

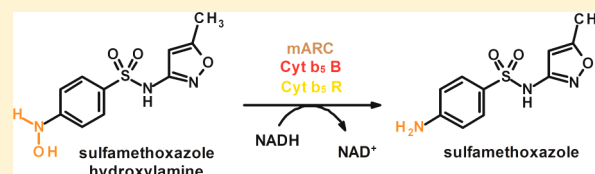
Reduction of Sulfamethoxazole Hydroxylamine (SMX-HA) by the Mitochondrial Amidoxime Reducing Component (mARC)

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ABSTRACT: Under high dose treatment with sulfamethoxazole (SMX)/trimethoprim (TMP), hypersensitivity reactions occur with a high incidence. The mechanism of this adverse drug reaction is not fully understood. Several steps in the toxification pathway of SMX were investigated. The aim of our study was to investigate the reduction of sulfamethoxazole hydroxylamine (SMX-HA) in this toxification pathway, which can possibly be catalyzed by the mARC-



containing N-reductive enzyme system. Western blot analyses of subcellular fractions of porcine tissue were performed with antibodies against mARC-1, mARC-2, cytochrome *b*₅ type B, and NADH cytochrome *b*₅ reductase. Incubations of porcine and human subcellular tissue fractions and of the heterologously expressed human components of the N-reductive enzyme system were carried out with SMX-HA. mARC-1 and mARC-2 knockdown was performed in HEK-293 cells. Kinetic parameters of the heterologously expressed human protein variants V96L, A165T, M187 K, C246S, D247H, and M268I of mARC-1 and G244S and C245W of mARC-2 and N-reductive activity of 2SF, D14G, K16E, and T22A of cytochrome *b*₅ type B were analyzed. Western blot analyses were consistent with the hypothesis that the mARC-containing N-reductive enzyme system might be involved in the reduction of SMX-HA. In agreement with these results, highest reduction rates were found in mitochondrial subcellular fractions of porcine tissue and in the outer membrane vesicle (OMV) of human liver tissue. Knockdown studies in HEK-293 cells demonstrated that mARC-1 and mARC-2 were capable of reducing SMX-HA in cell metabolism. Investigations with the heterologously expressed human mARC-2 protein showed a higher catalytic efficiency toward SMX-HA than mARC-1, but none of the investigated human protein variants showed statistically significant differences of its N-reductive activity and was therefore likely to participate in the pathogenesis of hypersensitivity reaction under treatment with SMX.

INTRODUCTION

The mARC-containing N-reductive enzyme system consists of one of the two mARC paralogues, mARC-1 (NP_073583.3) or mARC-2 (NP_060368.2), cytochrome *b*₅ type B (NP_085056.2), and NADH cytochrome *b*₅ reductase (NP_015565.1).¹ This enzyme system is able to catalyze the reduction of N-hydroxylated substrates in the presence of NADH.² Several studies provided evidence that these structures are reduced independent of the rest of their structural formula like benzamidoxime, *N*-hydroxymelagatran, *N*-hydroxysulfonamides, *N*-hydroxy-valdecoxib, *N*-hydroxylated nucleobases and nucleosides, upamostat (Mesupron), *N*-oxides, oximes, or *N*-hydroxyamidinohydrazones.^{3–8} This variety of substrates underlines the role of the mARC-containing enzyme system in drug development and detoxification.^{7,9} A putatively physiologic substrate of the mARC-containing N-reductive enzyme system is the nitric oxide precursor *N*^ω-hydroxy-L-arginine (NOHA), which is involved in the complex regulation of nitric oxide biosynthesis.¹⁰ All substrates are reduced under aerobic conditions.² Recently, the involvement of the mARC-containing N-reductive enzyme system in the reduction of nitrite to NO under anaerobic conditions was demonstrated.¹¹

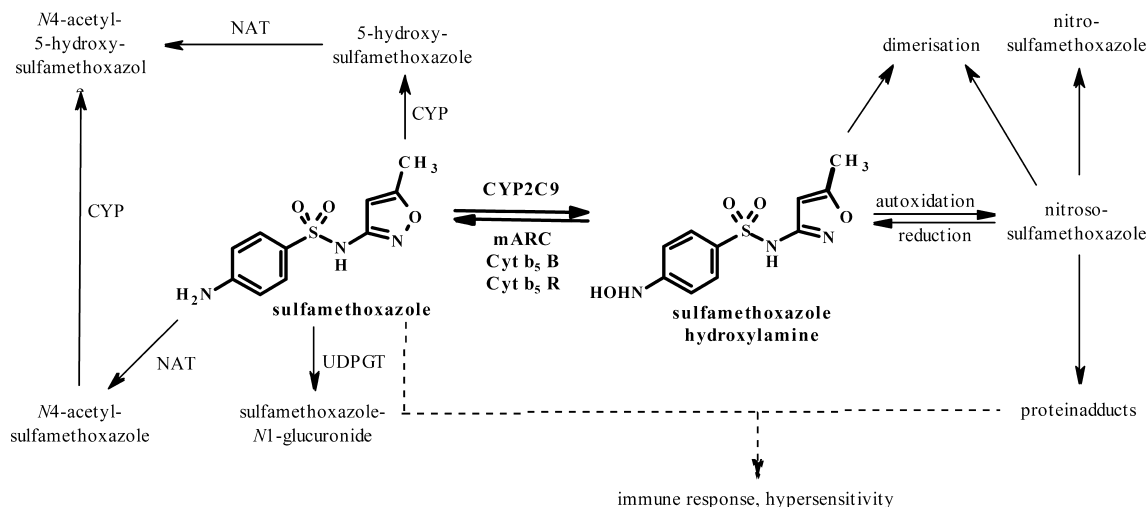
Metabolites of approved drugs or the parent drugs themselves that contain a primary aromatic amine structure are often associated with a high incidence of idiosyncratic drug reactions.¹²

For example, the 5-amino-2-nitrobenzotrifluoride metabolite of the approved drug flutamide is a primary aromatic amine metabolized to *N*-hydroxy-5-amino-2-nitrobenzotrifluoride, which is considered to be involved in the hepatotoxicity of flutamide.¹³ SMX is an example of an approved drug containing a primary aromatic amine structure that is metabolized to SMX-HA, a metabolite causing hypersensitivity reactions.

