Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 335 (2007) 63–69
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

A novel method for assessing inhibition of ibuprofen chiral inversion and its application in drug discovery

Anita Reddy [a,*], Muhammed Hashim [a], Ziqiang Wang [b], Lara Penn [c], Charles J. Stankovic [a], Douglas Burdette [a], Narayanan Surendran [a], Hongliang Cai [a]

[a] *Preclinical Discovery-Development, Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI 48105, USA*
[b] *Chemistry, Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI 48105, USA*
[c] *Bioanalytical Research, Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI 48105, USA*

Received 9 August 2006; accepted 27 October 2006
Available online 14 December 2006

Abstract

An inhibition assay to assess the potential for chiral inversion of compounds was developed using *R*(−)-ibuprofen as the probe substrate. Inhibition of the chiral inversion of *R*(−)-ibuprofen by structurally similar compounds in cyropreserved rat hepatocytes was studied using chiral HPLC and LC/MS methods for the chromatographic separation and detection of enantiomers. Concept validation of this assay was performed with three commercially available compounds and four Pfizer compounds. The results of these studies demonstrated that compounds that are structurally similar to ibuprofen inhibited the formation of *S*(+)-ibuprofen, suggesting that they may undergo similar enzymatic chiral inversion pathways or compete for the same enzyme active sites. Additionally, an application of this assay in early drug discovery for a specific class of compounds was demonstrated. Thirty-three in-house compounds were screened for their chiral inversion potential utilizing this assay to investigate the structure activity relationship (SAR) for this class of compounds.

Keywords: Chiral inversion; Inhibition; Ibuprofen; Enantiomer

1. Introduction

Approximately 25% of drugs are marketed as either racemates or mixtures of diastereoisomers. In recent years, research has been intensified to understand the molecular mechanism by which stereoselective biological activities of chiral molecules occur (Drayer, 1986; Hubbard et al., 1986; Hutt and O'Grady, 1996; Landoni and Soraci, 2001; Tanner, 2002). Multiple isomeric forms arising from a drug may be residual from compound synthesis or result from chiral inversion either due to chemical instability or biological catalysis. However, for a given compound the potential for chiral inversion is difficult to predict. While chemical instability may be identified by critical review of the structure or *in vitro* stability experiments, catalyzed chiral inversion can result from action by diverse enzymes acting through a range of mechanisms (Wsol et al., 2004). These mechanisms include: (i) oxidoreduction (androsterone by 3($\alpha \rightarrow \beta$)-hydroxysteroid epimerase); (ii) sulfoxide/sulfide and *N*-oxide/tertiary amine equilibriums (mediated by Cytochrome P450 or other reductases and monooxygenases); (iii) cofactor independent conjugation (glutamate racemase, praline racemase, diaminopimelate racemase, aspartate racemase, and mandelate racemase); or (iv) cofactor dependent conjugation (2-methylacyl racemase for fatty acids and acyl-CoA synthetase for ibuprofen and fenoprofen).

It is well known that different stereoisomers frequently differ in terms of their biological activities and pharmacokinetic profiles and that the use of such mixtures may contribute to the adverse effects of the drug (Muller et al., 1990; Reist et al., 1998). Because of these known differences, the stereochemistry of new chemical entities (NCEs) has been an area of focus for both the pharmaceutical industry and regulatory authorities in recent years. The assessment of the chiral inversion potential of NCEs may improve pharmacokinetic and safety properties,

* Corresponding author at: Pfizer Global Research and Development, 2800 Plymouth Road, 025/2-36A, Department of Pharmacokinetics, Dynamics, and Metabolism, Ann Arbor, MI 48105, USA. Tel.: +1 734 622 1294; fax: +1 734 622 1459.
E-mail address: anita.reddy@pfizer.com (A. Reddy).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.11.006

thereby improving the odds of clinical success. In addition, the increase in the number of chiral compounds moving from the file through development indicates a need to identify and assess the potential for chiral inversion and to experimentally track the inversion liability as they progress.

A typical analytical approach in the pharmaceutical industry is to develop an enantiomeric separation method via chromatography that requires a significant resource investment (since an analytical method needs to be developed for each compound or compound class) and hence is often conducted later in the drug discovery and development process. *In vivo* studies are more physiologically relevant but lack the throughput required for implementation in early drug discovery and development. Alternatively, *in vitro* tools (for example, isolated hepatocytes) can provide the needed data earlier but may lack appropriate level of predictivity.

2-Arylpropionic acid (APA) derivatives, the so-called profens, are known to undergo metabolic chiral inversion *in vivo* (Knihinicki et al., 1991; Landoni and Soraci, 2001). The mechanism by which this occurs is well understood and two of the enzymes involved have been isolated and characterized. Within the APA derivatives, ibuprofen is the most studied and has been shown to undergo unidirectional chiral inversion in different animal species and humans (Kaiser et al., 1976; Jamali, 1988; Baillie et al., 1989; Sanins et al., 1990; Sanins et al., 1991; Xiaotao and Hall, 1993). The enzymatic chiral inversion of ibuprofen is a three step mechanism involving the formation of the acyl-CoA thioester by stereoselective activation of *R*-(−)-enantiomer in the presence of acyl-CoA synthetase (CoA) and enzymatic epimerization of the *R*-thioester to the *S*-(+)-thioester followed by the formation of *S*-(+)-enantiomer by hydrolysis of *S*-(+)-thioester (Sanins et al., 1991). Compounds demonstrating the same chiral inversion mechanisms as that of *R*-(−)-ibuprofen should inhibit the ibuprofen inversion and result in a decrease in the amount of *S*-(+)-ibuprofen formed.

