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Reduction of *N*-hydroxy-sulfonamides including *N*-hydroxy-valdecoxib (*N*-hydroxy-4-[5-methyl-3-phenyl-4-isoxazolyl]-benzenesulfonamide) by the molybdenum-containing enzyme mARC*

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Running title: Reduction of *N*-hydroxy-sulfonamides

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Nonstandard abbreviations

BzSA	benzenesulfonamide
COX	cyclooxygenase
mARC	mitochondrial amidoxime reducing component
m.p.	melting point
NSAID	Non-steroidal anti-inflammatory drug
NOHBSA	*N*-hydroxy-benzenesulfonamide
NOHTSA	*N*-hydroxy-4-toluenesulfonamide
TSA	4-toluenesulfonamide

Abstract

Purification of the mitochondrial enzyme responsible for reduction of *N*-hydroxylated amidine prodrugs led to the identification of two newly discovered mammalian molybdenum-containing proteins, the *mitochondrial amidoxime reducing components* mARC1 and mARC2 (Gruenewald *et al.*, 2008). These 35-kDa proteins represent a novel group of molybdenum proteins in eukaryotes as they form a molybdenum cofactor dependent enzyme system consisting of three separate proteins (Havemeyer *et al.*, 2006). Each mARC protein reduces *N*-hydroxylated compounds after reconstitution with the electron transport proteins cytochrome b_5 and b_5 reductase. In continuation of our drug metabolism investigations (Havemeyer *et al.*, 2006, Gruenewald *et al.*, 2008) we present data from reconstituted enzyme systems with recombinant human and native porcine enzymes showing the reduction of *N*-hydroxy-sulfonamides (sulfohydroxamic acids) to sulfonamides: The *N*-hydroxy-sulfonamide *N*-hydroxy-valdecoxib (*N*-Hydroxy-4-[5-methyl-3-phenyl-4-isoxazolyl]-benzenesulfonamide) represents a novel COX-2 inhibitor and is therefore a drug candidate in the treatment of diseases associated with rheumatic inflammation, pain, and fever. It was synthesized as an analogue of the known COX-2 inhibitor valdecoxib (4-[5-methyl-3-phenyl-4-isoxazolyl]-benzenesulfonamide) (Talley et al., 2000). *N*-Hydroxy-valdecoxib had low in *vitro* COX-2 activity but showed significant analgesic activity *in vivo* and a prolonged therapeutical effect compared to valdecoxib (Erdelyi *et al.*, 2008). In this report, we demonstrate that *N*-hydroxy-valdecoxib is enzymatically reduced to its pharmacologically active metabolite valdecoxib. Thus, *N*-hydroxy-valdecoxib acts as prodrug that is activated by the molybdenum-containing enzyme mARC.

Introduction

The enzymatic processes involved in the *N*-oxidative pathway are very well understood, whereas reduction pathways have been investigated in depth. The reduction of nitrogen-containing groups like aromatic nitro compounds, hydroxamic acids, oximes, tertiary *N*-oxides, azo compounds, and *N*-hydroxyguanidines by the molybdenum hydroxylases aldehyde oxidase and xanthine oxidase have been described previously (Kitamura, *et al.*, 2006, Dambrova *et al.*, 1998). However, the activities were often only described for *in vitro* investigations under the exclusion of oxygen. The *in vivo* relevance is thus questionable. Microsomal and mitochondrial reductions of hydroxylamines and amidoximes that are O_2-insensitive have been investigated by different groups (Kadlubar and Ziegler, 1974; Kurian *et al.*, 2006, Kurian, *et al.*, 2004; Andersson *et al.*, 2005, Clement *et al.*, 2005) and it is well accepted that cytochrome b_5 and its reductase are involved in the reduction activities mentioned. The membrane-bound forms of these electron transport proteins are located in the mitochondrial outer membrane and the endoplasmatic reticulum (Borgese *et al.*, 1993).

