Studies on the In Vitro Inversion of Flurbiprofen

ANNA LOMBARD,[1]* VALERIA ROSSETTI,[1] AND MARIA CHIARA CASSONE[2]
[1]*Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Italy*
[2]*Dipartimento di Anatomia, Farmacologia e Medicina Legale, Università di Torino, Italy*

ABSTRACT This paper reports in vitro studies on the metabolic inversion of flurbiprofen (FL), an arylpropionic acid antiinflammatory agent (2-APA). The inversion was studied with both *rac*-FL and R-FL, by incubation with rat hepatic microsomes, in the presence of either CoASH and ATP or NADPH. The two isomers of the drug were separated as their (+)-(R)-1-phenylethylamides by direct phase high-performance liquid chromatography on a silica gel column with an achiral mobile phase. The inversion was more pronounced in the presence of CoASH and ATP for both the racemate and the R-isomer, which supports the key role of CoA thioesters in the metabolic inversion of profens. The inversion observed in the presence of NADPH suggests that, when the incubation is run with hepatic microsomes, a CYP450-mediated pathway is also active. In order to get more insight into the CYP450-mediated inversion pathway, we studied the effect of irradiating microsomes with a low dose of He-Ne laser radiation (0.2 J). Such irradiation caused a significant increase in inversion at all times studied and normalized the anomalous value of inversion observed at 15 min in this pathway. *Chirality 9:317–319, 1997.*

KEY WORDS: NSAID metabolism; rat liver microsomes; metabolic pathway; He-Ne laser; (+)-(R)-1-phenylethylamides; HPLC separation

Flurbiprofen (FL), (2-[2-fluoro-2-biphenyl]-propionic acid) is an arylpropionic acid antiinflammatory agent (2-APA) with a chiral center in the molecule, which therefore exists in two enantiomeric forms. Although the antiinflammatory activity resides almost exclusively in the S-isomer, the drug is used clinically as the racemate.

As for other chiral drugs, to avoid the undesirable effects of the distomer on the pharmacokinetics of the active isomer, the use of a single isomer would be desirable. It is therefore of interest to study the underlying mechanisms of chiral inversion in order to develop enzymatic or microbial systems for the preparation of enantiomerically pure drugs.

Metabolic inversion of FL has been studied in vivo[1–3] and in vitro.[4–6] It is generally accepted that, in the rat, chiral inversion of the (–)R-enantiomers of 2-arylpropionic acids in the clinically active (+)-S-enantiomers involves the formation of a CoA thioester intermediate, catalyzed by a hepatic microsomal long-chain fatty acid CoA ligase.[5–7]

In previous studies on the in vitro metabolic inversion of other profens,[8–11] we observed metabolic inversion from the R- to the S-isomer, after incubation with rat liver microsomes in the presence of an NADPH-generating system. The inversion was more pronounced in phenobarbital-treated rats, suggesting an involvement of the CYP450 microsomal oxidizing system.

The first aim of this study, therefore, was to further elucidate the mechanisms of metabolic inversion of FL, by incubating both *rac*-FL and R-FL with rat liver microsomes in the presence of either CoASH and ATP or NADPH. Experiments were run with RS- and R-FL in order to evidence a possible influence of the S-isomer on the process.

In the presence of NADPH we observed an anomalous time course for the inversion at 15 min; to gain more insight into the mechanism of chiral inversion in the presence of NADPH, we decided to investigate the effect of low doses of He-Ne laser radiation on the in vitro metabolic inversion of FL by the rat hepatic microsomal oxidizing system. Chiral inversion is an endoergonic process involving several enzymes and cofactors, and it has been demonstrated that low-power laser radiations, at low doses, can modulate the activity of some enzymes since they bring about chemicophysical changes in cofactors and enzyme molecules.[12]

MATERIALS AND METHODS

Rac-flurbiprofen (*rac*-FL) was obtained from Sigma (Milan, Italy); (–)-(R)-FL was kindly supplied by Boots Pharmaceuticals (Nottingham, UK). Rat hepatic microsomes were prepared by a standard differential centrifugation procedure from the livers of male Wistar albino rats (body weight approx. 250 g).

