CHARACTERIZATION OF HUMAN CYTOCHROME P450 ENZYMES INVOLVED IN THE METABOLISM OF cPLA$_2$α INHIBITORS WITH 1-INDOL-1-YLPOPAN-2-ONE SKELETON

Westfälische Wilhelms-Universität Münster

Jörg Fabian, Matthias Lehr

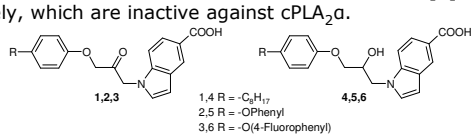
Institute of Pharmaceutical and Medicinal Chemistry, University of Münster

Hittorfstr. 58-62, 48149 Münster, Germany

Introduction:

Inhibitors of cytosolic phospholipase A_2α (cPLA$_2$α) are assumed to provide a novel therapeutic with application in many inflammatory diseases [1]. Certain 1-indol-1-ylpopan-2-ones, such as compounds **1**[2] and **2** [3], are potent inhibitors of cPLA$_2$α.

An important part of the pharmacophore of these substances is the activated electrophilic ketone moiety, which is supposed to form covalent binding interactions with a serine of the active site of cPLA$_2$α. In earlier investigations metabolic reduction of the ketone by rat liver microsomes led to the alcohols **4** [4] and **5**, respectively, which are inactive against cPLA$_2$α.



As inter-species differences and variations of CYP isoforms are known [5] we now investigated, which human CYP450 isoenzyms are responsible for metabolism of compound **2** by inhibitor approach [6].

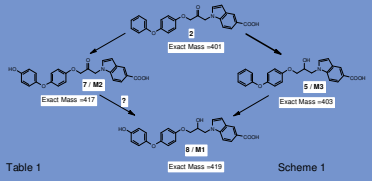
Incubation procedure:

2 µl of analyte solution (final concentration 25 µM) and -where required- 1 µl inhibitor solution dissolved in DMSO were added to potassium phophate buffer (100 mM, pH 7.4 / 3 mM $MgCl_2$) containing 20 pmol BD Supermix™ cDNA-expressed CYP450 isoenzymes (1A2, 2C8, 2C9, 2C19, 2D6 and 3A4) cocktail to give a final volume of 200 µl. The reaction was started by the addition of 50 µl NADPH solution (15 mM) and carried out at 37 °C in a shaking water bath for 2h. Reference incubations were performed without NADPH. The incubations were stopped by the addition of ice-cold 375 µl MeCN with 0.5 % HCOOH (*v/v*). The samples were centrifuged and the supernatants were analysed. The analyte solution were shown to be stable at 10 °C for 24 h.

For LC-MS investigations 250 µl of ice-cold MeCN was used as stop solution. After acidification with 625 µl of 0.1 M phosphoric acid liquid-liquid-extraction of the samples with 2 x 2.5 ml diethylether was accomplished. The separated organic layers were concentrated under a stream of nitrogen and the residue was reconstituted in 300 µl mobile phase.

Inhibitor	CYP450-isoenzymes	IC_{50}-value Literature [µM]	Assay concentration [µM]
8-Phenylthiophylline	1A2	0.7	20
Quercetine dihydrate	2C8	6.2	10/50/100/300
Sulfaphenazole	2C9	0.4	20
Tranylcypromine	2C19	12.5	125
Quinidine	2D6	0.030	2
Ketoconazole	3A4	0.04	2
Liponic acid	NADPH-Reductase		5000

Table 1



Scheme 1

LC-Analysis:

A 100 µl of sample was injected onto Kromasil 100-5 ODS (150 x 3.2 mm, with securityguard™cartridge) column (Phenomenex) at a flow rate of 0.6 ml/min, column oven temperature of 30 °C and a UV detection wavelength of 235 nm. The mobile phase consisted of MeCN / 20 mM ammonium formiate 20:80 (0.2% HCOOH) (A) and MeCN / 20 mM ammonium formiate 85:15 (0.2% HCOOH) (B). The following gradient was applied (A%): 0 min: 90, 23 min: 10, 24 min: 10, 25 min: 90, 35 min: 90.

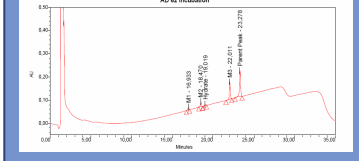


Fig. 1

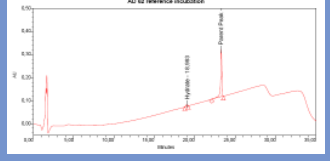


Fig. 2

Results - Metabolism:

The chromatogram of the reference incubation (Fig. 2) displays the hydrate [4] and the analyte peak of **2**. The incubation (Fig. 1) resulted in the formation of metabolites (**M1**: 16.9 min, **M2**: 18,5 min, **M3**: 22,0 min, Scheme 1). M 3 was the main metabolite with about 30 % relative peak area. M2 was formed by about 10 % and M1 derived out of **5** at circa 6 %. Approximately 53 % of **2** were not metabolised within 120 min.

