Microbial Metabolism of Rofecoxib, A Selective COX-2 Inhibitor

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Abstract: Microbial transformation studies of rofecoxib (**1**), a potent selective cyclooxygenase-2 inhibitor, using sixty microorganisms, mainly fungi and actinomycetes, have revealed that it was metabolized by three microorganisms. Using a standard two-stage screening technique, *Cunninghamella echinulata* var. *echinulata* ATCC 9244, *Mucor griseocyanus* ATCC 1207b, and *Rhizopus oryzae* ATCC 34121 showed two common more polar metabolites **2** and **3**. Moreover, it was apparent that *Cunninghamella echinulata* var. *echinulata* was the most efficient microorganism to almost completely metabolize **1**, and hence, was selected for preparative scale fermentation. These metabolites were characterized on the basis of their spectral data as 4′-hydroxyrofecoxib (**2**) and 3′-hydroxyrofecoxib (**3**).

Keywords: Rofecoxib, *Cunninghamella echinulata* var. *echinulata*, 4′-hydroxyrofecoxib, 3′-hydroxyrofecoxib, COX-2 inhibitor.

INTRODUCTION

The enzyme prostaglandin cyclooxygenase, COX, catalyzes the rate-limiting cyclization step in the formation of prostanoids from arachidonic acid [1,2]. Two isoforms of the COX enzyme have been characterized; COX-1 and COX-2 [3]. The discovery of COX-2 has provided the rationale for the development of a new class of the non-steroidal anti-inflammatory drugs (NSAIDs), the selective COX-2 inhibitors, coxibs. These agents were developed on the premise that selective inhibition of COX-2 would result in decreased inflammation without the adverse gastrointestinal effects associated with inhibition of COX-1 [4-6]. Rofecoxib and celecoxib were the first selective COX-2 inhibitors approved by the US Food and Drug Administration for the treatment of rheumatoid arthritis, osteoarthritis and for relief of acute pain associated with dental surgery and primary dysmenorrhea. Rofecoxib is a diarylfuranone derivative containing a phenylsulphone moiety that interacts with COX-2 side pocket [7]. This interaction represents an important determinant for COX-2 selectivity. In fact, rofecoxib almost completely inhibits monocyte COX-2 activity without affecting COX-1 activity [8].

On September 30, 2004, Merck and Co. voluntary withdrew rofecoxib, VIOXX®, from the market due to increased risk of cardiovascular toxicities associated with the drug [9]. It is not clear whether these toxicities are due to rofecoxib high COX-2 selectivity. It was shown that COX-2 inhibitors decrease vascular prostacyclin PGI_2 production and may disrupt the homeostatic mechanisms that limit the effects of platelets activation. Therefore, the mechanism of this cardiovascular toxicity could lie in the inhibition of COX-2 itself, and thus be a class effect. On the other hand, it may be due to its unique chemical structure, its pharmacokinetics and/or the presence of toxic metabolites [9]. In order to explore more this hypothesis, rofecoxib metabolites need to be prepared in quantities enough to be identified and evaluated for possible activity and/or toxicity.

In drug research and development, metabolism is of a pivotal importance due to the interconnectedness between pharmacokinetic and pharmacodynamic processes. The phenomenon of metabolism originates from the fact that mammals usually treat any exogenous drug as non-self and work hard to weaken the effect and/or the concentration of this "foreign" substance through enzymatic machinery. However, xenobiotic metabolic system sometimes generates products more reactive and biologically destructive than the original substrate. The metabolism process generates new chemical entities, metabolites, which may have distinct pharmacodynamics and toxicodynamic properties of their own [10].

Human metabolism of rofecoxib is primarily mediated through reduction by cytosolic enzymes. The principal metabolic products are the *cis*- and *trans*-dihydro derivatives of rofecoxib. An additional metabolite was recovered as the glucuronide of the hydroxy derivative, a product of oxidative metabolism. Cytochrome P450 plays a minor role in metabolism of rofecoxib. This was concluded since rofecoxib disposition was not affected by the inhibition of CYP 3A activity after the administration of ketoconazole.

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However, induction of general hepatic metabolic activities by administration of the non-specific inducer rifampin, produces a 50% decrease in rofecoxib plasma concentrations (VIOXX® insert).

The use of microbial systems as *in vitro* models for drug metabolism in humans was first formalized by Rosazza and Smith [11], and was defined as the use of microorganisms, bacteria, yeasts, and fungi, to facilitate the study of xenobiotic biotransformation in mammals, including man.

