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The stereospecificity of flobufen metabolism in isolated guinea pig hepatocytes

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Abstract

Background: Flobufen (F) is an original nonsteroidal anti-inflammatory drug with one center of chirality. 4-Dihydroflobufen (DHF), compound with two chiral centers, is the main metabolite of F in microsomes and cytosol in all standard laboratory animals. This work describes the biotransformation of F enantiomers and DHF stereoisomers in isolated male guinea pig hepatocytes. Guinea pigs were chosen with respect to similarities in F metabolism as in Man found earlier. R-F, S-F, (2R;4S)-DHF, (2S;4R)-DHF, (2S;4S)-DHF and (2R;4R)-DHF, structurally very similar compounds, served as substrates in order to observe their interaction with enzymes. Stereospecificity of the respective enzymes was studied *in vitro*, using hepatocytes monolayer. Chiral HPLC using R,R-ULMO column as chiral stationary phase was used for detection and quantitation of metabolites.

Results: (2R;4S)-DHF and (2S;4S)-DHF were the principle stereoisomers detected after incubation with *rac*-F, R-F and S-F. The ratio of (2R;4S)-DHF/(2S;4S)-DHF ranged from 1.1 to 2.4 depending on the substrate used. (2R;4S)-DHF was the major stereoisomer found after incubation with (2S;4S)-DHF and (2R;4R)-DHF. (2S;4S)-DHF was the principle stereoisomer found after incubation with (2R;4S)-DHF and (2S;4R)-DHF. Besides DHF stereoisomers, other metabolites (M-17203, UM-1 and UM-2) were also detected after incubation of hepatocytes monolayer with F. Interestingly, these metabolites were not found in incubation of all F forms and DHF with fresh liver homogenate.

Conclusions: Different activities and stereospecificities of the respective enzymes were observed for each substrate in primary culture of hepatocytes. Cell integrity is crucial for formation of secondary metabolites M-17203, UM-1 and UM-2.

Background

Anti-inflammatory drugs are currently the most widely used among pharmaceutical drugs. Nonsteroidal anti-inflammatory drugs (NSAIDs) form a significant part of this group. Their therapeutic effects are accompanied by a series of adverse effects [1]. NSAIDs in clinical use (e.g. ibuprofen, diclofenac, ketoprofen) or compounds for potential use still undergo investigation of their biotransformation [2,3]. Searching for metabolites and observation of their further fate in the organism lead to a detailed description of their metabolic pathways in order to better understand their desired and adverse effects. One of these NSAIDs is flobufen, 4-(2',4'-difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid (F). F is one of the outcomes of the study of the relationship between arylalkanoic acids and living organisms towards the end of the 1980's [4,5]. F, together with fenbufen [6], belongs to the group of aryloxobutyric acids, which are structurally related to arylpropionic acids (e.g.: ibuprofen, flurbiprofen, ketoprofen) [7]. F mechanisms of action and biological activities have already been reported [8,9].

Metabolism of F was already tested in different species [10]. In the *in vitro* experiments conducted on rats, mice, guinea pigs, mini-pigs, rabbits and dogs, 4-dihydroflobufen, 4-(2',4'-difluorobiphenyl-4-yl)-2-methyl-4-hydroxybutanoic acid (DHF) was discovered to be the main metabolite [9,10]. In addition to DHF, another metabolite, 2-(2',4'-difluorobiphenyl-4-yl)-acetic acid (M-17203), was found in isolated hepatocytes [9], in urine and in faeces of rats [9,10]. DHF seems to be transformed to its secondary metabolite M-17203. The biological activities of DHF and M-17203 have already been reported [11,12].

F and DHF are chiral compounds with one and two asymmetric carbons, respectively. Our first study was focused on the biotransformation of F in rats [9]. Unfortunately, preliminary *in vivo* experiments in Man (unpublished data) revealed differences in F metabolites excreted by rat and Man. The following preliminary *in vivo* experiments revealed that the guinea pig is the most convenient and the nearest species for description of F metabolism in Man.

Our last study described chiral metabolism of F in guinea pig [manuscript submitted for publication]. Investigation of F biotransformation *in vitro* (microsomes and cytosol) showed DHF stereoisomers as the only metabolites in these two subcellular fractions. *In vivo* experiments revealed the formation of several other metabolites: M-17203, UM-1 and UM-2. These results indicate that these metabolites are formed in some other liver cell compartment, in intact liver cell or in extrahepatic tissue.

This work reports about the investigation of F metabolism in primary culture of hepatocytes, because it represents a more comprehensive experimental system for evaluation of drug metabolism [13]. Fresh liver homogenate and primary culture of hepatocytes were compared in order to prove the key role of cell integrity in the formation of M-17203. Primary culture of hepatocytes was also used in order to determine stereospecificity of DHF stereoisomer formation using individual enantiomers of F as substrates and mutual chiral inversion among DHF stereoisomers.

Results

Primary culture of hepatocytes

Incubation with rac*-F*

Primary cultures of hepatocytes were incubated with *rac*-F in five concentrations (25, 50, 75, 100, 200 μM). All four DHF stereoisomers and M-17203 were detected. Their clearances are summarized in Table 1. The production of all DHF stereoisomers culminated between 2 and 4h of incubation. The ratio of the most produced stereoisomers, (2R;4S)-DHF/(2S;4S)-DHF, did not change strongly and ranged from 1.3 to 1.9. Formation of M-17203 increased several times from 8 to 24 h of incubation and the shape of the curve (production of M-17203 vs. time) predicted growing production after 24 h. In addition to DHF stereoisomers and M-17203, two other unknown metabolites, marked as UM-1 and UM-2, were detected. UM-2 was detected already after 2 h of incubation while UM-1 was detected only after 24 h of incubation. UM-1 and UM-2 production is summarized in Table 2.