Investigation of the aerobic reduction of amidoxime structures led to the discovery of a hitherto unknown molybdenum-containing enzyme system (Havemeyer *et al.*, 2006). It was named “mitochondrial amidoxime reducing component” (mARC), because initially *N*-reduction of amidoxime structures was studied with this enzyme purified from mammalian liver mitochondria. After recombinant expression of human mARC (Gruenewald *et al.*, 2008) it became clear that besides sulfite oxidase, xanthine oxidoreductase and aldehyde oxidase, a fourth molybdenum-containing enzyme exists. The human genome encodes for two homologous mARC-proteins, mARC1 and mARC2 (designated as MOSC1 and MOSC2 in the databases). Both mARC homologues are able to reduce benzamidoxime as a model substrate in a reconstituted enzyme system together with NADH cytochrome b_5 and its reductase. We were able to demonstrate that this molybdenum-containing enzyme system is

responsible for amidoxime/*N*-hydroxyguanidine prodrug reduction to the pharmacologically active drug (Gruenewald *et al.*, 2008).

Although *N*-hydroxylated sulfonamides (sulfohydroxamic acids) have considerable potential to treat a variety of disorders, the reductive biotransformations of the compounds have been largely ignored. For example *N*-substituted sulfonamides could act as NO donors (Shirota *et al.*, 1999), carbonic anhydrase (Mincione *et al.*, 1998), and tyrosinase inhibitors (Khan *et al.*, 2005). The recent development of a selective COX-2 inhibitor with a better adverse event profile using valdecoxib as a lead compound led to the corresponding *N*-hydroxylated sulfonamide (Erdelyi *et al.*, 2008). *N*-Hydroxy-valdecoxib is also a known metabolite of valdecoxib in humans (Yuan *et al.*, 2002). Recent *in vivo* studies of analgesic and anti-inflammatory effects, showed that the *N*-hydroxylated analogue is not only more potent it also has a prolonged activity in comparison to valdecoxib. Contrary to expectations, *in vitro* COX-2 inhibitory potency of *N*-hydroxy-valdecoxib was low compared to valdecoxib (Erdelyi *et al.*, 2008). This data suggests that *N*-hydroxy-valdecoxib is bioactivated by reduction *in vivo.* Thus, *N*-hydroxy-valdecoxib could serve as a prodrug (Fig. 1). If enzymatic metabolism is necessary for the pharmacological effect of valdecoxib, it is necessary for further drug development to identify the reductive enzymes involved so as to be able to evaluate potential interspecies variability, genetic polymorphisms, and drug-drug interactions.

In the current study, the *N*-reductive substrate metabolism of *N*-hydroxy-sulfonamides in the recombinant and purified native porcine liver mARC containing enzyme system is characterized by evaluating their kinetic parameters K_m and V_{max}. Furthermore, conversion rates of *N*-hydroxy-sulfonamide reduction by porcine liver mitochondria were compared with results obtained with purified native and recombinant enzymes.

In continuation of our drug metabolism studies this report demonstrates that the described enzyme system is also able to reduce *N*-hydroxy-sulfonamides such as *N*-hydroxy-valdecoxib to their corresponding sulfonamides.

Methods

Chemicals. *N*-Hydroxy-benzenesulfonamide (NOHBSA) and *N*-hydroxy-4-toluenesulfonamide (NOHTSA) were synthesized according to the following procedure: 2.0 g (29 mmol) hydroxylamine-HCl was dissolved in refluxing methanol and a solution of 0.6 g (26 mmol) sodium in 12.0 ml ethanol was added. After cooling, the precipitated sodium chloride was filtered off and in the remaining solution 1.7 g (10 mmol) of benzenesulfonyl chloride or 1.9 g (10 mmol) of toluenesulfonyl chloride was added. Most of the methanol was evaporated. The remainder was filtered and the solution was evaporated to dryness *in vacuo*. The solid thus obtained was extracted three times with 5.0 ml warm diethylether. Evaporation of the solvent afforded NOHBSA (m.p. 120°C, Smith and Hein, 1960) or NOHTSA (m.p. 143-144°C, Przybylski J and Kupryszwski, 1975). In the same way the corresponding sulfonamides benzenesulfonamide (BzSA) and 4-toluenesulfonamide (TSA) were obtained: 1.0 ml (8 mmol) benzenesulfonyl chloride or 1.5 g (8 mmol) toluenesulfonyl chloride were heated with 5.0 ml concentrated ammonia solution for 15 min. Upon dilution with water the corresponding sulfonamides precipitated (BzSA , m.p. 153°C (Johnson *et al.*, 1978) and TSA, m.p. 141-142°C (Mcfarland *et al.*, 1987)). The solid was recrystallized with ethanol and washed. *N*-Hydroxy-valdecoxib and valdecoxib were prepared according to the literature (Erdelyi *et al.*, 2008, Talley *et al.*, 1996). Benzamidoxime (*N*-hydroxy-benzenecarboximidamide) was synthesized from benzonitrile and hydroxylamine as described previously (Krüger, 1885). All other chemicals were obtained from Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or Promochem (Wesel, Germany).