Incubation

Incubation was run for various times (5, 15, 60 min) at 37°C. The incubation mixture (total volume 3.0 ml) con-

Contact Grant sponsor: National Research Council (CNR); Contract Grant sponsor: M.U.R.S.T.
*Correspondence to: Dr. Anna Lombard, Dipartimento di Scienza e Tecnologia del Farmaco, Via Pietro Giuria, 9, 10125 Torino, Italy.
Received 1 August 1996; Accepted 22 October 1996

tained *rac*-FL (0.5 m*M*); microsomal protein (10 mg/ml) in 50 m*M* Tris buffer, pH 7.4; and either (a) CoASH (1.3 m*M*), ATP (20 m*M*), $MgCl_2$ (17 m*M*) or (b) an NADPH-generating system: NADP (3.0 µ*M*), glucose-6-phosphate (15 µ*M*), glucose-6-phosphate dehydrogenase (10.5 µg protein), $MgCl_2$ (30 µ*M*). Microsomes were obtained from two pools of livers from three rats; six to nine replicate measurements were carried out on the extract of each incubation mixture.

A nonincubated standard was run since previous experiments have shown that the enantiomeric ratio of the standard was not altered by incubation in buffer in the absence of microsomes.

Incubation With Irradiated Microsomes

One milliliter of the microsomal suspension was irradiated with a 5 mW He-Ne laser in a 1 cm^2 cuvette on the whole surface in dim light, at a distance of 15 cm, at room temperature. Irradiation time was 40 sec, giving a total dose of 0.2 J. The incubation was run for various times (5, 15, 60 min) at 37°C in the presence of the NADPH-generating system (see above) with irradiated microsomes. Microsomes were obtained from two pools of livers from three rats; incubation was run in duplicate; three replicate measurements were carried out on the extracts of each incubation mixture. A nonincubated standard was run in parallel.

Extraction of Enantiomers and Evaluation of Enantiomeric Ratio

Enantiomers were extracted with CH_2Cl_2 from acidified incubation aliquots and converted to (+)-(R)-1-phenylethylamides, following the procedure previously described.[13] The diastereoisomeric amides were separated by direct phase high-performance liquid chromatography (HPLC) on a silica gel column with an achiral mobile phase. Separations were performed on a Millipore (Bedford, MA) isocratic HPLC apparatus consisting of a model 501 pump system, a Rheodyne model 7125 injector with a 20 µl injection loop, a model 484 UV/vis detector, and a model 745 integrator. A LiChroCart silica gel 60 column (250 × 4 mm ID, particle size 5 µm) (Merck, Darmstadt, Germany) was used with a LiChroCart silica gel 60 (4 × 4 mm ID, 5 µm) guard column; the mobile phase was hexane-isopropanol (93:7) at room temperature; flow rate was 1.0 ml/min; detection was at 251 nm. Percentage net inversion was calculated, correcting for excess of S-isomer in the standard.

Statistical Evaluation

The statistical significance of the differences between groups was evaluated by variance analysis; differences were considered significant for $P \leq 0.05$.

RESULTS

Metabolic Inversion Pathway

Under the conditions used, the two stereoisomeric 1-phenylethylamides were separated with retention times of 8.1 min (S-amide) and 13.6 min (R-amide). As can be seen in Table 1, after 5 and 15 min of incubation, the inversion was more pronounced in the presence of coenzyme A and ATP (6.05% net inversion after 5 min and 5.85% after 15 min) than with NADPH (3.75% after 5 min and 1.97% after 15 min). On the contrary, after 60 min incubation, the inversion was more pronounced in the presence of NADPH than in the presence of CoASH and ATP (3.39% and 2.71%, respectively), but the difference was statistically not significant.

TABLE 1. FL chiral inversion *in vitro* after incubation of the racemate with rat liver microsomes for different times in the presence of CoASH + ATP or NADPH

Incubation time (min)	CoASH + ATP		NADPH	
	% S-isomer	% net inversion	% S-isomer	% net inversion
5	56.06 ± 0.39[a,b]	6.05	54.91 ± 0.22	3.75
15	55.96 ± 1.45[a,b]	5.85	54.02 ± 0.31	1.97
60	54.39 ± 0.97[a,c]	2.71	54.73 ± 0.44	3.39

Nonincubated standard: 53.03 ± 0.58 % S-isomer

Values are means of six to nine measurements ± SD. For the standard, the value is the mean of 10 measurements ± SD. Statistical significance: CoASH + ATP vs. standard.
[a] $P \leq 0.001$; NADPH vs. standard, $P \leq 0.001$; CoASH + ATP vs. NADPH.
[b] $P \leq 0.001$.
[c] not significant.