Incubation with R-F

R-F, in the same concentrations as *rac*-F, was incubated with hepatocytes. All four DHF stereoisomers and M-17203 were detected. The production of all DHF stereoisomers culminated again between 2 and 4 h of incubation. Formation of M-17203 had the same characteristic increase from 8 to 24 h of incubation as in incubation with *rac*-F (see Table 1). M-17203 was produced to a lesser extent than after incubation with *rac*-F. (2R;4S)-DHF was the most produced stereoisomer, as expected. Significant amounts of (2S;4S)-DHF, which is the direct product of S-F, were also detected. The ratio of (2R;4S)-DHF/(2S;4S)-DHF ranged from 1.5 to 4.1. UM-1 and UM-2 were formed in lesser amounts than after incubation with *rac*-F. Results are summarized in Table 3.

Incubation with S-F

S-F, in the same range of concentrations as *rac*-F and R-F, was incubated with hepatocytes. All four DHF stereoisomers and M-17203 were detected. The maximum of DHF stereoisomer formation was between 2–4 h, and retarded formation of M-17203 displayed the same tendency as for incubation with *rac*-F and R-F (see Table 1). M-17203 was produced to a greater extent than after

Table 1: Biotransformation of *rac*-F, R-F and S-F in isolated guinea pig hepatocytes.

Substrate	Incubation time [h]	Apparent actual clearance [$h^{-1} \times 10^{-3}$]				
		M-17203	(2R;4S)-**DHF**	(2S;4R)-**DHF**	(2S;4S)-**DHF**	(2R;4R)-**DHF**
rac-**F**	2	1.8 ± 0.7	111.2 ± 8.6	10.8 ± 0.1	57.5 ± 5.0	2.3 ± 0.0
	4	1.4 ± 0.2	67.7 ± 0.5	7.1 ± 0.4	42.4 ± 0.6	0.8 ± 0.1
	8	1.6 ± 0.1	22.8 ± 1.3	1.6 ± 0.2	12.8 ± 1.0	0.1 ± 0.0
	24	3.8 ± 0.8	4.2 ± 0.2	0.3 ± 0.0	3.6 ± 0.1	-
R-**F**	2	1.6 ± 0.5	147.1 ± 11.8	1.6 ± 0.4	38.0 ± 3.8	6.3 ± 0.5
	4	1.2 ± 0.1	91.1 ± 2.7	3.4 ± 0.1	33.6 ± 1.4	2.0 ± 0.1
	8	1.4 ± 0.1	32.3 ± 0.8	1.0 ± 0.8	12.9 ± 0.1	0.2 ± 0.0
	24	2.9 ± 0.0	7.6 ± 1.4	0.2 ± 0.0	4.5 ± 0.5	-
S-**F**	2	2.9 ± 0.2	58.1 ± 3.2	13.3 ± 0.7	56.9 ± 2.6	-
	4	2.4 ± 0.3	47.3 ± 1.5	10.2 ± 1.0	44.6 ± 2.1	0.5 ± 0.3
	8	2.0 ± 0.1	20.0 ± 0.4	3.7 ± 0.1	15.8 ± 0.2	0.1 ± 0.0
	24	3.0 ± 0.2	4.3 ± 0.5	0.6 ± 0.0	4.9 ± 0.4	0.4 ± 0.1

Apparent actual clearance (Cl_{aa}) of **F** metabolites in the isolated hepatocytes after incubation with 100 μM *rac*-**F**, R-**F** and S-**F** as substrates. Cl_{aa} is expressed as the ratio of actual velocity (mol/l/h) and substrate concentration (mol/l), (mean value ± S.D., n = 4).

incubation with *rac*-F or R-F. Interestingly (2R;4S)-DHF remained the most produced stereoisomer. (2S;4S)-DHF was produced in smaller yields than after incubation with *rac*-F and R-F, within 8 h of incubation. Its concentration after 24 h of incubation remained at the same level as after 8 h of incubation, while the concentrations of other DHF stereoisomers decreased. The ratio of (2R;4S)-DHF/(2S;4S)-DHF ranged from 1.0 to 1.5. The formation of UM-1 and UM-2 in time was the same as after incubation with *rac*-F and R-F, but their concentrations were higher compared to the previous incubations. Results of their production are summarized in Table 4.

Incubation with (2R;4S)-DHF

Primary cultures of hepatocytes were incubated with (2R;4S)-DHF in concentrations 50 and 100 μM. The maximum of DHF stereoisomer formation was between 2 h and 4 h of incubation. Mild growth of M-17203 appeared within 8 h of incubation and it increased several times between 8 h and 24 h of incubation. Results are summarized in Table 5. Chiral inversion at carbon 2 and significant production of (2S;4S)-DHF were found in hepatocytes after incubation with (2R;4S)-DHF. While its concentration decreased between 4 h and 8 h, it started to grow again from 8 h until 24 h. UM-1 was detected only after 24 h incubation while UM-2 was detected again after 2 h of incubation. Growing production of UM-2 was observed within the whole 24 h incubation period (Table 6). UM-2 was found as the principle metabolite after 24 h incubation.

