Aceclofenac spares cyclooxygenase 1 as a result of limited but sustained biotransformation to diclofenac

Objective: **The mechanism of action of aceclofenac is currently unclear. This study investigated whether biotransformation to metabolites (4′-hydroxy-aceclofenac, diclofenac, 4′-hydroxy-diclofenac) contributes to inhibitory effects on the cyclooxygenase (COX) isozymes in vitro and ex vivo.**

Methods: **In vitro investigations were performed with human whole blood and human blood monocytes. A randomized crossover study was performed in volunteers receiving 100 mg aceclofenac or a sustained-release resinate formulation of 75 mg diclofenac to assess the pharmacokinetics and the ex vivo inhibition of COX-1.**

Results: **In short-term in vitro assays, neither aceclofenac nor 4′-hydroxy-aceclofenac affected COX-1 or COX-2, whereas diclofenac and 4′-hydroxy-diclofenac inhibited both isoforms. In long-term in vitro assays, aceclofenac and 4′-hydroxy-aceclofenac suppressed both COX isoforms. However, this inhibition was paralleled by a conversion to diclofenac and 4′-hydroxy-diclofenac, respectively. Maximal plasma concentrations of diclofenac after oral administration of aceclofenac (0.39 μmol/L) or diclofenac (1.28 μmol/L) were sufficient for a greater than 97% inhibition of COX-2 (50% inhibitory concentration, 0.024 μmol/L) and a 46% (aceclofenac treatment) or 82% inhibition (diclofenac treatment) of COX-1 (50% inhibitory concentration, 0.43 μmol/L). Moreover, ex vivo COX-1–dependent thromboxane B_2 synthesis was inhibited significantly less by aceclofenac than by diclofenac.**

Conclusions: **Inhibition of COX isozymes by aceclofenac requires conversion into diclofenac. Although 100 mg aceclofenac yielded diclofenac concentrations substantially lower than 75 mg diclofenac, these were sufficient for a sustained block of COX-2 but caused a minor and shorter inhibition of COX-1 than 75 mg diclofenac. In conclusion, both COX-1–sparing and COX-2–inhibitory actions of aceclofenac may rest in its limited but sustained biotransformation to diclofenac. (Clin Pharmacol Ther 2003;74:222-35.)**

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The pharmacologic activity of nonsteroidal anti-inflammatory drugs (NSAIDs) is attributed to inhibition of the cyclooxygenase (COX) enzymes, which catalyze the first step of the synthesis of prostanoids.[1-5] Whereas the majority of side effects of NSAIDs (eg, gastrointestinal ulceration and bleeding) are attributed to suppression of the COX-1 isoform, inhibition of COX-2–dependent prostanoid production is held responsible for the anti-inflammatory, analgesic, and antipyretic effects of NSAIDs (for review, see references 6 and 7). At therapeutic doses, conventional NSAIDs inhibit both COX-1 and COX-2, although they vary in their potencies against the 2 isozymes.[8]

Over the past years, several approaches have been pursued to reduce gastrointestinal toxicity associated with the use of NSAIDs. Apart from the specific COX-2 inhibitors celecoxib and rofecoxib, another NSAID, aceclofenac, has been launched as an anti-inflammatory and analgesic drug with efficacy similar to that of other NSAIDs.[9-17] On the basis of data from clinical trial reports, aceclofenac seems to possess an improved gastrointestinal tolerability,[11,12,18-23] al-

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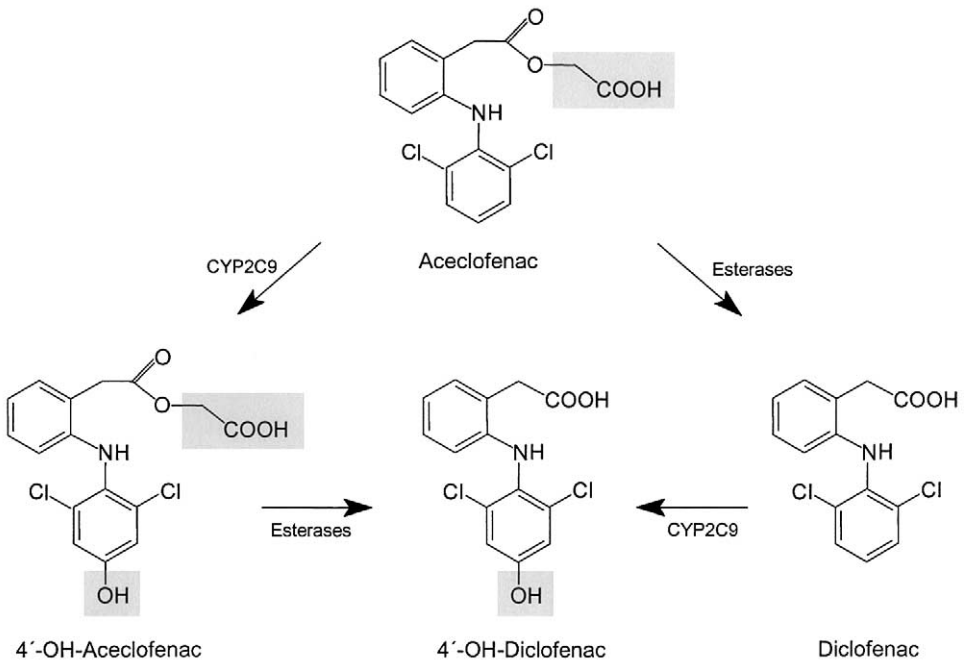

Fig 1. Biotransformation of aceclofenac in humans.

though the safety of this drug remains to be established in large endoscopic studies. Aceclofenac (2-[(2',6'-dichlorophenyl)amino]phenylacetoxyacetic acid) contains an additional esterified acetoxy side chain as compared with the structurally related diclofenac. The mode of action of aceclofenac and the reason for its improved gastrointestinal tolerability are currently unknown. It has been suspected that aceclofenac may act predominantly by suppressing the synthesis of proinflammatory cytokines or by other mechanisms independent of COX inhibition (for review, see reference 24). However, these actions cannot explain the entire pharmacologic activity of the compound. Conflicting data have been published on the ability of aceclofenac to inhibit COX enzymes.[25-28] Inhibition of COX-2 expression by interference with cytokine signaling[28-30] has been proposed to account for inhibition of prostaglandin formation during long-term treatment with aceclofenac. On the other hand, biotransformation to active metabolites may provide an explanation for the pharmacologic activity of aceclofenac. In vivo, aceclofenac is 4'-hydroxylated by cytochrome P450 2C9 or hydrolyzed at the acetoacetoxy side chain to yield 4'-hydroxy-aceclofenac, diclofenac, and 4'-hydroxy-diclofenac (Fig 1).[31,32] 4'-Hydroxy-aceclofenac has been shown to be the main metabolite in plasma and urine after oral administration of aceclofenac.[31,32] However, another metabolite, diclofenac, has been largely neglected thus far. Although diclofenac is a minor metabolite of aceclofenac,[31,32] the compound has previously been proved to inhibit the COX-2 enzyme even at nanomolar concentrations.[8] No study published as yet has addressed the question as to whether the concentrations of diclofenac generated in vivo after aceclofenac administration are sufficient to inhibit either or both of the COX isoforms. Moreover, differences in the COX-2 selectivity profiles of clinically used doses of aceclofenac and diclofenac have not been evaluated until now.

