The Involvement of the Mitochondrial Amidoxime Reducing Component (mARC) in the Reductive Metabolism of Hydroxamic Acids

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ABSTRACT
The mitochondrial amidoxime reducing component is a recently discovered molybdenum enzyme in mammals which, in concert with the electron transport proteins cytochrome b5 and NADH cytochrome b5 reductase, catalyzes the reduction of N-oxygenated substrates. This three component enzyme system plays a major role in N-reductive drug metabolism. Belonging to the group of N-hydroxylated structures, hydroxamic acids are also potential substrates of the mARC-system. Hydroxamic acids show a variety of pharmacological activities and are therefore often found in drug candidates. They can also exhibit toxic properties as is the case for many aryl hydroxamic acids formed during the metabolism of arylamides. Biotransformation assays using recombinant human proteins, subcellular porcine tissue fractions as well as human cell culture were performed. Here the mARC-dependent reduction of the model compound benzhydroxamic acid is reported in addition to the reduction of three drugs. In comparison with other known substrates of the molybdenum dependent enzyme system (e.g., amidoxime prodrugs) the conversion rates measured here are slower, thereby reflecting the mediocre metabolic stability and oral bioavailability of distinct hydroxamic acids. Moreover, the toxic N-hydroxylated metabolite of the analgesic phenacetin, N-hydroxyphenacetin, is not reduced by the mARC-system under the chosen conditions. This confirms the high toxicity of this component, as it needs to be detoxified by other pathways. This work highlights the need to monitor the N-reductive metabolism of new drug candidates by the mARC-system when evaluating the metabolic stability of hydroxamic acid-containing structures or the potential risks of toxic metabolites.

Introduction
Hydroxamic acids are a class of substances with a variety of biologic activities, including antibiotic (Barb et al., 2007; Halouska et al., 2014) and anti-inflammatory activities (Brodden et al., 1975), along with inhibitory properties toward metalloproteinases (Dalvie et al., 2008; Verma, 2012) and histone-deacetylases (HDACs) (Dokmanovic et al., 2007; Zhang and Zhong, 2014). The latter is of particular interest, as inhibitors of HDACs show antineoplastic activities and are currently applied in cancer treatment with new candidates under development (Wagner et al., 2010; Zhang and Zhong, 2014). Examples are vorinostat (suberoylanilide hydroxamic acid) which is marketed as Zolinza for the treatment of T-cell lymphoma and Panabinostat (Farydak) which has just been approved (Raedler, 2016).

Applying compounds with hydroxamic acids in therapy requires a reasonable level of metabolic stability. One expectable metabolic pathway, besides conjugation reactions, is hydrolysis to the corresponding carboxylic acid (Liu et al., 2014). As shown in Fig. 1, another likely metabolic conversion is the reduction of hydroxamic acids to the corresponding amide (Lowenthal, 1954; Hirsch and Kaplan, 1961; Kitamura and Tatsumi, 1985; Kiesel et al., 2013). For example, metabolism studies of CP544439, a hydroxamic acid-containing matrix metalloproteinase inhibitor, revealed the formation of the amide, the glucuronide and the carboxylic acid as the main metabolites of the orally administered drug (Dalvie et al., 2008). Besides their pharmacological advantages, aryl hydroxamic acids have been shown to possess toxic and mutagenic properties (Miller et al., 1961; Vaught et al., 1981). For example, the analgesic drug “Phenacetin” is N-hydroxylated during metabolism to yield N-hydroxyphenacetin (Hinson and Mitchell, 1976; Wirth et al., 1980). After the withdrawal of the market because it was found to induce severe renal papillary necrosis and tumors of the renal pelvis and bladder in humans (Liu et al., 1972; Bengtsson et al., 1978), N-hydroxyphenacetin has been held responsible for these severe risks of toxic metabolites.
Reagents and Cell Lines

Unless otherwise stated all chemicals were purchased from Carl Roth GmbH & co. KG (Karlsruhe, Germany), Sigma Aldrich (St. Louis, MO) or Fluka (Buchs, Switzerland) and used without further purification. Methanol (HPLC grade) was from JT Baker (Deventer, Netherlands). 4-nitrophenetole, 4-butoxyphenylacetic acid and sodium carboxylate hydrochloride and 4-fluorophenoxybenzene were from abcr (Karlsruhe, Germany). Ethyl 4-aminotetrahydro-2H-pyran-4-carbohydroxamic acid) and its metabolites see Supplementary Information Data.

