Myelotoxicity of thienopyridines

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Dedication

To my Dad

Acknowledgements

It is a difficult task to acknowledge all people who influenced my life during the last three years and contributed, in different ways, to this thesis. I am very grateful to all my friends and colleagues; however, I would like to add some personal words.

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Table of contents

This thesis is presented in the form of three scientific papers that have either been published or are in preparation. Reference lists for each paper are presented at the end the relevant section. A reference list covering the general introduction and discussion is at the end of the thesis.

Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AK	Adenylate kinase
ASA	Aspirin
CCA	Clopidogrel carboxylate
CAPRIE	Clopidogrel versus Aspirin in Patients at Risk Ischemic Events
CFU-GM assay	Colony-forming unit granulocyte/macrophage assay
Clp	Clopidogrel
COX-1	Cyclooxygenase-1
CURE	Clopidogrel in Unstable angina Recurrent Events
CYP	Cytochrome P450
Cys	Cysteine
Cyt C	Cytochrome C
DCF	Dichlorofluorescein
DCFH-DA	2′,7′- Dichlorofluorescein-diacetate
DEM	Diethly-maleate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithiobis (2-nitrobenzoic acid)
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenas
GP	Glycoprotein
GSH	Glutathione
GSSG	Oxidised glutathione
hMNC	Human mono nuclear cells

- HCl Hydrochloric acid
- HSC Hematopoietic stem cells
- HL-60 Human promyelocytic leukemia cells
- IMDM Iscove's modified Dulbecco's medium
- KCl Potassium chloride
- LC/MS Liquid Chromatography/Mass Spectrometry
- MAO Monoamine oxidase
- $MgCl_2$ Magnesium chloride
- MI Myocardial infarction
- MPO Myeloperoxidase
- NaCl Sodium Chlorie
- NAPDH β-nicotinamide adenine dinucleotide phosphate
- PBS Phosphate saline buffer
- PCI Percutaneous intervention
- PI Propidium iodide
- Psr Prasugrel
- ROS Reactive oxygen species
- R-130964 Active metabolite of clopidogrel
- R-95913 Inactive thiolactone metabolite of prasugrel
- R-138727 Active metabolite of prasugrel
- rhCYP3A4 Recombinant human CYP3A4
- mRNA Messenger ribonucleic acid
- RT-PCR Real time polymerase chain reaction
- SD Standard deviation
- STS Staurosporine
- Tcp Ticlopidine
- TMRE Tetramethylrhodamine ethyl ester
- TNB 5-thio-2-nitrobenzoic acid
- TTP Thrombocytopenic purpura

- TXA_2 Thromboxane A2
- UR-4501 Active metabolite of ticlopidine
- VWF Von Willebrand factor

1. Summary

Ticlopidine, clopidogrel and prasugrel are thienopyridine derivatives used for inhibition of platelet aggregation in several cardiovascular diseases. Besides hepatotoxicity, also bone marrow toxicity may limit their use. The mechanisms associated with myelotoxicity of thienopyridines are currently unclear. Uncovering the mechanism by which thienopyridines lead to these adverse effects is therefore of great importance. In this thesis, three different cell systems which include human hematopoietic stem cells isolated from umbilical cord blood, neutrophils isolated from peripheral blood and human promyelocytic leukemia cell line were used. The main aims of this thesis were to investigate whether the mother substance and/or the active metabolites are responsible for myelotoxicity and whether the inactive clopidogrel metabolite clopidogrel carboxylate contributes to myelotoxicity. Apart from the well-known CYP-mediated metabolism of thienopyridines in liver, MPO present in neutrophils also metabolise clopidogrel, ticlopidine, prasugrel, and clopidogrel carboxylate. In contrast, clopidogrel carboxylate was not metabolized by human CYP3A4. MPO-dependant metabolism of clopidogrel, ticlopidine, prasugrel, and clopidogrel carboxylate showed dose dependent cytotoxicity. Similarly, CYP3A4-mediated metabolism showed dose dependent cytotoxicity of all drugs except clopidogrel carboxylate. Taking into account the pharmacokinetics in humans, we conclude that the myelotoxic principle of clopidogrel is most probably associated with metabolite formation from clopidogrel carboxylate by myeloperoxidase. For ticlopidine and prasugrel the mother substance itself and metabolites formed by myeloperoxidase are myelotoxic. Both mother substance and metabolites formed by MPO or CYP3A4 showed mitochondrial mediated cytotoxicity.

2. Introduction

2.1 Thrombosis

Thrombosis is the formation of a blood clot within a blood vessel, obstructing the flow of blood. A blood clot can occur anywhere in the body's bloodstream. There are two forms of thrombosis, venous thrombosis and arterial thrombosis. Venous thromboembolism is a blood clot that develops in veins. Arterial thrombosis is the formation of a thrombus within an artery, that develops on the chronic lesions of the atherosclerosis and causes myocardial infarction and stroke.[1] It is the most common causes of mortality in developed countries. Platelets play a central role in formation of thrombosis.

2.1.1 Platelets

Platelets play a central role in the haemostatic process. The normal function of platelets is to arrest the bleeding from wounds by adhesion and rapid cellular activation, following the coagulation cascade to form a haemostatic plug. [1]

Platelets also play a key role in pathological counter part, formation of thrombosis. Platelets, which adhere to the vessel wall at sites of endothelial-cell activation, contribute to the development of chronic atherosclerotic lesions, and when these lesions rupture, they trigger the acute onset of arterial thrombosis.[2,3] It is well established that inappropriate platelet activation and thrombus formation plays a major role in onset and progression of arterial thrombotic disorders and therefore antiplatelet therapy is the basis of treatment and prevention.[2]

Platelets are anucleated cells produced by bone marrow derived cell called megakaryocytes. During the maturation of megakaryocytes, the cell undergoes

nuclear endomitosis, organelle synthesis, and dramatic cytoplasmic maturation. The cytoplasm becomes compartmentalized and plasma membrane ruptures releasing fragments, which are condensed to form a disk shaped platelets circulating in blood for about 10 days.[4] Platelets provide a circulating source of chemokines, cytokines, and growth factors, which are preformed and packaged in storage granules.

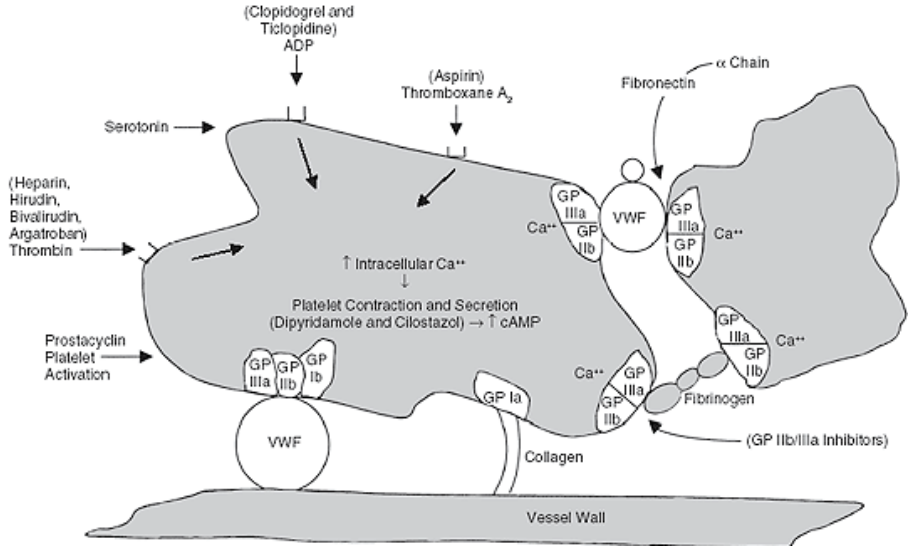


Figure 1 Initiation and amplification of platelet aggregation.

Platelets, when circulating through vessels with an intact, healthy endothelium remains in unactivated state. However, in response to vessel trauma, platelets encounter molecules that trigger its activation, particularly collagen. Platelets spontaneously adhere to collagen and von Willebrand factor (VWF) via their respective receptor, glycoprotein (GP) IIb/IIIa (Figure 1). Following platelet activation, the GPIIb/IIIa receptor undergoes a conformational change allowing the activated platelets to adhere together via bridges formed by the GPIIb/IIIa complex and proteins such as fibrinogen, fibrin. The fibrinogen that connects two platelets via these receptors is found in the blood and also a little is released from the alpha granules.[5] The binding of VWF by the VWF receptor also causes the activated platelets to adhere to collagen under the broken endothelium. In addition,

thromboxane A_2 (TXA_2), Adenosine diphosphate (ADP) and thrombin are other factors that trigger the activation of platelets by changing their shape and release of the contents of their storage vesicles (Figure 2). All these agonists act by common pathway, which leads to increased intracellular calcium concentration through direct ion flux or release of stored calcium.[2] These calcium-dependent processes include change in platelet shape and activation of phospholipase A_2, leading to increase arachidonic acid, which is then converted by cyclooxygenase into TXA_2. The release of TXA_2 causes the releases of ADP from Platelet granules act as positive feed back mediator perpetuating the cycle.[5] Therefore, both ADP and TXA_2 are important targets for antiplatelet therapy. The activated platelets also stimulate the coagulation cascade, by accelerating GPIIb/IIIa receptor affinity for fibrinogen. Fibrinogen, the primary polypeptide involved in platelet aggregation, promotes the cross-linking of adjacent platelets, leading to the formation of a platelet-rich thrombus.

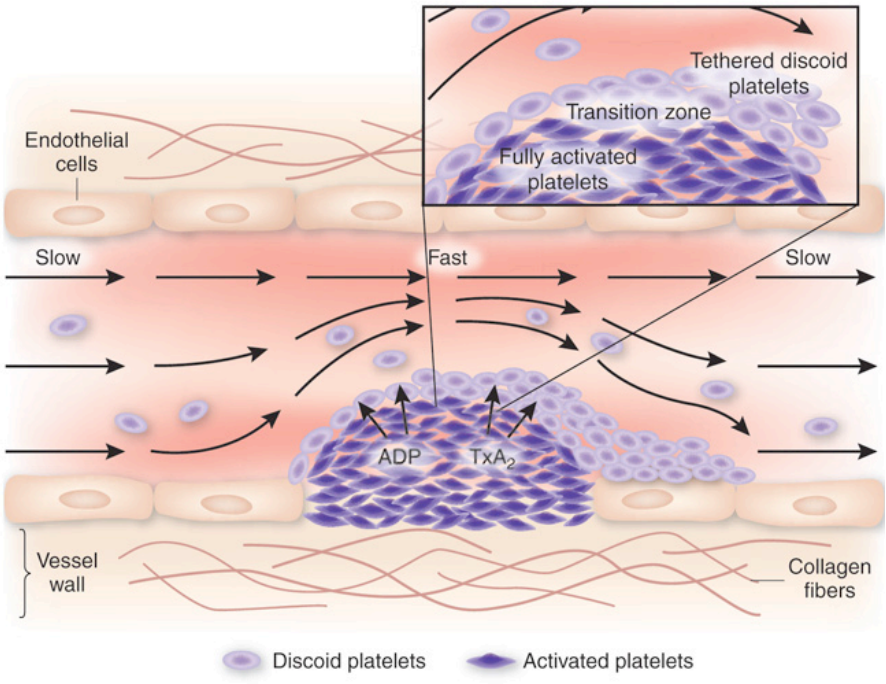
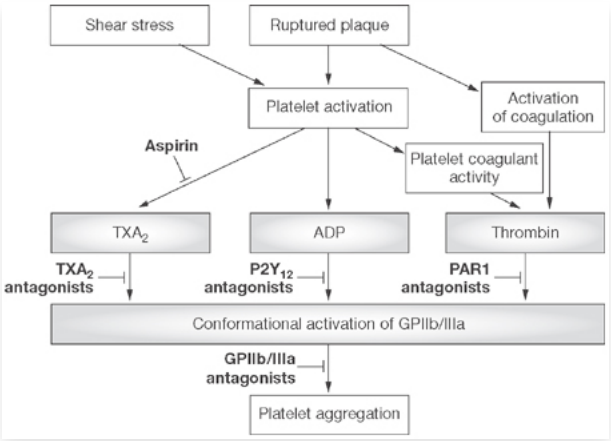


Figure 2 ADP and TxA_2 released by the core of activated platelets help to turn on discoid (unactivated) platelets, causing them to become activated as well. ADP = Adenosine diphosphate, TxA_2 = thromboxane A_2.

2.1.2 Antiplatelet drugs

Antiplatelet drugs are intended to prevent and or reverse platelet aggregation in arterial thrombosis, most prominently in myocardial infarction and ischemic stroke. Antiplatelet agent that critically block thromboxane A_2 synthesis, those that block receptors for ADP, thrombin and thromboxane A_2, and agents that block fibrinogen and other ligands from binding to activated glycoprotein IIb/IIIa receptors have been developed (Figure 3 and Box 1).[6] Aspirin is the most widely used and cost effective drug in prevention of platelet aggregation. Other well established antiplatelet strategies include P2Y12 inhibitors and GPIIb/IIIa antagonists. However, the pathways that promote thrombosis are critical to hemostasis, treatment with antiplatelet drugs is usually associated with an increased risk of bleeding. Furthermore, several agonists could initiate platelet activation, increased antithrombotic efficacy could reasonably be obtained by blocking more than one pathway of platelet activation, but such strategies incur the cost of an increased risk of bleeding.



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 3 Various sites of action of platelet inhibitors.

Box 1 Various antiplatelet agents and corresponding drug classes.

