Biochemical Pharmacology, Vol. 43, No. 7, pp 1465–1471, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

INHIBITION KINETICS OF HEPATIC MICROSOMAL LONG CHAIN FATTY ACID-CoA LIGASE BY 2-ARYLPROPIONIC ACID NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

KATHLEEN M. KNIGHTS* and MICHAEL E. JONES†
Dept of Clinical Pharmacology and †Dept of Anatomy and Histology, The Flinders University of South Australia, Bedford Park 5042, Australia

(*Received* 19 *November* 1991; *accepted* 9 *January* 1992)

Abstract—Microsomal long chain fatty acid CoA ligase (EC 6.2.1.3) has been implicated in the formation of CoA thioesters of xenobiotics containing a carboxylic acid moiety. In this study we have demonstrated that the microsomal enzyme from rat liver exhibits biphasic kinetics for the formation of palmitoyl-CoA, i.e. there are high affinity low capacity ($K_{m_{high}}$, 1.6 μM, $V_{max_{high}}$, 12.9 nmol/mg/min) and low affinity high capacity ($K_{m_{low}}$, 506 μM, $V_{max_{low}}$, 58.3 nmol/mg/min) components. Inhibition of the high affinity isoform was studied using the *R* and *S* enantiomers of ibuprofen, fenoprofen, ketoprofen and naproxen. The high affinity component of palmitoyl-CoA formation was competitively inhibited by *R*-fenoprofen (K_i 15.4 μM) while *R*-ibuprofen exhibited mixed inhibition kinetics. In contrast the *R* and *S* enantiomers of ketoprofen and naproxen were non-competitive inhibitors. This diversity of inhibition kinetics observed argues in favour of a binding site in addition to the catalytic site. A competitive interaction with the high affinity form correlated with literature evidence of enantiospecific chiral inversion and "hybrid" triglyceride formation for the *R* enantiomers of fenoprofen and ibuprofen. Paradoxically, *R*-ketoprofen which is extensively inverted in rats was a non-competitive inhibitor of palmitoyl-CoA formation by the high affinity isoform suggesting that it may not act as an alternate substrate. The results of this study clearly indicate that formation of *R*-2-arylpropionate-CoAs is not fully explained by interaction with the high affinity isoform of a microsomal long chain (palmitoyl) CoA ligase and therefore the involvement of other isoforms cannot be discounted.

A common chemical feature of the 2-arylpropionate (2-APA‡) non-steroidal anti-inflammatory drugs (NSAIDS) is a chiral centre and with the exception of naproxen they are administered to man as racemates (*RS*) although anti-inflammatory activity resides almost exclusively in the (+)*S* enantiomer. In humans and animals enantiospecific metabolic inversion of the inactive (−)*R* to the pharmacologically active (+)*S* enantiomer exhibits marked substrate and species variability [1]. Ibuprofen and ketoprofen are inverted extensively in the rat [2, 3] while inversion of naproxen and fenoprofen has been demonstrated in the rabbit [4, 5]. In relation to ketoprofen however, the rat data contrasts with man where minimal inversion of *R*-ketoprofen has been reported [6].

In 1981 Nakamura *et al.* [2] proposed that the mechanism of inversion of ibuprofen involved enzymic activation of the *R* but not the *S* enantiomer to a CoA thioester intermediate. Subsequent studies have both supported this general mechanism and confirmed the enantioselectivity of CoA thioester formation [7, 8]. In addition to a pivotal role in the overall inversion process, the *R*-2-APA-CoA intermediate is also a substrate for the enzymes of lipid synthesis. Ibuprofen, fenoprofen and ketoprofen have been shown in rats to be incorporated into "hybrid" triglycerides both *in vivo* and *in vitro* [9–11].

It has been demonstrated that rat hepatic microsomal long chain fatty acid CoA ligase (EC 6.2.1.3) catalyses the stereospecific formation of *R*-fenoprofen and *R*-ibuprofen CoAs [7, 8]. In addition, other studies have implicated this enzyme in the formation of CoA thioesters of 3-phenoxybenzoic acid [12], ciprofibrate, clofibric acid and nafenopin [13]. Although the primary role of the ligase is activation of long chain fatty acids to acyl-CoA thioesters, it is apparent that this enzyme is also responsible for the formation of CoA esters of xenobiotic carboxylic acids. In the case of the *R*-2-APAs this step is a prerequisite for both inversion and incorporation into triglycerides. The aim of the present study was to examine the hypothesis that the substrate specificity of the rodent microsomal long chain CoA ligase determines both the incidence and enantiospecificity of chiral inversion, and hence explains the formation of triglycerides containing an arylpropionate moiety.