As can be seen from Table 2, in the case of R-FL, as in the case of *rac*-FL, after 5 and 15 min incubation, the inversion was higher in the presence of coenzyme A and ATP (3.32% net inversion after 5 min and 3.59% after 15 min) than with NADPH (1.85% after 5 min and 1.66% after 15 min). After 60 min incubation, the inversion, on the contrary, was more pronounced in the presence of NADPH than in the presence of CoASH and ATP (2.15% and 0.57%, respectively). Inversion in the presence of the latter cofactors was statistically not significant.

He-Ne Laser Irradiation of Microsomes

The experimental data obtained showed that He-Ne laser irradiation of microsomes with a 0.2 J dose, under the experimental conditions described, caused a statistically significant increase of FL inversion after 5, 15, and 60 min of incubation in the presence of NADPH (Table 3).

DISCUSSION

The metabolic inversion of FL after incubation with rat liver microsomes was more pronounced in the presence of CoASH and ATP than in the presence of NADPH, which is in agreement with the mechanism of metabolic inversion of profens involving arylpropionylCoA thioester formation, as suggested by Nakamura et al.[14] This is consistent with acyl-CoA synthetase playing a key role in the inversion process. However, the less conspicuous, but still statistically significant, inversion observed after incubation of R-FL and the increase of percentage of the S-isomer after incubation of *rac*-FL in the presence of NADPH suggest that, in vitro, an oxidative pathway is also active in the presence of rat microsomal preparations.

The inversion of R-FL observed in our studies is in accordance with the results of Knihinicki et al.[3] and Jamali et al.[1] in vivo in the rat and with the formation of the CoA

TABLE 2. Chiral inversion of R-FL in vitro after incubation with rat liver microsomes for different times in the presence of CoASH + ATP or NADPH

Incubation time (min)	CoASH + ATP		NADPH	
	% S-isomer	% net inversion	% S-isomer	% net inversion
5	6.46 ± 0.27	3.32	4.99 ± 0.43	1.85
15	6.73 ± 0.71	3.59	4.80 ± 0.44	1.66
60	3.72 ± 0.21	0.58	5.29 ± 0.44	2.15
Nonincubated standard: 3.14 ± 0.141 (% S-isomer)				

Values are means of six to nine measurements ± SD. For the standard, the value is the mean of eight measurements ±SD. Statistical significance: CoASH + ATP vs. standard, NADPH vs. standard, and CoASH + ATP vs. NADPH: $P < 0.001$.

TABLE 3. FL metabolic chiral inversion in vitro after incubation of the racemate in the presence of NADPH with rat liver microsomes irradiated with He-Ne laser, total dose 0.2 J

Incubation time (min)	Irradiated microsomes		Nonirradiated microsomes	
	% S-isomer	% net inversion	% S-isomer	% net inversion
5	55.12 ± 0.20[a,b]	4.62	54.68 ± 0.31	3.84
15	55.09 ± 0.11[a,c]	4.66	54.00 ± 0.34	2.48
60	54.96 ± 0.21[a,b]	4.40	54.43 ± 0.28	3.34
Nonirradiated standard: 52.76 ± 0.18 % S-isomer				

Values are means of six measurements ± SD; the value for the standard is the mean of five measurements ±SD. Statistical significance: irradiated vs. standard.
[a] $P \leqslant 0.001$; nonirradiated vs. standard, $P \leqslant 0.001$; irradiated vs. nonirradiated.
[b] $P \leqslant 0.01$.
[c] $P \leqslant 0.001$.

thioester of R-FL in vitro by rat liver microsomes reported by Knights et al.[7] The findings of other authors on the metabolic inversion of 2-APAs in vitro are, however, divergent. Mayer et al.[4] found no chiral inversion of R-FL after incubation with subcellular rat liver preparations. According to Knihinicki et al.,[5] FL does not undergo inversion by rat liver, kidney, or small intestine homogenates, presumably as it is unable to act as a substrate for acyl-CoA synthetase and cannot, in consequence, be activated to the corresponding CoA thioester. Porubeck et al.[6] reported inversion of R-FL only after incubation of the thioesters but not after incubation of the free drug.

Differences between the results observed in vitro by various authors may be due to differences in experimental conditions (cellular subfractions, cofactor concentration, presence of inhibitors, protein and drug concentration, etc.).

The anomalous time course of the inversion of FL observed in the presence of NADPH, namely, the decrease of the percent net inversion after 15 min of incubation in comparison to 5 and 60 min, is difficult to explain on the basis of these data. Interestingly, the lower value of inversion at 15 min is normalized by laser irradiation, which also caused an increase of inversion at all of the times studied.

