Doctoral Thesis

Metabolism of amiodarone - Biotransformation of mono-N-desethylamiodarone in-vitro

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Metabolism of Amiodarone - Biotransformation of mono-N-Desethylamiodarone *in-vitro*
For
Jian
and
my parents
Acknowledgements

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Abbreviations

AMI amiodarone
AMI-EtOH deaminated amiodarone (X2)
CYP cytochrome P450 enzyme
cCYP cDNA-expressed human cytochrome P450 enzyme
DDEA di-N-desethylamiodarone (X2)
ESI electron spray ionization
HPLC high-performance liquid chromatography
HLM human liver microsomes
NMR nuclear magnetic resonance
NO- nitric oxide
NO-MDEA N-nitroso-monodesethylamiodarone
MDEA mono-N-desethylamiodarone
3'OH-MDEA n-3'-hydroxybutyl-N-desethylamiodarone (X1)
MDZ midazolam
α'OH-MDZ α-hydroxymidazolam
MS mass spectrometry
MS/MS tandem mass spectrometry
TLC thin layer chromatography
Zusammenfassung

Teil I:


Bei der Inkubation von MDEA mit menschlichen Lebermikrosomen wurde vergleichsweise weniger 3'OH-MDEA gebildet wie im Experiment mit Kaninchenlebermikrosomen. Dafür wurde die Bildung von zwei weiteren Metaboliten X2 und X3 gemessen. Die chemischen Strukturen wurden mittels HPLC-ESI-MS/MS und durch den Vergleich (Retentionszeiten mittels HPLC-UV) mit synthetisierten Referenzprodukten charakterisiert. Man fand heraus, dass MDEA durch Deaminierung zu AMI-EtOH (X2) und durch Dealkylierung zu DDEA (X3) metabolisiert wird. Die entsprechenden Michaelis-Menten parameter waren $K_m = 21.7 \pm 3.4$, $32.6 \pm 14.3$ und $29.4 \pm 13.1 \mu M$ und $V_{max}$: $17.6 \pm 3$, $64.3 \pm 35.4$ und $13.7 \pm 5.3 \text{ pmol/min/mg prot.}$ ($n = \ldots$)
3) für die Bildung von 3’OH-MDEA, AMI-EtOH und DDEA. Ein Vergleich der Inkubation von MDEA mit humanen, Kaninchen-, Ratten- und Schweine-Lebermikrosomen hat verdeutlicht, dass MDEA bei allen getesteten Spezies zu allen drei charakterisierten Metaboliten abgebaut wird.

Bei der Abklärung der Frage, welche Enzyme in die Metabolisierungsreaktionen involviert sind wurden folgenden Methoden eingesetzt: Koinkubation mit CYP-spezifischen Substraten, Korrelation mit CYP3A4-spezifischen α'OH-Midazolam Bildungsraten und die Inkubation mit cDNA exprimierten P450 Isozymen. Die Experimente ergaben, dass MDEA nicht nur durch CYP3A4, sondern auch durch andere P450 Isozyme (CYP1A1, 1A2, 2A6, 2D6) metabolisiert wird. Aufgrund der experimentell gezeigten Aktivität der Isoenzyme und deren prozentualer Verteilung im Lebergewebe, ist CYP3A4 vermutlich das wichtigste Enzym für die Bildung der drei untersuchten Reaktionen in vivo.

Die neuen Metaboliten müssen noch auf ihre toxische und pharmakologische Wirkung untersucht werden. Dabei wird ein Augenmerk vor allem auf die Verbesserung der Therapie mit AMI gelegt, mit dem Ziel, Nebenwirkungen rationeller behandeln zu können.

Teil II:
AMI wird rasch zu MDEA, einem sekundären Amin, dealkyliert. Da AMI als Medikament für Herzrhythmusstörungen auch mit NO-freisetzenden Nitrovasodilatatoren verabreicht werden kann, besteht die Möglichkeit der Bildung des entsprechenden N-Nitrosamins N-nitroso-monodesethylamiodaron (NO-MDEA).

Dabei wurde NO-MDEA synthetisiert und die Strukturaufklärung ergab, dass NO-MDEA eine Mischung aus zwei Konfigurationsisomeren syn- und anti-NO-MDEA ist. 2 μM MDEA wurden in 10 mM phosphatpuffer pH 7.4 zusammen mit Nitroprussid-Natrium (NO• Freisetzung) bei Raumtemperatur inkubiert. Die Reaktionsprodukte wurden mit Dichlormethane extrahiert und mittels HPLC gemessen. Die HPLC-ESI-MS Analyse der Reaktionslösung im Vergleich mit synthetisiertem NO-MDEA ergab, dass MDEA bei pH 7.4 durch freigesetztes NO• nitrosyliert wird. Weitere Experimente ergaben, dass Thiol-Gruppen in Lösung die Geschwindigkeit der Reaktion hemmen können. Da S-Nitrosothiole aber NO auch wieder freisetzen können, kann in vivo die Nitrosylierung von MDEA nicht ausgeschlossen werden.
Summary

Part I:
Amiodarone (AMI) is one of the most widely used drugs for the treatment of atrial and ventricular tachyarrhythmias. Treatment of patients with AMI is also associated with adverse effects some of which may be severe and lead to the discontinuation of the therapy. Adverse effects include pulmonary, thyroid, hepatic, neurologic, ocular, dermatologic or gastrointestinal toxicity. Several studies have supported that some toxic effects may depend on the amount of either acute or maintenance and cumulative dose. In the human body AMI is rapidly dealkylated to its main pharmacologically active metabolite mono-N-desethylamiodarone (MDEA). AMI and MDEA are lipophilic compounds and accumulated in various tissues. The terminal elimination half life is comparably long with \( t_{1/2} = 53 \) days for AMI and \( t_{1/2} = 61 \) days for MDEA.

It was the aim of this work to further investigate the metabolism of AMI since the ongoing metabolism of this drug is not known in detail. The incubation of MDEA with liver microsomes isolated from different species was chosen as a model. The products were extracted and measured by HPLC-UV.

At the incubation with rabbit liver microsomes MDEA was rapidly metabolized to the first unknown metabolite denoted as XI. This Product was isolated and its structure was characterized by NMR and HPLC-ESI-MS/MS. It was found that MDEA was hydroxylated at the 3-position of the n-butyl-chain. Therefore, XI was denoted as 3'OH-MDEA.

At the incubation of MDEA with human liver microsomes (HLM) the amounts of 3'OH-MDEA formed were comparably lower than with rabbit liver microsomes. However, MDEA was found to be metabolized to two additional metabolites X2 and X3. The chemical structures were characterized by HPLC-ESI-MS/MS and by comparing those with synthesized reference products (Retention times by HPLC-UV). The corresponding Michaelis-Menten parameters for the respective formation rates of 3'OH-MDEA, AMI-EtOH and DDEA were \( K_m = 21.7 \pm 3.4, 32.6 \pm 14.3 \) und \( 29.4 \pm 13.1 \) \( \mu M \) und \( V_{max} = 17.6 \pm 3, 64.3 \pm 35.4 \) und \( 13.7 \pm 5.3 \) pmol/min/mg prot. \( (n = 3) \). The comparison of the incubations of MDEA with human, rabbit, rat and pig liver microsomes has shown that with all tested species MDEA is metabolized to all three newly characterized metabolites.
The enzymes involved into the metabolic reactions were investigated by the following means: Coincubation with CYP-specific substrates, correlation with CYP3A4-specific formation rates of $\alpha$-OH-midazolam and incubation with cDNA expressed P450 isozymes. The experiments revealed that MDEA is metabolized not only by CYP3A4 but also by other CYP enzymes (CYP1A1, 1A2, 2A6, 2D6). Considering the experimental activity of the used isozymes and the expression levels of those in liver tissue one may suggest that CYP3A4 is the most relevant enzyme for the three investigated reactions in vivo.

The new metabolites still have to be tested for their toxicological and pharmacological activity. Concerning this issue the main focus will be the improvement of therapy with AMI with the aim to treat adverse effects in a more rational way.

**Part II:**
AMI is rapidly dealkylated to MDEA, a secondary amine. Since AMI is an antiarrhythmic agent and may be administered together with nitrovasodilators, it may be possible that MDEA reacts to the corresponding N-nitrosamine which is N-nitroso-monodesethylamiodarone (NO-MDEA).

Thus NO-MDEA was synthesized and the characterization of its chemical structure revealed that NO-MDEA is a mixture of two configurational isomers syn- and anti-NO-MDEA. 2 $\mu$M MDEA were incubated in 10 mM phosphate buffer pH 7.4 together with 10 mM sodium nitroprusside (NO$^\bullet$ release) at room temperature. The reaction products were extracted with dichloromethane and measured by HPLC. The analysis of the reaction solution in comparison with the synthesized reference products by HPLC-ESI-MS revealed that MDEA is nitrosylated at pH 7.4 in aqueous solutions. Further experiments have shown that thiol groups dissolved in the reaction solution may inhibit the rates of the reaction. As S-nitrosothiols may also release NO$^\bullet$ the nitrosylation of MDEA in vivo has to be taken into consideration.
Chapter I: 
Biotransformation of MDEA Using in-vitro Models

1. Introduction

1.1. Amiodarone in Medical Use

Amiodarone (AMI) was originally developed as an antianginal drug [1, 2] before its antiarrhythmic action was discovered in 1969 [3]. In 1985 AMI (Cordarone) was approved in the United States as a drug for the treatment of life-threatening ventricular arrhythmias [4]. The clinical use of other antiarrhythmic agents belonging to the class I antiarrhythmics (Vaughan Williams classification; sodium channel blockers) was based on the suppression of ventricular ectopic beats [5]. Later cardiac arrhythmia suppression trials (CAST) [6-8] showed, however, that treatment of patients with flecainide, encainide or morcizine did not have beneficial effects concerning the mortality form
sudden cardiac death. This finding had a substantial impact on clinical practice [9]. Therefore, class III antiarrhythmic agents such as AMI or sotalol gained more importance in clinical use [5, 10]. Larger randomized clinical trials such as the European Myocardial Infarct Amiodarone Trial (EMIAT) or the Canadian Amiodarone Myocardial Infarction Arrhythmia Trial (CAMIAT) have shown that AMI treatment causes significant postmyocardial infarction reductions in arrhythmia mortality by maintaining the sinus rhythm [11-13]. This may indicate a favourable trend in total mortality and sotalol displayed similar results [14]. Nowadays, AMI is the most frequently prescribed drug in the United States [15].

AMI is counted to the class III antiarrhythmics due to its function in action potential duration prolongation. However, AMI is an unique drug since it also shows class I pharmacological effects and minor class II effects [12]. Class III agents may block potassium channels and it has been shown that AMI inhibits the delayed rectifier potassium current (I_K) [16]. Other studies have shown that AMI also blocks the sodium inward current (I_Na) [3, 17-19] and the calcium inward current (I_Ca) [20-22]. These electrophysiological effects may promote a decrease of the maximum rate of voltage of the action potential (V_max) [23-25]. Consequently AMI may prolong the repolarisation in myocardial tissue and it was shown to have an antifibrillatory effect. Nevertheless, the molecular mechanism of action of AMI has still not been resolved in detail.
1.1.1. Pharmacokinetics and Metabolism

Many differences between the pharmacokinetics of AMI and those of other drugs may be attributed to the drug's very high lipophilicity [27]. The bioavailability of AMI was shown to be very variable ranging from 22 to 86 % [28, 29]. It has been reported that AMI undergoes extensive enterohepatic circulation before distribution into the central compartment [30-32]. In the liver AMI is rapidly dealkylated to mono-N-desethylamiodarone (MDEA) [33-35]. Examination of hepatic and portal vein samples after oral administration of AMI indicates a large first pass effect [30]. AMI peak concentrations are reached within 3-7 hours and the drug is highly bound to proteins (~96-99%; predominantly to albumin) [36] but also to β-lipoprotein [36, 37].

The drug and its metabolite are then distributed into the central, deep and peripheral compartment [30]. A population pharmacokinetic study has shown that a two compartment model is most suitable to describe AMI therapy with values for volumes of distribution/bioavailability 

\[ V_1/F = 882 \text{ L} \] \[ V_2/F = 12'700 \text{ L} \pm 58 \% \] 

\( (V_1: \text{volume of distribution for central compartment}; \ V_2: \text{peripheral volume of distribution}) \) [38].

Previously the estimated total body volume of distribution \((V_d)\) was reported to be \(4936 \pm 3290 \text{ L} \) [30]. Values of \(V_d\) for acute and steady-state administration of AMI were estimated to be \(1.3 - 65.8 \text{ L/kg}\) and \(5.0 \text{ L/kg}\), respectively [32].

Tissue distribution of AMI and MDEA has been investigated in animal models and humans. Due to their high lipophilicity AMI and MDEA are extensively accumulated in tissues. Highest levels have been found in adipose tissue, lung, liver and lymph nodes which are denoted as deep compartments. Lowest levels have been found in brain, thyroid and muscles (peripheral compartments) [39-42]. Further data revealed that the cumulative dose of AMI correlated with AMI and MDEA concentrations in various parts of myocardial tissue [42].

Additional correlation has been found between cumulative dosage of AMI and plasma levels of AMI and MDEA [42]. Same observations were made between the amount of chronic dosage and AMI and MDEA plasma levels [40, 44] ranging from 1.06 to 3.9 mg/L for AMI and from 1.04 to 2.92 mg/L for MDEA [30].

These characteristics of AMI and MDEA implicate difficulties in determination of the appropriate dosage. Administration of AMI is either orally or by intravenous infusion. Short term AMI infusion is used to rapidly stabilise cardiac rhythm and achieve an
initial loading for oral therapy as maintenance. Oral administration is used for long-term management of life-threatening arrhythmias. Starting daily doses of AMI therapy range between 800 and 1600 mg in order to saturate the body stores and to reach the desired steady state concentration. The saturation amount of the body stores is estimated to be at about 15 g AMI. Under steady-state conditions the dosage has to be chosen to achieve the therapeutic window and to avoid incidence of adverse effects. They range from 200 up to 400 mg per day. According to newer studies the steady state dosage is kept rather low [45].

The terminal elimination half-lifes (t½) of AMI and MDEA vary markedly among individuals. The compounds are primarily eliminated through bile (liver) or intestine. T½-values after single dose application of chronic dosage range between 3-21 hours and between 20 and 120 days, respectively [33]. Mean values of terminal AMI (MDEA) elimination were estimated to 53 ± 24 days (61 ± 8 days) [40], 47 ± 13 days (54 ± 23 days) [46] or 52 days (60 days) [47].

As mentioned above MDEA is the main metabolite of AMI [48]. Further metabolic reactions have been proposed such as further dealkylation [49, 30], O-dealkylation, deiodination or hydroxylation [31]. Studies have shown that the primary dealkylation is mainly catalyzed by the cytochrome P450 3A4 isozyme with contributions of other P450 isozymes [34, 36]. A more recent study has shown that also other P450 isozymes such as CYP1A1, CYP1A2, CYP2D6, CYP2C8 and CYP2C19 catalyzed AMI-N-deethylolation [50]. CYP3A4 and CYP2C8 were estimated to be the most relevant enzymes in vivo for the corresponding reaction (CYP2C8 at low AMI concentrations). Glucuronidation of the metabolites and biliary excretion is thought to be the final elimination process for AMI-therapy [30].
1.1.3. Adverse Effects

One of the major problems of AMI therapy is the avoidance of side effects. In some studies the occurrence of side-effects was reported to be up to 80% of all treated patients and withdrawal was up to 41% for patients receiving 400 mg/day [51]. A more recent study has shown that low dosage AMI treatment (mean dosage 152-326 mg/day) had lower incidence of AMI discontinuation with 22.9% compared to placebo with 15.4% [52].

Pulmonary Toxicity

The most serious noncardiac side effect of AMI-therapy is pulmonary toxicity [51]. It was initially reported in 2-17% of patients while more recent estimates indicate an incidence of not more than 3% [45, 53-55]. This lower frequency may be brought into relation with the current trend to prescribe daily doses of 400 mg or less [56]. However, pulmonary toxicity is probably the most feared adverse effect.

Symptoms of pulmonary involvement in clinics may include dyspnea, hypoxemia and elevation of the erythrocyte sedimentation rate [57]. The pathogenesis of AMI-induced pulmonary toxicity likely results from direct toxic effects of the drug or its metabolites, as well as indirect inflammatory and immunologic processes [58]. Direct toxicity is likely because it is related in part to dosage and duration of therapy and many patients with this adverse reaction have no evidence of an inflammatory or immune response [58].

The agent may cause several types of pulmonary toxicity [58]. One type is the hypersensitivity pneumonitis which usually appears within the first few weeks of therapy causing nonspecific symptoms such as fever, dyspnea and cough.

In a more delayed stage of therapy a more severe pulmonary reaction may occur with an incidence of 6% between 3 to 12 months. With patients receiving AMI for over one year the incidence may increase to 15-17% [56]. This adverse reaction is generally insidious and associated with dyspnea, nonproductive cough, fatigue or occasional low-grade fever. In more serious cases it may lead to respiratory failure [58, 59].
**Thyroid Toxicity**

Thyroid hormone function in humans plays an important role in different biochemical processes such as the metabolism of food into energy at molecular level and heat, influencing the energy metabolism in mitochondria, rapid protein synthesis or mitochondrial gene transcription. The thyroid hormone exists in two major forms which are L-thyroxine (T4) and triiodothyronine (T3). T4 is deiodinated to the active form of the thyroid hormones T3 by enzymatic catalysis. The similarity of T4 to AMI analogues concerning the molecular structure of the diiodophenyl part implicated inhibitory effects of AMI analogues on either T4 deiodination [60] or binding to the thyroid hormone α1-receptor [61]. AMI treatment may cause hypothyroidism as well as hyperthyroidism [56].

Many patients taking AMI show deviations from normal thyroid function [62]. More serious thyroid abnormalities occur in 30% of patients and clinically important hypothyroidism and hyperthyroidism were reported in 2-10% [63]. Management of AMI induced hyperthyroidism is more complicated than management of hypothyroidism. If AMI therapy allows its discontinuation it may be attempted, although hyperthyroid and cardiac symptoms may worsen while the antiadrenergic effects of the drug wear off [64].

**Hepatotoxicity**

Elevations in liver function test results (levels of serum transaminase: alanine aminotransferase, aspartate aminotransferase) in patients receiving AMI occur with an incidence between 14 and 82% [63, 65]. Those enzyme elevations are generally mild and return to normal conditions after dose reduction or drug discontinuation. Severe hepatotoxicity appears with an incidence of 1-3% [63, 65, 66]. It has been reported that AMI-induced liver injury may even lead to fatalities [67, 68]. Appearance of severe hepatotoxicity is reported anytime from 1 week to several months after starting the AMI-therapy. Symptoms include nausea, vomiting, malaise, fatigue, anorexia, weight loss and hepatomegaly [69].
**Cardiovascular Toxicity**

Cardiovascular adverse reactions were reported in 14% of patients receiving AMI [70]. In one clinical study the risk of developing adverse reactions was related to serum AMI concentrations, being highest in patients with concentrations exceeding 2.5 mg/L [71]. Aggravation of arrhythmias appears to be significantly less common than in the case of class I agents [72]. However, in the Canadian Amiodarone Myocardial Infarction Trial (CAMIAT), the frequency of proarrhythmia was only 0.3% with AMI compared with 3% with placebo [12]. Intravenous administration produces hypotension unrelated to dosage in about 28% of patients [73].

**Neurologic Toxicity**

Neurologic side-effects were reported in 20-40% of patients treated with AMI [56]. Symptoms include tremor, ataxia, peripheral neuropathy, malaise-fatigue, sleep disturbances, dizziness and headaches. These side-effects are often dose related and commonly reported during the loading period [51]. During long-term therapy, tremor and sleeplessness appear in patients with an incidence of 30 and 28%, respectively, but they usually respond positively to a reduction of dosage [66].

**Ocular Toxicity**

The most common effect of ocular toxicity appears as corneal microdeposits in almost all patients (70-100%) who remain on AMI therapy over two months [74, 75]. Lens microdeposits may occur in 50-60% of patients [75]. Signs of visual disturbances include photophobia, blurred vision, blue-green halo vision especially at night or while looking at bright objects, dry eyes or itching. Corneal microdeposits resolve within 3-7 months after AMI discontinuance [74].

**Dermatologic Toxicity**

Several types of dermatologic reactions are associated with AMI-therapy. They include allergic rash, photosensitivity and an unusual blue-gray skin discoloration [51]. While photosensitivity is a common complaint, blue-gray skin discoloration occurs less
frequently, but may be related to dosage and duration of therapy. It occurs up to 2 years after the start of treatment [66].

**Gastrointestinal Effects**

Up to one quarter (25 %) of AMI-treated patients may display gastrointestinal effects which include nausea or loss of appetite and occur mostly during oral loading [51]. Abdominal discomfort and vomiting have also been reported.

**Drug-Drug Interactions**

Drug-drug interactions in AMI related therapy are an important issue. Numerous drugs were reported to interact with AMI [76, 77]. Increased levels of coadministered drugs were reported in several cases such as aprindine, digoxin, flecainide, phenytoin, procainamide, quinidine or warfarin [4]. In the case of coadministering other antiarrhythmics with AMI the narrow therapeutic window of those drugs [78] requires monitoring of plasma levels.

The AMI-digoxin interaction has received most attention since they may be severe [4]. It has been found that giving single digoxin doses to volunteers before and during administration of oral AMI increased the plasma concentration of digoxin and its urinary recovery without change in its renal clearance [79]. However, other studies suggested that AMI may reduce renal clearance of digoxin [80, 81]. The authors concluded that AMI increases the bioavailability of digoxin.

Another common interaction of AMI is the one with warfarin [82]. AMI may potentiate its effect by interfering with hepatic metabolism [51]. Interactions with hepatic metabolism was also associated with increased levels of quinidine [83], procainamide [83], phenytoin [84, 85] and flecainide [86].

Comedication of AMI may cause marked prolongation of QT intervals and occasionally drug induced torsades de pointes especially with class I antiarrhythmics [83, 87]. The assumption that AMI may affect the hepatic metabolism of coadministered drugs was successfully supported by \textit{in vitro} investigations. As shown in Table 1 AMI and its primary metabolite MDEA inhibited a wide range of important metabolic enzymes [88].
**Table 1:** Inhibitory types of AMI and MDEA for human CYP activities [88].