MATERIALS AND METHODS

The enantiomers [(−) *R* (97.3%) and (+) *S* (98.4%)] of ibuprofen were obtained from the Boots Co. (Nottingham, U.K.), *S*-naproxen (98.4%) from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and the *R* enantiomer (97%) from Syntex Research (Palo Alto, CA, U.S.A.). The *R* and *S* enantiomers of fenoprofen (98.1 and 97.4%,

* Corresponding author: Dr K. M. Knights, Dept of Clinical Pharmacology, School of Medicine, The Flinders University of South Australia, Bedford Park 5042, Australia. Tel. (61) 8-204-5053; FAX (61) 8-277-0085.

‡ Abbreviation: 2-APA, 2-arylpropionate; DTT, dithiothreitol; CoASH, coenzyme A.

respectively) and ketoprofen (98 and 95%, respectively were obtained by resolution of racemic compounds as described by Hayball and Meffin [5] and Abas and Meffin [14]. The purity of the *R* and *S* enantiomers was determined using an enantiospecific HPLC method [15]. ATP, CoASH, dithiothreitol (DTT) and Triton X-100 were purchased from Sigma, and palmitic acid and [^{14}C]-palmitic acid from Calbiochem (La Jolla, CA, U.S.A.) and Amersham International (Amersham, U.K.), respectively. All other chemicals were obtained from commercial sources. A series of [^{14}C]palmitic acid standards, sp. act. 7 and 20 mCi/mmol, were prepared in the range 0.25 μM–1 mM and 0.25–10 μM, respectively. For determination of radioactivity all samples were added to Beckman EP scintillant (10 mL) and counted using a Beckman LS3801 liquid scintillation system.

Animals and preparation of microsomal long chain fatty acid CoA ligase

Animal studies were approved by the Flinders Medical Centre Animal Ethics Review Committee. Male hooded Wistar rats (200–250 g) were housed under standard conditions of temperature and light cycles and allowed food and water *ad. lib.* Animals (N = 6) were stunned and decapitated and the livers removed rapidly and perfused with ice-cold Tris–HCl (50 mM, pH 7.4, 20 mL). Each liver was then chopped finely and homogenized in Tris–HCl (50 mM, pH 7.4, 25 mL), sucrose (0.25 M), DTT (5 mM) and EDTA (5 mM) using a motorized Potter–Elvehjem homogenizer. The homogenates were centrifuged (10 min, 600 *g*, 4°) and the supernatants aspirated and recentrifuged (10 min, 10,000 *g*). The resulting supernatants were centrifuged (60 min, 105,000 *g*) and the pellets combined and resuspended in buffer solution (100 mL) minus sucrose. The remainder of the isolation procedure was as stated by Merrill *et al.* [16]. Protein concentration was determined using a modified Lowry procedure described by Bensadoun and Weinstein [17] and the activity of the enzyme assessed using [^{14}C]palmitic acid according to the technique of Krisans *et al.* [18]. Aliquots (0.5 mL) of the enzyme preparation were stored at −70° until required.

In vitro *studies*

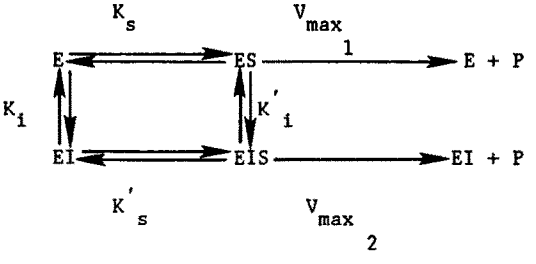
Characterization of enzyme. Preliminary studies established the linearity of palmitoyl-CoA formation with respect to protein concentration and duration of incubation. The standard incubation medium (0.2 mL) contained Tris–HCl (150 mM, pH 7.4), $MgCl_2$ (6.2 mM), Triton X-100 (0.05%), EDTA (2 mM), ATP (2.5 mM), CoASH (600 μM), DTT (1 mM) and [^{14}C]palmitic acid (0.25 μM–1 mM, 7 mCi/mmol). The medium was pre-warmed (1 min, 37°) and the reaction initiated by the addition of 20 μL enzyme protein (25 μg/mL). The incubation was terminated after 20 min and [^{14}C]palmitoyl-CoA extracted and quantified as described previously [18].