Preparation of subcellular fractions. Mitochondria and microsomes were prepared from porcine liver (Clement *et al.*, 2005, Hovius *et al.*, 1990).

Purification of native enzymes. Cytochrome b_5 was purified from porcine liver microsomes as described previously (Taniguchi *et al.*, 1984; Clement *et al.*, 1997). NADH cytochrome b_5 reductase was purified from porcine liver microsomes similar to the procedure described for the purification of NADH P450 reductase (Yasukochi and Masters, 1976). MARC was purified from porcine liver mitochondria as described previously (Havemeyer *et al.*, 2006)

Expression and purification of recombinant human mARC-1 and mARC-2. Full-length open reading frames of human *mARC1* and *mARC2* cDNAs of 1011 and 1105 base-pairs, respectively, were truncated by PCR to remove the coding sequence for the putative NH_2-terminal mitochondrial targeting sequences (according to GenPept accessions NP_073583 and NP_060368). The resulting 286 (mARC1) and 285 (mARC2) amino acids encoding cDNAs were cloned into the pQE80 expression plasmid (Qiagen, Hilden, Germany) and proteins were expressed in *E. coli* TP1000 cells (Palmer *et al.*, 1996) and purified as described previously for full-length mARC1 (Gruenewald *et al*., 2008).

Expression and purification of the N-terminally truncated recombinant proteins is described by Kotthaus *et al.* (paper submitted).

Expression and purification of recombinant human cytochrome b_5 and cytochrome b_5 reductase. Expression of C-terminally truncated human cytochrome b_5 (GenPept accession NP_085056) and human cytochrome b_5 reductase isoform 2 (GenPept accession NP_015565) from expression plasmid pQE80 (Qiagen, Hilden, Germany) in *Escherichia coli* DL41 cells and purification of the resulting recombinant protein was performed according to Kurian *et al.* (2004). For some incubation studies commercial available cytochrome b_5 (MoBiTec GmbH, Göttingen, Germany) was used.

Determination of protein concentration. Protein concentrations were determined using a BCA protein assay Kit (Pierce, Rockford, USA) following the manufacturer's instructions or using Roti Quant solution (Roth, Karlsruhe, Germany) according to Bradford (1976).

Enzyme assays. Cytochrome b_5 was estimated from the difference spectra between an oxidized and NADH reduced preparation (Estabrook and Werringloer, 1978). *NADH cytochrome b_5 reductase* was determined by a modification of the ferricyanide reduction assay (Mihara and Sato, 1978).

SDS-PAGE - SDS-PAGE was carried out using a separation gel containing 12% polyacrylamide (Laemmli, 1970). Silver staining was performed according to the manufactor's directions (Silver Staining Kit, Protein Plus One, GE Healthcare). Standards and samples were pretreated with ß-mercaptoethanol for 5 min at 100°C.

Molybdenum Cofactor Analysis—To identify the molybdenum cofactor coordinated by the purified mARC enzyme (porcine liver), the *nit-1* reconstitution assay was used as described previously (Havemeyer *et al*., 2006; Nason *et al*., 1971).

