowed to clot. Serum concentrations of TXB_2 (open symbols) were assessed by ELISA. For COX-2 inhibition lornoxicam (0.03 to 3 µM) was incubated with heparinized blood together with LPS (10 µg) for 24 h. Plasma concentrations of PGE_2 (closed symbols) were measured by ELISA. Values are presented as mean ± SD of the percentage of eicosanoid production of controls treated with vehicle only.

Table 2. Inhibition of eicosanoid formation in HEL cells (COX-1) and LPS-stimulated Mono Mac 6 cells (COX-2) by lornoxicam and other NSAIDs.

Compound	IC_{50} for COX-1 [μM] ± SD (n)	p-Value[a]	IC_{50} for COX-2 [μM] ± SD (n)	p-Value[a]	Ratio COX-1/COX-2	p-Value[b]
Lornoxicam	0.003 ± 0.002 (5)	–	0.008 ± 0.003 (5)	–	0.38	n.s.
Tenoxicam	0.32 ± 0.20 (3)	< 0.05	0.13 ± 0.016 (3)	< 0.01	2.44	n.s.
Piroxicam	0.45 ± 0.50 (3)	< 0.01	0.77 ± 0.22 (3)	< 0.05	0.58	n.s.
Meloxicam	1.46 ± 0.25 (3)	< 0.01	0.10 ± 0.027 (3)	< 0.05	14.21	< 0.05
Diclofenac	0.0006 ± 0.0001 (3)	< 0.05	0.017 ± 0.003 (3)	< 0.05	0.04	< 0.05
Ketorolac	0.025 ± 0.007 (3)	< 0.05	0.039 ± 0.006 (3)	< 0.01	0.64	n.s.
Indomethacin	0.0045 ± 0.0014 (3)	n.s.	0.045 ± 0.010 (3)	< 0.05	0.10	< 0.05
Tenidap	0.021 ± 0.009 (3)	< 0.05	0.70 ± 0.16 (3)	< 0.05	0.03	< 0.05
Ibuprofen	1.07 ± 0.32 (3)	< 0.05	1.12 ± 0.47 (3)	< 0.01	0.95	n.s.
Aspirin	9.58 ± 0.61 (4)	< 0.01	16.0 ± 5.8 (4)	< 0.01	0.60	n.s.

n.s. = not significant.
[a] p-value by t-test comparing lornoxicam with each of the other NSAIDs.
[b] p-value by t-test, COX-2 < COX-1 (meloxicam and tenoxicam); p-value COX-1 < COX-2 for all other compounds.
n = number of independent experiments.

Table 3. Inhibition of eicosanoid formation in clotted whole blood (COX-1) and LPS-stimulated whole blood (COX-2) by lornoxicam and other NSAIDs.

Compound	IC_{50} for COX-1 [μM] ± SD (n)	p-Value[a]	IC_{50} for COX-2 [μM] ± SD (n)	p-Value[a]	Ratio COX-1/COX-2	p-Value[b]
Lornoxicam	0.13 ± 0.022 (3)	–	0.13 ± 0.03 (3)	–	1.0	n.s.
Meloxicam	3.5 ± 1.7 (3)	< 0.05	0.74 ± 0.33 (3)	< 0.05	4.7	< 0.05
Ketorolac	0.11 ± 0.03 (3)	n.s.	0.06 ± 0.01 (3)	n.s.	4.0	< 0.05
Indomethaciin	0.24 ± 0.05 (3)	< 0.05	0.13 ± 0.02 (3)	n.s.	1.8	< 0.05
Tenidap	4.4 ± 1.2 (3)	< 0.01	2.16 ± 0.26 (3)	< 0.01	2.0	< 0.01

n.s. = not significant.
[a] p-value by t-test comparing lornoxicam with each of the other NSAIDs.
[b] p-value by t-test, COX-2 < COX-1.
n = number of independent experiments.