The primary objective of this work was to develop an *in vitro* assay based on inhibition of chiral inversion of *R*-(−)-ibuprofen using cryopreserved rat hepatocytes and HPLC/LC/MS methods to identify the potential for enzymatic chiral inversion of compounds which are structurally similar to ibuprofen. The secondary aim was to investigate the application of the assay in early drug discovery for structure activity relationship (SAR) determination. Three model compounds (ketoprofen, fenoprofen and thalidomide) and four Pfizer compounds (PF1, PF3, PF4, PF5) were used for concept validation. Additionally, 33 Pfizer compounds from a single chemical series were identified and screened to investigate the potential application of this assay in early drug discovery for assessment of structure activity relationship (SAR).

2. Methods

2.1. Chemicals

R-(−)-Ibuprofen was purchased from BIOMOL Research labs, Inc. (Plymouth Meeting, PA). ^{14}C labeled *R*-(−)-ibuprofen was purchased from ARC (St. Louis, MO), ^{3}H labeled *R*-(−)-ibuprofen was synthesized in-house. Ketoprofen, fenoprofen, and thalidomide were purchased from Sigma–Aldrich, Inc. PF1, PF3, PF4 and PF5 were obtained from Pfizer Research and Development. Cryopreserved hepatocytes and hepatocyte isolation kits were purchased from XenoTech, LLC (Lenexa, KS).

2.2. Instruments and materials

The high performance liquid chromatography system was equipped with an injector/autosampler and Pump (Perkin-Elmer, Wellesley, MA), Radio HPLC Detector β-Ram Model 3 (IN/US Systems, Inc., Tampa, FL) and Laura3 build91 operating software. A 0.46 cm i.d. × 15 cm, Chiracel OJ-RH Column (Chiral Technologies, Inc., West Chester, PA) was used to achieve chromatographic separation.

The LC–MS/MS system consisted of a Waters Micromass Quattro II triple quadrupole mass spectrometer (Waters, Milford, MA) outfitted with a model series 200 LC pump and autosampler (Perkin-Elmer, Norwalk, CT), Daicel Chiralcel OJ-RH chromatography column (part number 17794A, 5 μm, 150 mm × 2.1 mm i.d. (Chiral Technologies, Inc., Exton, PA), model 250 column heater (Cera, Marietta, CA), and Masslynx (version 3.4) operating software.

2.3. Cross species hepatocytes preparation

Cryopreserved hepatocytes were purchased from XenoTech, LLC (Lenexa, KS). XenoTech protocol and XenoTech Hepatocyte Isolation Kit (XenoTech LLC, Lenexa, KS) were used to thaw and prepare cryopreserved hepatocytes for the assays. First, 50 mL of Tube A (media containing Percoll^{TM} solution used in initial cell isolation) and 50 mL of Tube B (media used to wash the isolated hepatocytes) were pre-warmed at 37 °C. Subsequently, cryopreserved hepatocytes were removed from the liquid nitrogen freezer and immediately placed in 37 °C water bath for 1.5 min ± 15 s (for 1.5 mL vials) or 2 min ± 15 s (for 4.5 mL vials). Thawed hepatocytes were gently poured into Tube A, re-suspended and subsequently centrifuged at room temperature (RT) for 5 min at 90 × *g*. The supernatant was discarded and the media in Tube B was added to the cells. The resulting mixture was re-suspended and centrifuged at RT for 3 min at 60 × *g*. Following removal of the supernatant, the cells were re-suspended in an appropriate volume of pre-warmed Leibovitz L-15 media (In Vitrogen Corporation, Carlsbad, CA). Trypan blue solution (Tube C from the hepatocyte isolation kit) was used for cell count and viability calculations. Cell viability of >75% was set as criteria in selection of hepatocytes to ensure quality of this assay.

2.4. Incubation conditions for radiometric HPLC analysis

Incubations were carried out in 96-well plates in a total volume of 250 μL with cryopreserved rat hepatocytes (stock of 0.25×10^6/mL cell density). Preliminary inhibition experiments were done with 1 μM *R*-(−)-ibuprofen along with radiolabeled (^{3}H) *R*-(−)-ibuprofen and 0.1 or 0.5 mM test compounds. Two types of controls, one with substrate in the absence of cells and

Table 1
Incubation conditions for cryopreserved rat hepatocyte cell density optimization, time linearity, apparent K_m determination and IC_{50} determinations (analyzed by LC/MS/MS)

Study	Cell density stock (million/mL)	Concentration of substrate *R*-ibuprofen (μM)	Time points (minutes)
Time linearity	0.5	1	15, 30, 60, 120, 180
Cell density optimization	0.1, 0.25, 0.5, 1.0, 2.0	1	30
K_m determination	0.25	0.02, 0.05, 0.1, 0.5, 1.0, 2.5, 5, 10, 20, 30	30
IC_{50} determination of inhibitors	0.25	1	30

the other containing cells without any substrate or test compounds were used. After 30 min at 37 °C, incubations were quenched with 100 μL ice-cold acetonitrile. Samples were then centrifuged for 10 min at 4000 rpm and the supernatants were recovered and analyzed by radiometric HPLC method.