This study, therefore, investigated the contribution of metabolites to the action of aceclofenac. To address this issue, we first performed an in vitro analysis to clarify the effects of aceclofenac and its metabolites 4'-hydroxy-aceclofenac, diclofenac, and 4'-hydroxy-diclofenac on the activity of the COX isozymes under conditions of both short- and long-term incubation. The second and major objective of this study was to compare the kinetics of the COX inhibitors diclofenac and 4'-hydroxy-diclofenac and the concomitant inhibition of the COX enzymes in healthy volunteers who received recommended clinical doses of either 100 mg aceclofenac or 75 mg diclofenac. Here, we show that

aceclofenac per se does not inhibit the COX isozymes and that it interferes with the activity of both isoforms only after metabolic conversion to clinically relevant concentrations of diclofenac. The extent and the kinetics of this bioactivation were associated with a virtually complete COX-2 blockade for sustained periods but a pronounced COX-1–sparing effect as compared with the sustained-release diclofenac formulation.

METHODS

In vitro investigations

Materials

Aceclofenac was provided by Almirall Prodesfarma (Barcelona, Spain). 4'-Hydroxy-aceclofenac and 4'-hydroxy-diclofenac were provided by Novartis (Basel, Switzerland). A23187 was purchased from Alexis Deutschland GmbH (Grünberg, Germany). Aspirin, diclofenac sodium, Histopaque-1077, ketoprofen, and lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6) were obtained from Sigma (Deisenhofen, Germany). RPMI-1640 medium was purchased from Life Technologies (Eggenstein, Germany). Prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) enzyme immunoassay kits were from Cayman (Ann Arbor, Mich). For HPLC analysis, acetonitrile (gradient grade), disodium hydrogen phosphate dodecahydrate, *n*-hexane, methanol (gradient grade), phosphoric acid, potassium dihydrogen phosphate, and tetrahydrofuran were purchased from Merck (Darmstadt, Germany). Diethylether (HPLC grade) was obtained from Fluka (Sigma-Aldrich, Taufkirchen, Germany).

Effects of aceclofenac and its metabolites on COX-1 and COX-2 activity in short-term assays

COX-1 assay. Blood was drawn from healthy male volunteers who had not taken any NSAID or other drug for 2 weeks before blood sampling. Aliquots of whole blood without anticoagulant were immediately transferred to glass tubes containing test agent or vehicle, respectively. Blood was allowed to clot for 1 hour at 37°C.[8,33] Serum was separated by centrifugation, and serum TxB_2 levels were determined.

COX-2 assay. Mononuclear cells were isolated from heparinized human whole blood from healthy male volunteers by density gradient centrifugation with Histopaque-1077 as described previously.[34] Cells seeded in 48-well culture plates at a density of 1×10^6 cells per well were allowed to adhere for 3 hours. After removal of nonadherent cells by washing, adherent monocytes were cultured in RPMI-1640 medium. Incubations were performed under serum-free conditions in a humidified incubator at 37°C and 5% carbon dioxide. For assessing the effect of test compounds on COX-2 activity, monocytes were treated with aspirin (250 μmol/L) for 2.5 hours to inactivate endogenous COX activity. Thereafter cells were extensively washed and subsequently incubated with LPS (10 μg/mL) for 18 hours to induce COX-2. After extensive washing and medium change, the respective test compound was added to the cultures, followed by a 15-minute incubation period. Arachidonic acid (30 μmol/L) was subsequently added, and the incubation was continued for a further 15 minutes. Finally, the cell culture supernatants were removed and analyzed for PGE_2.

Effects of aceclofenac and its metabolites on COX-1 and COX-2 activity in long-term assays

COX-1 assay. Heparinized venous blood samples from healthy male volunteers were treated with test agents or vehicle for 24 hours. Fifteen minutes before harvest of the plasma, the calcium ionophore and stimulator of arachidonic acid liberation A23187 (50 μmol/L) was added to the blood samples. Plasma was separated by centrifugation, and TxB_2 levels were determined.

COX-2 assay. Aliquots of heparinized whole blood from healthy male volunteers were incubated with LPS (10 μg/mL) plus test agent or vehicle for 24 hours at 37°C. [8,33] The contribution of platelet COX-1 activity was suppressed by the addition of aspirin (10 μg/mL) at the start of the incubation. Plasma was separated by centrifugation, and PGE_2 levels were determined.

Stability of aceclofenac and 4'-hydroxy-aceclofenac in human whole blood

Sample preparation. Samples were prepared by adding 0.5 mL sodium fluoride solution (40 mg/mL), 1 mL 1-μmol/L phosphoric acid, and 0.1 mL internal standard stock solution (0.05 mg ketoprofen/1 mL acetonitrile/0.01-mol/L phosphate buffer solution [pH 2.5] [95:5 (vol/vol)]) to 1 mL plasma. Subsequently, 5 mL *n*-hexane/diethylether (50:50 [vol/vol]) was added and samples were extracted for 30 minutes under constant shaking. After centrifugation, the organic layer was transferred into a glass tube and evaporated to dryness under a nitrogen stream. The residue was dissolved in 120 μL of a solution consisting of 72% 0.01-μmol/L phosphate buffer, 15% acetonitrile, 10% methanol, and 3% tetrahydrofuran (final pH 2.5). Plasma calibration standards (10 to 10,000 ng/mL) were prepared by spiking plasma with aceclofenac and metabolites. Stock solutions of analytes were prepared in acetonitrile and stored at −80°C for a maximum of 1 month.

Chromatographic and detection conditions. Analytes were separated by use of a reversed phase column

(250/4 Nucleosil 120-5 C18; Machery-Nagel, Düren, Germany) and a C18 precolumn insert. The column temperature was maintained at 30°C. Elution of aceclofenac and metabolites was achieved at a flow rate of 1 mL/min. A gradient separation was used, with solvent A being 20% [vol/vol] 0.005-mol/L phosphate buffer and 80% [vol/vol] acetonitrile and solvent B being 88% [vol/vol] 0.01-mol/L phosphate buffer and 12% [vol/vol] acetonitrile. The gradient steps were as follows: 0 to 24 minutes, isocratic at 4% solvent A; 24 to 25 minutes, linear gradient from 4% to 12% solvent A; 26 to 40 minutes, isocratic at 12% solvent A; 40 to 41 minutes, linear gradient from 12% to 15% solvent A; 41 to 60 minutes, isocratic at 15% solvent A; 60 to 61 minutes, linear gradient from 15% to 4% solvent A. The program ended with a 15-minute re-equilibration at 4% solvent A. Ultraviolet absorption was at 282 nm. The limits of detection were 10 ng/mL (aceclofenac, 4'-hydroxy-aceclofenac, diclofenac) and 25 ng/mL (4'-hydroxy-diclofenac).