Inhibition of [^{14}C]*palmitoyl-CoA formation.* Samples were incubated using the standard reaction mixture as described. [^{14}C]Palmitic acid (20 mCi/mmol) was added at concentrations of 0.25, 0.5 and 1 μM and inhibition of palmitoyl-CoA formation determined in the presence of either the *R* and *S* enantiomers of ibuprofen and fenoprofen (5–25 μM) or ketoprofen and naproxen (2.5–10 μM). The enantiomers were added in a minimal volume (10 μL) of dimethyl sulphoxide (2% w/v) and there was no evidence of alteration in palmitoyl-CoA formation due to the vehicle only.

Kinetic analysis

Values for K_m and V_{max} were calculated using MKMODEL, an extended least-squares regression modelling program [19], using initial parameter estimates obtained from Eadie–Hofstee plots of the data. Equations modelling either a single-enzyme or a two-enzyme system were fitted to the data as appropriate.

A preliminary analysis of the data from the inhibition studies was carried out by the method of Dixon [20]. Subsequent analysis was by the direct non-linear least-squares method using the Marquardt–Levenberg algorithm as implemented in the Harwell Subroutine Library procedure VA05A. In the most general case, the reaction scheme was represented as:



It has been assumed throughout that the formation of product is slow relative to the formation of the complexes EI, ES, and EIS and that these complexes therefore exist in concentrations determined by [E], [I], and [S] and by the constants K_s, K_i, K_i' and K_s'. Although only three of the four constants are independent ($K_sK_i' = K_s'K_i$), four were retained for symmetry. To avoid ambiguity, the assumptions underlying each physical model and the corresponding equation to which the data was fitted during parameter estimation are stated (Appendix). The types of inhibition modelled were partially mixed inhibition, fully mixed inhibition, competitive inhibition and non-competitive inhibition. We have not modelled specifically the cases of partially mixed inhibition, which can be described as partially uncompetitive ($1/K_i = 0, K_i' \neq 0$), partially non-competitive ($K_s = K_s', V_{max_2} = 0$) or partially competitive ($1/K_s' \neq 0), V_{max_1} = V_{max_2}$), because they are less commonly encountered. They are, in any case, allowed for within the most general model, that of partially mixed inhibition.

Each experiment was carried out with the *R* and *S* enantiomers, and the best-fit set of parameters calculated for each. In the most general case, the

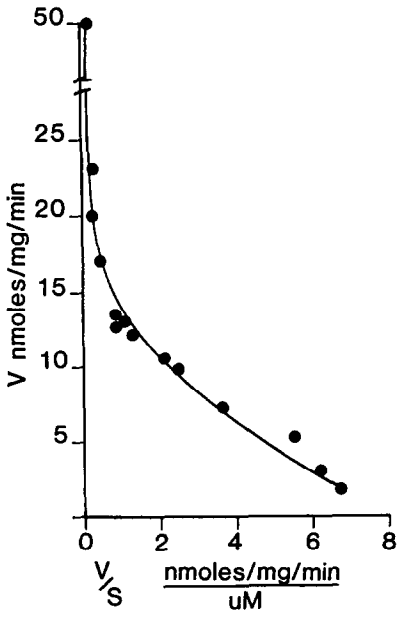


Fig. 1. A representative Eadie–Hofstee plot for palmitoyl-CoA formation by rat hepatic microsomal long chain CoA ligase. For experimental details see text. Points are experimentally determined values while the solid line is the computer-generated curve of best fit.