GP, glycoprotein; PAR, protease-activated receptor; TXA_2, thromboxane A_2.[6]

2.2 Aspirin

Aspirin is an ancient drug synthesized over a century ago with multiple pharmacological actions and therapeutic indications. Aspirin exerts its antiplatelet effect by irreversible acetylation and inhibition of cyclooxygenase-1 (COX-1), the enzyme that catalyses the first step in the synthesis of thromboxane A_2, a potent platelet agonist.[7] Aspirin inhibits COX-1 activity by transferring its acetyl moiety to a serine residue (Ser-530) located at the substrate channel and close to the catalytic centre. This acetyl group prevents the contact of arachidonic acid with the active site Tyr-385 (Figure 4). Since acetylation of serine is irreversible, inhibition of platelet TXA_2 production by aspirin persists for the entire life span of a platelet (7-10 days). Platelet COX-1 is sensitive to aspirin, and so low-dose of aspirin is sufficient to inhibit that platelet aggregation.[8]

Aspirin is effective in the treatment of arterial thrombosis and reduced the risk of vascular death by 15%, nonfatal myocardial infarction by 30% and nonfatal stroke by 25% in a broad range of patients. However, room for improvement remains, as aspirin inhibits the synthesis of only one platelet agonist (thromboxane A_2), its limited efficacy as an antithrombotic agent is not surprising. The other mediators in platelet activation (ADP, collagen, fibrinogen) overcome the antiplatelet effect of aspirin. Hence the identification of more potent antiplatelet agent is necessary to prevent the side effects in patients with higher risk.[9]

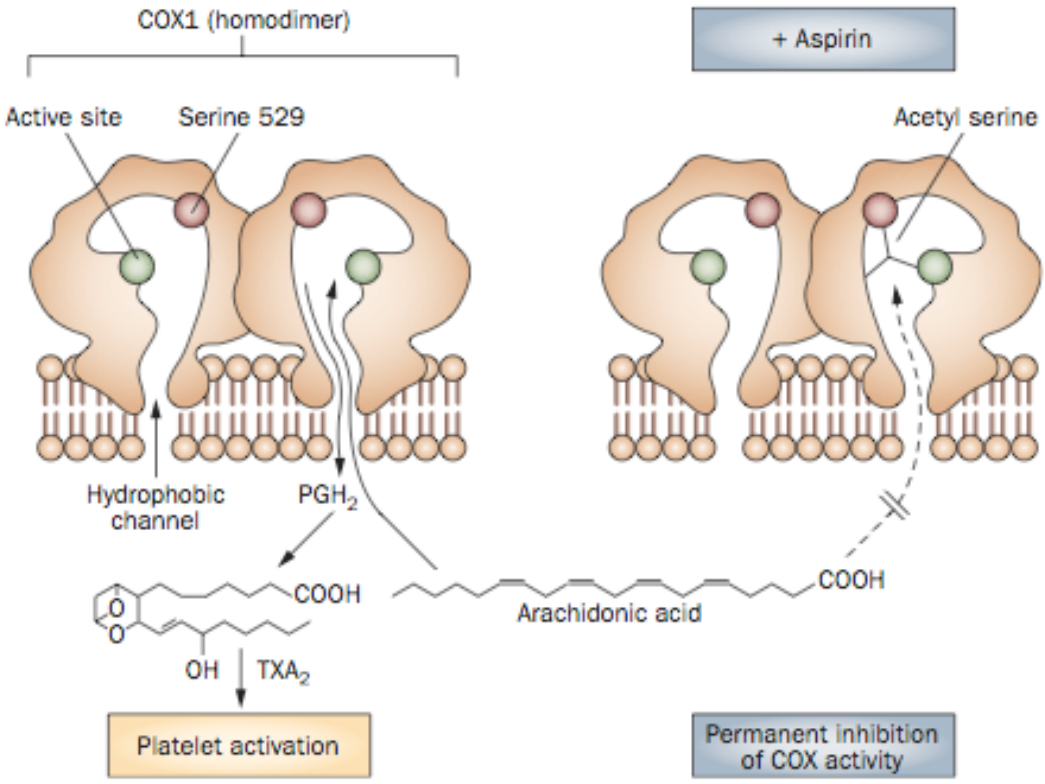


Figure 4 Mechanism of aspirin inhibition of cyclooxygenase-1.[9]

TX A_2, thromboxane A_2; PGH2, prostaglandin H2; COX-1, cyclooxygenase-1.

2.3 Thienopyridines

Thienopyridines are a class of ADP-receptor antagonists, which inhibits platelet P2Y12 receptor irreversibly. These are potential antiplatelet agents used in treatment and prevention of cardiovascular diseases especially in patients with acute coronary syndrome after stent implantation.[10] Thienopyridines are prodrugs, which need to be activated to exert their mode of action. The drugs include ticlopidine (Ticlid), clopidogrel (Plavix) and prasugrel (Efient). In patients with a broad spectrum of cardiovascular disease, these ADP antagonists are more effective than aspirin in the prevention of major cardiovascular events (MI, stroke or death).[11]

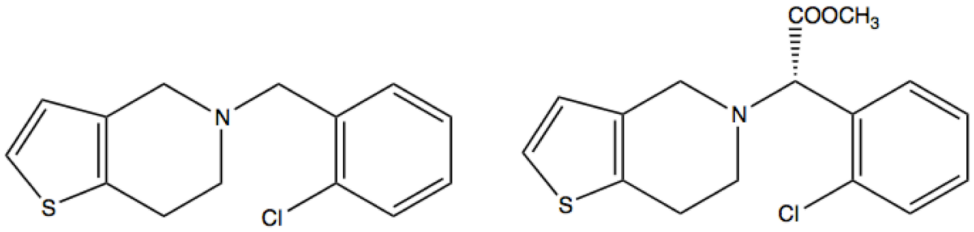
2.3.1 Ticlopidine

Ticlopidine, a first generation of thienopyridine was discovered in 1972 through a battery of screening tests performed *in vivo* while looking for anti-inflammatory compounds. The anti aggregation properties of ticlopidine in platelets led to its development as an antithrombotic drug years later. Ticlopidine was introduced to the market in 1978 in a very narrow therapeutic indication for prevention of thrombosis during coronary stent placement. The drug has been found to significantly reduce infarction size in acute myocardial infarcts and was an effective antithrombotic agent in cerebrovascular disease, venous thrombosis, and arteriosclerosis, with an efficacy that is similar to aspirin.[12] In patients, ticlopidine at a daily dose of up to 500 mg per day inhibit 50-70% of platelet aggregation with no further increase of inhibition at higher dose. Though ticlopidine was efficacious in secondary prevention of ischemic stroke, however, its use was also associated with significant and sometimes fatal adverse dyscrasias. The adverse side effects of ticlopidine include neutropenia/agranulocytosis, thrombotic thrombocytopenic purpura (TTP), aplastic anaemia and bleeding complications. This drug is rarely used nowadays for patients allergic to other antiplatelet drugs.[5]

2.3.2 Clopidogrel

Clopidogrel, the second generation of thienopyridines antiplatelet agent with similar efficacy, has largely replaced ticlopidine due to its better tolerability profiles and is the antiplatelet treatment for prevention of stent thrombosis. The patent for clopidogrel was first submitted in 1987, and clopidogrel was approved in western countries in 1997 for the prevention of ischemic stroke, myocardial infraction, and vascular death in patients with atherosclerosis (CAPRIE trial= Clopidogrel versus Aspirin in Patients at Risk Ischemic Events). [13] After the CURE trial (Clopidogrel in Unstable angina Recurrent Events), the use of clopidogrel in addition to standard therapy (including ASA) was approved for the reduction of atherothrombotic events

in patients with acute coronary syndromes. [14] In patients, standard clopidogrel dose of 300 mg was administered to prevent stent thrombosis, followed by a maintenance dose of 75 mg per day. Clopidogrel at a daily dose of 75 mg per day inhibits platelet aggregation by 40-60%. [15]



Ticlopidine Clopidogrel

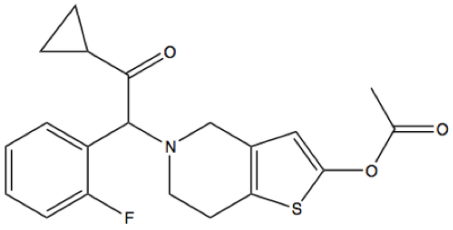
Figure 5 Chemical structures of ticlopidine and clopidogrel

Clopidogrel (Plavix) the second generation of thienopyridines is structurally related to ticlopidine (Figure 5). Structurally, the presence of a methoxycarbonyl group on the benzylic position provides an increased pharmacological activity and a better safety (lower incidence of adverse effects such as neutropenia and TTP) and tolerability profile compared with those seen with ticlopidine.[16] The S-enantiomer of clopidogrel has essential pharmacological activity when compared to R-enantiomer lacking antithrombotic activity in animal experiments. Clopidogrel exhibited potent dose-dependent antithrombotic activity in several models of arterial thrombosis and was 50-100 fold more potent than ticlopidine or aspirin.[12] Yet, because of insufficient clopidogrel-induced platelet inhibition, a significant number of patients remain at risk for subsequent death, MI, stent thrombosis, and stroke.

2.3.3 Prasugrel

Prasugrel, the third available thienopryrine, is 10 times more potent than clopidogrel in preclinical studies (Figure 6).[17] Prasugrel has been shown has been shown in

preclinical studies to be more potent and to have a more rapid onset of action than clopidogrel. A comparable efficiency and safety has been shown between prasugral and clopidogrel in JUMBO-TIMI study.[18] The TRITON-TIMI study has evaluated that prasugrel treatment reduced the death rate in cardiovascular diaseases, myocardial infraction and strokes in acute coronary sydromes when compared to clopidogrel.[6] However certain populations with acute coronary syndrome (ACS) may be at higher risk for major bleeding leading to fatal events when using prasugrel.



Prasugrel

Figure 6 Chemical structure of prasugrel.

Prasugrel at 60 mg loading dose and 10 mg per day maintaince dose inhibited 80% of platelet aggregation when compared to 300 mg of clopidogrel inhibiting 40-60% of platelet aggragation. Prasugrel has demonstrated a greater platelet inhibition and a decreased incidence of ischemic events compared with clopidogrel, but with an increased risk of fatal bleeding events. [19]

2.4 Metabolism of thienopyridines

The thienopyridines are prodrugs needing enzymatic bio activation by cytochrome P450 (CYP) in order to exert their anti-platelet effects. The active metabolites produced by the thienopyridines contain a thiol group, which covalently modifies and inactivates the ADP receptor on the platelet surface in a highly specific and irreversible manner.[12,20] All the thienopyridines must undergo a two-step metabolism to exert anti-platelet activity. Metabolism of ticlopidine to its active metabolite involves a two-step process mediated by liver cytochrome P450 enzymes (CYP). Ticlopidine after oral administration is oxidised to 2-oxo ticlopidine, which is then hydrolysed to generate the active metabolite (UR-4501).[21]

After oral administration of clopidogrel, undergoes rapid hydrolysis by esterases to form an inactive carboxylic acid derivative in the gut (approximately 85% of clopidogrel is converted to this metabolite).[22,23] only approximately 15% of clopidogrel is converted to active metabolite in 2-step CYP-dependent process. In the first step clopidogrel is oxidised to generate the intermediate metabolite, 2-oxo-clopidogrel with the involvement of CYP2B6 and CYP1A2. Regarding the second step, the formation of active metabolite (R-130964) was catalysed by CYP3A4 and CYP2B6 (Figure 7).[24,25] In addition CYP2C9 and CYP2C19 may also metabolize clopidogrel to a lower extent. The active metabolite contains a thiol group, which binds covalently to ADP receptor on platelet to inhibit the platelet aggregation.[23]

Prasugrel is rapidly converted to inactive thiolactone metabolite (R-95913) by plasma esterases, which then requires just 1 CYP-mediated step to form the active metabolite (R-138727).[26] These differences in metabolism explain why prasugrel is more efficient and reliable compared to clopidogrel. Because the rate and amount of active metabolite formed determines the rate and final extent of ADP receptor blockade on platelets. The prasugrel 60 mg loading dose produces more rapid and greater inhibition of the platelet function than a clopidogrel 600 mg loading dose; similarly

the prasugrel 10 mg daily maintenance dose produces greater inhibition than the clopidogrel 150 mg daily (double dose) maintenance dose.[27]

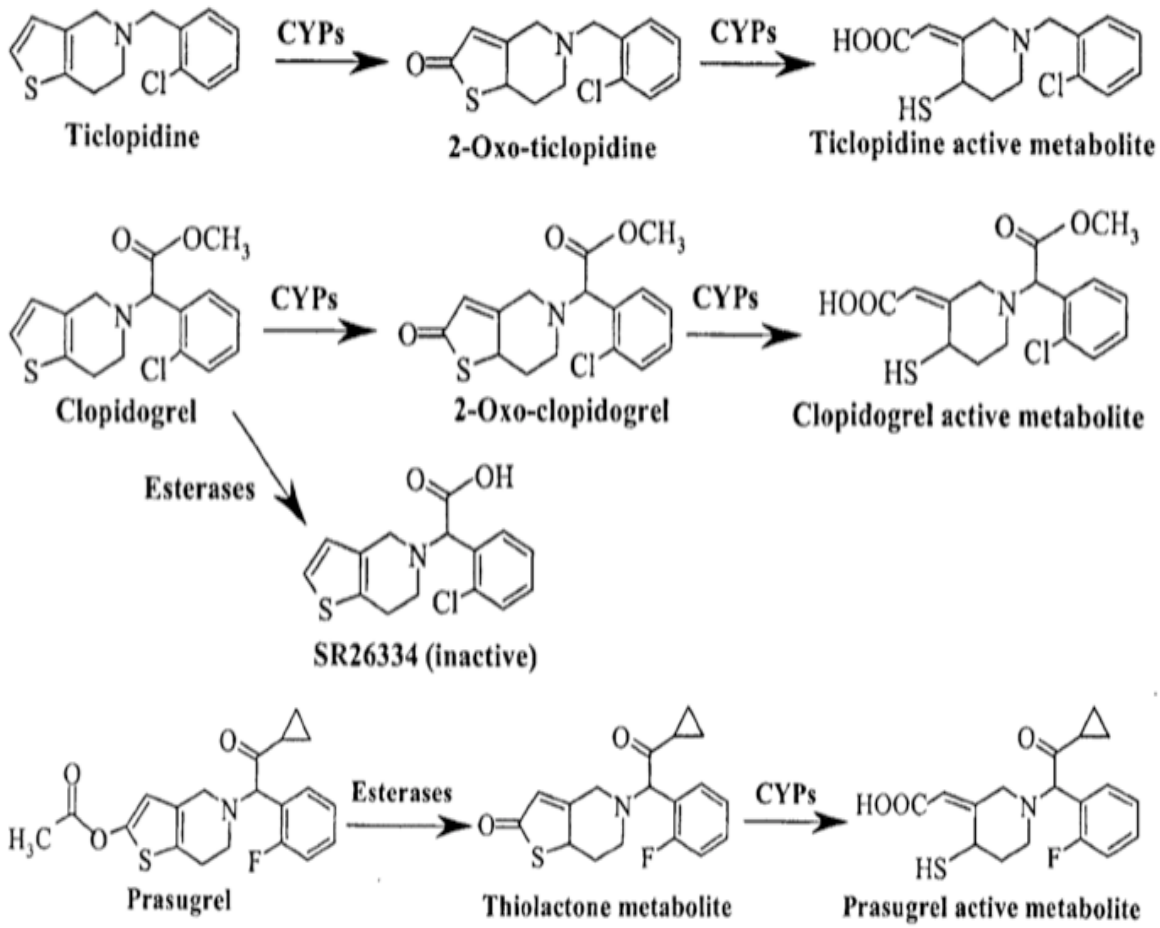


Figure 7 Metabolic pathways of ticlopidine, clopidogrel and prasugrel.[34] CYPs: Cytochrome P450.