Incubation with (2S;4R)-DHF

Primary cultures of hepatocytes were incubated with (2S;4R)-DHF in concentrations 50 and 100 μM. The characteristics of formed DHF stereoisomers and time course of M-17203 formation were very similar to results obtained from the previous incubation with (2R;4S)-DHF, and they are summarized in Table 5. Significant production of (2S;4S)-DHF and (2R;4S)-DHF revealed chiral inversion at both carbons 2 and 4. UM-1 was only detected after 24h of incubation (Table 6). UM-2 remained the principle metabolite and it was even produced to a larger extent than after incubation with (2R;4S)-DHF.

Incubation with (2S;4S)-DHF

Primary cultures of hepatocytes were incubated with (2S;4S)-DHF in concentrations 50 and 100 μM. Concentration and time characteristics of DHF stereoisomers and M-17203 formation were very similar to the results obtained from the previous incubations with DHF stereoisomers. They are summarized in Table 5. A significant amount of (2R;4S)-DHF was produced, due to chiral inversion at carbon 2. This inversion at carbon 2 (S to R) proceeded to a higher extent within 24 h compared to reverse inversion (R to S) after incubation with (2R;4S)-DHF. UM-1 was only detected after 24 h incubation

Table 2: UM-1 and UM-2 production after incubation with *rac*-F.

	peak area (AU × s × 10^4)			
	incubation time (h)			
	2	4	8	24
		25 μM		
UM-1	-	-	-	5.1 ± 0.1
UM-2	23.6 ± 1.1	46.8 ± 2.4	66.8 ± 1.2	120.0 ± 1.2
		50 μM		
UM-1	-	-	-	17.3 ± 1.1
UM-2	27.9 ± 0.4	67.3 ± 2.6	127.1 ± 6.7	185.2 ± 4.1
		75 μM		
UM-1	-	-	-	24.8 ± 4.0
UM-2	29.8 ± 1.7	79.3 ± 3.4	164.1 ± 10.2	250.0 ± 27.0
		100 μM		
UM-1	-	-	-	41.9 ± 10.3
UM-2	30.9 ± 3.6	91.8 ± 2.7	206.8 ± 13.2	331.5 ± 26.2
		200 μM		
UM-1	-	-	-	42.6 ± 2.3
UM-2	23.4 ± 2.0	102.9 ± 6.1	277.2 ± 12.1	521.3 ± 34.1

UM-1 and **UM-2** production in isolated guinea pigs hepatocytes after incubation with *rac*-**F** (mean value ± S.D., n = 4).

Table 3: UM-1 and UM-2 production after incubation with R-F.

	peak area (AU × s × 10^4)			
	incubation time (h)			
	2	4	8	24
		25 μM		
UM-1	-	-	-	10.0 ± 0.6
UM-2	18.4 ± 2.1	39.5 ± 2.7	55.2 ± 3.3	111.4 ± 14.3
		50 μM		
UM-1	-	-	-	17.4 ± 2.3
UM-2	23.8 ± 1.9	56.3 ± 2.7	100.7 ± 2.2	172.9 ± 0.6
		75 μM		
UM-1	-	-	-	24.2 ± 3.3
UM-2	22.0 ± 0.5	57.6 ± 4.2	129.5 ± 1.6	234.1 ± 5.0
		100 μM		
UM-1	-	-	-	36.2 ± 5.6
UM-2	19.3 ± 1.3	61.7 ± 3.1	144.8 ± 7.9	263.7 ± 2.5
		200 μM		
UM-1	-	-	-	47.6 ± 4.6
UM-2	16.1 ± 2.1	60.4 ± 2.9	178.3 ± 9.2	398.2 ± 17.2

UM-1 and **UM-2** production in isolated guinea pigs hepatocytes after incubation with R-**F** (mean value ± S.D., n = 4).

(Table 6). UM-2 remained the principle metabolite and it was even produced to a larger extent than after the incubations with (2R;4S)-DHF and (2S;4R)-DHF.

Table 4: UM-1 and UM-2 production after incubation with S-F.

	peak area (AU × s × 10^4)			
	incubation time (h)			
	2	4	8	24
		25 μM		
UM-1	-	-	-	11.8 ± 0.6
UM-2	20.5 ± 1.3	48.8 ± 4.3	58.2 ± 3.8	135.6 ± 7.8
		50 μM		
UM-1	-	-	-	23.8 ± 1.5
UM-2	30.5 ± 2.5	68.7 ± 4.4	119.7 ± 9.3	209.5 ± 10.3
		75 μM		
UM-1	-	-	-	22.7 ± 3.6
UM-2	33.8 ± 1.6	78.1 ± 4.0	156.3 ± 6.3	270.3 ± 0.5
		100 μM		
UM-1	-	-	-	20.3 ± 12.6
UM-2	35.1 ± 4.5	89.6 ± 0.7	188.1 ± 3.5	333.8 ± 7.8
		200 μM		
UM-1	-	-	-	54.3 ± 0.5
UM-2	45.2 ± 6.6	116.2 ± 0.1	298.8 ± 0.5	549.6 ± 22.5

UM-1 and **UM-2** production in isolated guinea pigs hepatocytes after incubation with S-**F** (mean value ± S.D., n = 4).