Determination of COX-2 messenger ribonucleic acid levels in human blood monocytes

Incubation protocol. Monocytes were isolated as described earlier with the exception that mononuclear cells were plated onto 24-well culture plates at a density of 1×10^6 cells per well. Monocytes were incubated with LPS (10 μg/mL) alone or in combination with the respective test compound for 4 hours in a humidified incubator at 37°C and 5% carbon dioxide. Afterward, cells were lysed and total ribonucleic acid was isolated with the RNeasy total RNA Kit (Qiagen, Hilden, Germany).

Quantitative reverse transcriptase–polymerase chain reaction. β-Actin (internal standard) and COX-2 messenger ribonucleic acid (mRNA) levels were determined by quantitative real-time reverse transcriptase–polymerase chain reaction as described previously.[34,35]

Statistics

All data are presented as mean ± SEM. Concentration response curves were fitted by use of a sigmoidal regression with variable slope, and 50% inhibitory concentration (IC_{50}) values were derived by use of PRISM version 3.0 (GraphPad, San Diego, Calif).

Pharmacokinetics of aceclofenac and its metabolites and ex vivo inhibition of COX activity

Subjects and study design

Twelve male volunteers, aged 21 to 29 years (mean age, 25 years) with a mean weight of 80.0 ± 2.6 kg (mean ± SEM), participated in the study. The study protocol was approved by the Ethics Committee of the University of Erlangen-Nürnberg, Erlangen, Germany. All volunteers gave written informed consent before study participation. All volunteers were healthy on the basis of medical history, physical examination, and routine laboratory screening results. Subjects were not permitted to take any other medication (including aspirin or other NSAIDs) within 2 weeks before and throughout the study. Volunteers with a history of coagulation disorders, a bleeding tendency, drug allergy, or gastrointestinal disorders were excluded from participation in the study. In a 1-way crossover design the subjects were randomly assigned to either 100 mg aceclofenac (Beofenac; UCB GmbH, Kerpen, Germany) or 140 mg diclofenac cholestyramine (INN, colestyramine) (sustained-release formulation; equivalent to 75 mg diclofenac sodium; in the following referred to as 75 mg diclofenac) (Voltaren Resinat; Novartis Pharma GmbH, Nürnberg, Germany) in an open-labeled fashion. The second drug was administered after a washout period of 14 days. Drugs were administered as a single oral dose between 8 and 9 AM after an overnight fast. Doses were chosen on the basis of clinical trials and the recommendations of the manufacturers.[11,12,36,37] Intake of food was delayed for 2 hours after drug administration. Peripheral venous blood samples were taken from each volunteer immediately before and at 20 and 40 minutes and at 1, 1.5, 2, 3, 4, 5, 6, 9, 12, 24, and 48 hours after administration of the drug. For determination of aceclofenac/metabolites or diclofenac/4'-hydroxy-diclofenac, heparinized blood samples were spun and, after addition of the esterase inhibitor sodium fluoride, plasma aliquots were snap-frozen. Until further analysis, plasma samples were stored at −80°C for a maximum of 1 month. Whole blood without anticoagulant was used for ex vivo COX-1 assays. Blood was drawn via an indwelling forearm vein catheter immediately before and up to 12 hours after the administration of the medication. Blood samples obtained 24 and 48 hours after administration were drawn by forearm vein puncture with a butterfly cannula.

Analysis of drugs and metabolites

Plasma samples were analyzed for aceclofenac, 4'-hydroxy-aceclofenac, diclofenac, and 4'-hydroxy-diclofenac (aceclofenac treatment) or diclofenac and 4'-hydroxy-diclofenac (diclofenac treatment) by use of the sample preparation and chromatographic conditions described earlier.

Table I. Apparent in vitro potencies of aceclofenac, 4'-hydroxy-aceclofenac, diclofenac, and 4'-hydroxy-diclofenac as inhibitors of COX-1 and COX-2 activity in short-term and long-term assays

	Short-term assays		*Long-term assays*	
	COX-1 IC_{50} (μmol/L)	*COX-2 IC_{50} (μmol/L)*	*COX-1 IC_{50} (μmol/L)*	*COX-2 IC_{50} (μmol/L)*
Aceclofenac	No inhibition*	No inhibition*	3.59 ± 0.54	1.65 ± 0.46
4'-Hydroxy-aceclofenac	No inhibition*	No inhibition*	12.73 ± 3.53	25.35 ± 7.98
Diclofenac	0.43 ± 0.13	0.0054 ± 0.0028	0.16 ± 0.03	0.024 ± 0.007
4'-Hydroxy-diclofenac	8.28 ± 0.93	0.72 ± 0.40	1.63 ± 0.56	0.76 ± 0.03

IC_{50} values are expressed as mean ± SEM of 3 blood donors. COX, Cyclooxygenase; IC_{50}, 50% inhibitory concentration.
*No inhibitory effect was noticed at concentrations up to 100 μmol/L.

Biochemical analysis

Ex vivo COX-1 assay. Immediately after blood sampling, whole blood samples without anticoagulant were incubated for 1 hour at 37°C and subsequently centrifuged, and serum TxB_2 levels (index of COX-1 activity) were determined.

Pharmacokinetic analysis

Plasma concentration–time curves were evaluated by noncompartmental analysis with WinNonlin version 3.3 (Pharsight, Mountain View, Calif). Maximal plasma concentration and time to maximal plasma concentration were obtained directly from the individual plasma concentration–versus–time curves. The terminal half-life was calculated as $\ln 2/\lambda_z$ by log-linear regression analysis of the plasma concentration–time curves in the terminal phase, in which λ_z denotes the time constant of the terminal slope. The area under the plasma concentration–time curve up to the last quantifiable plasma concentration (AUC_t) was determined according to the linear trapezoidal method. The calculated concentration-time profiles at steady state were approximated by superposition of the average concentration-time curves for thirty 12-hour dose intervals. Concentration-time profiles were calculated via a cubic spline function with exponential extrapolation. The superposition was estimated by the following formula:

$$C_{ss}(t) = \sum_{i=0}^{29} C_{spline}(t + i \cdot 12 \text{ hours})$$

Calculated steady-state concentrations (C_{ss}) at time 0 were approximately equal to the steady-state concentrations at 12 hours.

Pharmacodynamic analysis

The degree of COX-1 inhibition was calculated as the percentage change in serum TxB_2 concentrations measured at different time points after administration relative to predose serum TxB_2 levels. Maximal observed TxB_2 inhibition and the time to reach it were obtained directly from the effect-versus-time curves. The areas within the effect-time curves (AWECs) up to 12 hours after drug administration were calculated by use of the linear trapezoidal rule. For assessing the correlation between diclofenac plasma concentrations and changes from baseline serum TxB_2 levels, a plasma concentration response curve was fitted by use of a sigmoidal regression with variable slope, and the ex vivo IC_{50} value for COX-1 inhibition was derived by use of PRISM version 3.0 (GraphPad). The calculation of the theoretic inhibitions of COX activity after single-dose administration and at steady state was performed with use of the in vitro IC_{50} values of diclofenac or 4'-hydroxy-diclofenac for inhibition of COX activity, the Hill coefficient of the respective IC_{50} curve, and the respective plasma concentration of diclofenac or 4'-hydroxy-diclofenac.