Fig. 1. Metabolism of hydroxamic acids. Hydroxamic acids can either undergo reduction to the corresponding amide or hydrolysis to the corresponding carboxylic acid. A hydrolysis of the amide to the carboxylic acid might be possible.

Fig. 2. Structures of all studied compounds.
Synthesis of DS92. DS92 was synthesized by a modified procedure of the method described in the literature (Suzuki et al., 2002). Suberic acid (5.00 g, 28.7 mmol) was slowly added to neat freshly distilled aniline (2.70 ml, 30.1 mmol) and the mixture was stirred at 185°C and left at the same temperature for 2 hour. The mixture was cooled to room temperature and a NaOH 2 N aqueous solution was added to set the pH of the mixture at ~8. The suspension was filtered and the filtrate was acidified to pH 2, obtaining a white precipitate that was collected and washed in warm water (50°C). The insoluble part was filtered and washed with hot water, yielding DS92 as white pure precipitate (3.93 g, 55%). Rf = 0.35

Synthesis of DS116. DS116 was obtained by a fast two-step synthesis starting from the carboxylic acid derivative DS92, which is transformed using thionyl chloride into the corresponding acyl chloride, further reacted into the target according to an already described procedure (Wright and Corbett, 1993). To a solution of 8-oxo-8-(phenylamino)octanoic acid (0.30 g, 1.2 mmol) in dry DCM (3 ml) thionyl chloride (330 μl, 3.60 mmol) was slowly added to obtain a solution of 8-oxo-8-(phenylamino)octanoic acid chloride (0.39 g, 2.20 mmol) in 1 ml of water and the mixture was stirred at 20°C for 3 hours. The solution was heated to reflux for 3 hour and later a stream of nitrogen was used for the removal of solvent and the excess of thionyl chloride. To the crude product was added a solution of 8-oxo-8-(phenylamino)octanoic acid (0.30 g, 1.2 mmol) in dry DCM (3 ml) thionyl chloride (330 μl, 3.60 mmol) was slowly added to obtain a solution of 8-oxo-8-(phenylamino)octanoic acid chloride (0.39 g, 2.20 mmol) in 1 ml of water and the mixture was stirred at 20°C for 3 hour. The suspension was filtered and the filtrate was acidified to pH 2, resulting in a white precipitate that was collected and washed in warm water (50°C). The insoluble part was filtered and washed with hot water, yielding DS92 as white pure precipitate (3.93 g, 55%). Rf = 0.35

Synthesis of Vorinostat. Vorinostat was synthesized according to Mai et al. (2001). Retention time for HPLC was 7.8 minutes.

In vitro N-Reductive Activity Assay. In vitro biotransformation assay was carried out at 37°C in a shaking water bath. Incubation mixture consisted of 100 μg of porcine subcellular fractions in 100 mM potassium phosphate buffer, pH 6.0 or in case of recombinantly expressed human proteins of 7.5 μg mARC1 or mARC2, CYB5R3 resulting in 75 pmol heme and CYB5R3 resulting in 75 pmol FAD in 20 mM MES buffer, pH 6.0. Different substrate concentrations were used and for CP544439, vorinostat and bufexamac 4.0%, 4.8% and 8.0% DMSO were added respectively. After 3-hour-incubation, the reaction was started by adding 1 ml NADH, resulting in a total volume of 150 μl. Incubation was stopped after 15 minute by adding 150 μl of cold methanol. Afterward, samples were shaken for 5 minutes at room temperature and centrifuged for 5 minute with 9500*g at room temperature. Supernatants were analyzed by HPLC. For the determination of kinetic parameters with recombinantly expressed human protein the DMSO concentration was kept at the same level and only the substrate concentration was modified.

HPLC Analysis for Incubated Samples. The flow rate was kept at 1.0 ml/min and the injection volume was 10 μl for all performed HPLC analysis. All benzhydroxamic acid and CP544439 related samples were measured on a Waters e2695 Separation Module with a Waters 2998 Photodiode Array Detector and Waters Empower 2 Build 2154 as integration software.