2.5. Incubation conditions for LC/MS analysis

Subsequent to the preliminary radiolabeled *R*-(−)-ibuprofen inhibition incubations and radiometric HPLC analysis, IC_{50} determinations were performed with 12 concentrations of the compounds using an LC/MS method for sample analysis. Time linearity, cell density and K_m determination experiments were performed to establish the optimized conditions for the assay. Experimental conditions are shown in Table 1. Incubations were carried out in 96-well plates in total volume of 250 μL with cryopreserved rat hepatocytes (stock of 0.25×10^6/mL cell density) and 1 μM *R*-(−)-ibuprofen. Similar to the radiometric analysis, two types of controls, one containing substrate in the absence of cells and the other only cells without any substrate or test compounds were used. Incubations were quenched with 100 μL ice-cold acetonitrile after 30 min at 37 °C. Samples were subsequently centrifuged for 10 min at 4000 rpm and the recovered supernatants were analyzed by an LC/MS method.

2.6. HPLC conditions

A 20 μL aliquot of incubate was injected onto the chromatography column. Chromatographic separation of *R* and *S* enantiomers of ibuprofen was achieved in a 10 min HPLC run at RT using an isocratic mobile phase consisting of 85/15 (v/v) mixture of methanol + 0.1% acetic acid/H_2O + 0.1% acetic acid at a flow rate of 0.8 mL/min. A typical chromatogram of the enantiomeric separation of ibuprofen is shown in Fig. 1.

2.7. LC/MS/MS conditions

A 75 μL aliquot of incubate was spiked with 50-μL aliquots of 4000 ng/mL ketoprofen in a 50/50 (v/v) mixture of methanol/water. A 3 μL aliquot of quenched incubate was injected onto the chromatography column. Chromatographic separation was achieved under isocratic conditions maintained at 26 °C using mobile phase consisting of a 80/20 (v/v) mixture of methanol with 0.1% acetic acid/water with 0.1% acetic acid at a flow rate of 200 μL/min. The source block temperature of mass spectrometry was set to 100 °C and the desolvation temperature was set to 250 °C. Multiple reaction monitoring (MRM) parameters of ibuprofen were set as follows: ESI polarity: negative; MRM transition (*m/z*): 205.0 → 161.1; collision energy: 8 eV; cone voltage: 20 V; dwell: 0.4 s.

2.8. In vivo methods

Drug was administered orally (PO) and intravenously (IV) to Sprague Dawley rats, Beagle dogs and Cynomolgous monkey at a dose of 5 mg/kg. The PO dose was administered as suspension in 5:95 PEG-200: 0.5% methylcellulose in water (w/v) and IV dose was administered as a solution in 5% DMA/5% Pharmasolve/45% PEG-400/45% 50 mM lactic acid. Serial blood samples (for plasma) were collected over 48 h at 0, 0.5, 1, 2, 4, 6, 8 and 24 h post oral administration and at 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h following intravenous administration

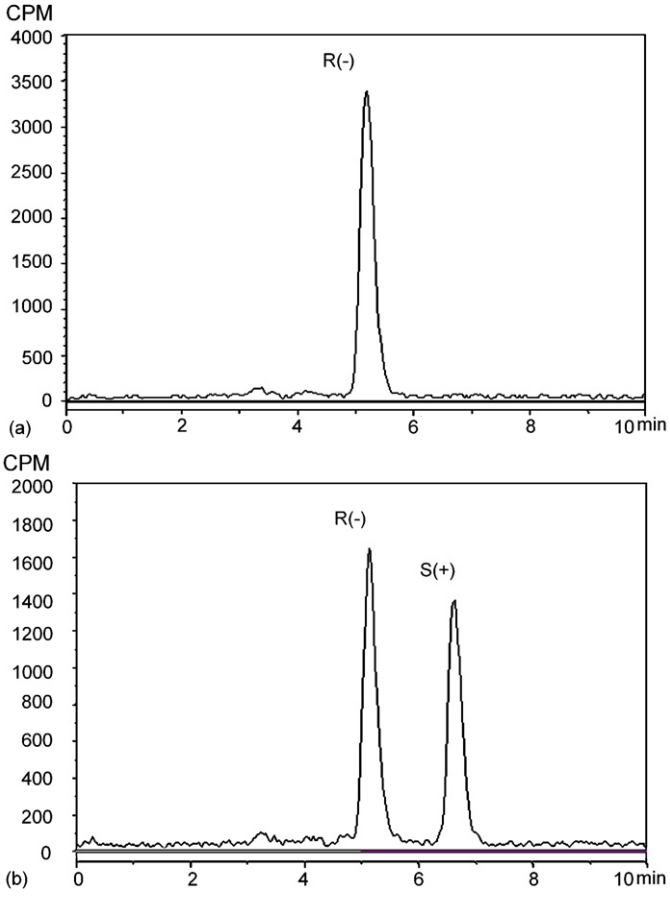


Fig. 1. (a) Typical chromatogram of *R*-ibuprofen incubated without hepatocytes and (b) incubated with hepatocytes.