SMX is administered in a fixed combination with TMP for the treatment of uncomplicated urinary tract infections and in higher doses for the prophylaxis of *Pneumocystis jirovecii* pneumonia. Patients receiving high-dose SMX/TMP show a significantly higher incidence of adverse drug reactions; especially HIV-infected patients show more frequent and more severe reactions, for example, the potentially life threatening Stevens-Johnson syndrome (SJS).^{14,15} Actually, SMX/TMP is also one alternative in the treatment of

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Scheme 1. Possible Participation of the mARC-Containing Enzyme System in the Metabolism of SMX^a

^aMetabolism and toxification pathways of SMX according to van der Ven et al.²⁴ and Sanderson et al.⁴⁸ Abbreviations: CYP, cytochrome P450; Cyt b₅ B, cytochrome b₅ type B; Cyt b₅ R, NADH cytochrome b₅ reductase; NAT, N-acetyltransferase; UDPGT, uridine 5'-diphosphoglucuronosyltransferase.

community acquired methicillin-resistant *Staphylococcus aureus* (MRSA) skin and soft-tissue infections.¹⁶

Much research is done to explain the mechanism of adverse drug reactions under SMX, especially the hypersensitivity reaction. Polymorphisms in several genes encoding enzymes in the metabolic pathway of SMX were investigated, for example, CYP2C9, GSTM1, GSTT1, GSTP1, and NAT2, the slow acetylator genotype and phenotype, CYB5A, CYB5R3, and NAT2, the NAT1 alleles *10 and *11, NAT2, and the glutamate cysteine ligase catalytic subunit (GCLC).^{17–23} All findings of these and many other studies enable a multifactorial pathogenesis and not one explanatory cause.

SMX is metabolically inactivated by cytochrome P450 monooxygenases, UDP-glucuronosyltransferases, and N-acetyltransferases 1 and 2.²⁴ The latter represent the enzymes of the main metabolic route of SMX. These enzymes acetylate the drug to the inactive metabolite N-acetyl-sulfamethoxazole.^{25,26} In addition, a toxification pathway is known that consists of several steps leading to a redox-cycle.²⁷ SMX can be oxidized by CYP2C9 to the toxic metabolite SMX-HA.^{25,28} In the following step, SMX-HA is autoxidized to the nitroso intermediate, which is highly reactive and able to bind to cellular proteins, finally leading to an immune response.^{25,29–31} On the other hand, nitroso-SMX can be reduced by glutathione to SMX-HA, which can be detoxified to SMX by reduction.²⁹ This step can possibly be catalyzed by the mARC-containing N-reductive enzyme (Scheme 1).

SMX-induced hypersensitivity is viewed as an idiosyncratic adverse drug reaction.²³ Polymorphisms in genes that encode proteins involved in the reduction of SMX-HA to SMX might be able to lead to increased toxicity. This reduction has been investigated intensively regarding participation of the enzymes cytochrome b₅ type A and NADH cytochrome b₅ reductase and regarding the influence of some variant proteins.^{19,32} Ten years ago, we already demonstrated that the reduction of SMX-HA is catalyzed by porcine and human subcellular liver fractions.³³ After discovering the mARC-containing enzyme system in our laboratory, which is responsible for the reduction of N-hydroxylated substrates, and based on the finding that it consists of the components mARC, cytochrome b₅ type B, and

NADH cytochrome b₅ reductase, we aimed to add a more detailed investigation of the reduction of SMX-HA with particular focus on a possible contribution of the mARC-containing enzyme system.^{1,34,35} Moreover, the influence of protein variants encoded by nonsynonymous SNPs on this reduction was likewise investigated in constitutive studies.

MATERIALS AND METHODS

Chemicals. SMX-HA was purchased from Dalton Pharma Services (Toronto, Canada) and SMX from Sigma-Aldrich (Steinheim, Germany).

Preparation of Subcellular Fractions. Subcellular fractions of porcine liver and thyroid gland (i.e., mitochondria, microsomes, cytosol, and homogenate and post nuclear supernatant (PNS), respectively) were prepared by gradient centrifugation.^{7,36} Human liver was obtained from the University of Kiel, Medical Department. Prior consent of the local ethics committee and from the donors before removal of the liver pieces was granted.³³ Subcellular fractions of human liver (i.e., outer membrane vesicle (OMV), mitochondria, and homogenate) were prepared from pooled livers of cancer patients in a similar manner to the method described by de Kroon et al.³⁷

SDS PAGE and Western Blot Analysis. Protein (10, 12, or 15 μg) of each subcellular fraction of porcine thyroid gland and 25 μg of protein of the total cellular protein of knockdown studies were separated on a 12.5% SDS-PA gel under reducing conditions.³⁸ Separated proteins were transferred to a PVDF membrane (Hybond-P, Amersham GE Healthcare), blocked, and incubated with primary antibodies against MOSC1 (=mARC-1, 1:10000 for the analysis of subcellular fractions and 1:500 for the analysis of knockdown; Abgent, San Diego, CA), MOSC2 (=mARC-2, 1:10000 for the analysis of subcellular fractions and 1:500 for the analysis of knockdown, Sigma-Aldrich, Taufkirchen, Germany), Cyt b₅ B (1:10000; Sigma-Aldrich, Taufkirchen, Germany), Cyt b₅ R3 (1:1000; Acris antibodies, Herford, Germany), VDAC2 (voltage dependent anion channel 2, 1:1000, Acris antibodies, Herford, Germany), calnexin (1:10000; Acris antibodies, Herford, Germany), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 1:8000; Sigma-Aldrich, Taufkirchen). Incubation with secondary horseradish peroxidase conjugated anti-rabbit IgG or anti-goat IgG antibodies (1:10000; Jackson Immune Research Laboratories, Suffolk United Kingdom) followed. Chemiluminescence was detected by using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, UNK) according to the manufacturer's protocol.