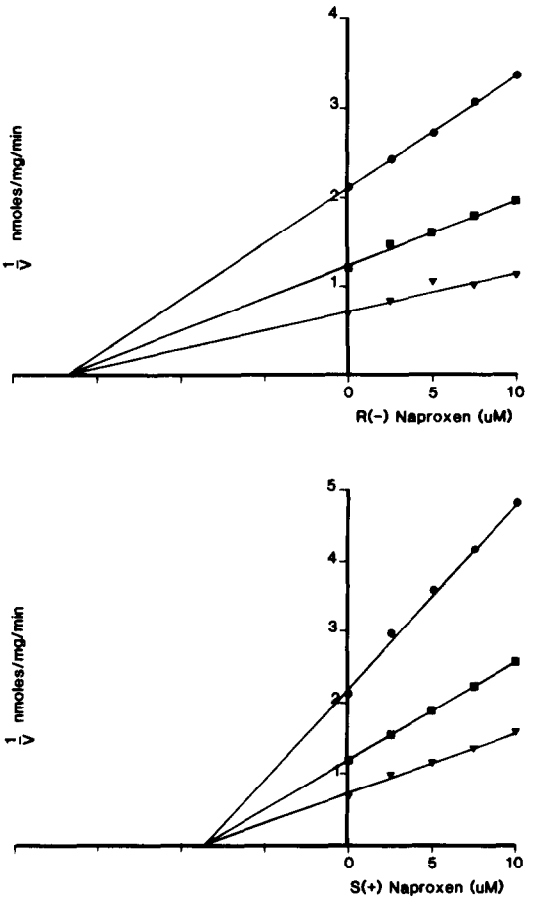


Fig. 2. Representative Dixon plot for the inhibition of palmitoyl-CoA formation by the *R* and *S* enantiomers of naproxen (2.5–10 μM). Substrate concentration of [^{14}C]palmitic acid: (●) 0.25, (■) 0.5 and (▼) 1 μM.

parameters V_{max_1}, V_{max_2}, K_s, K_i and K_s' were calculated for each enantiomer but, of these, V_{max_1} and K_s are properties of the enzyme and therefore independent of the inhibitor. Consequently, although each experiment generated five parameters, the two enantiomers as a pair generated not 10 but eight parameters. Data for both enantiomers were therefore analysed simultaneously, constraining the model to use, for both enantiomers, common values for the enzyme parameters V_{max_1} and K_s.

RESULTS

The enzyme from rat liver microsomes exhibited biphasic kinetics for the formation of palmitoyl-CoA. A representative Eadie–Hofstee plot of a combined enzyme preparation from six rats is shown in Fig. 1. The four parameters $V_{max_{high}}$, $K_{m_{high}}$ and $V_{max_{low}}$, $K_{m_{low}}$ were determined graphically from each plot and the data used as initial estimates in the MKMODEL program. Values for the Michaelis–Menten parameters for the high affinity low capacity component were $V_{max_{high}}$, 12.9 nmol/mg/min and $K_{m_{high}}$, 1.6 μM and for the low affinity high capacity component, $V_{max_{low}}$, 58.5 nmol/mg/min and $K_{m_{low}}$, 506.7 μM. Inhibition of the high affinity form of the enzyme was observed in the presence of *R*-fenoprofen (5–500 μM) while the low affinity component was unaffected (data not shown). Further investigation of the kinetics of inhibition was undertaken using 0.25, 0.5 and 1 μM palmitic acid. At these concentrations the high affinity component of the enzyme predominated at a ratio of 62, 54 and 45:1, respectively.

Plots of the reciprocal of velocity against inhibitor concentration are shown in Figs 2 and 3 for naproxen and ibuprofen, respectively. The intercept on the base line ($1/V = 0$) is consistent with non-competitive kinetics for both enantiomers of naproxen and a similar profile of interception was observed with the ketoprofen enantiomers. Interception above the base line for the *R* enantiomers of ibuprofen (K_i, 35.6 μM) and fenoprofen (K_i, 15.4 μM) is consistent with competitive kinetics while the intercepts below the base line for the (+)*S* enantiomers of these compounds suggests mixed inhibition. The range of K_i values for the competitive and mixed inhibitors (15.4–69.5 μM) indicates a difference in affinity of each of the inhibitors for the enzyme which in the case of mixed inhibition is a complex scenario. It should be noted that intercepts in the third quadrant are uncommon.