2.5 Mechanism of action of thienopyridines on ADP receptors

Adenosine diphosphate (ADP) plays a key role in haemostasis and thrombosis, as it stimulates platelet activation and, when secreted from platelet-dense granules, potentiates the aggregation response induced by other agents. [28] there are two receptors involved in ADP-induced platelet activation. The purinergic $P2Y_1$ receptor, is coupled to G-protein and intracellular calcium mobilization, mediates platelet shape change and initiates aggregation, while another, $P2Y_{12}$ receptor, also coupled

to G-protein is responsible for completion and amplification of the response (Figure 8).[29]

The P2Y12 receptor activation leads to a complex series of intracellular signalling events that yield in activation of GPIIb/IIIa receptor for the recruitment of other platelets to the site of injury subsequent to the adhesion of platelets to VWF and collagen, and granule release in the enhancement of the efficiency of platelet activation by other agonists such as thrombin and thromboxane A_2, which are generated as secondary platelet agonists.[30,31]

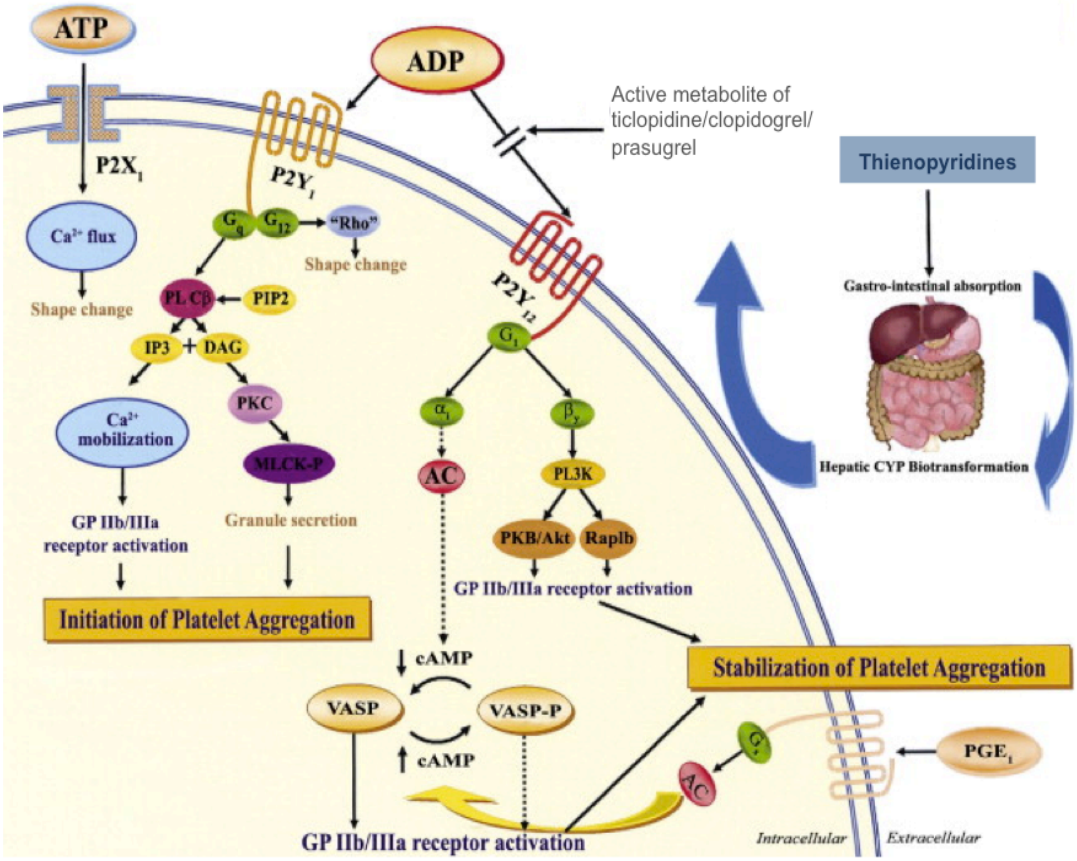


Figure 8 Mechanism of action of thienopyridines. The active metabolites irreversibly bind to the P2Y12 receptors on platelet surface to inhibit the platelet aggregation.[31]

The restricted tissue distribution of the $P2Y_{12}$ receptor makes it an attractive target for antithrombotic agents. The $P2Y_{12}$ receptor has been the target for antithrombotic thienopyridine compounds such as ticlopidine, clopidogrel and prasugrel.[12] The thienopyridines selectively and irreversibly bind to the $P2Y_{12}$ receptor inhibiting the platelet aggregation (Figure 8).[32] The reactive thiol group of active metabolites forms a disulphide bridge between cysteine residues (Cys 17 an Cys270) of $P2Y_{12}$ receptor leads to irreversible inhibition of platelets. The bloackde of $P2Y_{12}$ prevents the platelet degranulation and also inhibits the activation of GPIIb/IIIa receptor for stabilization of platelet aggregation for the life span of platelets.[33,34]

2.6 Adverse drug reactions

Thienopyridine therapy although increased inhibition of P2Y12 and improved platelet function efficacy when compared to aspirin, adverse drug reaction was also increased.

Ticlopidine, and effective antiplatelet agent with a mechanism of action distinct from aspirin, has been shown to be effective for the treatment of cerebro-vascular diseases and coronary arterial diseases. Its use was limited, due to severe adverse effect profile (Table 1).[35] Common adverse effects seen with ticlopidine include gastrointestinal disorders (indigestion, nausea and vomiting), rash and diarrhea.[35] However rare and serious hematologic dyscrasias include neutropenia[36-42], agranulocytosis[36,43-50], aplastic anaemia,[51-56] pancytopenia,[57,58] thrombocytopenia[59-66] and thrombotic thrombocytopenia purpura[59,67-72] Due to these unfavourable side-effect profile and relative lack of efficacy, ticlopidine is rarely used as a first line treatment, unless, otherwise the patients allergic for other antiplatelet drugs.[73]

Table 1: Case parameters for the various types of hematologic dyscrasia associated with ticlopidine therapy

Parameter	Agranulocytosis	Aplastic anemia	Neutropenia*	Pancytopenia	Thrombocytopenia*	TTP*
No. of case reports identified	15	29	64	4	4	95
No. of case reports that could be evaluated	15	29	14	4	3	26
Median age of patients (and range), yr	63 (26–87)	69 (51–85)	65 (40–83)	64 (59–78)	71 (67–72)	72 (37–82)
Sex, no.of patients female/male/unknown	9/6/0	19/10/0	3/7/4	3/1/0	0/3/0	9/12/5
Indication for ticlopidine therapy, no. of cases						
Stroke	7	11	5	–	3	5
Transient ischemic attack	1	5	2	1	–	2
Coronary artery disease	3	5	–	3	–	3
After coronary stent insertion	1	3	7	–	–	10
Peripheral arterial disease	1	3	–	–	–	4
Other	2	–	–	–	–	3
Other possible drug causes (no. of cases)	Digoxin (1) Dipyridamole (3) Enalapril (1)	ASA (3) Furosemide (1) HCTZ (1)	Allopurinol (2) Enalapril (3)	HCTZ (1)	–	–
Other possible nondrug causes (no. of cases)	–	Renal failure (1)	Renal failure (3)	–	–	–

Note: TTP = thrombotic thrombocytopenic purpura, HCT = hydrochlorothiazide.
*Excluded case reports failed to provide sufficient information of differential diagnostic value for analysis with the Bayesian Adverse Reaction Diagnostic Instrument.

Table 1 Case parameters of the various types of hematologic dyscrasias associated with ticlopidine therapy.[41]

Clopidogrel has emerged as a primary drug for the treatment and the prevention of arterial thrombosis in patients with cardiovascular disease.[10,74] The pharmacological activity of clopidogrel requires cytochrome P450 metabolism to generate a active metabolite to inhibit platelet function.[20,75] Although increased doses of clopidogrel provide increased inhibition of $P2Y_{12}$ and increased efficacy, adverse drug reaction also increased.

Clopidogrel is considered to be a safer alternative to ticlopidine due to its decreased incidence of myelotoxic adverse effects.[76] However, clopidogrel has been reported have myelotoxic adverse effects, most commonly effecting platelets (Table 2). Common adverse effects seen with clopidogrel include gastrointestinal disorders, bleeding, rash and diarrhea.[15,74,77] Myelotoxic adverse effects include severe neutropenia, TTP, pancytopenia and agranulocytosis.[78-82] Other severe adverse effects include liver injury,[75,83-87] serum sickness like reaction[88] and secondary inflammatory response syndrome.[89] Clopidogrel is still used as an effective thrombotic drug.

Diagnosis	Age (y)	Duration of Therapy	Other Drug Suspected Causes	Treatment	Time to Recovery
Leukopenia	58	8 d	none	N/A	7 d
Pancytopenia	90	20 d	none	N/A	8 d
Aplastic anemia	88	5 mo	none	N/A	N/A (death)
Aplastic anemia	68	3 mo	allopurinol	granulocyte colony-stimulating factor, cyclosporine	N/A (death)
Aplastic anemia	53	17 mo	phenytoin, sulfa-containing antibiotics	antithymocyte globulin	3 mo

N/A = not applicable.

Table 2 Reported cases of clopidogrel myelotoxicity.[90]

Prasugrel is a new, novel thienopyridine with a faster onset of action and more potent antiplatelet effect than other antiplatelet agents.[19] Prasugrel has been shown superiority over the currently used standard clopidogrel in reducing the risk of the composite end point of cardiovascular death, nonfatal MI, and nonfatal stroke in patients with stent thrombosis and cardiovascular diseases.[27,91,92] The greatest benefit appears to be in patients with diabetes mellitus. However, the enhanced antiplatelet activity and greater efficacy seen with prasugrel in clinical trials has been accompanied by increased bleeding risk (Table 3).[93] Thus FDA advisory committee approved prasugrel with guidance to physicians about increased risk in low-weight or elderly patients and avoidance of use in patients with prior or current stroke or transient ischemic attack, coronary artery bypass graft or other surgical or invasive procedures. This guidance of identification of patients at higher risk of bleeding events and attention to discontinuation of therapy before surgery help in therapeutic decisions and optimizes outcomes including the benefit-risk ratio.[94]

End Point	Prasugrel (N=6,741)	Clopidogrel (N=6,716)	Hazard Ratio for Prasugrel (95% CI)	p Value
	No. of Patients (%)			
Non–CABG-related TIMI major bleeding (key safety end point)	146 (2.4)	111 (1.8)	1.32 (1.03–1.68)	0.03
Related to instrumentation	45 (0.7)	38 (0.6)	1.18 (0.77–1.82)	0.45
Spontaneous	92 (1.6)	61 (1.1)	1.51 (1.09–2.08)	0.01
Related to trauma	9 (0.2)	12 (0.2)	0.75 (0.32–1.78)	0.51
Life-threatening	85 (1.4)	56 (0.9)	1.52 (1.08–2.13)	0.01
Related to instrumentation	28 (0.5)	18 (0.3)	1.55 (0.86–2.81)	0.14
Spontaneous	50 (0.9)	28 (0.5)	1.78 (1.12–2.83)	0.01
Related to trauma	7 (0.1)	10 (0.2)	0.70 (0.27–1.84)	0.47
Fatal	21 (0.4)	5 (0.1)	4.19 (1.58–11.11)	0.002
Nonfatal	64 (1.1)	51 (0.9)	1.25 (0.87–1.81)	0.23
Intracranial	19 (0.3)	17 (0.3)	1.12 (0.58–2.15)	0.74
Major or minor TIMI bleeding	303 (5.0)	231 (3.8)	1.31 (1.11–1.56)	0.002
Bleeding requiring transfusion	244 (4.0)	182 (3.0)	1.34 (1.11–1.63)	<0.001
CABG-related TIMI major bleeding	24 (13.4)	6 (3.2)	4.73 (1.90–11.82)	<0.001

Table 3 Rates of several bleeding end points were significantly higher for prasugrel compared with clopidogrel.[93]

2.7 Thienopyridine induced hepatotoxicity

The drug induced liver toxicity is difficult to diagnose especially for drugs with rare hepatic complications. Hepatic injury may occur from many drugs through a variety of mechanisms such as disruption of intracellular calcium homeostasis, derangement of CYP system or stimulation of multifaceted immune response against liver enzyme-drug adducts. Drug induced hepatotoxicity is a rare and serious complication in patients undergoing treatment with clopidogrel and ticlopidine. It if often difficult to determine the responsible drug for toxicity, since these patients are frequently under polymedication. The risk of incidence of abnormal liver functions due to ticlopidine was approximately 1% during clinical trails. In most of the published reports, the evidence of hepatotoxicity appears one to four months after the initiation of ticlopidine and recovers three months after the drug is discontinues.[95-97] The reactive metabolite formation followed by covalent binding is

believed to be associated with idiosyncratic toxicity through the immune mechanism. The ticlopidine irreversibly modifies CYP proteins in liver might be responsible for ticlopidine-induced idiosyncratic toxicity.[98,99] Several cases have been reported of cholestatic hepatitis or mixed hepatocellular and cholestatic hepatitis in patients undergone treatment with ticlopidine. [35,42]

Clopidogrel has been successfully used as a replacing drug in patients with ticlopidine induced hepatotoxicity. [100] However, clinically patients developed the cholestatic hepatitis or mixed hepatocellular and cholestatic hepatitis comparatively low to ticlopidine. The mechanism of clopidogrel-associated hepatotoxicity still remain unclear, hypersensitivity and direct toxicity have been postulated as potential mechanism. Clopidogrel triggers both dose-independent idiosyncratic and dose-dependent toxicity. [85] However, clopidogrel is still suggested as an effective and safe antithrombotic drug. The new novel thienopyridine, prasugrel though has been reported for increased bleeding risk, no case of hepatotoxicity has been reported.