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Inhibitory type</th>
<th>$K_i$ [µM]</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9</td>
<td>non-competitive</td>
<td>94.6 ± 15.4</td>
<td>S-warfarin 7-hydroxylase</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>mixed</td>
<td>45.1 ± 14.7</td>
<td>bufuralol 1-hydroxylase</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>non-competitive</td>
<td>271.6 ± 72.1</td>
<td>testosterone 6β-hydroxylase</td>
</tr>
</tbody>
</table>

**DEA**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Inhibitory type</th>
<th>$K_i$ [µM]</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>mixed</td>
<td>1.5 ± 0.2</td>
<td>7-ethoxyresorufin O-dealkylase</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>mixed</td>
<td>18.8 ± 4.3</td>
<td>phenacetin O-deethylase</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>non-competitive</td>
<td>13.5 ± 2.6</td>
<td>coumarin 7-hydroxylase</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>non-competitive</td>
<td>5.4 ± 0.3</td>
<td>7-benzylxysorufin O-dealkylase</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>mixed</td>
<td>2.3 ± 0.5</td>
<td>S-warfarin 7-hydroxylase</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>mixed</td>
<td>15.7 ± 4.6</td>
<td>S-mephenytoin 4'-hydroxylase</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>competitive</td>
<td>4.5 ± 2.6</td>
<td>bufuralol 1-hydroxylase</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>non-competitive</td>
<td>12.1 ± 3.2</td>
<td>testosterone 6β-hydroxylase</td>
</tr>
</tbody>
</table>

It has been observed that the inhibitory potential of MDEA is even higher than that of AMI. While AMI inhibited the cytochrome P450 enzymes CYP2C9, CYP2D6 and CYP3A4, MDEA additionally inhibited the enzymes CYP1A1, CYP1A2, CYP2A6, CYP2B6 and CYP2C19 [88]. Moreover, the $K_i$-values were lower in the case of coincubated MDEA for the three enzymes which were inhibited by both compounds.
1.2. Metabolism of Xenobiotics

1.2.1. Cytochrome P450 Enzyme System

Humans and animals are under the daily exposure of numerous xenobiotics which may vary in size and chemical structure. Uptake of xenobiotics by nutrition is usually at lower dosage than the administration of drugs. In the whole system of the body handling these compounds the cytochrome P450 enzyme system is responsible to turn lipophilic compounds into more water-soluble compounds by reactions such as oxidation, dealkylation, hydroxylation, epoxidation or deamination. The P450 enzymes consist of a porphyrine-iron complex and protein structure which determines the size and character of the active site.

The proposed catalytic cycle for cytochrome P450 enzymes [89] is not yet investigated in detail. However, some steps were experimentally determined to show reductive and oxidative reactions.

![Figure 1: Catalytic cycle for cytochrome P450 enzymes [90, 91]](image-url)
The catalytic activity of P450 enzymes include NADPH as a cofactor, NADPH-P450 reductase and cytochrome b5. Step 1 (enzyme substrate binding) is believed to be fast and occur at diffusion-limited rates [92, 93], whereas step 2 (reduction) is rate limiting and influenced by the concentration of the reductase and the phospholipid system [91]. Some conditions may require Mg^{2+}, phospholipid and either b5 or apo-b5 for rapid electron transfer (investigated with CYP3A4 [93]). To the investigated reaction steps also belong steps 3 (binding of O\textsubscript{2} to ferrous P450) and 4 (addition of second electron). The addition of oxygen is believed to be a rapid reaction while in step 4 the mechanism of the function of cytochrome b5 still remains unclear.

The nomenclature for the Cytochrome P450 enzyme system was simplified to CYP ("Cytochrome P450") as summarized in the last update of D.R. Nelson [94]. In addition the expression CYP is followed by an arabic number representing the family, then followed by a letter denoted to the subfamily (when two or more exist) and finally by another arabic number representing the individual gene within the subfamily. The whole gene family was described in 85 eukaryote (vertebrates, invertebrates, funghi and plants) and 20 prokaryote species. In total 74 gene families have been described of which 14 families exist in all mammals examined to date. These 14 families comprise 26 mammalian subfamilies of which 20 and 15 have been mapped in the human genome and mouse genome, respectively [94].

The P450 enzyme system is dedicated to biotransform substrates of diverse chemical structure and size [95]. This forced the nature to developed several enzymes with differing specificities for different structures. However, very few reactions are really specific for one single P450 enzyme. Isoforms such as CYP1A2, CYP2A6, CYP2C9, CYP2D6 or CYP3A4 may catalyze more lipophilic compounds, whereas CYP2E1 oxidizes ethanol to acetaldehyde.

The metabolic enzymes of the P450 system are expressed in different tissues of the body. However, the main centers of metabolism are the intestine and the liver. Thus the abundance of the P450 enzymes was investigated primarily in the liver. Contents of individual CYP proteins in native human liver microsomes were measured in different laboratories [96, 97, 98] and summarized [99] to be shown here in Table 2.
### Table 2: Nominal specific content of individual CYP proteins in native human liver microsomes [99]

<table>
<thead>
<tr>
<th>CYP form</th>
<th>pmol CYP / [mg]</th>
<th>(% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>45 (8)</td>
<td>42 (13)</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>68 (13)</td>
<td>42 (13)</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>39 (7)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>64 (12)</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>96 (18)</td>
<td></td>
</tr>
<tr>
<td>CYP2C18</td>
<td>&lt; 2.5 [98]</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>19 (4)</td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>10 (2)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>49 (9)</td>
<td>22 (7)</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>108 (20)</td>
<td>98 (29)</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>1 (0.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>534</strong></td>
<td><strong>344</strong></td>
</tr>
</tbody>
</table>

In the liver CYP3A4 is the most abundant P450 isozyme but closely followed by CYP2C9, CYP2A6 and CYP2C8. CYP1A2 is also expressed prominently while CYP1A1 was not measured. CYP1A1 is known to be expressed in very little amounts and to be an extrahepatic enzyme [100, 101]. These enzymes were extensively investigated due to their potential to activate carcinogens [102].

In metabolism of the currently applied drugs a major part of over 50 % is metabolized by CYP3A4 [95, 102]. This may be either caused by the diversity of its active site and also by its abundance in liver and intestine.

Cytochrome P450 enzymes may be induced by environmental chemicals. It has been observed that animals may develop tolerance to barbiturates after chronical exposure [103]. It was also reported that inducers are often substrates for the induced enzyme [104]. Enzyme induction enhances its detoxification. Many substrates or drugs such as phenobarbital, phenytoin, carbamazepine, rifampicin, dexamethasone may induce P450 enzymes [105, 106]. In the case of drugs with low therapeutic index enzyme induction may be disadvantageous and lead to loss of efficacy [104]. Induction response is dose dependent and reversible. The exact mechanism of the induction response is still not
known with the exception of certain P450 enzymes by polycyclic aromatic hydrocarbons such as TCDD (dioxin) via the Ah-receptor [107].

The structure of human CYP enzymes was not resolved in detail by lack of crystallisation. Due to this absence of structure and the diversity of substrate binding by P450 enzymes it is difficult to investigate the biotransformation of substrates on the molecular level. However, in the last years efforts have been made to understand metabolism on the molecular level and to be able to predict possible metabolic pathways. This may help to recognize toxical or cancerogenic effects of substrates in the early process of drug development.

Based on the crystallographic structure of four bacterial P450 enzymes such as P450 BM-3, P450cam, P450terp and P450eryF the structure of the human CYP3A4 was modeled in silico by Szklarz and Halpert [108]. The study revealed an enlarged active site of CYP3A4 allowing the binding and metabolism of large substrates (erythromycin [109], testosterone, aflatoxin B1 or cyclosporin A [110]). These results were followed by experiments using site directed mutagenesis in CYP3A4 of putative important amino acid residues for substrate binding [111-117].

Another approach was initialized by molecular modeling researchers with the aim to develop a pharmacophore for several important human CYP isozymes [118]. In detail pharmacophore modeling and the development of a three-dimensional quantitative structure activity relationship (QSAR) for P450 enzymes was based on the approach called comparative molecular field analysis (CoMFA) [119]. This method was developed to understand and interpret enzyme active sites in the absence of a crystal structure. The methodologies to apply these methods for cytochrome P450 enzymes was reviewed in detail elsewhere [120, 121].

For CYP1A2 the first models for theoretical investigation of theoretical enzyme-ligand interactions was published in 1993 [122], before the model was developed in more detail [123, 124]. In the case of CYP2A5 and 2A6 a QSAR model was developed after years of work [125-129]. By reviewing examples of metabolism of xenobiotics by CYP2B6 [130], a computational model was published [131] and for CYP2C9 structural requirements for substrate binding have been described as well [132, 133]. The derived model for CYP2D6 includes a hydrogen bond acceptor, a hydrogen bond donor and two to three hydrophobic regions [134-136]. CYP2E1 is involved in the metabolism of of many toxic and carcinogenic molecules such as low molecular weight
solvents and anesthetics. QSAR studies revealed a long hydrophobic access channel [137]. In the case of CYP3A4 a hydrogen bond acceptor atom was first found to be 5.5 to 7.8 Å from the site of metabolism and 3 Å from the the oxygen molecule associated with the heme [138]. More detailed QSAR models based on inhibitor data were later developed [139] showing three hydrophobes at distances of 5.2 to 8.8 Å from a hydrogen bond acceptor, three hydrophobes at distances of 4.2 to 7.1 Å form a hydrogen bond acceptor and an additional hydrophobe 5.2 Å from another hydrogen bond acceptor, or one hydrophobe at distances from 8.1 to 16.3 Å form the two furthest of three hydrogen bond acceptors. Substrate pharmacophore possessed two hydrogen bond acceptors, one hydrogen donor and one hydrophobic region [140].

Another approach for computational models for cytochrome P450 enzymes is a predictive model for aromatic oxidation and hydrogen atom abstraction [141]. Experimental data were combined with semiempirical molecular orbital calculations to predict activation energies for aromatic and aliphatic hydroxylations. Calculations were based on quantum mechanical model [142] studying the electronic state of the oxygen and the reactive intermediate [143]. Correlation ($R^2$) between experimentally obtained activation energies and predicted values was between 0.81 and 0.92 [141].
1.2.2. Enzyme Kinetics

Enzymatic catalysis [144] of chemical reactions is quantitatively characterized by the Michaelis-Menten equation (MM-equation), which is described in the following scheme 2.

\[ \text{Scheme 2: Michaelis-Menten kinetic model} \]

The reaction is simplified regarding the assumption that the enzyme reaction meets steady state and the enzyme-substrate complex formation is rapid equilibrium. Mathematical description of the Scheme 1 delivers equation (1)

\[ \frac{d[ES]}{dt} = k_1 [E][S] = - \frac{d[ES]}{dt} = (k_{-1} + k_p)[ES] \]  

which can be rearranged to the Michaelis-Menten equation (2)

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]

where \( K_m \) and \( V_{\text{max}} \) are defined as

\[ K_m = \frac{k_p + k_{-1}}{k_1} \]  

and

\[ V_{\text{max}} = k_p [E]_{\text{total}} \]

The relation of increasing substrate concentration and initial reaction velocity follows a characteristic hyperbolic saturation profile. Whereas the \( K_m \)-value can be associated with the affinity of the enzyme active site to the corresponding substrate, the \( V_{\text{max}} \)-value is a measure for the maximal velocity of the reaction. Moreover, resolution of the MM equation reveals that at substrate concentration at the \( K_m \) the reaction velocity is the half of \( V_{\text{max}} \). For appropriate analysis of kinetic data the substrate concentrations must be chosen around the \( K_m \)-value and the MM parameters are estimated by fitting the data points by non-linear least-square fitting using the MM-equation.

The Michaelis-Menten equation may also be transformed and plotted additionally in order to investigate the parameters with linear regression analysis. The most common way is the double-reciprocal plot (Lineweaver-Burk plot; 1934):
\[ \frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \times \frac{1}{[S]} \]  

(5)

where \(1/V_{\text{max}}\) is the intercept and \(K_m/V_{\text{max}}\) is the slope.

This equation (5) may be multiplied at both sides by \(vV_{\text{max}}\) and rearranged. Then one can obtain the equation (6)

\[ v = V_{\text{max}} - K_m \times \frac{v}{[S]} \]  

(6)

which represents the Eadie-Hofstee plot (\(v\) against \(v/[S]\)). \(v\) appears in both coordinates and errors in \(v\) affect both of them causing deviations towards or away from the origin.

Therefore, this kind of plotting is a very good method for the detection of systematic deviation from Michaelis-Menten behaviour [144]. The crossing points with the y- and x-coordinate represents \(V_{\text{max}}\) and \(V_{\text{max}}/K_m\), respectively.

### 1.2.3. Enzyme Inhibition

Inhibition of enzyme catalyzed reactions may be divided into reversible and irreversible inhibition which decrease the activity of the enzyme to zero and may be caused by traces of heavy metals.

As enzymes belonging to the cytochrome P450 system catalyze the biotransformation of drugs and possible drug-drug interactions are investigated \textit{in-vitro} the reversible types of inhibition have to be addressed in more detail. Several kinds of reversible inhibition are distinguished. The most important expressions of reversible inhibition are competitive, uncompetitive, noncompetitive or mixed type inhibition [145].

For competitive inhibition the substrate \(S\) and the inhibitor \(I\) compete for the same catalytically active site of the enzyme \(E\) as illustrated in the Scheme 3A. It may mainly occur with an inhibitor which has similar structure to the substrate.
Scheme 3: General kinetic reaction scheme for A) competitive inhibition, B) uncompetitive inhibition, C) non-competitive inhibition and D) mixed type inhibition [145].

The general kinetic structure of Scheme 3 leads to the equation (7) where $K_S$ is the dissociation constant for the substrate and $K_i$ the dissociation constant for the inhibitor. $K_m$ reduces to an apparent $K_{m\text{ app}}$ (increased) while $V_{\text{max}}$ remains unchanged with respect to inhibitor concentration [145].

$$v = \frac{V_{\text{max}} [S]}{K_S \left( 1 + \frac{[I]}{K_i} \right) + [S]} \quad (7)$$

For un-competitive inhibition ESI formation occurs only after formation of ES and both apparent $K_m$ and $V_{\text{max}}$ decrease with rising inhibitor concentration. Un-competitive inhibition is rare in practice, although the inhibition of CYP1A2-mediated phenacetin-O-deethylation by α-naphthoflavone (αNF) is an example [145]. Mathematical description of the reaction rate is described by equation (8).

$$v = \frac{V_{\text{max}} [S]}{K_S + [S] \left( 1 + \frac{[I]}{K_i} \right)} \quad (8)$$
While at the competitive inhibition only the ES or EI complex can be formed, non-competitive inhibition allows the coupling of ES or EI subsequently with another molecule to form ESI-complex that is believed not to lead to product formation. As an example P450 inhibition by monoclonal antibodies was reported to be non-competitive [145]. With increasing inhibitor concentration the apparent $K_m$ is unchanged while $V_{max}$ decreases.

$$v = \frac{V_{max}[S]}{K_s \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{K_i}\right)}$$  \hspace{1cm} (9)

Mixed type inhibition is reported for reaction catalyzed by P450 enzymes. First it is commonly observed that inhibition of P450 mediated reactions is partial with saturating levels of inhibitor ($0 < b < 1$, scheme 1) [145]. Then the dissociation constants $K_m$ and $K_i$ are altered after binding a second molecule to the enzyme leading to two different values for $K_i$ and $K_S$ ($K_i$, $aK_i$, $K_S$, $aK_S$), respectively. The existence of both situations in parallel is referred to as the mixed type inhibition [145] which is mathematically described by equation (10).

$$v = \frac{V_{max} \left(\frac{[S]}{K_s} + \frac{b[S][I]}{aK_sK_i}\right)}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[S][I]}{aK_sK_i}}$$  \hspace{1cm} (10)

Factors $a$ and $b$ represent the changes in $K_i$, $K_S$ and $V_{max}$ in the presence of inhibitor ($a \neq 0; 0 < b < 1$). The number of four unknowns in the equation (10) requires an adequate number of data points for regression analysis.

Inhibitory potency of P450 mediated reactions can be determined more easily by the IC$_{50}$ value which represents the concentration of the inhibitor that causes 50% inhibition of the activity of the corresponding reaction.
1.2.4. Atypical Enzyme Kinetics

The cytochrome P450 enzyme family was related to metabolism of xenobiotics in the early seventies [146-151] and enzyme kinetic analysis was applied as such. However, cytochrome P450 enzymes and particularly CYP3A4 are associated with a broad specificity for substrates of different size (up to 1'200 Dalton) and structure. Typical substrates are highly lipophilic and $K_m$ values range from 1 to 1'500 μM [145].

The model for CYP3A4 reveals a large active site with a well-defined access channel for substrates. Even the cyclosporin A molecule can be accommodated. This observation leads to the assumption that two smaller molecules may be accommodated in the same time by the CYP3A4 active site which may furthermore affect the kinetics of biotransformation. This hypothesis is supported by several kinetic observations [152-163].

Two models exist to explain atypical enzyme kinetics models. A major part of researchers are more familiar with the explanation of allosteric enzymes where binding of an effector causes conformational change of the protein structure and thus affect kinetics. The other model is called cooperativity which displays its characteristic in a sigmoidal enzyme kinetic curve (increasing reaction rate with increasing substrate concentration at low substrate concentration range) in contrast to hyperbolic Michaelis-Menten kinetics.

A first mathematical description of the degree of cooperativity was developed by Hill (1910) [164] and is expressed in the Hill equation (11). This was an empirical description of the cooperative binding of oxygen to haemoglobin.

$$ v = \frac{V_{\text{max}}S^n}{K_{0.5}^n + S^n} \tag{11} $$

As in the Michaelis-Menten equation $K_{0.5}$ refers to the substrate concentration where $v = 0.5V_{\text{max}}$. But this constant should not be called $K_m$. Rearrangement and taking the logarithm on both sides leads to equation (12).

$$ \log\left[\frac{v}{(V_{\text{max}} - v)}\right] = n\log S - n\log K_{0.5} \tag{12} $$

Plotting $\log\left[\frac{v}{(V_{\text{max}} - v)}\right]$ against $\log S$ should be a straight line with slope $n$ which is called the Hill-plot and allows evaluation of $n$ and $K_{0.5}$.

On the basis of kinetic models analysed using the method of King and Altman [165] more exact models for cooperative kinetics were proposed by Korzekwa et al. [153]
after experimental evidence of CYP3A4 mediated metabolism of e.g. aflatoxin B1 [154], steroids [162], carbamazepine [153, 166], amitriptyline [167], triazolam [168] and diazepam [155, 169].

Scheme 4: Proposed kinetic model for a P450 enzyme with two binding domains within the active site [145].

The model in Scheme 4 postulates that the two binding sites of the enzyme are of the same catalytic activity to the substrate. Development of the velocity equation (13) displays two values for $K_m$ ($K_{m1}$, $K_{m2}$) and $V_{max}$ ($V_{max1}$, $V_{max2}$), respectively.

$$v = \frac{V_{max1}[S] + V_{max2}[S]^2}{K_{m1} + K_{m2} + [S]^2}$$

(13)

The sigmoidal curve of cooperativity is discussed qualitatively assuming that after binding of the first substrate molecule to form ES the reaction rate is $V_{max1}$ (for saturated ES). With the increasing concentration of the substrate a second molecule is bound within the active site. The corresponding $V_{max2}$ is believed to be higher than $V_{max1}$. Affinity and metabolism of the two molecules are associated with the constants $K_{m1}$ and $K_{m2}$, respectively. However, conformational changes of the two sites with subsequent binding of a sigmoidal ligand may also be taken into account. Attempting that the substrate molecule can bind in two orientations provokes the reflection that binding of the two different binding sites does not affect the same catalytic activity. Thus a further model was developed in order to explain particularly CYP3A4 mediated metabolism.
Scheme 5: Proposed kinetic model for CYP3A4 with two binding domains in the active site [145]

As illustrated in Scheme 5 the number of different dissociation constants $K_S$ ($K_{S1}$, $K_{S2}$, $K_{S3}$, $K_{S4}$) and product formation rates increased to four each. The kinetic constants for product formation are written as $k_{p1}$, $k_{p2}$, $k_{p3}$ and $k_{p4}$ and the developed reaction velocity is shown as the following equation (14).

$$
\frac{\nu}{[E]_{\text{total}}} = \frac{k_{p1}}{K_{S1}} + \frac{k_{p2}}{K_{S2}} + \frac{[S]}{K_{S1}K_{S3}} (k_{p3} + k_{p4})
$$

(14)

Since the equation (14) contains eight constants its evaluation by nonlinear curve-fitting requires and sufficient number of experimental data points. However, equation (14) was applied by fitting CYP3A4-mediated enzyme kinetics of diazepam and its metabolites, temazepam and nordiazepam, as substrates [145]. The fitting data revealed that singly bound substrates (ES or SE) had poorer binding affinities and catalytic activity than SES or ESS. The increased binding affinity and catalytic activity after binding of the second molecule may be attributed to a more tight binding enabling a more active biotransformation of the substrate.
1.2.5. Substrate Inhibition

A decrease in the rate of product formation in the case of increased substrate concentration is determined as substrate inhibition. As a cause for this reaction binding of a substrate molecule to an inhibitory place at the enzyme is believed and models were developed to calculate the substrate inhibition kinetics accurately. The classical explanation is displayed in the next Scheme 6 where substrate S binds to the enzyme substrate complex SE to form SES.

\[
E + S \quad \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \quad ES \quad \quad \rightarrow \quad ES + S \quad \overset{k_p}{\rightarrow} \quad E + P
\]

Scheme 6: Model for substrate inhibition caused by excess substrate concentration [145].

Analysis of this model lead to the following mathematical description where \( K_{si} \) is the substrate inhibition constant and \( E_0 \) the total enzyme concentration.

\[
\nu = \frac{k_p E_0 [S]}{k_{-1} + k_p + S \left( 1 + \frac{[S]}{K_m} \right)} = \frac{V_{\text{max}} [S]}{K_m' + [S] + \frac{[S]^2}{K_{si}}}
\]

(15)

The constants \( K_m' \) and \( V_{\text{max}}' \) have the same mathematical definition as the Michaelis-Menten konstants but are used here in a different equation (15).

Since more detailed explanations have been found for CYP3A4 mediated metabolism the phenomenon of substrate inhibition was explained using reflections of cooperativity. Single binding of the substrate to the catalytic site of the enzyme may lead to higher product formation rate \( k_p \) than double binding of a substrate to the active site \( bk_{ps}, 0 < b < 1 \).
**Scheme 7**: Kinetic model for substrate inhibition based on two site model. Lowered product formation rate at binding of two substrates at the catalytic active site is described by the factor $b$ ($0 < b < 1$). Dissociation constants are defined by $K_S = k_1/k_1$, $K_i = k_2/k_2$, $aK_i = k_3/k_3$ and $aK_S = k_4/k_4$. [145].

The factor describes the lowering of the reaction rate and the inhibitory potency after binding of the second molecule to the active site at excess substrate concentration. From the model in Scheme 7 equation (16) can be derived as follows.

\[
V = \frac{V_{\text{max}}}{\frac{1}{K_S} + \frac{b[S]}{aK_iK_S}}
\]

The derived equation (16) was already applied for fitting substrate inhibition studies catalyzed by several cytochrome P450 isozymes (CYP1A2, 2C9, 2D6 and 3A4) with satisfactory correlation coefficients [145]. Cooperativity effects were observed predominantly at reactions catalyzed by CYP3A4 and data for cooperativity with other P450 isozymes are rare. Thus application of the newly derived equation (16) has to be discussed with cooperativity. However, both equations are models to describe substrate inhibitory effects and will deliver values which has to be discussed for their validity.
1.2.6. Activation

Activation is observed where the activity of a P450 enzyme towards one substrate can be increased by a second substrate [152, 153, 162, 170-172]. Since both molecules are substrates for the enzyme and do not compete with each other, the two-site model may be adapted to interpret the activation [153].

Activation may now be explained with favourable effect of the bound compound for the metabolism of the substrate. It is expected that the binding of the substrate is more tight in the presence of the activator and thus it is metabolized more rapidly. A simplified kinetic model where two different molecules can bind to the enzyme is shown in the next Scheme 8.

\[
\begin{align*}
\text{Scheme 8:} & \quad \text{Simplified kinetic scheme for the interaction between a substrate and an effector molecule for an enzyme with binding sites within the active site (S is the substrate, B the effector, E the enzyme and P the product) [153].} \\
& \quad \text{It was assumed that B is not metabolized and that product release is fast. With a further assumption of rapid equilibrium equations a form can be derived as has been already done earlier [173] for the substrate-effector model as shown by equation (17).} \\
& \quad \text{with equation (17).}
\end{align*}
\]

\[
v = \frac{V_{\text{max}} [S]}{K_m \left( 1 + \frac{[B]}{K_B} \right) + [S] \left( 1 + \frac{[B]}{aK_B} \right)}
\]  

(17)
In this equation the constants are expressed as $V_{\text{max}} = k_{25}[E_i]$ and $K_m = (k_{21}+k_{25})/k_{12}$ (kinetic constants for substrate metabolism), $K_B = k_{31}/k_{13}$ (binding constant for effector), $a$ is the change in $K_m$ resulting from the effector binding, and $b$ is the change in $V_{\text{max}}$ from the effector binding.