RESULTS

In vitro investigations

Effects of aceclofenac and its metabolites on COX-1 and COX-2 activity in short-term assays

In short-term incubation assays (ie, conditions with minimal biotransformation of aceclofenac), neither aceclofenac nor 4'-hydroxy-aceclofenac inhibited COX-1 or COX-2 activity (Table I). In contrast, diclofenac and 4'-hydroxy-diclofenac inhibited both isozymes, with diclofenac being about 19- and 133-fold more potent than 4'-hydroxy-diclofenac in inhibiting COX-1 and COX-2 activity, respectively (Table I). Only trace amounts of diclofenac (0.07% ± 0.01%, n = 3) or 4'-hydroxy-diclofenac (0.06% ± 0.01%, n = 3) were found in aqueous solutions of aceclofenac or 4'-hydroxy-aceclofenac immediately before addition to blood. After a 1-hour incubation of aceclofenac and 4'-hydroxy-aceclofenac in blood, HPLC analysis revealed

concentrations of 0.10% ± 0.08% (n = 3) diclofenac and 0.16% ± 0.10% (n = 3) 4'-hydroxy-diclofenac, respectively.

Effects of aceclofenac and its metabolites on COX-1 and COX-2 activity in long-term assays

In human whole blood exposed to the respective test compound for 24 hours, aceclofenac and all of its investigated metabolites inhibited COX-1–dependent TxB_2 formation and COX-2–dependent PGE_2 formation with the following order of potency: diclofenac > 4'-hydroxy-diclofenac > aceclofenac > 4'-hydroxy-aceclofenac (Table I). An analysis of the stability of aceclofenac and 4'-hydroxy-aceclofenac in human whole blood revealed substantial conversions to diclofenac (11.3% ± 2.8%, n = 3) or 4'-hydroxy-diclofenac (3.1% ± 0.6%, n = 3) in plasma of blood incubated in the presence of aceclofenac (10 μmol/L) or 4'-hydroxy-aceclofenac (10 μmol/L) for 24 hours. However, HPLC analysis revealed no quantifiable peaks of 4'-hydroxy-aceclofenac and 4'-hydroxy-diclofenac in plasma of blood incubated with aceclofenac for 24 hours.

Fig 2 shows the time-dependent conversion of aceclofenac to diclofenac in human whole blood that was paralleled by a time-dependent increase in COX-1 and COX-2 inhibition.

COX-1/COX-2 inhibition ratios of diclofenac and 4'-hydroxy-diclofenac

Whole-blood assays, based on the LPS-induced COX-2–dependent PGE_2 synthesis (referred to as COX-2 long-term assay in our study) and on the production of COX-1–dependent TxB_2 after blood clotting (referred to as COX-1 short-term assay in our study), are considered to provide the most meaningful index of selectivity. With the use of the ratio of IC_{50} values (COX-1/COX-2) derived from these assays (Table I), both diclofenac and 4'-hydroxy-diclofenac were revealed to be preferential inhibitors of COX-2, with selectivity ratios for COX-2 inhibition of 17.9 and 10.9, respectively.

Effect of aceclofenac and its metabolites on COX-2 expression in human blood monocytes

So that it could be investigated whether inhibition of COX-2–dependent PGE_2 synthesis by aceclofenac and 4'-hydroxy-aceclofenac observed in long-term assays was due to alterations in COX-2 expression, the influence of these substances on the expression of COX-2 mRNA by human blood monocytes was determined. Neither aceclofenac nor its metabolites inhibited LPS-induced COX-2 mRNA expression at concentrations causing significant inhibition of COX-2–dependent

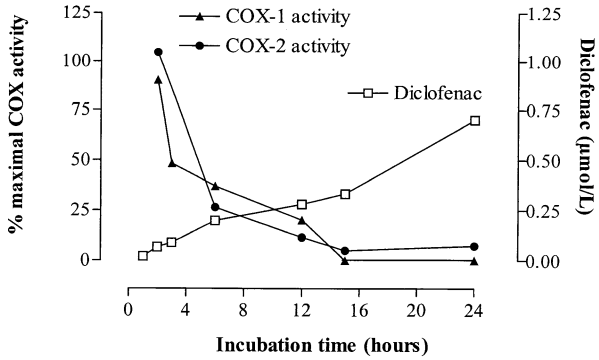

Fig 2. Time course of aceclofenac (10 μmol/L) conversion to diclofenac in human whole blood and inhibition of cyclooxygenase (COX)–1 and COX-2 activity determined in parallel. For determination of COX-1 activity, human blood aliquots were incubated with aceclofenac for the indicated times, including a 15-minute stimulation with A23187 (50 μmol/L) at the end of the incubation period. Thereafter plasma was collected for determination of thromboxane B_2 (TxB_2) levels. For the assessment of COX-2 activity, human blood was incubated with aceclofenac and lipopolysaccharide (LPS) for the indicated times. A23187 (50 μmol/L) was added to the blood samples 15 minutes before termination of the incubations to ensure measurable stimulations of COX-2–dependent prostaglandin E_2 (PGE_2) levels at short intervals after LPS stimulation. PGE_2 levels were determined in plasma thereafter. Values are means of 3 independent experiments.

PGE_2 synthesis. COX-2 mRNA levels determined by real-time reverse transcriptase–polymerase chain reaction were 123.3% ± 3.5% for aceclofenac (10 μmol/L, n = 3), 90.7% ± 6.4% for 4'-hydroxy-aceclofenac (10 μmol/L, n = 3), 112.8% ± 2.1% for diclofenac (0.1 μmol/L, n = 3), and 110.7% ± 5.6% for 4'-hydroxy-diclofenac (3 μmol/L, n = 3) relative to the stimulation (100%) elicited by LPS alone. In contrast, the use of dexamethasone (1 μmol/L) as a positive control significantly diminished LPS-induced COX-2 expression to 17.6% ± 2.3% of control (n = 3; $P < .05$, Student *t* test).

Pharmacokinetics of aceclofenac and its metabolites and ex vivo inhibition of COX activity

Tolerability of medications

All subjects completed the study, and no adverse events were observed.