- For the separation of benzbromarone (rt = 5.0 ± 0.2 minute) benzamidoxime (rt = 8.1 ± 0.2 minute) and benzoic acid (rt = 12.7 ± 0.1 minute) a Phenomenex Gemini NX-C18 (5 μm), 150 x 4.6 mm with a Phenomenex C18 4 x 3.0 mm pre-column was used. The mobile phase consisted of 50 mM KH₂PO₄ pH 6.0, 10 mM tetracylammonium chloride and 10% acetonitrile (v/v). Detection wavelength was 210 nm.

- For the separation of CP544439 (rt = 4.8 ± 0.3 minute), deoxy CP544439 (rt = 5.9 ± 0.2 minute) and the carboxylic derivative (rt = 7.0 ± 0.2 minute) a Waters Sunfire C18, 3.5 μm, 150 x 4.6 mm with a Phenomenex C18 4 x 3.0 mm pre-column was used. Solvent A (0.2% formic acid in H₂O (v/v)) and Solvent B (0.2% formic acid in acetonitrile (v/v)) were used. Starting with 60% A, the gradient changed linearly from 3 to 7% to 10% A. At 11 minute A was set to 60% over 0.5 minute. Total runtime was 16 minute. Detection wavelength was 247 nm. The column temperature was maintained at 25°C and sample storage temperature at 18°C.

- All vorinostat, bufexamac, N-hydroxyphenacetin and benzamidoxime related samples were measured on a Waters HPLC system consisting of a Waters 717 autosampler, a Waters 1525 pump and a Waters 2487 dual absorbance detector at room temperature. A Phenomenex Gemini NX-C18 (5 μm), 150 x 4.6 mm with a Phenomenex C18 4 x 3.0 mm pre-column was used. Solvent A (0.2% formic acid in H₂O (v/v)) and Solvent B (0.2% formic acid in acetonitrile (v/v)) were used. Starting with 60% A, the gradient changed linearly from 3 to 7% to 10% A. At 11 minute A was set to 60% over 0.5 minute. Total runtime was 16 minute. Detection wavelength was 247 nm. The column temperature was maintained at 25°C and sample storage temperature at 18°C.

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For separation of \( N \)-hydroxyphenacetin (rt = 7.8 ± 0.2 minute) and phenacetin (rt = 9.1 ± 0.0 minute) the mobile phase consisted of 1% formic acid in \( H_2O \) (v/v) and 22.5% acetonitrile (v/v). Detection wavelength was 245 nm. For separation of benzamidoxime (rt = 9.1 ± 0.3 minute) and benzamidine (rt = 15.9 ± 0.1 minute) the mobile phase consisted of 10 mM sodium 1-octanesulfonate and 20% acetonitrile. Detection wavelength was 229 nm.

Cell Culture

HEK-293 cells, derived from a female human source, 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.

siRNA Transfection and Design of Knockdown Experiments

HEK-293 cells were reverse transfected and mARC-protein down-regulated according to the previous described procedure (Plitzko et al., 2015).

\( N \)-Reactive Metabolism of Benzhydroxamic Acid in HEK 293 Cells

For \( N \)-reduction studies in HEK-293 the culture medium was removed, and cells were carefully washed and pre-incubated with substrate-free incubation buffer (Hanks’ balanced salt solution containing 10 mM HEPES, pH 7.4). After removing the substrate-free incubation buffer, the vital cells were then incubated with benzhydroxamic acid-containing incubation buffer (3 mM, 0.5% (v/v) DMSO) at 37°C for 180 minute. After the designated time, the culture supernatant was carefully removed, centrifuged to eliminate cellular debris and analyzed by HPLC as described above.

Total Cellular Protein Extraction

Cellular protein was harvested and protein contents determined as previously described (Plitzko et al., 2015).

Western Blot Analysis

SDS-PAGE and Western Blot analysis to verify down-regulation of mARC-protein in HEK-293 cells was carried out as described previously (Plitzko et al., 2015).

Statistical Analysis

Statistical analyses were carried out using the SigmaPlot 11 software (Systat Software Inc., Erkrath, Germany). The significance of observed differences was evaluated by Bonferroni test. A probability less than 5% was considered to be significant. All experimental values are given as means ± S.D.