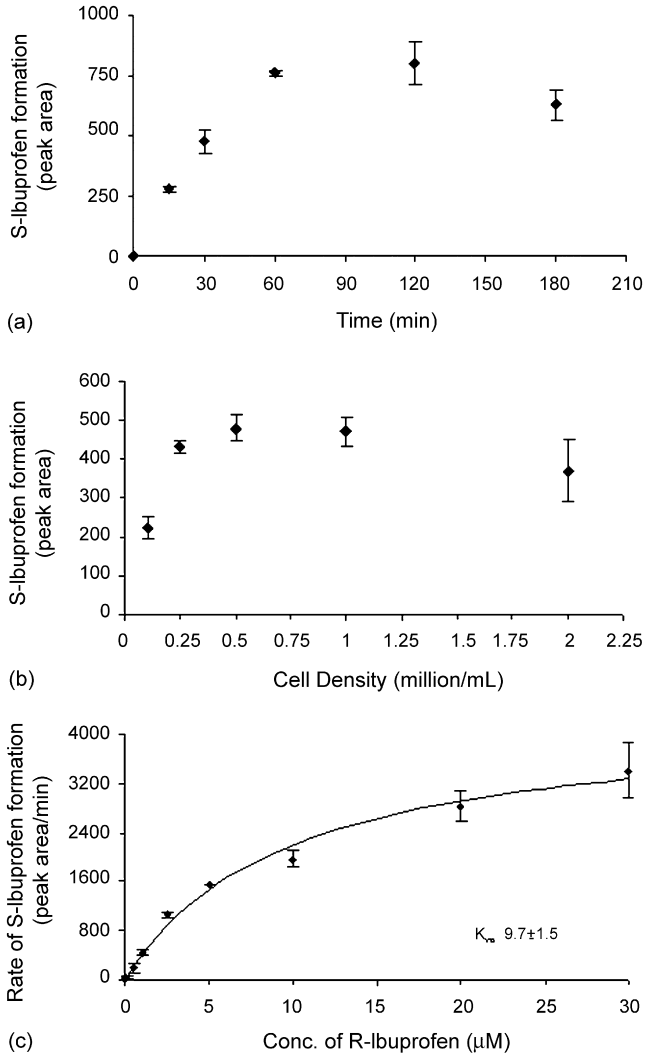


Fig. 2. The effect of time, cell density and substrate concentration in the chiral inversion of *R*(−)-ibuprofen to *S*(+)-ibuprofen in cryopreserved rat hepatocytes: (a) time linearity; (b) cell density determination; (c) apparent K_m determination.

for rats and 0, 0.5, 1, 2, 4, 6, 8, 24 and 48 h and at 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h for dog and monkey.

2.9. Data analysis

Substrate concentration versus activity data was analyzed to yield K_m and inhibitor concentration versus percentage of inhibition data was analyzed to yield IC_{50} using WinNonLin software (Pharsight Corporation, Mountain View, CA).

3. Results

3.1. Assay optimization

R(−)-Ibuprofen underwent metabolic chiral inversion in cryopreserved rat hepatocytes to the *S*(+) enantiomer and its formation was dependent on incubation time, cell density and substrate concentration (Figs. 1 and 2). In order to optimize the assay conditions, the effects of time linearity, cell density and substrate concentration on the enzymatic formation of *S*(+)-ibuprofen were investigated. When 0.5×10^6 cells/mL hepatocytes were incubated with 1 μM *R*(−)-ibuprofen for up to 3 h, the formation of *S*(+)-ibuprofen was found to be linear up to 60 min (Fig. 2a). Based on this finding, incubation time of 30 min was selected for the subsequent experiments. When hepatocytes were exposed to 1 μM *R*(−)-ibuprofen for 30 min at various cell densities, the formation of *S*(+)-ibuprofen increased linearly up to 0.5×10^6 cells/mL, but appeared to plateau thereafter (Fig. 2b). In view of these results, a cell density of 0.25×10^6 cells/mL was selected for the assay. Using this cell density, kinetics of inversion of (*R*)-ibuprofen to *S*(+)-ibuprofen was determined with a range of *R*(−)-ibuprofen concentrations (5–30 μM) using the cell density of 0.25×10^6 cells/mL and 30 min incubation time. The apparent K_m for this conversion was determined to be 10 μM. Therefore, a substrate concentration of 1 μM was chosen for all following IC_{50} determinations. The final assay conditions are shown in Table 2.