Effects of lornoxicam on cyclooxygenase-1/-2 activity in whole blood

In whole blood, lornoxicam inhibited COX-1-derived TXB_2 and COX-2-derived PGE_2 dose dependently resulting in almost identical inhibition curves (Fig. 2). This was also reflected by identical IC_{50} values of 0.13 μM for both COX isoenzymes. At 3 μM lornoxicam inhibited both COX isoenzymes completely. Indomethacin showed approximately equal inhibitory activities on both COX isoenzymes, whereas meloxicam and ketorolac were slightly COX-2 selective (Table 3). Thus, lornoxicam showed potent and balanced COX-1/-2 inhibition in whole blood.

Effect of lornoxicam on iNOS-mediated NO accumulation

Lornoxicam inhibited the accumulation of NO in supernatants of LPS-stimulated RAW 264.7 cells dose dependently with an IC_{50} of 65 μM (Table 4). Indomethacin was equipotent with lornoxicam, whereas piroxicam and diclofenac were approximately three times less potent than lornoxicam. Ketorolac, ibuprofen and naproxen inhibited NO formation only minimally at the concentrations tested.

Effects of lornoxicam on the formation of TNF-α, IL-1β, IL-6 and IL-8

We next investigated whether lornoxicam affected the production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and the chemokine IL-8 during LPS-stimulation of the human monocytic cells THP-1. For comparison we tested piroxicam as another member of the oxicam class of NSAIDs

Table 4. Inhibition of NO accumulation in supernatants of LPS-stimulated RAW 264.7 cells.

Compound	IC_{50} value n [μM] ± SD	p-Value[a]	
Lornoxicam	65 ± 3	9	–
Piroxicam	240 ± 38	3	< 0.001
Indomethacin	107 ± 29	3	< 0.001
Diclofenac	200 ± 23	3	< 0.001
Ketorolac	> 300	3	n.a.
Ibuprofen	> 300	3	n.a.
Naproxen	> 300	3	n.a.

n.a. = not applicable.
[a] p-value by t-test comparing lornoxicam with each of the other NSAIDs.
n = number of independent experiments.

Table 5. Effects of lornoxicam and piroxicam on the formation of TNF-α, IL-1β, IL-6 and IL-8 in LPS-stimulated THP-1 cells.

Cytokine	Compound	IC_{50} [µM]	% Cytokine formation (mean ± SD)			
			10 µM	30 µM	100 µM	300 µM
IL-6	Lornoxicam	54 ± 20	77.4 ± 11.0	57.7 ± 5.0	41.1 ± 4.7	11.5 ± 1.5
	Piroxicam	appr. 470	77.1 ± 14.9	97.1 ± 4.5	83.0 ± 18.5	59.1 ± 8.5
TNF-α	Lornoxicam	n.d.	113,2 ± 5.0	127.0 ± 5.2	143.0 ± 11.9	164.0 ± 25.2
	Piroxicam	n.d.	108.0 ± 9.9	105.0 ± 7.6	119.0 ± 9.1	134.0 ± 4.2
IL-1β	Lornoxicam	n.d.	89.3 ± 16.0	92.8 ± 7.5	114.0 ± 12.8	134.0 ± 2.4
	Piroxicam	n.d.	92.2 ± 8.8	98.2 ± 3.5	111.0 ± 8.3	106.0 ± 9.5
IL-8	Lornoxicam	n.d.	108.0 ± 6.6	122.0 ± 20.6	132.0 ± 19.9	125.0 ± 15.1
	Piroxicam	n.d.	106.0 ± 10.4	107.0 ± 8.0	111.0 ± 14.2	109.0 ± 7.3

n = 3.
n.d. = not determined.
appr. = approximately.

in parallel. The results are presented in Table 5. Lornoxicam dose dependently inhibited the IL-6 formation with an IC_{50} value of 54 µM (Fig. 3 and Table 5). At 300 µM 89% inhibition of IL-6 secretion was observed. The production of TNF-α, IL-1β and IL-8 was only slightly affected. At the highest concentrations used (300 µM) lornoxicam showed a weak stimulation of TNF-α , IL-1β and IL-8 production.