Expression, Purification and Determination of Protein, Molybdenum, Heme and FAD Content of Recombinant Proteins. Expression of mARC-1 and mARC-2 followed standard protocol. Variants of *MARCI*, *MARC2*, and *CYBSB* were generated by PCR mutagenesis using primers carrying the desired mutation. Accuracy of PCR mutagenesis was confirmed by DNA sequencing (GATC, Konstanz, Germany). mARC-1 and mARC-2 wild-type proteins, variants V96L, A165T, M187 K, C246S, D247H, and M268I of mARC-1, and variants G244S and C245W of mARC-2 were expressed in *Escherichia coli* TP 1000 cells, an *E. coli* strain that is able to express the eukaryotic form of the molybdenum cofactor (Moco). Expression of cytochrome *b₅* type B wild-type protein and variants 25F, D14G, K16E, and T22A was performed in *E. coli* DL41. Cells were supplemented with 1 mM aminolevulinic acid to support heme synthesis. Expression of NADH cytochrome *b₅* reductase was also performed in *E. coli* DL41. Expressions in *E. coli* DL41 followed the method described by Kurian et al.^{6,32,39}

Protein concentrations were determined by BCA assay (Pierce BCA Protein Assay Kits, Thermo Scientific, USA) after precipitating proteins using Compat-Able protein assay preparation reagent set (Thermo Scientific). Measurement of each duplicate was repeated twice.

Molybdenum content of the mARC proteins was measured by inductively coupled plasma mass spectrometry (ICP-MS). Measurement of each duplicate was repeated three times.

Heme content was determined by measurement of difference spectra according to Estrabrook and Werringloer, which is based on the reduction of cytochrome *b₅* type B in the presence of NADH cytochrome *b₅* reductase by NADH (Merck, Darmstadt, Germany).⁴⁰ The assay was performed in duplicate.

FAD content of NADH cytochrome *b₅* reductase was determined according to Whitby.⁴¹ The assay was performed in duplicate.

A detailed description of all methods was published.³⁹

Determination of mARC-Catalyzed N-Reduction of SMX-HA.

Incubation mixtures with subcellular fractions contained 0.01–0.2 mg of protein. Incubation mixtures with recombinant expressed proteins were adjusted on the cofactors and consisted of mARC containing 120 pmol of molybdenum, cytochrome *b₅* type B containing 60 pmol of heme, and NADH cytochrome *b₅* reductase containing 6 pmol of FAD. Incubation mixtures contained different substrate concentrations in 100 mM potassium phosphate buffer, pH 6.0 (subcellular fractions), or 20 mM 4-morpholineethanesulfonic acid (MES), pH 6.0 (recombinant proteins), 1 mM ascorbic acid, and 6.6% DMSO. Incubations were performed under aerobic conditions at 37 °C. After 3 min of preincubation, the reaction was started by addition of 1 mM NADH and stopped after 20 min by addition of ice-cold methanol. Precipitated proteins were separated by centrifugation (5 min, 10000 rpm), and the amount of SMX in the supernatant was determined by high-performance liquid chromatography (HPLC). Incubations were carried out in duplicate, each of which was measured twice. Each incubation was carried out in duplicate containing an external standard with an amount of 5 μM SMX, each of which was measured twice. Determination of kinetic parameters showed that the reduction followed Michaelis–Menten kinetics. Initially, data was checked for substrate inhibition by using Lineweaver–Burk plot. Kinetic parameters V_{max} and K_m were determined through Michaelis–Menten plot using SigmaPlot 11.0. In cases of substrate inhibition, kinetic parameters were calculated from data in the linear range of the Lineweaver–Burk plot.

HPLC Method for the Separation of SMX-HA and SMX. The HPLC system consisted of a Waters 1525 HPLC pump, a Waters 717 autosampler, and a Waters 2487 dual absorbance detector combined with Waters Breeze software, version 3.30. HPLC analysis was performed on a Waters Symmetry C18 column (5 μm, 250 mm × 4.5 mm) and a Phenomenex Security Guard Cartridge System C18 (4 mm × 3.0 mm). Elution was carried out isocratically with acetonitrile/water/pure acetic acid/triethylamine (30:68.5:1:0.5, v/v/v/v). pH was not adjusted. The flow rate was kept at 0.6 mL/min, and detection was carried out at 254 nm. The recovery rate and quantification limit of the metabolite SMX were determined using incubation mixtures with

defined concentrations of the synthetic reference substance (from 0.05 to 500 μM), which were incubated and worked up under the same conditions as the experimental samples without adding cofactor and using heat-denatured protein. The standard curves were linear in this range with regression coefficients of 0.999 for SMX. Quantification was performed on the basis of peak area. The obtained signals were compared with those of the same amount of SMX dissolved in the mobile phase. The recovery rate after incubation and sample work up was 104 ± 12%. The detection limit was about 0.5 μM. Characteristic retention times were 10.3 ± 0.5 min for SMX-HA and 12.9 ± 0.6 min for SMX.³³