It is anticipated that the microbial metabolism of rofecoxib would produce significant quantities of metabolites that would be difficult to obtain from either animal systems or chemical synthesis. Moreover, this work will also provide some new analogs that may serve as prospective candidates for their potential pharmacological action and/or cardiac toxicity evaluation, or as starting compounds for the semisynthesis of other derivatives.

MATERIALS AND METHODS

Rofecoxib used in this project was obtained as a gift (The Egyptian International Pharmaceutical Industries Co, 10th of Ramadan City, Egypt). The identity of the substrate was positively confirmed as a pure rofecoxib *via* NMR analyses. The microbial cultures used in this project were originally obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and from the US Department of Agriculture Northern Regional Research Laboratories (NRRL, Peoria, Illinois, USA). All the preliminary screening and preparative-scale experiments were carried out in medium α, which consists of, per liter of distilled water, glucose, 20 gm; NaCl, 5 gm; K_2HPO_4, 5 gm; yeast extract (BBL, Cockeysville, Maryland, USA), 5 gm; peptone (Difco, Detroit, Michigan, USA), 5 gm. TLC analyses were performed on precoated silica gel 60 F_{254} plates (Merck, New Jersey, USA). The adsorbent used for column chromatography was Si gel 60/230-400 mesh (EM Science, Humble, Texas, USA). All solvents used for chromatographic purposes were of analytical grade, solvent used for UV analysis was of spectral grade, and those used for extraction processes were general purpose reagents.

The compounds IR spectra were recorded as neat solids using an FTIR-4100 Jasco spectrophotometer (Jasco, Easton, Maryland, USA). UV spectra were measured in methanol using a UV-160 IPC UV-visible dual-beam spectrophotometer. The ^1H and ^{13}C NMR were obtained on a Bruker DPX-400 spectrometer (Bruker Bio Spin, Rheinstetten, Germany) operating at 400 and 100 MHz, respectively. Both ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, and the chemical shift values were expressed in δ (ppm) relative to the internal standard TMS. For the ^{13}C NMR spectra, the number of attached protons was determined by DEPT 135°. 2D NMR data were obtained using the standard pulse sequence of the Bruker DPX-400 for COSY, HSQC, and HMBC. HRMS was carried out using a Bruker Bioapex FTMS with Electrospray Ionization Spectrometer (Bruker Daltonic GmbH, Bremen, Germany).

Cultures and Fermentation Screening Procedure

A total of sixty microorganisms, mainly fungi and actinomycetes, Table **1**, have been screened for their ability to catalyze bioconversion of rofecoxib (**1**). Only three of them, *Cunninghamella echinulata* var. *echinulata* ATCC 9244, *Mucor griseocyanus* ATCC 1207b, and *Rhizopus oryzae* ATCC 34121, showed definite metabolism of rofecoxib. Stock Cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

All the preliminary screening and preparative-scale experiments were carried out as reported before [12], and according to a standard two-stage protocol [13]. Substrate **1** was prepared as a 15% solution in *N,N*-dimethylformamide (DMF) and added to the 24-h-old stage II culture medium of the microorganism at a concentration 0.3 mg/mL of medium. Substrate controls were composed of sterile medium to which the substrate (4 mg/100µL DMF) was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the substrate addition. After two weeks of incubation, each control was harvested and analyzed by TLC.

Culture Sampling and Chromatographic Conditions

Sampling was carried out by withdrawing 5 mL of culture and extracting it with 5 mL of ethylacetate. The concentrated organic phase was analyzed by TLC for the presence of metabolites. TLC analyses were performed using 5% MeOH in $CHCl_3$ as the solvent system. Visualization was accomplished by exposure to short wavelength UV (λ_{max} 254).

Preparative Scale Conversion of Rofecoxib by *Cunninghamella echinulata var. echinulata*

C. echinulata var. *echinulata* ATCC 9244 was grown in fifteen 1-L culture flasks each containing 200

mL of medium α. A total of 500 mg of **1** (in 5 mL DMF) were evenly distributed among the 24-h-old stage II culture. After two weeks, the incubation mixtures were checked by TLC. TLC revealed that most of **1** was transformed and two more polar metabolites were produced. The incubation mixtures were combined and filtered to remove the mycelia, and the filtrate was extracted with ethylacetate (3L x 4). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo* at 40 °C to afford a yellowish residue (0.7 gm). Part of this residue (0.4 gm) was purified by column chromatography over silica gel (45 g, 3.3 x 15 cm) using MeOH-$CHCl_3$ (5% v/v) as the eluting solvent system. Fractions of 20 mL each were collected and pooled on the basis of TLC analyses. Fractions 15-22 yielded pure 4'-hydroxyrofecoxib (**2**) (33 mg, 6.2% yield), with R_f = 0.41. Fractions 35-43 gave pure 3'-hydroxyrofecoxib (**3**) (12 mg, 2.3% yield), with R_f = 0.37.