Incubations with N-hydroxylated substrates. Incubations were carried out under aerobic conditions at 37°C in a shaking water bath. Incubation mixtures contained 0.5 mM or 2 mM substrate and 1.0 mM NADH in a total volume of 150 µl 100 mM potassium phosphate buffer, pH 6.0 or pH 6.3. After a preincubation period of 3 minutes at 37°C the reaction was initiated by addition of NADH and terminated after 15 – 30 minutes by addition of acetonitrile. Precipitated proteins were sedimented by centrifugation and the supernatant was analyzed by HPLC. Incubation mixtures with native mARC (porcine liver) consisted of 200 pmol cytochrome b_5 (porcine liver or recombinant protein), 0.05 units NADH cytochrome b_5 reductase (porcine liver), and 0.2 µg - 3 µg molybdenum enzyme. Minor contaminations of sulfonamide in the substrate were subtracted for calculation of the enzymatic conversion rates. Incubation mixtures with recombinant mARC1 or mARC2 consisted of 200 pmol cytochrome b_5 (recombinant protein), 0.05 units NADH cytochrome b_5 reductase (porcine liver or recombinant protein), and 10 - 30 µg molybdenum enzyme. Incubation mixtures of porcine

liver mitochondria contained 50 µg protein. Apparent kinetic parameters K_m and V_{max} were estimated using nonlinear regression analysis (Sigma Plot 5.0; SPSS Science, Chicago, IL).

HPLC Method for the separation of N-hydroxyvaldecoxib and valdecoxib. Separation was carried out isocratically with 62% (v/v) acetonitrile and 38% (v/v) water pH 3.2 using a Symmetry® C18 Column, 5 µm, 4.6 x 250 mm (Waters, Milford, USA) with a security guard cartridge system C18, 3 x 4 mm (Phenomenex, Torrance, USA) as precolumn at a flow rate of 1.0 ml/min. The effluent was monitored at 240 nm. The retention times were 20.0 ± 0.2 min (*N*-hydroxy-valdecoxib) and 17.6 ± 0.1 min (valdecoxib).

HPLC Method for the separation of NOHBSA and BzSA or NOHTSA and TSA . The separation was carried out isocratically with 5% (v/v) acetonitrile and 95% (v/v) 20 mM phosphate buffer pH 4.0 (separation of NOHBSA and BzSA) or 10% (v/v) acetonitrile and 90% (v/v) 20 mM phosphate buffer pH 4.0 (separation of NOHTSA and TSA) using a LiChroCART® 125-4 HPLC Cartridge with LiChrospher® RP-select B (5 µM) and a LiChroCART 4-4 guard column (Merck) as precolumn at a flow rate of 1.0 ml/min. The effluent was monitored at 220 nm or 226 nm. The retention times were 8.2 ± 0.5 min (NOHBSA), 11.4 ± 0.6 min (BzSA), 19.3 ± 0.4 min (NOHTSA) and 14.3 ± 0.3 min (TSA).

HPLC Method for the separation of benzamidoxime and benzamidine. The separation was carried out as previously described (Clement *et al.*, 2005).

Results and Discussion

N-Hydroxy-valdecoxib is a novel COX-2 inhibitor in preclinical development. Although it shows only low COX-2 activity *in vitro*, it has good analgesic and anti-inflammatory potency *in vivo* (Erdelyi *et al.*, 2008). Therefore, a simple *in vitro* assay in an early state of drug development was needed to confirm the presumed *in vivo* reduction of *N*-hydroxy-valdecoxib to its active sulfonamide structure (Fig. 1). In this report we demonstrate the reduction of several *N*-hydroxy-sulfonamides (Fig. 2) including *N*-hydroxy-valdecoxib to their corresponding sulfonamides by porcine liver mitochondria. The following V_{max} values were determined: 7.4 nmol sulfonamide/min/mg mitochondrial protein (reduction of *N*-hydroxy-valdecoxib, K_m = 0.6 mM), 15.6 nmol sulfonamide/min/mg mitochondrial protein (reduction of NOHTSA, K_m = 1.1 mM) and 20.6 nmol sulfonamide/min/mg mitochondrial protein (reduction of NOHBSA , K_m = 2.4 mM).

In conclusion, the analgesic and anti-inflammatory properties of *N*-hydroxy-valdecoxib together with its weak *in vitro* effect (Erdelyi *et al.*, 2008) could be explained by the reduction of *N*-hydroxy-valdecoxib to the pharmacological active sulfonamide. However, the formation of valdecoxib after *in vivo* administration of its *N*-hydroxysulfonamidederivative should also be demonstrated in further studies. In order to identify the enzyme system involved in the reduction of *N*-hydroxy-valdecoxib, the contribution of the molybdenum-containing enzyme system consisting of mARC, cytochrome b_5, and NADH cytochrome b_5 reductase was included in this study.