Cell Line and Reagents for Knockdown Experiments. HEK-293 human embryonic kidney cells were purchased from Cell Lines Service (Eppelheim, Germany). Opti-MEM, minimum essential medium, sodium pyruvate solution, sodium bicarbonate, minimum Eagles's medium nonessential amino acids, FBS, trypsin, L-glutamine, PBS, Lipofectamine RNAiMAX, Stealth Select RNAi siRNA targeting human MOSC1 (MOSC1HSS127704), and Stealth Select RNAi siRNA negative control were obtained from Invitrogen. ON-TARGETplus SMARTpool siRNA targeting human MOSC2 was purchased from Thermo Scientific. Complete protease inhibitor cocktail was acquired from Roche Applied Science (Mannheim, Germany).¹

Cell Culture. HEK-293 cells were maintained in minimum essential medium supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate. The cell line was incubated at 37 °C in 5% CO₂.¹

siRNA Transfection and Design of Knockdown Experiments.

HEK-293 cells were reverse transfected according to the manufacturer's transfection protocol from Invitrogen. Briefly, siRNA oligos were diluted in Opti-MEM in 24-well plates and incubated with transfection reagent to form liposome–siRNA complexes. The cells were trypsinized, counted, and suspended in culture medium. The cell suspension was then added to the liposome–siRNA complexes. Effects of the siRNA-mediated knockdown were examined on day 5 after transfection. Negative controls included a nontargeting siRNA control (scrambled siRNA) and transfection reagents without siRNA. Down-regulation of protein expression was verified by Western blot analysis.¹

Determination of the N-reduction of SMX-HA in HEK 293 cells.

For N-reduction studies, culture medium was removed, and cells were carefully washed and subsequently preincubated with substrate-free incubation buffer (Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4) at 37 °C for 10 min. Then 250 μL of incubation buffer containing 3 mM SMX-HA, 0.5% DMSO, and 1 mM ascorbic acid was added to each well simultaneously, and cells were incubated at 37 °C for 180 min. After the designated time, incubation medium was carefully removed and centrifuged to eliminate cellular contaminants and debris. The supernatant was analyzed by HPLC as described above.¹

Total Cellular Protein Extraction. To harvest cellular protein, the medium was removed, and cells were washed with ice-cold PBS and collected by centrifugation. After PBS was removed, cells were resuspended in ice-cold lysis buffer (1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0, protease inhibitor cocktail) and shaken for 60 min at 4 °C. Then cell lysates were cleared by centrifugation. The supernatant contained the total cellular protein extract.¹

Statistical Analysis. Data analysis of statistically significant differences was performed with SigmaPlot 11.0 using *t* test or one way ANOVA followed by a suitable post-hoc test with *p* < 0.05 considered significant.

RESULTS

HPLC Method for the Separation of SMX-HA and SMX.

The HPLC method for the separation and quantification of SMX-HA and SMX is based on methods published earlier.³³ Solubility of the SMX-HA was optimized according to Kurian et al.³² The method is characterized by an accurate quantification in the range of 0.05–500 μM SMX in the presence of protein matrix and the cosubstrate NADH/NAD⁺.

The separation of both compounds was achieved within 18 min. A representative chromatogram is shown in Figure 1.

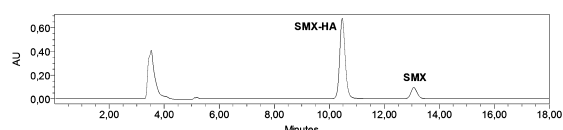


Figure 1. HPLC analysis for the separation of SMX-HA and SMX. Representative chromatogram of an incubation mixture containing human recombinant expressed proteins.

N-Reductive Activity Is Enriched in Mitochondria. The reduction of SMX-HA was investigated in subcellular fractions of (i) porcine liver and thyroid gland and (ii) human liver. Incubations with the purified subcellular fractions led to formation of SMX, which was quantified by HPLC. Mitochondrial fractions of both porcine tissues showed the highest reduction rates (Figure 2A,B). This is in agreement with the expression pattern of the components of the mARC-containing N-reductive enzyme system (Figure 2B). For positive control, incubations with our model substrate benzamidoxime were carried out under the same conditions as incubations with SMX-HA. In these control incubations, N-reductive activity was solely enriched in mitochondrial fractions as well. For both substrates, enrichment ratios were found in the same range. Investigation of subcellular fractions of human liver confirmed the result for porcine tissue. The highest conversion rate of the reduction of SMX-HA was found in OMV of human liver followed by conversion rate of the mitochondrial fraction. In the homogenate, N-reduction of SMX-HA was not detectable (Figure 3). Compared with the model substrate benzamidoxime conversion rates were lower for SMX-HA, but enrichment ratios were found in the same range as well as for the model substrate (data not shown).

Reduction of SMX-HA Requires the Presence of All Three Components of the Human N-Reductive Enzyme System. Studying the reduction of SMX-HA with recombinant expressed human enzymes revealed that the reaction was dependent on NADH and that the highest conversion rates were detectable with the complete enzyme system, that is, mARC-1 or mARC-2, cytochrome b_5 type B, and NADH cytochrome b_5 reductase. Lack of one component or the replacement of cytochrome b_5 type B for cytochrome b_5 type A resulted in a decreased or almost abrogated activity (Figure 4).

mARC-1 and mARC-2 Knockdown in HEK 293-cells. siRNA-mediated mARC knockdown studies were carried out in human cells to find out whether the mARC proteins were involved in the reduction of SMX-HA in these cells. Specific activities in HEK 293-cells were determined on day 5 after transfection. Knockdown of mARC-1 affected the specific activity of the reduction of SMX-HA significantly ($p < 0.001$) compared with negative control (NC). Single knockdown of mARC-2 did not have any influence on the reduction due to the low expression of mARC-2 in HEK 293-cells. Double knockdown of mARC-1 and mARC-2 affected the specific activity of the reduction of SMX-HA significantly ($p < 0.001$) compared with the single mARC-1 knockdown. The siRNA-mediated down regulations of the proteins of interest were verified by Western blot using anti-mARC-1, anti-mARC-2, and anticalnexin antibodies (Figure 5).