4'-hydroxyrofecoxib (**2**) was obtained as colorless needles; IR neat υ_{max} 3373 (OH), 1746 (C=O), 1595 (aromatic), 1445 (aromatic) cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 284 (4.14) nm; ^1H NMR ($CDCl_3$, 400 MHz) see Table **2**; ^{13}C NMR ($CDCl_3$, 100 MHz) see Table **2**; High resolution ESIMS *m/z* 330.0584 $[M]^+$ (calcd for $C_{17}H_{14}O_5S$ 330.0562). On the other hand, 3'-hydroxyrofecoxib (**3**) was obtained as colorless needles; IR neat υ_{max} 3373 (OH), 1746 (C=O), 1590 (aromatic), 1461 (aromatic) cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 281 (4.12) nm; ^1H NMR ($CDCl_3$, 400 MHz) see Table **2**; ^{13}C NMR ($CDCl_3$, 100 MHz) see Table **2**; High resolution ESIMS *m/z* 330.0584 $[M]^+$ (calcd for $C_{17}H_{14}O_5S$ 330.0562).

RESULTS AND DISCUSSION

A total of sixty microorganisms, mainly fungi and actinomycetes, Table **1**, have been screened for their ability to catalyze bioconversion of rofecoxib (**1**). Only three of them, *Cunninghamella echinulata* var. *echinulata* ATCC 9244, *Mucor griseocyanus* ATCC 1207b, and *Rhizopus oryzae* ATCC 34121, showed definite metabolism of rofecoxib. These cultures produced two more polar metabolites, **2** and **3**. Their structures are shown in Figure **1**. Thin layer chromatography, showed that *Cunninghamella echinulata* var. *echinulata* was the most efficient microorganism to almost completely metabolize rofecoxib, and hence, was selected for preparative-scale fermentation.

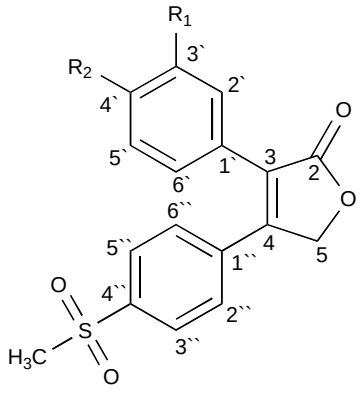
Table 1: Microorganisms Screened for their Ability to Metabolize Rofecoxib[a]

Absidia glauca ATCC 22752
Achlya racemosa ATCC 11392
Acrasis rosea ATCC 26823
Acrodictys erecta ATCC 24083
Acinetobacter calcoaceticus ATCC 14987
Agaricus campestris ATTCC 26815
Alcaligenes eutrophus ATCC 17697
Allomyces arbuscula ATCC 10983
Aspergillus flavus NRRL 501
Aspergillus fumigatus ATCC 26934
Aureobasidium pullulans ATCC 9348
Bacillus cereus var. fluorescens ATCC 13824
Beauvaria bassiana ATCC 7159
Bullera alba ATCC 18568
Calonectria decora ATCC 14767
Candida tropicalis ATCC 20021
Cantharellus cibarius ATCC 13228
Cellulomonas flavigena ATCC 482
Ceratobasidium cornigerum ATCC 38315
Coprinus species ATCC 16789
Cordyceps militaris ATCC 34164
Cryptococcus macerans Ziffer
Cunninghamella blakesleeana ATCC 8688a
Cunninghamella echinulata NRRL 3655
***Cunninghamella echinulata* var. *echinulata* ATCC 9244**
Cunninghamella elegans ATCC 9245
Cunninghamella species NRRL 5695
Cylindrocephalum aureum ATCC 12720
Debaryomyces polymorphus ATCC 20280
Dictyostelium mucoroides ATCC 2682
Eupenicillium javanicum ATCC 26879
Filobasidiella neoformans ATCC 10226
Fusarium oxysporum ATCC 7601
Fusarium solani ATCC 12823
Geotrichum amycelicum ATCC 24658
Gliocladium deliquescens ATCC 10097
Gongronella butleri ATCC 22822
Hansenula anomala ATCC 20170
Lipomyces lipofer ATCC 107242
Melanospora ornata ATCC 26180
Melanospora parasitica ATCC 18055
Mortierella zonata ATCC 13309

(Table 1). Continued.