Pharmacokinetics

Aceclofenac treatment. Time courses of plasma concentrations of aceclofenac and its metabolites

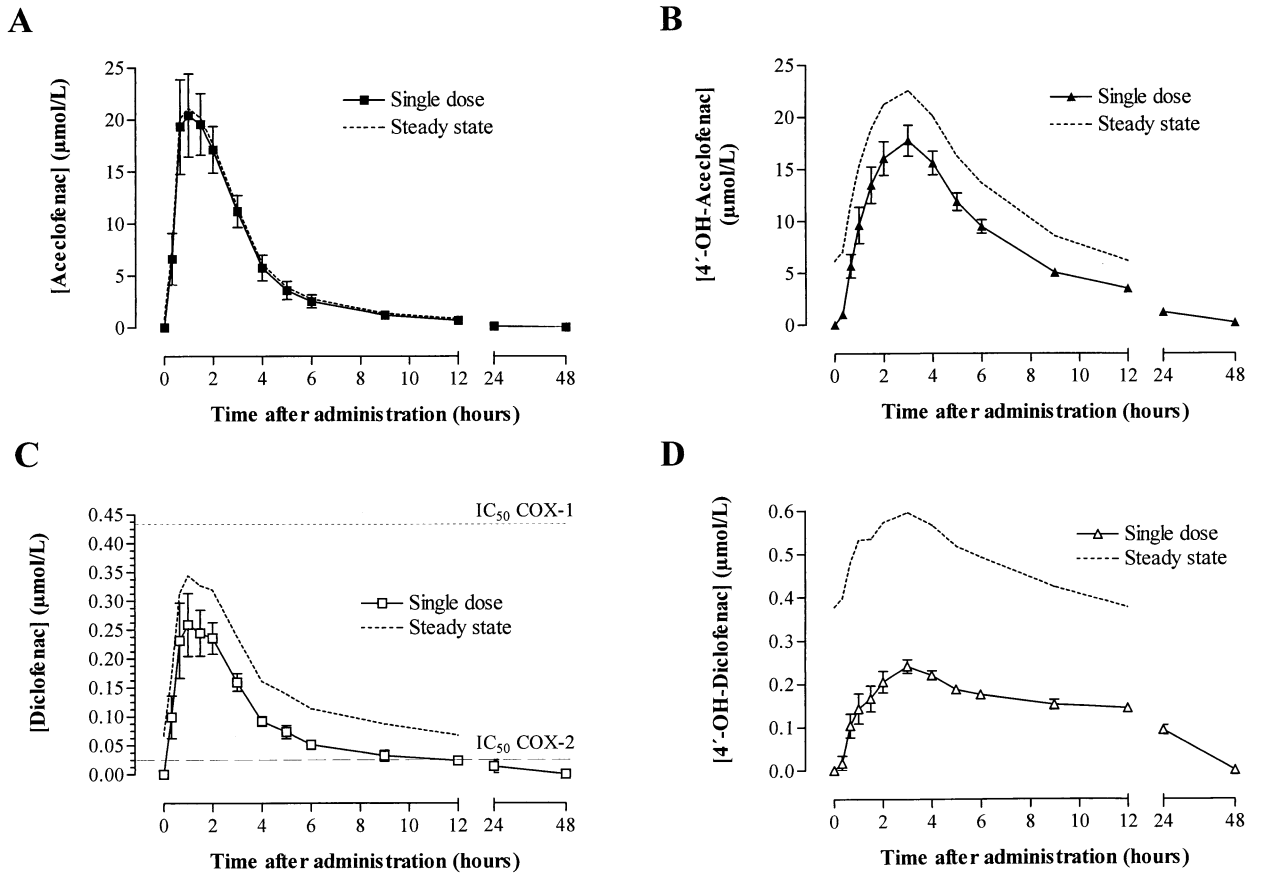

Fig 3. Plasma concentrations of aceclofenac (**A**) and metabolites (**B**, **C**, and **D**) after oral single-dose administration of 100 mg aceclofenac to 12 healthy male volunteers and calculated concentration-time curves at steady state. Values are mean ± SEM. *Superimposed horizontal lines* indicate the 50% inhibitory concentration (IC_{50}) values of diclofenac for COX-1 inhibition (short-term assay, *dotted line*) and COX-2 inhibition (long-term assay, *dashed line*) obtained in vitro (Table I). The *dashed curves* represent the calculated concentration-time profiles of aceclofenac and its metabolites at steady state.

after oral administration of 100 mg aceclofenac are shown in Fig 3. Average pharmacokinetic parameters are summarized in Table II. Plasma peak concentrations of diclofenac coincided with the maximal plasma levels of its parent compound, aceclofenac. However, in comparison with those of aceclofenac, the plasma levels of diclofenac declined more slowly, with an apparent terminal half-life of 7.0 hours (Table II). Plasma concentrations of diclofenac remained below the in vitro IC_{50} value for COX-1 inhibition (0.43 µmol/L) but were greater than or equal to the in vitro IC_{50} value for COX-2 inhibition (0.024 µmol/L) for 12 hours after administration (Fig 3, *C*). Diclofenac was still present in the blood at COX-2 inhibitory concentrations (0.014 µmol/L) 24 hours after administration of aceclofenac (Fig 3, *C*). Plasma concentrations of 4'-hydroxy-diclofenac declined in a slow and variable manner, with an apparent average terminal half-life of 25.7 hours (Fig 3, *D*, and Table II).

Diclofenac treatment. After single-dose administration of 75 mg diclofenac, average plasma concentrations of diclofenac remained above the in vitro IC_{50} value for COX-1 and COX-2 inhibition for at least 4 and 24 hours, respectively (Fig 4, *A*). The median AUC_t of diclofenac after administration of aceclofenac was 20% of the AUC_t of diclofenac after diclofenac administration (95% confidence interval, 15%-28%; $P <$

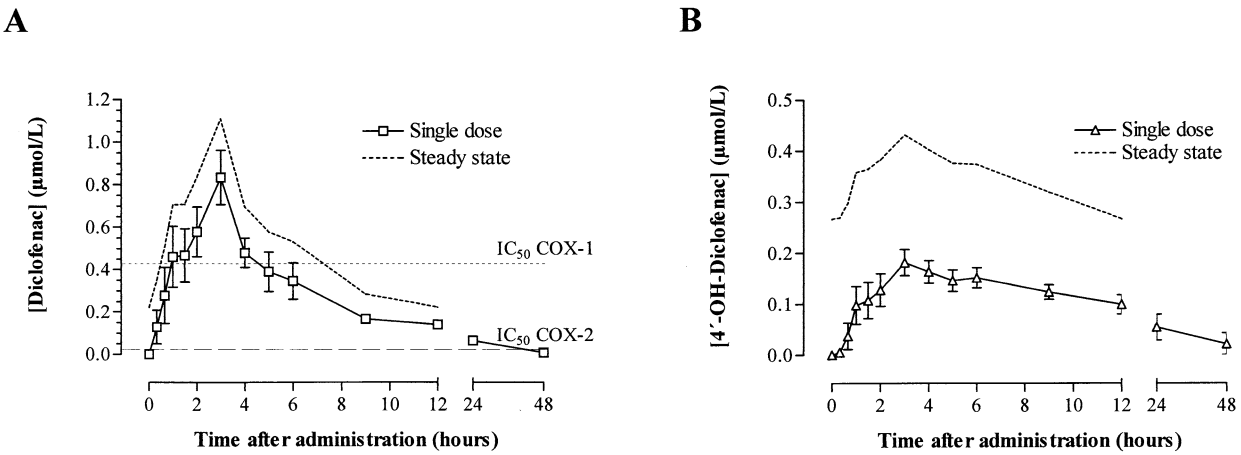

Fig 4. Plasma concentrations of diclofenac (**A**) and 4'-hydroxy-diclofenac (**B**) after oral single-dose administration of 75 mg diclofenac to 12 healthy male volunteers and calculated concentration-time curves at steady state. Values are mean ± SEM. *Superimposed horizontal lines* represent IC_{50} values of diclofenac for COX-1 inhibition (short-term assay, *dotted line*) and COX-2 inhibition (long-term assay, *dashed line*) obtained in vitro (Table I). The *dashed curves* represent the calculated concentration-time profiles of diclofenac and 4'-hydroxy-diclofenac at steady state.