Table 2
Optimized assay incubation conditions for the assay

Incubation time	30 min
Rat hepatocyte cell density	0.25×10^6 cells/mL
Substrate (*R*-ibuprofen) concentration	1 μM

3.2. Concept validation with commercial compounds

Three commercially available compounds were chosen to validate the assay, of which two (ketoprofen and fenoprofen) belong to 2-aryl propionic acid derivatives and are known to undergo similar enzymatic chiral inversion pathways as that of ibuprofen and the third chiral compound (thalidomide) is known to undergo chiral inversion through different pathway which

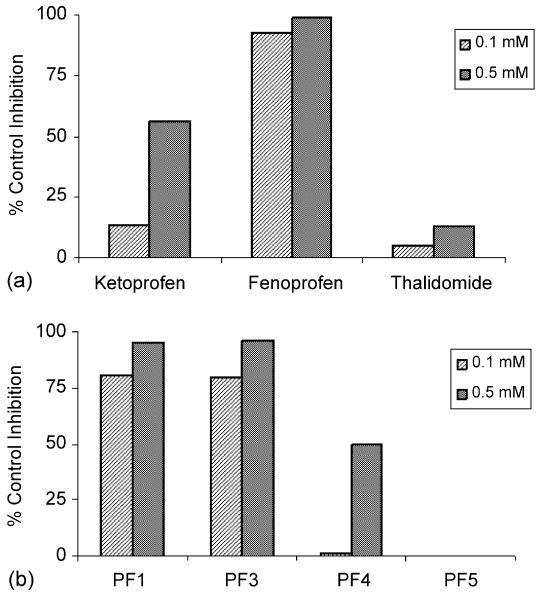
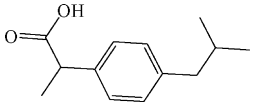
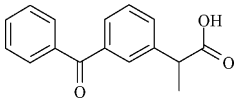
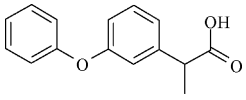
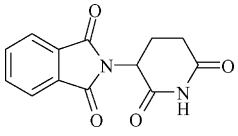
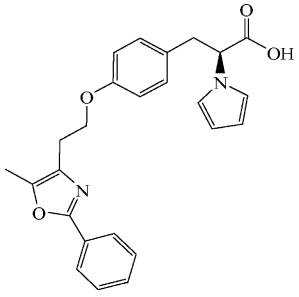
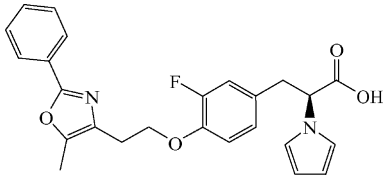
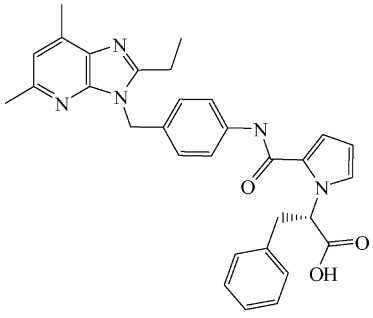
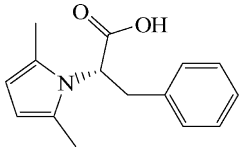


Fig. 3. Percent inhibition (relative to control *R*-ibuprofen to *S*-ibuprofen) when incubated with (a) model compounds and (b) Pfizer compounds at concentrations of 0.1 and 0.5 mM in cryopreserved rat hepatocytes. Data shown is the average of $n = 2$. Note: structures of the compounds in this figure are shown in Table 3.

serves as a negative control. As displayed in Fig. 3, ketoprofen and fenoprofen inhibited the *S*(+)-ibuprofen formation (56 and 99% at 0.5 mM, respectively), whereas, limited inhibition (12%) by thalidomide was observed. The data are consistent with IC_{50} values determined for ketoprofen and fenoprofen (89 and 2.2 μM, respectively), listed in Table 3.

Table 3
IC_{50} values of validation compounds for their ability to inhibit the formation of *S*-ibuprofen by cryopreserved rat hepatocytes using the validated assay conditions

Compound	Structure	IC_{50} (SE)
Ibuprofen		
Ketoprofen		89 μM (13.4)
Fenoprofen		2.2 μM (0.24)
Thalidomide		>500 μM
PF1		0.97 μM (0.47)
PF3		1.5 μM (1.16)
PF4		∼100 μM
PF5		No inhibition

3.3. Pfizer compounds

Three (PF1, PF3 and PF4) out of four Pfizer compounds significantly inhibited the inversion of (*R*)- to *S*(+)-ibuprofen (as demonstrated in Fig. 3b). No inhibition was observed with PF5. IC_{50} values of 1 and 1.5 μM were determined for PF1 and PF3, respectively (Table 3), show their similarity to fenoprofen in their potency to inhibit the ibuprofen inversion.

