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Normal-phase HPLC and HPLC–MS studies of the metabolism of a cytosolic phospholipase $A_2\alpha$ inhibitor with activated ketone group by rat liver microsomes

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Abstract

Inhibition of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) is assumed to provide a novel therapeutic approach for the treatment of many inflammatory diseases. 1-[3-(4-Octylphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**2**) is a potent inhibitor of $cPLA_2\alpha$. An important part of the pharmacophore of **2** is its activated electrophilic ketone moiety. Since it is known that activated ketones may be metabolically unstable, the metabolism of **2** by rat liver microsomes was investigated. For quantification of the metabolites normal-phase HPLC/UV on a cyano column was used, because under reversed-phase conditions with aqueous solvents **2** was partly transformed into its hydrate resulting in chromatograms with splitted peaks. Under the conditions applied about 30% of **2** were metabolized. The main metabolite was the alcohol **4** as shown by LC/MS^n.

Keywords: Metabolism; Rat liver microsomes; Activated ketone; Hydrate; Normal-phase chromatography; Cyano-phase

1. Introduction

Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) is an esterase that selectively cleaves the *sn*-2 position of arachidonoyl-glycerophospholipids of biomembranes to generate free arachidonic acid and lysophospholipids [1]. Arachidonic acid in turn is metabolized to a variety of inflammatory mediators including prostaglandins and leukotrienes. Lysophospholipids with an alkyl ether moiety at the *sn*-1 position can be acetylated to platelet activating factor (PAF), another mediator of inflammation. Thus, inhibition of $cPLA_2\alpha$ is considered as an attractive target for the design of new anti-inflammatory drugs [2,3].

Although there have been intense efforts for finding inhibitors of $cPLA_2\alpha$, no such compound has emerged to the market. The only $cPLA_2\alpha$ inhibitor reported to undergo clinical development as anti-inflammatory drug is the indole derivative efipladib from Wyeth [4]. Published compounds with high *in vitro* $cPLA_2\alpha$-inhibitory potency are thiazolidinediones of Shionogi [5] and propan-2-ones of AstraZeneca [6] such as compound **1** (Fig. 1). Recently, we have found that 1-[3-(4-octylphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**2**), which is structurally related to **1**, is also a potent inhibitor of $cPLA_2\alpha$ [7].

A common structural feature exhibited by **1** and **2** is the activated electrophilic ketone group. This moiety is an important part of the pharmacophore of the inhibitors. Its reduction leads to the inactive alcohols **3** and **4**, respectively (Fig. 1) [6,8,9]. Since it is known that activated ketones can be unstable towards keto-reduction [10], we tested the metabolic stability of **2** applying rat liver microsomes.

2. Experimental

2.1. Chemicals

Acetonitrile (HPLC grade), isohexane (HPLC grade), phosphoric acid (85%) were obtained from Baker (Deventer, Netherlands). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Geel, Belgium). Tetrahydrofuran (THF) (HPLC grade), diethyl ether, potassium dihydrogenphosphate,

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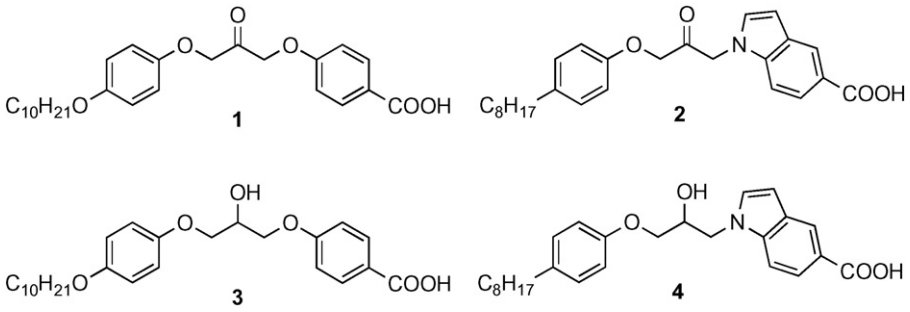

Fig. 1. Chemical structures of cPLA$_2\alpha$ inhibitors with activated ketone group (**1**, **2**) and their corresponding alcohol forms (**3**, **4**).

potassium hydrogenphosphate were acquired from VWR International (Darmstadt, Germany). Dimethylsulfoxide (DMSO) (p.a.), magnesium chloride hexahydrate (ultra) were purchased from Fluka (Buchs, Switzerland). Dihydronicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH) was acquired from Roth (Karlsruhe, Germany). 2-(*p*-Toluoyl)benzoic acid was obtained from Lancaster Synthesis (Morecambe, United Kingdom). Water was purified using a Bi 18 system from Heraeus (Hanau, Germany). 1-[3-(4-Octylphenoxy)-2-oxopropyl]indole-5-carboxylic acid (**2**) and 1-[2-hydroxy-3-(4-octylphenoxy)propyl]indole-5-carboxylic acid (**4**) were synthesized according to published procedures [7,9].