2.8 Thienopyridine induced myelotoxicity

Myelotoxicity is less frequent, occurring in up to 3% of the patients treated with ticlopidine [101,102] and approximately 1% of the patients treated with clopidogrel [80]. The major adverse effects associated with these drugs include neutropenia and agranulocytosis [39,44,78,103,104], thrombocytopenia [63,65,80], aplastic anaemia [105,106], thrombotic thrombocytopenic purpura [107,108], and the haemolytic uremic syndrome [109].

The patients with these adverse effects associated with thienopyridines, bone marrow investigations showed impaired myelopoiesis [79,110], compatible with a direct toxic effect on the bone marrow. The association of the thienopyridines with aplastic anaemia [105,106] also suggests a direct toxicity on the bone marrow.

The mechanisms associated with myelotoxicity of ticlopidine and clopidogrel are currently not entirely clear. Liu and Uetrecht have demonstrated that ticlopidine can be oxidized by myeloperoxidase (MPO) in neutrophil granulocytes to a reactive thiophene-S-chloride derivative, which may be associated with bone marrow toxicity.[111] The metabolites formed by MPO in bone marrow of ticlopidine, clopidogrel or the clopidogrel metabolite clopidogrel carboxylate are toxic for myelopoietic progenitor cells.[112] The toxicity is associated with the respective non-metabolized clopidogrel and ticlopidine circulating in peripheral blood or due to metabolism in bone marrow.[111,112]

3. Aims of the thesis

The main aim of the thesis was to investigate the molecular mechanism of thienopyridines induced myelotoxicity. Thienopyridine drug includes clopidogrel, ticlopidine and prasugrel. These are potent antiplatelet agents used for the treatment of several cardiovascular diseases. Thienopyridines are prodrugs that need enzymatic activation by cytochrome P450 in order to exert their antiplatelet effect. Ticlopidine use is limited due to its numerous adverse effects. Clopidogrel, the most frequently used drug has been reported to cause severe neutropenia and agranulocytosis. Prasugrel, the novel thienopyridine drug is associated with increased bleeding risk. The mechanisms associated with myelotoxicity of thienopyridines are currently not entirely clear.

The aim of the first project was to assess the toxicity of clopidogrel, its inactive metabolite clopidogrel carboxylate and ticlopidine on human hematopoietic stem cells derived from umbilical cord blood. Myeloperoxidase (MPO) of granulocytes in the bone marrow could produce potentially toxic metabolites. Using this as a guide, we investigated the toxicity of clopidogrel, ticlopidine and clopidogrel carboxylate in progenitor cells to explain if mother substance or metabolites formed by MPO present in neutrophils or CYP3A4 present in liver could be responsible for myelotoxicity. We used a standard colony forming unit assay to assess the toxicity due to MPO or CYP3A4 in progenitor cells.

The second aim of the thesis was to investigate the effects of clopidogrel, ticlopidine and clopidogrel carboxylate on peripheral blood cells. We have showed in our first project that clopidogrel, ticlopidine and clopidogrel carboxylate are metabolized by MPO present in neutrophils, which could lead to myelotoxicity. We aimed to investigate the molecular mechanism of the toxicity in neutrophils and lymphocytes isolated from human peripheral blood using *in vitro* techniques.

The final aim of the thesis was to study CYP3A4 and MPO dependent toxicity of thienopyridines. To answer this, we established two independent systems, namely HL-60 cells endogenously expressing MPO and spent HL-60 with no MPO where we stable induced overexpression of CYP3A4 in order to generate two independent metabolite systems. We aimed to investigate the molecular mechanism of toxicity due to MPO or CYP3A4 in the cell systems using *in vitro* techniques. We also aimed to detect the active metabolites produced by CYP3A4 and MPO when treated with thienopyridines.

4. Toxicity of clopidogrel and ticlopidine on human myeloid progenitor cells: importance of metabolites

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4.1 Summary

Ticlopidine and clopidogrel are thienopyridine derivatives used for inhibition of platelet aggregation. Not only hepatotoxicity, but also bone marrow toxicity may limit their use. Aims of the study were to find out whether non-metabolized drug and/or metabolites are responsible for myelotoxicity and whether the inactive clopidogrel metabolite clopidogrel carboxylate contributes to myelotoxicity. We used myeloid progenitor cells isolated from human umbilical cord blood in a colony-forming unit assay to assess cytotoxicity. Degradation of clopidogrel, clopidogrel carboxylate or ticlopidine (studied at 10 and 100μM) was monitored using LC/MS. Clopidogrel and ticlopidine were both dose-dependently cytotoxic starting at 10μM. This was not the case for the major clopidogrel metabolite clopidogrel carboxylate. Pre-incubation with recombinant human CYP3A4 not only caused degradation of clopidogrel and ticlopidine, but also increased cytotoxicity. In contrast, clopidogrel carboxylate was not metabolized by recombinant human CYP3A4. Pre-incubation with freshly isolated human granulocytes was not only associated with a myeloperoxidase-dependent degradation of clopidogrel, clopidogrel carboxylate and ticlopidine, but also with dose-dependent cytotoxicity of these compounds starting at 10μM. In conclusion, both non-metabolized clopidogrel and ticlopidine as well as metabolites of these compounds are toxic towards myeloid progenitor cells. Taking exposure data in humans into account, the myelotoxic element of clopidogrel therapy is likely to be secondary to the formation of metabolites from clopidogrel carboxylate by myeloperoxidase. Concerning ticlopidine, both the parent compound and metabolites formed by myeloperoxidase may be myelotoxic *in vivo*. The molecular mechanisms of cytotoxicity have to be investigated in further studies.

Keywords: Clopidogrel, ticlopidine, clopidogrel carboxylate, myelotoxicity, myeloperoxidase

4.2 Abbreviations

- clopidogrel carboxylate: major metabolite of clopidogrel produced by hydrolysis of the parent compound
- MPO: myeloperoxidase
- R-130964: active metabolite of clopidogrel
- UR-4501: active metabolite of ticlopidine
- rhCYP3A4: Recombinant human CYP3A4
- HSC: Hematopoietic stem cells
- CFU-GM assay: Colony-forming unit granulocyte/macrophage assay
- IMDM: Iscove's modified Dulbecco's medium
- MAO: Monoamine oxidase

4.3 Introduction

The thienopyridines ticlopidine and clopidogrel are important drugs used for the inhibition of platelet aggregation [1], especially in patients with acute coronary syndrome after stent implantation [2,3]. Ticlopidine is a first-generation thienopyridine, which has been widely used clinically. Currently, it is largely replaced by clopidogrel, mostly because clopidogrel has a better safety profile. The most important adverse drug reactions of ticlopidine are liver injury [4,5] and hematological toxicity including neutropenia and agranulocytosis [6] as well as aplastic anemia [7]. Although both liver injury [8,9] and hematological toxicity such as neutropenia, agranulocytosis and aplastic anemia [10,11] have also been described for clopidogrel, these adverse reactions appear to be less frequent and possibly also less severe compared to ticlopidine toxicity.

Both ticlopidine and clopidogrel are pharmacologically inactive prodrugs and need to be activated before they bind to $P2Y_{12}$-receptors on platelets and block them irreversibly [12,13]. In humans, both drugs are well absorbed from the gastrointestinal tract. Since clopidogrel is a methyl-ester, it is rapidly hydrolyzed by esterases to the carboxylate derivative (clopidogrel carboxylate). Clopidogrel carboxylate is the major metabolite of clopidogrel (approximately 85% of clopidogrel is converted to this metabolite [12]). Clopidogrel carboxylate is pharmacologically inactive [14], but could theoretically contribute to the toxicity of clopidogrel. Only approximately 15% of clopidogrel is converted in a two-step procedure involving different hepatic cytochrome P450 (CYP) enzymes [14-16] into the active metabolite R-130964. R-130964 contains a thiol group and can bind covalently to $P2Y_{12}$-receptors on platelets [17,18]. Ticlopidine is not an ester and can therefore not be metabolized by esterases. Similar to clopidogrel, ticlopidine is activated in a two-step procedure by different CYPs mainly in the liver. Activation of ticlopidine involves oxidation in position 2 of the thiophene ring and ring opening, eventually leading to the active metabolite UR-4501

[19]. Similar to clopidogrel, the active metabolite of ticlopidine contains a thiol group, which can bind irreversibly to $P2Y_{12}$-receptors on platelets.

The mechanisms associated with hematotoxicity of ticlopidine and clopidogrel are currently not entirely clear. Since aplastic anemia has been reported for both drugs [7,11], it is clear that both substances can act directly on cells in the bone marrow. Theoretically, toxicity could be associated with the respective non-metabolized compounds, which may be metabolized in bone marrow. This possibility has been investigated by Liu and Uetrecht [20] who showed that myeloperoxidase (MPO) of granulocytes in the bone marrow can oxidatively produce potentially toxic metabolites. Another possibility is toxicity associated with metabolites formed outside of the bone marrow, e.g. in the liver. Such metabolites are detectable in the systemic circulation [21] and can therefore reach the bone marrow. Clopidogrel carboxylate, the major metabolite of clopidogrel, may also be associated with myelotoxicity, since it reaches high plasma concentrations [12]. Based on these considerations, we decided to assess the toxicity of clopidogrel and ticlopidine on human myeloid progenitor cells derived from umbilical cord blood. The specific questions we wanted to answer with our investigations were, (i) are the non-metabolized compounds toxic on hematopoietic progenitor cells, (ii) is toxicity increased after activation by CYP3A4 or MPO and (iii) does clopidogrel carboxylate contribute to bone marrow toxicity of clopidogrel.

4.4 Materials and methods

4.4.1 Materials

Clopidogrel hydrogen sulphate was extracted from commercially available tablets (brand name Plavix®) and clopidogrel carboxylate was obtained by alkaline hydrolysis of clopidogrel. These procedures were carried out by ReseaChem life science (Burgdorf, Switzerland). Both substances were >99% pure by NMR analysis. Ticlopidine was obtained from Sigma–Aldrich (Buchs, Switzerland). Recombinant human CYP3A4 (rhCYP3A4) and NADPH regeneration system were from BD Bio sciences (Franklin Lakes, NJ, USA). Cell culture plates were purchased from BD Biosciences (Franklin Lakes, NJ, USA). All other chemicals and media used were purchased from Sigma (Buchs, Switzerland) and GIBCO (Lucerne, Switzerland).

4.4.2 Quantification of clopidogrel, clopidogrel carboxylate and ticlopidine

Concentrations of clopidogrel, clopidogrel carboxylate and ticlopidine were determined using liquid chromatography coupled to tandem mass spectrometry. Chromatographic separation was done on a Shimadzu HPLC system consisting of a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), four Shimadzu LC-20 AD pumps (Shimadzu AG, Reinach, Switzerland) controlled by a Shimadzu CBM-20A unit, a Shimadzu CTO-20AD column oven and a six port valve VICI (VICI AG, Schenkon, Switzerland). Samples were loaded on a trapping column (Haipeek Targa C18 3µm, 2.1 mm x 20 mm, Prolab Reinach, Switzerland) with 100% of eluent A (water, 1% formic acid) at a flow rate of 0.6 mL/min, while the analytical column (Phenomenex Kinetex C18, 50 mm x 2.1 mm, 2.6 µm, Brechbühler Schlieren, Switzerland) was conditioned with 40% eluent A and 60% eluent B (methanol, 0.1% formic acid) at a flow rate of 0.3 mL/min. After 1.5 min, the valve was switched; the flow reduced to 0.3 mL/min and a linear gradient from 60 % to 95 % eluent B from

1.5 to 4 min was applied. The gradient was held at 95% for 1 min before returning to 100% eluent A. The valve was switched back and the trapping and analytical columns were conditioned for one minute before the next injection. The injection volume was 5µL and total run time was 6.0min. The trapping column, analytical column, and switching valve were placed in a column oven at 40°C. The HPLC system was interfaced with a triple quadrupole mass spectrometer (API2000, AB/MDS Sciex, Concord, Canada) equipped with an ESI source. The ion spray voltage was 5500 eV and the probe temperature was 350°C. The selected mass-to-charge (*m*/*z*) ratio transitions of the protonated analyte ions MH^+ used in selective reaction monitoring mode were as follows: clopidogrel 322→212; clopidogrel carboxylate 308→198; ticlopidine 264→156. The dwell time was set at 50 ms for all analytes.