Results

From all analyzed subcellular fractions, \( N \)-reductive activity in mitochondria was found to be enhanced compared with other tissue fractions for all \( N \)-unsubstituted hydroxamic acids (Fig. 3). These findings reflect the enrichment of mARC depending enzyme activities in this fraction as published earlier (Krompholz et al., 2012). In the microsomal and cytosolic fractions only minor/no \( N \)-reductive activity was detectable. By contrast, no reduction of \( N \)-hydroxyphenacetin could be detected after incubation with the

Fig. 3. \( N \)-reduction of benzhydroxamic acid, bufexamac, CP544439 and vorinostat in hepatic subcellular fractions. Biotransformation assay consisted of 100 µg protein, 1 mM NADH and either 1.0 mM vorinostat and 4.8% DMSO, 3.0 mM benzhydroxamic acid, 0.5 mM CP544439 and 4.0% DMSO or 1.0 mM bufexamac and 8.0% DMSO. Incubation was carried out for 15 minute and stopped by addition of methanol. Activities are means ± S.D. of two biologic determinations. (A) benzhydroxamic acid, (B) bufexamac, (C) CP544439, (D) vorinostat, *under limit of quantification, n.d. not detectable.
mitochondrial fraction and only minor reduction rates were detected within the cytosolic fraction (data not shown). Besides reduction to the amide, hydrolysis to the corresponding carboxylic acids was monitored and was found to be more pronounced in microsomes compared with other fractions; this indicates the involvement of a microsomal enzymatic system or a non-enzymatic reaction (Fig. 3, A and D). In the case of bufexamac and CP544439 the microsomal fraction was the only fraction where hydrolysis could be detected to a small extent (data not shown).

To prove the involvement of mARC in the reduction of hydroxamic acids, a cell based siRNA experiment was performed. In HEK-293 cells reductive conversion of benzhydroxamic acid to benzamide occurs in a time-dependent (data not shown) and substrate-dependent manner (Fig. 4A) and followed Michaelis Menten kinetics ($v_{\text{max}} = 0.06 \pm 0.01 \text{ nmol benzamide/min}^{-1} \cdot \text{mg protein}^{-1}$). By siRNA-mediated down-regulation of mARC1, benzamide formation decreased dramatically to approximately 30% compared with the negative control (Fig. 4B). Knockdown of mARC2 in HEK-293 cells did not affect the reduction of benzhydroxamic acid in HEK-293 cells. This same behavior is also observed with the model substrate benzamidoxime and is attributed to the low level of mARC2-protein expression in HEK-293 cells (Plitzko et al., 2013). Simultaneous knockdown of both mARC-proteins led to a small, further decrease in $N$-reductive activity than was observed in the mACR1-only knockout experiments. To further elucidate the differences between mARC1 and mARC2 the kinetic parameters $v_{\text{max}}$ and $K_{M}$ were determined with the in vitro reconstituted recombinant $N$-reductive system (Fig. 5). All hydroxamic acids were clearly reduced to the corresponding amides with the exception of $N$-hydroxyphenacetin. These results are consistent with previous observations in subcellular fractions. Benzhydroxamic acid, bufexamac, CP544439 and vorinostat were exclusively reduced to their corresponding amides; none of the corresponding carboxylic acid products were detected. The reductions obey Michaelis-Menten kinetics for both mARC-proteins. The calculated $K_{M}$ and $v_{\text{max}}$ values are presented in Table 1. The conversion rates of hydroxamic acids are lower in comparison with the model compound benzamidoxime (e.g., for vorinostat, $v_{\text{max}}$ is 15 times slower). For both incubation types with subcellular fractions and recombinant expressed proteins, highest $v_{\text{max}}$ Values were obtained for the model compound benzhydroxamic acid in comparison with all other studied hydroxamic acids.

According to the $v_{\text{max}}$ values, CP544439 and vorinostat are reduced to the same amount and smallest conversion rates were detected for bufexamac. Vorinostat, bufexamac and CP544439 required the use of DMSO as a solubilizer. Due to the negative influence of high DMSO concentrations in the incubation mixture (see Supplemental Data), only the minimum amount of DMSO required for solubility was added. In the case of benzhydroxamic acid $v_{\text{max}}$ was about four times higher with mARC2 than with mARC1 but the $K_{M}$ increased sevenfold. Only slight differences of the kinetic parameters for vorinostat and bufexamac were detected for both mARC forms, whereas CP544439 was exclusively reduced by mARC1. In accordance with the results obtained with subcellular liver fractions and RNAi studies all $N$-unsubstituted hydroxamic acids are reduced by mARC.