Piroxicam also inhibited the IL-6 production, albeit to a lesser extent, not significantly and not strictly concentration dependently. At the concentrations tested, a complete inhibition of IL-6 formation was not obtained. With an IC_{50} of approximately 470 µM piroxicam's inhibitory activity was found to be at least one order of magnitude lesser than that of lornoxicam. TNF-α production was slightly increased by piroxicam at the highest concentration (134% of control at 300 µM), whereas the production of IL-1β and IL-8 was not affected (Table 5). Cellular toxic effect by the compounds were monitored by measuring the concentration of lactate dehydrogenase (LDH) in the culture supernatants in parallel. The concentration of LDH were not increased in any of the experiments (data not shown).

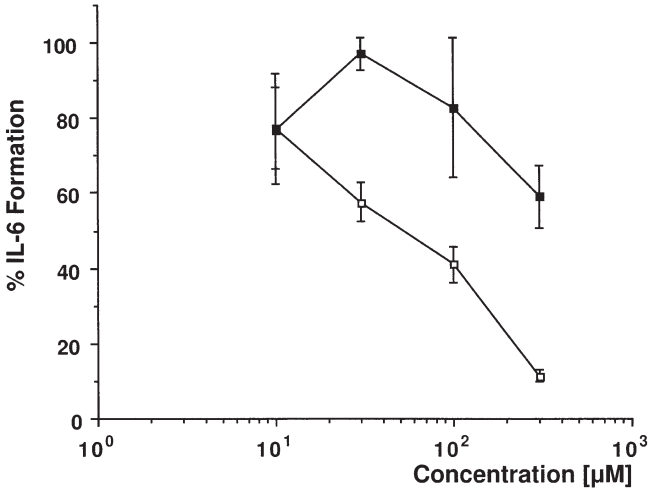

Fig. 3. Inhibition of IL-6 formation by lornoxicam and piroxicam during LPS-stimulated THP-1 cells. Lornoxicam (open symbols) or piroxicam (closed symbols) were incubated with the cells for 10 min at RT at concentrations ranging from 10 µM to 300 µM. Thereafter, LPS (5 µg/ml) was added and cells incubated for 15 h at 37 °C. Then, cell-free supernatants were harvested and subjected to IL-6 ELISA. Values are presented as mean ± SD of the percentage of IL-6 production of control cultures treated with vehicle only.

Effects of lornoxicam on IL-6 mRNA steady state levels

RT-PCR was performed to examine whether lornoxicam's IL-6 inhibitory activity was related to the inhibition of IL-6 gene transcription (Fig. 4). THP-1 cells were incubated with vehicle (DMSO) or with LPS in combination with vehicle and dexamethasone, or lornoxicam, or piroxicam. The isolated RNA was transcribed into cDNA. Using β-actin specific primers the cDNA together with a β-actin mimic competitor template was amplified to quantify β-actin specific cDNA. Then, about 0.12 attomole-eqivalents of β-actin cDNA were amplified with β-actin specific primers (Fig. 4a) or interleukin-6 specific primers (Fig. 4b). Figure 4b shows that the IL-6 mRNA steady state level is increased by LPS (upper panel, lane 2), and that this increase is completely inhibited by 10 nM of dexamethasone (upper panel, lane 3). Lornoxicam or piroxicam did not affect the IL-6 mRNA steady state levels detectably (Fig. 4b, lanes 4 and 5). This finding suggests that lornoxicam does not act on the level of induction and transcription of the IL-6 gene but points to an inhibition of one of the post transcriptional or translation processes of IL-6 production as a possible mechanism of action.