Additionally, 33 Pfizer compounds from a single chemical series were chosen to explore the ability of the assay to identify SAR for inversion. All of the compounds were initially screened in the HPLC based method using radiolabeled *R*(−)-ibuprofen at 0.1 and 0.5 mM compound concentrations for their potential to inhibit the *S*(+)-ibuprofen formation. Subsequently, compounds with >50% inhibition at 0.5 mM were tested in the LC/MS based method using a 10–12 point concentration range to determine the IC_{50} values (Table 5). Depending on the levels of inhibition seen in the initial HPLC based method, one of the following concentration ranges was used for the IC_{50} determination: (1) 0.01–10 μM; (2) 0.05–50 μM; (3) 0.001–2.0 mM.

4. Discussion

4.1. Concept validation

It is well documented that the enzymatic chiral inversion of ibuprofen is a three-step mechanism. Firstly, *R*-(−)-ibuprofen is conjugated to form an acyl-CoA thioester catalyzed by stereoselective acyl-CoA synthetase (CoA) which is a mitochondrial enzyme that has been purified from rat liver microsome preparations (Wechter, 1994). Secondly, the *R*-(−)-thioester is enzymatically epimerized to the *S*-(+)-thioester catalyzed by epimerase which is the best characterized of the three enzymes associated with the ibuprofen inversion (Bruggera et al., 2001). For epimerization to occur, C-2 of the ibuprofen must become planar via proton abstraction. Furthermore, although the epimerase is responsible for the inversion, Coenzyme A controls the overall extent and stereochemistry of the epimerization. Lastly, the *S*-(+)-thioester is hydrolyzed to the *S*-(+)-enantiomer enzymatically (Sanins et al., 1991). The exact identity of the hydrolase operative in the ibuprofen inversion is unknown.

To validate the concept of developing a chiral inversion inhibition assay using ibuprofen as the probe substrate, three commercially available compounds were initially chosen. Ketoprofen and fenoprofen belong to the family of 2-aryl propionic acid derivatives and are known to go through the same enzymatic chiral inversion pathway as ibuprofen. As expected, they inhibited the *S*(+)-ibuprofen formation indicating their interactions with one or more enzymes involved in the inversion of *R*-(−)-ibuprofen (Menzel et al., 1994). Consistent with literature reports, thalidomide, a chiral compound, which undergoes chiral inversion through a different pathway where its chiral inversion has been reported to be catalyzed by albumin, hydroxyl ions, phosphate and amino acids (Reist et al., 1998) did not inhibit ibuprofen inversion to a significant extent.

Table 4
In vivo inversion of PF1 and PF2: showing the chiral inversion predominantly from PF1 to its enantiomer PF2 with <0.3% from PF2 to PF1 in dogs

Species	Dose (mg/kg)	Route	AUC_{PF2}/AUC_{PF1} (% of parent)
Rat	5	PO	8.2
Dog	5	PO	154
Monkey	5	PO	29.6
Rat	5	IV	9.6
Dog	5	IV	115
Monkey	5	IV	30.2
Species	Dose (mg/kg)	Route	AUC_{PF1}/AUC_{PF2} (% of parent)
Dog	5	IV	<0.3

4.2. Assay application in early drug discovery

After concept validation using literature compounds, we sought to explore the applicability of the assay to drug discovery projects using Pfizer compounds. A compound series was chosen that contained a structurally similar chiral center to that of ibuprofen with an acidic proton. In a battery of *in vivo* studies, it was found that one of the lead compounds (PF1) in this series was predominantly converted to its enantiomer PF2, with the highest inversion in dogs and the reverse inversion being less than 0.3% (from PF2 to PF1) (Table 4). Consistent with the *in vivo* observations, PF1 was found to demonstrate time-dependent inversion in hepatocytes of different species (Fig. 4) and the highest inversion rate was detected in dog hepatocytes (similar to *in vivo* observation). While a rank order for *in vivo* inversion of PF1 to PF2 was discernible (rat < monkey < dog), the low rate of inversion in the *in vitro* hepatocyte system precluded a direct comparison of *in vivo* and *in vitro* data for this compound for the other species. The mechanism by which the compound underwent inversion was not currently known and we hypothesized that PF1 might use the same three step mechanism of ibuprofen inversion, simply because it is structurally similar at the chiral center to ibuprofen. Based on the hypothesis, several structurally similar analogs including PF1, PF3 and PF4 were chosen to evaluate whether they were able to inhibit the inversion of *R*(−)- to *S*(+)-ibuprofen in the *in vitro* hepatocyte system. Experimentally, PF1 had an IC_{50} of 1 μM against ibuprofen inversion indicating that PF1 was a potent inhibitor of ibuprofen inversion and that PF1 and ibuprofen likely share a common inversion mechanism, consistent with *in vitro* hepatocyte data (shown in Fig. 4). However, PF5 (Fig. 3), a compound that shares structural similarity with ibuprofen at the chiral center, showed no measurable inhibition towards ibuprofen inversion suggesting a potential SAR for this series. Subsequent to these initial studies, 33 additional compounds from this chemical series which share structural similarity with ibuprofen at the chiral center were selected and IC_{50} values were generated using the LC/MS based method for any compounds with >50% inhibition in the initial HPLC method to investigate the structure activity relationship. Based on the data from this set of compounds, two sites were identified that impact SAR around the potency to inhibit ibuprofen inversion (Table 5). For the first site (R1) on the aromatic group, replacement of hydrogen with bulkier groups such as methoxy reduced the potency to inhibit ibuprofen inversion by 2-fold and iodine or propyne caused 5-fold reduction and phenyl ring caused a 50-fold loss in potency (Table 5). At the second site (R2), replacement of hydrogen by a methyl group reduced the potency by 5-fold, whereas, a triflouromethyl replacement reduced the activity by 50-fold (Table 5). In the earlier case, the observed trend is probably due to steric hindrance of these substitutions when replaced with bulkier groups. Another plausible contributing factor is the electronegative inductive effect considering methoxyl being electron-donating whereas iodine, propyne or phenyl being electron-withdrawing. For the latter case the trend is likely due to combination of steric and electronegative inductive effects. The authors hypothesize that compounds with the acidic hydrogen replaced by other groups are not substrates for the ibuprofen inversion pathways, since they are lack of acidic hydrogen which is required for turnover (and conjugation) by acyl-CoA synthetase (to form thioester). However, as demonstrated by the data, they still had the potential to bind to acyl-CoA synthetase and therefore inhibit the overall inversion of ibuprofen.