Kinetic Parameters of Human mARC WT and Variants. Reduction of SMX-HA was carried out with recombinant

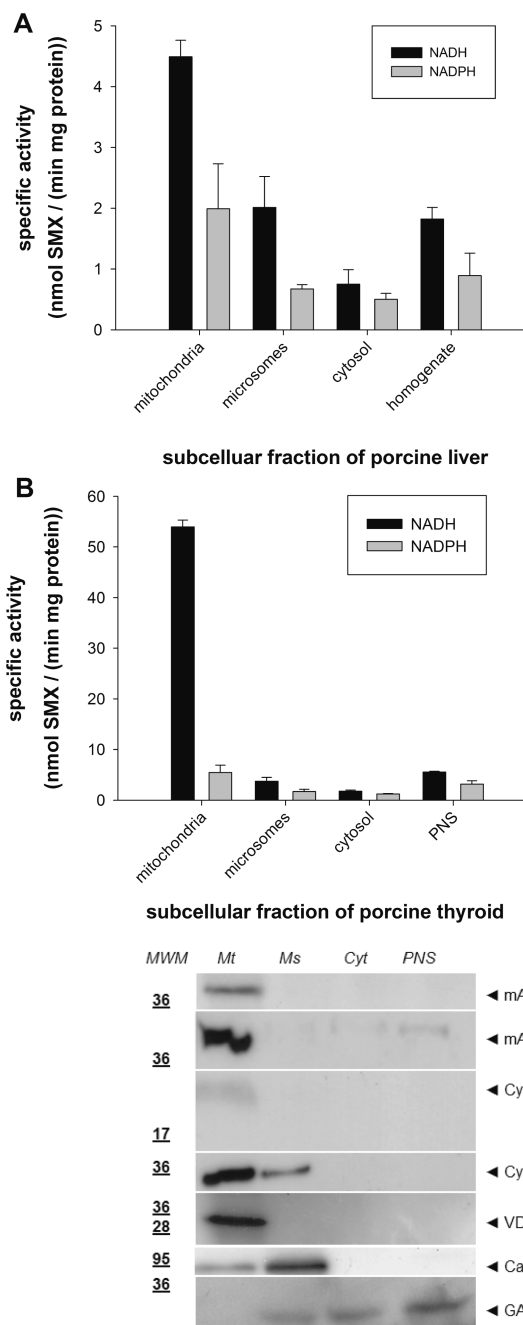


Figure 2. Reduction of SMX-HA is enriched in mitochondrial subfractions of porcine liver (A) and porcine thyroid gland (B). Protein (0.01–0.2 mg) was incubated with 5 mM SMX-HA and 1 mM NADH or NADPH in 100 mM potassium phosphate buffer, pH 6.0, containing 1 mM ascorbic acid and 3.3% DMSO under aerobic conditions. Incubation was carried out in duplicate, each measured twice by HPLC. Western blot analyses of subcellular fractions of porcine thyroid gland showed that the N-reductive activity was in agreement with the presence of the components of the N-reductive enzyme system. Abbreviations: MWM, molecular weight marker; Mt, mitochondria; Ms, microsomes; Cyt, cytosol; PNS, postnuclear supernatant; Hom, homogenate; VDAC, voltage dependent anion channel; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

human mARC wild-type (WT) proteins and variants, cytochrome b_5 type b and NADH cytochrome b_5 reductase and increasing amounts of substrate (0.1–10 mM) in the presence of NADH. Arithmetic means of V_{max} and K_m and their

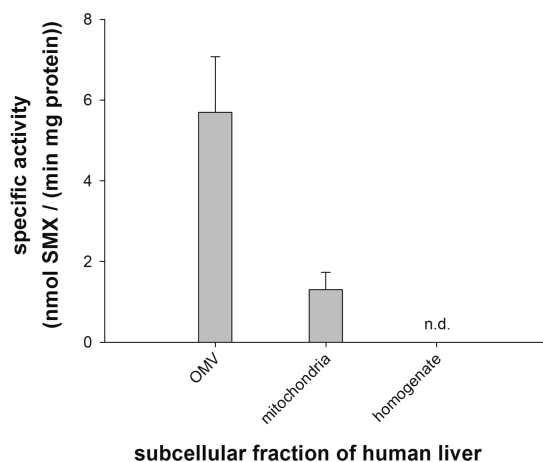


Figure 3. Reduction of SMX-HA is enriched in the mitochondrial subfraction and OMV of human liver. Protein (0.05 mg) was incubated with 5 mM SMX-HA and 1 mM NADH in 100 mM potassium phosphate buffer, pH 6.0, containing 1 mM ascorbic acid and 6.6% DMSO under aerobic conditions. Incubation was carried out in duplicate, each measured twice by HPLC. Abbreviations: OMV, outer membrane vesicle; n.d., not detectable.