Incubation with (2R;4R)-DHF

Primary cultures of hepatocytes were incubated with (2R;4R)-DHF in concentrations 50 and 100 μM. Clearances of DHF stereoisomers and M-17203 production were very similar to all three previous cases. Results are summarized in Table 5. Intensive chiral inversion at carbon 4 led to significant production of (2R;4S)-DHF. Chiral inversion at this carbon seems to be almost unidirectional. UM-1 was detected only after 24 h of incubation (Table 6). UM-2 remained the major metabolite even when its production was the lowest compared to all three previous incubations with DHF stereoisomers.

Liver homogenate

Fresh liver homogenate from guinea pig was incubated with *rac*-F (0.5 and 1.0 mM) and with *rac*-DHF (0.12 and 0.24 mM). Individual DHF stereoisomers and F were detected and quantified. The results are summarized in Table 7. DHF stereoisomers and F were the only structures detected after incubations with *rac*-F or *rac*-DHF. No amounts of M-17203, UM-1 and UM-2 were detected either in incubations with *rac*-F or *rac*-DHF.

Discussion

Investigation of F metabolism in isolated guinea pig hepatocytes and in fresh liver homogenate, improved our knowledge of its fate in this species, which is related to F metabolism in Man. Description of the formation of principle metabolites could be useful for determination of the stereospecifities of the respective enzymes. Knowledge

Table 5: Biotransformation of individual DHF stereoisomers in isolated guinea pig hepatocytes

Substrate	Incubation time [h]	Apparent actual clearance [$h^{-1} \times 10^{-3}$]				
		M-17203	(2R;4S)-**DHF**	(2S;4R)-**DHF**	(2S;4S)-**DHF**	(2R;4R)-**DHF**
(2R;4S)-**DHF**	2	1.0 ± 0.2	218.2 ± 0.5	2.6 ± 0.0	21.3 ± 0.6	1.5 ± 0.4
	4	1.2 ± 0.1	93.7 ± 1.2	1.6 ± 0.1	19.5 ± 0.5	2.3 ± 0.1
	8	1.1 ± 0.1	24.2 ± 2.0	0.8 ± 0.0	6.7 ± 0.0	0.2 ± 0.0
	24	3.1 ± 0.1	6.9 ± 1.4	0.2 ± 0.0	3.0 ± 0.4	0.1 ± 0.0
(2S;4R)-**DHF**	2	1.7 ± 0.0	16.7 ± 0.4	115.6 ± 1.8	36.8 ± 1.3	1.8 ± 0.3
	4	1.9 ± 0.1	20.1 ± 0.4	47.1 ± 1.1	34.7 ± 0.4	1.4 ± 0.3
	8	1.7 ± 0.0	9.6 ± 0.1	7.7 ± 0.5	14.3 ± 0.1	0.1 ± 0.0
	24	3.2 ± 0.2	2.1 ± 0.6	1.2 ± 0.5	6.0 ± 0.5	—
(2S;4S)-**DHF**	2	2.1 ± 0.2	35.1 ± 1.7	5.2 ± 0.4	179.2 ± 6.0	—
	4	2.1 ± 0.0	26.2 ± 0.3	4.3 ± 0.0	54.8 ± 1.2	0.2 ± 0.0
	8	1.3 ± 0.1	9.0 ± 0.5	2.1 ± 0.3	12.7 ± 0.8	—
	24	3.8 ± 0.1	2.2 ± 0.5	0.1 ± 0.0	5.0 ± 0.6	—
(2R;4R)-**DHF**	2	—	122.4 ± 3.5	19.6 ± 0.5	12.3 ± 0.3	84.6 ± 6.4
	4	0.8 ± 0.0	71.6 ± 1.6	11.7 ± 0.4	15.1 ± 0.1	8.0 ± 0.7
	8	1.4 ± 0.1	21.5 ± 1.3	2.2 ± 0.1	8.2 ± 0.3	0.4 ± 0.0
	24	3.3 ± 0.3	3.8 ± 0.3	0.3 ± 0.0	2.7 ± 0.1	—

Apparent actual clearance (Cl_{aa}) of DHF metabolites in the isolated hepatocytes after incubation with 100 μM individual **DHF** isomers as substrates. Cl_{aa} is expressed as the ratio of actual velocity (mol/l/h) and substrate concentration (mol/l), (mean value ± S.D., n = 4).

from these experiments could also contribute to better understanding of the relationship between mutual inversions among individual DHF stereoisomers.

Metabolism of F in isolated hepatocytes

The concentration range of substrates with respect to their solubility and toxicity was the limiting factor for designed experiments. The dependence of metabolite production on proposed substrate concentrations did not show saturation characteristics, therefore, no kinetic constants; such as apparent Michaelis constant, apparent maximal velocity and apparent intrinsic clearance could be calculated. Metabolism of F in primary cultures of guinea pig hepatocytes differed qualitatively from that in microsomes and cytosol. In addition to DHF stereoisomers, three other metabolites, M-17203, UM-1 and UM-2, were found (Table 2,3,4). Exact chemical structure of UM-1 and UM-2 has not been defined yet, but their UV-spectral properties confirm their relation to structures close to M-17203 and DHF (thanks to delocalisation of π electrons on C_4 in the side chain of the structure) (Fig. 1). It is thought that this metabolite could be a product of second phase biotransformation (a conjugate) or a compound like M-17203 and DHF but with the side carbon chain substituted in another way. Metabolism of the structurally related compound ibuprofen adverts to hydroxylation of the side chain [2,14]. The structure could also be derived from fenbufen metabolites found in Man [6] because fenbufen is structurally the most related compound to F.