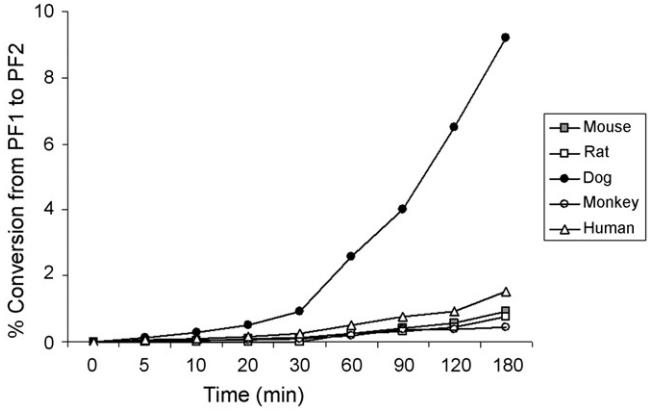
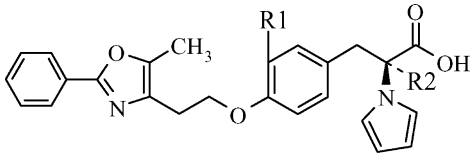


Fig. 4. *In vitro* chiral inversion of PF1 to PF2 in hepatocytes of different species.

Table 5
An example of SAR in a chemical series (based on their potencies to inhibit the inter-inversion of *R*(−)-ibuprofen to *S*(+)-ibuprofen) and the IC_{50} values



Compound	R1	R2	IC_{50} (μM)
PF6	H	H	2.04
PF7	O-CH3	H	4
PF8	I	H	9.1
PF9		H	9.9
PF10		H	100
PF6	H	H	2.04
PF11	H	CH_3	18.7
PF12	H	CF_3	100

As the recent advances in medicinal chemistry have led to an increasing number of chiral compounds in pharmaceutical libraries, the FDA has increased its expectations around mechanistic information for chiral compounds that reach the clinic. Analytical approaches require chemical standards for calibration, a method of detection, and a means to generate samples. Enantiomeric separation via chromatography requires significant investment in method development. Chemical synthesis for chiral investigation diverts resources from lead seeking. Optical rotation techniques can suffer from a lack of sensitivity and require very pure samples. In all cases, the analytical method must be tailored to each compound or compound class. Samples obtained from animal studies are more physiologically relevant, but provide limited throughput and are not consistent with the rapid turnaround and resource sparing nature of early drug discovery. *In vitro* and cell-based analyses (e.g. enzymes, isolated hepatocytes) may not be as predictive of clinical performance but can provide directive data earlier, balancing throughput and quality to justify cost/benefit and strategic considerations. For structurally similar compounds (assuming the identical inversion mechanism applies), an inhibition assay will allow single method development with a standard substrate. Data from such methods can be used to flag the compounds that have potential for chiral inversion. However, caution should be taken when interpreting such inhibition data. As previously mentioned, this *in vitro* tool is an interaction assay. As such it will provide information on whether the compounds of interest interact with the enzymes or pathways, which lead to the inversion of *R*-(+)-ibuprofen. It will not be able to distinguish whether compounds are substrates for the three enzymes. Further experiments will be needed in order to obtain such information (e.g. metabolite identification of the enantiomers of the compounds of interests).

5. Conclusions

An assay to identify the chiral inversion potential of compounds structurally similar to ibuprofen was developed using cryopreserved rat hepatocytes, *R*(−)-ibuprofen as substrate and chiral LC/MS methods for the chromatographic separation and detection of *R*(−) and *S*(+) enantiomers. Ketoprofen, fenoprofen, PF1, PF3 and PF4 inhibited the *S*(+)-ibuprofen formation supporting the concept behind the assay. The SAR for this assay was further explored using 33 compounds from one chemical series. The SAR for this series was found to be sensitive to substitutions both at the inversion site and at a site removed from the inversion site. Therefore, this assay can not only be used to identify the chiral inversion potential of compounds, but also can be used to examine and optimize the structure activity relationship in a class of compounds by recognizing the sites of chiral inversion sensitivity and potential structural remedies for chemistry modification. In conclusion, concept validation of the chiral inversion inhibition assay with the commercial compounds as well as Pfizer compounds was successfully completed and this assay provides a tool to assess the risk of chiral inversion for NCEs that are structurally similar to ibuprofen in the early stages of drug discovery.