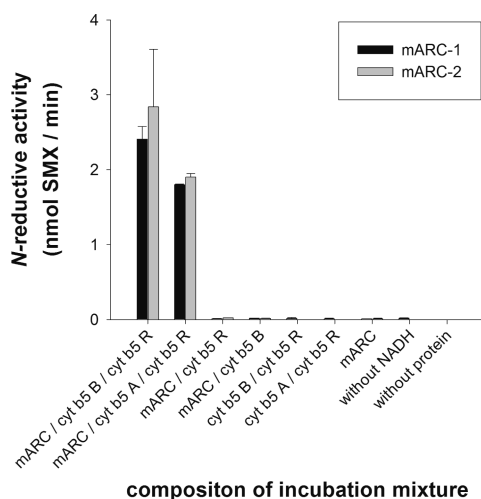


Figure 4. Reduction of SMX-HA requires the presence of all three components of the human N-reductive enzyme system. mARC containing 120 pmol of molybdenum, cytochrome b_5 containing 60 pmol of heme, and NADH cytochrome b_5 reductase containing 6 pmol of FAD were incubated with 3 mM SMX-HA and 1 mM NADH in 20 mM MES buffer, pH 6.0, containing 1 mM ascorbic acid and 6.6% DMSO under aerobic conditions. Data are means of duplicates \pm SD.

standard deviations are shown in Table 1. One way ANOVA did not reveal any statistical differences in the kinetic parameters of the variants compared with WT.

Catalytic efficiency was calculated as k_{cat}/K_m from kinetic parameters. Arithmetic means of the values and their standard deviations are shown in Figure 6. Catalytic efficiency of human mARC-1 WT was $410 \pm 175 \text{ s}^{-1} \text{ M}^{-1}$. Statistically, catalytic efficiency of human mARC-2 WT ($840 \pm 260 \text{ s}^{-1} \text{ M}^{-1}$) was significantly different compared with mARC-1 WT ($p < 0.001$, t test, Sigma-Plot 11.0).

N-Reductive Activity of Incubation Mixtures Containing Human mARC and Cytochrome b_5 Type B Variants. All combinations of human mARC-1 variants V96L, A165T, M187 K, C246S, D247H, and M268I, mARC-2 variants G244S

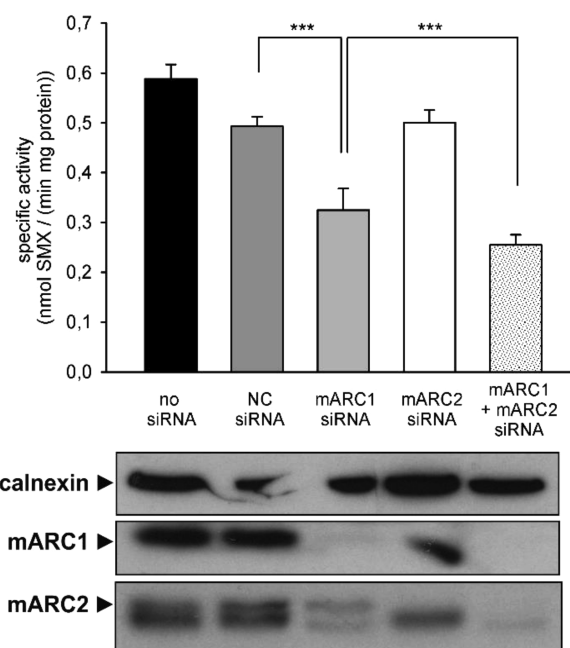


Figure 5. mARC-1 and mARC-2 knockdown in HEK-293 cells. HEK-293 cells were transfected with 20 nM of scrambled (NC) siRNA or mARC-1 or mARC-2 siRNA. For simultaneous knockdown, 10 nM mARC-1 and 10 nM mARC-2 siRNA were transfected. Specific activities in cells were determined as described in Materials and Methods on day 5 after transfection. Results are presented as means \pm SD ($n = 3$): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The siRNA-mediated down regulation of the proteins of interest was verified by Western blot using anti-mARC-1, anti-mARC-2, or anticalnexin (loading control) antibody.

Table 1. V_{max} and K_m Values of the Reduction of SMX-HA by Human mARC WT and Variants

mARC protein	number of protein lots	V_{max} (nmol SMX/(min mg protein))	K_m (mM SMX-HA)
mARC-1			
mARC-1	4	95 ± 40	0.8 ± 0.5
V96L	1	105 ± 24	0.8 ± 0.3
A165T	2	72 ± 17	0.7 ± 0.4
M187 K	2	90 ± 28	1.0 ± 0.6
C246S	2	73 ± 26	1.0 ± 0.6
D247H	2	77 ± 44	0.7 ± 0.2
M268I	1	103 ± 5	0.9 ± 0.2
mARC-2			
mARC-2	3	135 ± 31	0.5 ± 0.2
G244S	1	130 ± 26	0.6 ± 0.2
C245W	2	88 ± 24	0.5 ± 0.1

and C245W, and human cytochrome b_5 type B variants 2SF, D14G, K16E, and T22A were incubated with SMX-HA. Each incubation contained a control with an incubation mixture of the same mARC and cytochrome b_5 type B WT proteins and N-reductive activities obtained with protein variants were related to these controls. By this, it became evident that none of the cytochrome b_5 type B variants affected the N-reductive activity more than mARC variants alone (data not shown).