For this purpose the native mARC enzyme was purified from porcine liver mitochondria. The SDS-PAGE (Fig. 3) analysis showed an electrophoretically pure protein with the expected molecular weight of 35 kDa (Havemeyer *et al.*, 2006). The native enzyme was further characterized by its ability to reduce benzamidoxime in a reconstituted enzyme system together with NADH cytochrome b_5 and its reductase following Michaelis-Menten kinetic

(K_m = 0.2 mM and V_{max} = 2.0 µmol/min/mg total protein). Additionally the presence of Moco was verified. The most sensitive assay for the detection of biologically active Moco is the so-called *nit-1* assay (Nason *et al.*, 1971), which is based on the ability of any eukaryotic Moco source to complement the apo-NADPH nitrate reductase in crude extracts of the *N. crassa nit-1* mutant, which is deficient in Moco biosynthesis, thus reconstituting apo-NADPH nitrate reductase activity. The purified mARC enzyme was able to reconstitute apo-NADPH nitrate reductase activity with a specific activity of 1.7 ± 0.2 µmol nitrite/mg/min, confirming that this enzyme is a Moco-binding protein.

The reduction of *N*-hydroxy-valdecoxib to valdecoxib by native mARC (purified from porcine liver mitochondria) followed Michaelis-Menten kinetic with K_m = 1.5 mM and V_{max} = 51.2 nmol/min/mg total protein. Thus, the specific activity was 7-fold enriched in the purified system compared to porcine liver mitochondria, with similar Michaelis-Menten constants (K_m values)

Involvement of mARC in *N*-hydroxy-sulfonamide reduction was further verified by using recombinant enzymes which also catalyzed this transformation (Table 1, 2). Either no or only very little activity was detected if the two component enzyme system consisted of only cytochrome b_5 and its reductase was incubated (max. 1.7 ± 0.5 nmol sulfonamide/min/mg total protein). Regarding the K_m values, no substrate specificity for *N*-hydroxy-sulfonamides with both mARC homologues could be detected, but efficiency of catalysis (V_{max}/K_m) is higher for mARC-1 than for mARC-2 (Table 2).

It is not surprising, that conversion rates of the recombinant molybdenum enzymes were 2 – 30-fold lower than that of purified native mARC protein from porcine liver mitochondria (Table 1) or than that of mitochondria. These varying rates result may be due to the different origins of the mARC proteins (cDNA-expressed versus purified from porcine liver), which may be crucial for the absolute enzymatic activity. Perhaps, imperfect folding and/or a lack of

post-translational modifications will effect the activity. Furthermore, removal of the predicted N-terminal mitochondrial targeting sequences from recombinant mARC enzymes as well as removal of the hydrophobic anchor sequences of cytochrome b_5 and its reductase may influence efficiency. It can be assumed that electron transfer proteins must interact in a highly specific manner to allow effective electron transfer and that the hydrophobic domains afford an optimal complex formation of the enzymes. Nevertheless, the role of hydrophobic domains as well as the optimal stoichiometry of the three enzymes affecting conversion rates requires further analysis.

We cannot exclude the possibility that additional *N*-reductive enzymes may still play a minor role in the activation of *N*-hydroxy-valdecoxib. However, drug-drug interactions are unlikely to occur, because the prodrug-activation principle is not dependent on P450 enzymes.

In summary, sufficient *in vitro* metabolism of *N*-hydroxy-sulfonamides including *N*-hydroxy-valdecoxib could be detected using recombinant enzymes. Therefore the current study confirms the usefulness of the described recombinant enzymes for obtaining *in vitro* data for drug metabolism (Table 1, 2).

The reduction of *N*-hydroxy-sulfonamides supports previous studies (Gruenewald *et al.*, 2008) stating that mARC plays a key role in the reductive biotransformation of structurally diverse *N*-hydroxylated compounds.

Thus, the molybdenum-containing enzyme mARC is a protein should be added to the list of prominent drug-metabolizing enzymes.