Increased metabolism by binding of a second substrate molecule is called homotropic cooperativity. If activation is caused by the binding of second molecule which is not the substrate but an other effector the effect is called heterotropic cooperativity [174].

One of the best known effectors for CYP3A4 catalyzed reactions is $\alpha$-naphthoflavone [158, 175, 176] for already mentioned cooperative substrates such as aflatoxin B1 or testosterone.

In addition differential kinetics were defined to explain further observed effects. Moreover, an effector may inhibit the biotransformation of a substrate to one metabolite while activating the formation of another metabolite [154, 158].
2. Methods

2.1. Analytical Methods

2.1.1. HPLC-UV Analysis

a) HPLC-System A

Instrumentation:
Pump: Merck Hitachi L-7100
Autosampler: Merck Hitachi L-7200
UV-Detector: Merck Hitachi L-7400
Interface: Merck Hitachi D-7000
Super Fraction Collector: Merck Hitachi L-7655
Computer: Compaq Deskpro
Software: Merck HSM

Method 1: Measurement of Amiodarone Derivatives:

Column: Merck LiChroCART 250-4 (Cat. 50'839)
LiChrospher 60; RP-select B (5 μm)
Mobile Phase: 19 % (v/v) 20 mM phosphate buffer pH 6 (NaH₂PO₄)
66 % (v/v) Methanol
15 % (v/v) Acetonitril
Flow Rate: 1 ml/min
Pressure: 170 bar
UV-Detection: 242 nm
Temperature: 25° C
Biotransformation Methods

Method 2: Measurement of Midazolam Derivatives:

Column: Merck LiChroCART 250-4 (Cat. 50'839)  
LiChrospher 60; RP-select B (5 μm)

Mobile Phase:  
32 % (v/v) 10 mM phosphate buffer pH 5.5 (NaH₂PO₄)  
30 % (v/v) Methanol  
38 % (v/v) Acetonitril

Flow Rate: 1 ml/min
Pressure: 240 bar
UV-Detection: 245 nm
Temperature: 25°C
b) HPLC-System B

**Instrumentation:**

- **Pump:** GBC LC 1150 HPLC Pump (Dandenong, Australia)
- **Autosampler:** Gynkotek GINA Autosampler Model 160 (Germering, Deutschland)
- **UV-Detector:** GBC LC 1205 UV/Vis Detector
- **Column Thermostat:** Jetstream 2 Plus
- **Degasser:** Henggeler DG-4
- **Computer:** Compaq Deskpro
- **Software:** Merck HSM

**Method 1: Measurement of Amiodarone-Derivatives**

- **Column:** Macherey-Nagel CC 250/4 Nucleosil 100-5 Protect 1 (Macherey-Nagel, 4702 Oensingen, Switzerland)
- **Mobile Phase:**
  - 625 g Methanol
  - 290 g H₂O bidest.
  - 1.2 ml NH₃ conc.
- **Flow Rate:** 1.2 ml/min
- **UV-Detection:** 242 nm
- **Temperature:** 45° C

**Method 2: Measurement of Midazolam-Derivatives**

- **Column:** Merck LiChroCART 125-4 (Cat. 1.50829) LiChrospher 60, RP-select B (5 μm)
- **Mobile Phase:**
  - 362 ml methanol
  - 210 ml i-propanol
  - 0.06 ml HClO₄ (perchloric acid)
  - 35 ml H₂O bidest.
- **Flow Rate:** 1.3 ml/min
- **UV-Detection:** 245 nm
- **Temperature:** 45° C
c) **HPLC-System C**

**Instrumentation:**

- **System:** LKB liquid chromatograph (16 126 Bromma, Sweden)
- **Pump:** HPLC 2150 pumps (2×)
- **Controller:** 2152 HPLC controller
- **UV-Detector:** 2152 UV detector
- **Super Fraction Collector:** LKB Superac 2211
- **Software:** WinChrom version 1.3 (Dandenong, Victoria 3175, Australia)

**Method 1: Measurement of Amiodarone-Derivatives**

- **Column:** Nucleosil 100-5 Protect 1 (250 × 4 mm; 5 μm particle size)  
  (Macherey-Nagel, 4702 Oensingen, Switzerland)
- **Mobile Phase:** 300 g Methanol  
  90 g H₂O bidest.  
  0.25 g NH₃ 25%.
- **Flow Rate:** 1.2 ml/min
- **Pressure:** 190 bar
- **UV-Detection:** 242 nm
- **Temperature:** 45°C

**Method 2: Semi-preparative Fraction collection of X1**

- **Column:** Nucleosil-100-7C18 (125 × 10 mm)  
  (Macherey-Nagel, 4702 Oensingen, Switzerland)
- **Mobile Phase:** 300 g Methanol  
  65 g H₂O bidest.  
  0.50 g NH₃ 25%.
- **Flow Rate:** 2 ml/min (0 - 15 min)  
  3 ml/min (15 - 20 min; wash step)
- **UV-Detection:** 242 nm
- **Temperature:** 22°C
- **Fraction collection:** all 5 s between 12 and 14 min of run time
2.1.2 HPLC-ESI-MS/MS Measurement of Amiodarone Derivatives

**Instrumentation:**
- **Liquid Chromatograph:** HP 1100 HPLC system (Hewlett Packard, Palo Alto, CA, USA)
- **Mass Spectrometer:** Bruker ESQUIRE-LC quadrapole ion-trap instrument (Bruker-Franzen GmbH, Bremen, Germany)
- **Electrospray ion source:** Hewlett Packard

**HPLC-Assay:**
- **Column:** RP-C8 Waters Symmetry 150x2 mm (Waters, Milford, MA 01757, USA)
- **Mobile Phase:**
  - 300 g Methanol
  - 90 g H$_2$O bidest.
  - 0.25 g NH$_3$ 25%
- **Flow Rate:** 0.2 ml/min
- **Pressure:** 70 bar
- **UV-Detection:** 242 nm
- **Temperature:** 40° C

**Measurement:**
- **Nitrogen nebulizer gas:** 20 psi
- **Nitrogen dry gas:** 6 l/min
- **dry temperature:** 300° C
- **capillary voltage:** 4000 V
- **end-plate voltage:** 3500 V
- **capillary exit voltage:** 132 V
- **skimmer 1 voltage:** 42 V
- **acquisition:** at normal resolution, 0.6 amu at the half peak height
- **mass range:** m/z 100 - 1000 (m/z 60 cut-off value)
HPLC-ESI-MS/MS and ESI-MS/MS analysis using direct flow injection technique

Flow: 0.24 ml/h (continuous flow through ESI-interface)
Nitrogen nebulizer gas: 15 psi
Nitrogen dry gas: 6 l/min
dry temperature: 250°C
capillary voltage: 4200 V
end-plate voltage: 3700 V
capillary exit voltage: 100 V
skimmer 1 voltage: 35 V
acquisition: at normal resolution, 0.6 amu at the half peak height
mass range: m/z 100 - 800 (m/z 60 cut-off value)
Isolation width: monoisotopic (m/z 0.7)
fragmentation cut-off: "Fast Calc"
fragmentation amplitude: 0.9 V
2.1.3. NMR-, IR- and UV/VIS-Measurement of Amiodarone-Derivatives

a) NMR-Measurements

NMR. $^1$H-NMR and $^{13}$C-NMR spectra of chemical synthesis products were recorded on a Bruker AMX-300 spectrometer at 298 K. Spectra of products isolated from biochemical incubations were recorded at 295 K on a Bruker DRX600 spectrometer equipped with a 5-mm broadband inverse probe with an actively shielded z-gradient coil. Proton and carbon 90° pulse lengths were 7 μ and 13.4 μ, respectively. Unknown substance (150 µg) was dissolved in 0.2 ml CD$_3$OD (NMR-microtube, Shigemi, Tokyo, Japan). All spectra were referred to the solvent peaks ($^1$H at 3.31 ppm and $^{13}$C at 49.15 ppm).

The 1D-DPFGSE-TOCSY spectrum was recorded with a z-filter and a mixing time of 100 ms (MLEV17). The selective excitation was achieved with a 40% truncated Gaussian-shaped pulse of 16-ms duration.

The 2D-TOCSY spectrum was acquired with 2048 points in $f_2$, 512 complex points in $f_1$, four scans per increment, and 100 ms mixing time (MLEV17). Prior to Fourier transformation, the data matrix was zero-filled to 2048*1024 points and weighted with an unshifted sine function along both dimensions.

The DQF-COSY spectrum was acquired with 4096 points in $f_2$, 512 complex points in $f_1$, eight scans per increment, and $t_{1\text{max}}$ of 53.3 ms. Prior to Fourier transformation, the data matrix was zero-filled to 4096*1024 points and weighted with an unshifted sine function along both dimensions.

The gs-HSQC (echo-antiecho version) spectrum was acquired with 1646 points in $f_2$, 300 complex points in $f_1$, and 64 scans per increment. Carbon decoupling during proton acquisition was achieved by applying a garp pulse train. Prior to Fourier transformation, $t_1$ time domain data were doubled by linear prediction followed by zero-filling to 2048 points. The final data matrix was weighted with a sine function along both dimensions.
The gs-HMBC spectrum was acquired with 2048 points in f2, 260 points in f1, 128 scans per increment, and a 60-ms delay for evolution of long-range couplings. Prior to Fourier transformation, t1 time domain data were extended once by linear prediction, followed by zero-filling to 2048 points. The final data matrix was weighted with a cosine function along both dimensions.

b) IR
Infrared-spectra were recorded on a Perkin-Elmer FT-IR System 2000 (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) in a range between 400 and 4000 cm\(^{-1}\). Samples were prepared in KBr-plates.
2.2. **Identification of New Products in AMI Metabolism**

2.2.1. **Identification of 3’OH-MDEA**

2.2.1.1. **Biosynthesis of 3’OH-MDEA**

Since a sufficient amount for NMR measurement was desired (0.1 mg) MDEA was incubated under empirical conditions where 0.25 mg of MDEA hydrochloride (dissolved in 0.2 ml of ethanol) were added to a system of 8 mg of rabbit liver microsomal proteins suspended in 20 ml of 0.1 M sodium phosphate buffer pH 7.4 (PB7.4) and a NADPH generating system consisting of 150 mg of DL-isocitric acid, 0.8 ml of isocitric dehydrogenase and 0.1 g of MgCl₂*6 H₂O. The reaction was started by the addition of 80 mg of NADPH dissolved in 1 ml of ice-cooled PB7.4. The production of the unknown substances was reactivated after 4 hours by adding 50 mg of NADP dissolved in 0.5 ml of PB7.4 and prolonging the incubation time up to 8 hours. The final volume of the incubation was 40 ml.

2.2.1.2. **Purification of 3’OH-MDEA**

The incubation medium was (40 ml), centrifuged (10'000 g at 4° C for 10 min) and extracted three times with 20 ml of diethylether. The protein pellet was washed five times with 3 ml of methanol. The organic phases were separated by centrifugation, pooled and evaporated to dryness at 22° C. The residue was dissolved in 0.5 ml of ethanol and 5 ml of n-hexane and applied to silica cartridge (Chromafix 400 mg; Macherey-Nagel, Oensingen, Switzerland). Then the stationary phase was washed successively with 10 ml of n-hexane, 35 ml of n-hexane:isopropanol:aq.NH₃ 25 % (30.0:2.5:0.5 w/w) and 50 ml of n-hexane:diehtylether:acetic acid (52.8:14.2:1 w/w). Finally the unknown product (X1) was eluted with three portions of 2 ml of chloroform:methanol:acetic acid:water (95.5:39.5:5.25:3 w/w). The fractions were pooled and evaporated under reduced pressure at 22° C).
The residue was then dissolved in 0.5 ml chloroform and applied under atmospheric pressure to a NH$_2$-Sep-Pak cartridge (100 mg, Waters, Milford, MA 01757, USA) which has been previously conditioned successively with 5 ml methanol, 3 ml ethanol, 3 ml $n$-hexane, and 3 ml chloroform. The extraction device was then washed with 6 ml $n$-hexane and 5 ml acetic acid (2%) in diethyl ether. The unknown products were then eluted with three portions (2 ml) of chloroform:2-propanol (2:1 v/v). The fractions were pooled and evaporated to dryness under reduced pressure and stored under nitrogen at -20°C.

The residue was dissolved in 0.5 ml methanol and purified by means of semi-preparative HPLC (HPLC-system C, Method 2). The retention times of the major unknown product and MDEA were 12.6 min and 19.8 min, respectively. Between 12 min and 14 min, the eluent was collected, pooled, and evaporated to dryness under the reduced pressure at 22°C. Finally, the residue was suspended in 2 ml PB7.4, and the unknown products were extracted three times with 3 ml diethyl ether. The hydrochloride salt was obtained by adding 1 ml 1% HCl in methanol and evaporating to dryness.

2.2.1.3. Hydrogen-deuterium (H/D) exchange

The H/D exchange experiments were performed by dissolving MDEA, unknown product in 99.96 % CD$_3$OD (Cambridge Isotope Laboratories, Woburn, MA, USA) and waiting for 10 min to achieve a complete exchange of hydrogen atoms.

Characterization of the Chemical Structure of 3’OH-MDEA:
The isolated product was subjected to HPLC-ESI-MS/MS and NMR analyses as described previously (page 51).
2.2.2. Identification of X2 and X3

MDEA (20 μM) was incubated with 0.4 mg/ml HLM in the presence of a NADPH generating system (4.5 U/ml isocitric dehydrogenase, 7 mM DL-isocitric acid, 6 mM MgCl₂ and 400 μM NADP) in 0.1 M phosphate buffer pH 7.4 as described beneath. In order to investigate the chemical structure of the unknown compounds by ESI-MS/MS, fractions were collected manually at 7.8 and 10.6 min, respectively (HPLC-system B, method 1). The compounds were purified and concentrated by diethyl ether extraction and evaporation to dryness. The samples were kept at -20°C and analyzed within 12 hr.

The chemical structures of X2 and X3 were identified by
a) comparing the HPLC-UV retention times of X2 and X3 with those of synthesized reference products,
b) comparison of quasi-molecular ions ([M+H]⁺) obtained from LC-ESI-MS,
c) comparison of fragmentation patterns obtained from HPLC-ESI-MS/MS analysis.
2.3. Chemical Synthesis of Amiodarone Metabolites

Scheme 9: Synthesis of AMI-derivatives: a) PPh₃*HBr, CH₃CN, reflux, 3 h; b) overnight, RT; c) valeric acid (10), DCC, DMAP, CH₂Cl₂, overnight, RT; d) 1,4-dioxan, NEt₃; e) 4-methoxybenzoylchloride (11), FeCl₃, toluene, 6 h; f) AlCl₃, toluene; g) I₂,
Biotransformation Methods

NaOAc*3 H₂O, methanol, 74° C, 0.5 h; h) 2-chlorehtylamine, NaH, DMF, 115° C, 3 h; i) tertbutyl-N-(2-bromo)-ethylcarbamate (12), NaH, DMF, 95° C, 5 h; j) CF₃COOH, CHCl₃, 60° C, 3 h.

The synthesis of various AMI derivatives was achieved starting from 2-hydroxybenzylalcohol (1). The proceeding to synthesize a 2-n-butylbenzofuran (3) by a coupling of a carboxylic acid to the triphenylphosphonium salt (2) followed by an intramolecular Wittig reaction is based on previous work by Le Corre and Hercouet [177, 178]. Synthetic procedures were followed descriptions by McAllister et al. [179]. The following steps to the synthesis of 2-n-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)benzofuran (6) was strictly performed according the methods of Grain et al. [180]. The final steps had to be adapted to AMI-EtOH (7) and DDEA (9) synthesis. Preparation of (7) by etherification of (6) with 2-chloroethanol was achieved by previous preparation of (6) to a nucleophile by NaH. In the case of preparing (9) the electrophil was first protected by di-tertbutyldicarbonate (Boc₂O) in order to rise its boiling point. Subsequent acidic deprotection gave (9). They products were successfully used to identify formed metabolites in extracts of biological incubation mixtures.

2-Hydroxybenzyltriphenylphosphonium bromide (2).

Synthesis of (2) was performed following a previously described method [179]. First 1.24 g (9.98 mmol) of 2-hydroxybenzylalcohol (1) were introduced into a 250 ml flask and dissolved in 100 ml of acetonitrile. Then 3.43 g (9.98 mmol) of triphenylphosphine hydrobromide was added and the solution was stirred at 100° C (reflux) for 3 h before the solution was allowed to cool down to room temperature. Then the solution was stirred overnight. The solvent was removed under vacuo and the residue was recrystallised in ethanol. 3.79 g were obtained as white crystals, mp: 242° C, IR: 1H-NMR: δH[300 MHz, CDCl₃] 4.50 (1H, s, -CH₂-), 4.54 (1H, s, -CH₂-), 6.57 (1H, t, J 7.4 Hz), 6.94 (1H, dd, J 7.8, 7.6 Hz), 7.25 (1H, d, J 7.3 Hz), 7.51-7.55 (15 H, Ar-H), 7.70 (1H, d, J 7.3 Hz).
n-Butylbenzofuran (3).
Synthesis of (3) was realized following the method of McAllister et al. [179]. 0.65 g (1.45 mmol) of the phosphonium salt (2) were introduced into a 250 ml flask followed by 0.2126 g (1.74 mmol) of DMAP and 0.1481 g (1.45 mmol; d=0.938 g/ml; 0.166 ml) of valeric acid (10) and dissolved in 50 ml of dichloromethane. Then 0.343 g of Dicyclohexylcarbodiimid (DCC, 1.67 mmol) were added and dissolved in 15 ml of dichloromethane and the solution was stirred overnight (20 h) at room temperature under nitrogen. After evaporation the residue was dissolved in 1,4-dioxane and 0.84 ml (6.03 mmol) of triethylamine were added before the mixture was stirred under reflux (110° C) and nitrogen overnight (15-16 h). The solvent was evaporated after the conversion was controlled by TLC (n-hexane/ethylacetate 1:1). The Rf-value of (3) was about 0.95. The product was then purified by column chromatography (n-hexane/ethylacetate 95:5). After the evaporation of the fractions a colorless liquid was obtained with a yield of 0.2037 g (1.169 mmol; 80 %).
NMR: δH[300 MHz, CDCl3] 1.04 (3H, t, 77.3 Hz, =CH3), 1.52 (2H, m, J 7.5 Hz, -CH2-), 1.82 (2H, m, J 7.6 Hz, -CH2-), 2.85 (2H, t, J 7.6 Hz, -CH2-), 6.45 (1H, s, -H), 7.25 (1H, ddd, J 7.3, 7.2, 1.2 Hz, -H), 7.27 (1H, ddd, J 8.2, 7.3, 1.8 Hz, -H), 7.48 (1H, d, J 5.7 Hz, -H), 7.54 (1H, d, J 7.9 Hz -H).

2-n-butyl-3-(4-methoxybenzoyl)benzofuran (4).
As described previously [180] 2-n-butylbenzofuran (3) (0.45 g; 2.58 mmol) was introduced into the flask followed by 1.5 ml of dry toluene. Before the addition of 4-methoxybenzoylchloride (0.45 g; 2.64 mmol) the solution was purged with nitrogen. Then the solution was cooled with an ice/NaCl mixture to -10° C and anhydrous FeCl3 was added. After stirring at -10° C for 15 minutes the solution was allowed to warm up to room temperature and stirred for 6 hours. The mixture was cooled down to 0° C before 0.57 ml of water were added and was left at room temperature for 30 minutes. The 4-methoxybenzoic acid was filtered off and the organic phase was washed first with diluted hydrochloric acid (30 ml HCl 37 % dilute to 100 ml) and then with water (each three times). Then the organic phase was dried with Na2SO4 and evaporated.
Yield: 0.693 g (2.34 mmol; 91 %; Ref.: about 100 % [178])
NMR: δH[300 MHz, CDCl3] 0.91 (3H, t, J 7.3 Hz, =CH3), 1.36 (2H, m, J 7.5 Hz, -CH2-), 1.79 (2H, m, J 7.6 Hz, -CH2-), 2.93 (2H, t, J 7.6 Hz, -CH2-), 3.91 (3H, s, -CH3), 6.97 (2H, d, J 8.8 Hz, -H), 7.21 (1H, ddd, J 7.5, 7.3, 0.9 Hz, -H), 7.28 (1H, ddd, J 8.2, 7.2,
1.4 Hz, -H), 7.35 (1H, d, J 7.8 Hz, -H), 7.48 (1H, d, J 7.9 Hz, -H), 7.84 (2H, d, J 8.8 Hz, -H).

2-n-butyl-3-(4-hydroxybenzoyl)-benzofuran (5).

According to a previously published method [180] (4) (0.693 g; 2.25 mmol) was dissolved in 1.7 ml (1.464 g) of toluene and cooled down to 0° C. While stirring AlCl₃ (0.5933 g; 4.45 mmol) was added to get the 1:1 complex. Further 1.34 ml (1.155 g) of toluene were added before the mixture was heated to 130° C and 1.3 g of toluene were distilled off while 1 hour. The mixture was cooled down to 75° C and 1.4 ml of hydrochloric acid solution (46.2 ml HCl 36% in 600 g H₂O) were added. Finally the toluene phase was washed with water (3 times), dried over Na₂SO₄ and evaporated. Yield: 0.7907 g (85 %; Ref.: 86 % [178]).

NMR: δH[300 MHz, CDCl₃] 0.90 (3H, t, J 7.3 Hz, =CH₃), 1.36 (2H, m, J 7.5 Hz, -CH₂-), 1.79 (2H, m, J 7.6 Hz, -CH₂-), 2.93 (2H, t, J 7.7 Hz, -CH₂-), 6.44 (1H, s, OH), 6.91 (2H, d, J 8.7 Hz, -H), 7.19 (1H, ddd, J 7.7, 7.3, 1.1 Hz, -H), 7.28 (1H, ddd, J 8.3, 7.8, 1.2 Hz, -H), 7.37 (1H, d, J 7.8 Hz, -H), 7.48 (1H, d, J 7.7 Hz, -H), 7.81 (2H, d, J 8.7 Hz, -H).

2-n-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)-benzofuran (6).

Following the method of Grain and Jammot [180] sodium acetate trihydrate (0.878 g; 6.46 mmol), iodine (1.57 g; 6.187 mmol) and methanol were introduced into the flask and heated up to 30 - 35° C. (5) (0.7907 g; 2.69 mmol) was dissolved in few methanol and pipetted to the reaction solution which was then stirred at 70-74° C for 30-45 minutes. Then 1 ml of a 5 M sodium hydroxyde solution was added to the mixture which was stirred under reflux (80-90° C) for 2 h. After putting off the heating 0.86 g of a sodium bisulfite solution (35° Bé) were added during 20 minutes. About 2 ml were evaporated and the solution was cooled down to 75 - 80° C. Then 0.537 g water, 0.578 hydrochloric acid and 4.3 g toluene were added before the mixture was heated for 10 minutes at 85° C (evaporation of SO₂). The phases were separated and the toluene phase was washed with water, sodium bisulfite and twice with water. After drying the organic phase was evaporated and the product was recrystalized in toluene.