Discussion

NSAIDs reduce pain and swelling in inflamed tissues. Their mechanism of action is at least shared by the inhibition of prostaglandin synthesis which is mediated by the enzyme COX [37, 38]. COX exists in two isoforms, COX-1 and COX-2, each with distinct expression pattern in various cell types [39–42]. This is thought to reflect distinct functions. Thus, COX-1 has been suggested to provide a physiologic level of prostaglandins for normal platelet, stomach and kidney function. In contrast, COX-2 has been found to be high-

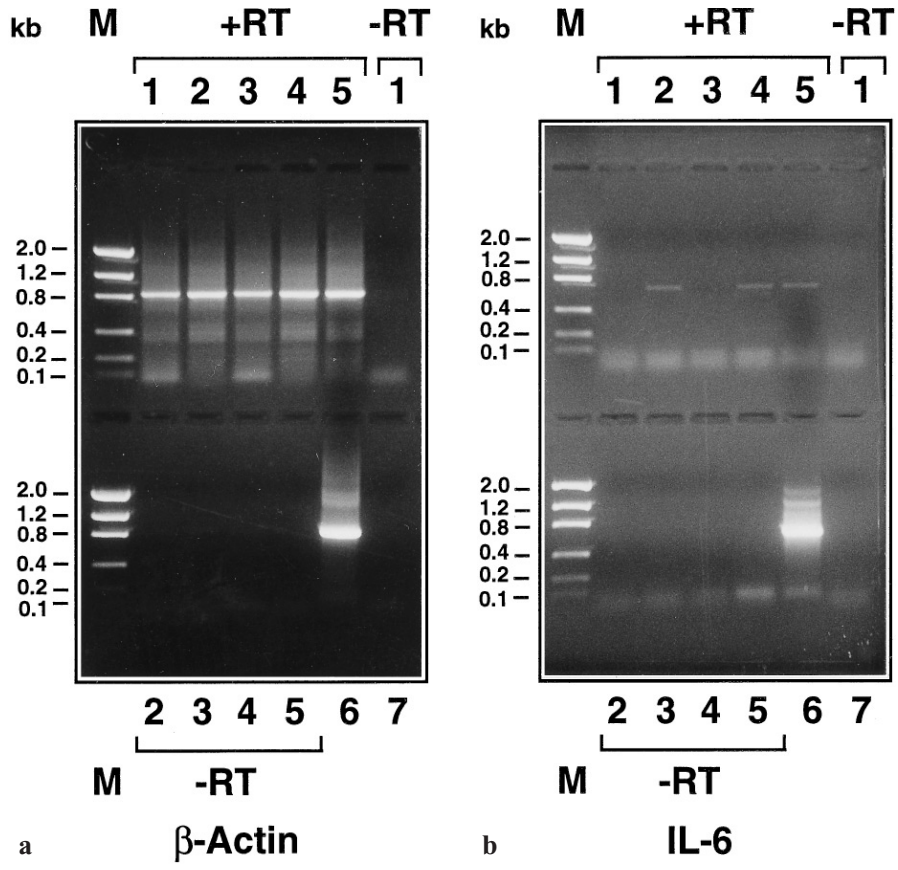

Fig. 4. Assessment of mRNA levels of IL-6 normalised on β-actin mRNA. RT-PCR with β-actin specific primers (a) or IL-6 specific primers (b) was performed with (+ RT) or without (– RT) added reverse transcriptase using RNA prepared from THP-1 cells that were cultured in the presence of DMSO (lane 1), DMSO and LPS (lane 2), LPS and dexamethasone (lane 3), LPS and lornoxicam (lane 4), LPS and piroxicam (lane 5). Lane 6 shows amplicons that were obtained using either β-actin specific cDNA (a) or IL-6 specific cDNA (b) as templates. Lane 7 represents the reagent control, and to lane M a DNA size marker was applied.

ly induced at inflammatory sites in animals [43–46] as well as in patients with inflammatory diseases [47]. Hence, COX-2 is considered to be responsible for pro-inflammatory prostaglandin formation [4, 48]. In addition, COX-2 has also been discovered to be involved in the regulation of physiologic functions (for recent reviews see [15, 16]). In the kidney, COX-2 derived prostaglandins seem to mediate the renin production that in turn regulates sodium balance and fluid volume [77]. In the female genital tract, COX-2 derived prostaglandins have been suggested to control ovulation and after fertilisation the implantation of the embryo in the uterine endometrium [78]. In the spinal cord and in the brain COX-2, is thought to provide prostaglandins that may modulate postsynaptic signalling of excitatory neurones [79].