Table II. Pharmacokinetic parameters of parent compounds and metabolites after oral administration of aceclofenac (100 mg) or diclofenac (75 mg)

	C_{max} (µmol/L) [ng/mL]	t_{max} (h)	AUC_t (µmol · h/L) [ng · h/L]	$t_{1/2}$ (h)
Aceclofenac (100 mg)				
Aceclofenac	30.5 ± 2.8 [10,809 ± 975]	1.6 ± 0.3	75.0 ± 4.7 [26,546 ± 1,668]	4.8 ± 0.2
4'-Hydroxy-aceclofenac	20.1 ± 0.9 [7,447 ± 350]	2.3 ± 0.3	151.3 ± 6.5 [56,022 ± 2,390]	8.5 ± 0.3
Diclofenac	0.39 ± 0.04 [116 ± 13]	1.6 ± 0.3	1.3 ± 0.3 [398 ± 78]	7.0 ± 1.8
4'-Hydroxy-diclofenac	0.28 ± 0.01 [86 ± 5]	2.5 ± 0.3	3.6 ± 0.2 [1,116 ± 59]	25.7 ± 3.6
Diclofenac (75 mg)				
Diclofenac	1.28 ± 0.11 [378 ± 33]	2.7 ± 0.5	5.9 ± 0.5 [1,749 ± 159]	13.0 ± 2.1
4'-Hydroxy-diclofenac	0.25 ± 0.02 [78 ± 8]	3.8 ± 0.9	2.9 ± 0.8 [899 ± 249]	21.3 ± 7.9*

Values are mean ± SEM from 12 healthy male volunteers.
C_{max}, Maximal plasma concentration; t_{max}, time to maximal plasma concentration; AUC_t, area under plasma concentration–time curve; $t_{1/2}$, terminal half-life.
*Because the terminal half-life of 4'-hydroxy-diclofenac of 2 volunteers could not be calculated, the indicated value represents the mean of 10 volunteers.

.001, Wilcoxon signed rank test). Again, the metabolite 4'-hydroxy-diclofenac underwent a slow elimination (Fig 4, *B*, and Table II).

Pharmacodynamics

Ex vivo COX-1 assay. Mean baseline values of serum TxB_2 were 447.8 ± 50.8 ng/mL for aceclofenac treatment and 394.2 ± 31.4 ng/mL for diclofenac treatment. Time courses of inhibition of ex vivo coagulation-induced TxB_2 synthesis in blood from aceclofenac- and diclofenac-treated volunteers are shown in Fig 5, *A*. In aceclofenac-treated volunteers maximal inhibition of TxB_2 generation (36.2% ± 4.9%) was observed 2.1 ± 0.5 hours after administration. In diclofenac-treated volunteers, maximal suppression of TxB_2 production (78.7% ± 2.6%) was evident 2.8 ± 0.4 hours after dosing. Moreover, administration of diclofenac was associated with a suppression of ex vivo COX-1 activity that lasted significantly longer than that after administration of aceclofenac. The median AWEC after administration of aceclofenac (107.7% ± 15.2% · h) was 25% (95% confidence interval, 13%-47%; $P < .001$, Wilcoxon signed rank test) of the AWEC after diclofenac administration (402.1% ± 42.7% · h).

Correlation between diclofenac plasma concentrations and ex vivo COX-1 inhibition. The relationship of diclofenac plasma concentrations to ex vivo COX-1

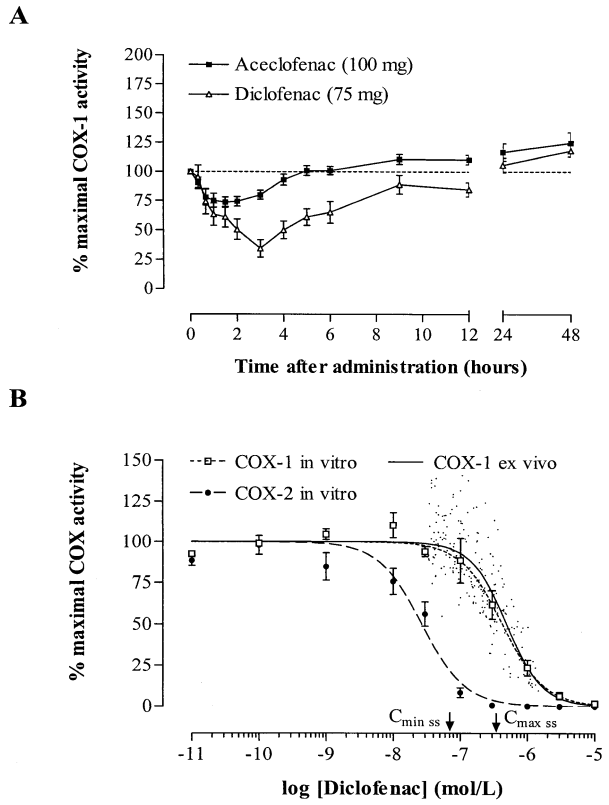

Fig 5. Time-dependent inhibition of TxB_2 generation ex vivo (index of COX-1 activity) after oral administration of 100 mg aceclofenac or 75 mg diclofenac to 12 healthy male volunteers (**A**), and relationship between ex vivo inhibition of TxB_2 generation and plasma concentrations of diclofenac achieved after oral administration of aceclofenac and diclofenac (**B**). Values are mean ± SEM in **A**. TxB_2 is expressed as the percentage of predosing values in **A**. In **B**, the *solid curve* indicates the relationship from the inhibition of TxB_2 synthesis and diclofenac plasma concentrations and is based on the data (scatter plot) from both aceclofenac- and diclofenac-treated volunteers. Superimposed are concentration-effect curves for COX-1 inhibition (short-term assay, *dotted line*) and COX-2 inhibition by diclofenac (long-term assay, *dashed line*) obtained in vitro (Table I). *Arrows* on the *x*-axis indicate estimated mean minimal ($C_{min\ ss}$) and maximal ($C_{max\ ss}$) plasma concentrations of diclofenac at steady state.

inhibition after administration of aceclofenac and diclofenac was examined graphically and explored by estimating the diclofenac plasma concentration required to produce 50% inhibition (IC_{50}) of TxB_2 generation. The calculated ex vivo IC_{50} value for COX-1 inhibition by diclofenac was 0.49 μmol/L (Fig 5, *B*). This value compares favorably with the in vitro IC_{50} value of diclofenac for COX-1 inhibition (0.43 μmol/L). Superimposed on the same graph are concentration-effect curves for COX-1 inhibition (dotted line) and COX-2 inhibition by diclofenac (dashed line) obtained in vitro (Table I).