Yield: 843.4 mg (1.545 mmol; 57 %)

NMR: δH[300 MHz, CDCl₃] 0.93 (3H, t, J 7.3 Hz, =CH₃), 1.36 (2H, m, J 7.3 Hz, -CH₂-), 1.79 (2H, m, J 7.6 Hz, -CH₂-), 2.86 (2H, t, J 7.7 Hz, -CH₂-), 6.21 (1H, s, OH), 7.25
(1H, ddd, J 7.4, 7.3, 0.8 Hz, -H), 7.32 (1H, ddd, J 8.3, 7.3, 1.3 Hz, -H), 7.41 (1H, d, J 7.9 Hz, -H), 7.49 (1H, d, J 7.7 Hz -H), 8.19 (2H, s, -H).

2-n-butyl-3-(3,5-diiodo-4-ß-hydroxyethoxybenzoyl)-benzofuran (7).

(6) (225 mg; 0.4 mmol) was introduced into the flask and dissolved in dry DMF (dimethylformamide). NaH (30 mg) was added in a single portion and the mixture was stirred for 2 hours at room temperature under nitrogen. Then 2-chloroethanol (166 mg; d = 1.202 g/ml; 138 µl; 2 mmol) was added and the reaction mixture was heated to 115°C. The reaction progress was controlled by TLC (n-hexane/ethylacetate 9:1) and removed after 3 hours. The Rf-value was 0.1. After evaporation of the solvent the product was dissolved in dichlormethane and the organic phase was washed with saturated sodium bicarbonate (sodium hydrogencarbonate, NaHCO₃), dried and evaporated. The product was purified by flash chromatography (silica gel) and obtained as viscous oil.

Yield: 34.1 mg (0.058 mmol; 14 %)

NMR: δH[300 MHz, CDCl₃] 0.93 (3H, t, 77.3 Hz), 1.38 (2H, m, 77.3 Hz), 1.78 (2H, m, J 7.6 Hz), 2.41 (1H, t, J 6.2 Hz), 2.86 (2H, t, J 7.7 Hz), 4.11 (2H, q, J 4.8 Hz), 4.27 (2H, t, J 4.3 Hz), 7.26 (1H, ddd, J 77.7, 7.2, 0.8 Hz), 7.32 (1H, ddd, J 8.1, 7.3, 1.3 Hz), 7.42 (1H, d, J 7.7 Hz), 7.49 (1H, d, J 7.8 Hz), 8.24 (2H, s); HPLC-ESI-MS [M + H]⁺ = m/z 591(91 %); ESI-MS/MS(m/z 591): m/z 547(40), m/z 373(54), m/z 201(6).

Tertbutyl-N-(2-chloro)-ehtylcarbamate (12)

2-Bromethylamine hydrobromide (1.92 g; 9.39 mmol), di-tert-butyldicarbonate (Boc₂O, 2.05 g; 9.39 mmol) and sodium hydroxide (0.41 g; 100 mmol) were dissolved in 9 ml H₂O and 10 ml isopropanol. The mixture was stirred overnight. Then the volume of solvent was reduced to about 10 ml by evaporation under reduced pressure and the product was extracted with 30 ml of dichlormethane. The organic phase was dried (Na₂SO₄) and evaporated. Thus the Boc-protected 2-bromethylamine was obtained as colorless oil. Yield: 1.69 g (7.55 mmol; 80 %).

2-n-butyl-3-(3,5-diiodo-4-ß-(O-tertbutyl)-carbamidoethoxybenzoyl)-benzofuran (8).

282 mg (0.4 mmol) of (2-n-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)-benzofuran) (6) were dissolved in dry DMF. NaH (37 mg; 1.2 mmol) was added in a single portion and
the mixture was stirred for 2 hours at room temperature under nitrogen. Then 116 mg (0.52 mmol) tert-butyl-N-(2-chloro)-ethylcarbamate (12) was pipetted into the reaction and the mixture was heated up to 95° C and stirred for 5 hours. The progress of the reaction was controlled by thin layer chromatography: silica gel; n-hexane:ethylacetate (8:2 v/v). The Rf values of the starting compound B2 and of the final product were 0.3 and 0.5, respectively. The solvent was then evaporated and the residue dissolved in dichloromethane before washing with 50 ml of a saturated solution of NaHCO3. The dichloromethane phase was dried (Na2SO4) and concentrated by evaporation under reduced pressure. Finally the product was obtained after purification by column chromatography (n-hexane:ethylacetate; 8:2 v/v). Yield: 57 mg (0.0823 mmol, 16 %).

2-n-butyl-3-(3,5-diiodo-4-ß-aminoethoxybenzoyl)-benzofuran (9).

2-n-butyl-3-(3,5-diiodo-4-ß-(O-tertbutyl)-carbamidoethoxybenzoyl)-benzofuran (8) (57 mg; 0.0823 mmol) was dissolved in CHCl3 and 20 equivalents of CF3COOH (142 mg; 1.24 mmol) were added. The reaction was maintained stirring under reflux (60° C) for 3 hours. Then the solution was washed with a saturated solution of K2CO3 and evaporated under reduced pressure. The product X3 was finally purified with flash chromatography (dichloromethane:methanol; 95:5 v/v). Yield: 23.2 mg (0.039 mmol; 7.5 %). NMR: δH[300 MHz, CDCl3] 0.93 (3H, t, J 7.3 Hz), 1.36 (2H, m, J 7.4 Hz), 1.78 (2H, m, J 7.6 Hz), 2.86 (2H, t, J 7.7 Hz), 3.26 (2H, t, J 5.0 Hz), 4.14 (2H, t, J 5.1 Hz), 7.25 (1H, ddd, J 7.4, 7.3, 1.2 Hz), 7.32 (1H, ddd, J 8.1, 7.3, 1.2 Hz), 7.42 (1H, d, J 7.7 Hz), 7.49 (1H, d, J 7.8 Hz), 8.23 (2H, s); HPLC-ESI-MS [M + H]+ = m/z 590(76 %); ESI-MS/MS(m/z 590): m/z 547(81), m/z 373(15), m/z 201(4).
2.4. Quantification of Amiodarone Derivatives

2.4.1. Preparation of Matrix Solution:

In order to take into account the matrix effects for the quantification of the AMI derivatives, three milliliters of denaturized rabbit liver microsomes (Sept99) were diluted in PB7.4 and extracted with 200 ml of diethylether. The diethylether phase was evaporated and the residue dissolved in 50 ml of the mobile phase for HPLC analysis. The amount of proteins was calculated to be the same as in the extracts of the microsomal incubations.

2.4.2. Calibration

The calibration was conducted on the basis of dilution. 3'OHDDEA, AMI-EtOH and DDEA were dissolved in dichloromethane to 5 mM and diluted with mobile phase (HPLC-system A, method 1) to solutions of 100 μM. Those solutions were then diluted with matrix containing solutions to solutions which contained all metabolites in a 10 μM concentration (prepared in triplicate). Further dilution gave standard solutions in triplicate containing 5, 2.5, 1, 0.5, 0.25 and 0.1 μM. Every solution was analyzed three times by injecting 80 μl resulting in analyzed amounts of 800, 400, 200, 80, 40, 20 and 8 pmol. Peak areas were estimated by integration and the data points were analyzed by linear regression analysis using Origin software (Redacom, Nidau, Switzerland) receiving a linear relationship between injected amounts and peak areas such as

\[ y = bx + a \]  \hspace{1cm} (18)

where b is the slope of the line and a the intercept of the y-axis. The obtained equation was used to calculate formed 3'OHDDEA, AMI-EtOH and DDEA in the biotransformation assay.

\[ x = \frac{y - a}{b} \]  \hspace{1cm} (19)

Confidence limits for the data sets were estimated by equation (20)

\[ CL(99\%) \text{ for } \mu = \overline{x} \pm ts \]  \hspace{1cm} (20)
where \( \bar{x} \) is the mean value of the estimations, \( \mu \) the "real value" (added amount) and \( s \) the standard deviation. The \( t \) value was taken from table [181] for 6 degrees of freedom and 95 % confidence limits.

2.4.3. Limit of Detection (LOD) and Limit of Quantitation (LOQ):

Measurement of the compounds after extraction from lipid and protein containing medium implicated difficulties in estimating the standard deviation of the noise directly from the HPLC-chromatogram. Thus another strategy was applied which is based on published data [182]. The solutions containing the metabolites were further diluted and low amounts (20, 8, 4, 2 and 0.8 pmol) were injected (three solutions per concentration analyzed in triplicate). The data points were fitted by linear least-square regression analysis using Origin software (Redacom, Nidau, Switzerland) in order to obtain a linear equation as described above. The statistic error \( s_{y/x} \) is calculated using the individual y-values (\( y_i \)) and the calculated y values \( \hat{y} \) using the linear equation and the x-values:

\[
s_{y/x} = \sqrt{\frac{\sum_{i} (y_i - \hat{y}_i)^2}{n-2}} \tag{21}
\]

One accepted definition in the literature is that the limit of detection is the analyte concentration giving a signal equal to the blank signal \( y_B \) plus three standard deviations of the blank \( s_B \).

\[
y - y_B = 3s_B \tag{22}
\]

In the present analysis \( s_{y,x} \) was used in the place of \( s_B \) with the assumption that each point in the HPLC-chromatogram has the same normally distributed variation (in the y-direction only) and thus the same standard deviation \( s_{y,x} \). Therefore the limit of detection was calculated from the corresponding signal \( y \) [182]. The limit of quantitation was estimated following the relation

\[
y - y_B = 10s_B = 10s_{y,x} \tag{23}
\]

expressed by equation (23).
2.4.4. Precision

Precision of the analysis was investigated by injecting 80 μl of the concentrations 2.5, 0.5 and 0.1 μM (200, 40 and 8 pmol) seven times at the same day. The results were then compared by recovery and relative standard deviation.
2.5. Incubation of MDEA with Rabbit Liver Microsomes

2.5.1. Preparation of Rabbit Liver Microsomes

Microsomes were prepared following the method of Meier et al. [183]. The samples from USZ were isolated from anesthetized rabbits and the tissues were transferred immediately to ice after weighing.

**Table 3:** Summary of available rabbit liver microsomes kindly donated either from Dr H. Cristina, Institute for Laboratory Animal Science, University of Zurich, Switzerland (USZ) or from Dr. P. Gallier, Laboratoire de Pharmacologie-Toxicologie INRA, 31931 Toulouse, France (INRA).

<table>
<thead>
<tr>
<th>Sample</th>
<th>conc. / [mg/ml]</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dez98</td>
<td>33.2</td>
<td>USZ</td>
</tr>
<tr>
<td>Feb99</td>
<td>47.0</td>
<td>&quot;</td>
</tr>
<tr>
<td>Mar99</td>
<td>33.0</td>
<td>&quot;</td>
</tr>
<tr>
<td>Juni99A</td>
<td>25.6</td>
<td>&quot;</td>
</tr>
<tr>
<td>Juni99B</td>
<td>17.7</td>
<td>&quot;</td>
</tr>
<tr>
<td>Sept99</td>
<td>44</td>
<td>&quot;</td>
</tr>
<tr>
<td>T1</td>
<td>7.4</td>
<td>INRA</td>
</tr>
<tr>
<td>T2</td>
<td>7.7</td>
<td>&quot;</td>
</tr>
<tr>
<td>R2</td>
<td>8.5</td>
<td>&quot;</td>
</tr>
<tr>
<td>R3</td>
<td>7.9</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The samples obtained from Dr. P. Gallier (Laboratoire de Pharmacologie-Toxicologie INRA, 31931 Toulouse, France) were isolated from cultured rabbit hepatocytes which were either cultivated under control conditions (T1, T2; without treatment of rifampicin) or treated with rifampicin. Protein concentrations were measured by a method described previously [184].
2.5.2. Incubation of MDEA in Rabbit Liver Microsomes

The incubation conditions were selected according the experience of Kronbach et al. [185] and optimized for the biotransformation of MDEA to 3'OH-MDEA as described below. MDEA was incubated together with a NADPH generating system consistent of isocitrate dehydrogenase, isocitric acid, magnesium chloride and NADP. The conditions are summarized in Table 4.

Table 4: Incubation conditions for the biotransformation of MDEA to 3'OH-MDEA with rabbit liver microsomes. The final volume was 200 µl.

<table>
<thead>
<tr>
<th>Component</th>
<th>conc. in react. mixt.</th>
<th>Origin / Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>microsomes</td>
<td>0.2 mg/ml</td>
<td>isolated by procedure described above</td>
</tr>
<tr>
<td>isocitr. dehyd.</td>
<td>4.5 U/ml (1 mg/ml)</td>
<td>60.3 U/ml; 13.4 mg prot./ml; 4.5 U/mg prot.</td>
</tr>
<tr>
<td>isocitric acid</td>
<td>7 mM</td>
<td>dissolved in PB7.4 (sol. A)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>6 mM</td>
<td>dissolved in PB7.4 (sol. A)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>400 µM</td>
<td>dissolved in PB7.4</td>
</tr>
<tr>
<td>MDEA</td>
<td>20 µM</td>
<td>dissolved in sol A</td>
</tr>
</tbody>
</table>

Microsomes, cofactors, proteins and the substrate were dissolved in 0.1 M phosphate buffer pH 7.4 (PB7.4). The reaction was started by the addition of NADP (40 µl). After vortexing the mixtures were incubated at 37°C for 15 minutes. The reactions were stopped by the addition of 1 ml of diethylether and by putting the reaction vessels on ice.
2.5.3. Sample Preparation

For the achievement of a better analysis by HPLC the AMI-derivatives were extracted from the reaction mixture as follows:

- extraction of the reaction mixture with 1 ml of diethylether each for 5 min. (3x)
- collection of the organic phase in vacutainer glass tubes
- evaporation of the organic phase by air flow
- dissolution of the residue by the addition of 100 μl of mobile phase (HPLC)
- vortexing of the dissolved residue
- centrifugation of the dissolved residue for 5 min at 14'000 rpm
- pipetting of the volume into the HPLC vial for injection by autosampler

2.5.4. Enzyme Kinetics of MDEA Metabolism in Rabbit Liver Microsomes

For the calculation of the Michaelis-Menten parameters the 3’OH-MDEA formation was investigated incubating 16 different concentrations of MDEA between 2.5 and 40 μM. The experiments were performed using rabbit liver microsomes Dez98, Feb99, T1 and R2 (Table 3). The apparent enzyme kinetic parameters were first estimated using Lineweaver-Burk plots (1/V vs. 1/S), and the values obtained were used as the first estimate for the nonlinear least-squares regression analysis using the Michaelis-Menten equation (2) and Origin software (Redacom, Nidau, Switzerland). K_m and V_max-values were averaged over samples Dez98, Feb99 and T1 (interindividual variability).

Eadie-Hofstee plots (V vs V/S) were then used to gain information regarding the involvement of the number of binding sites involved in the biotransformation. The inhibitory mechanisms of chemical inhibitors were investigated using Dixon plots (1/V vs inhibitor concentrations).
2.5.5. Increased Metabolic Activity after Induction of Rabbit CYP3A6

In addition to the enzyme kinetic experiments the activities of 3’OH-MDEA formation were compared between MDEA incubation with T1 and R3. Both rabbit liver microsomal samples were obtained from cultured rabbit hepatocytes which were either treated with control conditions (T1) or with rifampicin in order to induce CYP3A6 [186, 187] (R2). Due to the increased activity MDEA was incubated with 0.1 mg/ml of R2 microsomes compared to 0.2 mg/ml of T1.
2.5.6. Chemical Inhibition of MDEA Metabolism

The effect of a selection of cytochrome P450 (CYP) specific inhibitors and substrates was investigated for the hydroxylation of MDEA to 3’OH-MDEA. The compounds are summarized in Table 5.

Table 5: Cytochrome P450 specific inhibitors and substrates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specificity</th>
<th>Class</th>
<th>Dissolved in</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-naphthoflavone (αNF)</td>
<td>1A2</td>
<td>inhibitor</td>
<td>DMSO</td>
<td>[188, 189]</td>
</tr>
<tr>
<td>coumarine</td>
<td>2A6</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[190]</td>
</tr>
<tr>
<td>sulfaphenazole</td>
<td>2C9</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[191]</td>
</tr>
<tr>
<td>(S)-mephenytoine</td>
<td>2C19</td>
<td>substrate</td>
<td>ethanol</td>
<td>[190, 192]</td>
</tr>
<tr>
<td>quinidine</td>
<td>2D6</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[193, 194]</td>
</tr>
<tr>
<td>quinine</td>
<td>unspez.</td>
<td>substrate</td>
<td>ethanol</td>
<td>---</td>
</tr>
<tr>
<td>diethyldithiocarbamate (DDC)</td>
<td>2E1</td>
<td>inhibitor</td>
<td>PB7.4</td>
<td>[188]</td>
</tr>
<tr>
<td>troleandomycine (TAO)</td>
<td>3A4</td>
<td>inhibitor</td>
<td>DMSO</td>
<td>[188, 191]</td>
</tr>
<tr>
<td>ketoconazole</td>
<td>3A4</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[191, 195]</td>
</tr>
<tr>
<td>midazolam (MDZ)</td>
<td>3A4</td>
<td>substrate</td>
<td>ethanol</td>
<td>[185]</td>
</tr>
<tr>
<td>cyclosporin A</td>
<td>3A4</td>
<td>substrate</td>
<td>ethanol</td>
<td>[196, 197]</td>
</tr>
<tr>
<td>cimetidine</td>
<td>multiple</td>
<td>substrate</td>
<td>ethanol</td>
<td>[198]</td>
</tr>
<tr>
<td>SKF525A</td>
<td>unspez.</td>
<td>inhibitor</td>
<td>PB7.4</td>
<td>[199]</td>
</tr>
</tbody>
</table>

Before the addition to the reaction mixture the dissolved compounds were diluted in PB7.4 in order to keep the content of organic solvents not higher than 1.5 %. The inhibitors of substrates were added to the incubation solution by replacing the volume of PB7.4 with 20 µl of the solution containing the inhibitor. The incubation mixtures containing a concentration of inhibitor or substrate were compared to control reactions which contained the same residual concentration of organic solvent used to dissolve the compounds.
The HPLC peak area of the produced metabolite 3’OH-MDEA was compared to the corresponding control reaction and the amount of inhibition was calculated using the equation (24)

\[
\%I = \frac{v_0 - v_i}{v_0} \times 100
\]  

(24)

where \(v_0\) is the reaction rate of the control reaction and \(v_i\) is the reaction rate of the reaction containing the inhibitor [99].

### 2.5.7. Chemical Inhibition Kinetics of MDEA Metabolism

For assessing the mechanism of MDZ inhibition, four MDZ concentrations (50, 100, 150 and 200 μM) were added to three series of incubation samples containing 2.5, 5.0 and 10 μM MDEA. The inhibition type was analysed using Dixon plots.

### 2.5.8. Correlation of 1’OH-MDZ and 3’OH-MDEA Formation

MDEA hydroxylase activities were compared with those of MDZ using 9 rabbit liver microsomal samples (Table 3). MDZ was incubated according to previously published conditions [185] with a substrate concentration of 20 μM and 0.4 mg/ml protein concentration. As in the case of MDEA incubation the NADPH-generating system consisted of 4.5 U/ml (1 mg/ml) isocitric dehydrogenase, 7 mM DL-isocitric acid, 6 mM MgCl₂ and 400 μM NADP dissolved in PB7.4. The mixtures were incubated for 5 minutes at 37°C, stopped by the addition of diethlyether and extracted according to the procedure of MDEA. MDEA was incubated in a concentration of 25 μM and 0.2 mg/ml proteins. The formation rates of 3’OH-MDEA were correlated with those of α’OH-MDZ formation by linear least-square fitting using Origin software (Redacom, Nidau, Switzerland). Each data point is the mean of double incubation.
2.6. Incubation of MDEA with Human Liver Microsomes

2.6.1. Preparation of Human Liver Microsomes (HLM)

Three human liver samples were obtained from liver transplant patients from the University Hospital of Zurich. For the correlation study, 15 human liver microsomal samples (HLM) were received from Prof. U.A. Meyer Biocenter, University of Basel. The microsomal protein concentrations were measured using the method of Bensadoun et al. [184]. All HLM Samples are summarized in Table 6. Human cDNA expressed cytochrome P450 isozymes (cCYP) were obtained from Gentest Corp., Woburn, MA, USA. Isocitric dehydrogenase (from porcine heart) was obtained from Sigma, Buchs, Switzerland.

All samples were isolated by means of standard differential centrifugation [183]. The samples BH-22, BH-38 and BH-41 were prepared in our own laboratory following the same method as described here:
- homogenizing sample (50 - 150 mg) in 5 Vol. of 0.25 M sucrose (4° C)
  (10 strokes)
- centrifugation of the homogenized samples by 17'000 rpm for 56 minutes
- centrifugation of the homogenized samples by 10'000 g for 5 minutes
- resuspend pellets in 0.5 ml 0.25 M sucrose
- centrifuge suspension at 10'000 g for 5 minutes
- combine supernatants
- fractionate in 180 µl aliquots
- centrifuge at 148'000 g for 12 minutes
- resuspend pellets in 0.1 M Na-pyrophosphate pH 7.4
- centrifuge at 148'000 g for 12 minutes
- repeat washing step
- resuspend final pellets in 0.1 M NaPO₄ pH 7.4
- store at -80° C
**Table 6:** Collection of used human liver microsomal samples (KPT: Clinical Pharmacology and Toxicology, University Hospital of Zürich; Biocenter Basel: Group of Prof. U.A. Meyer; BH-22, 38 and 41 were prepared to microsomes after delivery)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Character</th>
<th>Prot.conc. / [mg/ml]</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM1</td>
<td>Liver transpl. pat.</td>
<td></td>
<td>KPT</td>
</tr>
<tr>
<td>HLM2</td>
<td>&quot;</td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>HLM3</td>
<td>&quot;</td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-17</td>
<td>3A4 low</td>
<td>8.22</td>
<td>Biocenter Basel</td>
</tr>
<tr>
<td>BH-20</td>
<td>&quot;</td>
<td>8.46</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-24</td>
<td>&quot;</td>
<td>4.09</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-37</td>
<td>&quot;</td>
<td>24.60</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-43</td>
<td>&quot;</td>
<td>5.37</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-18</td>
<td>3A4 medium</td>
<td>13.38</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-19</td>
<td>&quot;</td>
<td>28.37</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-22</td>
<td>&quot;</td>
<td>17.51</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-31</td>
<td>(no 2D6)</td>
<td>11.88</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-21</td>
<td>3A4 high (no 2C19)</td>
<td>14.99</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-23</td>
<td>&quot;</td>
<td>15.29</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-34</td>
<td>&quot;</td>
<td>6.56</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-35</td>
<td>&quot;</td>
<td>4.43</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-38</td>
<td>no 2D6</td>
<td>20.94</td>
<td>&quot;</td>
</tr>
<tr>
<td>BH-41</td>
<td>no 2C19</td>
<td>22.19</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
2.6.2. Incubation of MDEA

The incubation conditions were selected according the experience of Kronbach et al. [185]. The reaction time and protein concentration were optimized. The final reaction conditions are described in Table 7.