Confirmatory evidence of non-competitive and competitive inhibition from Dixon plots was obtained from secondary plots of slopes versus reciprocal

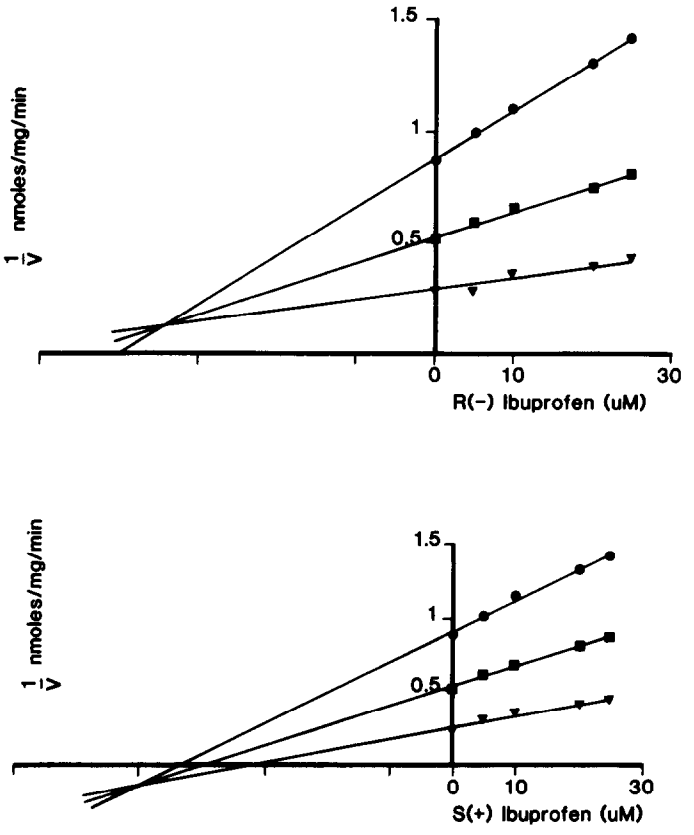


Fig. 3. Representative Dixon plot for the inhibition of palmitoyl-CoA formation by the *R* and *S* enantiomers of ibuprofen (5–25 μM). Substrate concentration of [^{14}C]palmitic acid: (●) 0.25, (■) 0.5 and (▼) 1 μM.

substrate concentration [21]. However, because of the diversity of kinetics encountered the experiments were repeated twice and gave identical results. Therefore, more detailed examination of the data was undertaken by direct fitting of the data to the equations representing the general mechanisms of enzyme inhibition as described (Appendix). The data from both the graphical and mathematical models are detailed in Table 1. The K_i values and the types of inhibition ascertained from Dixon and secondary plots closely equated with the same models when using the least squares method of analysis.

DISCUSSION

In this study microsomal long chain CoA ligase from rat liver exhibited biphasic kinetics for the formation of palmitoyl-CoA indicating the presence of two isoforms capable of forming long chain fatty acid CoA thioesters. The high affinity form exhibited a K_m of 1.6 μM which is within the range of K_m values reported by others for a rat microsomal enzyme [22, 23]. The presence of two long chain fatty acid CoA ligases has been demonstrated previously using mutant strains of *Candida lipolytica* [24]. However, to date, this is the first report of the existence of two isoforms of the rodent liver microsomal enzyme. Only formation of palmitoyl-CoA catalysed by the high affinity form of the enzyme was inhibited by *R*-fenoprofen and graphical and numerical analyses confirmed that the inhibition was of a competitive nature. A K_i of 15.4 μM for *R*-fenoprofen is consistent with the observation that fenoprofen supports a rate of xenobiotic triacylglycerol formation equivalent to that of conventional triglyceride synthesis [25]. These data clearly indicate that *R*-fenoprofen may act as an alternative substrate for the microsomal ligase and thus provides an explanation as to the mechanism of inhibition of endogenous triacylglycerol synthesis [26].

In contrast, when analysed by both methods the (+)*S* enantiomers of fenoprofen (K_i, 69.5 μM) and ibuprofen (K_i, 52.4 μM) exhibited mixed inhibition kinetics with $K'_s < K_s$. For fully mixed inhibition the Dixon plots are linear and intercept at the common point, $[I] = -K_i$ and $1/V = (1 - K_s/K'_s)/V_{max}$, which will be below the horizontal axis if $K_s > K'_s$. This in turn is not exclusively a condition for the binding of substrate because of the constraint $K_s/K'_s = K_i/K'_i$; it implies only that the binding of any one ligand (substrate or inhibitor) facilitates the binding of the other. Accepting a profile of mixed inhibition for the *S* enantiomers of ibuprofen and fenoprofen implies that there were two or more sites on the enzyme with binding at one influencing the affinity at the other. A two-site hypothesis has been

Table 1.