NSAIDs have been demonstrated to inhibit both COX isoenzymes to various extents [4, 30, 49, 50]. The NSAID lornoxicam was previously found to inhibit COX at nanomolar concentrations in rat polymorphonuclear leukocytes in vitro [1]. A discrimination of the two COX isoenzymes was not then possible. Hence with the discovery of COX-2, we asked ourselves whether lornoxicam would affect COX-1 or COX-2, or both of the COX isoenzymes.

COX-1 in the absence of COX-2 has been demonstrated to be present in human platelets and in cells of the human erythroleukemic cell line HEL [31, 40]. Testing lornoxicam's COX-1 inhibitory potential employing these cells i. e. the arachidonic acid-induced aggregation of human platelets and the formation of TXB_2 in HEL cells confirmed the potent COX inhibitory activity of lornoxicam observed earlier [1]. Lornoxicam's inhibitory activity was greater than that of other oxicams and structurally unrelated NSAIDs. Similar IC_{50} values for lornoxicam and the other oxicams were obtained in both assays, although compound incubation time and principle of measurement was different (see Materials and Methods). Diclofenac, indomethacin and tenidap elicited more pronounced COX-1 inhibition in the erythroleukemic cells than in platelets. This could be explained by the different incubation periods in both assays, since inhibition of COX by indomethacin and some other NSAIDs was found to be time-dependent [51, 52].

To assess lornoxicam's potency against COX-2, we also employed two intact cell assays and compared these results with the ones from the COX-1 inhibition experiments. In LPS-stimulated murine macrophages (J774.2 cells) lornoxicam and diclofenac inhibited COX-2 approximately equipotently, whereas the other NSAIDs showed markedly less inhibitory effects (Table 1). This was also observed in the human COX-2 assay using LPS-stimulated monocytic cells

(Mono Mac 6 cells) (Table 2). The comparison of the IC_{50} values of murine COX-2 with the ones from either human COX-1 assay indicated selectivity of all compounds for COX-1 (Table 1). This is in contrast to the comparison of human COX-2 inhibition with human COX-1 inhibition of either of the two COX-1 assays. The human COX-1/human COX-2 comparison revealed equipotent inhibition of both human COX isoenzymes by lornoxicam (Table 2). This was also observed for the other NSAIDs tested, with the exception that tenidap and diclofenac showed some COX-1 selectivity, and that tenoxicam and meloxicam showed some preference for COX-2 (Tables 1, 2). Of all tested oxicams and structurally unrelated NSAIDs lornoxicam was the most potent COX-1/-2 inhibitor in the intact cell assays.

Our IC_{50} values of the classical NSAIDs on both human COX-1 and human COX-2 in intact cells are in good agreement with the observations made by others [49] who used similar intact cell assays for the assessment of human COX-1/-2 inhibition. Our murine COX-2 inhibition data are also in line with the IC_{50} values reported earlier [30]. The differences between their and our murine COX-2 IC_{50} values to the IC_{50} values in human COX-2 testing suggests that comparisons of potencies of compounds against COX-1 or COX-2 should be made within the same species and within the same test set-up in order to arrive at meaningful selectivities for the compounds tested for.

