# Drug interactions and hepatotoxicity of clopidogrel

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*To Michel*

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## Summary

Clopidogrel (Plavix®) is an antiplatelet drug, which is clinically used in combination with aspirin to reduce cardiovascular events in patients undergoing percutaneous coronary intervention or in patients suffering from acute coronary syndromes. Clopidogrel is a prodrug requiring enzymatic activation by cytochrome P450 (CYP) isoenzymes in order to inhibit platelet aggregation. Drug-interactions can affect clopidogrel activation and therefore cause interference with its pharmacodynamic effect. Clopidogrel is generally well tolerated but hepatotoxicity associated with clopidogrel treatment has been reported.
Amiodarone (Cordarone®) is a class III antiarrhythmic drug used for the treatment of a wide spectrum of cardiac arrhythmias. Amiodarone's therapeutic use is limited due to its numerous side effects such as liver toxicity. Recent *in vitro* investigations revealed that the N-desethyl metabolites of amiodarone may be partially responsible for the hepatotoxicity. Since CYP3A4 is responsible for amiodarone metabolism, CYP3A4 induction may represent an important risk factor in the clinic.

In the first project we investigated potential drug interactions of CYP inhibitors or substrates with the activation of clopidogrel or the inhibition of clopidogrel's antiplatelet effect. Using human liver microsomes (HLM) or specific supersomes we reproduced activation of clopidogrel *in vitro*. We found that CYP3A4 is primarily responsible for the metabolism of clopidogrel. At low concentrations (< 10 μM) CYP2C19 may contribute to clopidogrel activation to a certain degree. Additionally, HLM in co-incubation with platelets freshly prepared from human blood, allowed us to investigate the pharmacodynamic effect of clopidogrel *ex vivo*. We showed that activated clopidogrel dose-dependently inhibited platelet aggregation, whereas platelet aggregation occurred in absence of HLM. Potent CYP3A4 inhibitors exhibited strong inhibitory effects on clopidogrel activation and on clopidogrel's antiplatelet effect. Additionally, statins metabolized by CYP3A4 impaired clopidogrel activation and its antiplatelet effect. In contrast, CYP2C19 inhibitors did not affect clopidogrel activation probably because clopidogrel itself inhibits CYP2C19 at concentration ≥10 μM.

In the second project, we investigated whether the reactive metabolite of clopidogrel is responsible for the observed hepatotoxicity and studied the corresponding mechanism. Our liver toxicity models involved HepG2 cells that overexpress human CYP3A4 or were supplemented with CYP3A4 supersomes. These cellular systems were able to generate the

active metabolite of clopidogrel, which was associated with cytotoxicity. Co-incubation with ketoconazole, a CYP3A4 inhibitor, attenuated the toxic effect, thereby confirming the CYP3A4 dependency of clopidogrel activation. Cytotoxicity was associated with the induction of an oxidative stress reaction and promoted apoptosis via a mitochondrial pathway. In contrast, the carboxylate metabolite of clopidogrel, which is generated by esterases after oral administration, did not cause cytotoxicity.

The aim of the third project was to study the role of CYP3A4 in amiodarone-induced hepatotoxicity. Experiments were conducted using the same cellular activation systems as for clopidogrel, since CYP3A4 is also responsible for amiodarone metabolization in the liver. The systems proved to be powerful screening tools for CYP3A4-mediated toxicity of xenobiotics. We demonstrated that amiodarone metabolization is CYP3A4 dependent and generates MDEA and DDEA. Accordingly, MDEA and DDEA are primarily responsible for the observed cytotoxicity by increasing ROS production, by inducing mitochondrial damage and cytochrome c release and by promoting late apoptosis/necrosis. Therefore, we concluded that induced activity of CYP3A4 is a risk factor for hepatotoxicity associated with amiodarone.

## Abbreviations

| | |
|---|---|
| ADP | Adenosine-5'-diphosphate |
| AK | Adenylate kinase |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| CAPRIE | Clopidogrel versus aspirin in patients at risk ischemic events |
| COX-1 | Cyclooxygenase 1 |
| CYP | Cytochrome P450 |
| Cys | Cysteine |
| Cyt C | Cytochrome C |
| CURE | Clopidogrel in unstable angina to prevent recurrent events |
| DCF | Dichlorofluorescein |
| DCFH-DA | 2',7'-dichlorofluorescein-diacetate |
| DDEA | Di-N-desethylamiodarone |
| DEM | Diethyl-maleate |
| DMEM | Dublecco's Modified Eagle Medium |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DTNB | 5,5'-dithiobis (2-nitrobenzoic acid) |
| EGFP | Enhanced green fluorescent protein |
| FACS | Fluorescence-activated cell sorting |
| FDA | US Food and Drug Administration |
| GP | Glycoprotein |
| GSH | Glutathione |
| GSSG | Oxidized glutathione |
| GTP | Guanosine triphosphate |
| HEPES | N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) |
| HepG2 | Human hepatocyte cell line |
| HLM | Human liver microsomes |
| HMG-CoA | 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors |
| $H_2O_2$ | Hydrogen peroxide |
| HPLC | High performance liquid chromatography |
| IPA | Inhibiton of platelet aggregation |
| IS | Internal Standard |

| | |
|---|---|
| JUMBO-TIMI 26 | Joint utilization of medications to block platelets optimally study |
| KCl | Potassium cholride |
| KCZ | Ketoconazole |
| LTA | Light transmission aggregometry |
| MDEA | Mono-N-desethylamiodarone |
| $MgCl_2$ | Magnesium chloride |
| MPA | Maximal platelet aggregation |
| NaCl | Sodium chloride |
| NADPH | β-nicotinamide adenine dinucleotide phosphate |
| NMR | Nuclear magnetic resonance |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| PAR-1 | Protease-activator receptor 1 |
| PBS | Phosphate buffered saline pH 7.4 |
| PCI | Percutaneous coronary intervention |
| PPIs | Proton pump inhibitors |
| PPP | Platelet-poor plasma |
| PRP | Platelet-rich plasma |
| ROS | Reactive oxygen species |
| SEM | Standard error of the mean |
| ThioTEPA | N,N′,N″-triethylenethiophosphoramide |
| TNB | 5-thio-2-nitrobenzoic acid |
| TRAP | Thrombin receptor activating peptide |
| $TXA_2$ | Thromboxane $A_2$ |
| VASP | Vasodilator-stimulated phosphoprotein |
| vWF | Von Willebrand factor |

# 1. Introduction

## 1.1. Platelets

Platelets play a central role in the haemostatic process, including the recognition of an injury site, the recruitment of additional platelets by intracellular signaling, initial adhesion to each other and interaction with the coagulation cascade to form a haemostatic plug. Inappropriate platelet activation and thrombus formation can give rise to clinical complications in arterial atherosclerosis and thrombosis. It is well established that the formation of platelet-rich thrombi plays a major role in the onset and progression of arterial thrombotic disorders and therefore antiplatelet therapy is the basis of treatment and prophylaxis. Thus, many antiplatelet approaches have been followed, aiming to interfere with one or more of the different events in thrombus formation.

Platelets are fragments of large bone-marrow-derived cells called megakaryocytes. During the maturation of a megacaryocyte, its cytoplasm becomes compartmentalized and its plasma membrane ruptures. The membranes associated with each fragment then condense to form anucleate, disk-shaped platelets, which have a lifespan of approximately 7-10 days [1, 2].

In hemostasis, resting platelets circulate through blood vessels without receiving activation signals from other cells [3]. However, in response to vessel trauma, platelets spontaneously adhere to newly exposed adhesive proteins, particulary collagen and von Willebrand factor (vWF) via their respective receptors, glycoprotein (GP) VI and GPIb/V/IX. GPIb is present on the surface of non-activated platelets and is the major receptor for platelet adhesion. Platelets are activated instantly by various agonists such as thrombin, adenosine diphosphate (ADP), collagen and thromboxane $A_2$ ($TXA_2$), causing the platelets to change their shape and release the contents of their storage vesicles. It is thought that all of these agonists act by a common pathway, which leads to increased intracellular calcium concentration through direct ion flux or the release of stored calcium [2, 4]. These calcium-dependent processes include the phosphorylation of myosin light chain (associated with a change in platelet shape) and activation of phopsholipase $A_2$, leading to increased arachidonic acid, which is then converted by cyclooxygenases into $TXA_2$. The release of $TXA_2$ causes the release of ADP from platelet granules, which in turn stimulates the arachidonic acid pathway further, thus perpetuating the cycle [4]. Therefore, both $TXA_2$ and ADP are attractive targets for antiplatelet therapy.

Platelet aggregation is the consequence of the signaling cascade initiated by GPIIb/IIIa receptor engagement on the surface of activated platelets. In turn, platelet activation is accompanied by conformational changes of the GPIIb/IIIa receptor, which increases its

affinity for fibrinogen. Fibrinogen, the primary polypeptide involved in platelet aggregation, subsequently promotes the cross-linking of adjacent platelets, leading to the formation of a platelet-rich thrombus [2, 4].

### 1.1.1. Assaying platelet function

The assessment of platelet function is clinically important, because changes in platelet adhesion behavior can have a direct impact on both hemostasis and thrombosis.

Platelet functionality has been investigated in multiple ways in both clinical and research settings. For patients with cardiovascular diseases, platelet function tests are mainly used to predict clinical outcomes or for monitoring the antiplatelet therapy. Several kinds of platelet function tests have been developed over the years and among the available methods, light transmission aggregometry (LTA) is considered the gold standard for the study of patients with platelet function disorders. LTA was developed in 1962 by the researchers Born and O'Brien independently [5, 6] and measures the increase in light transmission through platelet-rich plasma (PRP) that occurs when platelets are aggregated upon binding to an agonist.

Several platelet agonist ligands are used to stimulate platelet suspensions in a light transmission aggregometer. The most commonly used agonists include ADP, epinephrine, collagen, arachidonic acid, and the thromboxane $A_2$ analogue U46619. Another commonly used reagent as experimental positive control is ristocetin, which induces non-specific aggregation. Many other agonists are available for studies of platelet function in the clinical setting and more commonly, for research purposes (e.g. thrombin, the thrombin receptor (PAR-1) activating peptide (TRAP), vasopressin and others) [7].

### 1.1.2. Light Transmission Aggregometer

A light transmission aggregometer is a photometer consisting of a light source, a cuvette holder with a rotating magnet (which drives a small stirrer placed in the platelet suspension), a thermostat heater (which maintains the temperature of the sample), and a photoelectric cell that measures the light transmission across the platelet suspension (Figure 1). Upon addition of a platelet agonist, aggregation is reflected by increased light transmission through the cuvette.

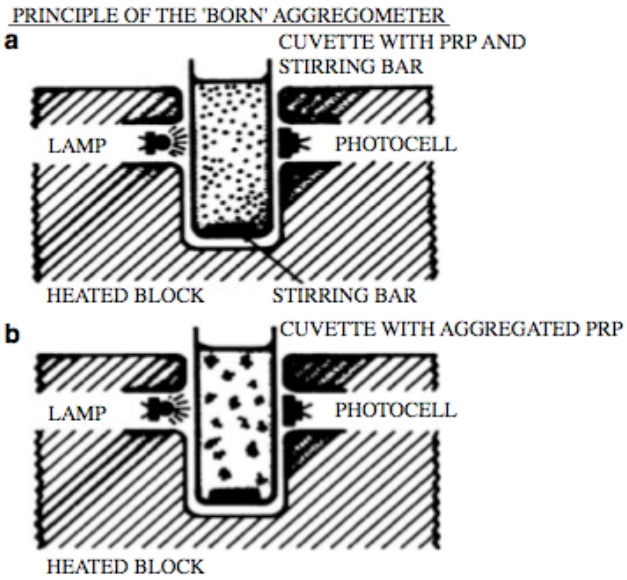


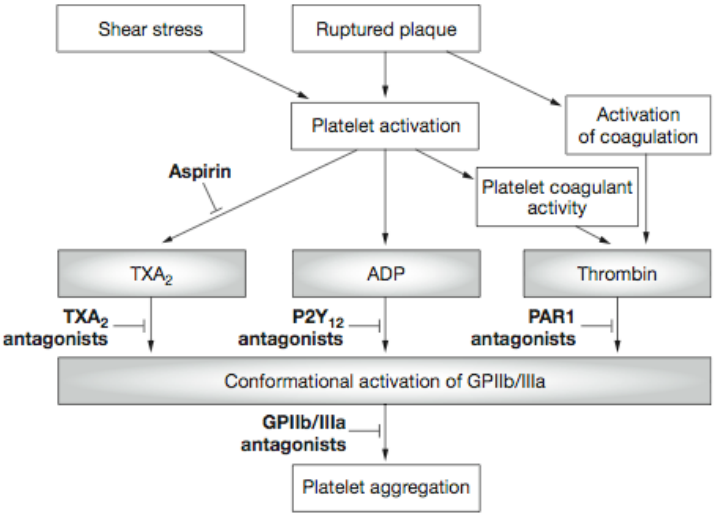

**Figure 1** Principle of LTA: as platelets aggregate in plasma, transmitted light increases [8].

Advantages of the LTA method include the feasibility to monitor aspirin, thienopyridines and platelet glycoprotein (GP) IIb/IIIa inhibitor therapy [9]. On the other hand, large sample volumes, long processing times and complex sample preparation are required. Although LTA is a commonly accepted method to test platelet function, yet there is no international standardization. Variability arises from numerous preanalytical and analytical steps that influence the results of LTA (e.g. blood sampling, use of anticoagulants, platelet count, temperature, pH, aggregometer stir speed, time). Since great experimental expertise is required only specialized laboratories are competent to reproducibly conduct LTA experiments [7].

Flow cytometry analysis of platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation is a relatively new assay for the analysis of platelet function [10]. VASP is an intracellular protein, which is non-phosphorylated at basal state. Dephosphorylation of VASP occurs following stimulation of the ADP receptor $P2Y_{12}$. Conversely, inhibition of the $P2Y_{12}$ receptor by clopidogrel induces phosphorylation of VASP [11]. Levels of VASP phosphorylation/dephosphorlyation thus reflect $P2Y_{12}$ inhibition/activation. Therefore, measurement of the VASP phosphorylation state is highly sensitive and specific for thienopyridine treatment. Several studies demonstrated a good correlation between VASP and LTA assays [12-15].

## 1.2. Antiplatelet drugs

Medical therapies targeting various molecules in the platelet activation pathways have been developed to prevent platelet aggregation. These agents are called antiplatelet drugs. Aspirin (acetylsalicylic acid) remains the most widely used and cost-effective drug in the prevention of platelet aggregation since the discovery of its effect over 40 years ago [8]. Other clinically well-established antiplatelet strategies include clopidogrel or GPIIb/IIIa antagonists. Newly developed compounds, which are being tested in clinical studies or are still in experimental preclinical phases include either alternative $P2Y_{12}$ inhibitors (chapter 1.3.3) and drugs that interact with alternative targets (Figure 2 & Box 1).




**Box 1** A summary of agents that inhibit platelet aggregation.

**$P2Y_{12}$ antagonists**
Clopidogrel
Prasugrel
AZD6140
Cangrelor

**Thromboxane antagonists**
Terutroban

**Protease-activated receptor 1 antagonists**
E5555
SCH530348

**Glycoprotein IIb/IIIa antagonists**
Abciximab
Tirofiban
Eptifibatide

**Figure 2** Sites of action of platelet inhibitors. Platelet aggregation can be inhibited by targeting cyclooxygenase (COX)-1 (aspirin) and therefore blocking the production of $TXA_2$, or by targeting platelet receptors (e.g. the $TXA_2$ receptor, $P2Y_{12}$, PAR1, and GPIIb/IIIa receptors) and thus blocking the action of platelet agonists. The various antiplatelet agents and corresponding drug classes are listed in **Box 1**. GP, glycoprotein; PAR, protease-activated receptor; $TXA_2$, thromboxane $A_2$.

### 1.2.1. Aspirin

Aspirin exerts its antiplatelet effect by irreversible acetylation of platelet cyclooxygenase (COX)-1 at serine residue 529 [16, 17]. This enzyme is responsible for the conversion of arachidonic acid to eicosanoids, which are precursors of the prostaglandins thromboxane $A_2$ ($TXA_2$) and prostacycline (prostaglandin $I_2$) (Figure 3). $TXA_2$ is a potent vasoconstrictor and platelet agonist that is released when platelets are activated. Platelet activation leads to activation of phospholipase $A_2$, which cleaves membrane phospholipids to release arachidonic acid. Arachidonic acid is converted to $TXA_2$ by sequential actions of COX-1 and thromboxane synthase present in platelets. Secreted $TXA_2$ binds to a specific Gq-coupled thromboxane receptor and recruits and activates surrounding platelets as part of a positive feedback mechanism. One study has shown that production of $TXA_2$ was prevented by a low dose (100 mg) of aspirin [18]. Prostacyclin is a vasodilator and platelet inhibitor synthesized by platelets and vascular endothelium. While aspirin permanently inhibits the rate-limiting step of $TXA_2$ production by COX-1 acetylation for the entire life span of platelets (7-10 days), the inhibited COX-1 of vascular endothelial cells is replaced with functional enzymes and maintains the synthesis of prostacyclin. Therefore, aspirin inhibits platelets by both reducing available $TXA_2$ and by increasing prostacylin relative to $TXA_2$. However, other mediators in platelet activation (ADP, collagen, fibrinogen) can overcome the antiplatelet effect of aspirin.

Aspirin has been shown to play a key role in the secondary prevention of atherothrombotic events [16] and the antithrombotic Trialists'Collaboration [17] confirms that aspirin therapy in patients with atherosclerotic vascular disease reduces non-fatal myocardial infarction by one third, non-fatal stroke by one quarter and vascular mortality by one sixth.

Although aspirin is a cost-effective therapy, a considerable number of patients who take aspirin continue to experience atherothrombotic complications [19]. Hence, the identification of more potent antiplatelet drugs is necessary especially to prevent these side effects in patients at higher risk.

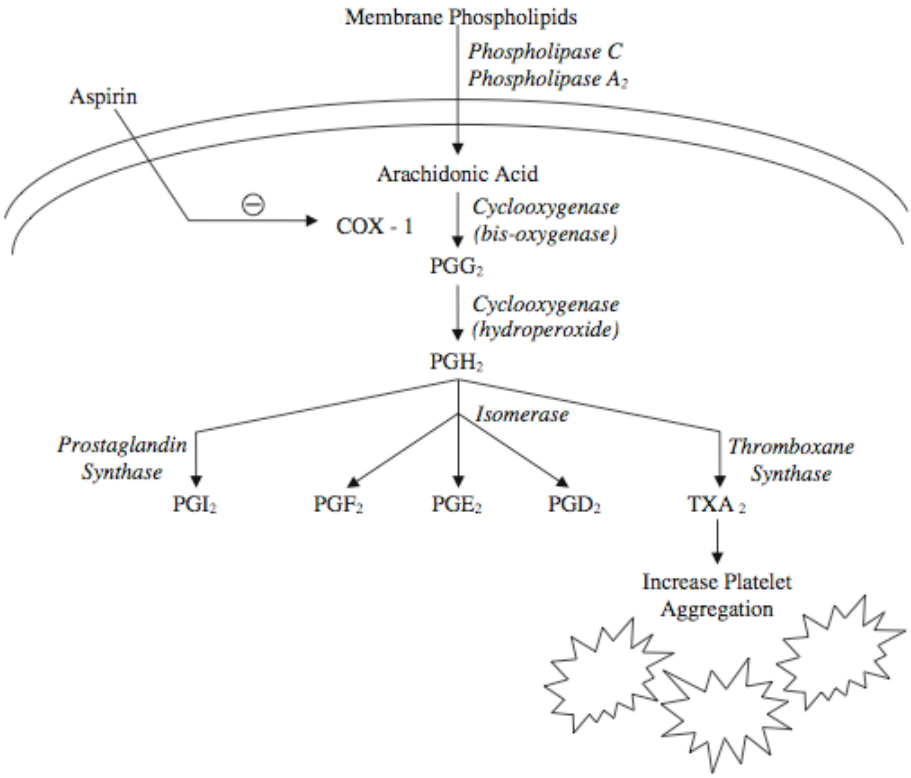



**Figure 3** Aspirin irreversibly acetylates COX-1 resulting in inhibition of $TXA_2$. $TXA_2$ = Thromboxane $A_2$, COX-1 = Cyclooxygenase-1, PG = Prostaglandin, $PGI_2$ = Prostacyclin.

### 1.3. Thienopyridines

The introduction of thienopyridines as potential adjunctive antiplatelet therapy has contributed significantly to the prevention and treatment of acute coronary thrombotic episodes. Thienopyridines belong to the family of ADP-receptor antagonists, which irreversibly inhibit the platelet receptor $P2Y_{12}$ (chapter 1.3.2).

***Ticlopidine*** (first-generation thienopyridine) was discovered in 1972 through numerous screening tests performed *in vivo* while looking for anti-inflammatory compounds. The discovery of ticlopidine's antiplatelet properties led to its development as an antithrombotic drug some years later. In patients, ticlopidine doses of 250, 375 and 500 mg per day inhibit platelet aggregation by 20-50%, 30-60% and 50-70%, respectively. Doses higher than 500 mg per day do not further increase the inhibition [20, 21]. Ticlopidine has been shown to be effective in peripheral artery disease, unstable angina and cerebrovascular disease [22]. However, due to its unfavorable side-effect profile (neutropenia, thrombocytopenia, bleeding complications)

and relative lack of efficacy, this drug is rarely used nowadays, except for patients allergic to other antiplatelet drugs [23, 24].

The patent for ***clopidogrel*** (second-generation thienopyridine) was first submitted in 1987, and clopidogrel was approved in western countries in 1997 for the prevention of ischemic stroke, myocardial infarction, and vascular death in patients with symptomatic atherosclerosis (CAPRIE trial= Clopidogrel versus Aspirin in Patients at Risk Ischemic Events) [25]. In 2002, after the CURE (Clopidogrel in Unstable angina to prevent Recurrent Events) trial [17], the use of clopidogrel in addition to standard therapy (including aspirin) was approved for the reduction of atherothrombotic events in patients with acute coronary syndromes. The standard clopidogrel regimen administered to prevent stent thrombosis is a 300 mg loading dose followed by a 75 mg daily maintenance dose [26]. Recent studies have used a 600 mg clopidogrel-loading dose based on superior pharmacodynamic effects compared to 300 mg [27-29]. A clopidogrel dose of 75 mg per day inhibits platelet aggregation by 40-60% at steady state [20, 30].

The clopidogrel molecule carries a methoxycarbonyl group on the benzylic position (Figure 4), which provides increased pharmacological activity and a better safety (lower incidence of adverse effects such as neutropenia and thrombotic thrombocytopenic purpura) and tolerability profile compared to ticlopidine. Clopidogrel is an S-enantiomer and the corresponding R-enantiomer is lacking antithrombotic activity in animal experiments, indicating that the S-configuration is essential for pharmacological activity. In several models of arterial thrombosis, clopidogrel exhibited potent, dose-dependent antithrombotic activity, and was approximately 50 to 100 fold more potent than ticlopidine or aspirin, respectively [22].

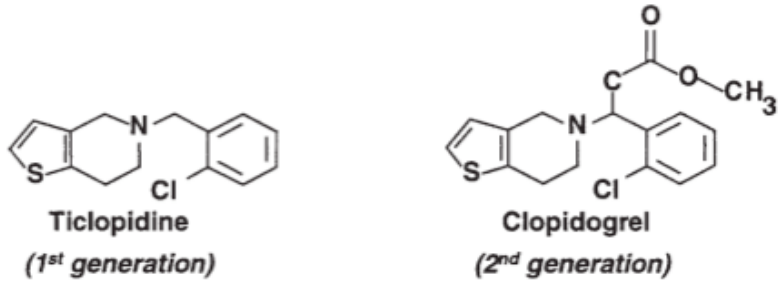



**Figure 4** Chemical structures of thienopyridines (ticlopidine and clopidogrel).

### 1.3.1. Metabolism of thienopyridines

The thienopyridines in current clinical use are prodrugs, requiring hepatic bioactivation by the cytochrome P450 (CYP) isoenzymes in order to generate the active metabolite. The transient intermediate contains a thiol group, which covalently modifies and inactivates the ADP receptor $P2Y_{12}$ on the platelet surface in a highly specific and irreversible manner [22, 31]. At least 20 metabolites of ticlopidine have been identified. It was proposed that among those, UR-4501 (containing a carboxylic acid and a thiol group as a result of 2-oxo-thiophene ring opening) is the molecule responsible for the *in vivo* activities of ticlopidine (Figure 5) [32].

After oral administration of clopidogrel, the majority (85%) is metabolized by esterases to form an inactive carboxylic acid derivative (carboxylate metabolite of clopidogrel, SR 26334) [33-36]. The conversion of clopidogrel to its active metabolite (R 130964) has been described as two-step, CYP-dependent process via the formation of 2-oxo-clopidogrel (Figure 5). In these steps, CYP3A4 seems to have a major role, with inferior involvement of CYP2B6 and CYP1A2. In addition, CYP2C9 and CYP2C19 may also metabolize clopidogrel to a lower extent [31, 37-39].

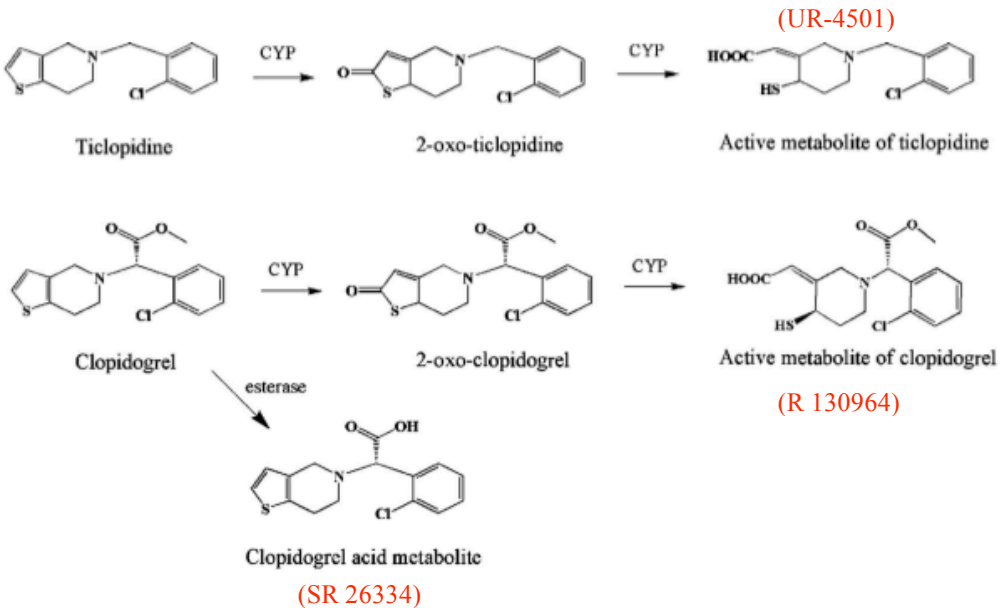



**Figure 5** Metabolic pathways of ticlopidine and clopidogrel. CYP: cytochrome P450.

Being a substrate for cytochrome P450 raises the possibility that thienopyridines function as competitive inhibitors of CYP isoenzymes. In fact, potent inhibition of the activities of cytochrome P450 has been demonstrated *in vitro*: CYP2B6, CYP2C19 and CYP2D6 for

ticlopidine [40-44]; CYP2C19 by 2-oxo-ticlopidine [41, 44]; CYP2B6 and CYP2C19 by clopidogrel [43, 44]. Ticlopidine is known to inhibit the CYP2C19 mediated metabolism of phenytoin [45] and omeprazole [46]. In a clinical setting it has also been reported that both clopidogrel and ticlopidine significantly inhibit CYP2B6-catalyzed bupropion hydroxylation, a monocyclic antismoking and antidepressant drug, in the clinical setting. Richter et al. [43] proposed previously that the mechanism-based inhibition of CYP2B6 by clopidogrel might be caused by disulfide bond formation between the active metabolite and the enzyme. Recent studies however supposed that this mechanism-based inhibition is due to chemically reactive metabolites produced during conversion of clopidogrel to the 2-oxo-clopidogrel [44, 47].