4.4.3 Isolation of myeloid progenitor mononuclear cells from human umbilical cord blood

The Ethics Committee of the State of Basel-Stadt approved the study protocol for the isolation of human mononuclear cells (hMNCs). Human umbilical cord blood cells were obtained from the obstetric unit of the University Hospital Basel and were processed within 12h after birth. hMNCs were isolated by low density gradient centrifugation using 50 mL Leucosep® tubes (Huber & Co, Reinach, Switzerland), according to the manufacturer's protocol. The hMNCs were washed twice with Iscove's modified Dulbecco's medium (IMDM) and stored in liquid nitrogen. The cell viability and recovery was assessed by counting cells with trypan blue exclusion (viability exceeded 90% for all preparations).

4.4.4 Experimental design

The test compounds (10 or 100µM clopidogrel, clopidogrel carboxylate or ticlopidine) were used without pretreatment or after treatment with rhCYP3A4 or

neutrophil granulocytes (with or without their respective inhibitors) in a shaking water bath at 37°C. After 60min, the samples were centrifuged at 3000 x g for 5min and the collected supernatant was used either for cytotoxicity experiments or for quantification of the ingredients.

4.4.5 Colony-forming unit granulocyte/macrophage (CFU-GM) assay

For cytotoxicity experiments, the standard operating procedure established by Pessina et al. [22] was used to perform the CFU-GM assay. Briefly, prior to the CFU-GM assay, frozen aliquots of hMNCs were rapidly thawed in 37°C and diluted with IMDM medium containing 20% FBS. Cell viability was assessed by trypan blue exclusion and exceeded 90% for every experiment performed. For each experiment, an internal linearity control was performed to confirm the relationship between the cells seeded and the colonies obtained. For linearity control, 2.5×10^4, 5×10^4, 7.5×10^4 and 10×10^4 cells were seeded. For the cytotoxicity assays, 7.5×10^4 hMNCs were seeded in triplicate in 35mm petri dishes containing MethoCult H4534 (StemCell Technologies, Grenoble, France). The cells were seeded in the presence of different concentrations and conditions for each compound to be tested as mentioned above. Similar to the incubations containing drugs and/or inhibitors, control incubations contained 0.1% DMSO. It has been shown previously that 0.1% DMSO is not cytotoxic [23]. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in fully humidified air. After 14 days, the CFU-GM colonies were counted using the standardized scoring criteria described by Pessina et al. [22]. The colonies were counted using an inverted microscope in a random fashion by considering the aggregates containing 50 or more cells as a CFU-GM colony. 7.5×10^4 hMNCs produced 57±2 CFU-GM colonies. There was a linear relationship between the cells plated (2.5 - 10 x 10^4 hMNCs) and the colonies obtained ($r^2 = 0.992$).

4.4.6 Metabolism of clopidogrel, clopidogrel carboxylate and ticlopidine by rhCYP3A4

The reaction mixture (final volume of 1mL) contained test compounds (10 and 100μM), incubation buffer (0.1 M phosphate-buffered saline, pH 7.4), rhCYP3A4 (10pmol P450/mL) and a NADPH-regeneration system containing MgCl2 (3.3mM), NADP+ (1.3mM), glucose-6-phosphate (3.3mM) and glucose-6-dehydrogenease (0.4 U/mL). For inhibition studies, the reaction mixture was pre-incubated with 1μM ketoconazole for 15min prior to addition of test compounds. The suspensions were incubated in a shaking water bath at 37°C. Following the incubation with test compounds for different periods of time (0, 15, 30 and 60min), the reactions were stopped by the addition of 300 μl of methanol. Precipitated proteins were then removed by centrifugation at 3000 x g for 30min and supernatants were analyzed by LC–MS. The amount of test compounds metabolized after 15, 30 and 60min were calculated by subtracting the remaining from the initial amount (0min) present in the incubation.

4.4.7 Metabolism of clopidogrel, clopidogrel carboxylate and ticlopidine by freshly isolated human neutrophil granulocytes

Normal donor buffy coats were purchased from the local blood donation center, Basel (Switzerland). Neutrophils were isolated by a modification of the method described by Klebanoff et al. [24]. Briefly, the buffy coat was diluted 1:2 with IMDM medium and was mixed with 4% dextran (MW 500,000 kDa, Sigma) in 0.9% NaCl to allow erythrocytes to settle for 30min. The supernatant layer was washed with PBS and underlayed with 20 mL of cold Ficoll-Paque (GE Healthcare, Switzerland) and centrifuged at 500 x g for 25min. The supernatant was discarded and the pellet was gently resuspended with 10 mL of ice-cold 0.2% NaCl for 1min for hypotonic lysis of contaminating erythrocytes. Isotonicity was restored by adding an equal volume of 1.6% NaCl; the suspension was centrifuged at 350 x g for 5min and the pellet washed

twice with PBS. The pellet was resuspended in IMDM. Neutrophils were counted and the cell viability was assessed using trypan blue analysis.

To test whether thienopyridines are metabolized by granulocytes, 4.5 x 10^6/mL neutrophils were incubated with the test compounds (10 and 100μM) at 37°C for different periods of time (0, 15, 30 and 60min) with or without inhibitors (1μM ketoconazole for CYP3A4 [14] and/or 20 μM rutin for MPO [25]). Reactions were stopped by addition of 300 μl of methanol. The precipitated proteins were removed by centrifugation at 3000 x g for 30min and the supernatants were analyzed by LC–MS or used in the CFU-GM assays.

4.4.8 Metabolism of clopidogrel, clopidogrel carboxylate and ticlopidine by myeloperoxidase

MPO (5 U per assay) and hydrogen peroxide (0.5mM) were added to the reaction mixture (final volume of 1mL) containing test compounds (10 and 100μM) and incubation buffer (0.1M phosphate-buffered saline, pH 7.4), and the mixture was incubated in a shaking water bath at 37°C. Samples (100 μl) were collected at different time points after starting the reaction (0, 15, 30 and 60min) and the reactions were stopped by the addition of 300μl of methanol. Precipitated proteins were removed by centrifugation at 3,000g for 30min and supernatants were analyzed by LC-MS.

4.4.9 Expression of myeloperoxidase and CYP3A4 by neutrophil granulocytes and human mononuclear cells

Neutrophils or hMNCs were lysed on ice for 15min with 200 μl of NET lysis buffer (0.05M Tris–HCl pH 8.0, 50mM NaCl, 5mM EDTA, 1% NP-40 and a protease inhibitor tablet from Roche, Basel, Switzerland). After lysis, the mixture was vortexed and centrifuged for 10min at 4°C at 11,000 x g. The supernatant was

collected and the protein concentration determined using the pierce BCA protein assay kit (Darmstadt, Germany). Twenty μg of protein were separated on a denaturating SDS polyacrylamide gel (4%). The antibodies against MPO and CYP3A4 were used at a 1:1000 dilution (monoclonal anti-CYP3A4 from Epitomics, Danvers, USA and anti-MPO from Cell Signaling Technology, Allschwil, Switzerland). Peroxidase-labeled anti-rabbit IgG in combination with a chemiluminescent substrate (GE Healthcare, Amersham, UK) was used for quantification.

4.4.10 Statistics

Data are represented as means ± SD of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between many groups were tested by one-way ANOVA followed by Bonferroni's post hoc test to localize significant results in the ANOVA. Differences between many groups at different time points were compared using two-way analysis of variance (ANOVA) followed by the protected Bonferroni's post hoc test to localize significant results obtained in the ANOVA. A p-value <0.05 was considered as statistically significant.

4.5 Results

4.5.1 Role of CYP3A4 in the toxicity of thienopyridines on human myeloid progenitor cells

Thienopyridines are prodrugs requiring activation to exert their anti-platelet effects. In order to study metabolism of thienopyridines *in vitro*, we pre-incubated human recombinant CYP3A4 (rhCYP3A4) with the test compounds with or without the CYP3A4 inhibitor ketoconazole (1µM). Incubations of clopidogrel and ticlopidine with rhCYP3A4 showed a significant concentration-dependent decrease of parent compound, suggesting the generation of metabolites (Fig. 1A and B). Significant inhibition of clopidogrel and ticlopidine degradation with ketoconazole confirmed the suspected CYP3A4-dependent metabolite formation (Fig. 1A and B). Clopidogrel carboxylate, the major inactive metabolite of clopidogrel produced by hydrolysis with esterases [14], was not significantly metabolized by CYP3A4 (Fig. 1C).

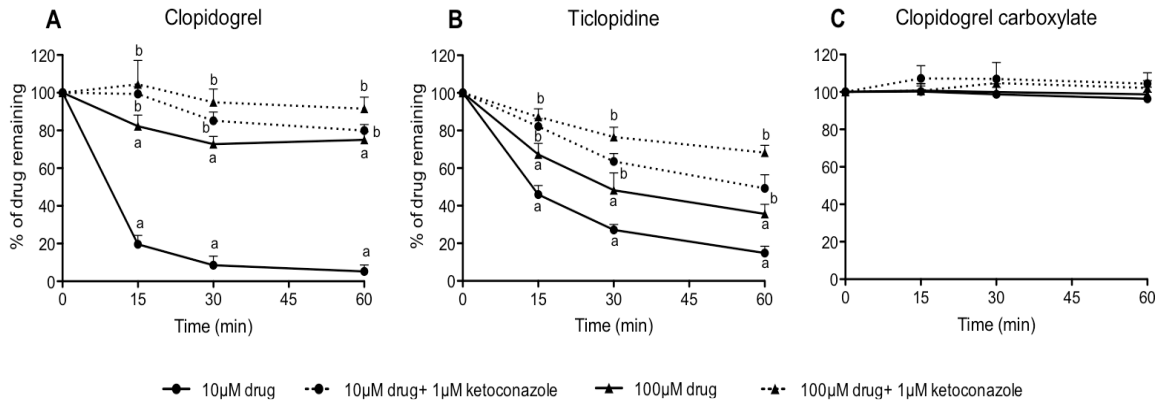

Figure 1 Metabolism of clopidogrel (A), ticlopidine (B) and clopidogrel carboxylate (C) by rhCYP3A4. Different concentrations of clopidogrel, ticlopidine or clopidogrel carboxylate were incubated with rhCYP3A4. Some incubations also contained the CYP3A4 inhibitor ketoconazole (1µM) as indicated in the figure labels. Data are expressed as the percentage of drug remaining during 60min of incubation. The results are the means + SD of three independent determinations. (a) $p<0.05$ versus respective incubations at time 0 min ; (b) $p<0.05$ incubations containing drug and 1µM ketoconazole versus respective incubations containing drug only.

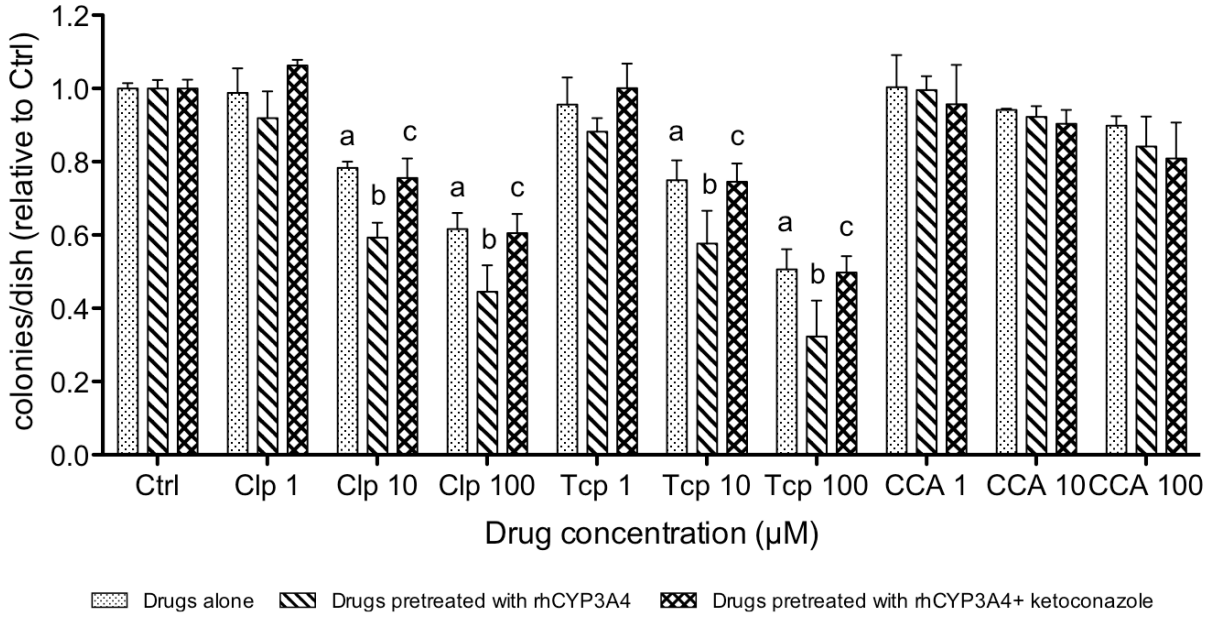

Figure 2_Cytotoxicity of clopidogrel, ticlopidine and clopidogrel carboxylate with and without pretreatment with rhCYP3A4. Human myeloid progenitor cells were exposed to the compounds mentioned above with or without previous incubation with rhCYP3A4. Some incubations also contained the CYP3A4 inhibitor ketoconazole (1μM) as indicated in figure labels. A colony-forming unit assay was performed and colonies were determined after 14 days of incubation as a measure of cytotoxicity. The results are the means + SD of three independent experiments in triplicate. (a) $p<0.05$ incubations containing drugs without pretreatment versus respective control incubations (Ctrl); (b) $p<0.05$ incubations containing drugs with rhCYP3A4 pretreatment versus respective incubations containing drugs without pretreatment; (c) $p<0.05$ incubations containing drugs pretreated with rhCYP3A4 and ketoconazole (1μM) versus respective incubations containing drugs pretreated with rhCYP3A4 only. Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylic acid.