DISCUSSION

The mARC-containing N-reductive enzyme system is localized in the outer mitochondrial membrane and able to catalyze the reduction of N-hydroxylated substrates.^{2,3,4,42}

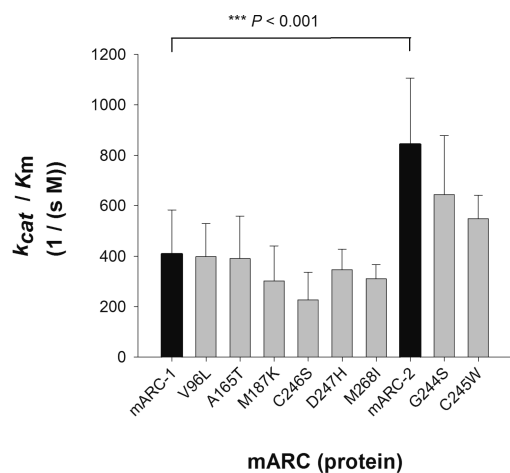


Figure 6. Catalytic efficiency of human mARC WT and variants. mARC containing 120 pmol of molybdenum, cytochrome b_5 containing 60 pmol of heme, and NADH cytochrome b_5 reductase containing 6 pmol of FAD were incubated with 0.1–10 mM SMX-HA and 1 mM NADH in 20 mM MES buffer, pH 6.0, containing 1 mM ascorbic acid and 6.6% DMSO under aerobic conditions. Catalytic efficiency was calculated from kinetic parameters. Data are means of duplicates \pm SD of the number of protein lots mentioned in Table 1. WT proteins are presented as black bars and variant proteins as gray bars.

A first assay for the proof of the participation of the mARC-containing N-reductive enzyme system is the enrichment of reductive activity in mitochondrial subcellular tissue fractions. Therefore, our studies with the substrate SMX-HA started with the investigations of subcellular tissue fractions of porcine liver and thyroid gland. The liver plays a major role in metabolic and detoxification pathways and represents the organ that is always investigated first regarding its N-reductive activity.⁷ The thyroid gland was chosen as an extrahepatic tissue showing noticeably high N-reductive activity in subcellular mitochondrial fractions.⁷ Furthermore, several publications report about hypothyroidism under treatment with SMX/TMP.⁴³ Probably effects of sulfonamides on the thyroid gland depend on the species to which the drug is administered. The thyroid gland of rodents shows a greater sensitivity to derangement by drugs, chemicals, and physiologic perturbations than the one of human beings. Sensitive species like rat, dog, and mice more frequently develop thyroid nodules after long-term treatment with sulfonamides than insensitive species like monkey, guinea pig, chicken and human beings.⁴⁴ Our study is just able to give a hint that the mARC-containing N-reductive enzyme system in porcine thyroid is able to reduce SMX-HA. Further studies are necessary to find whether it plays an important role. Our investigations with the substrate SMX-HA showed that N-reductive activity was only enriched in subcellular mitochondrial fractions of porcine liver and thyroid gland. Western blot analyses of all components of the N-reductive enzyme system showed that all of them were detectable in the subcellular mitochondrial fraction. Control stains for mitochondria, microsomes, and cytosol confirmed purity of the mitochondrial and cytosolic fraction. Only the microsomal fraction showed the slightest contamination with mitochondria and cytosol. N-reductive activity was in agreement with the expression pattern of the components of the mARC-containing N-reductive enzyme system (Figure 2).

Reductions that take place in microsomal fractions usually rely on NADPH as cosubstrate, whereas reductions that take place in mitochondrial fractions usually require NADH as cosubstrate as postulated in the hypothetical reaction cycle of amidoxime reduction by mARC.² Our results give evidence that the reduction of SMX-HA is an NADH dependent reaction (Figure 2).

The use of the porcine enzyme system in these initial experiments successfully led to the hypothesis of an involvement of the mARC-containing N-reductive enzyme system in this reaction. Following studies were necessary to verify this hypothesis for the human enzyme system.

In humans the main tissue involved in metabolism is the liver. For the investigation of subcellular fractions, OMV, mitochondria, and homogenate of human liver were available. Results showed that highest reduction rates for SMX-HA were detectable in OMV followed by mitochondria (Figure 3). This result is in agreement with the results for our model substrate benzamidoxime and with the results received from subcellular porcine fraction using further substrates.^{3,7,10}

Incubations with heterologously expressed human proteins easily allow differentiation between the single components of the N-reductive enzyme system and enable us to identify those components that are essential for reduction *in vitro*. Results showed that all three components of the N-reductive enzyme system, that is, mARC-1 or mARC-2, cytochrome b_5 type B, and NADH cytochrome b_5 reductase, had to be present for exhibition of N-reductive activity. mARC-1 and mARC-2 were indeed interchangeable, but the lack of one component led to almost absent N-reductive activity (Figure 4).

In past years, several studies investigated the reduction of SMX-HA to SMX with an enzyme system containing cytochrome b_5 type A and NADH cytochrome b_5 reductase.^{32,45} The influence of protein variants encoded by SNPs in the genes of these enzymes was ruled out, as well as the influence of ascorbate and glutathione on the expression levels of these enzymes.^{19,20,46,47} Our *in vitro* investigations likewise showed that it was possible to substitute cytochrome b_5 type B for cytochrome b_5 type A, albeit N-reductive activity was decreased. In this respect, however, recent knock down studies gave solid evidence that only cytochrome b_5 type B is part of the mARC-containing N-reductive enzyme system *in vivo*.^{1,35}

The most important experiments supporting our hypothesis analyzing the cell metabolism of SMX-HA *in vivo* were the knockdown studies of mARC-1 and mARC-2 in HEK-293 cells. Knockdown of mARC-1 in HEK-293 cells showed a significant decrease in the reduction of SMX-HA, whereas the knockdown of mARC-2 did not affect the reduction of SMX-HA in these cells. This result was also observed for the model substrate benzamidoxime and is attributed to the low expression of mARC-2 in HEK-293 cells compared with the expression of mARC-1.¹ Interestingly, effects of a mARC-2 knockdown were detectable when mARC-1 was also knocked down. The double knockdown led to a further significant decrease in the reduction rates of SMX-HA compared with the single knockdown of mARC-1 (Figure 5).