### Discussion

The mARC-containing three component enzyme system is responsible for the reduction of various $N$-hydroxylated compounds (Ott et al., 2015) and the results of our recent investigations demonstrate clearly that hydroxamic acids belong to this class of compounds. The hydroxamate moiety exhibits strong cation chelating properties and thereby possesses the ability to affect a variety of enzymes. Hydroxamic acid moieties are found in a multitude of drugs and drug candidates (Halouska et al., 2014; Zhang and Zhong, 2014). In addition, aryl hydroxamic acids have been shown to possess toxic and mutagenic properties (Miller et al., 1961; Vaught et al., 1981). Therefore, the investigation of the metabolic fate of hydroxamic acids is of particular relevance for further drug developments and for the understanding of detoxification pathways. We could demonstrate that hydroxamic acids can serve as substrates for the mARC-system.
The model compound benzhydroxamic acid, as well as three other drugs (vorinostat, bufexamac and CP544439) are reduced to the corresponding amides by porcine mitochondria and the reconstituted recombinant human mARC-system (Table 1). The N-reduction observed in vitro is also evident in intact human cell metabolism and is mARC-dependent as the siRNA-mediated down-regulation leads to a dramatic decrease in N-reductive activity (Fig. 4B). HEK-293 cells were chosen because of the high N-reductive activity in renal tissue (Krompholz et al., 2012). However, the N-reductive conversion of hydroxamic acids is lower compared with the model compound (benzamidoxime). Amidoximes, used as pro-drugs for amidines, are rapidly reduced in vivo (Clement et al., 1992). In the case of hydroxamic acids, reduction leads to inactivation because the amide is not able to form strongly chelating complexes. As metabolism studies for CP544439 reveal, the main metabolism pathways of hydroxamic acids in vivo are glucuronidation, reduction and hydrolysis (Dalvie et al., 2008). The reduction to the amide is of great physiologic relevance, especially in rats, where the amide is the most prevalent metabolite. It has been demonstrated that the aldehyde oxidase is capable of reducing hydroxamic acids to amides (Sugihara et al., 1983a,b; Sugihara and Tatsumi, 1986). Reduction of CP544439 was proposed to be catalyzed by this enzyme as well; studies with human cytosolic liver fractions using the artificial electron donator N-methylnicotinamide resulted in a very low conversion rate (1.6 pmol·min⁻¹·mg protein⁻¹) (Obach, 2004). The aldehyde oxidase is located in the cytosol, but we could not detect any N-reductive activity in the porcine liver cytosol under our tested conditions (Fig. 5C). Our determined conversion rate with the mitochondrial fraction for CP544439 is about 2.5 nmol·min⁻¹·mg protein⁻¹, which is more than 1500 times higher than the previous described cytosolic conversion rate. This finding indicates the important role of mARC in the reduction of hydroxamic acids to the amides. Interestingly, vorinostat has sufficient metabolic stability for therapy and is applied orally, but high doses of 400 mg/day are necessary (Mann et al., 2007). The enhanced lipophilicity of

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>mARC1</th>
<th>mARC2</th>
<th>Activities are means ± S.D. of two biologic determinations.</th>
</tr>
</thead>
<tbody>
<tr>
<td>vorinostat</td>
<td>1.45 ± 0.37</td>
<td>2.73 ± 0.18</td>
<td>46.3 ± 5.5, 19.3 ± 0.8</td>
</tr>
<tr>
<td>benzhydroxamic acid</td>
<td>0.31 ± 0.11</td>
<td>2.12 ± 0.28</td>
<td>313.5 ± 22.5, 13.4 ± 1.1</td>
</tr>
<tr>
<td>bufexamac</td>
<td>1.09 ± 0.15</td>
<td>1.07 ± 0.15</td>
<td>50.2 ± 0.4, 8.2 ± 0.7</td>
</tr>
<tr>
<td>CP544439</td>
<td>0.25 ± 0.06</td>
<td>2.12 ± 0.28</td>
<td>313.5 ± 22.5, 13.4 ± 1.1</td>
</tr>
<tr>
<td>N-hydroxyphenacetin</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>benzonoxime</td>
<td>0.63 ± 0.06</td>
<td>0.54 ± 0.03</td>
<td>674.8 ± 25.1, 549.6 ± 12.1</td>
</tr>
</tbody>
</table>

LOQ = 0.9 nmol·min⁻¹·mg protein⁻¹,

LOQ = 3.5 nmol·min⁻¹·mg protein⁻¹.
vorinostat compared with benzhydroxamic acid and benzamidoxime could be a pivotal characteristic. The low conversion rates determined for bufexamac, which is a very lipophilic other compound, support this theory (Fig. 5B). Studies to further elucidate structure-activity relationships should be done to evaluate whether lipophilicity is an important feature for enzymatic conversion of hydroxamic acids by the mARC-system and to detect candidates with better stability toward N-reduction.