As our data demonstrated that clopidogrel and ticlopidine could be metabolized by CYP3A4, we tested the role of metabolite formation in the toxicity towards hMPCs. At least for clopidogrel, it has been shown that the thiol group-containing, active metabolite R-130964 is present in the systemic circulation after oral ingestion of this drug [21]. Due to its reactivity, this metabolite could possibly be associated with myelotoxicity. As described in Section 2.5, hMPCs were grown in the presence of the

mentioned toxicants for 14 days, before the CFU-GM colonies were counted (Fig. 2). In comparison to control incubations, treatment with clopidogrel or ticlopidine was cytotoxic, reducing the number of colonies by approximately 25% (10μM) or 40–50% (100μM), with ticlopidine being numerically slightly more toxic than clopidogrel. After pretreatment with CYP3A4, both substances were more toxic than without pretreatment, reducing the number of colonies by another approximately 20%. This additional CYP3A4-associated cytotoxic effect could be completely prevented by the addition of ketoconazole to the incubations. In contrast, 1μM clopidogrel or ticlopidine and the carboxylate metabolite of clopidogrel were not toxic to hMPCs, even after pre-incubation with rhCYP3A4.

4.5.2 Role of neutrophil granulocytes in the toxicity of thienopyridines on human myeloid progenitor cells

After isolation, human neutrophils were incubated with clopidogrel, ticlopidine or clopidogrel carboxylate, and the resulting supernatant was either used for cytotoxicity experiments or subjected to LC–MS analysis. All the compounds tested were metabolized by human neutrophils (20–40% of the compound per h, depending on the compound and on the initial concentration) if no inhibitor was present (Fig. 3A–F). The addition of ketoconazole did not reduce the metabolism of clopidogrel, ticlopidine or clopidogrel carboxylate, indicating that CYP3A4 is not important in human neutrophils. In contrast, inhibition of MPO by rutin prevented metabolism of the three compounds almost completely, demonstrating that MPO is the most important enzyme metabolizing thienopyridines in granulocytes.

These findings are consistent with the protein expression data obtained in isolated granulocytes and hMNCs shown in Fig. 4. Neutrophil granulocytes express no CYP3A4, but they express MPO. In comparison, hMNCs express neither CYP3A4 nor MPO.

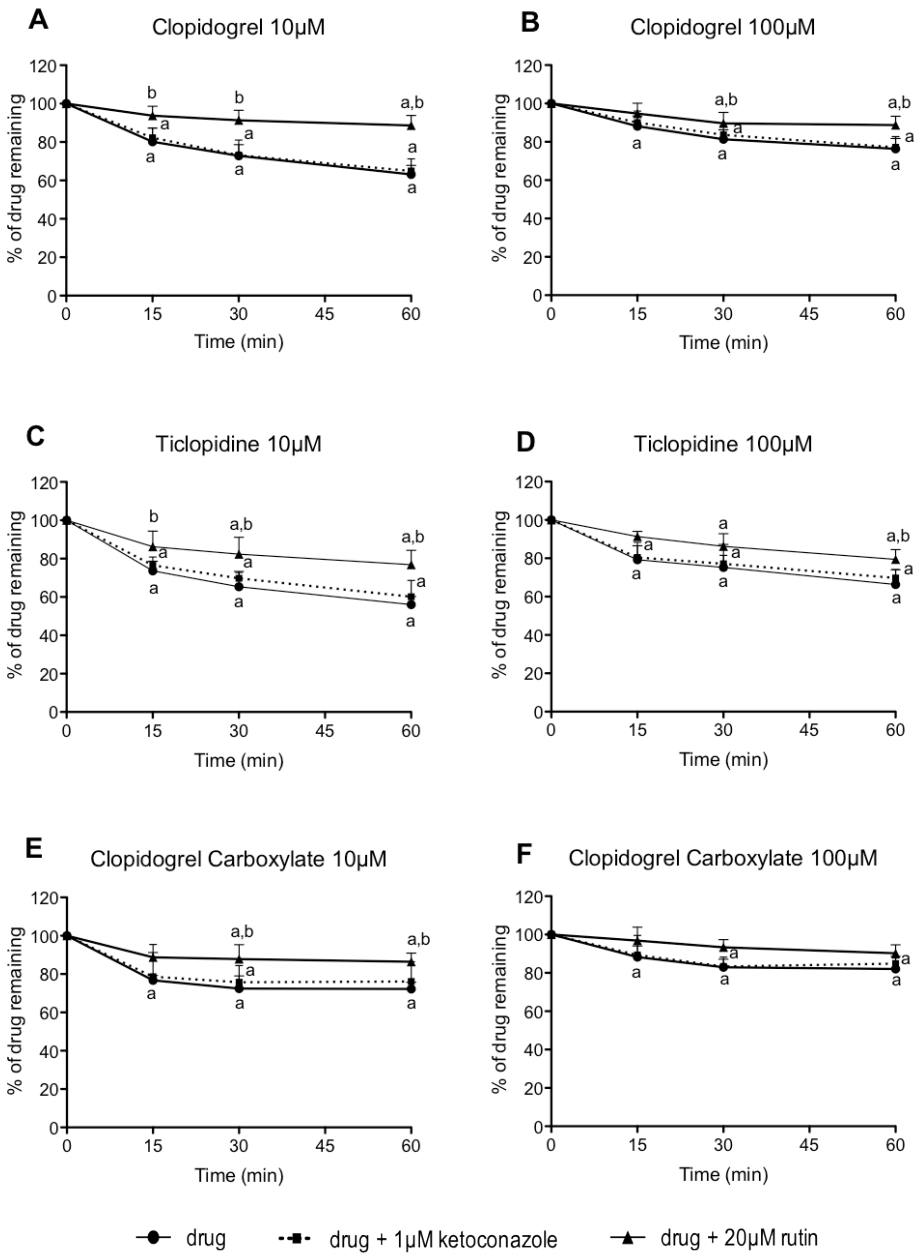

Figure 4 Metabolism of clopidogrel, ticlopidine and clopidogrel carboxylate by freshly isolated human neutrophil granulocytes. Clopidogrel 10μM (A), clopidogrel 100μM (B), ticlopidine10μM (C), ticlopidine100μM (D), clopidogrel carboxylate 10 μM (E) or clopidogrel carboxylate 100μM (F) were incubated with neutrophil granulocytes as described in Section 5.4. Some incubations contained also the CYP3A4 inhibitor ketoconazole (1μM) or the myeloperoxidase inhibitor rutin (20μM) as indicated in the figure labels. Data are expressed as the percentage of drug remaining during 60 min of incubation. The results are the means + SD of three independent determinations. (a) $p<0.05$ versus respective incubations at time 0 min; (b) $p<0.05$ incubations containing drug with 20μM rutin versus respective incubations containing drug only.

In further experiments, degradation of clopidogrel, ticlopidine and clopidogrel carboxylate by MPO was directly confirmed by incubating the individual compounds with isolated MPO (Fig. 6).

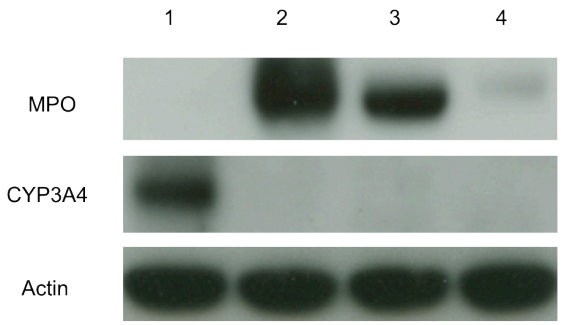

Figure 4 Myeloperoxidase and CYP3A4 protein expression in human neutrophil granulocytes and human mononuclear cells. The Western blot was carried out as described in Section 5.4. Actin was used as control to confirm the equal loading of samples. Primary human hepatocytes (lane 1) were used as a positive for CYP3A4 and HL-60 cells (lane 2) as a positive control for myeloperoxidase. Lane 3 and 4 contain human neutrophil granulocytes and human mononuclear cells, respectively.

Finally, the effect of pre-incubation of clopidogrel, ticlopidine and clopidogrel carboxylate with granulocytes on the cytotoxicity towards hMPCs was investigated (Fig. 5). Similar to the data presented in Fig. 2, clopidogrel or ticlopidine without pre-incubation with granulocytes were toxic to hMPCs starting at 10μM, whereas clopidogrel carboxylate was not toxic. Pre-incubation with granulocytes was associated with an additional cytotoxic effect for 10 and 100μM clopidogrel and also for ticlopidine when compared to the respective incubations without pre-incubation. This granulocyte-associated toxicity resulted in a further decrease of the colony number by 20–30%. Importantly, pre-incubation with granulocytes was associated with cytotoxicity also for 10 and 100μM clopidogrel carboxylate. Concomitant pre-incubation with rutin (20μM) completely prevented the toxicity associated with granulocyte incubation, indicating that metabolism by MPO produces toxic metabolites. In contrast, co-incubation with ketoconazole (1μM) did not prevent

cytotoxicity associated with granulocyte incubation (data not shown). Rutin and ketoconazole alone were not cytotoxic in any of the conditions investigated (data not shown). Low concentrations (1μM) of clopidogrel, ticlopidine or clopidogrel carboxylate were not cytotoxic, even after pre-incubation with granulocytes.

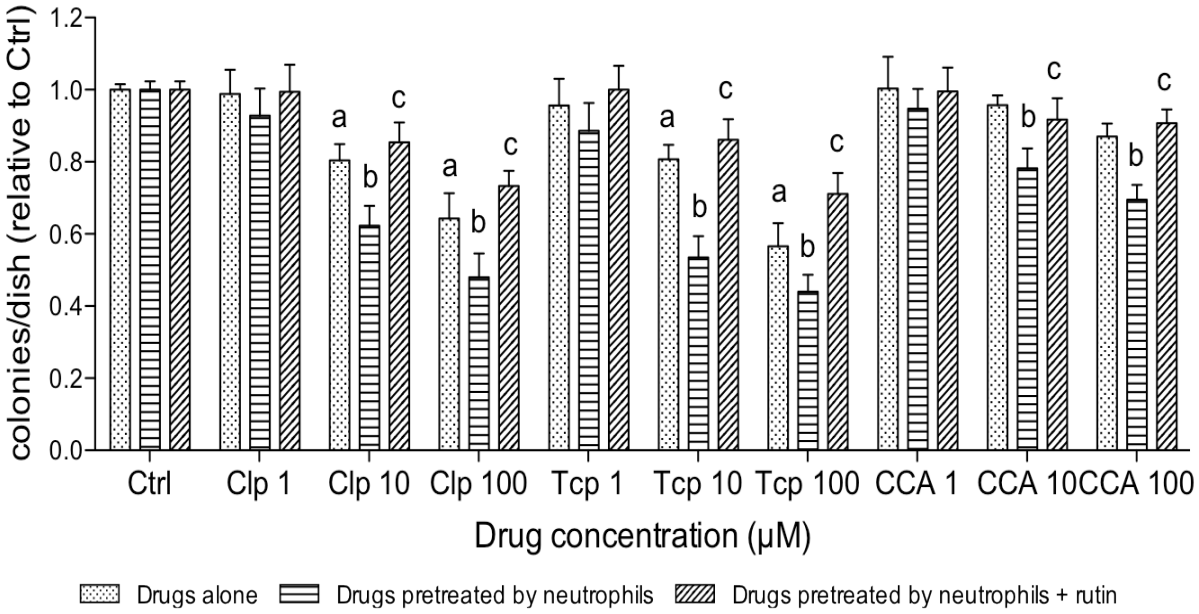

Figure 5 Results of the colony-forming unit assay showing cytotoxicity of clopidogrel, ticlopidine and clopidogrel carboxylate. Human myeloid progenitor cells were exposed to the compounds (concentrations indicated in the figure labels) with or without previous incubation with freshly isolated human neutrophil granulocytes. Some incubations contained also the CYP3A4 inhibitor ketoconazole (1μM) or the myeloperoxidase inhibitor rutin (20μM) as indicated in the figure labels. The results are the means + SD of three independent experiments in triplicates. (a) $p<0.05$ incubations containing drugs without pretreatment versus respective control incubations (Ctrl); (b) $p<0.05$ incubations containing drugs pretreated with neutrophils versus respective incubations containing drugs without pretreatment; (c) $p<0.05$ incubations containing drugs pretreated with neutrophils and rutin (20μM) versus incubations containing drugs pretreated with neutrophils only. Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylic acid.

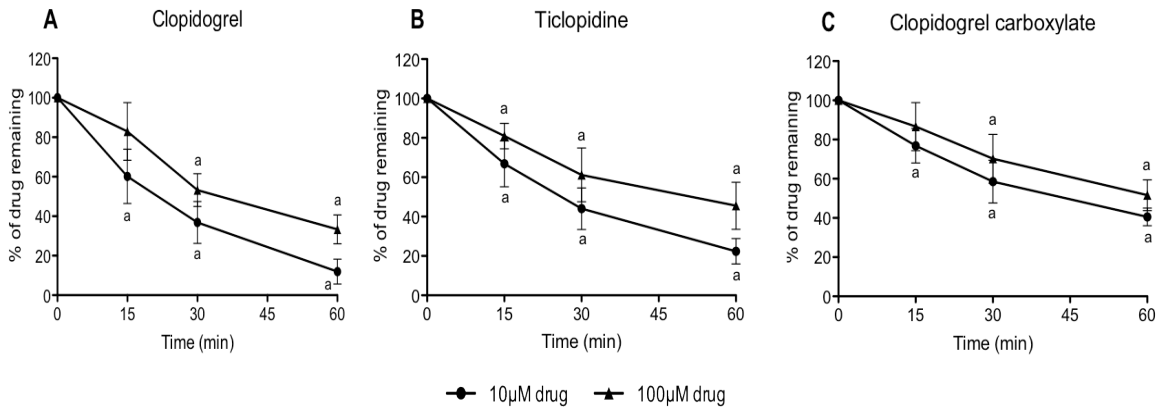

Figure 6 Metabolism of clopidogrel (A), ticlopidine (B) or clopidogrel carboxylate (C) by myeloperoxidase. Clopidogrel (10 and 100µM), ticlopidine (10 and 100µM) or clopidogrel carboxylate (10 and 100µM) were incubated with myeloperoxidase as described in Methods. The concentration of the respective non-metabolized compounds was determined at different time points as shown in the Figure. Data are expressed as the percentage of intact drug remaining at the specified time points. The results are the means + SD of three independent determinations. **a** $p<0.05$ versus respective incubations at time 0 min.