Table 7: Reactions were conducted in 0.1 M phosphate buffer pH 7.4 (PB7.4). The final volume was 400 µl.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Final conc.</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>microsomes</td>
<td>0.5 mg/ml</td>
<td>diluted in PB7.4</td>
</tr>
<tr>
<td>isocitr. dehydr.</td>
<td>4.5 U/ml (1 mg/ml)</td>
<td>dissolved aqueous glycerol solution</td>
</tr>
<tr>
<td>isocitric acid</td>
<td>7 mM</td>
<td>dissolved in PB7.4</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>6 mM</td>
<td>dissolved in PB7.4</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>400 µM</td>
<td>dissolved in PB7.4</td>
</tr>
<tr>
<td>MDEA</td>
<td>20 µM</td>
<td>dissolved in sol A</td>
</tr>
</tbody>
</table>

Microsomes, cofactors, proteins and the substrate were dissolved in 0.1 M phosphate buffer pH 7.4 (PB7.4). The reaction was started by the addition of NADP. After vortexing the mixtures were incubated at 37°C for 20 minutes. The reactions were stopped by the addition of 1 ml of diethylether and by putting the reaction vessels on ice.
2.6.3. Sample Preparation

For the achievement of a better analysis by HPLC the AMI-derivatives were extracted from the reaction mixture following the steps listed in the next lines:

- extraction of the reaction mixture with 1 ml of diethylether each for 5 min. (3x)
- collection of the organic phase in vacutainer glass tubes
- evaporation of the organic phase by air flow
- dissolution of the residue by the addition of 100 μl of mobile phase (HPLC)
- vortexing of the dissolved residue
- centrifugation of the dissolved residue for 5 min at 14'000 rpm
- pipetting of the volume into the HPLC vial for injection by autosampler
- injection of 80 μl of the solution into the HPLC

2.6.4. Enzyme Kinetics of MDEA Metabolism

Enzyme kinetic analysis was performed by incubating MDEA in the concentration range between 5 and 100 μM with HLM1, HLM2 and HLM3 in order evaluate the interindividual variability. The formation of metabolites formed by MDEA biotransformation was measured by HPLC-UV and the data points were analyzed by nonlinear least square fitting (Origin Software) using the Michaelis-Menten- and the Hill-equation in order to obtain the enzyme kinetic parameters (K_m, V_max, K_0.5). Additionally the data were analyzed by Eadie-Hofstee plots.
2.6.5. Chemical Inhibition of MDEA Biotransformation

Several substrates are known to have an inhibitory effect on the catalytic activity of specific cytochrome P450 isoforms. A selection of the most important P450 specific inhibitors and substrates was coincubated with MDEA. The used compounds are described in the Table 8.

Table 8: Cytochrome P450 specific inhibitors and substrates used for the chemical inhibition of MDEA incubation in HLM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spezificity</th>
<th>Class</th>
<th>Dissolved in</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-naphthoflavone (αNF)</td>
<td>1A2</td>
<td>inhibitor</td>
<td>DMSO</td>
<td>[188, 189]</td>
</tr>
<tr>
<td>coumarine</td>
<td>2A6</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[190]</td>
</tr>
<tr>
<td>sulfaphenazole</td>
<td>2C9</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[191]</td>
</tr>
<tr>
<td>(S)-mephenytoine</td>
<td>2C19</td>
<td>substrate</td>
<td>ethanol</td>
<td>[190, 192]</td>
</tr>
<tr>
<td>quinidine</td>
<td>2D6</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[193, 194]</td>
</tr>
<tr>
<td>quinine</td>
<td>unspez.</td>
<td>substrate</td>
<td>ethanol</td>
<td>---</td>
</tr>
<tr>
<td>diethyldithiocarbamate (DDC)</td>
<td>2E1</td>
<td>inhibitor</td>
<td>PB7.4</td>
<td>[188]</td>
</tr>
<tr>
<td>midazolam (MDZ)</td>
<td>3A4</td>
<td>substrate</td>
<td>ethanol</td>
<td>[185]</td>
</tr>
<tr>
<td>ketoconazole</td>
<td>3A4</td>
<td>inhibitor</td>
<td>ethanol</td>
<td>[191, 195]</td>
</tr>
<tr>
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<td>inhibitor</td>
<td>DMSO</td>
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</tr>
<tr>
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<td>multiple</td>
<td>substrate</td>
<td>ethanol</td>
<td>[198]</td>
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<td>multiple</td>
<td>inhibitor</td>
<td>PB7.4</td>
<td>[199]</td>
</tr>
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</table>

The compounds were dissolved in organic solvent (assigned in Table 8) or PB7.4 and diluted in PB7.4 to the desired concentration. Each diluted solution was added to the reaction mixture in order to keep the content of the organic solvent below 1.5 % (v/v). The reactions containing an inhibitor were compared to the corresponding control reactions with the same amount of organic solvent which was used to dissolve the
inhibitor. After incubation the formation of metabolites was analysed by HPLC-UV. The degree of inhibition was calculated as the percent inhibition (%I) using equation (24).

2.6.6. Chemical Induction/Inhibition of Enzyme Kinetics

As α-naphthoflavone (αNF) is a mechanism based activator of CYP3A4 [158, 175, 176] and as an inhibitor of CYP1A2 [188, 189]. Thus the activation of the formation of metabolites from MDEA incubation in HLM was investigated by coincubating 20 μM αNF with the reaction mixture. MDEA was incubated in a concentration range between 0.5 and 80 μM. Those incubations were compared to control experiments containing the same content of organic solvent (1 %(v/v) DMSO).
2.6.7. MDEA Metabolism in cDNA Expressed CYP Isoenzymes

Using the same incubation conditions as those for HLM, commercially available cDNA expressed P450 isozymes (1 mg/ml) were incubated with 20 μM MDEA for 1 hour. The biotransformation products were then isolated by diethyl ether extraction and analyzed by HPLC-UV assay as described above. The isozymes used were CYP1A1, CYP1A2, CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. In addition enzyme kinetic studies were performed with selected active P450 isoforms such as 1A1, 2D6 and 3A4. MDEA concentrations were varied between 20 and 100 μM.

2.6.8. Correlation MDEA Biotransformation with 1’OH-MDZ-Formation

The formation rates of three new MDEA derivatives were investigated in 15 HLM samples (BH-17 to BH-43) using the HPLC-UV assay (HPLC-System A; Method 1). Based on the $K_m$ values estimated previously, 20 μM MDEA were used in this study. The results were then expressed in pmol/min/mg protein and compared with the formation rates of α-hydroxymidazolam (α’OH-MDZ) performed as described by Kronbach et al. [185] (0.4 mg/ml HLM; 4.5 U/ml isocitric dehydrogenase, 7 mM DL-isocitric acid, 6 mM MgCl₂ and 400 μM NADP in PB7.4). The midazolam concentration was 20 μM (determined by previous enzyme kinetic experiment) and the final volume was 200 μl. After 10 minutes the reactions were stopped by the addition of 200 μl of methanol containing 10 μM flurazepam as internal standard and the samples were analyzed directly by HPLC-UV (HPLC-System A; Method 2) after centrifugation (13’000 rpm).

The data points (mean of three determinations) were collected and the correlation between the formation rates of the measured metabolites and those of α’OH-MDZ were analyzed by linear regression using Origin Software (Redacom AG, Nidau, Switzerland).
2.7. **Species Comparison of MDEA Biotransformation**

The incubation conditions were selected according the experience of Kronbach et al. [185]. HLM (HLM1) and rabbit liver microsomes (Dez98) were received or isolated as described previously (page 58). Rat liver microsomes were isolated by the procedure reported previously [183] and described above. Pig liver microsomes were a kind gift of Biopharmacy Laboratory of the Institute of Pharmaceutical Sciences (Swiss Federal Institute of Technology, Zürich). Protein concentrations of HLM, rabbit and rat liver microsomes were estimated as described previously [184] and diluted before adding to the reaction mixture. Proteins (0.5 mg/ml) were incubated together with 4.5 U/ml isocitric dehydrogenase, 7 mM D/L-isocitric acid, 6 mM MgCl₂, 400 μM NADP and 20 μM MDEA as final concentrations in 0.1 M phosphate buffer pH 7.4. The final volume was 400 μl and the mixtures were incubated for 20 minutes at 37°C. The reactions were stopped by the addition of 1 ml of diethyl ether and by putting the reaction vessels on ice. The reaction mixtures were extracted with 3 times 1 ml of diethyl ether each and the organic phase was evaporated to dryness. Then the residues were dissolved with 100 μl of mobile phase for HPLC-Analysis (HPLC- System A, Method 1) of which 80 μl were injected into the chromatograph.
3. Results

3.1. Identification of New Products in Amiodarone Metabolism

3.1.1. Identification of the Chemical Structure of X1

3.1.1.1. Biosynthesis and purification of the major unknown product X1.

Upon incubation of MDEA with rabbit liver microsomes in the presence of the NADPH-generating system at pH 7.4, at least three unknown compounds, X1, X2, and X3, with respective retention times of 4.26, 5.67, and 6.07 min, were observed (Figure 2). The substrate MDEA eluted at 7.23 min. The signal at 4.26 min had the highest intensity and may correspond to the major degraded product. The reaction reached steady state after 4 h. After one reactivation cycle (see Methods), 5–7% of the initial MDEA present was biotransformed into unknown products, as judged by HPLC system B (Method 1).

![Figure 2: HPLC-Chromatogram (System B, Method 1) of the products of MDEA oxidation by rabbit liver microsomes (0.2 mg/ml prot.) (a), rat liver microsomes (2 mg/ml prot.) (b) and blank control (c).](image-url)
When the incubation medium was centrifuged at high speed, MDEA and its derivatives were mainly located in the protein precipitate. Therefore, they may be isolated efficiently by washing the precipitate with methanol (five times). However, this operation was not selective for the drug as lipids were also co-extracted. Separation of the drug from contaminants required considerable efforts.

The first purification step was performed using a silica cartridge. Under basic conditions, MDEA was retained less than its degraded products on the silica; thus, the unreacted MDEA may be washed out using a mixture of hexane:2-propanol:aq.NH₃ 25%. Thereafter, mixtures of hexane:diethyl ether:acetic acid and chloroform:methanol:acetic acid:water were applied successively to the silica cartridge for eluting cholesterol, glycerides, and phospholipids [200]. HPLC system C (Method 1) was used for assaying the benzofuran derivatives in the eluents and revealed that they coeluted with phospholipids. Thus, a further purification step using NH₂-sorbent and chloroform:2-propanol as eluent was necessary. Under these conditions, phospholipids were retained on the sorbent [201], whereas MDEA and its derivatives were not. Thereafter, semi-preparative HPLC was used to separate X₁ from X₂, X₃, and a trace of MDEA. The fractions corresponding to the major signal were collected and evaporated to dryness. Re-injected into the HPLC system C (Method 1), X₁ was detected as one signal at 4.26 min. In order to accumulate the required amount of X₁ for NMR analysis, X₁ was isolated from ten incubations and the residues were combined.

3.1.1.2. UV spectrum of X₁.

The UV spectrum of the purified X₁ in methanol showed the following characteristics: 208 nm, 242 nm (maxima); 223 nm (minimum); and 275 nm, 282 nm (shoulders). It was comparable with that of MDEA.HCl in methanol [202]. Thus, its concentration may be calculated using the molar extinction coefficient of MDEA.HCl at 241 nm (E = 440.000 liter/mole/cm) [202], and the total amount of X₁ was estimated to be 0.15 mg. The compound was stable (as assayed by UV spectra measurement) in methanol at 22°C or dry-stored at -20°C for at least 10 days.
3.1.1.3. MS Results.

Since HPLC-ESI-MS was operated using a low flow rate (0.2 ml/min), the method was modified to that described under methods. When the incubation medium was extracted with diethyl ether and immediately analyzed, the same chromatogram as that shown in Figure 2 was obtained. The UV signals of X1, X2, X3, and MDEA displaced to 4.4, 5.4, 6.0, and 7.45 min, respectively (data not shown). ESI-MS spectra revealed the quasi-molecular ions ([M+H]+) at m/z 618 and 634 for MDEA and X1, respectively (Figures 3a,b). Compared with MDEA, the molecular ion of X1 showed a mass shift of +16 u, suggesting that the MDEA molecule may have been oxygenated. The mass spectra of the minor products X2 and X3 revealed that their quasi-molecular ions were at m/z 591 and 590, respectively.

In this study, only the chemical structure of X1 was investigated. For this, MDEA and off-line purified X1 were dissolved separately in methanol (unlabeled) and D4-methanol (labeled), and analyzed using ESI-MS and ESI-MS/MS successively. These H/D exchange experiments allowed us to investigate the number and the position of the exchangeable hydrogen atoms in the structures of MDEA and X1.

ESI-MS analysis revealed a mass shift of +2 u for labeled MDEA (m/z 618 to 620, Figure 3a insert), suggesting that, as expected, the quasi-molecular ions of MDEA contained an exchangeable hydrogen atom in addition to its site of protonation (deuteration), whereas X1 contained two exchangeable hydrogen atoms in addition to its site of protonation (deuteration) (mass shift m/z 634 to 637, Figure 3b insert). Thus, based on the observations that X1 contained an additional oxygen and an additional exchangeable hydrogen atom, one may infer that its structure could have a hydroxy (OH) group.
Figure 3: ESI-MS spectra of DEA and purified X1. a) ESI-MS of DEA in methanol and in $d_4$-methanol (insert); b) ESI-MS of X1 in methanol and in $d_4$-methanol (insert); c) ESI-MS/MS of DEA; d) ESI-MS/MS of X1; e) ESI-MS/MS of DEA in $d_4$-methanol; f) ESI-MS/MS of X1 in $d_4$-methanol.

The combination of ESI-MS/MS data obtained from unlabeled and labeled MDEA and X1 may allow the position of the OH function in the X1 structure to be determined. In fact, the ESI-MS/MS spectrum of unlabeled X1 showed the presence of fragments at $m/z$ 563, 545 (base peak), 503, 417, 376, 373, 291, and 249 (Figure 3d), whereas, under
identical analysis conditions, the MDEA molecule gave only two fragment ions at \( m/z \) values of 547 and 373 (Figure 3c). However, analyzing \([d_2-X1+D]^+\) showed three major daughter ions at \( m/z \) 566, 546 (peak base), and 374 and other low-intensity signals at \( m/z \) 504, 418, 377, 292, and 250 (Figure 3f); whereas, \([d_1-MDEA+D]^+\) gave two major fragments at \( m/z \) 549 and 374 (Figure 3e).

![Fragmentation pathways](image)

**Figure 4:** Fragmentation pathways leading to the fragment ions observed in Fig. 3f) for \([d_2-X1+D]^+\) at \( m/z \) 637

Plausible fragmentation pathways leading to the observed fragments in Figure 3f is summarized in Figure 4. The origin of the fragment ions at \( m/z \) 549 (Figure 3e) and 566 (Figure 3f) may be explained by the loss of 71 u from the quasi-molecular ions \([d_1-\]

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68
MDEA+[D]⁺ and [d₂-XI+[D]]⁺ (Figure 4a), respectively. This indicated that the hydroxylation of MDEA did not take place on the -CH₂-CH₂-NH-ethyl chain. Interestingly, the fragment ions b at m/z 374 (Figure 4b) were found for both structures of MDEA and XI (Figure 3, e and f). This indicated that the diiodobenzene ring of XI was unchanged.

In ESI-MS/MS analysis, XI gave more signals than its precursor MDEA. This was probably due to the instability of the ions a at m/z 566 (Figure 4). In fact, they may lose D₂O (-20 u) to give the fragment ions c at m/z 546 (observed in Figure 3f). In turn, ions c may be further deiodinated to fragment ions at m/z 418 and 292 (Figure 4c). These data suggested strongly that the benzofuran moiety of XI was not hydroxylated, because this type of "dehydration" cannot occur on an aromatic group. Additionally, a careful examination of the low-intensity signals of XI at m/z 504, 377, and 250 (Figure 3f) supported the hypothesis that, due to the presence of an additional OH-group, the ions a may lose their n-hydroxybutyl moiety to give the fragment ions d at m/z 504. In turn, the latter were deiodinated to fragment ions at m/z 377 and 250 (Figure 4d). These data supported the hypothesis that the OH-function of XI may be found on the n-butyl side chain. Nevertheless, the exact position of the OH function on this part of the molecule cannot be deduced from the MS data. Thus, further NMR analyses were necessary.
3.1.1.4. NMR Spectroscopic Results.

NMR experiments were exclusively performed with the off-line purified XI isolated from the rabbit liver microsome experiments. The comparison of the $^1$H-NMR spectra of MDEA and XI revealed that the chemical shifts and coupling constants of the signals corresponding to protons of the 3,5-diiodo-4-ethyl-aminoethoxybenzoyl moiety and those of the benzene ring of benzofuran group were unchanged (Table 9, the carbon atoms of the XI molecule were arbitrarily numerated). However, the proton signals of the $n$-butyl group of XI were shifted and split (Figure 5b). The signals at 1.76 and 2.82 ppm, corresponding to protons attached to the carbon atom at the 3- and 4-positions of MDEA, respectively, were missing in the proton spectrum of XI (Figure 5a,b). Additionally, in the spectrum of XI, a doublet at 1.17 ppm was newly observed, suggesting that the methyl group of $n$-butyl must now be adjacent to a CH-group rather than a -CH$_2$-group. Unfortunately, due to impurities (phospholipids), the structure for XI cannot be drawn definitively from these $^1$H-NMR data (Figure 5b). Thus, a 1D-TOCSY experiment (selective excitation of the signal at 1.17 ppm) was performed to find out which spin system the methyl group at 1.17 ppm belongs to. Our results showed clearly that the multiplets at 3.70, 3.00, 2.92, and 1.94-1.85 ppm built up a new spin system (Figure 5c). Moreover, the splitting of these multiplets suggested that a chiral center was present in the $n$-butyl side chain of the XI molecule.
In order to be able to unambiguously assign the new signals, 2D NMR (HSQC, HMBC, DQF-COSY) experiments were run. In the HSQC spectrum, a major correlation between the proton at 3.7 ppm and a carbon at 67.5 ppm has been found. This data indicated that an oxygen atom is bound to the n-butyl moiety of MDEA and was in agreement with our previous ESI-MS/MS data. Finally, the interpretation of all HMBC correlations supported strongly and definitively the hypothesis that the new oxygen atom of X1 was found on the 2-position on the n-butyl side chain (see the structure of Table 9).
Table 9: The carbons of the molecule are arbitrarily numerated. The chemical shifts are in ppm and the coupling constants (J) are in Hz.

<table>
<thead>
<tr>
<th></th>
<th>¹H NMR</th>
<th>¹³C NMR</th>
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<th>¹H NMR</th>
<th>¹³C NMR</th>
</tr>
</thead>
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<td>C-H (7)</td>
<td>7.54, d, J = 8.3</td>
<td>112.3</td>
</tr>
<tr>
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<td>23.7</td>
<td>C-H (8)</td>
<td>7.34, ddd, J = 8.3, 7.3, 1.3</td>
<td>126.2</td>
</tr>
<tr>
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<td>1.76, quin, J = 7.6</td>
<td>31.2</td>
<td>C-H (9)</td>
<td>7.25, ddd, J = 7.7, 7.3, 0.9</td>
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</tr>
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<td>1.42, t, J = 7.3</td>
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</tr>
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<tr>
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<td>11.6</td>
<td>C (17)</td>
<td></td>
<td>161.5</td>
</tr>
</tbody>
</table>

In summary, our MS and NMR data suggest that, in liver microsomes isolated from untreated rabbits, MDEA is biotransformed to 2-(3-hydroxybutyl)-3-[4-(3-ethylamino-1-oxapropyl)-3,5-diiodobenzoyl]-benzofuran (3OH-MDEA).
3.1.2. Characterization of the Chemical Structure of X2 and X3

Under the described conditions, reversed phase HPLC-UV analysis (HPLC-System A, Method 1) of the diethyl ether extract of MDEA-HLM incubation revealed three signals which eluted at 10.6 (X1), 7.8 (X2) and 19.2 (X3) min. These retention times were shorter than that of MDEA (23.8 min) (Figure 6) suggesting that MDEA had been biotransformed to more polar compounds. Our ESI-MS analysis also indicated that their quasi-molecular ions ([M+H]+) were at \( m/z \) 634, 591 and 590, respectively. It has been reported that in rabbit liver microsomes, MDEA is biotransformed to 3’OH-MDEA [206]. In the present studies, the chemical structure of this compound was supported by chemical synthesis. In fact, the synthetic 3’OH-MDEA [203] coeluted with X1 at 10.6 min and both of them had identical MS behaviors. Thus, using commonly available techniques such as UV, MS, NMR (described above) and chemical synthesis [203], the structure of the first hydroxylated derivative of AMI: 3’OH-MDEA is fully documented.

![Figure 6](image_url)

**Figure 6:** The HPLC-UV analysis shows that in human liver microsomes, MDEA (20 \( \mu \)M) was biotransformed to 3’OH-MDEA (10.6 min) and two newly unknown X2, X3 products, which coeluted with the synthetic products of AMI-EtOH (7.81 min) and DDEA (19.25 min). The retention time of MDEA was 23.8 min.
Biotransformation Results

Compared to rabbit liver microsomes, the MDEA hydroxylase activity in HLM was weak and additionally, due to the limited amount of available HLM, it was not possible to collect enough X2 and X3 for NMR analysis. In the current studies, based on LC-ESI-MS/MS data the suspected compounds were synthesized and their MS behaviors were compared with those of unknowns. Based on the similarity in: (1) HPLC retention time; (2) value of [M+H]+ ions; and (3) MS fragmentation pattern, the identity of the unknowns may be established.

In order to clarify the chemical structure of X2 and X3, the extract of the incubation medium was injected repeatedly into the chromatograph (HPLC-System A; Method 1) and the eluent was collected manually at 7.8 min (for X2) and 19.2 (for X3) min. The compounds were then purified, concentrated by diethyl ether extraction and analyzed. Our direct ESI-MS/MS analysis revealed that X2 and X3 showed quasi-molecular ions ([M+H]+) at m/z 591 and 590, respectively and both of them gave daughter ions at m/z 373 and 547, while the ion at m/z 591 also was fragmented to a characterized ion at m/z 201. Based on previous data [204] (and own data shown above), one may infer that the 3,5-diiodo-4-hydroxybenzoyl and the 2-n-butyl-(3,5-diiodo-4-hydroxybenzoyl)-benzofuran moieties of MDEA were unchanged. Probably only its diethylamine function would have been modified during the incubation.

For investigating the chemical structure of X2, the retention time and MS-profile of X2 were compared with those of the previously synthesized AMI analogues [60]. It was found that its retention time of 7.8 min, its [M+H]+ at m/z 591 and the presence of the daughter ions at m/z 373, 547 in the MS fragmentation were identical to those of the analogue B21 [2-n-butyl-3-(3,5-diiodo-4-ß-hydroxyethoxybenzoyl)-benzofuran] (Figure 7A, B). Thus one may assume that in HLM, the amine function of MDEA has been oxidized to an alcoholic one (AMI-EtOH).
**Figure 7:** LC-ESI-MS analysis of unknowns X2 (A) and X3 (C) revealed that their [M + H]^+ were at m/z 591 and 590, respectively. Their corresponding ESI-MS/MS analysis showed the daughter ions at m/z 201, 373, 547 (B) and 373, 547 (D). These fragment ions suggest strongly that the 2-n-butyl-3-(4-iodo-4-β-hydroxybenzoyl)-benzofuran rest of MDEA was not modified during the incubation.