In the case of N-hydroxyphenacetin no reduction to the amide by mARC was observed. This is the first investigated hydroxamic acid studied by us so far which is not reduced. However, this could be an explanation for the high toxicity of this compound as it needs to be detoxified by other pathways. The most apparent difference between N-hydroxyphenacetin and the other studied hydroxamic acids is the substitution of the nitrogen’s hydrogen with the sterically demanding phenyl group. Due to this steric hindrance mARC might not be able to bind and reduce such kind of substrates. This hypothesis needs to be proved by further structure activity studies.

In conclusion, to properly evaluate the metabolic stability of new hydroxamic acid containing drug candidates, metabolism by the mitochondrial mARC-system must be considered.

Acknowledgments
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Authorship Contributions
Participated in research design: Scheidig, Havemeyer, Clement. Conducted experiments: Ginsel, Plitzko.
Contributed new reagents or analytical tools: Ginsel, Fronek, Stolfia, Jung. Expression and purification of recombinant proteins: Kubitsa.
Performed data analysis: Ginsel, Plitzko.
Wrote or contributed to the writing of the manuscript: Ginsel, Plitzko, Clement.

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Supplemental Data

Article title: The Involvement of the Mitochondrial Amidoxime Reducing Component (mARC) in the Reductive Metabolism of Hydroxamic Acids

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Journal title: Drug Metabolism and Disposition
Legends to supplemental figures

Supplemental figure 1. Chemical synthesis for CP544439 (7) and related compounds

Supplemental figure 2. Influence of the DMSO concentration on the reduction of benzhydroxamic acid via mARC1. Marks are means ± standard deviation of two biological determinations.

Supplemental figure 3. Influence of the DMSO concentration on the reduction of benzhydroxamic acid via mARC2. Marks are means ± standard deviations of two biological determinations.
Synthesis of 4-(4-fluorophenoxy)benzene-sulfonyl chloride (2). 2 was prepared after the patent from Van Eck-Smit BLF, Pinas VA, and Windhorst AD, Radiolabelled MMP Selective Compounds, WO2009139634, 19.11.2009.

Synthesis of ethyl 4-((((4-(4-fluorophenoxy)phenyl)-sulfonylamino)tetrahydropyran-4-carboxylate (4). Ethyl 4-aminotetrahydro-pyran-4-carboxylate hydrochloride (3) was coupled with 2 to give 4 according to the patent from Reiter, Lawrence Alan, (4-arylsulfonylamino)-tetrahydropyran-4-carboxylic acid hydroxamides, US6087392, 21.10.1999.

Synthesis of 4-(((4-(4-fluorophenoxy)phenyl)-sulfonylamino)tetrahydropyran-4-carboxylic acid (5). 4 was treated with NaOH to give 5 according to the patent from Reiter, Lawrence Alan, (4-arylsulfonylamino)-tetrahydropyran-4-carboxylic acid hydroxamides, US6087392, 21.10.1999.

\[ ^1H-\text{NMR (300 MHz, DMSO-d}_6) \delta 12.64 (s, 1H), 8.07 (s, 1H), 7.76 (d, J = 9.0 Hz, 2H), 7.33 – 7.27 (m, 2H), 7.21 – 7.16 (m, 2H), 7.08 (d, J = 9.0 Hz, 2H), 3.49 – 3.43 (m, 2H), 3.39 – 3.31 (m, 2H), 1.90 – 1.76 (m, 4H) \text{ ppm.} \]

\[ ^{13}C-\text{NMR (75 MHz, DMSO-d}_6) \delta 174.2, 160.3, 158.9 (d, J = 240.9 Hz), 151.0 (d, J = 2.4 Hz), 137.0, 128.8, 122.0 (d, J = 8.7 Hz), 117.1 (d, J = 3.4 Hz), 116.8, 62.4, 58.1, 32.7 \text{ ppm.} \]

LC-MS (ESI), \textit{m/z} 396.0 [M+H]\(^+\).