### 1.3.2. Mechanism of action of thienoypridines on ADP receptors

Adenosine diphosphate (ADP) is one of the most important mediators of both physiological hemostasis and thrombosis as mentioned in chapter 1.1 [48]. After platelet activation, ADP is released from its intracellular storage granules and further activates neighboring platelets, thereby amplifying this process.

There are two main purinergic receptor types in the membrane: $P2X_1$ and P2Y. $P2X_1$ is a ligand-gated ion channel that utilizes adenosine triphosphate (ATP) as an agonist and mediates extracellular calcium influx leading to altered platelet shape (Figure 6). There are two known P2Y receptors, $P2Y_1$ and $P2Y_{12}$, both are (GTP) dependent G-protein coupled receptors which utilize ADP as agonist. Engagement of $P2Y_1$ receptor leads to a series of signaling events that result in a weak and transient phase of platelet aggregation. In contrast, activation of $P2Y_{12}$ receptor leads to a complex series of intracellular signaling events that yield in activation of the glycoprotein (GP) IIb/IIIa receptor, granule release, amplification of platelet aggregation and stabilization of the coagulated cells [48, 49].

Thienopyridines selectively and irreversibly inhibit the $P2Y_{12}$ receptor [22]. The reactive thiol group of the active metabolite forms a disulfide bridge between one or two cysteine residues (Cys17 and Cys270) of the $P2Y_{12}$ receptor, resulting in its irreversible inhibition for the life span of the platelet [50]. In fact, platelet $P2Y_{12}$ blockade prevents platelet degranulation and the release of prothrombotic and inflammatory mediators from the activated platelet, and also inhibits the transformation of the GPIIb/IIIa receptor that binds fibrinogen and links platelets.

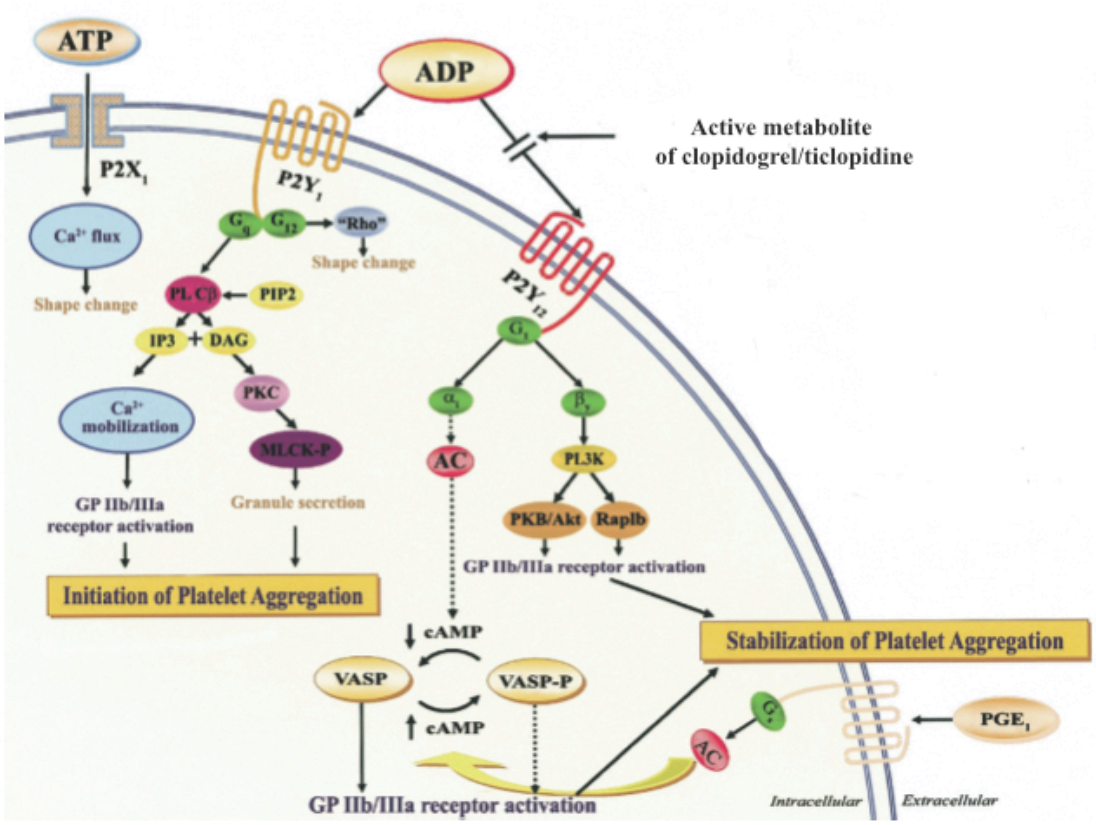



**Figure 6** Mechanism of action of clopidogrel and ticlopidine. The active metabolite irreversibly inhibits the ADP-$P2Y_{12}$ receptor on platelet surface. Activation of the $P2X_1$ and $P2Y_1$ receptor leads to alteration in shape and initiates a weak and transient platelet aggregation. The binding of ADP to the $P2Y_{12}$ receptor leads to a complex series of intracellular signaling events that yield in activation of the glycoprotein (GP) IIb/IIIa receptor, granule release, amplification of platelet aggregation and stabilization of the coagulated cells [48].

### 1.3.3. New ADP-receptor antagonists

Among the many new antiplatelet compounds that are currently being developed, different new $P2Y_{12}$ antagonists are likely to be introduced into clinical routine soon. The third orally available thienopyridine prodrug, prasugrel and the two non-thieropyridine antagonists of $P2Y_{12}$, cangrelor (intravenous administration) and AZD6140 (orally applicable) are currently being tested in phase III studies or are already being launched by the manufacturers, after having shown promising results in phase II studies in terms of efficiency and safety [51, 52].

Prasugrel is a prodrug similar to clopidogrel, which only after hepatic metabolism turns into a clinically active compound. In preclinical evaluation, it is 10 times more potent than other thienopyridines [51, 52]. Comparable efficiency and safety between prasugrel and clopidogrel was shown in the JUMBO-TIMI 26 study [53]. In the recently published TRITON-TIMI 38

study, prasugrel treatment was associated with a significantly reduced rate of the primary end point, which was death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke in patients with acute coronary syndromes, compared to clopidogrel [53]. However this advantage did not result in a difference in overall mortality, and, more importantly there was an increase in rates of major bleeding, including fatal bleeding [53].

AZD6140 and cangrelor are both reversible, nonthienopyridine $P2Y_{12}$ inhibitors, which showed more rapid onset and cessation of action than clopidogrel and reduced bleeding risk in patients with acute coronary syndromes [51, 52].

### 1.4. Variability in responsiveness to oral antiplatelet therapy

Patients who are suffering from acute coronary syndrome or are undergoing percutaneous coronary intervention receive antiplatelet therapy to reduce the risk of atherothrombotic complications. Clopidogrel in combination with aspirin is the current standard of care for reducing cardiovascular events in these patients [54-56]. However, the response to clopidogrel varies among patients, and clopidogrel 'resistance' has been observed. Such variations have repeatedly been associated with adverse cardiovascular outcomes in patients undergoing percutaneous coronary intervention [57, 58]. The occurrence of ischemic events despite dual antiplatelet therapy is a serious clinical problem and may origin from drug non-responsiveness.

#### 1.4.1. Variability in individual responsiveness to aspirin

Emerging evidence of aspirin 'resistance' in the recent literature may have substantial clinical implications. Aspirin 'resistance' has been defined either as the failure of aspirin to prevent individuals from clinical thrombotic complications or as the failure to produce an expected response on a laboratory measurement of platelet activation or aggregation [29].

The prevalence of reported aspirin 'resistance' can vary considerably and may in fact be overestimated when patient non-compliance to aspirin therapy is excluded and COX-1 specific laboratory analyses are performed. Using arachidonic acid-induced COX-1 specific platelet aggregation, Tantry et al. [59] reported a 0.4% incidence of aspirin non-responsiveness in patients undergoing PCI and Schwartz et al. [60] reported a 0.5% incidence in patients with a history of myocardial infarction. This stands in contrast to 20-35% incidence of aspirin 'resistance' when determining non COX-1 specific platelet aggregation regardless of compliance [61, 62].

Recent observations have demonstrated that the primary cause of aspirin failure is poor compliance to medication [59, 63]. Another important factor is whether the patients are receiving concomitant administration of non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, which interfere with the irreversible inactivation of platelet COX-1 by aspirin. Other potential mechanisms of aspirin resistance that have been proposed are redundant platelet activation pathways, increased COX-2 activity and polymorphism of platelet GPIIIa and COX-1 [29].

### 1.4.2. Variability in individual responsiveness to clopidogrel

Accruing data show that the variability of individual responsiveness to antiplatelet therapy applies to clopidogrel as well. However, the nature of variability of clopidogrel responsiveness is different compared to aspirin, since these drugs exhibit substantially different pharmacologic profiles. The occurrence of ischemic events despite the use of clopidogrel suggests that inadequate response to treatment affects certain patients. This risk may be further enhanced in patients who have inadequate response to both, aspirin and clopidogrel therapy [29]. Standardized definitions to individual responsiveness to clopidogrel are required but still lacking. The prevalence of clopidogrel non-responsiveness has been reported to be in the range of 5-40% [29, 58].

The mechanisms underlying interindividual variability in response to clopidogrel have not been defined but are probably multifactorial [48]. Potential factors are shown in Figure 7 and include genetic, clinical, and cellular factors. Among these, genetic polymorphisms of CYP isoenzymes, which have a major role in generating the active metabolite of clopidogrel, presumably affect the individual responsiveness more severely than downstream targets, such as platelet membrane receptors.

Furthermore, clinical factors also have a major role in variable response profiles to clopidogrel [29, 48, 64]. Drug-drug interactions (e.g., CYP3A4 metabolizing statins and omeprazole) may interfere with the pharmacodynamic effects of clopidogrel (chapters 1.4.2.3 & 1.4.2.4). Similar to aspirin, inadequate prescription or poor compliance may play a pivotal role. Certain clinical scenarios such as diabetes and elevated body mass index are also associated with reduced clopidogrel responsiveness. This may explain why such patients have a greater likelihood of developing recurrent thrombotic complications despite clopidogrel usage [29, 48, 64].

Cellular factors may also play a role in clopidogrel response variability. These include faster platelet turnover, increased platelet exposure to ADP, reduced CYP activity, upregulation of

P2Y-dependent signaling ($P2Y_1$ and $P2Y_{12}$), and the upregulation of P2Y-independent pathways [29, 48, 64].

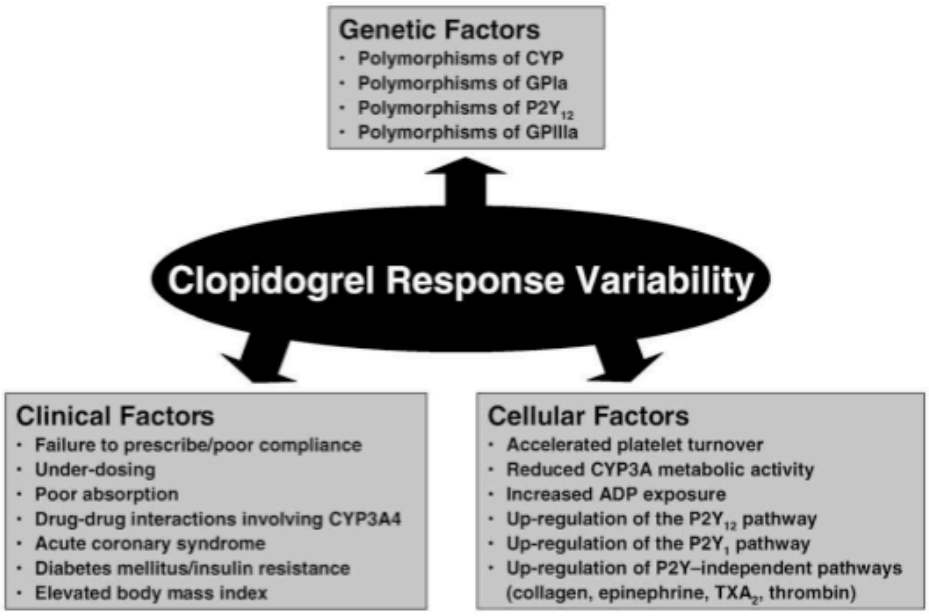



**Figure 7** Proposed mechanisms leading to variability in individual responsiveness to clopidogrel. ADP = adenosine diphosphate; CYP = cytochrome P450; GP = glycoprotein; $TXA_2$= thromboxane $A_2$.

1.4.2.1. Cytochrome P450 polymorphisms and response to clopidogrel

Clopidogrel is a prodrug that becomes converted by cytochrome P450 (CYP) enzymes for its antiplatelet effect. One important mechanism for response variability to clopidogrel involves *genetic polymorphisms* that alter expression and therefore the enzymatic activity of CYP isoenzymes. In some cases, CYP polymorphisms may cause insufficient enzymatic activity to convert clopidogrel into the active metabolite.

Clopidogrel metabolism is predominantly performed by the CYP3A system, which consists of the 3A4 and 3A5 isoenzymes [37]. Many research groups have failed to show an association between CYP3A4 variants and enzymatic activity. In contrast, CYP3A5 is polymorphically expressed and shows marked differences between ethnic populations. Single nucleotide polymorphisms *CYP3A5*3* and *CYP3A5*6* can cause alternative splicing or protein truncation, which results in the absence of CYP3A5 from tissues. When CYP3A5 is expressed (in subjects with at least one *CYP3A5*1* allele), it may account for more than 50% of the total CYP3A activity in the liver [65]. Suh et al. [66] reported an increased frequency of atherothrombotic events within 6 months after coronary angioplasty among patients with the

CYP3A5 nonexpression genotype (*CYP3A5*3*) who were receiving clopidogrel therapy. However, further studies failed to show an association between the CYP3A5 genetic polymorphism and the antiplatelet effect of clopidogrel *ex vivo*, either in patients or in healthy subjects [67-69]. Likewise, the results of the FAST-MI [70] study did not support a role for CYP3A5 genetic polymorphism in the clinical response.

CYP2C19 is another key enzyme of clopidogrel activation [67, 69]. Genetic polymorphisms of CYP2C19 modulate clopidogrel pharmacokinetics and pharmacodynamics in healthy volunteers [39, 69], as well as in patients [71]. Compared to wildtype CYP2C19 expression, subjects carrying one or two CYP2C19 loss-of-function alleles exhibit lower plasma concentrations of the active clopidogrel metabolite and a decreased antiplatelet effect of clopidogrel in *ex vivo* aggregation tests. Those findings are supported by recent data from Mega et al [72] and the FAST-MI study [70]. Both of these studies demonstrated a > 3-fold increase in the risk of adverse cardiovascular events among patients undergoing percutaneous coronary intervention who were homozygous or heterozygous for any of the *CYP2C19* alleles known to result in a nonfunctional protein (*CYP2C19*2, *3, *4, *5*).

In summary, these studies suggest that polymorphisms associated with decreased function of CYP2C19 or CYP3A4/5 contribute accordingly to a reduced response to clopidogrel.

Recent reports have suggested that polymorphisms of other targets not directly involved in clopidogrel metabolism may also be involved in the response variability. A minor haplotype of the $P2Y_{12}$ receptor was found to be associated with increased platelet reactivity in healthy subjects [48, 64, 73]. However, these findings could not be confirmed by other studies testing patients treated with clopidogrel [70, 71]. A potential role for a genetic polymorphism of the GPIIb/IIIa receptor has been demonstrated in small sample size studies but also failed to be confirmed by others [48, 74].

#### 1.4.2.2. Drug-drug interactions and response to clopidogrel

A drug-drug interaction is considered clinically relevant when it occurs between two commonly co-administered agents and results in the need for dosage adjustment or other medical intervention [75]. Theoretically, a clinically relevant drug interaction may exist between clopidogrel competing with other drugs metabolized by CYP3A (e.g. statins) and CYP2C19 (e.g. PPIs). If the CYP3A4 or CYP2C19 catalyzed activation of clopidogrel is compromised, this most likely results in a reduced antiplatelet effect, which may or may not be of clinical relevance. The outcome of competitive interaction is generally not predictable from theoretical considerations or *in vitro* studies on CYP inhibition and $K_i$ values obtained. The

outcome of an interaction depends on the relative concentration of the drugs at the enzyme level, their relative affinity for the CYP isoenzyme-binding site *in vivo* and the individual capacity of the enzymes [75].

#### 1.4.2.3. Drug-drug interactions involving CYP3A4

Clopidogrel and 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors (statins) are frequently co-administered in patients with cardiovascular disease (acute coronary syndrome, cerebral vascular disease, peripheral arterial disease) [76]. Potentially unfavorable drug-drug interactions between clopidogrel and statins, which contribute to the phenomenon of clopidogrel response variability has been a recent topic of debate. As both clopidogrel and several statins (atorvastatin, lovastatin and simvastatin) are metabolized by CYP3A4, the antiplatelet effect of clopidogrel may be compromised by their co-administration [77, 78]. In 2003, Lau et al [77] first described this interaction between clopidogrel and atorvastatin: in 44 patients undergoing PCI with stent implantation, concomitant treatment with atorvastatin, a CYP3A4 substrate, was associated with reduced antiplatelet activity of clopidogrel. They also showed a reduction in clopidogrel's antiplatelet activity associated with the use of erythromycin and troleandomycin, two potent CYP3A inhibitors; however, the application of rifampin, a CYP3A inducer, was associated with enhanced antiplatelet activity. To confirm these observations, Lau et al. conducted another study, in which they found further evidence for a relationship between CYP3A4 and clopidogrel activity [78]. Neubauer et al. [79] observed in 47 patients undergoing elective PCI that pretreatment with atorvastatin or simvastatin was associated with a reduction in clopidogrel efficiency.

However, subsequent studies investigating the interaction of CYP3A4-metabolized statins with the antiplatelet effects of clopidogrel failed to confirm the initial findings [80-85]. The clinical significance of the lipophilic statin-clopidogrel drug-drug interaction has not been definitely demonstrated and remains controversial [86].

Importantly, if competing CYP3A4 substrates interact with clopidogrel activation, such an interaction may become clinically more meaningful when CYP inhibitors are applied. Inhibitors include certain calcium antagonists, azole fungostatics and/or macrolide antibiotics. Indeed recent studies suggest that concomitant treatment with CYP3A4 inhibitors decreases the ability of clopidogrel to inhibit platelet aggregation [38, 66, 87]: a study by Siller-Matula [87] showed that co-administration of calcium-channel blockers, another frequently used class of cardiovascular drugs, reduced the antiplatelet effect of clopidogrel. A drug-drug interaction study showed that the potent CYP3A4 inhibitor ketoconazole significantly reduced the

generation of clopidogrel's active metabolite. Moreover, concomitant treatment with ketoconazole decreased platelet inhibition in response to clopidogrel as measured by LTA [38]. Another study showed that the CYP3A4 inhibitor itraconazole significantly decreased the ability of clopidogrel to inhibit platelet aggregation [66].

#### 1.4.2.4. Drug-drug interactions involving CYP2C19

Patients receiving dual antiplatelet treatment with aspirin and clopidogrel are commonly treated with proton pump inhibitors (PPIs) aiming to reduce the risk of gastrointestinal tract bleeding while taking dual-antiplatelet therapy [88]. Hepatic metabolization of PPIs is predominantly mediated by CYP2C19 and CYP3A4 [89, 90] and it has been suggested that a potential drug-drug interaction at the level of the hepatic CYP system exists [88, 91]. Importantly, PPIs differ in their metabolization properties as well as in their potential for drug-drug interactions [92]. Whereas omeprazole is the PPI with the highest affinity for CYP2C19 and is therefore predominantly metabolized by this isoenzyme, pantoprazole and esomeprazole exhibit a high affinity for both CYP2C19 and CYP3A4. Moreover, pantoprazole is metabolized to a significant extend by a conjugating enzyme, a cytosolic sulfotransferase, and therefore has the lowest potential for drug-drug interactions [90, 92]. Due to its specific dependence on CYP2C19 compared to other PPIs, a number of studies have shown that omeprazole has a considerable potential for drug interactions [88, 92-96]. It has recently been reported that omeprazole decreases the antiplatelet effect of clopidogrel probably due to the inhibition of CYP2C19 [88, 96]. Since all PPIs are metabolized by CYP2C19 to a varying degree, they hypothesized that the reported omeprazole-clopidogrel interaction may not be a class effect. Indeed, contrary to the omeprazole-clopidogrel interaction, the intake of pantoprazole, esomeprazole or lansoprazole was not associated with a reduced platelet inhibition by clopidogrel [21, 93, 97].

To date, substantial controversy regarding the clinical outcome of patients taking clopidogrel and PPIs remains. The US Food and Drug Administration (FDA) recently released a safety review about the potential interaction between these two medications [98]. However, there was insufficient data to make any recommendations, highlighting the need for additional studies to evaluate the effectiveness of clopidogrel when used together with PPIs.

## 1.5. Hepatotoxicity under clopidogrel treatment

Drug-induced liver disease occurs approximately in 1 in every 1000 patients to 1 in every 10'000 patients at therapeutic doses [99]. It is often difficult to determine the responsible drug since these patients are frequently under polymedication. Hepatic injury can occur from many drugs through a variety of mechanisms such as disruption of intracellular calcium homeostasis, derangement of the CYP system or stimulation of a multifaceted immune response against liver enzyme-drug adducts [99]. Drug induced liver injury is difficult to diagnose especially for drugs with rare hepatic complications.

Common adverse effects seen with clopidogrel include gastrointestinal disorders (indigestion, nausea, vomiting), bleeding, rash and diarrhea [20, 25, 30]. However, rare and serious complications such as hepatotoxicity [100-110], thrombotic thrombocytopenic purpura [111], pancytopenia [112], systemic inflammatory response syndrome [100] and serum sickness-like reaction [113] have also been reported in recent years. Until now, thirteen cases [100-110] have been published that describe clopidogrel-induced hepatotoxicity. The time between clopidogrel intake and hepatic injury ranged from 4 days to 6 months after commencement of clopidogrel therapy and all patients except one completely recovered when the drug was discontinued (Figure 8). Clinically, patients developed cholestatic hepatitis or mixed hepatocellular and cholestatic hepatitis similar to ticlopidine [114-118]. In one case a patient had a systemic inflammatory response syndrome indicated by rash, leucopenia, tachycardia and liver injury[113].

Though, the mechanisms of clopidogrel-associated hepatotoxicity still remain unclear. Hypersensitivity and direct toxicity have been postulated as potential mechanisms. Based on the available clinical-pathological observations, it appears likely that clopidogrel triggers both dose-independent idiosyncratic and dose-dependent toxic reaction [103, 104, 110].

Interestingly, a cross-hypersensitivity reaction between ticlopidine and clopidogrel has not been described so far. Ticlopidine-induced hepatotoxicity is well known and documented in the literature [114-119]. Clopidogrel has been successfully used as replacing drug in three patients with ticlopidine-induced hepatotoxicity [120]. However, the vice versa strategy to use ticlopidine in patients with clopidogrel-induced hepatotoxicity has not been attempted, mainly due to the fact that ticlopidine has been associated with a higher rate of neutropenia and elevated transaminases than clopidogrel.

Clopidogrel is still advertised as an effective and safe antithrombotic drug. Due to its low incidence of hepatotoxicity, monitoring of the liver function for patients receiving clopidogrel is not performed routinely. Therefore, clopidogrel should be used with caution in pre-existing liver disease and discontinued in case of other hepatic disorders such as jaundice or hepatitis.

**Characteristics of Patients Who Developed Clopidogrel-Induced Liver Injury**

| Age (yrs) | Sex | Daily Dose (mg) | Onset of Liver Injury (days) | Previous Liver Injury | Concomitant Drugs | Pattern of Liver Injury | Rechallenge Test | Histologic Finding | Follow-up Recovery |
|---|---|---|---|---|---|---|---|---|---|
| 50 | F | 75 | 12 | No | Yes | SIRS | NA | NA | Yes |
| 56 | M | 75 | 60 | NA | Yes | Mixed | Positive | NA | Yes |
| 59 | F | 75 | 3 | NA | Yes | Hepatocellular | Positive | NA | Yes |
| 80 | M | 75 | 43 | No | Yes | Mixed | Not performed | Cholestatic hepatitis and focal cell necrosis | Yes |
| 74 | M | 75 | 37 | NA | Yes | Mixed | Not performed | NA | Yes |
| 59 | M | NA | 4 | NA | Yes | Hepatocellular | Positive | NA | Yes |
| 89 | F | 75 | 60 | NA | Yes | Mixed | Not performed | NA | Yes |
| 77 | M | NA | 180 | NA | Yes | Mixed | NA | NA | Yes |
| 81 | F | 75 | 21 | No | Yes | Mixed | Not performed | NA | Yes |
| 63 | M | 75 | 30 | NA | Yes | Mixed | Not performed | NA | Yes |
| 84 | F | 75 | 56 | No | Yes | Mixed | Not performed | Drug-induced liver injury pattern | Yes |
| 65 | F | 75 | 150 | NA | Yes | Hepatocellular | Not performed | Drug-induced liver injury pattern | Died |
| 78 | F | 75 | 23 | No | Yes | Mixed | Positive | NA | Yes |

SIRS = systemic inflammatory response syndrome; NA = data not available; mixed = hepatocellular and cholestatic injury.

**Figure 8** Characterization of patients who developed clopidogrel induced liver injury [110].

## 2. Aims of this thesis

The aim of the first project was to study drug-interactions with clopidogrel. Initially, we designed an *in vitro* system using human liver microsomes (HLM) or supersomes in order to study the activation of clopidogrel and the conceivable activation of the carboxylate metabolite. We wanted to examine drugs that inhibit the hepatic activity of CYPs, since interference with clopidogrel likely occurred at the level of the cytochromes. We were also interested in CYP substrates, which are frequently co-administered with clopidogrel (e.g. statins, PPIs). In a second step, we expanded our system to investigate the effect of inhibitors on clopidogrel's antiplatelet effect. Using the LTA method we monitored platelet aggregation in response to HLM mediated clopidogrel activation and interacting drugs.

In the second project we focused on the hepatotoxic potential of clopidogrel and ticlopidine *in vitro*. To address this, we established two bioactivating systems, namely HepG2 cells stably overexpressing CYP3A4 and HepG2 wt cells in co-incubation with CYP3A4 supersomes in order to generate the active metabolite of clopidogrel. We aimed to detect this active metabolite, which is carrying a reactive thiol group and possibly binds glutathione. Since we accomplished to measure a drop in the glutathione pool, it's likely to attribute the toxic effect to the active metabolite. Furthermore, we used standard methods to study cytotoxicity and the corresponding mechanism.

The aim of the third project was to study CYP3A4 dependent hepatic toxicity of amiodarone. We hypothesized that amiodarone hepatotoxicity is based on the CYP3A4-dependent generation of the two toxic metabolites MDEA and DDEA. We used the same cellular activating systems to investigate the conversion of amiodarone to its N-desethyl metabolites MDEA and DDEA. We intended to detect the generation of MDEA and DDEA in a quantitative way using HPLC. Furthermore, we wanted to study the mechanism of cytotoxicity of activated amiodarone in CYP3A4 overexpressing HepG2 cells.

# 3. Drug interactions with biotransformation and antiplatelet effect of clopidogrel *in vitro*

Running head: Drug interactions with clopidogrel *in vitro*

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## 3.1. Abstract

Background and purpose: The conversion of clopidogrel to its active metabolite, R-130964, is a two-step cytochrome P450 (CYP)-dependent process. The current investigations were performed to characterize *in vitro* the effects of different CYP inhibitors on the biotransformation and on the antiplatelet effect of clopidogrel.

Experimental approach: Clopidogrel biotransformation was studied using human liver microsomes (HLM) or specific CYPs and platelet aggregation using human platelets activated with ADP.