Correlation between diclofenac and 4'-hydroxy-diclofenac plasma concentrations and in vitro inhibition of COX isozymes. On the basis of maximal plasma concentrations of diclofenac measured after single-dose administration of aceclofenac or diclofenac (Table II) and IC_{50} values for COX-1 inhibition (short-term assay) and COX-2 inhibition (long-term assay) by diclofenac (Table I), the calculated inhibitions of the COX enzymes were 46.4% (COX-1) and 97.6% (COX-2) for the aceclofenac group and 81.8% (COX-1) and 99.5% (COX-2) for the diclofenac group. Maximal plasma concentrations of 4'-hydroxy-diclofenac were not sufficient to suppress COX-1 activity in either group. In the case of 4'-hydroxy-diclofenac, theoretic inhibitions of COX-2 activity were 6.3% for the aceclofenac group and 4.7% for the diclofenac group.

Correlation between calculated steady-state plasma concentrations of diclofenac and 4'-hydroxy-diclofenac and in vitro inhibition of COX isozymes. On the basis of estimated minimal and maximal plasma concentrations of diclofenac derived from the average steady-state concentration-time curves (Fig 3, *C*, and Fig 4, *A*), the calculated inhibitions of the COX enzymes at trough and peak concentrations were 7.1% to 42.1% (COX-1) and 79.8% to 97.2% (COX-2) for the aceclofenac group or 28.4% to 78.6% (COX-1) and 95.0% to 99.4% (COX-2) for the diclofenac group. Again, estimated minimal and maximal plasma concentrations of 4'-hydroxy-diclofenac left COX-1 activity in either group unaltered. However, concentrations of 4'-hydroxy-diclofenac at steady state could well contribute to COX-2 inhibition in the aceclofenac group (13.2%-34.3%) but to a lesser extent in the diclofenac group (5.6%-18.0%).

DISCUSSION

The mechanism of action responsible for decreased prostanoid synthesis by aceclofenac has been elusive. Here, we show that aceclofenac itself is inactive in terms of COX inhibition but interferes with prostanoid formation in vivo and in vitro after conversion to its active metabolite diclofenac. The amount of diclofenac generated after administration of 100 mg aceclofenac can cause an almost complete inhibition of the COX-2 enzyme but only a partial and short-term suppression of the COX-1 isoform. In comparison with a 75-mg dose

of a sustained-release diclofenac formulation, aceclofenac was associated with a pronounced COX-1–sparing effect.

In the in vitro approach of this study, aceclofenac and its major metabolite, 4'-hydroxy-aceclofenac, were shown to be virtually devoid of any inhibitory effect on the COX isoforms when tested under short-term incubation conditions that prevent hydrolysis of both ester compounds. On the other hand, under the very same experimental conditions, inhibition of COX-1 and COX-2 activity was observed in the presence of the aceclofenac metabolites diclofenac and 4'-hydroxy-diclofenac. However, after long-term incubation of aceclofenac and 4'-hydroxy-aceclofenac in human blood, inhibition of COX-1– and COX-2–dependent prostanoid synthesis was observed, along with a substantial conversion of aceclofenac to diclofenac and of 4'-hydroxy-aceclofenac to 4'-hydroxy-diclofenac. The absence of 4'-hydroxy-aceclofenac and 4'-hydroxy-diclofenac in plasma after a 24-hour incubation with aceclofenac suggests that ester hydrolysis, rather than oxidative metabolism, is the predominant metabolic pathway in this system. A causal relationship between inhibition of the activity of either COX isozyme by aceclofenac and the generation of its active metabolite, diclofenac, was corroborated by time-course experiments showing that the time-dependent conversion of aceclofenac to diclofenac paralleled an increase in COX-1 and COX-2 inhibition. The inhibitory effect of aceclofenac on COX-2 activity in the long-term whole-blood assay is in line with previous reports demonstrating inhibition of COX-2–dependent prostaglandin synthesis by aceclofenac on long-term exposure to chondrocytes,[29] osteoarthritic cartilage,[38] and human synovial cells.[25,26] With respect to the failure of aceclofenac to interfere with the activities of the COX enzymes in short-term assays, our data support the results obtained in ovine isoforms.[25] In our hands aceclofenac and 4'-hydroxy-aceclofenac did not alter LPS-induced COX-2 expression in human blood monocytes, suggesting that suppression of COX-2 gene expression does not contribute to inhibition of COX-2–dependent prostaglandin synthesis in this model. These data are in line with a recent study showing that NSAIDs, although suppressing COX-2 mRNA levels at later times of monocyte activation by inhibiting a PGE_2-elicited positive feedback on COX-2 expression, are devoid of a direct inhibitory effect on the induction of COX-2 at the transcriptional level.[34] In accordance with previous findings,[5,39-41] COX-2 expression was inhibited by the glucocorticoid dexamethasone, which was used as a positive control.

Whole-blood assays, based on the synthesis of coagulation-induced TxB_2 as an index for COX-1 and on LPS-induced PGE_2 as an index of COX-2 activity (referred to as COX-1 short-term assay and COX-2 long-term assay in our study), are considered to provide the most meaningful index of COX selectivity.[8,33,42] In these assays the selectivity of COX inhibition is measured in a physiologic milieu, with the binding of drugs to plasma proteins taken into account. With use of the ratio of IC_{50} values (COX-1/COX-2) derived from these assays, the selectivity ratios for the inhibition of COX-2 by the aceclofenac metabolites diclofenac and 4'-hydroxy-diclofenac were 17.9 and 10.9, respectively. The aim of the in vivo approach of this study was therefore to determine whether pharmacologically relevant concentrations of the preferential COX-2 inhibitors diclofenac and 4'-hydroxy-diclofenac are reached in the plasma of aceclofenac-treated volunteers. Moreover, we investigated whether the extent and time course of this bioactivation are associated with an improved COX-2 selectivity profile as compared with the administration of a sustained-release diclofenac resinate formulation. So that these questions could be addressed, a 1-way crossover study was performed in volunteers randomized to clinically recommended analgesic and anti-inflammatory clinical doses of aceclofenac or diclofenac.[11-13] The pharmacokinetic parameters of aceclofenac were in agreement with previously published data.[43,44] Aceclofenac was metabolized to 3 main metabolites, 4'-hydroxy-aceclofenac, diclofenac, and 4'-hydroxy-diclofenac, with 4'-hydroxy-aceclofenac being the major circulating metabolite. After administration of aceclofenac, maximal plasma concentrations of diclofenac (0.39 μmol/L), as well as the calculated average steady-state concentrations of diclofenac, remained below the in vitro IC_{50} value of diclofenac for COX-1 inhibition (0.43 μmol/L) but were about 1 order of magnitude above the in vitro IC_{50} value for COX-2 blockade (0.024 μmol/L). Estimation of steady-state concentrations revealed diclofenac plasma levels above the IC_{50} value for COX-2 inhibition for the whole 12-hour dose interval, with a theoretic COX-2 inhibition of 80% to 97%. These data suggest that long-term treatment with aceclofenac is associated with a sustained inhibition of the COX-2 isozyme explaining the effective analgesic and anti-inflammatory activity of the drug.[9-17] However, because aceclofenac undergoes time-dependent hydrolysis to the potent COX-2 inhibitor diclofenac in blood, it was not possible to determine inhibition of COX-2 activity by aceclofenac ex vivo by use of the 24-hour lasting COX-2 whole-blood assay.

