Reflections on flurbiprofen eyedrops
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CHAPTER 5

FLURBIPROFEN AND ENTIOMERS IN OPHTHALMIC SOLUTION TESTED AS INHIBITORS OF PROSTANOID SYNTHESIS IN HUMAN BLOOD

Nicolaas J. van Haeringen, Adriaan A. van Sorge, Jan L. van Delft, and Valérie M.W. Carballosa Coré-Bodelier

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ABSTRACT

The purpose of this study was to assess the selectivity and potency of the non-steroidal anti-inflammatory drug (NSAID), flurbiprofen, and its enantiomers in their inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). An assay was used with freshly drawn heparinized human whole blood, incubated with 25 mM calcium ionophore A 23187 during 60 min to produce thromboxane B$_2$ (TXB$_2$) by activity of COX-1 in platelets. Incubation with E.Coli lipopolysaccharide (LPS) during 24 hours produced prostaglandin E$_2$ (PGE$_2$) by induction of COX-2 in monocytes, suppressing any possible contribution of COX-1 activity by addition of acetylsalicylic acid. Concentration inhibition curves were determined with racemic, S(+), and R(-) flurbiprofen in final concentrations ranging from $10^{-3}$ to $10^{-10}$ M. The stereoselectivity of S(+) flurbiprofen vs. R(-) flurbiprofen, expressed as the reciprocal of the ratio of the concentrations giving 50% inhibition (IC$_{50}$), is 340 for COX-1 and 56 for COX-2. The selectivity for COX-1 vs. COX-2, expressed as the reciprocal ratio of the IC$_{50}$, was 16 for racemic, 32 for S(+), and 5.3 for R(-) flurbiprofen. Meloxicam in the same assay showed COX-2 selectivity with a ratio of 0.19.

INTRODUCTION

Cyclooxygenase (COX; prostaglandin-endoperoxide synthase, E.C.1:14.99.1), the rate limiting enzyme in the production of proinflammatory prostaglandins, exists in two isoforms: COX-1, the constitutive form, and COX-2 the inducible form. COX-1 has clear physiological functions such as in the protection of the stomach, kidney, and vessel walls, whereas COX-2 is induced by inflammatory stimuli and by cytokines in migratory and other cells, leading to inflammatory conditions. The therapeutic use of non-steroidal anti-inflammatory drugs (NSAIDs), in general, is based on their ability to inhibit the COX-2 activity, while inhibition of COX-1 might explain the undesired side effects such as gastric and renal toxicity and hematological disorders.

On the basis of their inhibitory activity on COX-1 and COX-2 at least three major aspects of NSAIDs are distinguished: 1) preferential COX-1 inhibition, 2) preferential COX-2 inhibition, and 3) nonpreferential inhibition. Selectivities, varying between COX-1 preferential (1-3) and nonpreferential (1,4,5), have been reported for flurbiprofen, possibly because of methodological variations. The assay systems to investigate the potency and selectivity of NSAIDs include intact cells, broken cells, purified enzymes and microsomal preparations of recombinantly expressed enzymes. Some of the reasons for variation have been identified such as the use of animal or human cells, microsomal preparations, incubation time of the assay or protein binding of the NSAID.
Whole blood, using COX-1 in platelets and induced COX-2 in monocytes represents an in vitro system as close to physiological conditions as possible. It has been shown to be a satisfactory system for testing the inhibitory action of NSAIDs on COX-1 and COX-2 activity (3,6-11) accounting for differences in plasma protein binding (12) and cellular accumulation. Platelets are known to contain COX-1 and the concentration of the enzyme remains largely stable, but two- to four-fold increases can occur in response to stimulation by hormones or growth factors (13,14). COX-2, however, cannot be brought to expression in platelets in response to lipopolysaccharide (LPS) (15). Therefore, platelets stimulated by calcium ionophore represent a useful assay system for COX-1 activity, measuring thromboxane production as the most distinguished prostanoid. Normal peripheral blood monocytes express COX-1 but can be induced to express COX-2 by LPS to 10 - 20-fold after 6-24 hours (15,16). This represents a useful assay system for COX-2 activity, measuring PGE₂ or thromboxane as metabolite, when suppressing any possible contribution of COX-1 activity by addition of aspirin (acetylsalicylic acid). Aspirin acetylates platelet COX-1, thereby causing irreversible loss of its cyclooxygenase activity (17). The inactivation of COX-1 is complete by 20 min (18) and excess of aspirin itself is hydrolyzed in blood with a half-life time of 30 min (10) before expression of COX-2 by LPS is fully developed.

The NSAID flurbiprofen, as used in eyedrops, is on the one hand effective against inflammatory responses of the eye (19-21) caused by activity of the COX-1 of the iris (22); on the other hand in systemic use, it is effective in arthritis (23) caused by induction of COX-2 (24,25).