Key results: Experiments using HLM or specific CYPs (3A4, 2C19) revealed that at clopidogrel concentrations ≥10 μM, CYP3A4 is primarily responsible for clopidogrel biotransformation. At a clopidogrel concentration of 40 μM, ketoconazole showed the strongest inhibitory effect on clopidogrel biotransformation and clopidogrel-associated inhibition of platelet aggregation with $IC_{50}$ values of 0.03 ± 0.07 μM and 0.55 ± 0.06 μM, respectively. Clarithromycin, another CYP3A4 inhibitor, impaired clopidogrel biotransformation and antiplatelet activity almost as effectively as ketoconazole. The CYP3A4 substrates atorvastatin and simvastatin both inhibited clopidogrel biotransformation and antiplatelet activity, but less potently than ketoconazole. In contrast, pravastatin showed no inhibitory effect. Since clopidogrel itself inhibited CYP2C19 at concentrations <10 μM, the CYP2C19 inhibitor lansozprazole affected clopidogrel biotransformation only at clopidogrel concentrations ≤10 μM. The carboxylate metabolite of clopidogrel is no CYP substrate and did therefore not affect platelet aggregation.

Conclusions and implications: At clopidogrel concentrations >10 μM, CYP3A4 is mainly responsible for clopidogrel biotransformation, whereas CYP2C19 contributes only at clopidogrel concentrations ≤10 μM. CYP2C19 inhibition by clopidogrel at concentrations >10 μM may explain the conflicting results between *in vitro* and *in vivo* investigations regarding drug interactions with clopidogrel.

Key words

Clopidogrel, carboxylate metabolite of clopidogrel, CYP3A4, CYP2C19, drug interactions

## 3.2. Introduction

The drugs or drug classes currently used for the inhibition of platelet aggregation include acetylsalicylic acid, glycoprotein IIb/IIIa receptors antagonists and the thienopyridine derivatives. The thienopyridines, in particular clopidogrel, have become standard drugs for the management of patients following percutaneous coronary intervention (PCI) and stent placement [121]. In addition, clopidogrel is also used in patients after acute coronary syndromes without PCI and in aspirin intolerant patients.

Clopidogrel is a prodrug requiring hepatic biotransformation for pharmacological activity [122]. The active metabolite of clopidogrel (R-130964) contains a thiol group, which binds irreversibly to a free cysteine in the P2Y12 receptor and blocks activation by ADP [50]. In humans, >85 % of clopidogrel is metabolized by esterases to a carboxylic acid metabolite (clopidogrel carboxylate, SR 26334) [33-36], which is considered to be inactive. The rest is converted to the active metabolite (R-130964) in a two-step, CYP-dependent process proceeding via the formation of 2-oxo-clopidogrel. Initial studies suggested that CYP3A4 plays a prominent role in these metabolization steps, with lesser involvement of CYP2C19, CYP2B6, CYP1A2, CYP2C9 [31, 37-39]. A more recent *in vitro* study indicates that CYP2C19 is the most important CYP for the conversion of clopidogrel to 2-oxo-clopidogrel and CYP3A4 for the conversion of 2-oxo-clopidogrel to the active metabolite R-130964 [123].

Studies conducted *in vitro* [37] and *ex vivo* [77, 79] suggested that the CYP3A4 substrate atorvastatin may attenuate the platelet inhibitory effect of clopidogrel due to interference with clopidogrel biotransformation by CYP3A4. These observations resulted in an intense debate about the clinical relevance of drug-drug interactions by lipophilic statins, drugs prescribed frequently to patients ingesting clopidogrel [124]. This controversy remains currently unresolved, since subsequent studies investigating effects of atorvastatin and/or simvastatin on antiplatelet effects of clopidogrel failed to confirm the initial findings [80-85]. More recent *in vivo* studies suggest an important role for both CYP3A4 and CYP2C19 regarding biotransformation and pharmacological activity of clopidogrel. Both concomitant treatment with strong CYP3A4 inhibitors [38, 66, 87] as well as a reduced activity of CYP2C19 in patients with CYP2C19 single nucleotide polymorphisms were associated with an impaired pharmacological activity of clopidogrel [70, 72, 125]. Similar to CYP3A4, CYP2C19 substrates and/or inhibitors impaired biotransformation and/or pharmacological activity of clopidogrel in some [88, 91, 126], but not all, studies [37, 127, 128].

Considering the uncertainties regarding drug interactions with clopidogrel, we undertook the current study to determine concentration-dependent effects of inhibitors and/or substrates of various CYPs on in vitro biotransformation and antiplatelet activity of clopidogrel.

## 3.3. Materials and Methods

### 3.3.1. Materials

Clopidogrel hydrogensulfate was isolated from commercially available tablets (Plavix®, Sanofi Aventis, Geneva, Switzerland) and the carboxylate metabolite of clopidogrel was obtained by saponification of clopidogrel (ReseaChem life science, Burgdorf, Switzerland). The purity was >99% for both substances as assessed by nuclear magnetic resonance spectroscopy (NMR). Atorvastatin was obtained from Sequoia Research Products Ldt. (Pangbourne, UK). NADPH regeneration system, pooled human liver microsomes (HLM; same batch was used for all experiments), recombinant human CYP3A4 supersomes (rhCYP3A4) and rhCYP2C19 were from BD Biosciences Gentest (Woburn, MA, USA). The CYP nomenclature conforms to BJP's Guide to Receptors and Channels [129]. Acetonitrile LiChrosolv for HPLC use was obtained from Merck (Darmstadt, Germany). All other chemicals used were purchased from Sigma or Fluka (Buchs, Switzerland).

### 3.3.2. Kinetic studies of clopidogrel or the carboxylate metabolite of clopidogrel with HLM or rhCYP

The incubation mixture (final volume 250 µl) contained varying concentrations of clopidogrel (5-100 µM), incubation buffer (0.1M potassium phosphate, pH 7.4), reduced glutathione (5 mM), NADPH-regenerating system containing $MgCl_2$ (3.3 mM), $NADP^+$ (1.3 mM), glucose-6-phosphate (3.3 mM) and glucose-6-phosphate dehydrogenase (0.4 U/ml) and either HLM, rhCYP3A4 or rhCYP2C19. Preliminary studies were performed to determine time and protein concentration producing a linear rate. For HLM, 10 min incubation and 0.25 mg/ml protein were selected, and for rhCYP3A4 10 min incubation and 10 pmol P450/ml. The concentrations of the substrates (clopidogrel or clopidogrel carboxylate) are given in the Figures. The final volume of methanol (solvent for clopidogrel) did not exceed 1.0 % of the total incubation volume and was identical in all incubations including controls. Each reaction mixture was equilibrated for 4 min at 37°C in a shaking thermomixer. The reaction was initiated by adding the NADPH-regenerating system and the system incubated for the

respective time at 37°C. Reactions were stopped by addition of 100 µl of chilled acetonitrile (containing 6.5 µM naproxen as internal standard) and cooled on ice for 10 min. Precipitated proteins were removed by centrifugation at 10,000 g for 10 min and supernatants were analyzed by HPLC as described below. The fraction of substrate metabolized was calculated as the difference between the measured and initial clopidogrel or clopidogrel carboxylate concentration expressed as a percentage of the initial concentration.

### 3.3.3. *In vitro* inhibition of clopidogrel metabolism

CYP inhibition studies were performed in the presence of the respective inhibitors or substrates following the same incubation procedure as described for the kinetic experiments. Stock solutions containing inhibitors (see figures) were prepared in methanol or water. The final volume of methanol did not exceed ≤1.0% of the total incubation volume and was identical in all incubations including controls. The inhibitor concentrations are given in the Figures. $IC_{50}$ values were calculated by non-linear regression analysis using the software program GraphPad Prism version 4.00 (San Diego, CA, USA).

### 3.3.4. HPLC analysis of clopidogrel and the clopidogrel carboxylate

Clopidogrel concentrations were determined using a LaChrom® high performance liquid chromatography (HPLC) system equipped with an UV detector operating at a wavelength of 235 nm, a column oven, a quaternary pump and an autosampler. The column temperature was maintained at 32°C and the injection volume was 30 µl. Separation was done on a Nucleosil 50-5-C18 column equipped with a corresponding guard column using a gradient of solvent A (sodium phosphate 0.01 M, pH 3.0: acetonitrile; 50:50 v/v) and solvent B (sodium phosphate 0.01 M, pH 3.0: acetonitrile; 20:80 v/v). The gradient started at 80% A and 20% B for 2.5 min, changed to 100% B for 3.5 min and finally returned to the starting conditions for 4 min. The flow rate was 1 ml/min and the total run time 10 min. The variability of the method was < 10% at high and low clopidogrel concentrations. Calibration curves were performed in a concentration range of 1.0-43.0 µg/ml.

The carboxylate metabolite of clopidogrel was determined using the same HPLC system as described above, but with a different mobile phase. The mobile phase consisted of a gradient of solvent A (sodium phosphate 0.01 M, pH 3.0: acetonitrile; 50:50 v/v), solvent B (sodium phosphate 0.01 M, pH 3.0: acetonitrile; 20:80 v/v) and solvent C (sodium phosphate 0.01 M, pH 3.0: acetonitrile; 80:20 v/v). The gradient started at 20% A and 80% B for 2.5 min, changed to 100% B for 3.5 min and returned to the starting conditions for 4 min. The flow rate was 1 ml/min and the total run time was 10 min. Clopidogrel carboxylate was quantified

by comparison to a standard curve. Variability and calibration curve range were identical to clopidogrel.

### 3.3.5. *Ex vivo* inhibition of platelet aggregation by activated clopidogrel

Isolation of platelet rich plasma (PRP) and platelet aggregation experiments were performed according to Born and Cross [5]. The platelet count in PRP samples was adjusted with platelet-poor plasma (PPP) to 200-250 $x10^9$ platelets per L. Clopidogrel or clopidogrel carboxylate were activated in a mixture containing clopidogrel, HLM (0.25 mg/ml), incubation buffer (0.1 M potassium phosphate, pH 7.4) and NADPH-regenerating system for different periods of time. Inhibitors (dissolved in methanol, concentrations in Figures) were evaporated to dryness at 37°C before addition of the same biotransformation mixture as above containing clopidogrel or clopidogrel carboxylate. In order to test the effect of glutathione (GSH), biotransformation experiments were performed also in the presence of 5 mM GSH. To assess platelet aggregation, 120 µL incubation mixture (activated clopidogrel or clopidogrel metabolite) was added to the same volume of platelets and preincubated at 37°C for 15min. Platelet aggregation was stimulated with ADP (final concentration 2.5 µM) and recorded with an APACT4 aggregometer (LABiTec, Ahrensburg, Germany) as the maximal percentage in light transmittance of the reaction mixture. The percentage of inhibition of platelet aggregation (IPA) was calculated from the observed maximum platelet aggregation (MPA) as follows [38]:

$$IPA(\%) = \frac{(MPA_{baseline} - MPA_{postdose}) \times 100}{MPA_{baseline}}$$

### 3.3.6. Molecular modeling studies

Molecular Modeling was performed on a structure of human cytochrome 3A4 (PDBcode: 1W0G). All calculations were done on a Dell Precision 670 workstation using the program Moloc (www.moloc.ch). Clopidogrel was docked manually into the active site of the enzyme, which was previously modified such that an oxygen atom was placed at the position completing the octahedral geometry of the central $Fe^{2+}$ of the heme. Multiple positions of clopidogrel were tried and subsequently optimized with the force field integrated in Moloc. All atoms of the structure were considered for calculations but only substrate atoms were allowed to move. An analogous procedure was applied for the more hydrophilic clopidogrel carboxylate.

### 3.3.7. Kinetic and statistical analysis

Kinetic parameters of clopidogrel biotransformation were calculated according to the Michaleis-Menten equation using nonlinear regression (GraphPad Prism version 4.00; San Diego, USA):

$$v = \frac{V_{max} \times S}{K_m + S}$$

v and S are biotransformation rate and substrate concentration, respectively, $V_{max}$ the maximal biotransformation rate and $K_m$ the Michaelis-Menten constant. $IC_{50}$ values for inhibitors were calculated by non-linear regression analysis (GraphPad Prism version 4.00; San Diego, USA). *P* values were calculated using one-way analysis of variance (ANOVA) with Dunnet's multiple comparison test for post hoc analysis. Data are presented as mean ± SEM. A *p* value < 0.05 was considered to be significant.

## 3.4. Results

### 3.4.1. *In vitro* metabolism of clopidogrel and the carboxylate metabolite of clopidogrel by HLM and rhCYP

We used human liver microsomes (HLM) and the recombinant human enzymes CYP3A4 (rhCYP3A4) and CYP2C19 (rhCYP2C19) for this purpose. Clopidogrel was metabolized in a concentration-dependent fashion following Michaelis-Menten kinetics by both HLM and rhCYPs. In the presence of HLM, the apparent $K_m$ was 23.1±3.7 µM and $V_{max}$ of 34.1±2.1 nmoles/min/mg protein (Fig. 1A). In the presence of human rhCYP3A4, the corresponding values were of 45.9±12.4 µM ($K_m$) and 0.62±0.09 nmoles/min/pmol P450 ($V_{max}$) (Fig. 1B).

As shown in Fig. 1C, clopidogrel biotransformation by rhCYP2C19 was observed only at clopidogrel concentrations <20 µM, indicating that clopidogrel is an inhibitor of CYP2C19 at higher concentrations. Its conversion rate at 10 µM clopidogrel was estimated to be 0.07 nmoles/min/pmol P450, a value approximately 10 times lower than the $V_{max}$ obtained for rhCYP3A4.

To the best of our knowledge, there are no published data regarding possible biotransformation of clopidogrel carboxylate, the main metabolite of clopidogrel [33, 36]. In none of the two in vitro systems tested, concentrations of clopidogrel carboxylate decreased measurably during incubation, indicating that clopidogrel carboxylate is metabolized neither by HLM (Fig. 1D), nor by rhCYP3A4 (data not shown).

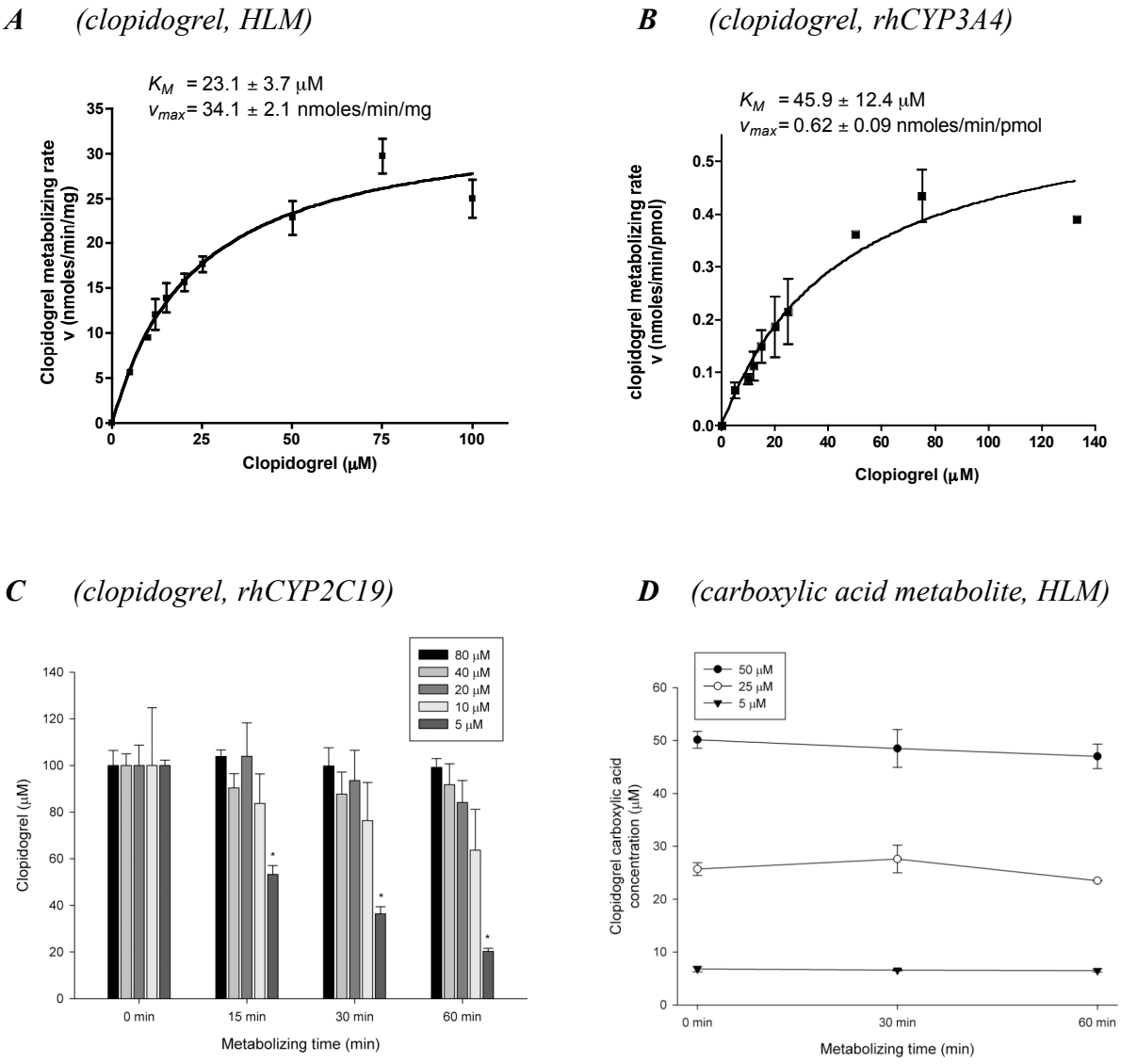


**Figure 1**

*Biotransformation of clopidogrel and its carboxylate metabolite*. Kinetic parameters for clopidogrel metabolism were determined in the presence of human liver microsomes (HLM) or supersomes containing (rhCYP3A4 or rhCYP2C19). Where possible, data were described using the Michaelis-Menten model. Increasing concentrations of clopidogrel ***(A, B, C)*** or of its carboxylate derivative ***(D)*** were incubated with HLM, rhCYP3A4 or rhCYP2C19 and analyzed by HPLC. The biotransformation of clopidogrel in the presence of CYP3A4 showed a clear saturation and could be described by Michaelis-Menten kinetics ***(A,B)***. CYP2C19 activates clopidogrel only at concentrations <20 μM ***(C)***. The carboxylate metabolite of clopidogrel is not metabolized by HLM ***(D)***.

### 3.4.2. Effect of specific CYP inhibitors and CYP substrates on clopidogrel biotransformation

The inhibition of clopidogrel (40 μM) metabolism by various CYP inhibitors and substrates was investigated using HLM (Fig. 2). The oxidation of clopidogrel was significantly impaired by the CYP3A4 inhibitors ketoconazole and clarithromycin already at concentrations in the nanomolar range. Ciprofloxacin, a strong inhibitor of CYP1A2 and a weak inhibitor of CYP3A4 [130], reduced clopidogrel oxidation by 35% at 500 μM, but not at lower concentrations. In contrast, inhibitors of CYP2C9 (sulfaphenazole), CYP2D6 (quinidine), CYP2B6 (N,N′,N″-triethylenethiophosphoramide, thioTEPA) and CYP2C19 (omeprazole) revealed no significant effect on clopidogrel biotransformation by HLM. For amiodarone, we found no significant inhibition of clopidogrel biotransformation in a concentration range of 1 to 100 μM (data only partially shown).

Due to the importance of CYP2C19 for clopidogrel biotransformation [70, 72, 125], CYP2C19 inhibitors were investigated in more detail (Fig. 3). At a clopidogrel concentration of 40 μM, neither the proton pump inhibitors (PPIs) omeprazole (up to 100μM) and lansoprazole (up to 100 μM), nor ticlopidine, inhibited clopidogrel biotransformation by HLM. In contrast, at 5 μM clopidogrel, lansoprazole affected clopidogrel biotransformation in a concentration-dependent manner, reaching significance at 100 μM.

Since atorvastatin has been suspected to impair clopidogrel biotransformation [77], we investigated the impact of the CYP3A4 substrates atorvastatin and simvastatin on clopidogrel biotransformation by HLM. Both statins significantly inhibited clopidogrel biotransformation at the maximal concentration tested (10 μM). In contrast, pravastatin (no CYP3A4 substrate) showed no inhibitory effect at this concentration (Fig. 4).

Inhibition of clopidogrel biotransformation by CYP inhibitors or substrates was confirmed by the determination of the corresponding $IC_{50}$ values (Table 1). Ketoconazole showed a slightly stronger inhibitory effect ($IC_{50}$ 0.03 μM) than clarithromycin ($IC_{50}$ 0.33 μM). An $IC_{50}$ for ciprofloxacin could not be determined, since 50% inhibition were not reached up to 500 μM. Simvastatin and atorvastatin both revealed dose-dependent inhibition of clopidogrel biotransformation with $IC_{50}$ values of 1.28 μM and 16.9 μM, respectively.

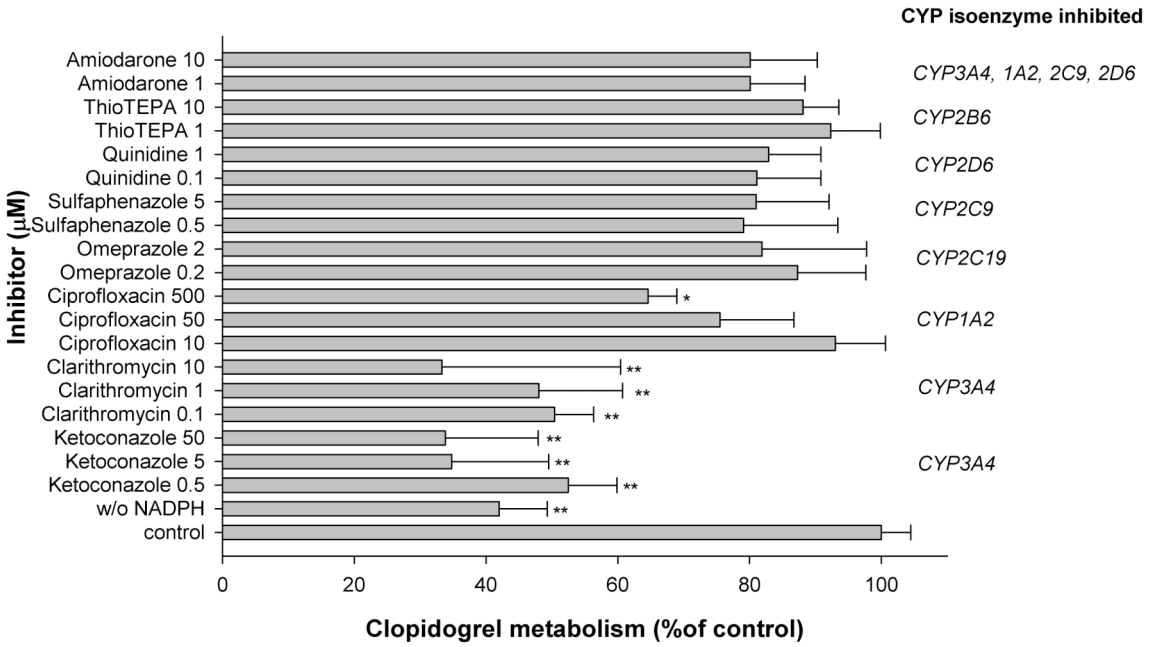


**Figure 2**

*Effect of various CYP450 inhibitors on clopidogrel biotransformation*. Clopidogrel (40 µM) was co-incubated in the presence of human liver microsomes (HLM) with different concentrations of CYP inhibitors. The CYP isoenzymes affected by the respective inhibitor are indicated in *italic*. Data are expressed as the percentage of clopidogrel activated in the presence of the inhibitor compared to biotransformation without inhibitor (100%). Data points consist of five individual determinations. Data are presented as mean±SEM. **P*<0.05, ***p*<0.001 versus control incubations.

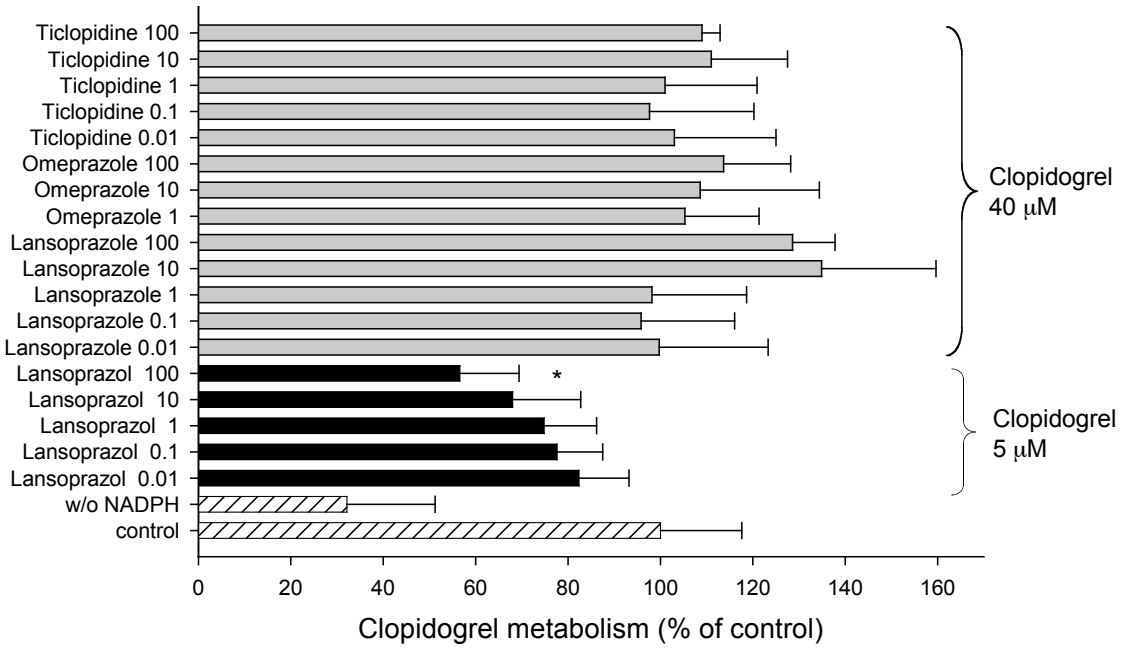


**Figure 3**

*Effect of CYP2C19 inhibitors on clopidogrel biotransformation.* Clopidogrel (40 or 5 μM) was co-incubated with different concentrations of CYP2C19 inhibitors in the presence of human liver microsomes (HLM). Data are expressed as the percentage clopidogrel biotransformation in the presence of the inhibitor compared to biotransformation without inhibitor (100%). Data points consist of three individual determinations. Data are presented as mean±SEM. ***P*<0.001 versus control incubations.