Acknowledgements

The authors would like to acknowledge Dr. Joanne Brodfuehrer, Dr. Christopher Lepsy, Dr. Cho-Ming Loi, Kelly Rose, John Hollembaek (Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Global Research and Development) for providing us the *in vivo* and *in vitro* information on chiral inversion of PF1.

References

Baillie, T.A., Adams, W.J., Kaiser, D.G., Olanoff, L.S., Halstead, G.W., Harpootlian, H., Van Giessen, G.J., 1989. Mechanistic studies of the metabolic chiral inversion of (*R*)-ibuprofen in humans. J. Pharmacol. Exp. Ther. 249, 517–523.

Bruggera, R., Reichel, C., Garcia Alia, B., Brune, K., Yamamoto, T., Tegeder, I., Geisslinger, G., 2001. Expression of rat liver long-chain acyl-CoA synthetase and characterization of its role in the metabolism of *R*-ibuprofen and other fatty acid-like xenobiotics. Biochem. Pharmacol. 61, 651–656.

Drayer, D.E., 1986. Pharmacodynamic and pharmacokinetic differences between drug enantiomers in humans: an overview. Clin. Pharmacol. Ther. 40, 125–133.

Hubbard, J.W., Ganes, D., Lim, H.K., Midha, K.K., 1986. Chiral pharmacology and its consequences for therapeutic monitoring. Clin. Biochem. 19, 107–112.

Hutt, A.J., O'Grady, J., 1996. Drug chirality: a consideration of the significance of the stereochemistry of antimicrobial agents. J. Antimicrob. Chemother. 37, 7–32.

Jamali, F., 1988. Pharmacokinetics of enantiomers of chiral non-steroidal anti-inflammatory drugs. Eur. J. Drug Metab. Pharmacokinet. 13, 1–9.

Kaiser, D.G., Vangiessen, G.J., Reischer, R.J., Wechter, W.J., 1976. Isomeric inversion of ibuprofen (*R*)-enantiomer in humans. J. Pharm. Sci. 65, 269–273.

Knihinicki, R.D., Day, R.O., Williams, K.M., 1991. Chiral inversion of 2-arylpropionic acid non-steroidal anti-inflammatory drugs—II. Racemization and hydrolysis of (*R*)- and (*S*)-ibuprofen-CoA thioesters. Biochem. Pharmacol. 42, 1905–1911.

Landoni, M.F., Soraci, A., 2001. Pharmacology of chiral compounds: 2-arylpropionic acid derivatives. Curr. Drug Metab. 2, 37–51.

Menzel, S., Sauernheimer, C., Brune, K., Geisslinger, G., 1994. Is the inversion from *R*- to *S*-ketoprofen concentration dependent? Investigations in rats in vivo and in vitro. Biochem. Pharmacol. 47, 1267–1270.

Muller, N., Payan, E., Lapicque, F., Bannwarth, B., Netter, P., 1990. Pharmacological aspects of chiral nonsteroidal anti-inflammatory drugs. Fundam. Clin. Pharmacol. 4, 617–634.

Reist, M., Carrupt, P.A., Francotte, E., Testa, B., 1998. Chiral inversion and hydrolysis of thalidomide: mechanisms and catalysis by bases and serum albumin, and chiral stability of teratogenic metabolites. Chem. Res. Toxicol. 11, 1521–1528.

Sanins, S.M., Adams, W.J., Kaiser, D.G., Halstead, G.W., Baillie, T.A., 1990. Studies on the metabolism and chiral inversion of ibuprofen in isolated rat hepatocytes. Drug Metab. Dispos. 18, 527–533.

Sanins, S.M., Adams, W.J., Kaiser, D.G., Halstead, G.W., Hosley, J., Barnes, H., Baillie, T.A., 1991. Mechanistic studies on the metabolic chiral inversion of *R*-ibuprofen in the rat. Drug Metab. Dispos. 19, 405–410.

Tanner, M.E., 2002. Understanding nature's strategies for enzyme-catalyzed racemization and epimerization. Acc. Chem. Res. 35, 237–246.

Wechter, W.J., 1994. Drug chirality: on the mechanism of *R*-aryl propionic acid class NSAIDs. Epimerization in humans and the clinical implications for the use of racemates. J. Clin. Pharmacol. 34, 1036–1042.

Wsol, V., Skalova, L., Szotakova, B., 2004. Chiral inversion of drugs: coincidence or principle? Curr. Drug Metab. 5, 517–533.

Xiaotao, Q., Hall, S.D., 1993. Modulation of enantioselective metabolism and inversion of ibuprofen by xenobiotics in isolated rat hepatocytes. J. Pharmacol. Exp. Ther. 266, 845–851.