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Footnotes

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Legends for Figures

FIG. 1. Prodrug principle of *N*-hydroxy-valdecoxib

FIG. 2. Reduction of *N*-hydroxy-sulfonamides

N-hydroxy-benzenesulfonamide (NOHBSA) to benzenesulfonamide (BzSA), (R = - H)

N-hydroxy-4-toluenesulfonamide (NOHTSA) to toloulsulfonamide (TSA), (R= -CH_3)

FIG. 3. SDS-PAGE and silver staining of purified mARC (porcine liver)

Native mARC (0.5 μg) purified by ion exchange chromatography was electrophoresed on a 12 % gel. (M = molecular weight marker; masses are indicated in kDa)

TABLE 1

Reduction of N-hydroxy-sulfonamides

substrate	specific activity nmol sulfonamide/min/mg total protein		
	native mARC	mARC-1	mARC-2
N-hydroxyvaldecoxib	32.3 ± 2.5	4.0 ± 0.2	1.7 ± 0.2
NOHBSA	30.9 ± 2.4	2.1 ± 0.1	0.9 ± 0.1
NOHTSA	6.2 ± 0.6	3.0 ± 0.1	3.3 ± 0.4

A complete incubation mixture consisted of 200 pmol cytochrome b_5, 0.05 units NADH cytochrome b_5 reductase, and 2,4 - 3 µg native mARC (purified from porcine liver mitochondria) or 10 -30 µg mARC-1 or mARC-2 (human, recombinantly expressed), 0.5 mM *N*-hydroxyvaldecoxib or 2 mM NOHBSA respectively NOHTSA and 1.0 mM NADH in a total volume of 150 µl 100 mM potassium phosphate buffer. Sample preparation and HPLC analysis were described at *Materials and Methods*. Data are means ± SD of two determinations.

TABLE 2

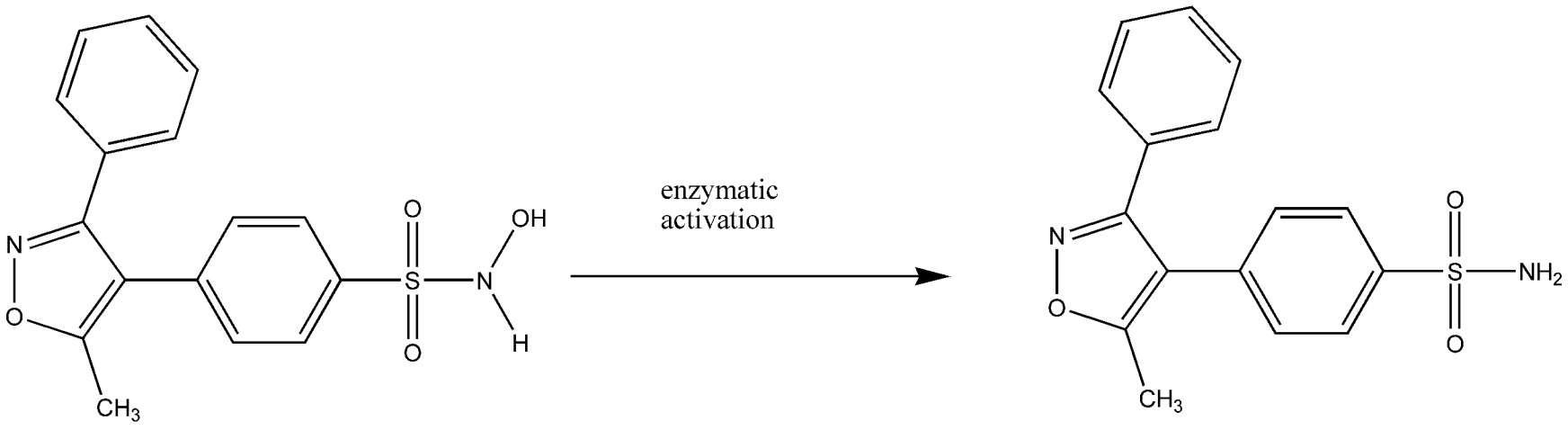
Kinetic parameters of the reduction of N-hydroxy-sulfonamides to sulfonamide

substrate	Parameters	mARC-1	mARC2
N-hydroxy-valdecoxib	K_m[a]	0.5	0.4
	V_{max}[b]	4.6	1.3
	efficiency[c]	9.2	3.3
NOHBSA	K_m[a]	1.0	1.9
	V_{max}[b]	6.4	8.1
	efficiency[c]	6.4	4.3