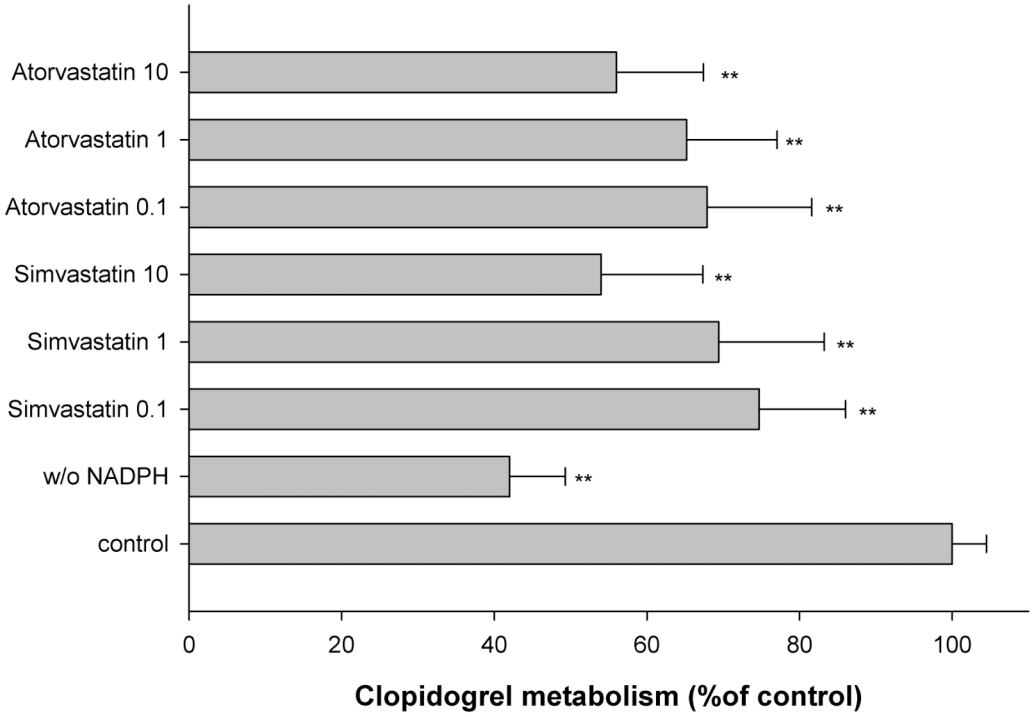


**Figure 4**

*Effect of statins on clopidogrel biotransformation*. Clopidogrel (40 μM) was co-incubated with different concentrations of statins in the presence of human liver microsomes (HLM). Data are expressed as the percentage clopidogrel biotransformation compared to the biotransformation without inhibitor (100%). Data points consist of five individual determinations. Data are presented as mean±SEM. **P*<0.05, ***P* <0.001 versus control incubations.

### 3.4.3. Inhibition of platelet aggregation by activated clopidogrel

Further, we developed a test system for analyzing platelet aggregation in incubations containing HLM, the drugs investigated and human platelets (Fig. 5A). Clopidogrel inhibited platelet aggregation concentration-dependently, reaching 67% at 200 µM. In contrast, clopidogrel without biotransformation by HLM showed no significant inhibition of platelet aggregation. To demonstrate the formation of an active metabolite, we investigated the antiplatelet effect of clopidogrel in the presence of 5 mM glutathione. Since glutathione is known to affect formation of and breaking of disulfide bonds in cells [131], we hypothesized that it could trap the newly formed thiol group of activated clopidogrel [50]. As expected, addition of glutathione significantly decreased the effect of clopidogrel on platelet aggregation (Fig. 5B).

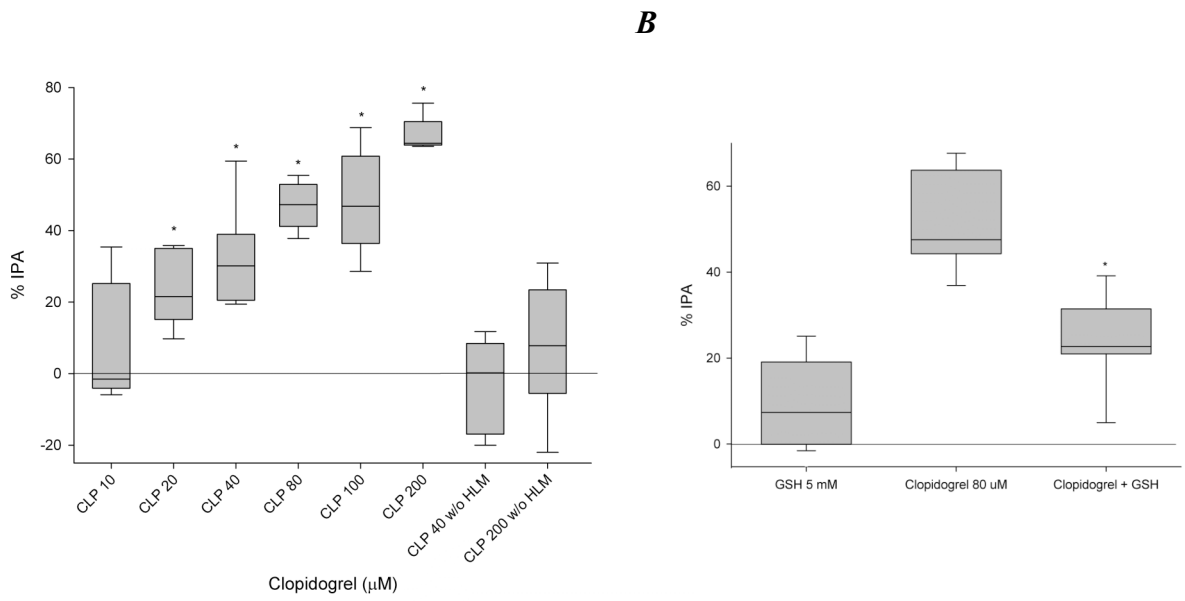


**Figure 5**

*Effect of activated clopidogrel on platelet aggregation*. ***A***: Increasing concentrations of clopidogrel (Clp; 10-200 µM) activated by human liver microsomes (HLM) were incubated with platelet rich plasma (PRP). Platelet aggregation was determined in response to 2.5 µM ADP by light transmittance aggregometry. Clopidogrel incubated in the absence of HLM served as a negative control. ***B***: In the presence of 5 mM glutathione (GSH), the effect of clopidogrel on platelet aggregation was significantly decreased. GSH itself had no effect on platelet aggregation. Results are expressed as percentage inhibition of platelet aggregation (IPA), calculated from the maximum platelet aggregation in the presence of the solvent (1% methanol). Data are presented as box-plots with the median indicated by the line within the box (n = 8 to 12). **P*<0.001 versus (*A*) Clp 40 µM w/o HLM or (*B*) clopidogrel 80 µM.

### 3.4.5. Interaction of clopidogrel with CYP3A4

In order to investigate the reason for the different binding affinity of neutral clopidogrel and its much more hydrophilic carboxylate derivative to CYP3A4, we manually docked both compounds into the active site of CYP3A4. As shown in Fig. 6B, the neutral and hydrophobic clopidogrel fits smoothly into the hydrophobic active site of CYP3A4, which is optimized to recognize and bind hydrophobic substances. In contrast, the slightly smaller carboxylate metabolite contains a polar and solvated carboxylate functionality that does not bind in a productive way to the hydrophobic catalytic site of CYP3A4 (not shown).

***A*** ***B***

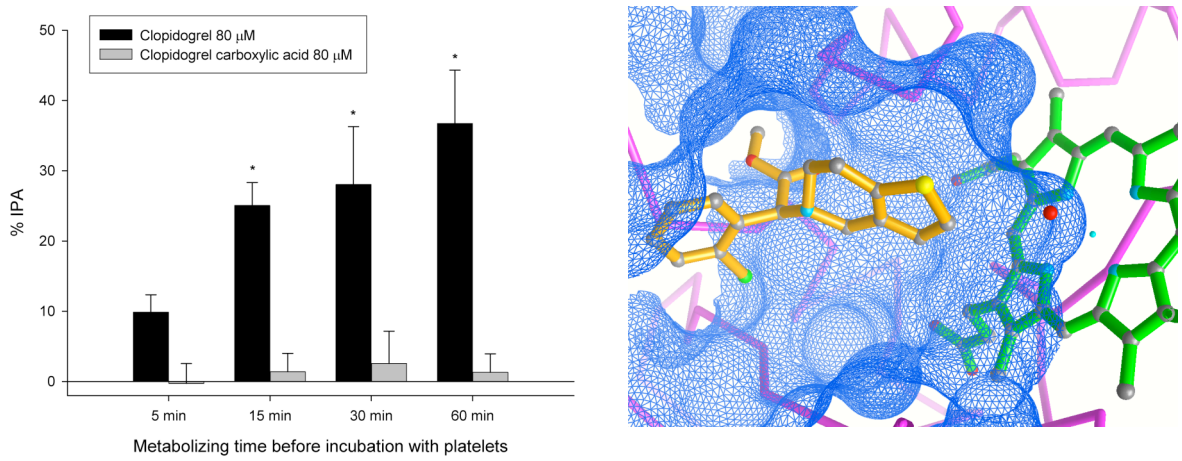


**Figure 6**

*Time dependency of the effect of clopidogrel or clopidogrel carboxylic acid (80 µM) on platelet aggregation and interaction of clopidogrel with CYP3A4.* ***A:*** Clopidogrel or clopidogrel carboxylate were incubated with human liver microsomes (HLM) for 5, 15, 30 or 60 min. At the indicated time points, aliquots were incubated with platelet-rich plasma and platelet aggregation determined in response to 2.5 µM ADP. Clopidogrel inhibits platelet biotransformation time-dependently whereas the carboxylate derivative does not affect platelet aggregation. Results are expressed as the percentage inhibition of platelet aggregation (% IPA), calculated from the maximum platelet aggregation obtained in the presence of the vehicle (1% methanol v/v). Data are presented as mean±SEM (n= 4). **P*<0.05, ** $p$ <0.01 clopidogrel or clopidogrel carboxylate vs. incubations containing no clopidogrel (not shown). ***B***: Clopidogrel (orange) interacts with the active site (blue net) of CYP3A4 (backbone, magenta). A heme molecule with the $Fe^{2+}$ (cyan dot in center of heme) is shown in green. Note that the activated oxygen (red ball) above the $Fe^{2+}$ of the heme is placed in an ideal position to interact with the 2-carbon of clopidogrel (arrow). In contrast, the clopidogrel carboxylate (not shown) carries a polar and solvated carboxylate group preventing it from bringing the 2-carbon close enough to the activated oxygen and the $Fe^{2+}$ of the heme, which are crucial conditions for its subsequent biotransformation.

### 3.4.6. Effect of CYP3A4 inhibitors and/or substrates on clopidogrel-associated IPA

Finally, we addressed the question whether an inhibition of clopidogrel biotransformation by CYP3A4 inhibitors or substrates is associated with an inhibition of platelet aggregation. As shown in Table 1, ketoconazole turned out to be the most potent inhibitor with an $IC_{50}$ of 0.55 μM, confirming the results obtained in the biotransformation experiments. The inhibitory effect of clarithromycin was comparable ($IC_{50}$ 0.95 μM), whereas the statins were less effective inhibitors.

**Table 1**

$IC_{50}$ (μM) values for inhibition of clopidogrel biotransformation by human liver microsomes (HLM) and for the antiplatelet effect of clopidogrel in the presence of CYP3A4 inhibitors or substrates. The assay conditions are described Methods. The clopidogrel concentration was 40 μM for all incubations. Data are expressed as mean±SEM of n=4-8 experiments.

| | **$IC_{50}$ for biotransformation by HLM (μM)** | **$IC_{50}$ for antiplatelet effect (μM)** |
|---|---|---|
| Ketoconazole | 0.03 ± 0.07 | 0.55 ± 0.06 |
| Clarithromycin | 0.33 ± 0.09 | 0.95 ± 0.04 |
| Simvastatin | 1.28 ± 0.09 | 1.29 ± 0.02 |
| Atorvastatin | 16.9 ± 0.3 | 3.83 ± 0.07 |

### 3.5. Discussion

In our studies, clopidogrel was metabolized in a concentration-dependent manner in all incubations containing CYP3A4 (Fig. 1A,B), whereas supersomes containing rhCYP2C19 metabolized by clopidogrel only at substrate concentrations ≤10 µM. Regarding the inhibition of CYP2C19 by clopidogrel, our data are in accordance with recent studies [44, 132], showing that clopidogrel is a mechanism-based inhibitor of CYP2C19 with an $IC_{50}$ in the low micromolar range.

Clopidogrel is rapidly and efficiently absorbed from the GI tract [33], but more than 85% of the drug is converted to its carboxylate metabolite during the first passage across intestine and liver [36]. Assuming that the maximal concentration of the carboxylate derivative of clopidogrel in plasma approximately corresponds to the maximal clopidogrel concentration in the liver (no data on the clopidogrel concentration in the liver are available), a maximal concentration of 5-20 µmol/L is reached in hepatocytes after ingestion of 75 mg clopidogrel [33, 36]. Taking into account that clopidogrel inhibits CYP2C19 at concentrations >10 µM without affecting CYP3A4 and that, after oral ingestion of 75 mg, the hepatocellular clopidogrel concentration will drop below 10 µM with time, it can be expected that *in vivo* both CYP3A4 and CYP2C19 contribute to clopidogrel biotransformation. These considerations therefore help to explain why both, strong inhibitors of CYP3A4 [38, 66, 87] and genetic variants of CYP2C19 [70, 72, 125], are associated with impaired antiplatelet activity of clopidogrel.

In contrast to the recent study of Kazui et al. [123], in the current study, clopidogrel was biotransformed not only by HLM, but also by rhCYP3A4, indicating that CYP3A4 can also perform the conversion of clopidogrel to 2-oxo-clopidogrel. While the discrepancy is scientifically interesting and should be resolved by further studies, its clinical impact is minimal since many CYPs are involved in the biotransformation of clopidogrel [31, 37-39, 123].

The good correlation between the inhibition of clopidogrel biotransformation and the antiplatelet effect by clopidogrel suggests that our systems are able to predict drug interactions with clopidogrel. In our study, ketoconazole, a potent inhibitor of CYP3A4, showed the strongest inhibitory effect on clopidogrel biotransformation and inhibition of platelet aggregation. These data are in good agreement with a clinical study demonstrating that ketoconazole not only decreases clopidogrel biotransformation but also its antiplatelet activity [38].

Amiodarone is a drug prescribed often with clopidogrel and possibly interferes with its biotransformation [78]. It is mainly metabolized by CYP3A4 in humans and is an inhibitor of CYP2C9, CYP2D6 and CYP3A4 [133]. Surprisingly, amiodarone did not affect clopidogrel

biotransformation in our *in vitro* system up to concentrations of 100 µM. This finding may be explained by the fact that the *in vivo* generated desethylamiodarone, the major metabolite of amiodarone, is a more potent inhibitor of human CYPs than amiodarone itself [133]. A prolonged incubation time would possibly have been necessary to generated this metabolite and detect inhibitory effects of amiodarone in our system.

Ciprofloxacin, a well known CYP1A2 inhibitor, significantly inhibited clopidogrel biotransformation at a concentration of 500 µM, but not at lower concentrations. In the studies demonstrating inhibition of CYP1A2, ciprofloxacin concentrations in the range of 10-200 µM were used [134, 135]. On the other hand, McLellan et al. [130] reported that ciprofloxacin significantly decreases the activity of CYP3A4 when used at high concentrations (~2 mM), which is in accordance with our findings.

Our investigations with statins are in good agreement with results presented by Lau et al.[77, 79] and by Clarke and Waskell [37]. Atorvastatin and simvastatin are both metabolized by CYP3A4 and significantly inhibited clopidogrel biotransformation *in vitro*, whereas pravastatin showed no such inhibitory effect (Figure 4). Additionally, we could demonstrate that the inhibitory effect of atorvastatin and simvastatin on clopidogrel biotransformation resulted in an impaired antiplatelet effect of clopidogrel (Table 1). In agreement with our findings, Lau et al. demonstrated in their *ex vivo* study a dose-dependent attenuation of the clopidogrel-associated antiplatelet effects by atorvastatin [77]. The results of another small *ex vivo* analysis using simvastatin confirmed the occurrence of a clopidogrel-statin interaction [79]. In contrast, other studies investigating the influence of CYP3A4-metabolized statins on antiplatelet effects of clopidogrel failed to confirm these findings [80-85]. Taking into account our findings, the exact time-points when clopidogrel and reversible CYP inhibitors such as statins are ingested may play a crucial role for the occurrence and possible manifestation of drug-drug interactions. Similar to the data reported by Clarke and Waskel [37], the CYP2C19 inhibitors omeprazole, lansoprazole and ticlopidine did not influence clopidogrel metabolism in our *in vitro* system at clopidogrel concentrations ≥20 µM. Our data are also in agreement with a recent clinical study by Siller-Matula et al.[128] and with the reanalysis of the TRITON-TIMI 38 trial [127], both showing no significant effect of different proton pump inhibitors (PPIs) on the antiplatelet effect of clopidogrel. However, they are in disagreement with other clinical [88] or epidemiological studies [91, 126] indicating that PPIs may interfere with CYP2C19 activities and impair the antiplatelet effect of clopidogrel. As shown in Figure 3, PPIs such as lansoprazole can inhibit clopidogrel biotransformation if the clopidogrel concentration is sufficiently low. As discussed above, in the liver of patients treated with clopidogrel, the clopidogrel concentration can be assumed to drop to levels at which inhibition of clopidogrel

biotransformation by PPIs become potentially significant. PPIs with a strong inhibition of CYP2C19 such as lansoprazole, omeprazole, esomeprazole and rabeprazole [90] should therefore best be avoided in patients treated with clopidogrel, especially when they are treated also with CYP3A4 inhibitors.

In conclusion, we could demonstrate that CYP3A4 is the most important CYP isoenzyme for clopidogrel biotransformation at clopidogrel concentrations >10 μM, since clopidogrel inhibits CYP2C19 at high concentrations. At concentrations ≤10 μM, CYP2C19 starts to contribute to clopidogrel biotransformation and the clopidogrel biotransformation can be inhibited by PPIs. The concentration-dependent interaction pattern between CYP inhibitors, clopidogrel and CYP3A4 and CYP2C19 helps to explain the often diverging results regarding clopidogrel biotransformation and pharmacological activity between studies conducted *in vitro* and *in vivo*.

# 4. Hepatotoxicity of clopidogrel

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## 4.1. Abstract

Clopidogrel has largely replaced ticlopidine due to its superior safety profile and faster antiplatelet effect. However, rare but serious complications such as hepatotoxicity have been reported for clopidogrel lately. The mechanism of clopidogrel associated liver toxicity is still unclear. Activation of clopidogrel generates an active metabolite carrying a mercapto group, which may be responsible not only for the therapeutic effect, but also for the toxicity of clopidogrel. The current study investigates whether the reactive metabolite of clopidogrel is responsible for the observed hepatotoxicity and analyzes the corresponding mechanism. Clopidogrel (100 µM) showed cytotoxicity in experiments using the hepatocyte *HepG2* cell line that overexpress human CYP3A4 (herein referred to as *CYP3A4 cells*) or in co-incubation with CYP3A4 supersomes (referred to as *3A4 supersomes*). In contrast, wild type *HepG2* cells (referred to as *HepG2 wt cells)* or *HepG2 wt cells* in combination with control supersomes (referred to as *control supersomes*) were not affected. Accordingly, co-incubation with 1 µM ketoconazole, a CYP3A4 inhibitor, attenuated the toxic effect of clopidogrel in *CYP3A4 cells* and *3A4 supersomes*, indicating that the generated reactive metabolite is responsible for the observed toxicity. Activated clopidogrel (100 µM) triggered an oxidative stress reaction, leading to a decrease in the intracellular glutathione content. Furthermore, activated clopidogrel is inducing mitochondrial damage and cytochrome c release, which promotes apoptosis in *CYP3A4 cells*. The carboxylate metabolite of clopidogrel, which is the major metabolite of clopidogrel in the plasma, does not show a toxic effect in *CYP3A4 cells*, *3A4 supersomes* or in *HepG2 wt cells* or *control supersomes*. In contrast to clopidogrel, ticlopidine was not activated by *CYP3A4 cells* and therefore revealed no toxicity under our experimental conditions.

In conclusion, this study identifies the reactive metabolite of clopidogrel to be responsible for clopidogrel-induced toxicity in *CYP3A4 cells* and *3A4 supersomes*. The induction of an oxidative stress reaction, which promotes apoptosis by a mitochondrial-dependent pathway represents an important mechanism for hepatotoxicity associated with clopidogrel.

## 4.2. Introduction

In patients who undergo percutaneous coronary intervention or are suffering from acute coronary syndromes, clopidogrel plus aspirin is the first-line antiplatelet therapy to reduce cardiovascular events [54-56]. Clopidogrel, like ticlopidine, is a thienopyridine derivative that irreversibly inhibits platelet aggregation by selectively binding to the adenosine-5'-diphosphate (ADP) $P2Y_{12}$ receptor on the platelet surface. After oral administration, clopidogrel is rapidly absorbed and is metabolically activated by the cytochrome P450 (CYP) system [37, 38, 43, 78]. Clopidogrel has largely replaced ticlopidine because of its superior safety profile and more efficient antiplatelet effects [23, 136]. Common adverse effects associated with clopidogrel include gastrointestinal disorders (dyspepsia, nausea, vomiting), bleeding, rash and diarrhea [20, 25, 30]. In addition, more serious adverse reactions such as thrombotic thrombocytopenic purpura [111], pancytopenia [112], systemic inflammatory response syndrome [100], serum sickness-like reaction [113] and hepatotoxicity [100-110] have been observed in recent years. Until now, thirteen patients with clopidogrel-associated hepatotoxicity [100-110] have been reported. In these patients, the time between start of clopidogrel therapy and appearance of hepatic injury ranged from 4 days to 6 months. The pathomechanism of clopidogrel associated liver injury is still unclear. Recent publications argue that clopidorel can cause similar liver injury like ticlopidine [114-118]. Clinically, the type of liver injuries associated with clopidogrel are either cholestatic or mixed hepatocellular and cholestatic. Hypersensitivity and direct toxicity have been suggested as responsible mechanisms.

Activation of clopidogrel generates an active metabolite carrying a mercapto group, which covalently binds to the platelet's $P2Y_{12}$-receptor [22, 31]. Most likely, this active metabolite interacts with other cellular proteins such as glutathione. The production of reactive metabolites in the liver may exert a direct toxic effect by functional modification of the target or by immunological mechanisms [99].

We performed this study to investigate the hepatotoxic potential of clopidogrel and its carboxylate metabolite in a hepatocyte cell line. We used two systems to assess the toxicity of activated clopidogrel: *HepG2* cells overexpressing human CYP3A4 (*CYP3A4 cells*) and HepG2 cells in combination with CYP3A4 supersomes (*3A4 supersomes*). Additionally, we also investigated cytotoxicity of ticlopidine using the same cellular systems as for clopidogrel.

## 4.3. Materials and Methods

### 4.3.1. Materials

Clopidogrel hydrogensulfate was isolated from commercially available tablets (Plavix®, Sanofi Aventis, Geneva, Switzerland) and the carboxylate metabolite of clopidogrel was obtained by saponification (ReseaChem life science, Burgdorf, Switzerland). The purity was >99% for both substances as assessed by NMR spectroscopy. Human CYP3A4 supersomes (with supplementation of cytochrome $b_5$ and cytochrome P450 reductase) and insect cell control supersomes were from BD Gentest (Woburn, MA, USA). Cell culture supplements were purchased from GIBCO (Paisley, UK). Cell culture plates were purchased form BD Bioscience (Franklin Lakes, NJ). NADPH was ordered from Sigma-Aldrich (Switzerland) and the ToxiLight® BioAssay Kit from Cambrex Bio Science Rockland Inc. (USA). Acetonitrile LiChrosolv® for HPLC use was obtained from Merck (Darmstadt, Germany). All other chemicals used were purchased from Sigma or Fluka (Buchs, Switzerland).

### 4.3.2. Cell lines and cell culture

The hepatoma cell line HepG2 was provided by Professor Dietrich von Schweinitz (University Hospital Basel, Switzerland). HepG2 cells stably transduced with human CYP3A4 (*CYP3A4 cells*) were prepared as described (chapter 5.4.1 & 5.4.2). *HepG2 wt cells* and *CYP3A4 cells* were cultured in Dulbecco's modified Eagle's medium (DMEM; with 2mmol/L GlutaMAX®, 1.0g/L glucose and sodium bicarbonate) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 10mM HEPES buffer, pH 7.2 and non-essential amino acids. The culture conditions were 5% $CO_2$ and 95% air atmosphere at 37°C.

### 4.3.3. Treatment of CYP3A4 cells and HepG2 wt cells

*HepG2 wt cells* and *CYP3A4 cells* were passaged at 80-85% confluency, using trypsin. 50'000 cells/well were allowed to adhere overnight in 96-well culture plates. Stock solutions of test compounds (clopidogrel, ticlopidine, the carboxylate metabolite of clopidogrel, ketoconazole, glutathione and diethyl-maleate) were prepared in DMSO or water. The reaction volume was 200 µl and DMSO concentrations never exceeded 0.2%. Cells were incubated with 0.1% Triton X as positive control and 0.2% DMSO as negative control. Drug treatment was performed for 24 h at 37°C and 5% $CO_2$.

### 4.3.4. Treatment of 3A4 supersomes and control supersomes

Human CYP3A4 supersomes™ (referred to as *3A4 supersomes)* and insect cell control supersomes™ (referred to as *control supersomes)* were used to activate clopidogrel or ticlopidine. Cells were passaged and prepared for drug treatment similar as for the *3A4 cells* and *HepG2 wt cells*. Test compounds were supplied to the cells in the presence of 20 pmol/ml CYP3A4 supersomes or control supersomes and 1 mM NADPH.

### 4.3.5. Cytotoxicity assay (Adenylate kinase release)

The loss of cell membrane integrity resultsis reflected in the release of adenylate kinase (AK), which can be detected using the firefly luciferase system (ToxiLight® BioAssay Kit, Cambrex Bio Science, Rockland, ME). After 24 h of incubation, 100µl assay buffer was supplied to 20 µL supernatant from drug-treated cells in the presence or absence of ketoconazole (concentrations indicated in the Figures) and luminescence was measured after 5 minutes.

### 4.3.6. Quantification of clopidogrel metabolism using HPLC

450'000 *HepG2 wt* or *CYP3A4 cells/well* were seeded in 24-well plates and allowed to adhere overnight. Supersomes were used as mentioned above. Following the incubation with clopidogrel (10, 50 and 100 µM) for different periods of time (0, 6,12 and 24 h), 120 µl acetonitrile containing internal standard (6.5 µM naproxen) was added and cells were detached with a cell scraper. Cells were lysed by freeze-thaw cycles and the cleared lysate was subjected to HPLC analysis.

We used a LaChrom® system HPLC equipped with an UV 7400 detector operating at a wavelength of 235 nm, a 7360 column oven, a 7100 quaternary pump and a 7200 autosampler. The column temperature was maintained at 32°C and the injection volume was 30 µl. Separation was performed on a Nucleosil 50-5-C18 column equipped with the corresponding guard column with a mixture of sodium phosphate (0.01 M, pH 3.0) and acetonitrile (50:50 v/v, solvent A) and sodium phosphate (0.01 M, pH 3.0) and acetonitrile (20:80 v/v, solvent B). The gradient was starting with 80% solvent A and 20% solvent B for 2 min, then 100% solvent B for 4 min followed by equilibration with 80% solvent A and 20% solvent B for 4 min. The flow rate was 1 ml/min and the run time 10 min. The variability of the method was <10% at high and low clopidogrel, the carboxylate metabolite of clopidogrel or ticlopidine concentrations. Calibration curves were performed at a concentration range of 1.0-43.0 µg/ml. There were no interfering peaks using cell culture medium. The clopidogrel concentration was determined by comparison to a standard curve. The amount of clopidogrel

metabolized after 6,12 and 24 h was calculated by substracting the remaining clopidogrel from the initial amount (zero time point) present in the incubations.

### 4.3.7. Determination of intracellular GSH and GSSG after drug treatment

Determination of glutathione (GSH) and oxidized glutathione (GSSG) was performed using the enzymatic recycling assay of Tietze [137], with the modifications described by Griffith et al [138] as follows: cells were co-incubated with clopidogrel, ticlopidine or the carboxylate metabolite of clopidogrel for 24 h. Diethyl-maleate (DEM), a glutathione depleter, and $H_2O_2$ (0.3%) served as positive controls. After incubation cells were detached, suspended in 1 ml 1mmol/l bathophenanthrolinedisulfonic acid in 10% perchloric acid and sonicated for 30 sec (sonicator from Heat Systems Ultrasonics Inc., Farmingdale, NY, USA, setting 4.5). After centrifugation, the pellet was used for protein determination (BCA protein assay kit, Pierce, Rockford, IL, USA) and the supernatant for the determination of glutathione. Total glutathione was determined by lowering the pH with triethanolamine. For the determination of GSSG, derivatization with 2-vinylpyridine was performed prior to pH adjustment. For the enzymatic reaction, NADPH (0.21 mmol/l), DTNB (0.6 mmol/l), glutathione reductase (1 U/ml) and were mixed with the sample or standard, respectively. The formation of TNB was measured at 412 nm using a plate reader and normalized to the standard curve.