Enantiomer	Graphical analysis: Inhibition type	Graphical analysis: K_i (μM)	Least-squares analysis: Inhibition type	Least-squares analysis: K_i (μM)	Least-squares analysis: V_{max_1}/K_s
R-Ibuprofen	Competitive	35.6	Competitive	28.4	4.6
	—	—	Mixed	59.5	4.2
S-Ibuprofen	Mixed	52.4	Mixed	51.1	4.2
R-Fenoprofen	Competitive	15.4	Competitive	16.6	3.9
S-Fenoprofen	Mixed	69.5	Mixed	52.8	4.2
R-Naproxen	Non-competitive	16.6	Non-competitive	15.2	2.1
	—	—	Mixed	19.5	2.0
S-Naproxen	Non-competitive	8.9	Non-competitive	7.6	2.1
	—	—	Mixed	9.8	1.9
R-Ketoprofen	Non-competitive	3.5	Non-competitive	4.1	2.8
S-Ketoprofen	Non-competitive	5.8	Non-competitive	5.2	3.3

Inhibition of palmitoyl-CoA formation by the *R* and *S* enantiomers of ibuprofen, fenoprofen, naproxen and ketoprofen. The type of inhibition and the apparent K_i (μM) determined from both graphical and numerical analyses are presented. The ratio V_{max_1}/K_s relates to the properties of the enzyme and is independent of the inhibitor. For experimental details see text.

proposed recently as an explanation for the observation that 1-pyrenedecanoic acid inhibits palmitic acid activation but not *vice versa* [27]. A profile of mixed inhibition must therefore be considered in conjunction with the finding in some studies of small quantities of the *R* enantiomer of ibuprofen following administration of the *S* antipode [28, 29].

The results of graphical analysis, suggesting that *R*-ibuprofen competitively inhibited palmitoyl-CoA (K_i, 35.6 μM) conflicts with the numerical analysis which suggests mixed inhibition with $K_s > K_s'$. Despite the apparent "mixed" profile, there is considerable evidence in the literature which documents both the chiral inversion of *R*-ibuprofen in rats [28, 30] and the enantiospecific incorporation of *R*-ibuprofen into rat adipose tissue triglycerides [9]. More recently, Knihinicki *et al.* [8] using the same enzyme preparation as reported in this study have demonstrated that *R*-ibuprofen is a substrate for the rat microsomal long chain CoA ligase; thus, competitive inhibition of palmitoyl-CoA formation would have been expected. This metabolic evidence argues strongly that *R*-ibuprofen binds in part (although not necessarily exclusively) to the catalytically active site and would act in part via a competitive mechanism. Although the mixed kinetics shown by (+)*S*-fenoprofen and (+)*S*-ibuprofen argue for there being a binding site in addition to the catalytic site, the metabolic evidence indicates that at least some of the inhibitors may bind at both sites. This implies an interaction more complex than even partially mixed inhibition, in which substrate and inhibitor are assumed to bind exclusively to their unique respective sites. This more complex scenario resolves, in part, the apparent conflict between the graphical and numerical analyses; both may be fitting to a model which is an oversimplification of reality. Least-squares analysis confirmed the apparent non-competitive inhibition for *R*- and *S*-ketoprofen, but suggested mixed inhibition with $K_i > K_i'$ for *R*- and *S*-naproxen. We suggest that the discrepancy between the two methods is more apparent than real. In these experiments the substrate concentration was typically one-twentieth of K_s and the added inhibitor was typically of the order of K_i. Accordingly $1/V_{max}$ represents about 2% of the height of the Dixon plot. With K_s/K_s' being of the order of 2, the intercept is less than 5% of the plot's height below the horizontal axis.

In this study *R*-ketoprofen was consistently found to be a non-competitive inhibitor of palmitoyl-CoA formation, again suggesting binding at a second site which in this case was not the catalytic site. However, evidence from rat studies of both extensive chiral inversion (>50%) of *R*-ketoprofen to the *S* antipode [3] and incorporation into triacylglycerols implies formation of the requisite CoA intermediate. At present, we can only speculate that the formation of *R*-ketoprofen-CoA is catalysed by an alternate isoform of a microsomal CoA ligase.