The compound X3 ([M+H]^+ at m/z 590) eluted at 19.2 min. Its relatively long retention time was rather close to that of MDEA (23.8 min) than to those of the hydroxylated derivatives (3’OH-MDEA and AMI-EtOH) and varied strongly with pH of the mobile phase (data not shown), suggesting that X3 was easily ionisable. From these data, it was suspected that X3 still possessed an amine function which would correspond to MDEA molecule with a loss of the ethyl chain (-28 amu). In order to confirm this hypothesis, di-N-desethylamiodarone (DDEA) was synthesized and compared. Data from HPLC-UV and HPLC-ESI-MS/MS analysis (Figure 6 and Figures 7C,D) supported the hypothesis that X3 and DDEA had the same chemical structure.

In summary, in HLM the known metabolite MDEA of AMI may be hydroxylated to 3’OH-MDEA, dealkylated to DDEA and deaminated to AMI-EtOH.
3.2. **HPLC-UV Quantification of AMI Metabolites**

3.2.1. Calibration

Due to the lack of sufficient amounts of reference substance the calibration was not conducted by weighing the products for every reference solution but by diluting a stock solution which was prepared in triplicate. Each stock solution was prepared by dissolving the compounds in organic solvent followed by its dilution in the matrix solution. All three identified metabolites were combined in one initial solution. The solution with the highest concentrations of the metabolites contained 10 µM of 3’OH-MDEA, AMI-EtOH and DDEA. Those solutions were then diluted to 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.05 µM and analyzed by injecting 80 µM into the HPLC-UV system A (Method 1) and B (Method 1). Each solution was injected three times and the linear relationship between the concentration of the injected solution and integrated peak areas of the detected metabolites is expressed in Figure 8.

![Figure 8](image)

**Figure 8:** Calibration curves for A) 3’OH-MDEA, B) AMI-EtOH and C) DDEA after triple injection of three solutions of every concentration (n = 7). D) HPLC chromatogram of the analysis of a solution containing 200 pmol of 3’OH-DEA, X2 and X3

Linear relationship equations were obtained by linear regression analysis of the data points. Those equations were then used to calculate the amounts of metabolites formed by incubation of MDEA with hepatic microsomes and a NADPH generating system. The correlation coefficients for 3’OH-MDEA, AMI-EtOH and DDEA measurement were $r = 0.99965$, $r = 0.99966$ and $r = 0.99936$, respectively.
3.2.2. Limit of Detection and Quantitation

The limits of detection (LOD) and quantitation (LOQ) were estimated based on nonlinear least-square fitting of data points of low-concentration samples and the calculation of the signal standard deviation [182]. The calculated values are summarized in Table 10.

Table 10: Limit of detection (LOD) and limit of quantitation (LOQ) values were estimated by measuring 3’OH-MDEA, AMI-EtOH and DDEA with HPLC-UV system A (Method 1).

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<th>Compound</th>
<th>LOD [pmol]</th>
<th>LOQ [pmol]</th>
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<td>3’OH-MDEA</td>
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<td>AMI-EtOH</td>
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</tr>
<tr>
<td>DDEA</td>
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</table>

The values were in good agreement with previous manual estimation. Therefore, the amounts injected for calibration started at 8 pmol. However, the LOQ-values of 3’OH-MDEA and DDEA quantitation were estimated to be higher than 8 pmol. This will be considered in the following results.
3.2.3. Recovery

For the calculation of the recovery the injected amounts of 800, 400, 200, 80, 40, 20 and 8 pmol were taken into account. The recovery is calculated from the results of three different solutions per concentration level which were analysed three times each.

Table 11: Recovery of 3’OH-MDEA, AMI-EtOH and DDEA analysis after triple measurement of three solutions of every concentration.

<table>
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<td>80</td>
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<td>3.34</td>
<td>97.79</td>
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<tr>
<td>0.5</td>
<td>40</td>
<td>40.68</td>
<td>6.08</td>
<td>101.69</td>
</tr>
<tr>
<td>0.25</td>
<td>20</td>
<td>19.09</td>
<td>5.86</td>
<td>95.46</td>
</tr>
<tr>
<td>0.1</td>
<td>8</td>
<td>8.72</td>
<td>16.68</td>
<td>109.02</td>
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<tr>
<td>DDEA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>800</td>
<td>869.63</td>
<td>1.08</td>
<td>108.70</td>
</tr>
<tr>
<td>5</td>
<td>400</td>
<td>405.88</td>
<td>4.06</td>
<td>101.47</td>
</tr>
<tr>
<td>2.5</td>
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<td>202.27</td>
<td>0.71</td>
<td>101.14</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
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<tr>
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<td>37.78</td>
<td>3.07</td>
<td>94.45</td>
</tr>
<tr>
<td>0.25</td>
<td>20</td>
<td>20.04</td>
<td>1.61</td>
<td>100.22</td>
</tr>
<tr>
<td>0.1</td>
<td>8</td>
<td>&lt; 9.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In the first column of the Table 12 the concentrations of the corresponding solutions is given and in the next column the effective amount of injected metabolites (80 μl of injection volume). Measurement of 8 pmol was considered only for AMI-EtOH since the LOQ of 3’OH-MDEA and DDEA was estimated higher. The high amount of organic solvents (composition of the mobile phase) prevented further difficulties quantificating AMI-derivatives.
3.2.4. Precision

Precision of injection was determined at three representative data concentration levels of 2.5, 0.5, and 0.1 μM (200, 40 and 8 pmol of injected amount). One solution of every concentration was analysed seven times to give the relative standard deviations (RSD) in Table 12.

**Table 12:** Precision of injection determined by analysing the same solution containing 200, 40 or 8 pmol of the metabolites 3’OH-MDEA, AMI-EtOH and DDEA (n=7). Confidence limits (CL) were calculated using equation (20).

<table>
<thead>
<tr>
<th></th>
<th>Introduced / pmol</th>
<th>Found / pmol</th>
<th>Recovery / [%]</th>
<th>RSD / [%]</th>
<th>CL (99%) / pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3’OH-MDEA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>242.201</td>
<td>121.10</td>
<td>3.74</td>
<td>± 8.37</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>40.968</td>
<td>102.42</td>
<td>3.66</td>
<td>± 1.38</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&lt; 10.700</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Introduced / pmol</th>
<th>Found / pmol</th>
<th>Recovery / [%]</th>
<th>RSD / [%]</th>
<th>CL (95%) / pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMI-EtOH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>216.230</td>
<td>108.11</td>
<td>1.98</td>
<td>± 3.97</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>40.956</td>
<td>102.39</td>
<td>2.47</td>
<td>± 0.93</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8.253</td>
<td>103.16</td>
<td>3.86</td>
<td>± 0.29</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Introduced / pmol</th>
<th>Found / pmol</th>
<th>Recovery / [%]</th>
<th>RSD / [%]</th>
<th>CL (95%) / pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DDEA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>223.614</td>
<td>111.81</td>
<td>3.04</td>
<td>± 6.28</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>042.028</td>
<td>105.07</td>
<td>2.28</td>
<td>± 0.89</td>
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<tr>
<td>8</td>
<td>&lt; 9.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Most RSD values are in the range between 1.98 % and 3.86 % and the calculated recovery is ranging from 102.39 to 111.81 %. Confidence limits (CL) were set to 95 % as the appropriate level for the analysis of biological solutions.
3.3. Biotransformation of MDEA in Rabbit Liver Microsomes

3.3.1. Introduction

In vitro incubations of MDEA with rabbit liver microsomes resulted in the rapid formation of 3’OH-MDEA. The cytochrome P450 (CYP) enzyme family was suspected to be responsible for this biotransformation. In this section of this work the involvement of the responsible enzyme was investigated. After the evaluation of the enzyme kinetic experiments the reaction mixture was coincubated with several CYP specific chemicals to investigate first questions if whether the CYP system is involved and if yes which isozyme or isozymes of the P450 enzyme family participate in the MDEA biotransformation. Results from the inhibitory experiments were then further investigated using induction or correlation methods.

3.3.2. Enzyme Kinetic Evaluation of MDEA Biotransformation

Since AMI-EtOH and DDEA were not fully characterized yet and since 3’OH-MDEA was the predominant metabolite by the incubation with rabbit liver microsomes, the investigation of the MDEA biotransformation with rabbit enzymes was limited for 3’OH-MDEA.
Figure 9: Enzyme kinetic analysis of the hydroxylation of MDEA to 3'OH-MDEA where A) is the HPLC-UV chromatogramm of MDEA incubation in rabbit liver microsomes, B) the enzyme kinetic analysis (Microsome Batch: Feb99) and C) the Eadie-Hofstee plot.

As can be seen in Figure 9A 3'OH-MDEA eluted at 10.4 minutes (HPLC-System A, Method 1) is the predominant metabolite formed by the incubation of MDEA in rabbit liver microsomes. In the enzyme kinetic analysis (Figure 9B) the data points were fitted nonlinear least-square fitting (Michaelis-Menten equation). The obtained parameters are summarized in Table 13.
Table 13: Apparent enzyme-kinetic parameters of the biotransformation of MDEA to 3'OH-MDEA in three samples of rabbit liver microsomes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Km [μM]</th>
<th>Vmax [nmol/min/mg prot.]</th>
<th>Vmax/Km [μl/min/mg prot.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dez99</td>
<td>7.97</td>
<td>0.38</td>
<td>47.68</td>
</tr>
<tr>
<td>T1</td>
<td>7.29</td>
<td>0.54</td>
<td>74.07</td>
</tr>
<tr>
<td>Feb99</td>
<td>5.11</td>
<td>0.74</td>
<td>144.81</td>
</tr>
<tr>
<td>Average</td>
<td>6.79</td>
<td>0.55</td>
<td>88.85</td>
</tr>
<tr>
<td>SD</td>
<td>1.49</td>
<td>0.18</td>
<td>50.23</td>
</tr>
</tbody>
</table>

The interindividual relative standard deviations (RSD) of the Km and Vmax values are comparably low with 22 and 33 %, respectively. Eadie-Hofstee plotting of the data may discover abnormalities from classical Michaelis-Menten kinetics (deviation from linear relationship). The data in Figure 9C appear to be linear with a correlation coefficient of $r = -0.96$. 
3.3.3. Chemical Inhibition of MDEA Biotransformation

Inhibitors and substrates for several active P450 isozymes were used for the inhibition of the hydroxylation of MDEA to 3’OH-MDEA. The collected results are shown in Figure 10.

![Inhibition of the hydroxylation of MDEA to 3’OH-MDEA by the coincubation of P450 specific inhibitors and substrates. Results are given as the percentage of reaction rate compared to the corresponding control reaction.](image)

**Figure 10:** Inhibition of the hydroxylation of MDEA to 3’OH-MDEA by the coincubation of P450 specific inhibitors and substrates. Results are given as the percentage of reaction rate compared to the corresponding control reaction.

Methimazole which is known to inhibit FMO enzymes did not have any effect on the investigated reaction in contrast to SKF525A (Proadifen). This observation may exclude the involvement of the FMO-system. Thus the reaction of interest was studied in more detail using cytochrome P450 (CYP) specific inhibitors and substrates. Ketoconazole [191, 195] and Cyclosporin A [196, 197] are known to be potent inhibitors for CYP3A. Midazolam (MDZ) is known to be selective for human CYP3A4/CYP3A5 [185]. The inhibitory effect of all of these compounds appears to be dose dependent. Troleandomycin (TAO) did not show any substantial inhibitory effects. Among the residual compounds several are specific for human P450 isozymes but may be also substrates for CYP3A. Quinidine, quinine and mephenytoin displayed the strongest inhibitory effect of those compounds which are not primarily associated with CYP3A4.
in humans. The effects of coumarin, sulfaphenazole, mephenytoin and DDC are low with regard to the used final concentration in the incubation mixture. The effect of α-naphthoflavone (αNF) may be interesting since it showed strong concentration dependence.

**Figure 11:** Dixon-plot for the inhibition of MDEA hydroxylase activity by Midazolam (MDZ) has an intersection above the abscissa. This pattern is indicative of competitive inhibition with a suspected $K_i = 10\text{-}15 \mu\text{M}$.

Further analysis of the inhibitory effect of MDZ on the MDEA hydroxylation was performed to evaluate the character of inhibition. The data shown in Figure 11 have to be interpreted carefully since two distinct intersections can be observed.
3.3.4. Induction of CYP3A6 in Rabbit Cell Culture

Rifampicin is a known inducer of CYP3A6 [186, 187]. It was our aim to see if an increase in CYP3A6 levels may influence the MDEA hydroxylase activity and the present experiment was conducted with microsomes which were obtained from Dr. Gallier (INRA, France). MDEA was then incubated with microsomes isolated from cultured rabbit hepatocytes under control conditions (T1) and with those which were isolated from cultured rabbit hepatocytes treated with rifampicin. The results are shown in Figure 12.

![Figure 12: Hydroxylation of MDEA to 3'OH-MDEA in microsomes which were isolated from either untreated (control) or rifampicin treated rabbit liver cell cultures.](image)

The treatment of rifampicin increased the activity of DEA hydroxylation up to 400% suggesting that induced levels of CYP3A6 may account for this effect. This observation also strongly indicates that CYP3A6 is responsible for MDEA biotransformation to 3'OH-MDEA. The enzyme kinetic parameters of the incubation with control and rifampicin induced microsomes were $K_m = 7.29$ and $10.48 \mu M$, $V_{max} = 0.54$ and $2.29$ nmol/min/mg prot., respectively.
3.3.5. Correlation of 3’OH-MDEA and 1’OH-MDZ Formation

Midazolam (MDZ) is known to be metabolized by CYP3A4 to 1’OH-MDZ in human liver microsomes [185]. Since the rabbit isoform CYP3A6 is the homologue to the human CYP3A4 the hydroxylation activity may correlate with the CYP3A6 content of the rabbit liver microsomal samples. The hydroxylation activity of MDEA and MDZ was correlated using 9 samples of rabbit liver microsomes and is shown in Figure 13.

![Graph showing correlation between 3’OH-MDEA and 1’OH-MDZ formation](image)

**Figure 13:** The hydroxylation activity of MDEA to 3’OH-MDEA was correlated with that of MDZ to 1’OH-MDZ using 9 samples of rabbit liver microsomes. The data points are the mean of duplicate determination and are expressed in nmol/min/mg prot.

The 3’OH-MDEA formation correlated with the midazolam-α-hydroxylase activity with $r = 0.69$. These data suggest that CYP3A6 may be possibly involved into the hydroxylation of MDEA.
3.4. Biotransformation of MDEA by HLM

3.4.1. Introduction

The change from rabbit to human microsomes was accompanied by major interest since the results may directly be brought into relation with AMI metabolism in humans and therefore may help to improve therapeutic conditions. The formation of 3’OH-MDEA with human liver enzymes was first directly compared with those of rabbit by conducting enzyme kinetic experiments using different samples of HLM. One has to consider that experiments with rabbit liver microsomes were focused on 3’OH-MDEA formation while the experiments with HLM AMI-EtOH and DDEA were discovered to have the same importance as 3’OH-MDEA and therefore, were characterized. Additional species comparison of MDEA biotransformation is illustrated in section 3.5., where the reaction is compared human, rabbit, rat and pig liver microsomes.

Similar methods as in the previous section (3.3.) were applied to identify the metabolic enzymes which are active in the biotransformation of MDEA to the three newly characterized metabolites in HLM. In comparison with the experiments with rabbit liver microsomes the coincubation of CYP specific chemicals to the reaction may allow better conclusions since their effect is better investigated for HLM and furthermore help to compare the used human and rabbit in vitro models.

3.4.2. Enzyme Kinetics

Among three investigated compounds, 3’OH-MDEA and DDEA may be considered as the primarily bio-oxidized products of MDEA. However, this is not the case for AMI-EtOH. The latter may be issued from both MDEA and DDEA by an oxidative deamination. Assuming that the secondary formation of AMI-EtOH from DDEA was much slower than the primary one (issued directly from MDEA), simple enzyme kinetic laws may be applied. Using 10 MDEA concentrations in the range of 5-100 μM, the formation rates of 3’OH-MDEA, AMI-EtOH, DDEA were fitted by the Michaelis-Menten equation using nonlinear least-square fitting program as shown in Figure 14.
Their relative kinetic parameters were $K_m = 21.7 \pm 3.4$, $32.6 \pm 14.3$ and $29.4 \pm 13.1$ μM; $V_{max}$: $17.6 \pm 3$, $64.3 \pm 35.4$ and $13.7 \pm 5.3$ pmol/min/mg protein, respectively (Table 14). The values were the mean of three estimations and the standard errors accounted for the inter-individual variability. The intrinsic clearance values ($V_{max} / K_m$) of MDEA decreased in order of AMI-EtOH > 3’OH-MDEA > DDEA and were much lower than 53 ml/min/mg protein of the amiodarone mono-N-dealkylation [34].

Eadie-Hofstee analysis of MDEA biotransformation (Figures 14.B,D,F) did not show a linear relationship in the case of 3’OH-MDEA and AMI-EtOH. The corresponding points data points in the low substrate concentration range may not be described by the hyperbolic enzyme kinetic law by Michaelis-Menten [154, 162]. These observations were supported by the Eadie-Hofstee analysis of the MDEA incubation with the residual HLM-samples (data not shown). On the other hand the linear relationship of $v$ vs $v/S$ for DDEA formation was observed among HLM1-HLM3.
**Figure 14:** Saturation kinetics of MDEA biotransformation with HLM3 to A) 3’OH-MDEA, C) AMI-EtOH and E) DDEA. Eadie-Hofstee plots for B) 3’OH-MDEA, D) AMI-EtOH and F) DDEA formation are shown.
**Table 14:** Apparent Michaelis-Menten parameters of the biotransformation of MDEA using three different samples of human liver microsomes. The intrinsic clearance values \((V_{\text{max}}/K_m)\) for 3’OH-MDEA, AMI-EtOH and DDEA formation were 0.81, 1.97 and 0.47 µl/min/mg prot.

<table>
<thead>
<tr>
<th>Sample</th>
<th>3’OH-MDEA [µM]</th>
<th>AMI-EtOH [µM]</th>
<th>DDEA [µM]</th>
<th>3’OH-MDEA [pmol/min/mg prot.]</th>
<th>AMI-EtOH [pmol/min/mg prot.]</th>
<th>DDEA [pmol/min/mg prot.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM 1</td>
<td>25.43</td>
<td>48.73</td>
<td>44.43</td>
<td>19.62</td>
<td>105.15</td>
<td>19.72</td>
</tr>
<tr>
<td>HLM 2</td>
<td>20.74</td>
<td>27.21</td>
<td>20.39</td>
<td>18.99</td>
<td>45.32</td>
<td>11.94</td>
</tr>
<tr>
<td>HLM 3</td>
<td>18.81</td>
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<td>23.50</td>
<td>14.09</td>
<td>42.33</td>
<td>9.55</td>
</tr>
<tr>
<td>Average</td>
<td>21.66</td>
<td>32.57</td>
<td>29.44</td>
<td>17.57</td>
<td>64.27</td>
<td>13.74</td>
</tr>
<tr>
<td>SD</td>
<td>3.40</td>
<td>14.26</td>
<td>13.07</td>
<td>3.03</td>
<td>35.44</td>
<td>5.32</td>
</tr>
</tbody>
</table>

In order to investigate this issue in more detail the data of MDEA biotransformation with HLM 3 were fitted with the Hill-equation. As shown in Figure 15 the nonlinear curve fit of 3’OH-MDEA and AMI-EtOH formation using the Hill equation is better than those using the MM-equation (Figure 14A, 14C) in the lower MDEA concentration range.
Figure 15: Nonlinear least-square fitting with the Hill equation \( v = V_{\text{max}} [S]^n / (S_{50}^n + [S]^n) \) of the biotransformation of MDEA to A) 3’OH-MDEA, B) AMI-EtOH and C) DDEA with HLM 3.

The resulting fitting parameters are summarized in Table 15. The exponent \( n \) is a measure for cooperativity and was estimated either by nonlinear least-square fitting or by Hill-plot (\( \log(v/(V_{\text{max}}-v)) \) vs. \( \log([S]) \)). It can be observed that the \( n \)-values for 3’OH-MDEA and AMI-EtOH formation are above 1. This is in good agreement with the nonlinear Eadie-Hofstee plots of this two reactions. As expected the \( n \)-value for DDEA formation is in the range of 1.
Table 15: Evaluation of the enzyme kinetic parameters of MDEA biotransformation with HLM using the Hill equation. The parameters were estimated by fitting the data points with the Hill equation \( v = \frac{V_{\text{max}} [S]^n}{K_{0.5}[S] + [S]^n} \). The n-values were additionally calculated by linear least square fitting of the Hill-plot (log\( v/(V_{\text{max}} - v) \) vs. log[S]) using the previously estimated \( V_{\text{max}} \)-values where n is the slope of the regression line. The results represent the mean of three different estimations (HLM 1-3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3’OH-MDEA</td>
</tr>
<tr>
<td><strong>nonlinear curve fit (Hill equation)</strong></td>
<td></td>
</tr>
<tr>
<td>( K_{0.5} ) (\mu M)</td>
<td>16.1 ± 3.1</td>
</tr>
<tr>
<td>( V_{\text{max}} ) (pmol/min/mg prot.)</td>
<td>13.8 ± 2.4</td>
</tr>
<tr>
<td>n</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td><strong>plot of log( v/(V_{\text{max}} - v) ) vs. log[S]</strong></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>
3.4.3. Chemical Inhibition of MDEA Biotransformation

After measuring enzyme kinetics the investigation of involved P450 isoforms was primarily attempted by screening the reactions by the coincubation of a selection of cytochrome P450 selective inhibitors and substrates. The data in Figure 16 for the inhibition of MDEA biotransformation in HLM shows similarities in the profile of inhibitory effects with the data presented in the section for MDEA incubation with rabbit liver microsomes. SKF525A, a non-selective but potent CYP inhibitor, inhibited all three reactions by an extent between 45 and 70 %.
Ketoconazole appeared to be the most potent inhibitor for 3'OH-MDEA, AMI-EtOH and DDEA formation by almost complete inhibition starting from 100 μM. However, Ketoconazole is specific for CYP3A4 below 1 μM [195]. Therefore, this result can not serve as evidence for the involvement of the corresponding enzyme. Other CYP3A4 related inhibitors (troleandomycin, TAO [188, 191]; after preincubation) and substrates (midazolam, MDZ) [185] inhibited all reactions by over 50%. TAO was reported to be one of the most selective inhibitors for CYP3A4 [191]. Those results indicate that CYP3A4 is involved in MDEA biotransformation. Moreover, cimetidine is not only known to inhibit CYP3A enzymes but also CYP1A2, CYP2C9 and CYP2D6 [198]. Sulfaphenazole, quinidine and diethyldithiocarbamate (DDC) are selective inhibitors for CYP2C9, CYP2D6 and CYP2E1, respectively. However, sulfaphenazole appears to be selective below 100 μM [193] and DDC was reported to inhibit several other important P450 isozymes [188]. While Sulfaphenazole did not show any significant effect, quinidine and DDC inhibited some reactions up to 50%. Considering the used concentrations of inhibitors those effects can be interpreted as non-specific. Quinidine was reported to selectively inhibit CYP2D6 [193, 194]. The inhibitory effect of quinidine may also be related to its pathway of biotransformation by CYP3A4. Mephenytoin as a selective CYP2C19 substrate [190, 192] inhibited weakly the DDEA
formation (20 %) and more extensively 3’OH-MDEA and AMI-EtOH formation (>50 %).