Flurbiprofen is a racemic mixture of S(+) and R(-) enantiomers, and its effect is largely attributed to the inhibitory action of the S(+) enantiomer, reportedly being about 100 - 10,000 times more effective than the R(-) enantiomer when measured on COX-1 or COX-2 (10,26-28). There are sufficient differences between the enzymes from different species, such that selectivity and potency established with animal enzymes is not always predictive of effects on human enzymes and no data are available on the potency or selectivity of the flurbiprofen enantiomers on COX-1 and COX-2 in human blood. In this study, we report dose effect inhibition curves for racemic, S(+) and R(-) flurbiprofen using the human whole blood assay. In comparison the preferential COX-2 inhibitor, meloxicam, was also investigated in respect of its enzyme selectivity.

**MATERIALS AND METHODS**

**Materials**

Flurbiprofen and the enantiomers were obtained from Duchefa Pharma bv, Haarlem, The Netherlands. The enantiomeric impurity of S(+) flurbiprofen was
<0.1% and of R(-) flurbiprofen 0.7% (29). Stock solutions of flurbiprofen were prepared in a phosphate buffer pH 7.4 with a concentration of 0.03% for racemic flurbiprofen and 0.015% for the enantiomers (for details see 29). Meloxicam was a gift from Boehringer Ingelheim, Germany. Calcium ionophore A 23187, dimethylsulfoxide (DMSO) and LPS, derived from E.Coli 0111:B4, were obtained from Sigma (St Louis, MO, USA). Assay kits for enzyme immune analysis of Thromboxane B2 (TXB2) and of prostaglandin E2 (PGE2) were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.).

Measurement of COX-1 Activity
For the determination of COX-1 activity 900 µl of freshly drawn heparinized human blood was incubated at 37°C with 2 µl of calcium ionophore A23187 (12.5 mM in DMSO) and 100 µl of phosphate buffered saline (PBS) for 30 min. Controls were prepared by incubation of 900 µl of blood with 2 µl of DMSO and 100 µl of PBS. The reaction was terminated by chilling quickly on ice. Plasma was separated by centrifuging, stored at -20°C, and TXB2 levels were determined.

Measurement of COX-2 Activity
For the determination of COX-2 activity, 900 µl of heparinized human blood was incubated at 37°C with 10 µl of acetylsalicylic acid (1 mg/ml in PBS), 2 µl of LPS (E.Coli 0111:B4, 5 mg/ml in DMSO), and 100 µl PBS for 24 hr. Controls were prepared by incubation of 900 µl of blood with 10 µl of acetylsalicylic acid, 2 µl of DMSO, and 100 µl PBS. The reaction was terminated by quickly chilling on ice. Plasma was separated by centrifuging, stored at -20°C, and PGE2 levels were determined.

Enzyme Inhibition by Flurbiprofen and by Meloxicam
The effect of flurbiprofen was studied by substitution of 100 µl of PBS in the assays, by 100 µl of an appropriate dilution in PBS of the ophthalmic solution, reaching final concentrations of 10^-3 to 10^-10 M. Meloxicam was studied using appropriate solutions in PBS. Inhibition of enzyme activity was calculated by the difference of TXB2- or PGE2 - release in the absence and the presence of flurbiprofen or meloxicam, expressed in percent of the noninhibited release. From concentration-response inhibition curves the concentration giving 50% inhibition was extrapolated and presented as IC50.

RESULTS

Addition of calcium ionophore A23187 to human blood resulted in the generation of large amounts of TXB2 in the plasma (128±16 ng/ml/30 min) caused by COX-1
activity of the platelets. Non-stimulated controls, to which only DMSO was added, produced 5 ± 0.6 ng TXB\(_2\)/ml/30 min. The production of PGE\(_2\) by LPS-challenged whole blood, caused by induction of COX-2, amounted 124 ± 19 ng/ml/24hr. Nonstimulated controls, to which only DMSO and aspirin was added, contained small amounts of PGE\(_2\) (1.3 ± 0.5 ng/ml/24hr).

Figure 1. Concentration-response curves for inhibition of the COX-1 activity of whole human blood for racemic flurbiprofen (\(\nabla\)), S(+) flurbiprofen (\(\Box\)) and R(-) flurbiprofen (\(\bigcirc\)). Concentrations are final concentrations in the assay blood mixture. Each point represents the average ± SEM of four to six determinations from blood of different individuals.

Figure 2. Concentration-response curves for inhibition of the COX-2 activity of whole human blood for racemic flurbiprofen (\(\nabla\)), S(+) flurbiprofen (\(\Box\)) and R(-) flurbiprofen (\(\bigcirc\)). Concentrations are final concentrations in the assay blood mixture. Each point represents the average ± SEM of four to six determinations from blood of different individuals.
Dose response inhibition curves of racemic flurbiprofen and its S(+) and R(-) enantiomers on the activity of COX-1 and COX-2 isoenzymes in the whole blood model are presented in figures 1 and 2. Results of meloxicam in the same assay are presented in figure 3. In table 1 the IC$_{50}$ values are presented together with the enzyme selectivity, expressed as the COX-2/COX-1 ratio, for racemic flurbiprofen, its R(-) and S(+) enantiomers and for meloxicam.