After ensuring that the mARC-containing N-reductive enzyme system is capable of reducing SMX-HA to SMX in cells, our studies continued with the *in vitro* investigation of human heterologous expressed mARC-1, mARC-2, and cytochrome b_5 type B protein variants and their possible influence on the N-reductive capacity. Six protein variants of human mARC-1, two of human mARC-2, and four of human

cytochrome b_5 type B, whose effects on the reduction of our model substrate benzamidoxime have already been analyzed, were investigated for their reductive capacity toward SMX-HA.^{1,39} The mARC-1 variants V96L, A165T, M187 K, C246S, D247H, and M268I, which did not influence the reduction of benzamidoxime, likewise showed unaltered activity when SMX-HA was used as substrate.³⁹ While the protein variants G244S and C245W of mARC-2 showed a statistically significant decrease in the catalytic efficiency of benzamidoxime reduction, only a non-statistically significant decrease in the catalytic efficiency of SMX-HA reduction was observed.³⁹ Briefly summarized, the results for the N-reductive activity of mARC variants toward the substrate SMX-HA are in agreement with the results concerning the model substrate benzamidoxime. Furthermore, K_m values for the reduction of SMX-HA were determined in a range of 0.5 to 1 mM SMX-HA. Under high-dose SMX/TMP treatment (3200 mg SMX per day) a concentration of 0.02–0.023 mM SMX-HA occurs in the blood volume for a typical adult (4.7–5 L), which fits in the determined kinetic parameters.

The human cytochrome b_5 type B variants S2F, D14G, K16E, and T22A did not influence the reduction of benzamidoxime and, in view of the results for the substrates benzamidoxime and SMX-HA for the mARC variants, a significant influence on the reduction of SMX-HA therefore seems to be unlikely.¹ Thus, N-reductive activity of the four protein variants of cytochrome b_5 type B were not only investigated together with mARC wild-type proteins but also with the respective mARC variants. The results showed that protein variants of cytochrome b_5 type B were not able to influence N-reductive activity of mARC wild-type proteins significantly. These results were expected and are consistent with those published for the substrate benzamidoxime. Additionally, it was not possible to induce a further decrease of N-reductive activity when protein variants of cytochrome b_5 type B were incubated together with protein variants of mARC. Thus, the protein variants of cytochrome b_5 type B are incapable of inducing a relevant effect on the reduction of SMX-HA as well.

All investigated protein variants were encoded by nonsynonymous SNPs in *MARC1*, *MARC2*, and *CYBSB* genes. A previous study determined genotype frequencies of these nonsynonymous SNPs in *MARC1* and *MARC2* in a Caucasian cohort.³⁹ Homozygous carriers of the variant genotype were only detectable for one SNP in *MARC1*, which encodes the protein variant A165T, with a frequency of 7.1%. Analyzing the kinetic parameters of this important variant did not show a statistically significant difference in the reduction of SMX-HA compared with the wild-type protein. Therefore, this investigation could not establish a connection between a polymorphism in *MARC1* and a decrease in reduction capacity of SMX-HA that might possibly underlie the pathogenesis of hypersensitivity reactions.

The mARC proteins, mARC-1 and mARC-2, show overlapping substrate preferences.² Results of incubations with heterologously expressed proteins showed that most of the investigated substrates, for example, N-hydroxylated nucleobases and nucleosides, including the model substrate benzamidoxime show a higher catalytic efficiency in the reduction when incubations were performed with mARC-1.^{6,7,39} The present investigation with SMX-HA is the second that shows a statistically significant increase of catalytic efficiency when incubations were performed with mARC-2.

Besides SMX-HA, only NOHA was reduced more efficiently by mARC-2 compared with mARC-1.¹⁰ These results demand a further explanation of the differences between mARC-1 and mARC-2, which have to be a necessary part of further investigations.

CONCLUSIONS

In this study, we demonstrated that the detoxification of SMX-HA to SMX is catalyzed by the mARC-containing N-reductive enzyme system. This hypothesis was proposed based on the first results of the investigations of porcine subcellular tissue fractions, further supported by investigations of subcellular fractions of human liver, and finally mARC-1 and mARC-2 knockdown studies using HEK-293 cells gave *in vivo* evidence. Thus, mARC plays an important role in the metabolism of SMX and its metabolites. Recent studies were not able to detect any effects of polymorphisms *MARC1*, *MARC2*, and *CYBSB* on the N-reductive activity using the model substrate benzamidoxime. The encoded human protein variants had not been investigated with the substrate SMX-HA so far. Therefore, the ten heterologously expressed human protein variants encoded by SNPs in *MARC1*, *MARC2*, and *CYBSB* were investigated in this study, and a decrease in the reduction of SMX-HA was excluded.

Our results allow the conclusion that the detoxification pathway of SMX-HA to SMX is catalyzed by the mARC-containing N-reductive enzyme system. *In vitro*, the heterologously expressed human protein variants do not affect the reduction of SMX-HA. It can be suspected that they might not participate in the pathogenesis of hypersensitivity reactions under treatment with SMX. But this hypothesis can only be formulated very vaguely because further effects that might be caused by influences on the expression levels of the proteins, by anchoring of the protein in the outer mitochondrial membrane, and by posttranslational modifications of the protein cannot be excluded so far.

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Notes

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■ ABBREVIATIONS

Cyt b_5 B/CYBSB, cytochrome b_5 type B; Cyt b_5 R/CYBSR3, NADH cytochrome b_5 type reductase; mARC/MARC, mitochondrial amidoxime reducing component; Moco, molybdenum cofactor; SMX, sulfamethoxazole; SMX-HA, sulfamethoxazole hydroxylamine; SNP, single nucleotide polymorphism; TMP, trimethoprim; WT, wild-type

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