### 4.3.8. Measurement of reactive oxygen species (ROS)

A fluorescence-based microplate assay [139] was used for the evaluation of oxidative stress in *CYP3A4 cells* and *HepG2 wt cells* treated with the test compounds. 2',7'-dichlorofluorescein-diacetate (DCFH-DA) is a membrane-permeable, nonpolar, and nonionic molecule. DCFH-DA is hydrolyzed in the cytoplasm by intracellular esterases to nonfluorescent DCFH, which can be oxidized to fluorescent dichlorofluorescein (DCF) in the presence of reactive oxygen species (ROS). Cells were simultaneously exposed to test compounds and to DCFH-DA (5 µM) and incubated for 6, 12 and 24 h. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microtiter plate reader (HTS 700 Plus Bio Assay Reader; PerkinElmer, Beaconsfield, Buckinghamshire, UK) in incubations containing cells and exposure medium.

### 4.3.9. Cytochrome c release detection by fluorescence microscopy

Microscop cover glasses (diameter 13mm; thickness 0.17mm) were activated with (3-aminopropyl) triethoxy-silane („Tespa"; Sigma No A3648) in acetone for 5min. 100'000 *HepG2 wt* or *CYP3A4 cells/well* were seeded in 24-well plates and allowed to adhere on

supplied microscopy cover glasses overnight. 100 µM of clopidogrel (Clp), the carboxylate metabolite of clopidogrel (Carboxy-Clp) and ticlopidine (Tcp) were added to the cells for 12 h. 100 µM benzbromarone served as positive control (data not shown) and 0.2% DMSO (DMSO ctrl) as negative control. Cells were stained with 50 nM Mitotracker Red CMXRos (Invitrogen No. M7512) for 30 min at 37°C and crosslinked with 4% formaldehyde (in PBS; Polysciences Inc.) for 15 min at 37°C. Permeabilization was performed with 0.2% TritonX-100 followed by blocking with 5% BSA (Sigma No. A2153). Immuno staining was performed using a biotinylated Cytochrome C antibody (BioLegend No. 612303) at 1/20 for 45 min in PBS 5% BSA.1/200 Streptavidin-Alexa647 (Molecular Probes No. S32354) plus 1/1'000 DAPI (Invitrogen No. D3571) was applied in PBS 2.5% BSA for 20min. Subsequently, cells were mounted on Superfrost glass slides (Thermo Scientific) using the FluorSave reagent (Calbiochem No. 345789). An Olympus IX81 inverted microscope was used for this analysis in combination with the CellR software (Olympus). Images were exported as 16-bit TIFF files. ImageJ software (http://rsbweb.nih.gov/ij/) was used to convert the images to 8-bit and create merged images. Photoshop was used to trim images and adjust the brightness.

### 4.3.10. Detection of apoptosis and necrosis using flow cytometry

200'000 cells/well were seeded in 24-well plates and allowed to adhere for 24 h before drug treatment. Drug treatment was performed for 24 h in the presence or absence of 1 µM ketoconazole (KCZ). *CYP3A4 cells* and *HepG2 wt cells* were stained for 15 min with 1/20 AnnexinV-PE (BD No. 556422) and 1/200 PI in a volume of 50 µl AnnexinV-binding buffer (10 mM Hepes, 150 mM NaCl, 2.5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$ in $H_2O$). Flow cytometry was carried out on a DAKO Cyan cytometer. Benzbromarone (100 µM) [140] and deoxycholic acid (200 µM) [141] were used as positive controls.

### 4.3.11. Statistical analysis

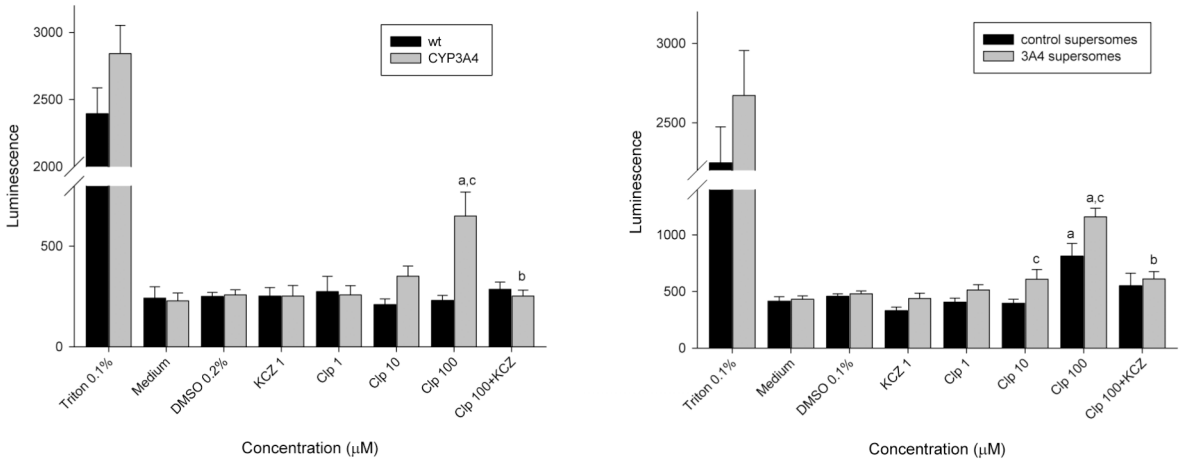
Data are presented as mean values±SEM of at least three experiments. Statistical analyses were performed using Sigma Stat release 3.5 (SYSTAT Software, Inc.). Differences between many groups at two levels were tested by two-way analysis of variance (ANOVA) followed by Dunnet's post hoc test if the data were normally distributed. In the case of not normally distributed data, Holm-Sidak statistics was performed. Differences between groups (e.g. control versus test compound incubations in *CYP3A4 cells*) was tested by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test if ANOVA showed significant differences. A $P$-value < 0.05 was considered as significant.

## 4.4. Results

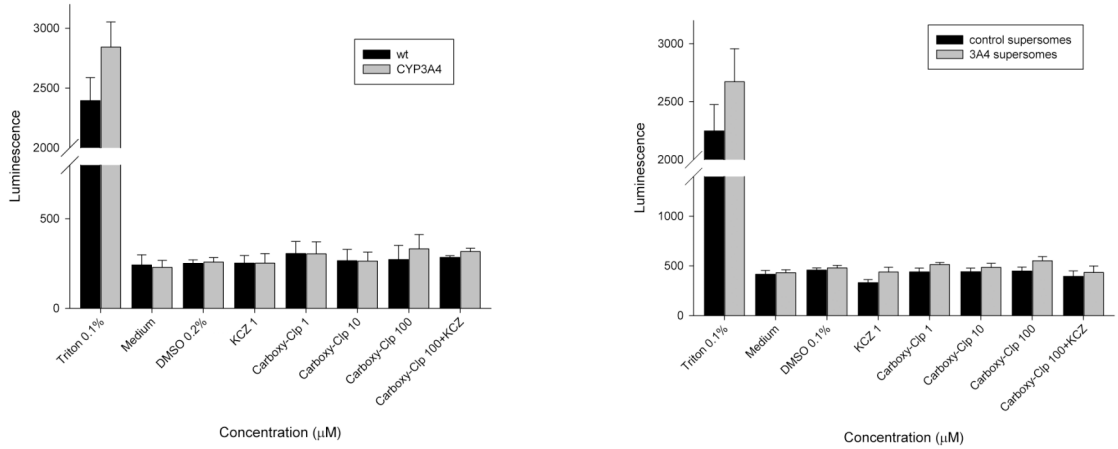
### 4.4.1. Cytotoxicity of clopidogrel in CYP3A4 cells and 3A4 supersomes

To study the cytotoxicity of clopidogrel we used *CYP3A4 overexpressing cells* and *3A4 supersomes* as previously described (chapter 5.4.1 & 5.4.2). This system was chosen since activation of clopidogrel is predominantly performed by human CYP3A4 [37, 78] (chapter 1.3.1). The toxicity of clopidogrel, the carboxylate metabolite of clopidogrel and ticlopidine was assessed using the ToxiLight® assay as described in methods. Clopidogrel showed dose-dependent toxicity in *CYP3A4 cells* (compared to *HepG2 wt cells*) and in *3A4 supersomes* (compared to *control supersomes*), confirming the generation of toxic metabolites by CYP3A4 after 24 h (Fig. 1A). To confirm the CYP3A4-dependent formation of toxic metabolites, clopidogrel (100 µM) was co-incubated with 1µM ketoconazole, a well characterized CYP3A4 inhibitor. Indeed, the clopidogrel induced cytotoxicity in *CYP3A4 cells* and *3A4 supersomes* could be prevented by ketoconazole. We also tested the carboxylate metabolite of clopidogrel, which is representing more than 85% of the circulating drug-related compound in plasma [33-36]. The carboxylate metabolite of clopidogrel showed no toxic effect (Fig. 1B) in both activating systems (*CYP3A4 cells, 3A4 supersomes*) and control systems (*HepG2 wt, control supersomes*) tested. The cellular toxicity was further assessed with ticlopidine (Tcp). As depicted in Fig. 1C, ticlopidine exhibited a significant toxic effect in *CYP3A4 cells* at 100 µM. However, ketoconazole could not attenuate this effect, suggesting that CYP3A4 is not the only responsible CYP-isoenzyme to activate ticlopidine.

***A*** *Clopidogrel (Clp)*




***B*** *Carboxylate meabolite of clopidogrel (Carboxy-Clp)*




***C*** *Ticlopidine (Tcp)*

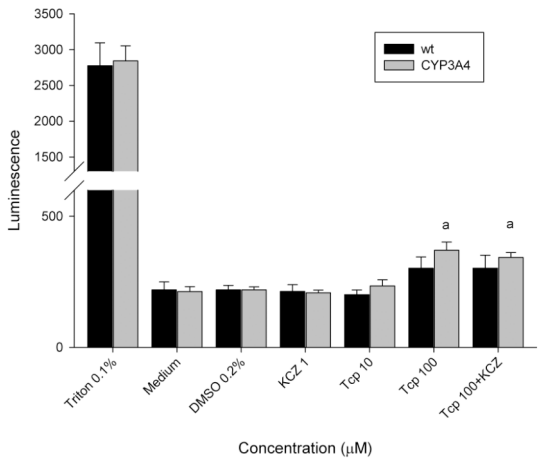


**Figure 1**

*Metabolic toxicity of* ***(A)*** *Clp,* ***(B)*** *Carboxy-Clp and* ***(C)*** *Tcp*. Cytotoxicity was studied in *CYP3A4 cells* compared to *HepG2 wt cells* (intracellular metabolic toxicity) and *3A4 supersomes* compared to *control supersomes* (extracellular metabolic toxicity) by measuring adenylate kinase release as described in methods. Triton-X (0.1%) was used as positive control and 1 µM ketoconazole (KCZ) to ensure involvement of CYP3A4. Adenylate kinase release was measured after 24 h. Mean values±SEM of at least six independent experiments are shown. **a** $p<0.05$ versus control incubations containing 0.2% DMSO; **b** $p<0.05$ versus co-incubation with 1 µM ketoconazole; **c** $p<0.05$ *CYP3A4 cells* versus *HepG2 wt cells* or *3A4 supersomes* versus *control supersomes*.

### 4.4.2. Quantification of clopidogrel metabolism by HPLC

The metabolism of clopidogrel in *CYP3A4 cells* and *3A4 supersomes* was quantified using HPLC. Cells were incubated with clopidogrel (10, 50 and 100 µM) for 6, 12 and 24 h and subjected to HPLC analysis. Clopidogrel metabolism was measured in *CYP3A4 cells* and compared to *HepG2 wt cells* (Fig. 2A). Similarly, clopidogrel conversion was tested in *3A4 supersomes* and compared to *control supersomes* (Fig. 2B). The amount of clopidogrel metabolized was calculated by substracting the remaining clopidogrel from the initial amount (zero time point). In *CYP3A4 cells* clopidogrel is time-dependently metabolized at all concentrations tested. After 24 h incubation, more than half of the clopidogrel amount is metabolized: 6.6 ± 1.6 µM (for 10 µM), 27.9 ± 2.4 µM (for 50 µM) and 65.9 ± 6.0 µM (for 100 µM), respectively. Co-incubation with ketoconazole (1 µM) showed a significant inhibition of clopidogrel metabolism in *CYP3A4 cells:* 3.1 ± 2.2 µM (for 10 µM), 14.2 ± 1.4 µM (for 50 µM) and 35.5 ± 1.8 µM (for 100 µM). These data confirm the ketoconazole-mediated prevention of clopidogrel toxicity (Fig. 1A). In *HepG2 wt cells* clopidogrel is not significantly metabolized since they are lacking CYP3A4 activity. Additionally, *3A4 supersomes* represent the activation system where clopidogrel is metabolized outside the cells (Fig. 2B). In *CYP3A4 cells*, clopidogrel is time-dependently metabolized by *3A4 supersomes*: 9.5 ± 0.8 µM (for 10 µM), 50.4 ± 1.6 µM (for 50 µM) and 70.2 ± 4.2 µM (for 100 µM), respectively. Interestingly, clopidogrel is almost completely metabolized after 24 h at low (10 µM) and intermediate (50 µM) concentrations in the presence of *3A4 supersomes*. However, this could not be observed at high (100 µM) concenrations of clopidogrel, where the effect of CYP3A4 was less pronounced. Ketoconazole inhibited clopidogrel metabolism in *3A4 supersomes*. C*ontrol supersomes*, which are lacking CYP3A4 activity, failed to metabolize clopidogrel (Fig. 2B).

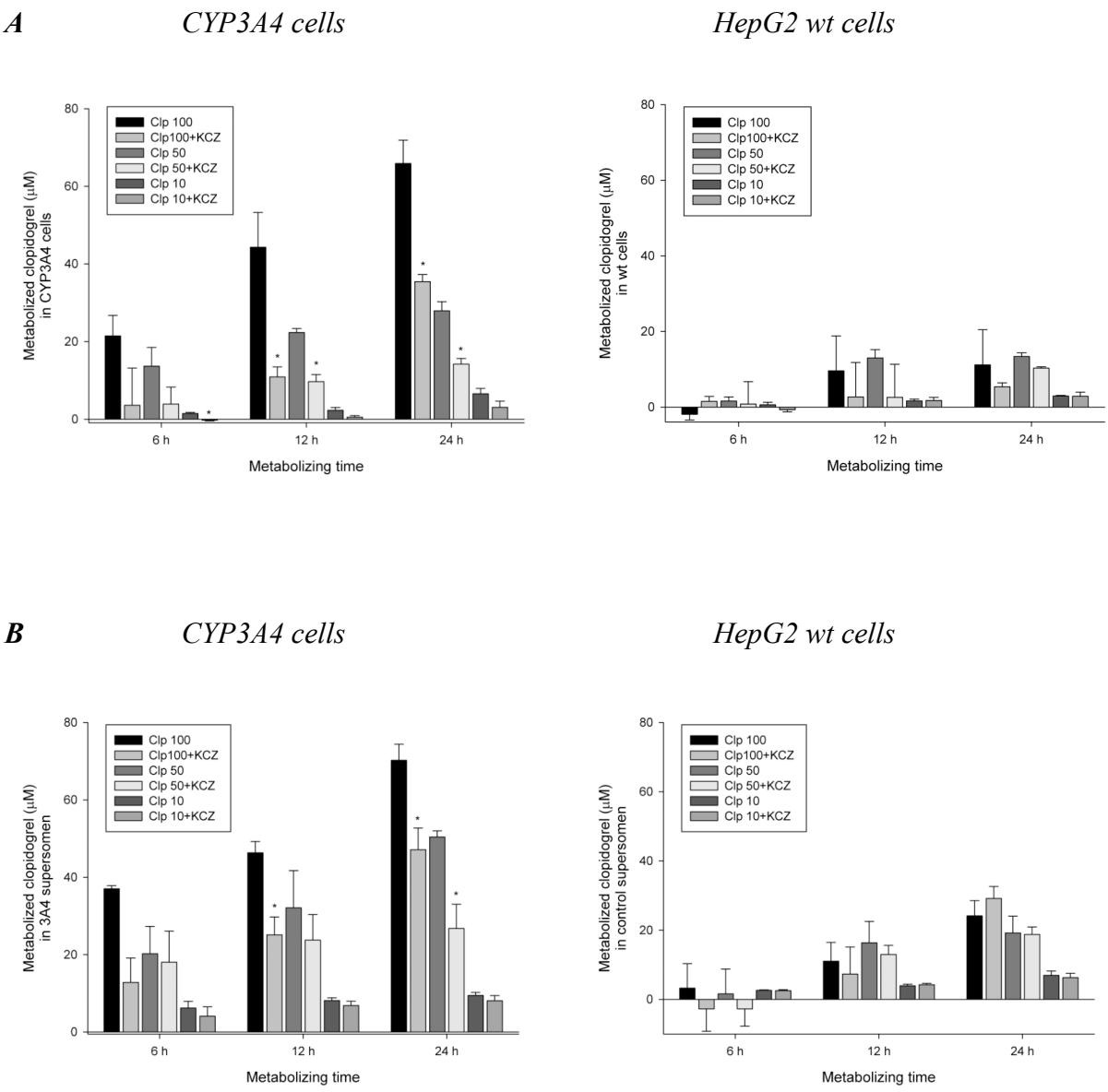



**Figure 2**

*Quantification of clopidogrel (Clp) metabolism by* ***(A)*** *CYP3A4 cells and* ***(B)*** *3A4 supersomes using HPLC*. The clopidogrel concentration (µM) in *CYP3A4 cells* (compared to *HepG2 wt cells*) and *3A4 supersomes* (compared to *control supersomes*) after incubation with 10, 50 and 100 µM for 6,12 and 24 h was quantified using HPLC. 1 µM ketoconazole (KCZ) confirmed involvement of CYP3A4. For quantification of clopidogrel metabolism standard curves were used. Clopidogrel conversion was normalized to the zero time point. Mean values±SEM of three independent experiments are shown. **p*<0.05 clopidogrel incubations versus co-incubation with 1 µM ketoconazole.

### 4.4.3. Cytotoxicity of activated clopidogrel in absence/presence of GSH

The active metabolite of clopidogrel contains a free and possibly reactive mercapto group, which is considered to bind to the ADP-receptor of platelets. Most likely, the free mercapto group also forms covalent bindings with other cellular proteins and peptides, such as glutathione. To test whether the active metabolite is responsible for the cytotoxicity observed in *CYP3A4 cells* we performed toxicity experiments in the presence or absence of glutathione (GSH, 10 μM). Diethyl-maleate (DEM), a glutathione-depleting agent was used as positive control. Co-incubation of DEM and GSH decreased the toxic effect of DEM on CYP*3A4*- and *HepG2 wt cells* (Fig. 3). Clopidogrel (100 μM) showed toxicity only in *CYP3A4 cells* and co-incubation with GSH attenuated the observed effect, consistent with the idea that the active metabolite of clopidogrel is responsible for cytotoxicity possibly at least in part through cellular GSH depletion. Significant toxicity was seen in both *CYP3A4*- and *HepG2 wt cells* with 1000 μM clopidogrel, although the effect was significantly enhanced in *CYP3A4 cells*. In the presence of GSH, the toxicity was decreased in *CYP3A4* cells to the level of *HepG2 wt cells*. However, some toxicity remained at such a high clopidogrel concentration. The carboxylate metabolite of clopidogrel showed no toxicity up to 1000 μM confirming previous experiments (Fig. 1B). The small toxic effect induced in *CYP3A4 cells* by ticlopidine could be prevented by GSH, similar to clopidogrel.

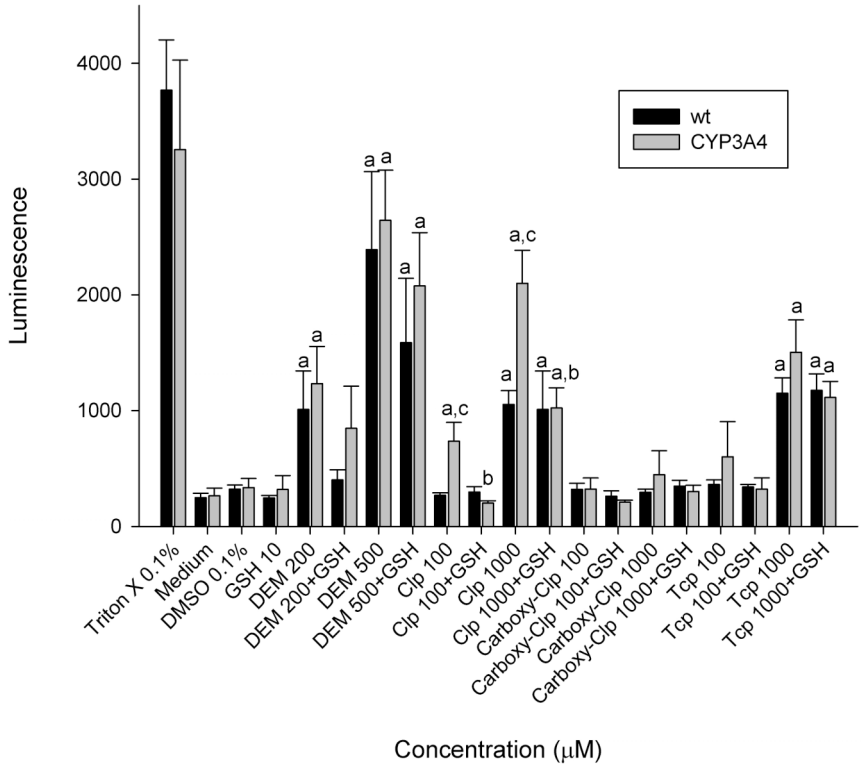



**Figure 3**

*Cytotoxicity of clopidogrel (Clp), the carboxylate metabolite of clopidogrel (Carboxy-Clp) and ticlopidine (Tcp) in the presence or absence of glutathione (GSH)*. Adenylate kinase release was measured after 24 h as described in methods. Mean values±SEM of four independent experiments are shown. **a** $p<0.05$ versus control incubations containing 0.2% DMSO; **b** $p<0.05$ versus co-incubation with GSH; **c** $p<0.05$ *CYP3A4 cells* versus *HepG2 wt cells*.

### 4.4.4. Glutathione pool in CYP3A4 cells after clopidogrel treatment

Measuring the glutathione pool in *CYP3A4 cells* allowed us do determine the redox status (Fig. 4). DEM 200 μM, which was used as a positive control, induced a decrease in cellular GSH and a corresponding increase in GSSG (data not shown), resulting in GSH/GSSG ratio of 1.2±0.5 in *CYP3A4 cells* (10.0±2.6 control), respectively. Clopidogrel and ticlopidine induced a concentration-dependent drop in their GSH/GSSG ratio in *CYP3A4 cells* (Fig. 4), whereas the carboxylate metabolite of clopidogrel failed to alter the GSH/GSSG ratio. Treatment with 100 μM clopidogrel was associated with a decrease in cellular GSH and an increase in GSSG (data not shown) and accordingly the GSH/GSSG ratio of 3.0 ± 0.5 was significantly decreased compared to the control.

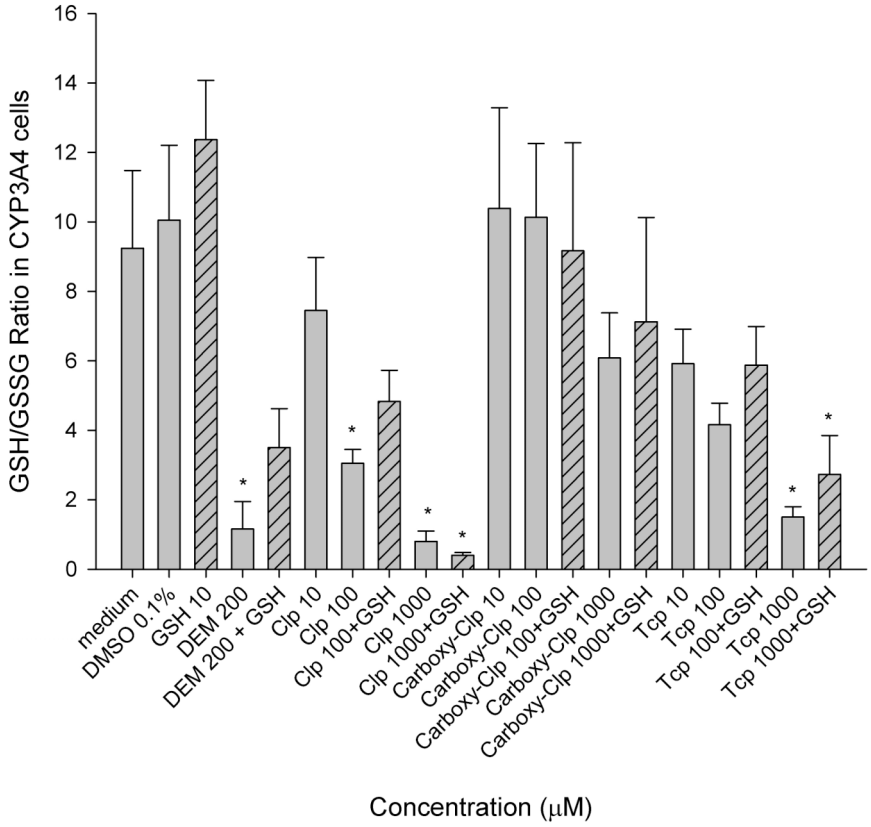



**Figure 4**

*GSH/GSSG Ratio of CYP3A4 cells treated with clopidogrel (Clp), the carboxylate metabolite of clopidogrel (Carboxy-Clp) or ticlopidine (Tcp) in the absence or presence of glutathione (GSH)*. GSH ratio (GSH/GSSG) was determined as described in methods. GSH = reduced glutathione (µg/mg protein), GSSG= oxidized glutathione (µg/mg protein). Diethyl-maleate (DEM), a glutathione-depleting agent, served as a positive control. Mean values±SEM of four independent experiments are shown. **p*<0.05 versus control containing 0.1% DMSO.

### 4.4.5. ROS production after clopidogrel treatment

The observed drop in the cellular glutathione content may be associated with a cellular stress reaction and with cellular accumulation of reactive oxygen species (ROS), since glutathione is needed for ROS degradation. Therefore, we determined the cellular ROS content using 2,7-dichlorofluorescein diacetate after incubation for 6,12 or 24 h with clopidogrel, the carboxylate metabolite of clopidogrel or ticlopidine as described in methods. There is a time-dependent increase of ROS formation in *CYP3A4 cells* in response to clopidogrel treatment (Fig. 5), whereas in *HepG2 wt cells* no increase in ROS formation was observed with neither of the drugs tested. After 24 h, ROS production was significantly increased in *CYP3A4 cells*

in response to clopidogrel (Fig. 5B). Co-incubation with ketoconazole attenuated the ROS production, consistent with the toxicity data. For ticlopidine small increase in ROS formation was observed in *CYP3A4 cells* and with ketoconazole co-incubation the increase was slightly diminished.