Our previous work with isolated microsomes and cytosol showed that (2R;4S)-DHF and (2R;4R)-DHF originated strictly from R-F and (2S;4R)-DHF with (2S;4S)-DHF originated only from S-F [manuscript submitted for publication].

This rule was also confirmed in this work, in the incubation with *rac*-F. (2R;4S)-DHF and (2S;4S)-DHF were determined as the main DHF stereoisomers originating from R-F and S-F, respectively. The amounts of the other two stereoisomers were negligible. This shows that reducing enzymes form stereospecificaly DHF stereoisomers with S configuration at carbon 4. All four DHF stereoisomers had the same characteristics: their production culminated between 2 h and 4 h and afterwards their concentrations decreased probably due to their following biotransformation.

Table 6: UM-1 and UM-2 production after incubation with individual DHF stereoisomers.

	peak area (AU × s × 10^4)			
	incubation time (h)			
	2	4	8	24
	substrate (2R;4S)-**DHF** 50 μM			
UM-1	-	-	-	18.0 ± 0.5
UM-2	8.7 ± 0.6	41.4 ± 3.7	72.1 ± 4.0	167.7 ± 8.0
	substrate (2R;4S)-**DHF** 100 μM			
UM-1	-	-	-	32.3 ± 0.2
UM-2	10.4 ± 0.1	48.2 ± 1.5	102.7 ± 6.1	228.6 ± 4.2
	substrate (2S;4R)-**DHF** 50 μM			
UM-1	-	-	-	31.1 ± 4.2
UM-2	10.0 ± 1.1	48.9 ± 5.0	95.4 ± 3.5	196.1 ± 11.7
	substrate (2S;4R)-**DHF** 100 μM			
UM-1	-	-	-	37.7 ± 3.3
UM-2	69.0 ± 0.5	43.0 ± 1.1	133.1 ± 7.3	237.5 ± 5.8
	substrate (2S;4S)-**DHF** 50 μM			
UM-1	-	-	-	18.9 ± 2.8
UM-2	71.7 ± 2.4	140.7 ± 5.5	171.3 ± 2.2	248.6 ± 15.8
	substrate (2S;4S)-**DHF** 100 μM			
UM-1	-	-	-	39.3 ± 2.2
UM-2	119.1 ± 7.5	228.1 ± 7.8	299.7 ± 18.7	401.5 ± 9.1
	substrate (2R;4R)-**DHF** 50 μM			
UM-1	-	-	-	21.9 ± 1.8
UM-2	11.2 ± 0.1	17.9 ± 0.1	69.7 ± 3.7	143.6 ± 8.3
	substrate (2R;4R)-**DHF** 100 μM			
UM-1	-	-	-	33.9 ± 3.3
UM-2	11.3 ± 8.1	28.3 ± 1.0	99.9 ± 4.4	193.4 ± 7.2

UM-1 and **UM-2** production in isolated guinea pigs hepatocytes after incubation with individual **DHF** stereoisomers (mean value ± S.D., n = 4).

Incubations with individual enantiomers of F should confirm the stereospecific formation of the respective DHF stereoisomers. Increment of (2R;4S)-DHF formation occurred as expected but also a considerable amount of (2S;4S)-DHF was formed. Due to this result, one assumption arose: inversion of R-F to S-F or inversion of (2R;4S)-DHF to (2S;4S)-DHF, or both cases together, could increase the final amount of (2S;4S)-DHF. The inversion of R-F to S-F has been already described in rats [15] and in ruminants [16]. This type of unidirectional inversion of R enantiomers of arylpropionic acids to Senantiomers has been reported many times [17–24]. The main enzyme responsible for this chiral inversion, 2-arylpropionyl-CoA epimerase, was even isolated and characterized in rats [25]. Long chain acyl-CoA synthetase seems to be the key enzyme at the beginning of the inversion process and it catalyses the formation of 2-arylpropionyl-CoA [26].

More interesting results were obtained in incubation with S-F. (2R;4S)-DHF remained the most produced DHF stereoisomer within 8 h of incubation. Inversion of S-F to R-F or inversion of (2S;4S)-DHF to (2R;4S)-DHF or combination of both of them must be taken into account. Inversion of S-F to R-F has already been suspected in rats [15]. Bi-directional inversions of ibuprofen and ketoprofen have already been described in both guinea pigs [27] and mice [28].

Inversions among DHF stereoisomers and substrate preference in the production of M-17203, UM-1 and UM-2 became clearer using individual DHF stereoisomers as substrates (Table 5, 6).

Incubation of isolated hepatocytes with (2R;4S)-DHF showed that it is inverted predominantly to (2S;4S)-DHF. This could easily explain the strong increase of (2S;4S)-DHF concentration after incubation with R-F.

Incubation with (2S;4R)-DHF showed that the main DHF stereoisomer, (2S;4S)-DHF, is produced almost two times more than the second one, (2R;4S)-DHF. The formed (2S;4S)-DHF probably served as substrate for the following enzymatic transformation to (2R;4S)-DHF, but fast inversion at carbon 4 (S to R) and slower inversion at carbon 2 (Sto R) explains why (2S;4S)-DHF is the most produced DHF stereoisomer during 24 h incubation.

Incubation with (2S;4S)-DHF proved that it is mainly inverted to (2R;4S)-DHF. With respect to the ways of inversion mentioned above, the equilibrium between (2R;4S)-DHF and (2S;4S)-DHF arises. This equilibrium is moved in the direction of (2R;4S)-DHF formation. Considering these facts, it can be explained why (2R;4S)-DHF is the principle DHF stereoisomer produced during incubation with S-F.