***Mucor griseocyanus* ATCC 1207b**
Penicillium chrysogenum ATCC 9480
Polyporus brumalis ATCC 34487
Rhizopogon species ATCC 36060
Rhizopus arrhizus ATCC 11145
***Rhizopus oryzae* ATCC 34121**
Rhizopus stolonifer ATCC 24795
Rhodococcus species ATCC 21146
Saccharomyces cerevisiae ATCC 2366
Saccharomyces pastorianus ATCC 2366
Saccharomyces lipolytica ATCC 16617
Sepedonium chrysospermum ATCC 13378
Septomyxa affinis ATCC 6737
Streptomyces griseus ATTC 13968
Streptomyces rimosus NRRL 2234
Streptomyces roseochromogenus ATCC 13400
Talaromyces ucrainicus ATCC 18352
Thamnidium elegans ATCC 18191

[a]Microorganisms in bold represent those gave positive results.



Rofecoxib (**1**); $R_1 = R_2 = H$

4'-Hydroxyrofecoxib (**2**); $R_1 = H$, $R_2 = OH$

3'-Hydroxyrofecoxib (**3**); $R_1 = OH$, $R_2 = H$

Figure 1: Rofecoxib and its two hydroxylated metabolites.

Metabolites **2** and **3** were isolated by solvent extraction, purified by chromatography and subjected to spectral analyses.

Metabolite **2** was isolated as colorless needles in a 6.2% yield. The molecular formula was found to be $C_{17}H_{14}O_5S$ on the basis of the ion peaks at *m/z* 330.0584 $[M]^+$, and NMR data. The IR spectrum showed a broad absorption at υ_{max} 3373 cm^{-1} suggesting the presence of a hydroxyl group. However, an intermediate absorption at υ_{max} 1746 cm^{-1} was also shown indicating the survival of the original α, β-unsaturated 5-membered ring lactone carbonyl group in the parent compound.

Most ^1H and ^{13}C NMR spectral data of **2** (Table **2**) were similar to those of **1** except for resonances attributed to the non-phenylsulfone aromatic ring. The ^{13}C NMR spectra revealed the presence of an oxygenated aromatic carbon signal at δ_C 157.7 which was concluded to be attached to a hydroxyl group as suggested by the IR spectrum (υ_{max} 3373 cm^{-1}) and by the presence of a D_2O exchangeable proton at δ_H 3.55. This hydroxyl group was determined from the HMBC spectra to be at C-4′ due to the presence of ^3J cross peaks between this carbon (δ_C 157.7, s) and two protons resonated at δ_H 7.58 as doublets with a *J* value of 8.1 Hz. This conclusion was supported by protons coupling patterns and constants that were identical to a *para*-substituted aromatic ring, Table **2**. COSY spectra showed a correlation between these protons and other two protons both resonated at δ_H 6.70 as doublets with a *J* value of 8.1 Hz. It is noteworthy the upfield shifts of carbons 1′, 3′, and 5′ ($\Delta\delta$5.0 ppm, 14.0 ppm, and 14.0 ppm, respectively) due to the resonance effect of the hydroxyl moiety, as an electron-donating group, on the aromatic ring. These data unambiguously determined that **2** is 4′-hydroxyrofecoxib (Figure **1**).

Metabolite **3** was isolated as colorless needles in a 2.3% yield. It was shown to possess identical molecular formula to **2**, $C_{17}H_{14}O_5S$, as derived from the presence of an ion peak at *m/z* 330.0584 $[M]^+$ and from the NMR data. The presence of an additional hydroxyl group and an intact α, β-unsaturated 5-membered ring lactone carbonyl group was concluded from the IR spectrum due to the presence of absorptions at υ_{max} 3373 and 1746 cm^{-1}, respectively.

^{13}C NMR showed seventeen resonances distributed as one methyl, one methylene, eight aromatic methines, and one oxygenated aromatic quaternary carbon. Other quaternary carbons were almost identical to those of the parent rofecoxib. These data showed that metabolite **3** is similar to **2**, i.e., another rofecoxib hydroxy derivative. Protons coupling patterns and constants were instrumental in determining the substitution pattern of the non-phenylsulfone aromatic ring as a *meta*-substituted aromatic ring, Table **2**. ^1H NMR spectrum showed that one aromatic proton resonated at δ_H 7.01 as a singlet and was assigned H-2′. On the other hand, H-5′ was shown to resonate at δ_H 7.58 as a double doublet with *J* values of 8.1 and