α-Naphthoflavone (αNF) is known as a specific inhibitor of CYP1A1 and CYP1A2 but not for one single of those isozymes [189]. In addition αNF was described to activate CYP3A4 catalyzed reactions [158, 175, 176]. In this study αNF activated the rate of all three investigated reactions. The strongest activation was observed in the case of AMI-EtOH formation by 150 % compared to control conditions. Coumarin is known to be a selective CYP2A6 substrate [190] but not a mechanism based inducer of P450 catalyzed metabolism. However, coumarin was able to activate all investigated reactions as well.
3.4.4. Chemical Activation of MDEA Biotransformation

Inhibition studies have shown that αNF may activate MDEA biotransformation to 3'OH-MDEA, AMI-EtOH and DDEA. In addition enzyme kinetic evaluation of MDEA biotransformation in HLM has shown that the Eadie-Hofstee-plots (v/S vs v) are not linear in any case (3'OH-MDEA, AMI-EtOH). Therefore MDEA was incubated in concentrations ranging from 2 to 60 μM together with 20 μM αNF compared with control conditions (addition of equivalent amount of DMSO used to dissolve αNF). The data points for 3'OH-MDEA, AMI-EtOH and DDEA formation were fitted by the Michaelis-Menten- and the Hill-equation and are shown in Figure 17.

3'OH-MDEA and DDEA formation rates were not elevated by αNF which was found to induce the biotransformation of AMI-EtOH. This results corresponds to the observations received by the inhibitory screening experiments where AMI-EtOH was induced by up to 150 % by 10 μM αNF. The combined fitting parameters are summarized in Table 16.
Figure 17: MDEA was coincubated with 10 μM αNF (■) and the saturation kinetics of A) 3’OH-MDEA, B) AMI-EtOH an C) DDEA were compared with control conditions (○). The formation of D) 3’OH-MDEA, E) AMI-EtOH and F) DDEA was fitted by the Hill-equation. ANP increased the formation rate of AMI-EtOH formation while no effect was observed in the case of 3’OH-MDEA and DDEA.
**Table 16:** Estimation of the enzyme kinetic parameters of MDEA biotransformation (data of $V_{max}$ are given in pmol/min/mg prot.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3’OH-MDEA</th>
<th>AMI-EtOH</th>
<th>DDEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>ANF</td>
<td>control</td>
</tr>
<tr>
<td><strong>nonlinear fitting by Hill-equation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{0.5}$ ($\mu$M)</td>
<td>10.5 ± 1.3</td>
<td>12.6 ± 1.7</td>
<td>27.1 ± 14.2</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>34.1 ± 2.7</td>
<td>34.0 ± 2.7</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>$n$</td>
<td>2.4 ± 0.6</td>
<td>2.1 ± 0.5</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

**plot of log[v/(V_{max}-v)] vs. log[S]**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3’OH-MDEA</th>
<th>AMI-EtOH</th>
<th>DDEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>ANF</td>
<td>control</td>
</tr>
<tr>
<td>$n$</td>
<td>2.0 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

**nonlinear fitting by MM-equation**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3’OH-MDEA</th>
<th>AMI-EtOH</th>
<th>DDEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>ANF</td>
<td>control</td>
</tr>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>22.2 ± 9.1</td>
<td>27.2 ± 10.1</td>
<td>46.5 ± 13.7</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>50.4 ± 8.8</td>
<td>51.9 ± 9.0</td>
<td>4.4 ± 0.7</td>
</tr>
</tbody>
</table>

The data were analyzed by Eadie-Hofstee plots ($v/S$ vs. $v$). These results are shown in the next Figure 18.

In the present experiments more points were chosen in the lower concentration range of MDEA metabolism in order to investigate cooperativity of the reactions in more detail. These show even stronger deviation from classical Michaelis-Menten behaviour as seen in the first section. ANP did not affect significantly the shape of the Eadie-Hofstee plot of any investigated reaction.
Figure 18: Eadie-Hofstee plots of the saturation kinetics of A) 3’OH-MDEA, C) AMI-EtOH and E) DDEA under control conditions compared with the corresponding experiments with coincubated 20 μM αNF (B: 3’OH-MDEA; D: AMI-EtOH; F: DDEA).
3.4.5. MDEA Biotransformation in cDNA expr. human CYP Enzymes

In order to investigate the identity of human CYP isoforms involved in the elimination of MDEA, the MDEA biotransformation in a selection of cDNA expressed P450 isoforms (cCYP) was performed. Our data (Figure 19) demonstrated that CYP3A4 and 2D6 catalyzed the MDEA biotransformation to all three investigated compounds. Additionally, CYP1A1, CYP1A2 and CYP2A6 were involved in the AMI-EtOH, DDEA formation, whereas no trace of 3'OH-MDEA was detected.

**Figure 19:** Catalytic activity of the human cDNA expressed CYPs in the biotransformation of MDEA to 3'OH-MDEA (A), AMI-EtOH (B) and DDEA (C). In addition an incubation of MDEA with HLM was compared with that of MDEA incubated with CYP3A4 (D). Peaks 1, 2, 3, and 4 were identified as 3'OH-MDEA, AMI-EtOH, DDEA and MDEA, respectively (Chromatograms: red: Incubation of MDEA with HLM; green: Incubation of MDEA with CYP3A4; blue: Incubation of MDEA with denatured CYP3A4).

As can be seen (Figure 19D) the extracts of the incubation of MDEA with either HLM or CYP3A4 resulted in a similar distribution of 3'OH-MDEA, AMI-EtOH and DDEA.
In summary, at least four human cCYPs were involved in the MDEA biotransformation. The importance of the most abundant P450 enzyme CYP3A4 may be further analyzed in combination with the correlation data in the following section.
3.4.6. Correlation of 1'OH-MDZ and MDEA-Metabolites Formation

With the aim to estimate the relative importance of CYP3A4 towards other CYP enzymes in the biotransformation of MDEA in humans, the correlation between the formation rates of 3'OH-MDEA, AMI-EtOH, DDEA and that of α-hydroxymidazolam (α’OH-MDZ) was investigated in 15 different HLM samples. Their formation rates (mean of three estimations) were measured by HPLC-UV and the correlation coefficients (r) were estimated by linear regression analysis. The correlation coefficients were $r = 0.83$ ($p<0.00014$), $0.89$ ($p<0.0001$) and $0.91$ ($p<0.0001$), respectively (Figure 20).

**Figure 20:** The positive correlations between the formation rates of 3'OH-MDEA (A), AMI-EtOH (B) and DDEA (C) and those of midazolam-α-hydroxylation (α'OH-MDZ) were investigated in 15 HLM samples. The correlation coefficients were 0.83, 0.89 and 0.91, respectively. Data points are the mean values of three determinations.
All three reactions showed positive correlation with the MDZ α-hydroxylase activity. The selectivity of this reaction for the CYP3A isoform may show a major involvement of CYP3A since all microsomal samples were diluted to the same protein concentration. Therefore, the α'OH-MDZ formation rate is a substitute measure for the CYP3A-content of the individual samples. The incubation of MDEA with CYP enzymes has shown that several isozymes are involved. Thus not only CYP3A enzymes may contribute to MDEA biotransformation. One can assume that CYP2D6 takes part in the formation of the characterized AMI-metabolites. However, its content is lower than that of CYP3A4 and it does not catalyze α'OH-MDZ formation. CYP1A1 showed metabolic activity in vitro but is not expressed in the liver. On the other hand CYP1A2 may be rather important in metabolizing MDEA to DDEA. CYP2C8 was not tested in our experiments but since it was shown to dealkylate AMI to MDEA it may probably also be active in MDEA biotransformation.
3.5. **Species Comparison of MDEA Biotransformation**

MDEA was incubated with HLM, rabbit, rat and pig liver microsomes in order to compare the abilities of different species to biotransform MDEA. The HPLC-UV chromatograms of the analyzed incubation mixtures is shown in Figure 21.

![HPLC-UV Chromatograms of MDEA incubations in a) rabbit, b) rat, c) pig and d) human liver microsomes. AMI-EtOH (Peak 1), 3’OH-MDEA (Peak 2), DDEA (Peak 3) and MDEA (Peak 4) are eluted at 6.7, 10.6, 19.6 and 24.5 minutes, respectively.](image)

**Figure 21:** HPLC-UV Chromatograms of MDEA incubations in a) rabbit, b) rat, c) pig and d) human liver microsomes. AMI-EtOH (Peak 1), 3’OH-MDEA (Peak 2), DDEA (Peak 3) and MDEA (Peak 4) are eluted at 6.7, 10.6, 19.6 and 24.5 minutes, respectively.

The 3’-hydroxylation of MDEA to 3’OH-MDEA was the predominant reaction using rabbit liver microsomes. In HLM 3’OH-MDEA is not formed by the same extent but all three discovered metabolites were produced in apparently similar amounts. It is interesting to note that all tested species may metabolize MDEA to the same identified products. Differences seem to be based on varying activities.
4. Discussion

4.1. Identification of Products of MDEA Biotransformation

The aim of our research on the AMI metabolism is to investigate the fate of the AMI molecule in humans. In patients and in-vitro experiments the drug is metabolized rapidly and abundantly to MDEA. For the first attempt in this project, the primary metabolite MDEA of AMI was used as substrate for in-vitro experiments. The elimination half-life of the drug is comparably long and the accumulation of AMI and MDEA in tissues [42, 205] in long-term treatment may be related to some severe toxic effects. Thus the better understanding of the metabolism of AMI may be relevant for clinical therapy.

Oxidative metabolism renders metabolites more lipophilic resulting in shorter retention times in RP-HPLC measurements. The extraction of lipids from the incubation mixtures led to an unstable baseline in the ESI-MS-detector and implicated a series of purification steps in order to purify the first metabolite (X1) for NMR analysis. As can be seen in the HPLC-UV chromatogram MDEA (Figure 2, Figure 9A) was biotransformed in rabbit liver microsomes predominantly to one unknown metabolite X1 which was subsequently characterized as 3’OH-MDEA (2-(3-hydroxybutyl)-3-[4-(3-ethylamino-1-oxapropyl)-3,5-diiodobenzoyl]-benzofuran) [206]. About 0.15 mg were isolated in order to conduct the NMR analysis. However, the purification was not achieved completely (signals in the range of 0.8 and 2.5 ppm; Figure 5).

Incubation of MDEA with HLM did not show a comparable strong hydroxylase activity as with rabbit liver microsomes. The metabolites X2 and X3 were already detected in experiments with rabbit liver microsomes but they were investigated in experiments with HLM. The further dealkylated product of MDEA was already found in dogs [49] and thus it may be suspected to be formed in humans as well. In fact X3 was identified as the dealkylated product DDEA. In contrast to the dealkylation reaction the deaminated metabolite AMI-EtOH was newly discovered.
4.2. Biotransformation of MDEA in Rabbit Liver Microsomes

In rabbit liver microsomes MDEA was rapidly hydroxylated to 3’OH-MDEA. Enzyme kinetic studies have shown high affinity of MDEA to the metabolizing center in relation to this reaction. Eadie-Hofstee plotting (Figure 9C) of the saturation kinetics was linear. Therefore, the Michaelis-Menten equation may be appropriate to analyze those kinetic data.

Experiments performed with chemical inhibitors have to be interpreted carefully since the inhibitors and substrates are reported to be specific for human or rat liver microsomes. The strong inhibitory effect of ketoconazole may not serve as evidence for the involvement of CYP3A6 as it is not specific in the used concentration [195]. However, substrates for the CYP3A isozyme such as midazolam (MDZ), quinidine, quinine and cyclosporin A inhibited the MDEA hydroxylation. Furthermore, MDZ inhibited competitively the 3’OH-MDEA formation and the rates of 3’OH-MDEA formation correlated moderately with those of α’OH-MDZ ($r = 0.69; p< 0.039$). The correlation results indicates that also other P450 isozymes may pariticipate in MDEA hydroxylation besides CYP3A. This assumption is also supported by the fact that CYP3A related inhibitors or substrates had a significant impact on the 3’OH-MDEA formation.

The involvement of CYP3A6 in the 3’-hydroxylation of MDEA can be determined by comparing the catalytic activities of microsomes isolated from rifampicin-induced rabbit hepatocytes with those isolated form untreated hepatocytes. In rabbits, the gene coding for this isoform is located on chromosome 6 in the q-ter region [186]. In earlier literature other names have been used for this enzyme such as P450IIIA4 [187], P450 LM3c [207] or LM3c [208] and it was reported to be involved in the metabolism of human CYP3A related compounds such as cyclosporin A [209] or midazolam [207].
The *in-vitro* data obtained in this study have to be used carefully to explain *in-vivo* observations. It has been reported that coadministration of rifampicin with AMI may increase clearance of MDEA and AMI [210]. Rifampicin is believed to induce CYP2B6, 3A4 and 3A5 in humans which was confirmed measuring mRNA levels in cultured human hepatocytes [211]. However, it has been found that the highest levels of CYP3A4 mRNA were found in some liver samples from patients who did not receive any putative inducers [212].

In conclusion the rabbit isozyme CYP3A6 appears to be mainly involved in the hydroxylation of MDEA. On the other hand the contribution of other P450 enzymes can not be completely ruled out. The comparison of these results with those of the MDEA biotransformation with HLM may deliver further insights of how the metabolic enzymes of rabbits and humans can be compared.
4.3. MDEA Biotransformation in HLM

Enzyme kinetics reveal that in HLM the apparent affinity of MDEA to the metabolizing centers is lower than to those in rabbit liver microsomes. The intrinsic clearance values suggest that MDEA is eliminated mainly by deamination while the dealkylation to DDEA is the slowest activity. The last statement is in agreement with previous observations telling that dealkylation of the secondary amines is less rapid than that of the tertiary amines [213].

Our data confirm that the hydroxylation of MDEA is significantly faster in rabbit liver microsomes [206, 214, 215] than that in HLM. In rabbit the MDEA tissue concentrations are lower than those of AMI [205] while in humans MDEA organ tissue concentrations are higher than those of the parent drug. This may just in part be the reason for the faster hydroxylation activity of MDEA in rabbit liver microsomes.

Scheme 10: Pathway of AMI metabolism
The comparison of the inhibition experiments between rabbit and human liver microsomes may illustrate the overlapping activities of the used inhibitors and substrates. Troleandomycin (TAO) is one of most specific inhibitors for CYP3A isoforms [188] but did not show any effect in rabbit experiments. Its coincubation in the used concentration (in HLM) may abolish the contribution of CYP3A4 which was in the range of up to 60 % in all three investigated reactions. The antimycotic agent ketoconazole will likely inhibit several CYP isozymes in the used concentration [195]. It is a model substrate combining ligand binding activity to the iron center and a lipophilic rest (binding to lipophilic active site residues) [102]. The inhibitors and substrates were used in same or similar concentrations as in the case with rabbit liver microsomes and in fact the distribution of effects is very similar between the two species. However, most of the observed effects may be explained as overlapping inhibitory effects. Interestingly, the coincubation of α-naphthoflavon (αNF) did not only inhibit the contribution of CYP1A1 and CYP1A2 but also activated the product formation rates of the investigated reactions. Activatory effects may be explained by its role as a mechanism based inducer of CYP3A. Further investigation of this issue just confirmed the activation of AMI-EtOH formation by the addition of αNF to an enzyme kinetic experiment.

The incubation of MDEA with cDNA expressed P450 isozymes allows to find out rapidly the identity of the involved enzymes in the biotransformation. Our data suggest that at least five CYPs such as CYP1A1, CYP1A2, CYP2A6, CYP2D6 and CYP3A4 may participate into the elimination of MDEA in humans. Due to the similarity to CYP3A4 CYP3A5 is believed to be active as well. In previous work CYP2C8 was shown to dealkylate AMI to MDEA and therefore, this enzyme may also catalyze MDEA biotransformation. Due to activity and expression levels CYP3A4 may be suspected as the major enzyme for MDEA biotransformation to the three characterized metabolites. This task was additionnally investigated by correlating their formation rates the CYP3A specific midazolam α-hydroxylase activity. The positive correlation coefficients may rather support the hypothesis that CYP3A enzymes may have major relevance in vivo. Furthermore, the relative abundance of the CYP3A isoform in the liver is 20-29 % of the total CYP content [96-99]. Other CYP isoforms may participate
in the *in vitro* experiments but the correlation experiment can not help to determine their relevance *in vivo* for the reactions of interest.

In conclusion, in human liver microsomes the main metabolite MDEA of AMI is biotransformed by hydroxylation to 3’OH-MDEA, by dealkylation to DDEA and by deamination to AMI-EtOH. Under our experimental conditions the MDEA deamination seems to be the dominant metabolic pathway and human CYP3A4 may be the main P450 isoform involved into the MDEA elimination. Further steps in the metabolism process may include conjugation and elimination through bile.

The discovery of new metabolites including the involved metabolic enzymes may be results of potentially clinical importance. The new metabolites may be included in the drug monitoring of AMI patients. In addition to their plasma and tissue concentrations those compounds may be analyzed for their toxical effects. Therefore, their pharmacological significance may be a relevant issue of ongoing AMI research with the aim to improve therapy. The toxic effects of AMI derivatives has already been investigated [216] revealing that the benzofuran derivative possibly is the part of the AMI-molecule which inhibits the electron transport complexes and oxidation reactions in rat liver mitochondria.
5. References


Biotransformation References


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References


Chapter 2:
N-Nitrosylation Potential of mono-N-Desethylamiodarone in Physiological pH

1. Introduction

1.1. Amiodarone

Amiodarone (AMI) is a potent drug for the treatment of supraventricular arrhythmias [1-3]. In humans AMI is rapidly dealkylated to its main metabolite mono-N-desethylamiodarone (MDEA) [4, 5]. Plasma concentrations of MDEA may be higher than those of AMI [6]. Furthermore, AMI and MDEA have a high plasma protein binding ratio [7] and are accumulated in tissues [8].

Supraventricular arrhythmias may be caused by a myocardial infarct and can include the coadministration of AMI with nitrovasodilators such as glycerol trinitrate, isosorbiddinitrate or sodium nitroprusside (SNP). The mechanism of its vasodilative
action is based on the release of nitric oxide which functions as an endothelium derived relaxing factor (EDRF) [9].

1.2. Nitrovasodilators

Glycerol trinitrate was first synthesized with the objective to find a new explosive in 1846 [10] prior the finding of its antihypertensive effect. In the following years further agents of this class were discovered and are in medical use until now to treat coronary artery disease (Figure 1).

**Figure 1:** A selection of the most widely used nitrovasodilators: A) glycerol trinitrate (GTN), B) pentaerythritol tetranitrate (PETN), C) sodium nitroprusside (SNP), D) isosorbiddinitrate (ISDN), E) isosorbidmononitrate (ISMO), F) Diazeniumdiolate (NONOate) and G) Molsidomin reacting to H) SIN1A which then may release NO by decomposition.

Until the early 1980s the mechanism of action was completely unknown and the drugs were commonly referred to as "direct-acting smooth muscle spasmolytic agents".
the observation was made that NO and certain nitro compounds activate guanylate cyclase and stimulate cyclic GMP formation [11]. With this knowledge the nitroso-compounds (Figure 1) were identified as potent vasodilators which liberate NO [12]. A series of synthesized S-nitrosothiols were found to be potent vascular smooth muscle relaxants in vitro, potent vasodilators in vivo and potent inhibitors of human platelet aggregation in vitro [13, 14]. A recent study was published, where the effect of S-nitrosothiols in the respiratory process of the body was investigated [15].

1.3. Release of NO from Nitrovasodilators

One hypothesis for the release of NO from vasodilators was that chemical reduction is responsible since nitroso- and nitro-compounds released NO better or only in the presence of thiols such as cysteine or glutathione [12, 16]. It is believed that enzymes may catalyze the release of NO but the process is not well understood [17]. Organic nitrates have an oxidation state of +5 and the nitrogen of released NO has an oxidation state of +2. In the case of sodium nitroprusside (SNP) an one electron reduction of the nitrogen atom is required for the release of NO. SNP may release NO by exposure to light [17, 18, 19]. But since light is absent in the body other processes have to act for the release chemically and thus a variety of agents and tissues such as vascular smooth muscle membranes have mediated NO release [20]. Another NO-releasing pharmaceutical agent is molsidomin which is metabolized to SIN-1 and the metabolite releases NO by radical cleavage. Release of NO from several nitrovasodilators was investigated for the dependence of time, temperature or reducing medium [21, 22]. It is believed that the various nitrovasodilators are metabolized differently [23]. In the case of glyceryl trinitrate an extensive first pass effect was observed. Therefore, it is difficult to detect the drug in human plasma after oral administration [24]. In the case of transdermal patch application glycerol trinitrate and its metaboltes 1,2-glycerol dinitrate and 1,3-glycerol dinitrate were measured in plasma over 24 hours [25, 26]. The organic nitrate isosorbide dinitrate disappears less rapidly form the plasma than glyceryl trinitrate but there is also a hepatic first-pass extraction [24]. A major problem concerning the long-therm therapy with organic nitrates involves development of tolerance. Different pathways have been studied in the case of the bioactivation of
organic nitrates [27]. In more detail it was found that glyceryl trinitrate is bioactivated by an enzyme called mitochondrial aldehyde dehydrogenase [28]. Another study has shown that isosorbiddinitrate is metabolized by cytochrome P450 enzymes [29].

1.4. Reactions of NO in Aqueous Solutions

Nitric oxide (NO⁻) is a small molecule which is soluble in water to the extent of 1 to 3 mM (30 - 90 mg/l) and which is oxidized rapidly to nitrite (NO₂⁻) and, under certain conditions, to nitrate (NO₃⁻). The half-life of NO⁻ in aqueous solutions is 3-5 seconds [30]. The reaction of nitric oxide in oxygenated solutions with secondary amines in physiological pH is described as follows [30, 31].

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \xrightleftharpoons{\text{k}_1} 2\text{NO}_2 \\
\text{NO} + \text{NO}_2 & \xrightleftharpoons{\text{k}_2, \text{k}_3} \text{N}_2\text{O}_3 \\
\text{N}_2\text{O}_3 + \text{H}_2\text{O} & \xrightleftharpoons{\text{k}_4, \text{k}_5} 2\text{HNO}_2 \xrightarrow{\text{k}_5} 2\text{NO}_2^- + 2\text{H}^+ \\
\text{N}_2\text{O}_3 + \text{R}_2\text{NH} & \xrightarrow{\text{k}_6} \text{R}_2\text{NNO} + \text{NO}_2^- + \text{H}^-
\end{align*}
\]

**Scheme 1:** Proposed chemical reaction of nitric oxide in aqueous solutions leading to either formation of nitrite or nitrosation of amines [30, 31].

Reaction (2) represents at least two elementary steps. The proposed reactions are based on the assumption that nitrous anhydride (N₂O₃) is the main agent for the nitrosation of either secondary amines or thiols. As can be seen N₂O₃ is hydrolysed to nitrous acid (HNO₂) which then dissociates. Thus this reactions appears to be pH-dependent and at basic pH nitrous anhydride (N₂O₃) will preferably react to nitrite. On the other hand nitrite can be converted to the reactive N₂O₃ at acidic pH and may explain its carcinogenic potential in the human body. This reaction mechanism may also be related to the highly acidic reaction conditions for the synthesis of N-nitrosamines since low pH...
will promote the equilibrium of reaction 3) to the side of N₂O₃. Consequently, the hydrolysis of N₂O₃ described by reaction (3) and the nitrosation of amines (reaction 4) are two competing reactions at higher pH.