Incubation with (2R;4R)-DHF showed that it is transformed predominantly to (2R;4S)-DHF. Therefore, it can be reasonably explained, why negligible concentration of (2R;4S)-DHF was found after incubation of *rac*-F, R-F, S-F, (2R;4S)-DHF, (2S;4R)-DHF and (2S;4R)-DHF. (2R;4R)-DHF is easily transformed to (2R;4S)-DHF. The concentration of (2R;4S)-DHF was 6 times higher after incubation with (2R;4R)-DHF than other DHF stereoisomers at the maximum of their production.

Taking all these results into account, we can conclude that DHF stereoisomers are stereoselectively utilized by the respective enzymes. Investigation of chiral inversion among individual DHF stereoisomers showed that (2R;4R)-DHF is the most convenient substrate for the enzymes.

M-17203 was selectively formed from S-F. M-17203 was produced two times higher than from R-F (Table 1). Incubations with individual DHF stereoisomers confirm

Table 7: Biotransformation of *rac*-F and *rac*-DHF in fresh liver homogenate.

incubation time (h)	activity nmol/mg protein					
	M-17203	(2R;4S)-**DHF**	(2S;4R)-**DHF**	(2S;4S)-**DHF**	(2R;4R)-**DHF**	**F**
			substrate *rac*-**F** 0.5mM			
1	-	11.1 ± 0.1	1.6 ± 0.1	3.9 ± 0.1	9.1 ± 0.2	89.2 ± 1.2
2	-	14.8 ± 0.1	3.2 ± 0.1	7.0 ± 0.1	16.0 ± 0.4	81.2 ± 1.2
4	-	18.3 ± 1.0	5.1 ± 0.3	7.7 ± 0.0	18.1 ± 0.4	69.5 ± 2.3
			substrate *rac*-**F** 1.0 mM			
1	-	14.2 ± 0.6	1.2 ± 0.0	4.5 ± 0.3	6.8 ± 0.3	138.4 ± 27.6
2	-	22.3 ± 0.6	2.9 ± 0.1	9.4 ± 0.3	15.1 ± 0.4	108.4 ± 5.8
4	-	30.7 ± 0.8	6.0 ± 0.2	10.5 ± 0.4	20.3 ± 0.5	106.4 ± 4.1
			substrate *rac*-**DHF** 0.12 mM			
1	-	5.4 ± 0.1	5.1 ± 0.1	5.7 ± 0.1	4.9 ± 0.1	0.3 ± 0.0
2	-	5.3 ± 0.2	5.5 ± 0.2	5.9 ± 0.3	5.1 ± 0.4	0.5 ± 0.1
4	-	5.4 ± 0.0	5.4 ± 0.0	4.7 ± 0.1	4.0 ± 0.0	1.1 ± 0.0
			substrate *rac*-**DHF** 0.24 mM			
1	-	12.2 ± 0.3	11.7 ± 0.3	12.5 ± 0.3	11.5 ± 0.3	0.6 ± 0.0
2	-	11.9 ± 0.2	11.9 ± 0.2	12.8 ± 0.2	11.8 ± 0.2	0.7 ± 0.1
4	-	12.1 ± 0.5	11.9 ± 0.5	11.4 ± 0.5	10.4 ± 0.5	2.0 ± 0.1

Activity of the respective enzymes in guinea pigs fresh liver homogenate after incubation with *rac*-**F** and *rac*-**DHF** (mean value ± S.D., n = 4).

the substrate dependence production of M-17203. We observed that DHF stereoisomers formed from S-F were stereoselectively chosen for M-17203 formation. (2S;4S)-DHF led to formation of M-17203 to the largest extent. Incubations with the other 3 DHF stereoisomers showed significantly lower production of M-17203 (Table 5).

Formation of UM-1 and UM-2 can be explained by a similar way. Productions of UM-1 and UM-2 were significantly higher after incubations with *rac*-F and S-F compared to R-F (Table 2,3,4). The stereoselectivity was also observed after incubation with individual DHF stereoisomers (Table 6). UM-1 and UM-2 were predominantly formed from (2S;4S)-DHF and (2S;4R)-DHF, which are stereoisomers originated from S-F. All enzymes producing UM-1, UM-2 and M-17203 prefered S-F as substrate.

Liver homogenate

Our previous work revealed formation *in vivo* of M-17203 from F in guinea pigs (in urine and in faeces), but also its absence in F incubations with subcellular fractions (microsomes and cytosol) [manuscript submitted for publication]. Results from hepatocytes showed that M-17203 arose in intact liver cells. Incubations of fresh liver homogenate with *rac*-F and with *rac*-DHF did not lead to any production of M-17203 (Table 7). In addition, no amount of UM-1 and UM-2 was detected. With respect to these results, we can conclude that the integrity of the hepatocytes is essential for the formation of M-17203, UM-1 and UM-2. Integrity of the cell as a critical point has already been proven in other enzymatic reactions, like chiral inversions of profens [21].

The achieved results from our investigations allow us to suggest a scheme for F biotransformation in hepatocyte primary culture of male guinea pigs (Fig. 1).