4.6 Discussion

Our study shows that both the non-metabolized compounds and metabolites are potentially important for the myelotoxicity of ticlopidine and clopidogrel. Both non-metabolized compounds were associated with concentration-dependent toxicity towards hMPCs, which was accentuated by pre-incubation with CYP3A4 or neutrophil granulocytes.

Our study showed that non-metabolized clopidogrel is toxic to hMPCs (Figs. 2 and 5), starting at a concentration of 10μM. By comparison, peak serum clopidogrel concentrations reached 38μg/L (approximately 0.1μM) in healthy in human volunteers who had ingested a clopidogrel loading dose of 600 mg [26]. The half-life of non-metabolized clopidogrel was approximately 1 hour in this study. Taking into account that a normal maintenance dose is 75mg per day and the short half-life of clopidogrel, the human exposure to non-metabolized clopidogrel appears to be too low to be associated with myelotoxicity. Even if the possible production of toxic metabolites from clopidogrel by MPO in bone marrow is taken into consideration, the clopidogrel exposure of the bone marrow appears to be too low to be toxicologically relevant.

The most important CYPs for clopidogrel activation are 3A4, 2B6, 2C19, 2C9 and 1A2 [14-16]. None of these CYPs has a high expression in human bone marrow [27,28], suggesting that clopidogrel cannot be activated in bone marrow. This is in agreement with our present study wherein we show that CYP3A4 is not expressed in hMNCs and that clopidogrel is practically not metabolized by hMPCs (Fig. 4). Our results reveal that pre-incubation of clopidogrel with rhCYP3A4 was associated with clopidogrel degradation (Fig. 1) and increased cytotoxicity (Fig. 2). These findings suggest the formation of toxic metabolites, possibly of the active clopidogrel metabolite R-130964 [14]. Since CYP-associated metabolism of clopidogrel does apparently not occur in bone marrow, CYP-associated metabolites formed in organs

outside the bone marrow (e.g. the liver) would have to be transported into the bone marrow to exert myelotoxicity. After a maintenance dose of 75 mg clopidogrel, the maximal plasma concentration of the active metabolite of clopidogrel was in the range of 30µg/L (approximately 0.1µM) with a half-life in the range of 1 h [21]. This concentration is considerably lower than the highest clopidogrel concentration used in our experiments, which was not cytotoxic (1µM). The possibility that bone marrow toxicity is associated with the active metabolite of clopidogrel *in vivo* appears therefore to be unlikely.

The myelotoxic element of clopidogrel is therefore most likely to be its major metabolite clopidogrel carboxylate. While clopidogrel carboxylate itself showed no toxicity on hMPCs in our experiments (Figs. 2 and 5), it was converted to toxic metabolites by MPO (Figs. 3 and 5). While the conversion of ticlopidine to potentially toxic metabolites by MPO has been reported previously [20], our study demonstrates for the first time that the same is also true for clopidogrel and, more importantly, for clopidogrel carboxylate. After oral ingestion of 75 mg clopidogrel, clopidogrel carboxylate reaches plasma concentrations in the range of 2.8mg/L (approximately 10µM), a concentration shown to be toxic in the presence of MPO (Fig. 5). The exposure of the bone marrow to clopidogrel carboxylate appears therefore to be high enough to explain clopidogrel-associated myelotoxicity by this mechanism.

As shown in Figs. 2 and 5, non-metabolized ticlopidine also showed a dose dependent toxicity towards hMPCs. In humans, ticlopidine is almost completely absorbed, reaches peak concentrations in the range of 900µg/L (approximately 3µM) after the usual ingested dose of 250 mg twice daily and is eliminated with a half-life in the range of 20 hours [29]. Since this concentration is close to the concentration showing cytotoxicity in our study, non-metabolized ticlopidine could be responsible for bone marrow toxicity in patients treated with this drug. In addition, Liu and Uetrecht (2000) were able to show that ticlopidine exposed to MPO could be converted to the reactive and therefore potentially toxic ticlopidine metabolite

thiophene-S-chloride. Ticlopidine thiophene-S-chloride is a strong electrophile which can react with electron donors and exert cytotoxicity. This is in line with our observations, showing that pre-incubation of ticlopidine with MPO-containing neutrophil granulocytes renders this compound more toxic for hMPCs. Myelotoxicity of ticlopidine can therefore be explained by both non-metabolized ticlopidine reaching the bone marrow and by the formation of ticlopidine thiophene-S-chloride by granulocytes in the bone marrow.

Ticlopidine is almost completely hepatically metabolized, mainly by CYP 3A4, 2C9 and 2B6 [30]. Important metabolites described are 2-oxoticlopidine, ticlopidine N-oxide, ticlopidine S-oxide and dyhydrothieniumpyridinium as well as thienopyridinium compounds [31,32]. The fact that the thienopyridinium metabolite can also be formed by monoamine oxidase possibly explains our observation that ticlopidine is partially degraded by hMPCs (Figs. 2 and 5). Similar to clopidogrel, the metabolites formed from ticlopidine by hepatic metabolism are reactive [31,32] and could therefore react with cellular components in the liver. Due to their reactivity it appears unlikely, however, that they can reach the bone marrow at high enough concentrations to contribute to the myelotoxicity of ticlopidine.

It has to be pointed out that we could only focus on direct and not immune-mediated toxicity in our study. In a patient who was treated with ticlopidine and developed agranulocytosis, ticlopidine was toxic on a lymphocyte-depleted bone marrow culture and signs of an allergic pathomechanism were absent, supporting our findings [33]. It cannot be excluded, however, that allergic mechanisms may also play a role, for instance associated with hapten formation by reactive metabolites [34].

4.7 Conclusions

Both the non-metabolized compounds and metabolites of clopidogrel and ticlopidine are toxic for human MPCs. Taking exposure data in humans into account, the most likely toxic element in the non-allergic myelotoxicity of clopidogrel is likely to be MPO-associated formation of toxic metabolites from clopidogrel carboxylate. For ticlopidine, non-allergic myelotoxicity can best be explained by the action of ticlopidine itself and/or by MPO-associated formation of toxic metabolites. Further studies are necessary to investigate the molecular mechanisms leading to cell death.

4.8 Acknowledgements

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5. Toxicity of clopidogrel and ticlopidine on human neutrophils and lymphocytes

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5.1 Summary

The thienopyridines ticlopidine and clopidogrel can cause neutropenia and agranulocytosis. The aim of the current investigations was to compare cytotoxicity of these compounds for human neutrophil granulocytes with the toxicity for lymphocytes and to characterize underlying mechanisms. For granulocytes, clopidogrel, ticlopidine and clopidogrel carboxylate were concentration-dependently toxic starting at 10μM. Cytotoxicity could be prevented by the myeloperoxidase inhibitor rutin, but not by the cytochrome P450 blocker ketoconazole. For lymphocytes, clopidogrel and ticlopidine were concentration-dependently toxic starting at 10μM. In contrast to granulocytes, cytotoxicity for lymphocytes could not be blocked by rutin or ketoconazole. Clopidogrel carboxylate was toxic for lymphocytes starting at 10μM, but without a clear concentration dependency. Cytotoxicity for lymphocytes could not be prevented by rutin or ketoconazole. Granulocytes metabolized clopidogrel, ticlopidine and clopidogrel carboxylate and the metabolism was inhibited by rutin, but not by ketoconazole. Metabolism of these compounds by lymphocytes was much slower and could not be inhibited by ketoconazole or rutin. In granulocytes, all compounds investigated decreased the mitochondrial membrane potential, were associated with cellular accumulation of ROS, mitochondrial loss of cytochrome c and induction of apoptosis. In conclusion, clopidogrel, ticlopidine and clopidogrel carboxylate are toxic for both granulocytes and lymphocytes. For granulocytes, cytotoxicity of these compounds is significantly increased after metabolization by myeloperoxidase. The current studies suggest a mitochondrial mechanism for cytotoxicity for both parent compounds and myeloperoxidase-associated metabolites.

Key words: clopidogrel, ticlopidine, clopidogrel carboxylate, myelotoxicity, granulocytes, lymphocytes.

5.2 Abbreviations

- MPO: myeloperoxidase
- CYP: cytochrome P450
- IMDM: Iscove's modified Dulbecco's medium
- PBS: phosphate-buffered saline
- FBS: fetal bovine serum
- PI: propidium iodide
- TMRE: tetramethylrhodamine ethyl ester
- DCFH-DA: dichlorodihydrofluorescein-diacetate

5.3 Introduction

The thienopyridines clopidogrel and ticlopidine block the ADP ($P2Y_{12}$) receptors on blood platelets and thereby efficiently inhibit platelet aggregation [1-3]. Thienopyridines are used widely in patients with cardiovascular diseases, in particular in patients with acute coronary syndrome [4-6], but also in patients with cerebrovascular or peripheral artery disease [7-9].

The most important adverse reaction of these drugs is bleeding [10,11]. In patients treated with the combination aspirin and clopidogrel, non-fatal bleeding episodes occur in approximately 7% of the patients per year and fatal bleeding in 0.6% [10]. Hematotoxicity is less frequent, occurring in up to 3% of the patients treated with ticlopidine [9,12] and approximately 1% of the patients treated with clopidogrel [13]. Important hematological adverse effects associated with these drugs include neutropenia and agranulocytosis [14-18], thrombocytopenia [13,19,20], aplastic anemia [21,22], thrombotic thrombocytopenic purpura [23,24], and the hemolytic uremic syndrome [25].

In patients with neutropenia or agranulocytosis associated with thienopyridines, bone marrow investigations have shown impaired myelopoiesis [26,27], compatible with a direct toxic effect on the bone marrow. The association of the thienopyridines with aplastic anemia [21,22] also suggests a direct toxicity on the bone marrow. The precise mechanism associated with myelotoxicity of these drugs is so far not entirely clear, however. Liu and Uetrecht have demonstrated that ticlopidine can be oxidized by myeloperoxidase (MPO) in neutrophil granulocytes to a reactive thiophene-S-chloride derivative [28], which may be associated with bone marrow toxicity. In line with this study, we have shown recently that metabolites formed by MPO in neutrophil granulocytes of ticlopidine, clopidogrel or the clopidogrel metabolite clopidogrel carboxylate are toxic for cultured human myelopoietic progenitor cells [29].

Another possible explanation for hemotoxicity of these compounds could be by cytochrome P450 (CYP)-associated production of toxic metabolites by cells in the bone marrow, circulating blood cells and/or hepatocytes. Both clopidogrel and ticlopidine are prodrugs needing activation to be active. Activation of clopidogrel and ticlopidine is achieved by oxidation of the thiophene ring and hydrolytic ring opening of the thioester formed [3,30]. The free mercapto-group can not only covalently bind a cysteine residue of the $P2Y_{12}$-receptor on platelets [2], but possibly also other proteins or peptides with free mercapto groups such as for instance glutathione [31]. The fact that both ticlopidine and clopidogrel are mechanism-based inhibitors of CYP2B6 and CYP2C19 [32,33] supports this hypothesis.

While we could demonstrate that metabolites formed by MPO or CYP3A4 from clopidogrel or ticlopidine are toxic for human myelopoietic progenitor cells [29], it is currently unclear whether also mature blood cells can be affected and which mechanisms are responsible for myelotoxicity of these compounds on a cellular level. We therefore isolated and enzymatically characterized the most abundant bone marrow cells from peripheral blood, neutrophil granulocytes and lymphocytes, and studied metabolism and toxicity of ticlopidine, clopidogrel and clopidogrel carboxylate by and for these cells.

5.4 Materials and methods

5.4.1 Materials

Clopidogrel hydrogen sulphate was extracted from commercially available tablets (brand name Plavix®) and clopidogrel carboxylate was obtained by alkaline hydrolysis of clopidogrel. These procedures were carried out by ReseaChem Life Sciences (Burgdorf, Switzerland). Both substances were >99% pure by NMR analysis. Ticlopidine was obtained from Sigma-Aldrich (Buchs, Switzerland). Cell culture plates were purchased from BD Biosciences (Franklin Lakes, NJ, USA). All other chemicals and culture media used were purchased from Sigma (Buchs, Switzerland) or GIBCO (Lucerne, Switzerland).

5.4.2 Cell lines and cell culture:

Normal donor buffy coats were purchased from the local blood donation center, Basel (Switzerland). Blood cells were isolated from the buffy coat by a modification of the method described by Klebanoff et al. [34]. Briefly, the buffy coat was diluted 1:2 with Iscove's modified Dulbecco's medium (IMDM) and mixed with 4% dextran (MW 500'000 kD) in 0.9% NaCl (Sigma, Buchs, Switzerland) to allow erythrocytes to settle for 30 min. The supernatant layer was washed with phosphate-buffered saline (PBS), underlayed with 20 ml of cold Ficoll-Paque (GE Healthcare, Switzerland) and centrifuged at 500 g for 25 min. The supernatant containing PBMCs and the pellet containing neutrophil granulocytes were washed with phosphate-buffered saline (PBS) and resuspended separately with 10 ml of ice-cold 0.2% NaCl for 1 min to induce hypotonic lysis of contaminating erythrocytes. Isotonicity was restored by adding an equal volume of 1.6% NaCl. The suspensions were centrifuged at 350 g for 5 min and the pellets were washed twice with PBS. The pellets were then resuspended in IMDM containing 10% fetal bovine serum (FBS). Cells were counted

and the cell viability was assessed using trypan blue analysis. For the experiments, 500,000 freshly isolated lymphocytes or neutrophils in 1 mL IMDM containing 10% FBS per well were seeded in 24-well plates. Stock solutions of test compounds (clopidogrel, clopidogrel carboxylic acid and ticlopidine) were prepared in DMSO. The test compounds were added at a concentration of 1 to 100μM in presence or absence of CYP3A4 inhibitor ketoconazole (1μM) [31] or the MPO inhibitor rutin (20μM) [35]. The DMSO concentration was 0.1% in all incubations, including control incubations. This DMSO concentration is not cytotoxic [36]. Staurosporine (STS, final concentration 1μM) was used as a positive control for cytotoxicity.