N-nitrosamines were usually synthesized under acidic conditions [32] but the nitrosation of secondary amines at higher pH values has been investigated as well. Challis et al. exposed different secondary amines to gaseous NO, N₂O₃, and N₂O₄ dissolved in acetonitrile [30]. N₂O₃ had the higher potential of N-nitrosation than N₂O₄ while with NO N-nitrosamine formation is comparably slow. Lately the formation of N-nitrosamines and N-nitramines was measured by the reaction of secondary amines and reactive nitrogen species including peroxynitrite (ONOO⁻) or the combination of nitric oxide (NO⁻) and superoxide anion (O₂⁻) [33]. It has been shown that the amount of formed N-nitroso products correlated with the amount of introduced nitric oxide and that superoxide anion inhibited this reaction. Before this investigation Lewis et al. [31] studied the kinetics of N-nitrosomorpholin formation and found that N-nitrosation occurs by the reaction of the secondary amine, introduced gaseous nitric oxide and oxygen. It was proposed that nitrous anhydride (N₂O₃) is a central reactive nitrogen oxide responsible for N-nitrosamine formation.

In human plasma NO⁻ may react rapidly with oxyhemoglobin to nitrate and methemoglobin [34, 35]. Another pathway of nitric oxide was found to be the binding to deoxy-hemoglobin to form nitrosyl-hemoglobin (HbFe(II)NO). It has been shown that nitrosyl-hemoglobin further reacts to S-nitrosohemoglobin where NO⁻ is bound to the β-93 cysteine of hemoglobin [36-38] which in contrast to the oxyhemoglobin reaction preserves NO⁻. The binding location of hemoglobin within erythrocytes prevents the rapid decay of the plasma NO⁻ concentration [39]. The formation of S-nitrosothiols depends on the rate of NO-release [40] suggesting that this is also the case for the nitrosylation of secondary amines. Furthermore, the formation of N-nitrosamines was investigated by incubating morpholine with macrophages. This experiment revealed that N-nitrosomorpholine was formed by released nitrogen species (derived from L-arginine) and not from the nitrite dissolved in the cell culture medium [41, 42].

The present study was designed to examine the potential of MDEA to react to the corresponding N-nitrosamine in physiological pH. N-Nitrosomonodesethylamiodarone was synthesized and characterized by HPLC-ESI-MS and NMR. The synthesized product was then used as a reference to measure the nitrosation of MDEA.
2.2. Methods

2.2.1. Chemicals

Mono-N-desethylamiodarone [2-n-butyl-3-(3,5-diiodo-4-ß-ethylaminoethoxybenzoyl)-benzofuran hydrochloride] was a kind gift of the Sanofi-Synthélabo Research Laboratories, Montpellier, France. Sodium nitroprusside and di-sodium hydrogen phosphate were obtained from Merck, Darmstadt, Germany. Dichloromethane, ethyl acetate, methanol and acetonitrile were purchased from Scharlau, Barcelona, Spain. Cysteine, N-acetylcysteine, amonium acetate, sodium acetate, sodium nitrite, sodium sulfate, acetic acid, sulfanilic acid, 1-naphthylamine, hydrochloric acid, n-Hexane and potassium carbonate were ordered from Fluka Chemicals, Buchs, Switzerland. Serum albumin (bovine) was purchased from Sigma Chemicals, Buchs, Switzerland.

2.2.2. HPLC-UV

HPLC-UV measurements were performed on a Merck-Hitachi (Darmstadt, Germany) La Chrom HPLC system equipped with a L-7100 pump, a UV-detector L-7400, an autosampler L-7200 and an interface D-7000 with a 250×4 mm Merck LiChroCART column packed with LiChrospher 60 RP-select B (particle size 5 μm) as a stationary phase. The mobile phase consisted of a phosphate buffer (pH 6; 20 mM)-methanol-acetonitrile (19:66:15, v/v/v) mixture with a flow rate of 1 ml/min. The detection wavelength was 242 nm and the pressure 170 bar (room temperature).
2.2.3. HPLC-ESI-MS

HPLC-ESI-MS experiments were performed on an HP 1100 HPLC system (Hewlett-Packard, Palo Alto, CA 94304, USA). The assay was operated under the following conditions: HPLC column (RP-C8 Waters Symmetry 150x2 mm; Waters, Milford, MA 01757, USA) maintained at 40°C; variable-wavelength detector setting at 242 nm. The mobile phase consisted of NH₄OAc (50 mM) as solvent A and acetonitrile as solvent B. The gradient started from 60 % solvent A and 40 % solvent B and increased to 100 % solvent B in 9 minutes. The final composition of 100 % solvent B and 0 % solvent A was kept until 30 minutes of total run time elapsed. The ESI-MS detector was interfaced directly to the output of the UV. ESI-MS conditions were similar to those reported previously [43]. The ion-polarity was operated with negative-mode (cap exit offset: -65 Volt; skimmer 1: -15 Volt). Acquisitions were performed in the range between m/z 50 and m/z 1000 and 6 spectra were averaged.

2.2.4. NMR-Measurements

¹H-, ¹³C- and inverse gated ¹³C-NMR spectra were recorded on a Bruker AMX-300 spectrometer (operating at 300.13 MHz for ¹H and at 75.47 MHz for ¹³C) at 298 K. DQF-COSY-, HMBC- and HSQC-spectra were recorded on a Bruker DRX-500 spectrometer (operating at 500.13 MHz for ¹H and at 125.77 MHz for ¹³C) at 295 K. The samples were measured in CDCl₃ and the residual resonance of the solvent was used as internal reference.
2.2.5. Synthesis of N-nitroso-monodesethylamiodarone (NO-MDEA)

NO-MDEA (2-n-butyl-3-(3,5-diiodo-4-ß-N-nitroso-ethylaminoethoxybenzoyl)-benzofuran) was synthesized according to methods described previously for N-nitrosodiphenylamine [44]: 16.2 mg (0.025 mmol) of MDEA hydrochloride (2-n-butyl-3-(3,5-diiodo-4-ß-ethylaminoethoxybenzoyl)benzofuran hydrochloride) were dissolved in 0.1 ml of dichloromethane before adding 0.3 ml of glacial acetic acid. Then 40 µl of a 5 M solution of NaNO₂ were pipetted into the solution and the progress of the reaction was monitored by TLC (n-hexane/ethylacetate 85:15 v/v). The Rᵣ-value of the formed product was 0.3. After 2 hours of reaction 10 ml of dichloromethane were added to the mixture which was then washed with 70 ml of saturated potassium carbonate. The organic phase was dried (Na₂SO₄) and evaporated under reduced pressure to give a viscous and colorless oil. Yield: 14.9 mg (0.023 mmol; 93 %). NMR (for numbering see Figure 4): δH[300 MHz, CDCl₃] syn-NO-MDEA: 0.92 (C(1)H₃, t, J 7.3 Hz, =CH₃), 1.37 (C(2)H₂-, m, J 7.6 Hz), 1.54 (C(21)H₃-, t, J 7.3 Hz), 1.78 (C(3)H₂-, m, J 7.6 Hz), 2.86 (C(4)H₂-, t, J 7.7 Hz), 4.09 (C(19)H₂-, t, J 4.5 Hz), 4.15 (C(18)H₂-, t, J 5.0 Hz), 4.46 (C(20)H₂-, q, J 7.3 Hz), 7.25 (1H, ddd, J 7.7, 7.3, 0.9 Hz), 7.32 (1H, ddd, J 8.3, 7.1, 1.1 Hz), 7.38 (1H, J 7.5 Hz) 7.49 (1H, d, J 8.3 Hz), 8.23 (C(15)H-, s); anti-NO-MDEA: 0.92 (C(1)H₃, t, J 7.3 Hz, =CH₃), 1.21 (C(21)H₃-, t, J 7.2 Hz), 1.37 (C(2)H₂-, hex, J 7.6 Hz), 1.78 (C(3)H₂-, quin, J 7.6 Hz), 2.86 (C(4)H₂-, t, J 7.7 Hz), 3.90 (C(20)H₂-, q, J 7.2 Hz, -CH₂-N), 4.41 (C(18)H₂-, t, J 5.1 Hz), 4.65 (C(19)H₂-, t, J 5.2 Hz), 7.25 (1H, ddd, J 7.7, 7.3, 0.9 Hz), 7.32 (1H, ddd, J 8.3, 7.1, 1.1 Hz), 7.38 (1H, J 7.5 Hz) 7.49 (1H, d, J 8.3 Hz), 8.23 (C(15)H-, s); δC [75.47 MHz, CDCl₃] syn-NO-MDEA: 13.7 (q, C(1)H₃), 14.1 (q, C(21)H₃), 22.5 (t, C(2)H₂), 28.2 (t, C(4)H₂), 30.0 (t, C(3)H₂), 43.6 (t, C(19)H₂), 49.2 (t, C(20)H₂), 69.1 (t, C(18)H₂), 90.6 (d, C(16)I)), 111.1 (d, C(7)H), 115.7 (s, C(12)), 121.0 (d, C(10)H), 123.9 (d, C(9)H), 124.7 (d, C(8)H), 126.3 (s, C(11)), 138.8 (s, C(14)), 140.7 (d, C(15)H), 153.6 (s, C(6)), 160.0 (s, C(17)), 166.3 (s, C(5)), 187.6 (s, C(13)); anti-NO-MDEA: 11.2 (q, C(21)H₃), 13.7 (q, C(1)H₃), 22.5 (t, C(2)H₂), 28.2 (t, C(4)H₂), 30.0 (t, C(3)H₂), 40.7 (t, C(20)H₂), 51.2 (t, C(19)H₂), 71.6 (t, C(18)H₂), 90.6 (d, C(16)I)), 111.1 (d, C(7)H), 115.7 (s, C(12)), 121.0 (d, C(10)H), 123.9 (d, C(9)H), 124.7 (d, C(8)H), 126.3 (s, C(11)), 138.8 (s, C(14)), 140.7 (d, C(15)H), 153.6 (s, C(6)), 160.0 (s, C(17)), 166.3 (s, C(5)), 187.6 (s, C(13));
LC-ESI-MS $[M-H]^+ = m/z$ 645 (100 %; ret. time 15.6-15.8 min), $m/z$ 645 (100 %; ret. time 16.0-16.2 min).

### 2.2.6. NO• release from SNP

A 10 mM solution of SNP in phosphate buffer (pH 7.4; 10 mM) was exposed to laboratory light conditions. After 0, 1, 2, 5, 10, 15, 20, 25, 30 and 50 min 1 ml of the solution were transferred to the prepared Griess-Reagent mixture consisting of 125 μl sulfanilic acid (30 mM; dissolved in 0.2 N hydrochloric acid), 125 μl 1-naphthylamine (40 mM; dissolved in 0.12 N hydrochloric acid), 125 μl sodium acetate solution (25%), 1 ml acetic acid (96%) and 125 μl of deionized water. The glass tubes were protected against light exposure to prevent further release of nitric oxide. A buffer solution without SNP was used as blank solution. The absorbance was measured at $\lambda = 525$ nM in a polystyrene cuvette. The system was previously calibrated with dissolved NaNO₂ in increasing concentrations. The data points were fitted by linear regression analysis using Origin Software (Redacom, Nidau, Switzerland).

### 2.2.7. N-Nitrosylation of MDEA

10 ml of phosphate buffer (pH 7.4; 10 mM) were introduced into a 50 ml flask and purged with nitrogen for 5 minutes. Then 10 ml of air purged phosphate buffer (pH 7.4; 10 mM) were pipetted into the flask (dilution of oxygenated solution to levels measured in arterial plasma [45]). MDEA was dissolved in ethanol and added to the stirred buffer solution to result in a concentration of 2 μM which corresponds to levels measured in human plasma [6]. The reaction was run in laboratory light conditions and was started by the addition of 59.6 mg of SNP (10 mM). After 10 minutes the reaction was stopped by extracting the solution with two times 20 ml of dichloromethane. The organic phase was collected in a 100 ml flask and evaporated under reduced pressure at 40 °C. Then the residue was dissolved in two times 3 ml of dichloromethane. The dichloromethane
solution was collected in a reaction tube and evaporated under a flow of nitrogen. The residue was dissolved in 200 μl of mobile phase (HPLC-UV), vortexed and centrifuged before injecting 70 μl into the HPLC.

2.2.8. Oxygen dependence

The oxygen dependence of the reaction was investigated by mixing varying volumes of air- or nitrogen-purged phosphate buffer (pH 7.4; 10 mM) assuming that the oxygen concentration in the air purged buffer solution corresponds to tabulated values of oxygen solubility in aqueous solutions at room temperature (250 μM). Removal of oxygen was achieved by purging the reaction solution with nitrogen for 10 minutes. The reactions were run and analyzed as described above.

2.2.9. Influence of high and low molecular weight thiols

After pipetting the oxygenated phosphate buffer (pH 7.4; 10 mM) to the nitrogen purged buffer solution the thiols such as serum albumin (792 mg; 600 μM), L-cysteine (6.1 mg; 2.5 mM) or N-acetylcysteine (8.2 mg; 2.5 mM) were added and dissolved by stirring before the addition of MDEA and SNP. The reactions were run and analyzed as described above. Reactions with serum albumin were extracted with diethylether instead using dichloromethane.

2.2.10. Peak identification by HPLC-ESI-MS

The identity of the measured peaks was additionally investigated by HPLC-ESI-MS. Therein, the AMI-derivatives of the solution (0.5 ml) were extracted with diethylether (3*1 ml) and the combined organic phase was evaporated to dryness. The samples were stored at -20°C and analyzed within 24 hours.
2.3. Results

2.3.1. Synthesis of NO-MDEA

The product of the N-nitrosylation of MDEA was a mixture of two compounds as can be seen in the $^1$H-NMR spectrum (Figure 2) and the HPLC-UV chromatogram (Figure 3).

Previous work has shown that N-nitrosamines contain partial double-bond character as predicted from one of their resonance structures ($\text{>N}^+=\text{N-O}^-$) leading to a high rotation barrier of the N-N-bond of ca 23-25 kcal/mol [46-49]. Thus it was suspected that the two peaks detected by HPLC represent the syn- and anti-forms of N-nitroso-monodesethylamiodarone (NO-MDEA) (Figure 4). $^1$H-NMR analysis of the synthesis product mixture resulted in the detection of eight signals for the protons 18-21 (arbitrarily numbered according to [43]). Based on chemical shifts and spin system
information, proton frequencies could be easily assigned to the butyl, the ethyl and the ethylene moieties. Therein, resonances were then assigned by standard correlation techniques. Thereafter, the carbon chemical shifts were determined from the HSQC and HMBC spectra. The complete assignment of $^1$H and $^{13}$C frequencies of the ethyl and ethylene moieties is presented in Table 1. As shown in Figure 2 the signals at 4.09 and 4.14 ppm are more intense than those at 4.41 and 4.65 ppm. We suspected that these pairs belong to either the syn- or the anti-configurational isomer of the molecule. Data from DQF-COSY spectrum did not show any cross correlation peaks between the two pairs of signals indicating that the protons are located in different molecules. The assignment of the signals to either the syn- or anti-configuration could only be achieved by comparing the $^1$H- and $^{13}$C-chemical shifts with published data. It is known that $\alpha$-protons and $\alpha$-carbons situated anti to the N-NO group are shifted further downfield than the atoms in syn-position [50]. $^{13}$C-NMR chemical shifts of carbon atoms in $\alpha$- or $\beta$-position to the nitrogen atom of anti- or syn-positioned ethylgroups are published [51]. The $\alpha$-carbon chemical shifts for heterocyclic compounds (syn: 39.2 ppm; anti: 50.2 ppm) were close to those published before [50] indicating that primarily the conformation of the nitroso-group influences the chemical shifts. HSQC, HMBC and DQF-COSY measurements support the assumption that the protons attached to C-18 resonate at 4.14 ppm in case of the syn- and at 4.41 ppm in case of the anti-isomer. These data suggest that the product mixture obtained from chemical synthesis can be explained by the equilibrium of two configurational isomers of the corresponding N-nitrosamine.

Table 1: $^1$H-NMR chemical shifts of the protons bound to carbons C(18) to C(21) of
N-Nitrosylation Results

Nitrosylated MDEA conformers expressed in ppm with the corresponding coupling constant in Hz (in parenthesis) in combination with chemical shifts obtained from $^{13}$C-NMR (in ppm relative to TMS).

<table>
<thead>
<tr>
<th>Carbon No.</th>
<th>$^1$H-NMR syn</th>
<th>$^1$H-NMR anti</th>
<th>$^{13}$C-NMR syn</th>
<th>$^{13}$C-NMR anti</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>4.15 ($J$ 5.0)</td>
<td>4.41 ($J$ 5.1)</td>
<td>69.1</td>
<td>71.6</td>
</tr>
<tr>
<td>19</td>
<td>4.09 ($J$ 4.5)</td>
<td>4.65 ($J$ 5.2)</td>
<td>43.6</td>
<td>51.2</td>
</tr>
<tr>
<td>20</td>
<td>4.46 ($J$ 7.3)</td>
<td>3.90 ($J$ 7.2)</td>
<td>49.2</td>
<td>40.7</td>
</tr>
<tr>
<td>21</td>
<td>1.54 ($J$ 7.3)</td>
<td>1.21 ($J$ 7.2)</td>
<td>14.1</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Integration of $^1$H-NMR peaks (Figure 2) revealed that the mixture consists of about 58% syn- and 42% anti-NO-MDEA. Assuming that the two NO-MDEA conformers have the same response in HPLC-UV analysis, the syn-NO-MDEA has the higher retention time compared to anti-NO-MDEA. In HPLC-ESI-MS analysis quasi-molecular ions of ([M-H]) at $m/z$ 645 were found for both peaks. This may correspond to the expected values for NO-MDEA.
Figure 3: HPLC-UV chromatogram of MDEA where peaks 1, 2, and 3 eluted at 6.1, 6.7 and 13.0 minutes and were identified as *anti*-NO-MDEA, *syn*-NO-MDEA and MDEA, respectively. Chromatogram a) represents the injection of synthesized NO-MDEA dissolved in mobile phase while b) is the chromatogram of the extract of the reaction of 2 μM MDEA with 10 mM SNP after 10 minutes (dissolved in mobile phase).
2.3.2. Formation of NO-MDEA

It is known that nitric oxide is released from SNP by exposure to light [17, 18]. As NO-reacts to nitrite in oxygenated aqueous solutions the release was calibrated indirectly using the Griess reaction. The release was estimated to be about 0.5 μM/min. A blank solution (protection from light) did not show any formation of nitrite suggesting that no NO- is released from SNP in darkness. The release per time was linear during the time of the measurement (30 minutes).

![Diagram of NO-MDEA formation](image)

**Figure 4.** Nitrosylation of MDEA is either obtained from chemical synthesis or by incubation of the substrate in 67 mM phosphate buffer pH 7.4 together with SNP. The mesomeric structure of the formed N-nitrosamine favours the configurational isomers denoted as syn- and anti-NO-MDEA (X).

As shown in Figure 3 the HPLC-UV analysis of the extracts of the reaction mixtures containing MDEA and SNP revealed the formation of two peaks at 6.1 and 6.7 minutes. Those coeluted with synthesized reference NO-MDEA. In HPLC-ESI-MS measurements the quasi-molecular of ions ([M-H]) of both peaks were detected at m/z 645 in agreement with the results for synthesized NO-MDEA. These results suggest that the peaks detected at 6.1 and 6.7 minutes of retention time correspond to anti- and syn-NO-MDEA, respectively.
Figure 5: MDEA was incubated in phosphate buffer (pH 7.4; 10 mM) with SNP under laboratory light conditions where (A) shows the time dependence of the nitrosylation of MDEA to syn- and anti-NO-MDEA. The reaction was further investigated concerning (B) the addition of varying concentrations of oxygen (n=3; for 125 μM oxygen: n=5) and (C) varying initial MDEA concentration.

The time dependence (Figure 5A) revealed a linear formation rate of combined syn- and anti-NO-MDEA for the initial 30 minutes. Under the present reaction conditions MDEA was nitrosylated by about 10 nM/min. No NO-MDEA peaks were detected at zero minutes of reaction indicating that the extraction procedure did not allow the reaction to proceed.

Surprisingly the variation of the oxygenation of the reaction solution (Figure 5B) did not cause major changes in the reaction yield. Moreover, the highest amounts of nitrosylated MDEA after 10 minutes of reaction time were achieved in nitrogen purged reaction buffer. However, it has been proposed that the nitrosylation of secondary amines does not run under strictly anaerobic conditions [31]. In the present experiment
traces of oxygen may have been sufficient since the release of NO⁻ was measured to be low.

The variation of the MDEA concentration (Figure 5C) has shown that the increase of the MDEA concentration may affect an increased production rate of the NO-MDEA conformers. This observation may as well be related to the limitation of the concentration of nitric oxide.

The influence of high and low molecular weight thiols was examined by adding either 0.6 mM serum albumin (bovine), 2.5 mM cysteine or 2.5 mM N-acetylcysteine to the reaction mixture. In this reactions the nytrosylated MDEA products were below the detection limits indicating that the nitric oxide merely formed S-nitrosothiols with the introduced thiol groups (results not shown).
2.4. Discussion

It was our aim to investigate the N-nitrosylation potential of MDEA in physiological solutions. MDEA is a secondary amine and the major metabolite of a cardiovascular drug. The reaction parameteres were adapted to physiological conditions concerning the concentrations of MDEA, phosphate and oxygen. However, changes of parameters such as the concentrations of phosphate and chloride may influence the nitrosylation of secondary amines in physiological conditions. In the present assay serum albumin as well as cysteine and N-acetylcysteine were successful to inhibit the nitrosylation of MDEA. It is known that thiols may be rapidly nitrosylated but may also release NO- [13].

As can be seen in the experiments of this work nitric oxide is a highly reactive molecule and the amount of MDEA nitrosylation appears to depend strongly on the presence of NO- in the reaction solution. The nitrosylating agent is believed to be N₂O₃ [31] while it was described that anions such as phosphate or chloride may acts as inhibitors of this reaction to form nitrosyl anions (XNO's).

Elevated levels of NO- may arise by the administration of nitrovasodilators which are metabolized rapidly [23-29]. One part of the released nitric oxide will be degraded by the reaction with oxyhemoglobin while another part will react with thiol groups. It has been found that thiol groups may function as carriers of NO- in the human body. The release of NO- from the S-NO's may result in a steady state level of nitric oxide in blood plasma. Consequently the nitrosylation of other thiol groups or secondary amines may occur.

Between 120 and 150 μM of oxygen have been found in human arterial blood. The present experimental conditions were adapted to this concentration. However, at low levels of NO- the nytrosylation of secondary amines may not be prevented by low oxygen levels.

The nitrosylation amount of MDEA will depend on its local plasma concentration together with peak NO- concentrations. Thiol groups may bind NO- before its releasing and thus may lower the reaction rate of NO-MDEA formation. N-Nitrosamines are more stable than S-nitrosothiols and therefore, reactions with longer duration time may
lead to an increased formation of those compared to the present experimental conditions.

MDEA plasma levels were measured to be 2 µM while a larger fraction of MDEA and its parent drug AMI is bound to serum albumin [7] or accumulated in tissues e.g. adipose, heart or lung tissue [8]. AMI's pulmonary toxicity is the most feared adverse effect. In addition the lung may as well be a way for NO-to enter the human body.

Finally one can state that the nitrosylation of a secondary amine such as MDEA may be taken into consideration. AMI and MDEA are highly lipophilic which leads to a long terminal elimination half life of these substrates. The conformers of NO-MDEA are more hydrophilic as can be seen from the significantly shorter retention times in RP-HPLC analysis. Therefore, they may be eliminated more rapidly.
2.5. References


Curriculum Vitae

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