Conclusions

This study described metabolic pathways of *rac*-F and its enantiomers and DHF-stereoisomers in isolated guinea pig hepatocytes. Different activities of the respective enzymes were observed for each substrate. Incubation with liver homogenate showed that hepatocyte integrity is essential for M-17203, UM-1 and UM-2 formation. Both chiral inversions of DHF stereoisomers and cell integrity, which are responsible for the formation of the three additional metabolites opens the question of the mechanism of their formation.

Methods

Chemicals

Rac-F, R-F and S-F, (2R;4S), (2S;4R), (2S;4S), (2R;4R)-DHF and M-17203 were obtained from Biotest (Prague, Czech Republic). Coenzymes ($NADP^+$, NADPH, NADH) and enzyme glucose-6-posphate dehydrogenase (Glc-6P-DH) were provided by Boehringer-Mannheim (Germany). Magnesium chloride ($MgCl_2$), trifluoroacetic acid (TFAA), insulin, powdered Williams E medium, powdered HAM 12 medium, fetal bovine serum, streptomycin, penicillin, rat tail collagen, collagenase, Trypan blue and glucose-6-posphate (Glc6P) were purchased from

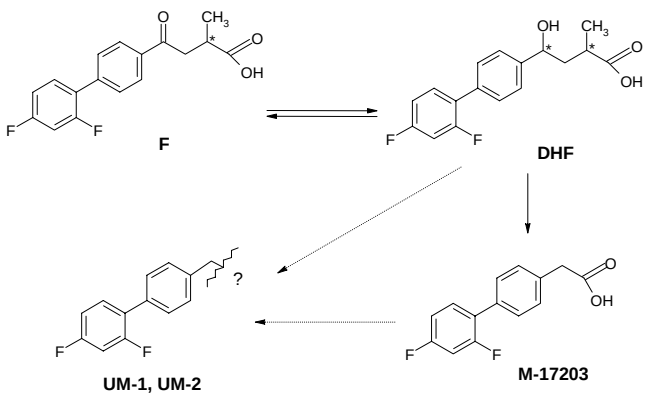

Figure 1
Proposed metabolic pathways of **F** in male guinea pigs isolated hepatocytes.

Sigma (Prague, Czech Republic). Dimethylsulfoxid (DMSO) and bovine serum albumin (BSA) were bought from Fluka (Prague, Czech Republic). Methanol (MeOH), 2-propanol (IPA) and n-hexane (all solvents HPLC grade) were from Merck (Prague, Czech Republic). Water from the Milli-Q-RG Ultra-Pure Water System from Millipore (Prague, Czech Republic) was used in all cases.

Animals

Male guinea pigs (*Cavia aperea* f. var. TRIC, 15–16 weeks, weight 650–700 g) were obtained from Biotest (Konarovice, Czech Republic). They were kept under standard conditions with free access to food and water. Experiments with animals were carried out according to Guide for the care and use of laboratory animals (Protection of Animals against Cruelty Act. No. 246/92 Coll., Czech Republic). The animals were killed by decapitation in total inhalation anesthesia. The liver tissue was removed from abdomen after bleeding out and underwent procedure of isolation of hepatocytes.

Preparation of liver homogenate

Liver tissue from 4 guinea pigs was homogenized with phosphate buffer pH 7.4 (PBS) in the ratio 1/6 (w/v) in Potter and Elvehjem homogenizer. This fresh homogenate was used for incubations.

Isolation of hepatocytes

Hepatocytes were obtained by a two-step collagenase method [29]. In the first step, the whole liver (10–15 g) was washed with 100 ml of solution without calcium, with aim of removing the rest of blood and making the cell-cell junction weaker. In the second step, hepatocytes were released by action of collagenase (50 mg/100 ml) in perfusion solution. Second perfusion lasted 6–8 min (recirculation system). Isolated hepatocytes were rewashed three times and mixed together with culture medium. The culture medium consisted of a 1:1 mixture of Ham F12 and Williams' E, supplemented as described [13,30]. The viabilities of cells measured by Trypan blue staining according to Sigma protocol were 75–90%. Three million of viable cells in 3 ml of culture medium were placed into 60 mm plastic dishes pre-coated with collagen. The foetal calf serum was added in culture medium (5%) to favour the cells attachment during the first four hours after plating. Then the medium was exchanged with a fresh one without serum. The cultures were maintained at 37°C in ahumid atmosphere of air and 5% CO_2.

Incubations

Liver homogenate: The substrates (*rac*-F, R-F and S F), dissolved in 10% of MeOH and 90% of PBS, were prepared in the concentrations 0.5 and 1.0 mM. Stock solutions of *rac*-DHF had to be made in another way because of its low solubility in water solution. Total weight of solid *rac*-DHF was first dissolved in 50% DMSO in water, then 0.1 and 0.2 ml of this solution were mixed with 0.9 and 0.8 ml of PBS, respectively. Final concentration of *rac*-DHF in incubation mixture was 0.12 and 0.24 mM. Stock solutions of coenzymes (NADH, NADPH) were prepared in concentration 6 mM (in redistilled water). NADPH regenerating system (NADPH-RS) was prepared in final concentration 6 mM $NADP^+$, 45 mM Glc6P, 8 U/ml Glc6P-DH, 49.4 mM $MgCl_2$ in 0.1 M PBS. Incubations were performed at 37°C for 1, 2 and 4 h. Final volume of reaction mixture was 3 ml (1.0 ml of substrate, 1.0 ml of coenzyme, 1.0 ml of liver homogenate). Incubation was stopped with cooling to 0°C and with addition of 1 ml 5 M H_3PO_4. Acidified incubation mixtures were extracted three times with distilled ethylacetate (EA). Extracts were evaporated and the dry samples were used for HPLC assessment.