2.2. Preparation of rat liver microsomes

Livers of male Sprague–Dawley rats were obtained from the Institute of Pharmacology of the Medicinal Faculty of the University of Münster. Rat liver microsomes were preparated adapted to published procedures [11–13].

Six livers (95 g total) were washed with cold potassium chloride solution (1.15%, m/v) and homogenized in an Elvehjem-Potter homogenizer with an equivalent volume of cold potassium buffer (pH 7.4; 0.1 M) containing sodium EDTA (0.5 mM). The homogenate was diluted to 285 ml and centrifuged at 10,000 × *g* at 4 °C for 20 min. The supernatant was centrifuged at 100,000 × *g* at 4 °C for 60 min. The resulting microsomal pellet was resuspended in 285 ml potassium phosphate buffer (pH 7.4; 0.1 M). For removing all residual cytosolic glutathione *S*-transferases [13] the suspension was centrifuged again at 100,000 × *g* at 4 °C for 60 min. The pellet was resuspended in potassium phosphate buffer (pH 7.4; 0.1 M) (1.2 ml buffer/2 g original tissue) and stored at −80 °C. The microsomal protein concentration was determined according to the method of Bradford [14] applying bovine serum albumin (BSA) as standard and finally adjusted to 15 mg/ml.

2.3. Preparation of standards

Stock solutions containing 5 mM of **2** and **4** were prepared in DMSO. Working solutions were prepared by diluting the stock solutions with appropriate amounts of DMSO. The internal standard 2-(*p*-toluoyl)benzoic acid was dissolved in acetonitrile at a concentration of 0.2 mM and stored at 4 °C.

2.4. Incubation procedures

Microsomal incubations were carried out using 2.25 mg/ml rat liver microsomal protein in potassium phosphate buffer (pH 7.4; 0.1 M) with 3 mM $MgCl_2$ and 1.9 mM NADPH in a final volume of 498 μl. The reaction was initiated by the addition of 2 μl of the stock solution of **2** (final concentration 20 μM) and the mixtures were incubated in a shaking water bath for 30 min. The metabolisation was stopped by addition of 100 μl internal standard solution and 500 μl of ice cold acetonitrile. After addition of 1.25 ml 0.1 M phosphoric acid the sample was extracted with 3 ml diethyl ether in a rotating mixing wheel for 10 min and subsequently centrifuged at 1400 × *g* for 5 min. The organic layer was separated and the extraction step repeated. Then the combined organic layers were concentrated under a stream of nitrogen. The residue was reconstituted in 200 μl of isohexane–THF (75:25, v/v) and an aliquote (50 μl) of this solution was injected onto the HPLC column. Reference incubations without NADPH were performed in the same way. The analyte solutions were shown to be stable at 10 °C for 24 h.

The microsomal incubation samples used for HPLC/MS-analysis contained 25 μM of **2**. The incubation time was 180 min.

2.5. HPLC/UV-analysis

The HPLC system consisted of a Bischoff 2250 HPLC pump gradient system (Leonberg, Germany) with a dynamic mixing chamber from Knauer (Berlin, Germany) coupled to a Midas Cool autosampler with column oven from Spark Holland (Emmen, The Netherlands) and a UV-detector 486 from Waters (Milford, USA). System control and data processing were performed using McDAcq32 Control software. The temperature of the autosampler was kept at 10 °C. Chromatographic separation and quantitation of **2** and its metabolites was achieved by normal-phase chromatography on a Lichrospher 100-5 CN column (250 mm × 4 mm) protected by a Lichrospher 100-5 CN guard column (4 mm × 4 mm) (Merck, Darmstadt, Germany). The HPLC mobile phases consisted of isohexane–THF–TFA (92:8:0.1, v/v/v) (A) and isohexane–THF–TFA (50:50:0.1, v/v/v) (B). HPLC separation was conducted at 25 °C applying a gradient with a flow rate of 0.75 ml/min. The initial composition was 10% B. The gradient was programmed linearly to 80% B over 15 min and held for 3 min. Finally, the gradient was linearly