Table 2: NMR Data (400 MHz, $CDCl_3$) for Rofecoxib (1) and Metabolites 2 and 3

Rofecoxib (1)			4′-Hydroxyrofecoxib (2)		3′-Hydroxyrofecoxib (3)	
#	δ_c, m[a]	δ_H (*J* in Hz)	δ_c, m	δ_H (*J* in Hz)	δ_c, m	δ_H (*J* in Hz)
2	173.1, C	-	173.1, C	-	173.1, C	-
3	129.6, C	-	129.6, C	-	129.6, C	-
4	154.0, C	-	154.0, C	-	154.0, C	-
5	70.9, CH_2	5.22, s	70.9, CH_2	5.22, s	70.9, CH_2	5.22, s
1′	130.1, C	-	125.1, C	-	133.9, C	-
2′	129.6, CH	7.42, br s	130.5, CH	7.58, d (8.1)	112.1, CH	7.01, s
3′	129.8, CH	7.42, br s	115.8, CH	6.70, d (8.1)	158.4, C	-
4′	129.8, CH	7.42, br s	157.7, C	-	115.1, CH	6.80, d (8.1)
5′	129.8, CH	7.42, br s	115.8, CH	6.70, d (8.1)	130.0, CH	7.58, dd (8.1, 8.1)
6′	129.6, CH	7.42, br s	130.5, CH	7.58, d (8.1)	121.5, CH	6.85, d (8.1)
1″	136.9, C	-	136.9, C	-	136.9, C	-
2″	129.1, CH	7.53, d (8.3)	129.1, CH	7.53, d (8.3)	129.1, CH	7.53, d (8.3)
3″	128.7, CH	7.94, d (8.3)	128.7, CH	7.94, d (8.3)	128.7, CH	7.94, d (8.3)
4″	142.6, C	-	142.6, C	-	142.6, C	-
5″	128.7, CH	7.94, d (8.3)	128.7, CH	7.94, d (8.3)	128.7, CH	7.94, d (8.3)
6″	129.1, CH	7.53, d (8.3)	129.1, CH	7.53, d (8.3)	129.1, CH	7.53, d (8.3)
CH_3	44.9, CH_3	3.09, s	44.9, CH_3	3.09, s	44.9, CH_3	3.09, s
OH	-	-	-	3.55, br s[b]	-	3.55, br s[b]

[a]Carbon multiplicities were determined by DEPT 135°.
[b]D_2O exchangeable protons.

8.1 Hz. This proton was further shown to possess three-bond correlation with the oxygenated aromatic carbon (δ_C 158.4, s, C-3′). Another important change in the NMR data was the upfield shifts of carbons 2′, 4′, and 6′ ($\Delta\delta$17.5 ppm, 14.7 ppm, and 8.1 ppm, respectively) due to the resonance effect of the hydroxyl moiety, as an electron-donating group, on the aromatic ring. Other NMR assignments were similar to those of rofecoxib, Table **2**. These data confirmed the identity of **3** as 3′-hydroxyrofecoxib (Figure **1**).

Several animal metabolites were previously reported including 5-hydroxyrofecoxib, 5-hydroxyrofecoxib-*O*-β-D-glucuronide, *cis*- and *trans*-3,4-dihydrorofecoxib, rofecoxib-3′,4′-dihydrodiol, and 4′-hydroxyrofecoxib sulfate [14]. Additionally, *cis*- and *trans*-3,4-dihydrorofecoxib, 5-hydroxyrofecoxib, 4′- and 5-hydroxyrofecoxib-*O*-β-D-glucuronide, rofecoxib-3′,4′-*trans*-dihydrodiol, rofecoxib-*erthyro*-3,4-dihydrohydroxy acid, and rofecoxib-*threo*-3,4-dihydrohydroxy acid were isolated and identified from human urine [15]. However, none of the microbial metabolites were reported before.

Although, compounds **2** and **3** were reported from total chemical synthesis, along with their acetyl derivatives [16], they were prepared using a tedious six-step reaction sequence giving only 5% cumulative yields. Additionally, full spectral identification of metabolites **2** and **3** were never provided in this study. Comparatively, in the current report, these metabolites were microbially produced using cheap and readily available microorganisms and medium constituents. The yield of the produced compounds can be proportionally increased by increasing the fermentation culture volume. Moreover, full spectral data and analysis of compounds **2** and **3** are provided herewith.

This study demonstrated the potential ability of microbial models to prepare known or novel metabolites in quantities that would be difficult to obtain from either mammals or chemical routes. The formation of rofecoxib metabolites **2** and **3** using the above-mentioned protocol made it possible to furnish these metabolites in significant quantities enough for cardiac toxicity evaluation. Such a study findings should help in explaining if rofecoxib cardiac toxicity is class-dependent, being a selective COX-2 inhibitor, or member-dependent due its unique chemical structure. Moreover, the produced metabolites might serve as starting compounds for the semisynthesis of other more promising inhibitors.

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