Hepatocytes monolayers (18–24 h after isolation) were incubated with *rac*-F or individual R-F or S-F (25–200 μM) and also with individual DHF stereoisomers (50 and 100 μM) as substrates. Aliquots of medium (0.5 ml) were collected in the intervals of 0, 2, 4, 8 and 24 h. All samples of medium were acidified with 0.1 ml of 5 M phosphoric acid and extracted with distilled EA. The extracts were evaporated to dryness under vacuum. The dry samples were redissolved in 400 μl of mobile phase prior to their HPLC injection.

Analytical assessment

HPLC method was used for detection and quantification of all metabolites and substrates. Under the following conditions, separation of DHF stereoisomers from each other, M-17203 and F was achieved in one chromatographic run: chiral stationary phase (silica modified with 3,5-dinitrobenzoyl diphenylethylendiamine, where π-π

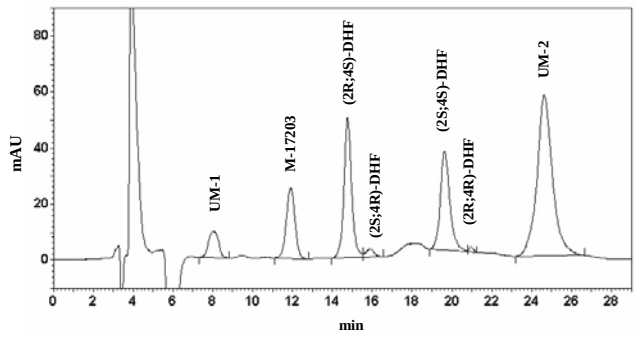

Figure 2
Chromatographic record of separation of **M-17203**, **DHF** stereoisomers, **UM-1** and **UM-2** in hepatocyte medium after 24 h incubation with *rac*-**F** (75 μM). Legend: Separation of **M-17203**, **DHF** stereoisomers, **UM-1** and **UM-2** in hepatocyte medium after 24 h incubation with *rac*-**F** (75 μM). **F** (R_t 23.1 min) was not detected after 24 h.

interactions are essential in the separation process), working in normal phase mode, was used; all analytical determinations were performed with the R,R-ULMO column (250 × 0.46 cm I.D.) from Regis Technologies (Morton Grove, Illinois, USA); mobile phase consisted of n-hexan:IPA:TFAA (98.5:1.5:0.1 v/v/v); flow rate 1.0 ml/min and a temperature of 25°C in the column compartment were set up; 240 nm was the optimal wavelength for DHF and M-17203 determination whereas 275 nm was the optimal wavelength for F determination.

100 μl of samples reconstituted in mobile phase for DHF separation (total volume 400 μl) was injected into the column. Each sample, made in triplicate, was measured individually. The final result represents the mean of these three individual measurements. Reproducibility of metabolites and substrate determination was tested during HPLC method development. The S.D. of consequent assessments was ≤ 2%. Student t-test was used for statistical evaluation of results. Extraction recovery of F enantiomers and DHF stereoisomers in pure medium was 85% (S.D. 2%). The calibration curves were prepared in the range 40 nM-50 μM for M-17203, 35 nM-50 μM for DHF stereoisomers and in the range 40 nM-100 μM for F. The measures of linearity (r^2) were 0.9998 for (2R;4S)-DHF, 0.9994 for (2S;4R)-DHF, 0.9989 for (2S;4S)-DHF, 0.9997 for (2R;4R)-DHF, 0.9996 for F and 0.9998 for M-17203. The limit of quantification (LOQ) for F enantiomers was 40 nM, for DHF stereoisomers 35 nM and for M-17203 40 nM. Optical purity was > 99% for individual F enantiomers, 99.1% for (2R;4S)-DHF, 100.0% for (2S;4R)-DHF, 96.1% for (2S;4S)-DHF and 97.0% for (2R;4R)-DHF. The successful separation process was characterized by the resolution (R_S) of individual compounds as follows: M-17203/(2R;4S)-DHF (5.0) (2R;4S)-DHF/(2S;4R)-DHF (1.7), (2S;4R)-DHF/(2S;4S)-DHF (4.8), (2S;4S)-DHF/(2R;4R)-DHF (1.8) and (2R;4R)-DHF/F (2.1).

Chromatographic instrumentation consisted of low-pressure gradient pump, degasser of mobile phase, autosampler, column thermostat and photodiode array detector. All components were products of Shimadzu (Prague, Czech Republic). Data acquisition and evaluation were performed using the Chromatography Laboratory Automated Software System Class VP (version 6.12) from Shimadzu (Prague, Czech Republic). Illustrative chromatogram resulting from these chromatographic conditions is shown in Fig. 2.

Apparent actual clearance for each metabolite was calculated as actual velocity divided by initial substrate concentration: $Claa = \frac{Va}{[Si]}$ (Claa – apparent actual clearance, Va – actual velocity [mol/l/h], [Si] – initial concentration of substrate).

Authors' contributions

RK carried out metabolic studies, analytical analyses, coordinated the study and drafted the manuscript. LSk and BS carried out isolation of hepatocytes and participated on conceiving of the study and drafting of the manuscript. JV carried out isolation of hepatocytes and participated on metabolic studies. LSch participated on metabolic studies. YB participated on composition and language revision of the manuscript. VW coordinated the study.

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