[a] mM

[b] nmol/min/mg total protein

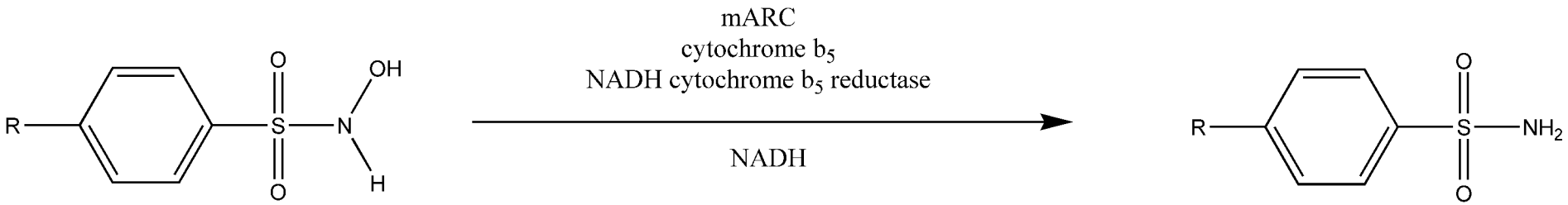
[c] efficiency of catalysis (V_{max}/K_m)



N-hydroxy-valdecoxib
- prodrug -

Fig. 1

valdecoxib
- active principle -



N-hydroxy-sulfonamide

sulfonamide

Fig. 2

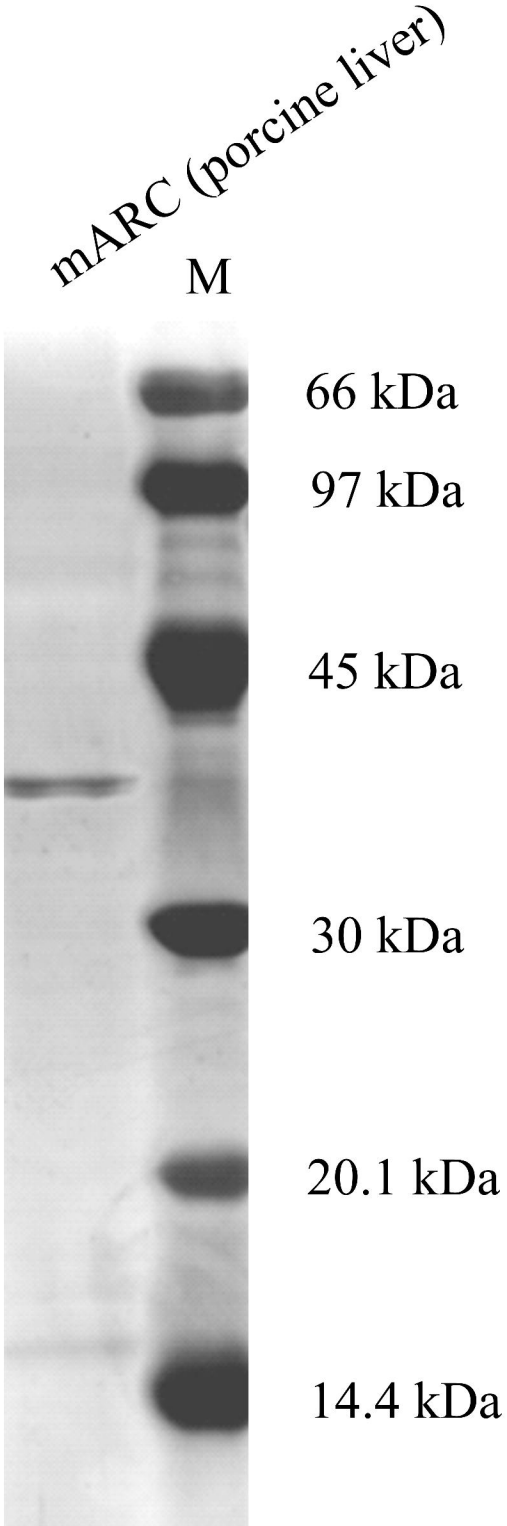


Fig. 3