\[ ^1H-\text{NMR (300 MHz, DMSO-d}_6) \delta 7.79 – 7.76 (m, 3H), 7.33 – 7.27 (m, 2H), 7.21 – 7.17 (m, 2H), 7.07 (d, J = 9.0 Hz, 2H), 7.02 (s, 1H), 6.89 (s, 1H), 3.50 – 3.30 (m, 4H), 1.91 – 1.82 (m, 2H) \text{ ppm.} \]

\[ ^{13}C-\text{NMR (75 MHz, DMSO-d}_6) \delta 174.2, 160.3, 158.9 (d, J = 240.9 Hz), 151.0 (d, J = 2.4 Hz), 136.7, 129.0, 122.0 (d, J = 8.7 Hz), 117.1, 116.8, 62.5, 58.8, 32.8 \text{ ppm.} \]

LC-MS (ESI), \textit{m/z} 395.1 [M+H]\(^+\).
Synthesis of 4-((4-(fluorophenoxy)phenyl)-sulfonylamino)tetrahydropyran-4-carboxyhydroxamic acid (7). For the synthesis of 7 the described method by Reiter, Lawrence Alan, (4-arylsulfonylamino)-tetrahydropyran-4-carboxylic acid hydroxamides, US6087392, 21.10.1999, was modified as followed: 5 (400 mg, 1.0 mmol) was dissolved in 4 ml DCM at room temperature. Oxalyl chloride (150 µl, 1.7 mmol) and 4 drops DMF were added. The solution was stirred overnight. After cooling down the flask to 0 °C O-(trimethylsilyl)hydroxylamine (350 µl, 2.3 mmol) and 300 µl pyridine were added and the mixture was stirred for one hour at 0 °C and additional two hours at room temperature. The reaction was stopped by addition of 12 ml 2 N HCl and one hour additional stirring at room temperature. The formation of the product (brownish spot with FeCl₃) was controlled by TLC (cyclohexane/ethyl acetate 8:2). The organic phase was separated and the aqueous layer was extracted with 20 ml ethyl acetate three times. The combined organic layers were washed with water and brine. After drying with anhydrous NaSO₄ the solvent was evaporated to a volume of 15 ml and stored overnight at 4 °C. The resulting crystals were rinsed with cold cyclohexane/ethyl acetate 1:1 and dried under high vacuum, yield 209 mg, 0.5 mmol (50 %).

1H-NMR (300 MHz, DMSO-d6): δ 10.37 (s, 1H), 8.69 (s, 1H), 7.80 – 7.74 (m, 3H), 7.33 – 7.24 (m, 2H), 7.22 – 7.15 (m, 2H), 7.07 (d, J = 8.9 Hz, 2H), 3.48 – 3.31 (m, 4H), 1.93 – 1.70 (m, 4H) ppm.

13C-NMR (75 MHz, DMSO-d6): δ 169.0, 160.2, 158.9 (d, J = 240.9 Hz), 151.2 (d, J = 2.3 Hz), 137.0, 128.9, 121.8 (d, J = 8.6 Hz), 117.3, 116.9 (d, J = 23.5 Hz), 62.5, 58.0, 32.9 ppm.

LC-MS (ESI), m/z 411.1 [M+H]^+.

Influence of DMSO addition to the incubation mixture:

For the studies of the DMSO-influence on the mARC1 and mARC2 activity, benzhydroxamic acid was chosen as the substrate and the quantification of benzamide as the outcome. All incubation mixture consisted of 3.75 µg mARC, 37.5 pmol b5 and 3.75 pmol NADH b5 reductase in 20 mM MES buffer, pH 6.0. Substrate concentration was set to 3 mM and incubation time was 15 minutes at 37 °C in a shaking water bath. After 3 minutes of pre-incubation the reaction was started by addition of 1 mM NADH, resulting in a total volume of 150 µl. The reaction was stopped with 150 µl of cold methanol, followed by 5 minutes shaking and 5 minutes centrifugation at 9.500 g. The supernatants were analyzed via HPLC.
Supplemental figure 2

The graph shows the relative area of formed benzamide in % as a function of DMSO [%]. The line has a correlation coefficient of $R^2 = 0.9503$. The data points are indicated with error bars.
Supplemental figure 3

Relative area of formed benzamide in %

DMSO [%]

R² = 0.9937