Testing of compounds in the environment of whole blood more closely resembles the in vivo situation than do intact cell assays. Therefore, in further experiments, we investigated lornoxicam's inhibitory potential on COX-1/-2 using whole blood from healthy volunteers [32]. Clotting of whole blood as a measure of COX-1 as well as the formation of PGE_2 in LPS-stimulated blood as a measure of COX-2 was markedly and equipotently inhibited by lornoxicam, albeit to about 20-fold less than in intact cell assays. This was also observed with the other compounds tested for comparison. The COX-2 preferential inhibitor meloxicam was 4.7-fold more selective for COX-2 and interestingly ketorolac also exhibited a similar selectivity for COX-2. These two results contrast with the selectivity results in the intact cell assays by us and others [30, 49]. The differing effects of NSAIDs in whole blood may be due to the complex environment of blood per se [32]. Thus, besides COX inhibition, other properties of the compounds could influence their inhibitory potency: effects on the mobilisation of arachidonic acid from intra- or extra-cellular sources [53], plasma protein binding [54], stability in plasma, or effects on COX-2 gene expression. The latter point has been a matter of debate, and was recently disproved for human macrophage COX-2 for aspirin, indomethacin and naproxen [55].

The inhibition of the synthesis of prostaglandins by NSAIDs or the specific neutralisation of PGE_2 by antibodies has been demonstrated to effectively reduce inflammatory symptoms such as oedema and pain [16–18]. However, prostaglandins do not seem to elicit inflammation per se. They instead amplify inflammatory symptoms elicited by other substances such as pro-inflammatory cytokines, histamine, bradykinin and NO [19, 56–58]. NO localised in high amounts in inflamed tissues has been shown to induce pain locally [59, 60] and enhances central as well as peripheral nociception [61]. Inflammatory NO is thought to be synthesised by the inducible isoform of nitric oxide synthase (iNOS). The NO inhibitory activity of lornoxicam in LPS-stimulated macrophages in vitro at µmolar concentrations suggests that lornoxicam may exhibit this potentially favourable activity in controlling inflammation and pain clinically. Indomethacin and piroxicam showed comparable but lesser inhibitory activities on NO levels in our experiments. Ketorolac, ibuprofen and naproxen did not show significantly reduced NO levels up to 300 µM. Inhibitory activities of the NO formation in stimulated rodent macrophages by indomethacin, ibuprofen and aspirin have been demonstrated previously [62]. The reported IC_{50} values for indomethacin at approximately 250 µmolar and for ibuprofen between 1 and 2 mM suggest that our test model is in good agreement with theirs, underscoring lornoxicam's potency in inhibiting NO formation. Lornoxicam's NO inhibitory activity in vitro at µmolar concentrations could translate to a clinically relevant activity complementing lornoxicam's potent COX-1/-2 inhibition.

Pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and chemokines such as IL-8 have been shown to control inflammation in vitro as well as in vivo [23, 24]. These cytokines are thought to be interlinked in a cascade being produced serially by e.g. macrophages during an inflammatory response. Furthermore, the development of hyperalgesic states during inflammation is thought to be mediated by pro-inflammatory cytokines [19]. Therefore, we investigated whether lornoxicam would possibly influence the formation of TNF-α, IL-1β, IL-6 and IL-8. Since prostaglandins have been shown to affect the formation of cytokines [18, 63], we examined whether lornoxicam would directly affect cytokine formation in an in vitro model that does not involve either COX-1 or COX-2 related prostaglandins. Cells of the human monocytic cell line THP-1 produce these cytokines without synthesising prostaglandins following direct stimulation with LPS [64 and our own unpublished observations], which is omitting any phorbol ester-induced differentiation [65]. Lornoxicam and piroxicam only slightly affected the production of TNF-α, IL-1β and IL-8. A marked effect was however seen with lornoxicam inhibiting the production of IL-6. Piroxicam also inhibited IL-6 production but, ten times less potently. Inhibition of IL-6 production was not paralleled by decreased levels of IL-6 specific mRNA. Therefore, lornoxicam and piroxicam do not seem to counteract LPS-stimulation, signal transduction or IL-6 gene transcription in this model. They rather seem to inhibit post-transcriptional or translational mechanisms of the IL-6 gene expression. The direct inhibition of IL-6 formation by lornoxicam may well complement the indirect IL-6 inhibition previously reported for NSAIDs in general which is mediated by the inhibition of PGE_2 formation by NSAIDs [18, 66]. Thus, direct inhibition of IL-6 formation may present a favourable additional anti-inflammatory and analgesic property of lornoxicam since IL-6 exhibits a plethora of pro-inflammatory effects and its levels have been correlated to inflammatory disease activity including hyperalgesia [67, 68].