In comparison with the diclofenac concentrations in plasma of aceclofenac-treated probands, diclofenac levels determined in plasma of volunteers receiving diclofenac had a significantly greater AUC_t and a slower decline in plasma concentrations. The latter finding is probably a result of the retarded absorption of the cholestyramine formulation. Consistent with the kinetic data, estimation of diclofenac plasma concentrations at steady state revealed a higher degree of COX-1 inhibition (28%-79%) in diclofenac-treated volunteers as compared with volunteers receiving aceclofenac (7%-42%). In line with a COX-1–sparing effect of aceclofenac, ex vivo TxB_2 synthesis was inhibited to a significantly lesser extent and for a shorter time period by 100 mg aceclofenac than by 75 mg diclofenac. Inhibition of COX-1 activity by diclofenac ex vivo showed a strong correlation to diclofenac plasma concentration, with the ex vivo IC_{50} value being almost identical to the in vitro IC_{50} value. For analysis of this plasma concentration-effect interrelationship, plasma concentrations of 4'-hydroxy-diclofenac were not included. This is justified by our kinetic data showing that maximal plasma concentrations of 4'-hydroxy-diclofenac were about 30-fold (aceclofenac-treated volunteers) and 33-fold (diclofenac-treated volunteers) lower than the IC_{50} value of 4'-hydroxy-diclofenac on COX-1. Moreover, no substantial inhibition of COX-1 was calculated for the estimated maximal concentrations of 4'-hydroxy-diclofenac at steady state. As with COX-1, a significant contribution of 4'-hydroxy-diclofenac to COX-2 inhibition after single-dose administration of aceclofenac or diclofenac is unlikely. However, the estimated steady-state plasma levels of 4'-hydroxy-diclofenac in aceclofenac-treated volunteers are approximately 2-fold higher, which will lead to a measurable contribution of this metabolite to COX-2 inhibition (13%-34%) during long-term use of aceclofenac. In comparison, the calculated steady-state concentrations of 4'-hydroxy-diclofenac after administration of diclofenac were associated with less COX-2 inhibition (6%-18%). Plasma concentrations of 4'-hydroxy-diclofenac determined in aceclofenac-treated volunteers may result from both hydroxylation of diclofenac and hydrolysis of 4'-hydroxy-aceclofenac.

The apparently improved COX-2 selectivity of aceclofenac as compared with diclofenac raises several questions. According to double-blind comparative trials, the efficacy of 100 mg aceclofenac twice daily is similar to that of 50 mg diclofenac 3 times daily in patients with rheumatoid arthritis[11] and osteoarthritis of the knee.[12,13] Moreover, a recently published double-blind study has shown that 100 mg aceclofenac twice daily is at least as effective as 75 mg resin-bound diclofenac twice daily in lowering pain in patients with acute low back pain who have degenerative spinal disorders.[45] On the other hand, aceclofenac would be expected to possess much lower anti-inflammatory and analgesic activity by virtue of the low plasma levels of its metabolite diclofenac compared with the levels of diclofenac obtained by the direct dosing of a clinically effective dose of diclofenac. There are several possible explanations concerning this matter. First, other mechanisms distinct from COX-2 inhibition could contribute to the activity of aceclofenac. Accordingly, aceclofenac has previously been shown to stimulate glycosaminoglycan synthesis in osteoarthritic cartilage, whereas diclofenac had no measurable effect.[30] Likewise, recent studies in rabbit articular chondrocytes[46] and human rheumatoid synovial cells[47] indicate that 4'-hydroxy-aceclofenac but not diclofenac possesses putative chondroprotective properties attributable to suppression of interleukin 1 (IL-1)–mediated promatrix metalloproteinase production and proteoglycan release. However, in a long-term clinical trial including aceclofenac- and diclofenac-treated patients with osteoarthritis, both drugs have been associated with suppression of IL-1 production by blood mononuclear cells,[28] raising doubts concerning the proposed selectivity of aceclofenac in suppressing cytokine formation. Another study has shown a stimulatory effect of aceclofenac on the synthesis of IL-1 receptor antagonist in human articular chondrocytes but unfortunately did not include a diclofenac control.[48] Clearly, more research is needed to understand how the proposed mechanisms may work in a coordinated fashion to contribute to the pharmacologic action of aceclofenac. Second, under long-term treatment with aceclofenac, 4'-hydroxy-diclofenac, which causes higher concentrations in aceclofenac- than in diclofenac-treated volunteers, may contribute to COX-2 inhibition by virtue of its higher plasma concentrations after multiple doses. Third, a wider clinical experience and further efficacy studies are required before the role of aceclofenac relative to diclofenac can be determined. However, apart from the efficacy concern, metabolic formation of diclofenac may be favorable through avoiding high initial plasma peaks of diclofenac that may confer a high degree of COX-1 inhibition. This advantage is certainly even more pronounced in comparison to enteric-coated tablets containing 75 mg diclofenac sodium, which have been shown to cause about 4-fold higher maximal diclofenac plasma concentrations than a resinate formulation of 75 mg diclofenac.[49]

The issue of whether the improved COX-2 selectivity profile of 100 mg aceclofenac may be of clinical relevance in view of previous observations reporting a lower incidence of gastric and duodenal mucosal damage[20] and less gastrointestinal bleeding[18] with oral aceclofenac than with oral diclofenac remains to be resolved. An improved gastrointestinal tolerability of aceclofenac was supported by the outcome of the recently published SAMM (Safety Assessments of Marketed Medicines) study, which showed a significantly lower incidence of gastrointestinal adverse events with aceclofenac (100 mg twice daily) than with diclofenac (75 mg twice daily), although significantly more aceclofenac recipients than diclofenac recipients had a history of dyspepsia at baseline.[21] Moreover, a meta-analysis of studies involving a total of 3574 patients revealed that a significantly greater proportion of patients receiving aceclofenac remained free from gastrointestinal symptoms after 3 to 6 months of treatment compared with patients treated with diclofenac or other NSAID comparators.[19] However, endoscopy was not performed in these studies to determine the ulcerogenic potential of aceclofenac. Thus the safety of aceclofenac awaits further confirmation in large prospective clinical trials with ulcers, bleeding, and the incidence of upper gastrointestinal perforation or obstruction used as primary end points.

Overall, this study demonstrates that aceclofenac itself does not possess any effect on the human COX isozymes in vitro but inhibits prostanoid formation by either isoform in vivo after conversion into its active metabolite, diclofenac. These data clearly contradict the notion that aceclofenac itself is principally responsible for its pharmacologic action. The time course and extent of the metabolic activation of aceclofenac in vivo result in plasma concentrations of the preferential COX-2 inhibitor diclofenac that leave COX-1 almost unimpaired but allow a nearly complete blockade of COX-2. Moreover, under long-term therapy with aceclofenac, 4'-hydroxy-diclofenac may contribute to COX-2 inhibition. The apparent COX-1–sparing effect of 100 mg aceclofenac as compared with a 75-mg dose of a sustained-release diclofenac formulation may contribute to the improved gastrointestinal tolerability of aceclofenac, but this has to be confirmed in future clinical studies.

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