***A*** *CYP3A4 cells* ***B*** *HepG2 wt cells*

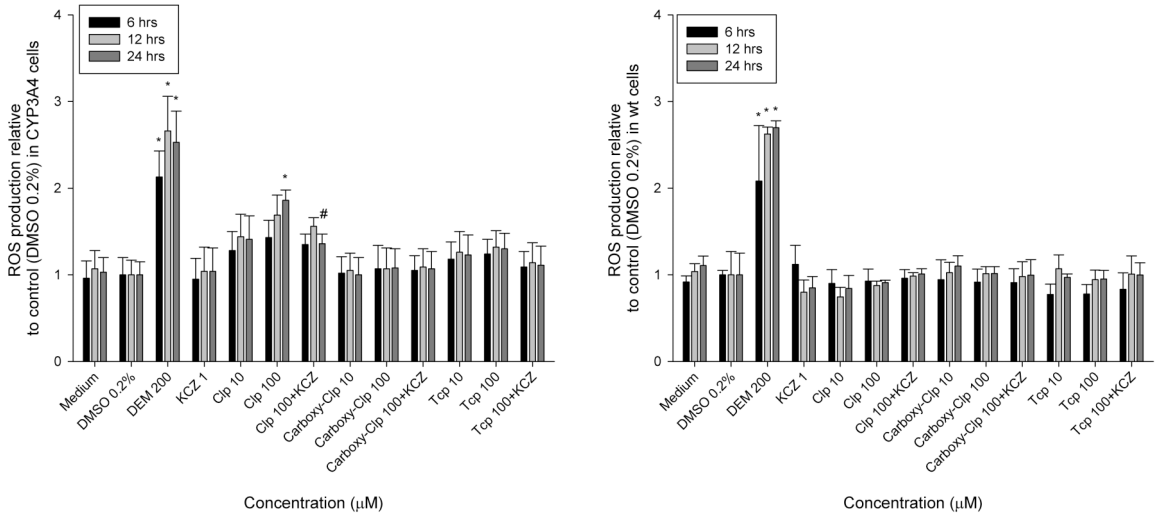



**Figure 5**

*ROS formation by* ***(A)*** *CYP3A4 cells and* ***(B)*** *HepG2 wt cells after treatment with clopidogrel (Clp), the carboxylate metabolite of clopidogrel (Carboxy-Clp) and ticlopidine (Tcp) for 6,12 or 24 h.* Diethyl-maleate (DEM) 200 μM served as a positive control. 1 μM ketoconazole (KCZ) was used as specific CYP3A4 inhibitor. Results are shown as fold increase to control (0.2% DMSO) and are indicated as mean values±SEM of four individual experiments in triplicate. **P*<0.05 versus control incubations (0.2% DMSO); # *p*<0.05 versus co-incubation with ketoconazole 1 μM.

### 4.4.6. Mitochondrial damage and cytochrome c release after clopidogrel treatment

ROS production can trigger the opening of the mitochondrial permeability transition pores, leading to a release of cytochrome c into the cytoplasm, a key event in mitochondrial-induced apoptosis. To investigate whether clopidogrel toxicity is mediated via this apoptotic pathway, mitochondrial structural damage and leakage of cytochrome c was assessed using immunofluorescence staining and microscopy. Clopidogrel induced mitochondrial damage and release of cytochrome c into the cytosol in *CYP3A4 cells*, indicated by the loss of co-localization in the merged image (Fig. 6). In contrast, *HepG2 wt cells* were only mildly

affected and cytochrome c still colocalized with mitochondria. Treatment with the carboxylate metabolite of clopidogrel as well as ticlopidine did not affect either of the cell lines. Benzbromarone (positive control) induced severe mitochondrial damage and cytochrome c release in both, *CYP3A4 cells* and *HepG2 wt cells* (data not shown).

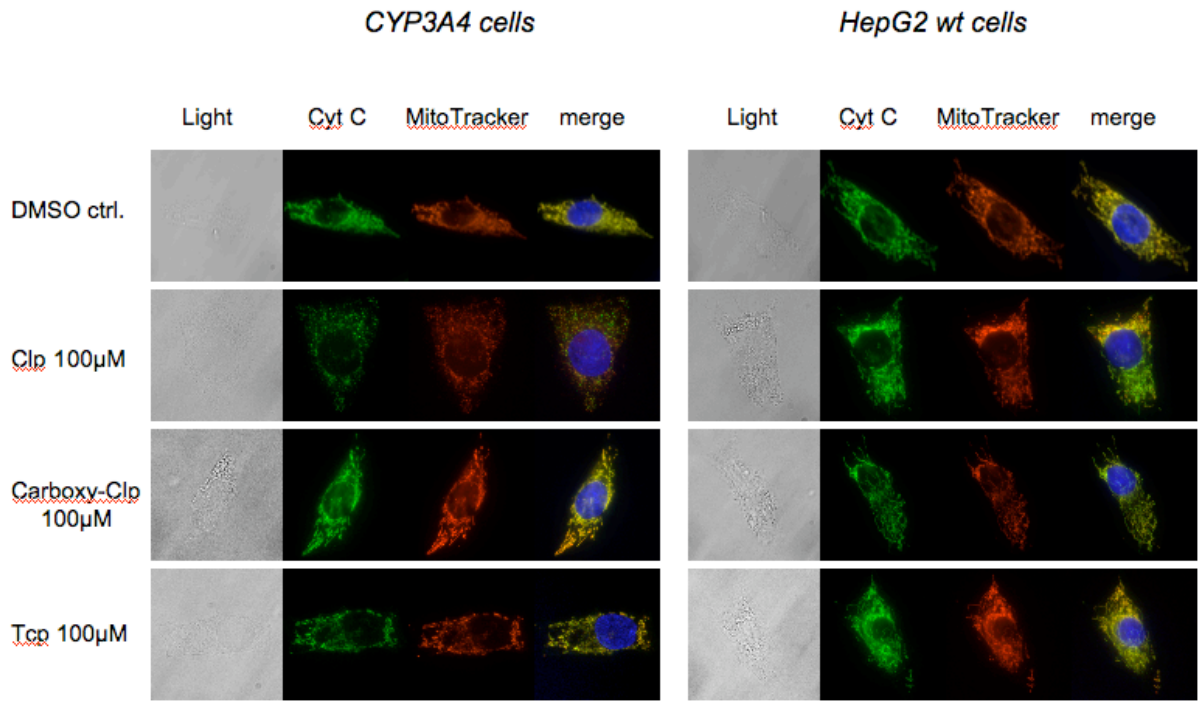



**Figure 6**

*Mitochondrial cytochrome c release after drug treatment in CYP3A4 cells and HepG2 wt cells.* Representative images of mitochondria (MitoTracker, red), cytochrome c (Cyt C, green) and their co-localization (merge, yellow). Cells were incubated with clopidogrel (Clp) 100 µM, the carboxylate metabolite of clopidgrel (Carboxy-Clp) 100 µM or ticlopidine (Tcp) 100 µM for 12 h and cytochrome c release was detected by immunofluorescence staining with a monoclonal antibody against cytochrome c. 0.2% DMSO (DMSO ctrl) was used as negative control and showed maximal co-localization. 100 µM benzbromarone (BB) was used as positive control (data not shown).

### 4.4.7. Determination of apoptosis and/or necrosis after clopidogrel treatment

Since mitochondrial damage is associated with apoptosis and/or necrosis [140, 142, 143] we assessed annexin V and propidium iodide staining of *CYP3A4* and *HepG2 wt cells* after drug treatment. As part of the early apoptotic process, phosphatidylserin is externalized and

therefore accessible to annexin V, which can be visualized by flow cytometry. During late apoptosis or necrosis, propidium iodide is able to enter cells across the disintegrated membrane and bind DNA, thereby rendering this process late apoptosis distinguishable from early apoptosis. Activated clopidogrel induced an increased percentage of early apoptotic *CYP3A4* but not *HepG2 wt cells* (Fig. 7). To a smaller extent clopidogrel also induced late apoptosis/necrosis in *CYP3A4 cells* ($p > 0.05$). In contrast *HepG2 wt cells* were not affected by the clopidogrel treatment. In agreement with our previous findings, co-incubation with ketoconazole attenuated the effect, again arguing that the CYP3A4-mediated generation of the clopidogrel metabolilte is responsible for the toxic effects and finally directing the cells into apoptosis. Treatment with the carboxylate metabolite of clopidogrel and ticlopidine did not induce apoptosis and/or necrosis in both *CYP3A4 cells* and *HepG2 wt cells*.

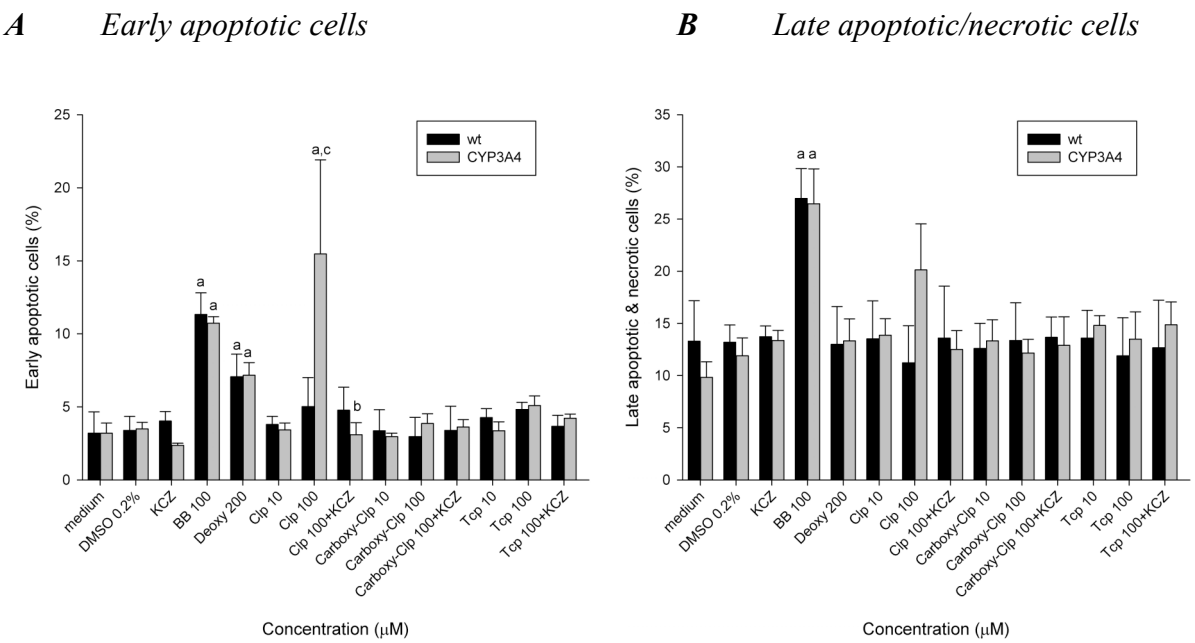



**Figure 7**

*Determination of apoptosis and/or necrosis after clopidogrel (Clp), the carboxylate metabolite of clopidogrel (Carboxy-Clp) and ticlopidine (Tcp) treatment.* Apoptosis was assessed using fluorescently labeled annexin V and propidium iodide followed by flow cytometry analysis. 200 µM Deoxycholate (Deoxy) was used as positive control for early apoptosis and 100µM benzbromarone (BB) as a positive control for early and late apoptosis/necrosis [140]. Mean values±SEM of three independent experiments are shown. **a** $p<0.05$ versus control incubations containing 0.2% DMSO; **b** $p<0.05$ versus incubations containing 1 µM ketoconazole; **c** $p<0.05$ *CYP3A4 cells* versus *HepG2 wt cells*).

## 4.5. Discussion

Clopidogrel is a generally well-tolerated antiplatelet drug although serious complications such as hepatotoxicity may rarely occur [100-110]. The mechanism of clopidogrel-induced hepatotoxicity is so far unknown. Clopidogrel is oxidized mainly by CYP 3A4 to an active metabolite carrying a mercapto group. This active metabolite is responsible for the therapeutic effect by inhibiting platelet aggregation but may, due to its reactivity, also be responsible for clopidogrel-induced toxicity, including hepatotoxicity.

To test this hypothesis, we studied hepatotoxicity of activated clopidogrel *in vitro* using *CYP3A4 overexpressing cells* and *3A4 supersomes* as activating systems. Our data showed that clopidogrel is metabolized similarly independent of whether intracellular (*CYP3A4 cells*) or extracellular (*3A4 supersomes*) activation was used. Consistent with the activation of clopidogrel we observed an increased toxicity in *CYP3A4 cells* and *3A4 supersomes*, suggesting that the generation of reactive metabolites is responsible for the observed toxic effect. Co-incubation with the CYP3A4 inhibitor ketoconazole prevented clopidogrel activation and attenuated the toxic effect of clopidogrel, thereby confirming CYP3A4 as specific clopidogrel activator. Furthermore, glutathione decreased the toxicity supporting the concept of a reactive metabolite of clopidogrel, which is at least in part responsible for the clopidogrel induced toxicity (Fig. 3).

The active metabolite of clopidogrel was shown to be the hydrolyzed derivative of the hydroxylated thiophene ring [122]. This metabolite carries a reactive thiol group, which can block the $P2Y_{12}$-receptor of platelets by forming disulfide bonds with its extracellular cystein residues [50]. In accordance, Richter et al [43] proposed that a similar mechanism may explain cytochrome P450 inhibition by ticlopdine and clopidogrel. Thiophene rings have been associated with a significant incidence of adverse reactions in other drugs such as ticrynafen (tienilic acid) [144]. We therefore speculate that the production of the reactive metabolite in the liver can be associated with „direct“ toxicity mainly due to the loss of function of the proteins (e.g. glutathione) modified by covalent binding of these reactive metabolites. Our findings support this oxidative stress reaction by showing a dose-dependent drop in the cellular glutathione pool in *CYP3A4 cells* by activated clopidogrel, most likely due to binding of glutathione to the active metabolite (Fig. 4). A drop in the cellular glutathione content is reflected in a cellular stress reaction and in cellular accumulation of reactive oxygen species (ROS), since glutathione is essential for ROS degradation. This was confirmed in our studies by showing that activated clopidogrel is time-dependently increasing ROS formation in *CYP3A4 cells*. Accumulation of ROS can lead to lipid peroxidation and protein carbonylation,

and as well to mitochondrial swelling due to induction of mitochondrial membrane permeability transition [140, 142]. Pore opening induces release of cytochrome c, which is located on the outer surface of the inner mitochondrial membrane. After release, cytochrome c is able to trigger the subsequent effector steps to induce apoptosis. Structural damage of mitochondria and subsequent release of cytochrome c by activated clopidogrel point towards a mitochondria dependent pathway that ends in apoptosis in *CYP3A4 cells* (Fig. 6).

Besides the described oxidative stress reaction due to depletion of glutathione, the interaction of cellular proteins with reactive metabolites could also lead to the formation of neoantigens, possibly triggering a hepatic immune response [145]. In a patient with developing hepatocellular injury during ticlopidine treatment, activation of T lymphocytes has been described, suggesting the involvement of the adaptive immune system [100]. Recent studies have shown that ticlopidine and clopidogrel are not solely activated by several CYPs but may also bind to certain CYPs after activation (mechanism-based inactivation of CYPs) [43]. Covalent modification of CYPs may form new antigens, which could represent a target for immunological reactions [145]. Such reactions have been described for hepatotoxicity associated with tienilic acid, where they are associated with the generation of anti-liver/kidney microsome (anti-LKM) antibodies [146, 147]. However, such antibodies have so far not been described in patients suffering from liver injury induced by ticlopidine or clopidogrel.

In humans, clopidogrel is predominantly metabolized by esterases to an inactive carboxylate metabolite of clopidgrel, which circulates in the plasma [33-36]. Regarding the carboxylate metabolite of clopidogrel, we neither observed toxicity in *CYP3A4 cells* or *3A4 supersomes* nor in *HepG2 wt cells* or *control supersomes*. It is therefore unlikely that this metabolite is responsible for the observed hepatotoxicity in patients treated with clopidogrel.

The mechanism of ticlopidine-induced hepatotoxicity remains still unknown. The involvement of direct toxicity of ticlopidine or one of its metabolites was proposed [148, 149]. Ticlopidine is metabolized through *N*-dealkylation, *N*-oxidation and/or oxidation of the thiophene ring by several CYP isoenzymes [32]. In our study, ticlopidine showed a clear cytoxicity at a concentration of 100 µM in *CYP3A4 cells*. This effect was not attenuated by ketoconazole, indicating that cytotoxicity is not associated by the formation of a metabolite by CYP3A4. Decrease in the GSH/GSSG ratio and the production of ROS were both less pronounced than with clopidogrel and mitochondrial damage as well as induction of apoptosis were not detectable by the cellular system used in our studies. Most probably, hepatotoxicity associated with ticlopidine is therefore caused by a metabolite, which is formed by a CYP different from CYP3A4.

In summary, activation of clopidogrel by CYP3A4 is associated with cytotoxicity. The most probable mechanism is depletion of glutathione by the active metabolite, leading to ROS production, cytochrome c release from mitochondria and cell death by apoptosis. Hepatotoxicity associated with ticlopidine cannot be explained by this mechanism.

# 5. The role of CYP3A4 in amiodarone-induced hepatotoxicity

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## 5.1. Abstract

Amiodarone (2-n-butyl-3-[3,5 diiodo-4-diethylaminoethoxybenzoyl]-benzofuran) is a class III antiarrhythmic drug, which is associated with potentially life-threatening liver toxicity. Recent investigations revealed that the N-desethyl metabolites of amiodarone may be at least partially responsible for the hepatic toxicity *in vitro*. Since cytochrome P450 (CYP) 3A4 is responsible for amiodarone N-deethylation in the liver, CYP3A4 induction may represent a risk factor. The aim of this study was to investigate the role of CYP3A4 in amiodarone-induced hepatotoxicity. Therefore we established and characterized stably transduced HepG2 cells overexpressing human CYP3A4 (herein referred to as *CYP3A4 cells*). Furthermore we used *HepG2 wt cells* co-incubated with human CYP3A4 supersomes as an extracellular activation system (referred to as *3A4 supersomes*). Amiodarone showed cytotoxicity in experiments using *CYP3A4 cells* or *3A4 supersomes*. In contrast, amiodarone was not cytotoxic when incubated with *HepG2 wt cells* or *control supersomes*. Co-incubation with ketoconazole, a CYP3A4 inhibitor, attenuated the toxic effect of metabolized amiodarone in *CYP3A4 cells* and *3A4 supersomes*, further demonstrating that the generated MDEA and DDEA are responsible for the observed toxicity. Accordingly, we detected MDEA and DDEA formation only in *CYP3A4 cells* and *3A4 supersomes* but not in *HepG2 wt cells* or *control supersomes*. In agreement with previous studies, metabolized amiodarone triggered the production of reactive oxygen species (ROS), induced mitochondrial damage and cytochrome c release, promoting late apoptosis/necrosis in *CYP3A4 cells*. This study adds further evidence to the hypothesis that a high activity of CYP3A4 is a risk factor for hepatotoxicity associated with amiodarone. Since CYP3A4 inducers are used frequently and hepatotoxicity associated with amiodarone can be fatal, our observations may be important for patients treated with this drug.

## 5.2. Introduction

Amiodarone (2-n-butyl-3-[3,5 diiodo-4-diethylaminoethoxybenzoyl]-benzofuran) is a class III antiarrhythmic drug with additional class I and II properties used in the treatment of a wide spectrum of cardiac arrhythmias [150]. Amiodarone's therapeutic use is limited because of its numerous side effects that include thyroidal [151], pulmonary [152], ocular [153] and/or liver toxicity [154, 155]. Amiodarone is a mitochondrial toxicant associated with uncoupling of oxidative phosphorylation and inhibition of the electron transport chain and β-oxidation of fatty acids [140, 141, 156-158]. The mechanisms leading to the toxicity of amiodarone are not completely understood, but most likely involve the accumulation of metabolites as well as the parent compound, finally resulting in cellular toxicity by impairing mitochondrial function [141]. Amiodarone is metabolized to mono-N-desethylamiodarone (MDEA) [159] and to di-N-desethylamiodarone (DDEA) [160] by N-deethylation. Waldhauser et al [141] reported that MDEA and DDEA are strong inhibitors of the respiratory chain and are both associated with ROS production. Moreover, they suggested that these metabolites are at least partially responsible for the hepatic toxicity in patients treated with amiodarone. Therefore, we hypothesized that induction of cytochrome P450 (CYP) 3A4, the main CYP isoenzyme responsible for amiodarone deethylation [161], may be a risk factor for hepatotoxicity associated with amiodarone. Since CYP3A4 inducers (e.g. antiepileptics such as phenytoin, phenobarbital and carbamazepine as well as rifampicin) are frequently used and amiodarone-mediated hepatotoxicity is potentially fatal [155], it appears to be important to reveal the cellular mechanisms leading to this type of toxicity in more detail.

In order to reach our aim, we established stably transduced cells overexpressing human CYP3A4 using the hepatocyte cell line HepG2 (*CYP3A4 cells*). This cellular system allowed us to investigate the metabolism of amiodarone and the generation of the two toxic metabolites MDEA and DDEA within the cells. Additionally, we employed an extracellular system including *HepG2 wt cells* and human CYP3A4 supersomes (*3A4 supersomes*) to assess possible differences to intracellular activation of amiodarone. An HPLC method allowed us to study and quantify the generation of MDEA and DDEA by *CYP3A4 cells* and by *3A4 supersomes*. Finally, the hepatocellular toxicity of amiodarone and derivatives described here could be compared to a previous study [141].

## 5.3. Materials and Methods

### 5.3.1. Materials

Amiodarone, mono-N-desethylamiodarone and L8040 (HPLC internal standard, IS) were purchased from Sanofi Recherche (Brussel, Belgium). Human CYP3A4 supersomes with supplementation of cytochrome $b_5$ and P450 reductase and insect cell control supersomes were from BD Gentest (Woburn, MA, USA). Cell culture supplements were purchased from GIBCO (Paisley, UK). Cell culture plates were purchased form BD Bioscience (Franklin Lakes, NJ). NADPH was obtained from Sigma-Aldrich (Switzerland) and the ToxiLight® BioAssay Kit from Cambrex Bio Science Rockland Inc. (USA). Methanol LiChrosolv for HPLC use was from Merck (Darmstadt, Germany). All other chemicals used were purchased from Sigma or Fluka (Buchs, Switzerland).

### 5.3.2. Cell lines and cell culture

The hepatocyte cell line HepG2 was provided by Professor Dietrich von Schweinitz (University Hospital Basel, Switzerland). HepG2 wild type cells (*HepG2 wt cells*), *GFP cells* (vector control) and HepG2 cells overexpressing human CYP3A4 (*CYP3A4 cells*) were cultured in Dulbecco's modified Eagle's medium (DMEM; with 2mmol/L GlutaMAX®, 1.0g/L glucose and sodium bicarbonate) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 10mM HEPES buffer, pH 7.2 and non-essential amino acids. The culture conditions were 5% $CO_2$ and 95% air atmosphere at 37°C.

### 5.3.3. Construction of the expression vector pCR2.1-CYP3A4

The coding region sequence of human CYP3A4 was obtained from NCBI's Nucleotide sequence database (Ref Seq NM_017460). cDNA was generated by RT-PCR from RNA extracted from human liver with the use of the SuperScript™III RT-PCR kit (Invitrogen, Life Technologies) according to the manufacturer's recommendations. The gene specific reverse primer (5'-TCAGGCTCCACTTACGGTGCCA-3') was designed. Forward and reverse oligonucleotide sequences were used as follows: forward 5'-TGATGCTCTCATCCCAGACTTGG-3' and 5'-TCAGGCTCCACTTAGGTGCA, respectively. The amplified product was cloned into the pCR®2.1-TOPO® vector (Invitrogen, Life Technologies) and transformed chemically into SURE®2Supercompetent cells (Strategene Europe, Amsterdam, NL) according to the manufacturer's protocols.

### 5.3.4. Production of lentivectors and transduction of HepG2 cells

The 1513-bp fragment containing the human CYP3A4 complete coding sequence was excised from the pCR®2.1-TOPO® vector and subcloned into the lentiviral pWpiresGFP vector. The vector envelope plasmid pMD2G, the packaging plasmid pCMCΔR8.91 and the pWpiresGFP vector were kindly provided by Dr. Didier Trono, University of Geneva. For the production of virions, pMD2G, pCMCΔR8.91 and the vector pWPhCYP3A4iresGFP (or empty vector pWpiresGFP) were transfected into 293T cells by calcium phosphate precipitation as described elsewhere [162]. After 12 h, the medium was replaced. The supernatant was harvested at 38 h post transfection, filtered and stored at -70°C.

### 5.3.5. Expression of human CYP3A4 in HepG2 cells

HepG2 cells ($0.5x10^5$) were seeded in six-well plates and incubated with viral supernatant (prepared as described above) in the presence of Polybrene® (Aldrich, Buchs, Switzerland). Successful transduction was assessed by fluorescence-activated cell sorting (FACS) analysis and cells expressing green fluorescent protein (EGFP) were separated and passaged. Expression of human CYP3A4 in the transduced HepG2 cells was determined with quantitative real-time PCR (QPCR). Total RNA was isolated using the RNeasy® (Qiagen, Basel, Switzerland) kit according to the manufacturer's recommendations. SuperscriptTMII in combination with oligo (dT) and Random Hexamer primers (Gibco BRL, Basel, Switzerland) was used for reverse transcription of 2 μg total RNA. Quantification was performed on an ABI PRISM 7700 Sequence Detector (PE Biosystems, Rotkreuz, Switzerland). Reporter probes hCYP3A4 and GAPDH FAM/TAMRA were from Eurogentec (Seraing, Belgium); the hCYP3A4 and GAPDH forward and reverse primers were from Microsynth (Balgach, Switzerland).

### 5.3.6. Human CYP3A4 protein overexpression in HepG2 cells

CYP3A4 expression was checked by Western blot using the polyclonal hCYP3A4 antibody (Daiichi pure chemicals, Tokyo, Japan). Cells ($10^6$) were lysed using 1% NP-40 (Nonidet P-40) and a protease inhibitor cocktail (Roche AG, Basel, Switzerland) plus 1 mM PMSF (Phenylmethylsulfonyl fluoride). Proteins were separated by electrophoresis in the presence of molecular weight standards (Gibco, Paisly, UK) on a 10% polyacrylamide sodium dodecyl sulfate (SDS) gel. Proteins were transferred onto a nitrocellulose membrane (BioradTrans-Blot, Hercules, CA). Membranes were incubated with the 1/500 diluted goat anti-human CYP3A4 antibody, a secondary peroxidase-conjugated anti-goat antibody (Jackson

Laboratories Inc) was used for chemiluminescence detection (ECL, Amersham International, Little Chalfont, UK) according to the manufacturer's protocol.

### 5.3.7. Functional characterization of CYP3A4 cells

CYP3A4 activity was measured using the P450-Glo™ Assay kit (Promega, Wallisellen, Switzerland). Cells (*HepG2 wt cells*, *GFP cells* and *CYP3A4 cells*) were seeded at $10^5$ cells per $cm^2$ in 96-well plates and allowed to adhere overnight. 50 µM rifampicin was used as induction control. After 72 h the P450-Glo™ Assay was performed according to the manufacturer's protocol using the P450-Glo™ luminogenic CYP450 substrate Luciferin-BE.

### 5.3.8. Prodrug treatment in CYP3A4 cells or HepG2 wt cells

*HepG2 wt* and *CYP3A4 cells* were passaged at 80-85% confluency, using trpysin. 50'000 cells/well were allowed to adhere overnight in 96-well culture plates. Stock solutions of test compounds (quinidine, amitriptyline, ketoconazole and amiodarone) were prepared in DMSO. The reaction volume was 200 µl and DMSO concentrations never exceeded 0.2%. Cells were incubated with 0.1% Triton X as positive control and 0.2% DMSO as negative control. Drug treatment was performed for 24 h at 37°C and 5% $CO_2$.

### 5.3.9. Prodrug treatment using 3A4 supersomes or control supersomes

Human CYP3A4 supersomes™ (referred to as *3A4 supersomes*) and insect cell control supersomes™ (referred to as *control supersomes*) were purchased from BD-Gentest. Cells were passaged and prepared for drug treatment similar as for the *3A4 cells* and *HepG2 wt cells*. Test compounds were added to the cells in the presence of 20 pmol/ml CYP3A4 supersomes or control supersomes and 1 mM of the co-factor NADPH.

### 5.3.10. Cytotoxicity assay (Adenylate kinase release)

The loss of cell membrane integrity is reflected in the release of adenylate kinase (AK), which can be detected using the firefly luciferase system (ToxiLight® BioAssay Kit, Cambrex Bio Science, Rockland, ME). After 24 h, 100 µl assay buffer was supplied to 20 µl supernatant from drug-treated cells in the presence or absence of ketoconazole (concentrations indicated in the Figures) and luminescence was measured after 5 min.