Figure 3. Concentration-response curves for inhibition of COX-1 (○) and COX-2 (●) activity of whole human blood for meloxicam. Concentrations are final concentrations in the assay blood mixture. Each point represents the average ± SEM of four to six determinations from blood of different individuals.

Table 1. Selectivities of NSAIDs for COX-1 and COX-2 in Human Blood.

<table>
<thead>
<tr>
<th></th>
<th>COX-1 IC$_{50}$ μM</th>
<th>COX-2 IC$_{50}$ μM</th>
<th>COX-2/COX-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>racemic flurbiprofen</td>
<td>0.14 ± 0.04</td>
<td>2.2 ± 0.2</td>
<td>16 ± 5</td>
</tr>
<tr>
<td>S(+) flurbiprofen</td>
<td>0.056 ± 0.030</td>
<td>1.8 ± 0.4</td>
<td>32 ± 24</td>
</tr>
<tr>
<td>R(-) flurbiprofen</td>
<td>19 ± 6</td>
<td>100 ± 32</td>
<td>5.3 ± 3.4</td>
</tr>
<tr>
<td>meloxicam</td>
<td>3.0 ± 1.1</td>
<td>0.56 ± 0.26</td>
<td>0.19 ± 0.16</td>
</tr>
</tbody>
</table>

IC$_{50}$ are mean values derived from the concentration response inhibition curves.
DISCUSSION

This is the first report to establish in the human whole blood assay the stereoselective inhibition of COX-1 and COX-2. The assays used were adapted from (3,8) and several authors of different research groups reported their results using this method, testing many NSAIDs, including racemic flurbiprofen (3,30) and meloxicam (31).

The whole blood system is very useful in that in vitro it may reflect better the in vivo effectiveness of NSAIDs with contributing factors as protein-binding. The prostanoid production in this assay is measured under conditions in which arachidonic acid is generated from endogenous lipid pools rather than added in artificially high (10 µM) exogenous concentrations as in other assays (28).

S(+) flurbiprofen, as in general for racemic mixtures of NSAIDs, is the more potent inhibitor of both COX isoenzymes than the R(-) isomer (9,10,32,33). The enantioselectivity of S(+) flurbiprofen, expressed as the ratio of the IC$_{50}$ of the R(-) and the S(+) isomer (R/S), amounts to 340 for COX-1 and 56 for COX-2. The difference in selectivity found in this study may be explained by a possible greater steric hindrance of the binding of R(-) flurbiprofen to COX-1 than to the COX-2 enzyme. The accessibility of the binding site for flurbiprofen, competing with arachidonate binding at the catalytic domain of the COX-1 structure, has been found to be more restricted than in the COX-2 structure (34-36).

On the other hand the inhibitory effect measured with the R(-) isomer has been ascribed to the small amount of S(+) present as impurity in the ineffective R(-) isomer (13) Theoretically the 0.7% impurity should give a R/S ratio of about 140 and a smaller ratio might be due to metabolic inversion of the R(-) into the S(+) isomer. Metabolic inversion, however, has been demonstrated to be absent in human blood (37).

Within the errors of the methods used, the inhibition curves of the various concentrations of flurbiprofen racemate and the S(+) isomer were found to run closely parallel, reflecting the two-fold (0.3 log-unit) greater concentration of the pure S(+) isomer as compared to the racemate (figures 1 and 2). These results correspond well with data obtained from eicosanoid production in ex vivo experiments in rats (26).

We confirmed the selectivity of flurbiprofen towards COX-1 as found by Young et al. (3), who also used the human blood assay. The ratio COX-2/COX-1 was 16 for racemic flurbiprofen, although the IC$_{50}$ values for both enzymes were about 4 times lower in the present study. The ratio COX-2/COX-1 was 32 for S(+) flurbiprofen and 5.3 for R(-) flurbiprofen, which is higher than found with guinea pig whole blood, where for S(+) flurbiprofen was found 1.0 and for R(-) flurbiprofen 0.48 (10). Genetic differences of human and guinea pig COX-2 are most likely responsible for these differences, as has also been suggested for human and murine COX-2 (38).

Meloxicam has been found in a number of other assay systems to be selective towards COX-2 (6,7,39,40). Figure 3 shows that meloxicam inhibits COX-2 in
human whole blood at concentrations that are at least 5 times lower than those required to inhibit COX-1. It was also observed that meloxicam (IC\textsubscript{50} 0.56 \times 10^{-6}M) displayed more potency towards COX-2 than S(+) flurbiprofen (IC\textsubscript{50} 1.8 \times 10^{-6}M).

REFERENCES


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