After spiking the incubation probe with substance **5**, it could be assumed that this compound was the main metabolite.

Results – Inhibition Investigation:

Inhibition assays (Table 1) revealed CYP 2C19 and 2C8 as the main metabolic pathways of **2** (Scheme 1). Quercetine inhibited the reduction of **2** and in minor the hydroxylation of the phenyl-function. Tranylcypromine exclusively avoided the hydroxylation of the aryl-group of **2** and **5**, respectively. As the 4´-fluorophenyl-derivate **3** did not show hydroxylation (Fig. 6), the 4´-position can be assumed as the preferred reaction site. CYP 3A4 is involved in hydroxylation reaction inferior.

LC/MS^n-Analysis:

LC/MS^n and counter synthesis were employed to verify the metabolites of **2** and **3**, respectively.

A 60 µl (70 µl at LC/MS^3) volume of reconstitued assay residue was injected onto Kromasil 100-5 ODS (150 x 3.2 mm, with securityguard™cartridge) column (Phenomenex) at a flow rate of 0.6 ml/min and a UV detection wavelength of 235 nm. The mobile phase consisted of MeCN / 20 mM ammonium formiate 20:80 (A) and MeCN / 20 mM ammonium formiate 85:15 (B). The following gradient was applied (A%): 0 min: 90, 23 min: 0, 24 min: 0, 25 min: 90, 35 min: 90. Three minutes after injection, the MS-detector (LCQ® Thermo-Finnigan) was activated and structure determination was performed using ESI-MS^n in negative mode.

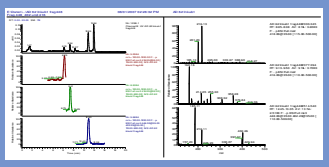


Fig. 3

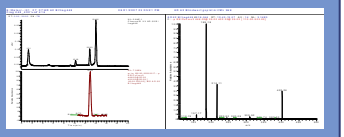
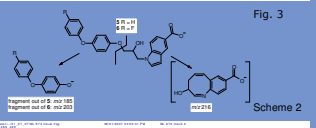


Fig. 4



Scheme 2

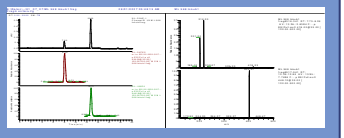


Fig. 5

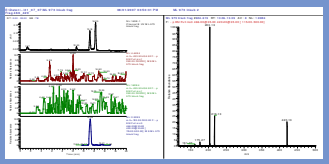


Fig. 6

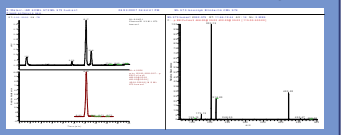


Fig. 7

Results - LC/MS^n

LC/MS^n investigations of the metabolism of **2** confirm the alcohol **5** (**M3**) as the main and **7/8** (**M2/M1**) as minor metabolites (Scheme 1). Representive ESI-MS$^{2/3}$ data of the incubation assay are shown in Fig. 3. Isolation and fragmentation of the formiate adduct of **5** at *m/z* 448 $[M+45]^-$ yields the daughter ion at *m/z* 402 $[M-H]^-$, which fragments mainly to MS/MS product ions at *m/z* 216 and 185. The assumed structures are represented in Scheme 2. The MS^3-spectrum of a reference probe spiked with **5** (Fig. 4) corresponds to latter fragmentation pattern. The MS^2 data of **8** (*m/z* 418) (Fig. 3) shows fragments at *m/z* 216 and 201. The product ion *m/z* 201 illustrates the hydroxylation at the phenyloxyphenyl-part. **7** can be detected at *m/z* 416.

Incubation of **5** forms the expected metabolite **8**, which was verified by the fragmentation pattern (Fig. 5).

Incubation of the 4`-fluorophenyl-derivate **3** yields **6** (*m/z* 420) and no further hydroxylation can be observed (Fig. 6). The fragmentation pattern of a spiked reference probe confirms **6** as the main metabolite (Fig. 7).

Literature:

[1] Lehr M., *Anti-Inflamm Anti-Allergy Agents Med Chem* **2006**, 5, 149–161
[2] Ludwig J. et al., *J Med Chem* **2006**, 49, 2611-2620
[3] Drews A. et al., unpublished results
[4] Fabian J., Lehr M., *J Pharm Biomed Anal* **2007**, 43, 601-605
[5] Zuber R. et al., *J Cell Mol Med* **2002**, 6, 189-98
[6] Bourrie M. et al., *J Pharmacol Exp Ther*. **1996**, 277, 321-32