### 5.3.11. Quantification of amiodarone, MDEA and DDEA using HPLC

450'000 *HepG2 wt* or *CYP3A4 cells*/well were seeded in 24-well plates and allowed to adhere overnight. Supersomes were used as mentioned above. After incubation with amiodarone (10,

25 or 50 µM) for 24 h cells, 30 µl of internal standard (IS) was added and cells were detached using a cell scraper. Cells were lysed by freeze-thaw cycles and the cleared lysate was subjected to HPLC analysis.

We used a Merck Hitachi HPLC equipped with a column oven (L7300), an autosampler (L7200) hold at 25°C, interface (L7000), UV-detector (L7400) operating at a wavelength of 254 nm, pump (L7100) and a Reprosil SI 80 column from Dr. Maisch GmbH (Ammerbuch / Germany). For the measurements in cell culture medium a guard column (LiChrospher® Si60 5 µm, Merck / Germany) was used. The method was based on a validated method used for the quantification of amiodarone and its major metabolite mono-N-desethylamiodarone (MDEA) in serum developed at our institution. This method was adapted for the quantification of amiodarone, MDEA and di-N-desethylamiodarone (DDEA). The variability of the method was < 10% at high and low concentrations of amiodarone, MDEA an DDEA. The mobile phase consisted of solvent A (97% methanol / 3% ammoniumsulphate buffer pH 8.7) and solvent B (90% methanol / 10% ammoniumsulphate buffer pH 8.7). The gradient changed according to the following conditions: after 50% A and 50% B for 5 min increase to 100% B in 5 min and back to 50% A and 50% B in 5 min. The flow rate was 1.5 ml/min and the total run time 15 min. The injection volume was 20 µl. Quantification of amiodarone and its metabolites was by comparison to a standard curve.

### 5.3.12. Measurement of reactive oxygen species (ROS)

A fluorescence-based microplate assay [139] was used for the evaluation of oxidative stress in *wt cells* and *CYP3A4 cells* treated with the test compounds. 2',7'-dichlorofluorescein-diacetate (DCFH-DA) is a membrane-permeable, nonpolar, and nonionic molecule. DCFH-DA is hydrolyzed in the cytoplasm by intracellular esterases to nonfluorescent DCFH, which is oxidized to fluorescent dichlorofluorescein (DCF) in the presence of reactive oxygen species (ROS). Cells were simultaneously exposed to test compounds and to DCFH-DA (5 µM) and incubated for 6, 12 and 24 h. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microtiter plate reader (HTS 700 Plus Bio Assay Reader; PerkinElmer, Beaconsfield, Buckinghamshire, UK) in incubations containing cells and exposure medium.

### 5.3.13. Cytochrome c release detection by fluorescence microscopy

Microscop cover glasses (diameter 13mm; thickness 0.17mm) were activated with (3-aminopropyl)triethoxy-silane („Tespa"; Sigma No A3648) in acetone for 5min. 100'000 *HepG2 wt* or *CYP3A4 cells/well* were seeded in 24-well plates and allowed to adhere on

supplied microscopy cover glasses overnight. Amiodarone (50µM) was added to the cells for 8 h. 100 µM benzbromarone served as positive and 0.2% DMSO (DMSO ctrl) as negative control. Cells were stained with 50 nM Mitotracker Red CMXRos (Invitrogen No. M7512) for 30min at 37°C and crosslinked with 4% formaldehyde in PBS (Polysciences Inc.) for 15min at 37°C. Permeabilization was performed with 0.2% TritonX-100 followed by blocking with 5% BSA (Sigma No. A2153). Immuno staining was perfored using a biotinylated cytochrome c antibody (BioLegend No. 612303) at a diution 1/20 in PBS containing 5% BSA for 45 min. Streptavidin-Alexa647 (Molecular Probes No. S32354) at a dilution 1/200 plus 1/1'000 DAPI (Invitrogen No. D3571) were applied in PBS 2.5% BSA for 20min. Subsequently, cells were mounted on Superfrost glass slides (Thermo Scientific) using the FluorSave reagent (Calbiochem No. 345789). An Olympus IX81 inverted microscope was used for analysis in combination with the CellR software (Olympus). Images were exported as 16-bit TIFF files. ImageJ software (http://rsbweb.nih.gov/ij/) was used to convert the images to 8-bit and to create merged images. Photoshop was used to trim images and to adjust the brightness.

### 5.3.14. Detection of apoptosis and necrosis using flow cytometry

200'000 cells/well were seeded in 24-well plates and allowed to adhere for 24h before drug treatment. Drug treatment was performed for 24 h in the presence or absence of 1 µM ketoconazole (KCZ). *CYP3A4 cells* and *HepG2 wt cells* were stained for 15 min with 1/20 AnnexinV-PE (BD No. 556422) and 1/200 propidium iodide in a volume of 50 µl AnnexinV-binding buffer (10mM Hepes, 150mM NaCl, 2.5mM $CaCl_2$, 5mM KCl, 1mM $MgCl_2$ in $H_2O$). Flow cytometry was carried out on a DAKO Cyan cytometer. Benzbromarone (100 µM) [140] and deoxycholic acid (200 µM) [141] were used as positive controls.

### 5.3.15. Statistical analysis

Data are presented as mean values±SEM of at least three experiments. Statistical analyses were performed using Sigma Stat release 3.5 (SYSTAT Software, Inc.). Differences between many groups at two levels were tested by two-way analysis of variance (ANOVA) followed by Dunnet's post hoc test if the data were normally distributed. In the case of not normally distributed data, Holm-Sidak statistics was performed. Differences between groups (e.g. control versus test compound incubations in *CYP3A4 cells*) was tested by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test if ANOVA showed significant differences. A $P$-value < 0.05 was considered as significant.

## 5.4. Results

### 5.4.1. Characterization of human CYP3A4 in stably transduced HepG2 cells

FACS analysis revealed successfully transduced HepG2 cells, which were co-expressing EGFP and CYP3A4 (data not shown). To verify CYP3A4 overexpression, we measured mRNA expression of human CYP3A4 using RT-PCR (Fig. 1A). Quantitative real time PCR showed a > 65-fold increase of CYP3A4 mRNA in the transduced cells (referred to as *CYP3A4 cells*). Expectedly transduction with the empty vector (referred to as *GFP cells*) showed no elevation of CYP3A4 mRNA. CYP3A4 protein expression was investigated from cellular lysates of *HepG2 wt, GFP* or *CYP3A4 cells* (Fig. 1B). Western blot analysis revealed increased CYP3A4 protein expression exclusively in *CYP3A4 cells*. Pretreatment with the CYP3A4-inductor rifampicin slightly increased human CYP3A4 protein expression. The functionality of the human CYP3A4 construct in HepG2 cells was assessed by measuring CYP3A4 activity using the P450-Glo$^{TM}$ Assay kit as described. Consistent with the RNA and protein expression data, *CYP3A4 cells* showed increased CYP3A4 activity (>2.5 fold) compared *HepG2 wt* and *GFP cells* (Fig. 1C). Pretreatment with rifampicin further increased CYP3A4 activity in all cell lines tested. These data indicate that *CYP3A4 cells* are a functional, intracellular activation system for CYP3A4.

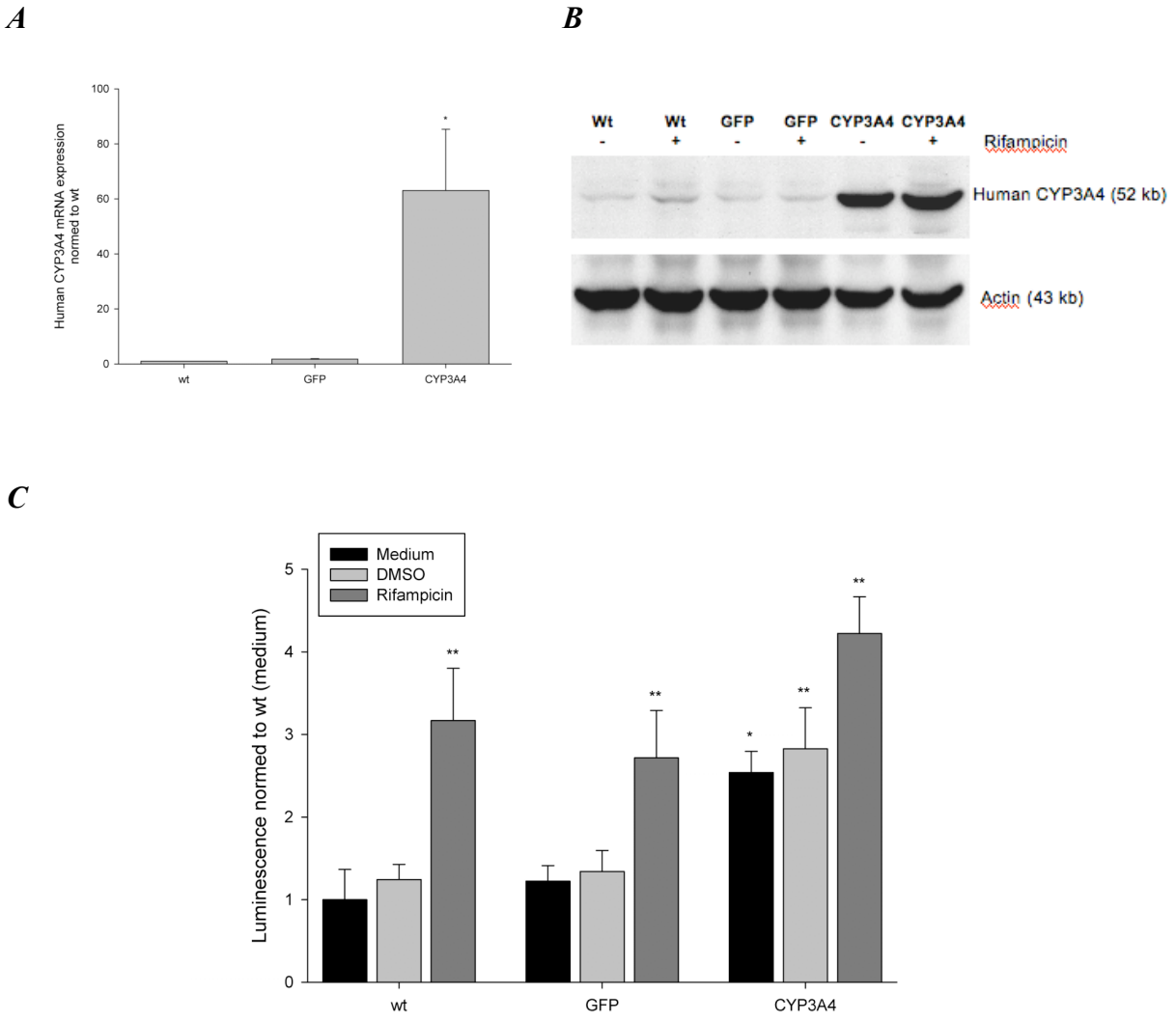


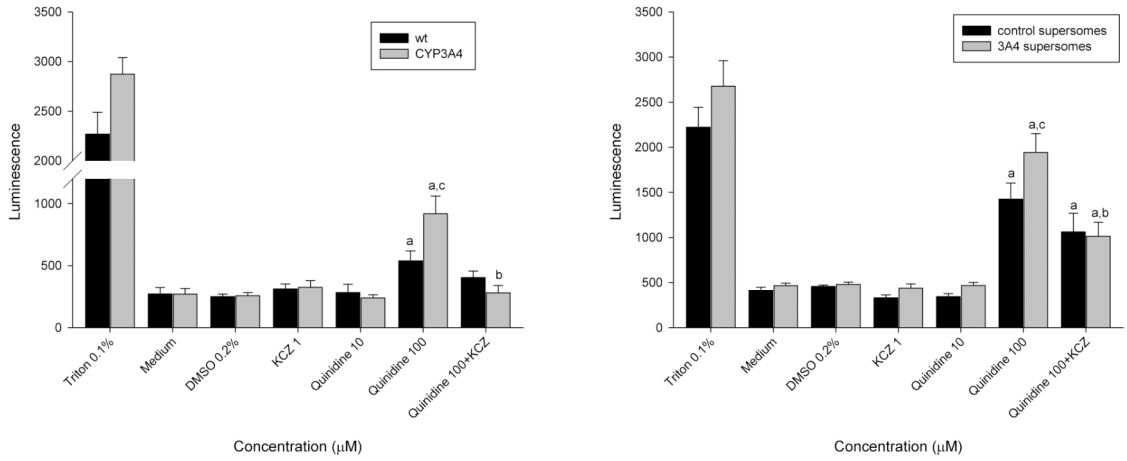

**Figure 1**

*Characterization of human CYP3A4 (hCYP3A4) in stably transduced HepG2 cells.* ***(A)*** Characterization of hCYP3A4 mRNA expression by quantitative PCR. Data are presented as fold change relative to the expression of hCYP3A4 in *wt cells* (n=4) **P< 0.01*. ***(B)*** Characterization of hCYP3A4 protein expression by Western blotting. Representative Western blot showing hCYP3A4 (52 kDa) and β-actin (43 kDa) protein expression in *HepG2 wt*, *GFP* and *CYP3A4 cells*. Human CYP3A4 was detected using a polyclonal hCYP3A4-antibody. Treatment with rifampicin 50µM is indicated. ***(C)*** Functional characterization of *CYP3A4 cells*. CYP3A4 activity was measured using the P450-Glo™ Assay kit. Cells were incubated with P450-Glo™ luminogenic CYP450 substrate Luciferin-BE and analysis was performed as described in methods. Luminescence was normalized to HepG2 *wt cells* incubated with medium. Mean values±SEM are shown of 6 experiments performed. **P*<0.05; ***P*<0.01 versus control.

### 5.4.2. Characterization of CYP3A4 cells to study metabolic toxicity

To determine the metabolic activity of *CYP3A4 cells* we tested different compounds that require CYP-mediated activation to manifest their cytotoxic effects (e.g. quinidine) [163]. *CYP3A4 cells* (= intracellular activation) were compared to HepG2 cells co-incubated with CYP3A4 cDNA expressing microsomes as a system with extracellular activation (*3A4 supersomes*). 100 µM quinidine was associated with a slight cytotoxicity in *HepG2 wt cells* (Fig. 2A). The toxic effect was significantly increased in *CYP3A4 cells*, confirming CYP3A4-mediated generation of toxic metabolites. Ketoconazole, a well described inhibitor of CYP3A4 activity, attenuated the effect. In the extracellular activation system, 100 µM quinidine also showed only slight cytotoxicity in the presence of control supersomes (Fig. 2B). Again, the effect was significantly increased using *3A4 supersomes*. Co-incubation with ketoconazole abolished the toxic effect of quinidine in *3A4 supersomes*. CYP3A4-mediated detoxification of amitriptyline was used as an additional control experiment (Fig 2 C,D) [163]. In both systems, the presence of CYP3A4 reduced the toxic effect of amitriptyline (1 µM amitriptyline in *CYP3A4 cells* and 50 µM amitriptyline in *3A4 supersomes*). Co-incubation of amitriptyline with 1 µM ketoconazole abrogated the effect. Taken together, these results demonstrate both activation systems can be used to investigate CYP3A4-mediated toxicity.

***A*** *intracellular activation* *extracellular activation*




***B*** *intracellular activation* *extracellular activation*

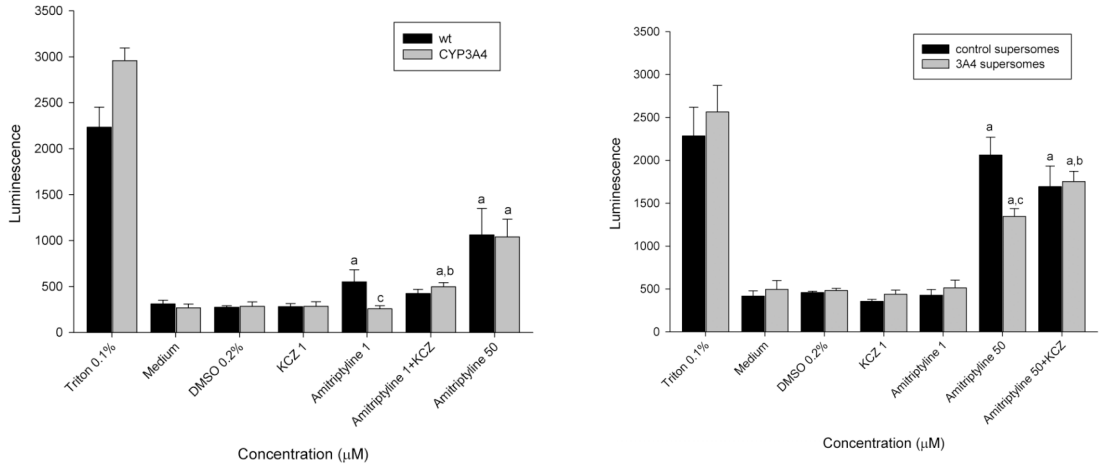



**Figure 2**

*Evaluation of metabolic toxicity in CYP3A4 cells.* ***(A)*** Metabolic toxicity of quinidine in *CYP3A4 cells* compared to *HepG2 wt cells* (intracellular activation) and *3A4 supersomes* compared to HepG2 cells incubated with *control supersomes* (extracellular activation). Adenylate kinase release was measured after 24 h exposure to quinidine (10, 100 µM). Cell lysis with Triton-X (0.01%) served as positive control. Co-incubation with 1 µM ketoconazole (KCZ) was used to specifically inhibit CYP3A4. ***(B)*** Metabolic toxicity of amitriptyline in *CYP3A4 cells* compared to *wt cells* and *3A4 supersomes* compared to *control supersomes*. Amitriptyline (1,10,100 µM) was added for 24 h before measuring cytotoxicity. Mean values±SEM are shown of at least six independent experiments. **a** $p<0.05$ versus control incubations containing 0.2% DMSO; **b** $p<0.05$ versus co-incubation with ketoconazole; **c** $p<0.05$ *CYP3A4 cells* versus *HepG2 wt cells*.

### 5.4.3. Metabolic toxicity of amiodarone

The toxicity of amiodarone on hepatocytes has been previously investigated [140, 141, 156-158, 164]. Waldhauser et al. [141] discovered that the two main metabolites MDEA and DDEA are at least partially responsible for the hepatocellular toxicity caused by amiodarone. The major metabolic conversion of amiodarone, N-deethylation to mono-N-desethylamiodarone (MDEA), is catalyzed by CYP3A [161]. Using *CYP3A4 cells* and *3A4 supersomes,* we aimed to investigate the metabolism of amiodarone and the subsequent generation of metabolites. Indeed, increasing amiodarone concentrations (10, 25 and 50 µM) induced corresponding cytotoxicity in *CYP3A4 cells* (Fig. 3A) Since 50 µM amiodarone failed to induce cytotoxicity in *HepG2 wt cells*, the effect could be specifically attributed to the generation of toxic metabolites by CYP3A4. Co-incubation with 1µM ketoconazole partly inhibited the cytotoxic effect of amiodarone in *CYP3A4 cells*. In the presence of *CYP3A4* or *control supersomes,* amiodarone promoted dose-dependent toxicity in both systems but was significantly more pronounced in the presence of *3A4 supersomes* (Fig. 3B). Co-incubation with ketoconazole lowered the CYP3A4-mediated effect to base line toxicity.

***A*** *intracellular activation* ***B*** *extracellular activation*

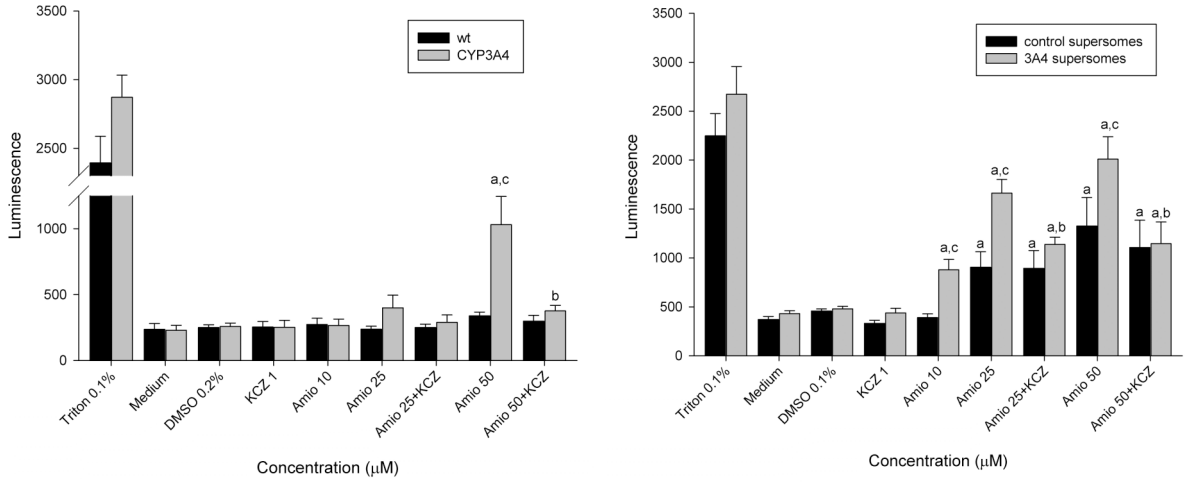



**Figure 3**

*Metabolic toxicity of amiodarone.* ***(A)*** Cytotoxicity of amiodarone in *CYP3A4 cells* (intracellular activation) or ***(B)*** *3A4 supersomes* (extracellular activation) by measuring adenylate kinase release as described in Figure 2. Triton-X (0.01%) mediated cell lysis served as positive control and 1 µM ketoconazole (KCZ) was used as specific CYP3A4 inhibitor. Drug treatment was performed for 24h. Mean values±SEM are shown of at least six independent experiments. **a** $p<0.05$ versus control incubations containing 0.2% DMSO; **b** $p<0.05$ versus co-incubation with ketoconazole; **c** $p<0.05$ *CYP3A4 cells* versus *HepG2 wt cells*.

### 5.4.4. Quantification of amiodarone metabolism using HPLC

The formation of the two main metabolites MDEA and DDEA can be detected and quantified by HPLC. Amiodarone, MDEA and DDEA concentrations were determined after 24 h of treatment with either 25 µM (A) or 50 µM (B) amiodarone (Table 1). The metabolites MDEA and DDEA were exclusively detected in *CYP3A4 cells* or *3A4 supersomes* after 24 h of amiodarone treatment. Accordingly, the amiodarone concentration decreased after 24 h. In contrast, *HepG2 wt cells* and *control supersomes* did not generate detectable concentrations of the metabolites independent of the initial amiodarone concentration.

***(A) 25 µM amiodarone treatment***

| | ***CYP3A4 cells*** | | | ***HepG2 wt cells*** | | |
|---|---|---|---|---|---|---|
| **Metabolizing time (h)** | amiodarone (µM) | MDEA (µM) | DDEA (µM) | amiodarone (µM) | MDEA (µM) | DDEA (µM) |
| **0** | 18.7 ±4.4 | 0 | 0 | 18.0 ±3.3 | 0 | 0 |
| **24** | **12.0 ± 1.9** | **7.9 ± 1.8** | **0.6 ± 0.6** | **17.2 ±1.3** | 0 | 0 |
| | | | | | | |
| | ***3A4 supersomes*** | | | ***Control supersomes*** | | |
| **Metabolizing time (h)** | amiodarone (µM) | MDEA (µM) | DDEA (µM) | amiodarone (µM) | MDEA (µM) | DDEA (µM) |
| **0** | 19.6±4.8 | 2.3±2.3 | 0 | 24.0±2.9 | 0 | 0 |
| **24** | **10.1±1.5** | **15.5±4.5** | **1.99±0.7** | **18.4±5.9** | 0 | 0 |

***(B) 50 µM amiodarone treatment***

| | ***CYP3A4 cells*** | | | ***HepG2 wt cells*** | | |
|---|---|---|---|---|---|---|
| **Metabolizing time (h)** | amiodarone (µM) | MDEA (µM) | DDEA (µM) | amiodarone (µM) | MDEA (µM) | DDEA (µM) |
| **0** | 45.8±5.2 | 0 | 0 | 54.7±4.3 | 0 | 0 |
| **24** | **33.9±2.6** | **15.5±2.8** | **2.3±0.8** | **34.7±2.1** | 0 | 0 |
| | | | | | | |
| | ***3A4 supersomes*** | | | ***Control supersomes*** | | |
| **Metabolizing time (h)** | amiodarone (µM) | MDEA (µM) | DDEA (µM) | amiodarone (µM) | MDEA (µM) | DDEA (µM) |
| **0** | 46.8±8.6 | 1.9±1.9 | 0.8±0.8 | 47.3±5.4 | 0 | 0 |
| **24** | **23.4±3.5** | **15.6 ± 3.1** | **3.0±0.6** | **43.0±8.5** | 0 | 0 |

**Table 1** Quantification of amiodarone metabolism by HPLC

### 5.4.5. ROS production of metabolized amiodarone

ROS formation can be a consequence of the inhibition of the electron transport chain [140], which has been shown for amiodarone as well as the two synthesized metabolites MDEA and DDEA in isolated rat hepatocytes [141]. We therefore investigated whether there is a difference in ROS formation by *CYP3A4 cells* compared to HepG2 wt cells incubated with amiodarone (Fig. 5). *CYP3A4 cells* and *HepG2 wt cells* were treated with amiodarone (25 and 50 µM) for 6, 12 and 24 h and ROS production was assessed as described in methods. Stress conditions were generated by depleting cellular glutathione with 200µM diethyl maleate (DEM) administration or by adding 0.3% $H_2O_2$ (data not shown). In *CYP3A4 cells*, 50µM amiodarone promoted a time-dependent increase in ROS formation (Fig. 5 A), whereas *HepG2 wt cells* did not show this behavior (Fig. 5B). Co-incubation with 1µM ketoconazole attenuated ROS production in *CYP3A4 cells*.

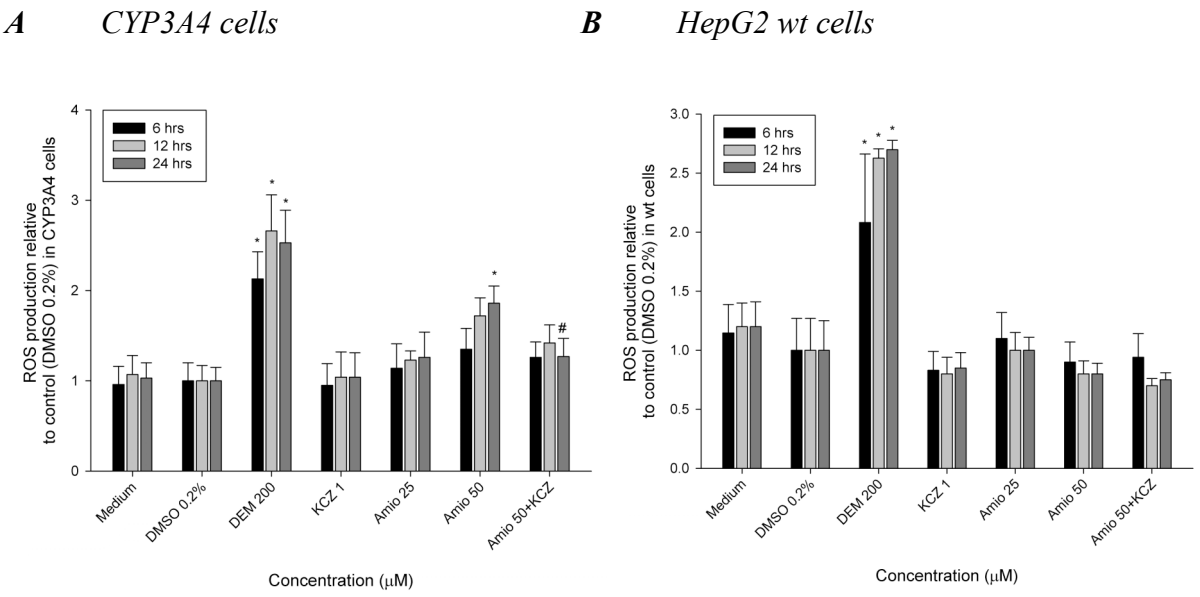



**Figure 5**

*ROS formation by* ***(A)*** *CYP3A4 cells and* ***(B)*** *HepG2 wt cells after treatment with amiodarone (Amio) for 6,12 and 24 h*. Diethyl maleate (DEM) 200 µM served as positive control. 1 µM ketoconazole (KCZ) was used as a specific CYP3A4 inhibitor. Results were normalized to control (0.2% DMSO) and mean values±SEM are shown of four individual experiments performed. **P*<0.05 versus control incubations containing 0.2% DMSO; # *p*<0.05 versus co-incubation with ketoconazole.