This rat study has established that a competitive type interaction with the high affinity form of the microsomal long chain palmitoyl-CoA ligase correlates with literature evidence from this species of both the enantiospecificity of chiral inversion and incorporation of the *R* enantiomers of ibuprofen and fenoprofen into triacylglycerols. However, *R*-ketoprofen was found to be a non-competitive inhibitor of palmitoyl-CoA formation suggesting that this compound interacts with the high affinity form of the enzyme but may not act as an alternate substrate. These data contrast with the evidence from rat studies of both chiral inversion and lipid incorporation of *R*-ketoprofen. In that regard the non-competitive kinetics exhibited by *R*-naproxen must await interpretation until such time as the involvement of other isoforms of rat microsomal long chain ligase in the formation of *R*-2-APA-CoA thioesters is either substantiated or refuted. Nevertheless, this study establishes that although all the enantiomers studied

interact with the enzyme, relatively few act as alternate substrates.

Acknowledgements—This research was supported by grants from the Arthritis Foundation and the National Health & Medical Research Council of Australia.

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APPENDIX

The most general scheme for inhibition considered here is that of partially mixed inhibition [31] which is modelled by the equation:

$$V = \frac{V_{max_1}[S] + V_{max_2}[S][I]/K_i'}{[S]\left(1 + \frac{[I]}{K_i'}\right) + K_s\left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

there being no constraints on the parameters other than that they should not be negative. This is a five-parameter model. In this most general case, plots of $1/V$ against inhibitor concentration, for constant substrate concentration, will intersect at the common point $[I] = -K_i$ and $1/V = (K_i' - K_i)/(V_{max_1}K_i' - V_{max_2}K_i)$. These lines will not in general be straight lines however unless $V_{max_2} = 0$ or K_i' is very large.

Direct least-squares fits of the data to this most general model were indistinguishable from those to the more constrained model of fully mixed inhibition (i.e. $V_{max_2} = 0$). This corresponds to, and is in agreement with, the graphical finding that the Dixon plot gave straight, not curved, lines for constant substrate concentration.

Setting $V_{max_2} = 0$ gives the next most general model, that of fully mixed inhibition described by:

$$V = \frac{V_{max_1}[S]}{[S]\left(1 + \frac{[I]}{K_i'}\right) + K_s\left(1 + \frac{[I]}{K_i}\right)} \quad (2)$$

in which there are four parameters to be estimated. In this, and hence in the particular cases considered below, a Dixon plot produces straight lines intersecting at the common point $[I] = -K_i, 1/V = (1 - K_i/K_i')/V_{max_1}$. We note that this intersection will be in the third quadrant if and only if $K_i/K_i' < 1$, i.e. if the binding of one ligand (substrate or inhibitor) facilitates the binding of the other. This seems to us to be the simplest model compatible with a Dixon plot exhibiting straight lines intersecting below the axis, as seen for *S*-ibuprofen.

More commonly encountered inhibition kinetics are the fully competitive and fully non-competitive pictures which may be considered to be particular cases of fully mixed inhibition. They share, therefore, Dixon plots in which straight lines intersect at a common point. For fully non-competitive, the binding of one ligand fails to influence the binding of the other so that $K_i = K_i'$. Accordingly:

$$V = \frac{V_{max_1}[S]}{([S] + K_s)(1 + [I]/K_i)} \quad (3)$$

and the intercept on the Dixon plot is at $1/V = 0$. Naproxen and ketoprofen show this picture.

The other familiar three-parameter model is fully competitive in which the binding of one ligand excludes the binding of the other so that K_s' and K_i' are very large, giving:

$$V = \frac{V_{max_1}[S]}{[S] + K_s\left(1 + \frac{[I]}{K_i}\right)}. \quad (4)$$

The intercept on the Dixon plot is in the second quadrant with $1/V = 1/V_{max}$. A second quadrant intersection is consistent with, but not conclusive of, this picture because such an intersection may take place below $1/V$ and still be in the second quadrant. Thus for *R*-ibuprofen (Fig. 3) the Dixon plot is consistent with competitive inhibition, but least squares analysis shows a 33% fall in residual error when the more general mixed inhibition model is fitted, suggesting that the form of inhibition is mixed for both enantiomers of ibuprofen. The 33% fall in residual error is accompanied by a large change in the estimated parameters for *R*-ibuprofen (Table 1). Because non-competitive inhibition is a particular case of mixed inhibition, data from a non-competitive inhibitor such as naproxen will also fit a mixed model with a small reduction in residual error and a small change in estimated parameters. The small changes apparent for naproxen in Table 1 do not argue for mixed inhibition.