The increase of microsomal enzyme activity induced by laser irradiation is consistent with previous observations on photostimulation of various enzymes by coherent or noncoherent radiations in vitro and in vivo.[15–17] Although the effects of coherent and noncoherent light on biological systems have been widely investigated, the mechanisms underlying photostimulation remain to be fully established.

LITERATURE CITED

1. Jamali, F., Berry, B.W., Tehrani, M.R., Russell, A.S. Stereoselective pharmacokinetics of flurbiprofen in humans and rats. J. Pharm. Sci. 77:666–669, 1988.
2. Mayer, J.M. Stereoselective metabolism of antiinflammatory 2-arylpropionates. Acta Pharm. Nord. 2:197–216, 1990.
3. Knihinicki, R.D., Day, R.O., Graham, G.G., Williams, K.M. Stereoselective disposition of ibuprofen and flurbiprofen in rats. Chirality 2: 134–140, 1990.
4. Mayer, J.M., Bartolucci, C., Maitre, J.M., Testa, B. Metabolic chiral inversion of antiinflammatory 2-arylpropionates: lack of reaction in liver homogenates and study of methine proton activity. Xenobiotica 18:533-543, 1988.
5. Knihinicki, R.D., Williams, K.M., Day, R.O. Chiral inversion of 2-arylpropionic acid nonsteroidal antiinflammatory drugs. 1—In vitro studies of ibuprofen and flurbiprofen. Biochem. Pharmacol. 38:4389–4395, 1989.
6. Porubeck, D.J., Sanins, S.M., Steffens, J.R., Grillo, M.P., Kaiser, D.G., Halstead, G.W., Adams, W.J., Baillie, T.A. Metabolic chiral inversion of flurbiprofen-CoA in vitro. Biochem. Pharmacol. 42:R1–R4, 1991.
7. Knights, K.M., Drew, R., Meffin, P.J. Enantiospecific formation of fenoprofen coenzyme A thioester in vitro. Biochem. Pharmacol. 37:3539–3542, 1988.
8. Lombard, A., Rossetti, V., Buffa, M., Gabriel, L., Miglietta, A. In vitro studies on the stereoselective inversion of R(–) to S(+)-ketoprofen. IRCS Med. Sci. 13:1025, 1985.
9. Rossetti, V., Lombard, A., Buffa, M., Cassone, M.C. Studies on the stereoisomeric inversion of ketoprofen in rats. Med. Sci. Res. 14:256–257, 1986.
10. Lombard, A., Rossetti, V., Buffa, M., Cassone, M.C. Stereospecific metabolism of indoprofen in rats. Med. Sci. Res. 18:209–210, 1990.
11. Rossetti, V., Lombard, A., Buffa, M., Miglietta, A., Olivero, A., Gadoni, E. The in vitro metabolic inversion of R(–) to S(+)-indoprofen. Eur. J. Drug Metab. Pharmacokinet. 17:183–186, 1992.
12. Karu, T.I. Photobiology of low-power laser therapy. In: Laser Science and Technology, vol. 8. Letokhov, V.S., Shank, C.V., Shen, Y.R., Walther, H., eds. Chur: Harwood Academic Publishers, 1989.
13. Rossetti, V., Lombard, A., Buffa, M. The HPTLC resolution of the enantiomers of some 2-arylpropionic acid anti-inflammatory drugs. J. Pharm. Biomed. Anal. 4:673–676, 1986.
14. Nakamura, Y., Yamaguchi, T., Takahashi, S., Hashimoto, S., Iwatani, K., Nagakawa, Y. Optical isomerization mechanism of R(–)hydratropic acid derivatives. J. Pharmacol. Biodynam. 4:s–1, 1981.
15. Hugh, D.H., Hunter, J.K. Photomodulation of enzymes. J. Photochem. Photobiol. B 10:3–22, 1991.
16. Ostuni, A., Passarella, S., Quagliariello, E.J. Photomodulation of glutamate dehydrogenase properties by red light. J. Photochem. Photobiol. B 20:101–111, 1993.
17. Rossetti, V., Lombard, A., Urciuoli, R., Cassone, M.C., Rolfo, P.M. Rat brain metabolism enzyme activity variations following He-Ne laser irradiation. Mol. Chem. Neuropathol. 15:185–191, 1991.