### 5.4.6. Mitochondrial damage and cytochrome c release caused by amiodarone

Production of ROS can be associated with mitochondrial disruption, leading to a release of cytochrome c into the cytoplasm, a key event in mitochondrial dependent apoptosis and/or necrosis [140]. We investigated mitochondrial damage and leakage of cytochrome c by fluorescence microscopy. For this, we labeled mitochondria with the fluorescent MitoTracker reagent and cytochrome c with a monoclonal antibody. As shown in Fig. 6, 50µM amiodarone induced mitochondrial damage and subsequent release of cytochrome c in *CYP3A4 cells* (as evidenced by a loss of co-localization of mitochondria and cytochrome c in the merged picture). In contrast, *HepG2 wt cells* exhibited strict co-localization of mitochondria and cytochrome c upon amiodarone treatment, demonstrating again the importance of CYP3A4 for amiodarone-associated toxicity. Benzbromarone (100 µM), a previously described mitochondrial toxin [140] induced mitochondrial damage and cytochrome c release in both, *Hep2 wt* and *CYP3A4 cells*.

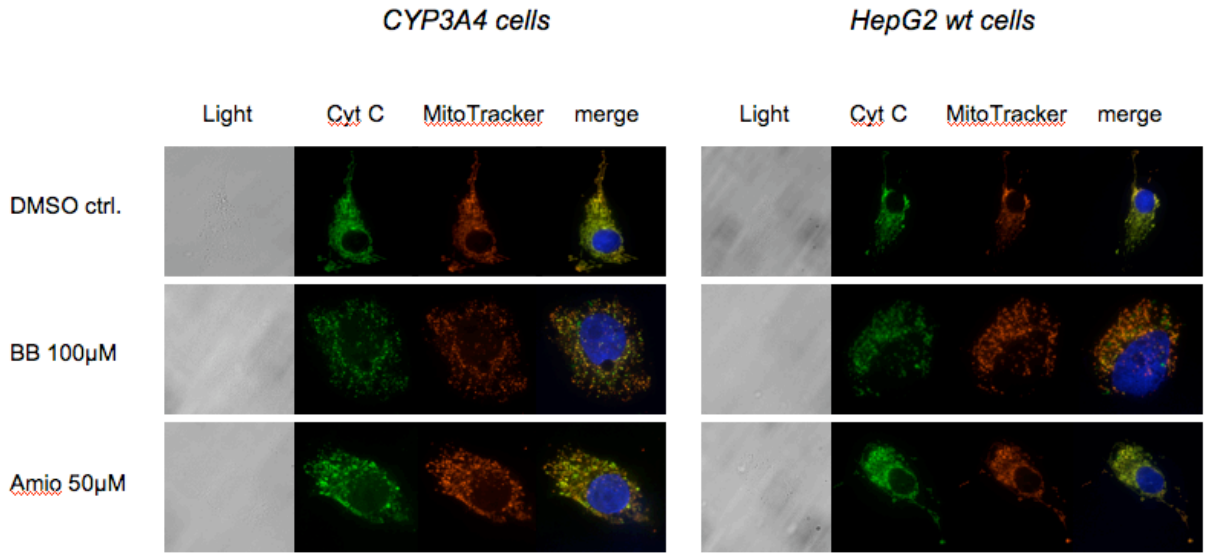



**Figure 6**

*Mitochondrial cytochrome c release detected by fluorescence microscopy. Representative images of mitochondria (MitoTracker, red), cytochrome c (Cyt C, green) and their co-localization (merge, yellow) are shown.* Cells were treated with 50 µM amiodarone for 8 h and cytochrome c release was detected by immunofluorescence staining with a monoclonal antibody against cytochrome c. 0.1% DMSO (DMSO ctrl) was used as negative control and showed maximal co-localization. Benzbromarone (BB) was used as positive control and caused loss of co-localization in both *CYP3A4* and *HepG2 wt cells*.

### 5.4.7. Determination of amiodarone-induced apoptosis and/or necrosis

The ability of metabolized amiodarone to induce early or late stages of apoptosis was investigated using annexin V/propidium iodide staining, which is often used to distinguish between the two [140]. *CYP3A4 cells* showed a significant increase in late apoptosis/necrosis after 24 h of amiodarone treatment (25 and 50 µM) (Fig. 6B). Co-incubation with 1 µM ketoconazole attenuated the effect. Interestingly, there is no significant increase in the percentage of early apoptotic cells (Fig. 6A). However, this result supports the previous finding, that most hepatocytes undergo late apoptosis/necrosis in the presence of 100 µM of amiodarone, MDEA or DDEA [141].

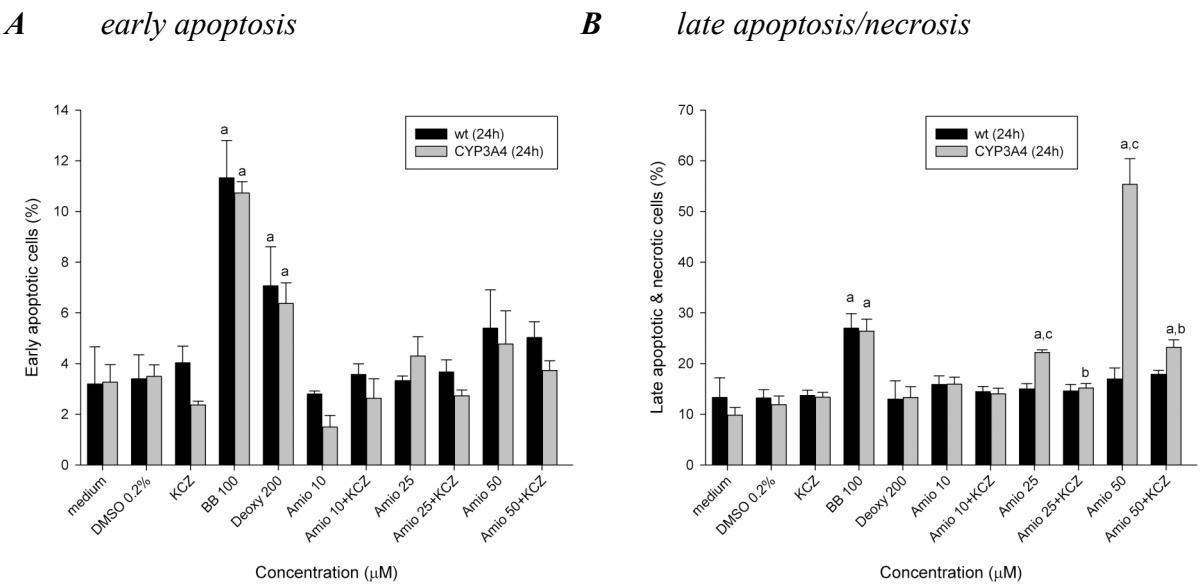



**Figure 7**

*Determination of apoptosis and/or necrosis following amiodarone treatment.* Apoptosis was assessed using fluorescence labeled annexin V and propidium iodide followed by flow cytometry analysis. 200 µM Deoxycholate (Deoxy) was used as a positive control for early apoptosis and 100 µM benzbromarone (BB) as a positive control for early and late apoptosis/necrosis [140]. 1 µM ketoconazole (KCZ) was used as specific CYP3A4 inhibitor. Mean values±SEM of three independent experiments are shown. **a** $p<0.05$ versus control incubations containing 0.2% DMSO; **b** $p<0.05$ versus co-incubation with ketoconazole; **c** $p<0.05$ *CYP3A4 cells* versus *HepG2 wt cells*.

## *5.5.* Discussion

Amiodarone (2-n-butyl-3-[3,5 diiodo-4-diethylaminoethocybenzoyl]-benzofuran) is metabolized through mono- [159] and bi-desalkylation [160] to the corresponding secondary MDEA and primary amine DDEA, respectively. The primary amine can subsequently be transaminated and oxidized to the corresponding acid or primary alcohol [160]. In a previous study we have demonstrated that amiodarone as well as the main metabolites MDEA and DDEA are hepatotoxic [141]. These compounds inhibit the respiratory chain, impair mitochondrial β-oxidation, and/or uncouple oxidative phosphorylation [140, 141, 158]. It is very likely that the metabolites (MDEA and DDEA) are at least partially responsible for the hepatic toxicity in patients treated with amiodarone. Therefore, induction of CYP3A4, the main cytochrome P450 isoenzyme responsible for amiodarone deethylation [161], may largely contribute to the observed hepatotoxicity. To address this question, we established two bioactivation systems, namely an intracellular (*CYP3A4 cells*; HepG2 cells overexpressing hCYP3A4) and an extracellular activating system (*3A4 supersomes*; HepG2 cells supplied with CYP3A4 supersomes). This allowed us to investigate whether there was a difference in toxicity depending on the site of metabolite formation (intra- or extracellular). At the same time, we could determine whether the metabolites were sufficiently stable in order to penetrate the cell membrane, possibly providing important information for the *in vivo* situation. To validate the two activation systems, we used the test compound quinidine [163], which requires metabolic activation through CYP3A4 to manifest cytotoxicity. 3-hydroxyquinidine is the main toxic metabolite of quinidine, causing heart, renal and a hepatic injury [165]. Quinidine showed dose-dependent cytotoxicity in both cell systems studied when compared to the control incubations (*HepG2 wt cells* and *control supersomes*) (Fig. 2A). Besides quinidine, we also tested amitriptyline [163] to assess the detoxification capacity of the two systems. Amitriptyline is associated with cardiac and CNS toxicity [163] and is detoxified by 10'-hydroxylation via CYP3A4. In both systems tested, amitriptyline exhibited a reduced cytotoxicity in the presence compared to the absence of CYP3A4 (Fig 2B). CYP3A4 dependency was further confirmed by using ketoconazole as CYP3A4 inhibitor. Our results clearly show that there is no difference between intra- and extracellular metabolite formation. Taken together, our data for quinidine and amitriptyline are consistent with those reported by Vignati et al. [163], who used transiently transfected HepG2 cells with CYP3A4 as well as HepG2 cells co-incubated with CYP3A4 supersomes.

We next tested the toxicity of amiodarone in *CYP3A4 cells* and *3A4 supersomes*, as amiodarone is mainly metabolized by N-deethylation via CYP3A4 [161]. Since Waldhauser et al. [141] has shown that both N-desethyl-metabolites are cytotoxic, we were interested whether the generation of these metabolites is the reason for cytotoxicity in *CYP3A4 cells* compared to *HepG2 wt cells*, which did not metabolized amiodarone. After incubation for 24 h with cells containing CYP3A4 (*CYP3A4 cells* or HepG2 co-incubated with CYP3A4 supersomes, *3A4 supersomes*) amiodarone showed a dose-dependent significant increase in cytotoxicity, suggesting the formation of toxic metabolites in both systems. Consistently we detected the generation of MDEA and DDEA exclusively *CYP3A4 cells* and *3A4 supersomes*, whereas *HepG2 wt cells* or *control supersomes* failed to do so. These results indicated that MDEA and/or DDEA are responsible for the cytotoxicity of amiodarone in both CYP3A4 containing systems. For further mechanistic investigations we used the *CYP3A4 cells* as test system due to the easier handling and cost saving.

The exact mechanisms leading to cytotoxicity of amiodarone are still not completely understood, but are assumed to involve accumulation of metabolites as well as the parent compound. Resulting cellular toxicity possibly arises from impaired mitochondrial function. Our findings on mitochondrial toxicity of amiodarone are in accordance with data from previous studies [141]. Several other studies have described hepatic mitochondrial toxicity of amiodarone *in vivo* [157] and *in vitro* [156] [140-142, 158, 164]. Taking into account that MDEA and DDEA are strong inhibitors of the respiratory chain and both are associated with ROS production and a remarkable cytotoxicity [141, 164], we addressed the question whether induction of CYP3A4 represents a risk factor for hepatotoxicity associated with amiodarone. With our established *in vitro* model (*CYP3A4 cells*, representing an CYP3A4 induction system) it should be possible to answer this question. Compared with amiodarone in *HepG2 wt cells*, where only a weak cytotoxicity was observed, ROS production, cytochrome c release, mitochondrial damage and induction of apoptosis and/or necrosis were much more pronounced in *CYP3A4 cells*. The involvement of CYP3A4 for the cytotoxic effect of amiodarone was further confirmed by using ketoconazole as an inhibitor of CYP3A4 mediated metabolism. Our data may be relevant for patients treated with amiodarone, since hepatotoxicity associated with this drug could be more pronounced in patients treated concomitantly with CYP3A4 inducers.

In conclusion, we were able to develop and characterize a cell-based system for studying metabolism and cytotoxicity of amiodarone, a well known mitochondrial toxin. While amiodarone itself revealed only a slight cytotoxicity in our cellular models, cytotoxicity of amiodarone was significantly increased after metabolism by CYP3A4. Since CYP3A4

inducers are used frequently and since hepatotoxicity associated with amiodarone is potentially fatal, our *in vitro* observations may be clinically important and should therefore be further investigated.

# 6. Discussion

Drug interactions remain an important concern in clinical practice and drug development. A number of clinical drug interactions have been attributed to the induction and/or inhibition of cytochrome P450s (CYPs). The clinical consequences range from lack of therapeutic efficacy to severe toxicity and, in extreme cases, to fatality. The CYP isoenzymes are a family of monooxygenases that catalyze the metabolism of a variety of endogenous and exogenous compounds, including xenobiotics, steroids and fatty acids. Among the human CYP enzymes CYP1A2, 2C9, 2C19, 2D6, and 3A4 have been described to contribute to the metabolism of the vast majority of drugs [166]. The human CYP3A subfamily includes CYP3A4, 3A5, 3A7 and 3A3, but CYP3A4 notably has the highest abundance in the liver (less than 40%) and metabolizes more than 50% of the clinically used drugs [167, 168]. This high metabolic activity and broad range of target substrates provides the basis for many drug interactions.
Drug interactions with clopidogrel are an important clinical factor contributing to the response variability of clopidogrel [29, 48, 64]. Clopidogrel is a prodrug that requires *in vivo* conversion mainly by CYP3A4 to an active metabolite in order to exert its antiplatelet effect [37, 38, 77]. Given the important role of CYP3A4 in the bioactivation of clopidogrel, drugs that inhibit this enzyme may reduce the antiplatelet effect of clopidogrel. Regarding drug interactions affecting clopidogrel activation, the currently available data are controversial. Therefore, this thesis investigates clopidogrel drug interactions, its cytotoxicity and how these parameters affect clopidogrel functionality.

In the first part of this thesis we designed an *in vitro* model to study drug interactions with clopidogrel. We utilized human liver microsomes since they include representative CYP enzymes expressed in the liver. Using HPLC, we could trace the microsome-mediated metabolization of clopidogrel in presence of various CYP inhibitors. In agreement with others[37, 38, 77] we found that clopidogrel is mainly metabolized by CYP3A4. We could confirm these findings by using CYP3A4 supersomes, which exclusively express CYP3A4. Furthermore, clopidogrel conversion was abolished by specific inhibition of CYP3A4 with ketoconazole. These experiments clearly show that clopidogrel metabolization is dependent on CYP3A4 and validate our *in vitro* model as powerful tool to study drug interactions. Clopidogrel metabolization correlated with functional assays, namely the aggregation of platelets, which is the primary pharmacodynamic effect of clopidogrel. Platelet aggregation was strictly depended on the clopidogrel dose and CYP3A4 inhibitors could interfere with its antiplatelet effect.

The carboxyl metabolite of clopidogrel defines 85% of drug-related compound in the body. This carboxylic acid derivative could theoretically be activated and contribute to the antiplatelet effect of clopidogrel. However, this metabolite was not metabolized by CYP3A4 in our systems and could not prevent platelet aggregation. We assume that the carboxyl metabolite does not bind the active site of CYP3A4 due to unfavorable electrostatic interactions, which may explain our findings. This was indirectly confirmed by Sanofi (the manufacturer of Plavix®), since they measured a pKa ≈ 4 for the carboxyl metabolite (unpublished communication).

Interestingly, atorvastatin and simvastatin interact with the metabolization of clopidogrel in our *in vitro* system. As a consequence, these statins also lowered the antiplatelet effect of clopidogrel in our experiments, which has been debated in the literature. On the other hand, pravastatin, which is not metabolized by CYP3A4, did not influence clopidogrel activation. Therefore these results argue for a drug interaction between clopidogrel and atorvastatin or simvastatin. However, it remains to be elucidated whether our findings can be extrapolated to a more physiological situation.

Clopidogrel is also activated by CYP2C19 in our experiments, but only at low concentrations (≤ 20µM). The concentration of clopidogrel in the liver is unknown. However, based on the carboxyl metabolite concentration in plasma, we estimated the clopidogrel concentration in hepatocytes to be in the range of 10-20 µM. This suggests that clopidogrel potentially interacts with both CYP3A4 and CYP2C19 under physiological conditions. Another hint for a role of CYP2C19 in converting clopidogrel comes from two studies [67, 125], which investigated patients with CYP2C19 polymorphisms. The authors found that the efficacy of clopidogrel was diminished due to the reduced contribution of CYP2C19. Along the same lines, the PPI omeprazole, which is primarily metabolized by CYP2C19 and to a lower degree by CYP3A4, interfered with the antiplatelet effect of clopidogrel [88]. This further argues that CYP2C19 is also involved in clopidogrel activation. Similarly, a recent clinical study reported that omeprazole diminished the antiplatelet effect of clopidogrel probably due to the inhibition of CYP2C19, although the authors did not explicitly investigate this interaction [93]. However, other PPIs with high affinities for both CYP3A4 and CYP2C19 did not show an interaction with clopidogrel [93, 97]. Therefore, the identification of CYP2C19 as clopidogrel metabolizing enzyme is challenging and its significance requires further investigation.

The first part of this thesis describes a powerful *in vitro* system to study drug interactions with clopidogrel. We demonstrated that drugs interfering with clopidogrel activation are responsible for a diminished antiplatelet effect associated with clopidogrel. However, further

investigations are necessary to estimate how predictable our *in vitro* findings are for the clinics.

The second part of this thesis specifically investigates the hepatotoxicity of clopidogrel. Since clopidogrel requires metabolic activation we decided to design a cellular activation system to study clopidogrel's toxicity.

Traditionally, primary hepatocytes were employed since they contain a great number of drug metabolizing enzymes in an active state. However, the use of human hepatocytes is limited due to poor availability and high costs. Moreover, experimental outcome is associated with significant variability [169, 170]. Therefore we selected the hepatocyte cell line HepG2 as representative model although they contain only limited CYP activity [171, 172]. Residual HepG2 cytochrome activity was not sufficient to mediate clopidogrel metabolism and therefore we stably transduced these cells with human CYP3A4. The resulting CYP3A4 overexpressing cell line metabolized clopidogrel in a time- and dose-dependent manner and could be inhibited with a specific CYP3A4 inhibitor. Similar results were obtained when HepG2 wild type cells were used in combination with CYP3A4 supersomes. This validates our cellular system to study CYP3A4 dependent clopidogrel activation and resulting cytotoxicity. In wild type cells, a toxic effect could only be measured at concentrations (1 mM) that obviously exceed the physiological concentrations of clopidogrel in the liver.

The metabolic activation of clopidogrel subsequently generated a toxic effect, which is most likely mediated by the active metabolite. Presumably this metabolite is highly reactive due to its thiol group, which prevents it from being directly detected in our assays. We approached this issue by measuring the decrease in the intracellular glutathione content. Indeed, the CYP3A4 mediated conversion of clopidogrel was associated with a drop in the cellular glutathione content. These results indicate that the reactive metabolite of clopidogrel is mostly responsible for the cytotoxicity.

In a next step, we aimed to identify a mechanism for the observed toxicity. We found that a mitochondrial dependent pathway leads to apoptosis in our CYP3A4 overexpressing cells. An oxidative stress reaction (as evidenced by decreased glutathione levels) promotes elevated ROS levels, cytochrome c release from damaged mitochondria and the exposure of early apoptotic markers on the cell surface. Importantly, the carboxyl metabolite, which is generated by esterases outside the liver, did not show detectable toxicity in our assays, therefore excluding this metabolite as candidate for cytotoxicity associated with clopidogrel.

Taken together, we could show that clopidogrel is converted in a CYP3A4 dependent way to a reactive metabolite, which is at least partly responsible for the cytotoxic effects. Our cellular system allowed us to study clopidogrel-associated toxicity in great detail and reveal the mechanism responsible for hepatic cell death. However, it remains important to compare these *in vitro* results to a more physiological situation, especially when CYP3A4 is not overexpressed.

Taking advantage of our *in vitro* activation system, we measured the cytotoxicity of the antiarrhythmic agent amiodarone in the third part of this thesis. Since amiodarone is mainly metabolized by CYP3A4 [161], we speculated that the *CYP3A4 cells* or *3A4 supersomes* could convert amiodarone to MDEA and DDEA, which would explain the cytotoxicity. Indeed, cytotoxicity of amiodarone was significantly increased after metabolism by CYP3A4 in both of our activation systems. By HPLC we detected the generation of MDEA and DDEA uniquely in CYP3A4 overexpressing cells and 3A4 supersomes. Therefore we provide direct evidence that these two metabolites are generated upon CYP3A4 induction and are responsible for the toxic effect associated with amiodarone. In contrast, amiodarone exhibits only a weak toxic effect in HepG2 wild type cells (in absence of CYP3A4 activity). Only the induced metabolism of amiodarone in CYP3A4 overexpressing cells led to an increase in ROS formation, mitochondrial damage and subsequent release of cytochrome c and finally triggered apoptosis and/or necrosis. Inhibiting CYP3A4 activity with ketoconazole significantly reduced all events related to amiodarone cytotoxicity, thereby proving that the effect was specific.

Our *in vitro* system reflects a clinical situation where CYP3A4 is induced. CYP3A4 inhibitors such as rifampicin or phenytoin are frequently administered concomitantly with amiodarone. Herein we give direct evidence that hepatotoxicity of amiodarone is more pronounced when CYP3A4 activity is increased.

In summary, our results attribute the cytotoxic effect of amiodarone in large part to excessive CYP3A4 enzymatic activity, which generates the metabolites MDEA and DDEA and likely represents a situation, where patients experience induced CYP3A4 activity. Since hepaotoxicity caused by amiodarone can even be fatal in rare cases [155], our observations contribute to the understanding of amiodarone cytotoxicity in the clinics and require further investigations.

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**169.** Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, Silber PM. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chemico-biological interactions.* Jun 1 1999;121(1):17-35.
**170.** Gomez-Lechon MJ, Donato MT, Castell JV, Jover R. Human hepatocytes as a tool for studying toxicity and drug metabolism. *Current drug metabolism.* Aug 2003;4(4):292-312.
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# 8. Curriculum Vitae

**Anja-Christina Zahno**

Born October 5, 1977
Swiss Citizen

## Education

| | |
|---|---|
| 02/2005-07/2009 | **PhD student**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland [directed by Prof.Dr.Stephan Krähenbühl, MD, PhD and PD Dr. med. Dimitrios Tsakiris] |
| 01/2008-12/2008 | **Visitor Graduate Student**, Department of Pharmaclogy, University of California San Diego, USA [directed by Prof. Paul Insel, MD and supported by the Swiss Cancer League] |
| 10/2000-11/2003 | **Master of Science (Pharmacy)**, University of Basel, Switzerland |
| 03/2003-08/2003 | **Diploma thesis**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland [directed by Prof.Dr.Stephan Krähenbühl, MD, PhD]<br>▪ Thesis topic: "Mitochondrial toxicity of statins" |
| 09/2001 | **Assistance-diploma of Pharmacy** |
| 08/2000-08/2001 | Practical year in the Pharmacy (Gurmels) and Hospital Pharmacy (Fribourg), Switzerland |
| 10/1998-7/2000 | **Basic Study of Pharmacy**, University of Fribourg, Switzerland |
| 09/1997-07/1998 | **Diploma of language french**, Alliance Française Versailles, France |
| 08/1993-07/1997 | **High school Type C** (mathematics, physics and chemistry), St. Michel in Fribourg, Switzerland |

## Professional Experiences

Since 09/2009 — **Post doc**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland

02/2005-07/2009 — **PhD student**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland [directed by Prof.Dr.Stephan Krähenbühl, MD, PhD and PD Dr. med. Dimitrios Tsakiris]

- Thesis topic: "Drug-drug interactions and hepatic toxicity of clopidogrel"
- Other tasks:
  - Clinical Pharmacological Service of the University Hospital Basel
    - Answering inquiries concerning pharmacological or toxicological questions
    - Presentation of current pharmacological problems/questions to medical professionals
  - Therapeutic Drug Monitoring (TDM)
    - Monitoring and Evaluation of drug concentrations and dose adjustments
  - Participant of Mentoring Program WIN 06/07, Novartis & University of Basel

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| 2008 | **Visitor Graduate Student**, Department of Pharmacology, University of California, San Diego, USA<br>▪ Research topic: "Targeting components of the cyclic AMP pathway as novel treatments for chronic lymphocytic leukemia"<br>▪ Other tasks:<br>o Mentored and trained 2 undergraduates lab internships in lab based techniques and presentation skills. |
| 2005-2007 | **Assistant at the practical course "Pharmacology and Toxicology"**, University Hospital Basel, Switzerland |
| 01/2007-07/2007 | **Supervisor of a master student in pharmacy**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland |
| 04/2004-12/2007 | **Pharmacist**, Pharmacy am Lindenplatz, Allschwil, Switzerland<br>▪ Customer care in the pharmacy<br>▪ Supervision of the working shifts |
| 2003-2004 | **Pharmacist**, Pharmacy Handschin, Gelterkinden, Switzerland<br>▪ Customer care in the pharmacy |
| 2002 | **Assistence pharmacist**, Central Station Pharmacy Hörning, Bern, Switzerland |
| 2001-2003 | **Assistence pharmacist** Pharmacy Gurmels, Gurmels, Switzerland |

## Professional Memberships

American Society for Pharmacology and Experimental Therapeutics (ASPET)
Swiss society of pharmacy (SAV)

## Congress Participations

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|---|---|
| **2010** | **Annual Research Meeting 2009, Department of Pharmaceutical Sciences, University Basel, Switzerland**<br>*poster presentation* (Winner of Best Poster) |
| **2009** | **Experimental Biology 2009, New Orleans, LA, USA**<br>*Oral presentation & poster presentation*<br>Winner of Best Abstract Award in ASPET Division for Clinical Pharmacology, Pharmacegenomics & Translational Medicine<br>Winner of a Graduate Student Travel Award in ASPET<br><br>**Annual Research Meeting 2009, Department of Pharmaceutical Sciences, University Basel, Switzerland**<br>Oral presentation<br><br>**SCAHT Symposium (Swiss Centre for Applied Human Toxicology), Geneva, Switzerland**<br>*poster presentation* |

## Publication Record

Zahno A, Brecht K, Bodmer M, Bur D, Tsakiris DA, Krähenbühl S. Drug interactions with biotransformation and antiplatelet effect of clopidogrel *in vitro*. BJP 2010; *in press*.

Zhang L, Murray F, Zahno A, Kanter JR, Chou D, Suda R, Fenlon M, Rassenti L, Cottam H, Kipps TJ, Insel PA. Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia. *PNAS 2008;* 105(49): 19532-37.

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Kaufmann P, Torok M, Zahno A, Waldhauser KM, Brecht K. Krahenbuhl S. Toxicity of statins on rat skeletal muscle mitochondria. *Cell Mol Life Sci 2006;* 63: 2415-25.