The potent COX-1/-2 inhibition by lornoxicam we observed in vitro is in good agreement with animal experiments where inhibition of COX-1-mediated prostaglandin synthesis is thought to be at least partly involved in compound efficacy such as acetylcholine-induced writhing, or where COX-2

inhibition such as carrageenan-induced paw oedema has been suggested to be causal for the anti-inflammatory effects [18, 45, 46]. Furthermore, lornoxicam's potent in vitro COX-1/-2 inhibition matches well the clinical observations of effective anti-inflammatory and analgesic activities [9–12]. However, whether clinically administered doses of lornoxicam are also clinically effective in inhibition of IL-6 and NO formation is unknown. The IC_{50} values for NO and IL-6 formation inhibition differ by four orders of magnitude to the IC_{50} values for COX inhibition from intact cells. The peak plasma concentration of a single orally-administered dose of 8 mg lornoxicam amounts to approximately 2 µM [69], which is well above the IC_{50} value for COX inhibition, but below the IC_{50} values for the inhibition of iNOS or IL-6 formation. In plasma, lornoxicam and other NSAIDs are bound to protein to more than 95% [6, 54]. Capillary leakage during inflammation leads to extravasion of protein-bound NSAID and the acidic tissue environment can facilitate the accumulation and cellular uptake of acidic NSAIDs such as lornoxicam [54]. Therefore, the concentrations of lornoxicam that inhibited the formation of IL-6 and NO in our in vitro experiments could be relevant, not to the plasma concentrations, but perhaps to concentrations in inflamed tissues.

COX-1 inhibition is thought to be responsible for the renal and gastrointestinal side effects of NSAIDs. The new COX-2 selective inhibitors currently being developed [16, 70] have been demonstrated to effectively suppress symptoms of inflammatory prostaglandin formation in animals [46, 71] as well as in man in the absence of classical NSAID side effects [70]. However, it is still a matter of discussion whether inflammation-related pain can be sufficiently treated with selective COX-2 inhibitors, since COX-2-mediated prostaglandin formation starts only after the transcriptional induction and synthesis of new COX-2 protein. Studies on acute pain have clearly shown involvement of COX-1-related prostaglandins in the periphery as well as in the spinal cord [72]. NSAIDs are effective in models of acute pain. In addition, NSAIDs have been observed to modify chronic inflammatory disease which is to some extent debated for COX-2 selective compounds [73]. Furthermore, studies in COX-1 and COX-2 gene knock-out mice involving topically-administered skin irritants such as phorbol esters indicated significant COX-1-mediated prostaglandin formation [74–76]. In these models, COX-2 was not required for this type of inflammation. A recent study using the murine chronic granulomatous tissue air pouch model pointed in the same direction, that the COX-2 selective inhibitors nimesulide and NS-398 failed to reduce granuloma development and vascularity whereas aspirin, indomethacin and steroids showed these effects [73]. In the carrageenan-induced rat paw oedema which is driven by COX-2-related prostaglandins a selective COX-1 inhibitor produced markedly decreased prostaglandin levels. Surprisingly this did not parallel substantial anti-inflammatory or analgesic effects. However, the administration of a COX-2 selective inhibitor was fully active in reversing oedema and hyperalgesia [80]. The differing effects of decreased prostaglandin levels in the periphery were reflected by the prostaglandin levels in the cerebrospinal fluids. The COX-1 selective inhibitor only marginally reduced elevated prostaglandin levels, whereas the COX-2 selective inhibitor reduced the elevated prostaglandin levels to normal [80]. Thus, COX-2 selective inhibitors may ultimately prove to be superior in the management of inflammatory disease and pain. Until then balanced COX-1/-2 inhibition, perhaps in combination with the inhibition of other pro-inflammatory principles, offers an effective therapeutic regimen in controlling acute and chronic inflammation, hyperalgesia and pain.

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