Table 1 Sequence of primers used for RT-PCR analysis. F: forward primer and R: reverse primer.

Gene	Primer sequence
GADPH	F: 5'-AGC CAC ATC GCT CAG ACA C-3' R: 5'-GCC CAA TAC GAC CAA ATC C-3'
MPO	F: 5'-CGT CAA CTG CGA GAC CAG-3' R: 5'-GTC ATT GGG CGG GAT CTT-3'
CYP1A1	F: 5'-TCC AAG AGT CCA CCC TCC-3' R: 5'-AAG CAT GAT CAG TGT AGG GAT CT-3'
CYP1A2	F: 5'-GTC ATT GGT GCC ATG TGC T-3′ R: 5'-GCT GAG CAT CTC ATC GCT ACT-3′
CYP1B1	F: 5'-ACG TAC CGG CCA CTA TAC ACT-3' R: 5'-CTC GAG TCT GCA CAT CAG GA-3'
CYP2A6	F: 5'-CCA CGG GAC TTC ATT GAC TC-3′ R: 5'-CGT GGT CAT CAC CAG GTT TT-3′
CYP2B6	F: 5'-GCA CCA CTC TCC GCT ACG-3′ R: 5'-TCA ATC TCC CTG TAG ACT CTC TCT G-3′
CYP2C8	F: 5'-CGG GAC TTT ATC GAT TGC TT-3' R: 5'-TGA ATT CTG ACT TTT GGT TGT CC-3'
CYP2C9	F: 5'-TGG ATG AAG GTG GCA ATT TT-3' R: 5'-AGG AAT AAA AAC AGC TCC ATG C-3'
CYP2C19	F: 5'-CAC TTT CTG GAT GAA GGT GGA-3' R: 5'-CCC TCT CCC ACA CAA ATC C-3′
CYP2D6	F: 5'-TGT GCC CAT CAC CCA GAT-3' R: 5'-AAG GTG GAG ACG GAG AAG C-3'
CYP2E1	F: 5′-CAA GCC ATT TTC CAC AGG A-3′ R: 5′-CAA CAA AAG AAA CAA CTC CAT GC-3′
CYP3A4	F: 5′-GAT GGC TCT CAT CCC AGA CTT-3′ R: 5′-AGT CCA TGT GAA TGG GTT CC-3′
CYP3A5	F: 5'-GGA GTT CCG CCC TGA AAG-3′ R: 5'-TCC AGT TCC AAA GGG TGT GT-3′
CYP5A1	F: 5'-GGA GAC CTT CAA CCC TGA AA-3′ R: 5'-AAG GGC AGG TAC GTG AAG G-3′
CYP26A1	F: 5'-GCA GCC ACA TCT CTG ATC ACT-3′ R: 5'-TGT TGT CTT GAT TGC TCT TGC-3′
CYP26B1	F: 5'-AAG GGA TCC ATG GGC TTC-3′ R: 5'-CTT GAA CAC GTT GCC ATA CTT C-3′
CYP26C1	F: 5'-CCC TCG ACC TAA TCA TTC ACA-3′ R: 5'-GAG CTC CAC AGC CGA CTC-3′

5.4.3 mRNA expression of myeloperoxidase and cytochrome p450 enzymes

Quantitative real time PCR was performed to check for the presence of CYPs potentially responsible for metabolism of thienopyridines. Neutrophils or lymphocytes were lysed with 350μl of RLT buffer (Qiagen, Hombrechtikon, Switzerland) and the lysate was transferred to Qiashredder columns and spinned for 2 min at 13,000 rpm. From the eluate, total RNA was extracted according to the manufacturer's protocol (Qiagen RNeasy Mini Extraction kit). cDNA was reverse transcribed from the isolated RNA using the Qiagen Omniscript system. For quantitative RT-PCR 10ng cDNA was used. Forward and reverse primers for all CYPs tested, MPO and GAPDH (Table 1 for primers) were purchased from Microsynth (Balgach, Switzerland). RT-PCR was performed on an ABI PRISM 7700 sequence detector (PE Biosystems, Rotkreuz, Switzerland). Quantification of mRNA expression levels was performed using the SYBR-Green fluorescence method (Roche, Basel, Switzerland).

5.4.4 Protein expression of myeloperoxidase and CYPs

Neutrophils or lymphocytes were lysed on ice for 15 min with 200μl NET lysis buffer (50mM NaCl, 5mM EDTA, 50mM Tris-HCl pH 8.0, 1% NP-40 and a protease inhibitor tablet from Roche [Basel, Switzerland]). The samples were centrifuged for 10min at 4°C at 11,000 g and the supernatant was collected. The protein concentration was determined using the Pierce BCA protein assay kit (Darmstadt, Germany). Proteins (20μg) were separated by electrophoresis on a denaturating SDS polyacrylamide gel (4%) in the presence of molecular weight standards. After separation, proteins were transferred onto a nitrocellulose membrane (BioradTransBlot, Hercules, CA). The membranes were blocked with PBS-Tween 20 containing 5% milk solution (w/v) for 1h at room temperature and washed twice with PBS-Tween 20. The blocked membranes were incubated overnight at 4°C with

5% milk solution containing primary antibodies against MPO (Cell Signaling Technology, Allschwil, Switzerland), CYP3A4, CYP2C19 (Epitomics, Danvers, USA), CYP2B6, CYP1A2 (Abcam, Cambridge, UK) or CYP2C9 (BD bioscience, Allschwil, Switzerland). All of the antibodies mentioned were diluted according to the manufacturer's instructions. Horseradish peroxidase-conjugated anti-rabbit or anti-goat antibodies (Jackson laboratories Inc, Suffolk, UK) were used in combination with a chemiluminescent substrate (ECL, Amersham, UK) for protein visualization.

5.4.5 Cytotoxicity

The cytotoxicity assay was performed to determine the percentage of dead (necrotic or late apoptotic) cells in the incubations. Propidium iodide (PI) is a red fluorescent dye used to distinguish living cells having intact cell membranes (exclude PI) from dead cells having disrupted cell membranes (permeable to PI). The cells were treated for 24h with the test compounds in the presence or absence of the CYP3A4 inhibitor ketoconazole (1μM) or the MPO inhibitor rutin (20μM). After the incubation, cells were centrifuged at 350 g for 5min and washed with PBS before PI staining (final concentration 5μg/ml). Flow cytometry analysis was carried out with a FACS Calibur™ using CellQuest Pro software (BD Bioscience, Allschwil, Switzerland).

5.4.6 Metabolism of ticlopidine, clopidogrel and clopidogrel carboxylate

Neutrophils or lymphocytes (4.5 x 10^6/ml in 24-well plates) were incubated with clopidogrel, ticlopidine or clopidogrel carboxylate (10 or 100μM) at 37°C for different periods of time (0, 6, 12 and 24 h) in the presence or absence of the CYP3A4 inhibitor ketoconazole (1μM) or the MPO inhibitor rutin (20μM). Reactions were stopped by the addition of 300μl of methanol and the precipitated proteins were removed by centrifugation at 3000 g for 30min. The samples were analyzed by LC-MS as described previously [29].

5.4.7 Mitochondrial membrane potential

To detect the changes in mitochondrial membrane potential, cells were exposed to tetramethylrhodamine ethyl ester (TMRE). Cells were incubated with drugs in presence or absence of ketoconazole (1μM) or rutin (20μM) for 24h. After the incubation, cells were washed twice with PBS and incubated with 100nM TMRE in PBS for 30min at room temperature. The fluorescence signal of the cationic dye was monitored by flow cytometry using a FACS Calibur™ equipped with CellQuest Pro software (BD Bioscience, Allschwil, Switzerland).

5.4.8 Reactive oxygen species (ROS)

Cellular ROS generation was measured using the 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) assay [37]. Cells (5 x 10^5 cells /ml) were co-incubated with drugs and 25μM DCFH-DA in the presence or absence of ketoconazole (1μM) or rutin (20μM). After an incubation of 24h, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland).

5.4.9 Cellular cytochrome c content

The cytochrome c content in the cells was analyzed using the method escribed by Waterhouse et al. [34,38] with some modifications. Briefly, after 24 h of drug treatment, the cells were washed with PBS and subsequently permeabilized on ice with 100μl of digitonin 50 μg/ml in PBS containing 100mM KCl for 10min. The cells were then fixed with paraformaldehyde (4% in PBS) at room temperature. After 30 min, the cells were washed twice with PBS and incubated for 1h in blocking buffer (PBS containing 3% BSA and 0.05% saponin). Afterwards, cells were incubated at 4°C overnight with a monoclonal anti-cytochrome c antibody (BD Pharmingen, Allschwil, Switzerland), which was diluted 1:500 in blocking buffer. Finally, cells

were washed twice with PBS and incubated with an Alexa Fluor®-labeled secondary antibody (Invitrogen, Basel, Switzerland) diluted 1:500 in blocking buffer for 1 h at room temperature and analyzed by flow cytometry.

5.4.10 Caspase 9 assay

Caspase 9 activity was determined in both cell lines using the Caspase-Glo® 9 assay according to the protocol of the manufacturer (Promega, Dübendorf, Switzerland). Cells (10^5 cells/well) were seeded in a white walled 96-well plate and treated with the drugs mentioned above in presence or absence of ketoconazole (1μM) or rutin (20μM). After 24h of incubation, an equal volume of assay buffer was added to the cells. The plates were then shaken at 400 rpm for 30sec and again incubated for 1h. Luminescence was measured using the Tecan Infinite pro 200 microplate reader.

5.4.11 Statistical analysis

Data are presented as mean ± SD of at least four independent experiments, except where indicated. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between groups (e.g. incubations containing drugs vs. control incubations) were tested by one-way ANOVA followed by Bonferroni's post hoc test to localize significant results in the ANOVA. Differences between many groups at two levels were compared using two-way analysis of variance (ANOVA) followed by the protected Bonferroni's post hoc test to localize significant results obtained in the ANOVA. A p-value <0.05 was considered as statistically significant.

5.5 Results

5.5.1 Expression of CYPs and MPO in human neutrophil granulocytes and lymphocytes

mRNA expression levels of multiple CYPs and MPO were analyzed in human neutrophils and lymphocytes. As shown in Table 2, the mRNA expression of CYPs was generally lower in neutrophils compared to lymphocytes. In both cell types, the CYP with the highest mRNA expression was CYP1B1, followed by CYP5A1 and CYP26B1. None of these CYPs has been associated with the activation of clopidogrel or ticlopidine. The CYPs associated with activation of ticlopidine and/or clopidogrel (CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP3A4) revealed only a low mRNA expression in both lymphocytes and neutrophil granulocytes. As expected, the mRNA expression of MPO was high in neutrophils and low in lymphocytes. The protein expression data were consistent with mRNA expression (Figure 1). Neutrophils did not express CYPs associated with the activation of clopidogrel or ticlopidine but had a high expression of MPO. In comparison, lymphocytes expressed neither CYPs nor MPO.

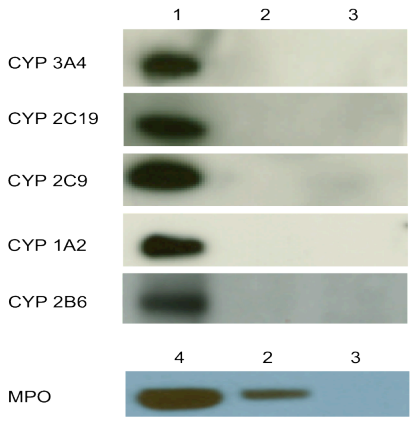

Figure 1 Protein expression of MPO and CYPs in human neutrophils and lymphocytes. The Western blot was carried out as described in Methods. Positive controls used were human microsomes (lane 1) for cytochrome P450 enzyme expression and HL-60 cells for myeloperoxidase expression (lane 4). Lanes 2 and 3 contain human neutrophil granulocytes and lymphocytes, respectively.

Gene	Lymphocytes (Ct)	Neutrophil granulocytes (Ct)
GADPH	18.9 ± 0.3	17.4 ± 0.3
MPO	26.8 ± 0.3	21.7 ± 0.4
CYP1A1	31.7 ± 0.1	33.1 ± 2.4
CYP1A2	30.3 ± 0.1	32.5 ± 1.2
CYP1B1	20.3 ± 0.7	20.8 ± 1.6
CYP2A6	31.7 ± 0.1	31.8 ± 0.1
CYP2B6	31.6 ± 0.9	30.6 ± 1.0
CYP2C8	29.4 ± 0.2	30.1 ± 0.4
CYP2C9	30.9 ± 0.1	N.D
CYP2C19	N.D	34.4 ± 1.0
CYP2D6	31.3 ± 0.1	33.6 ± 0.3
CYP2E1	29.7 ± 0.1	33.4 ± 1.2
CYP3A4	30.3 ± 0.1	35.0 ± 0.1
CYP3A5	30.1 ± 0.1	28.7 ± 0.7
CYP5A1	26.2 ± 0.1	23.9 ± 0.3
CYP26A1	30.9 ± 0.1	31.4 ± 2.0
CYP26B1	28.5 ± 0.4	24.6 ± 0.2
CYP26C1	N.D	N.D

Table 2 CYP mRNA expression in human lymphocytes and neutrophil granulocytes. Three independent experiments were performed as described in Methods. Data are expressed as the Ct value. N.D: not detectable (Ct value not determinable).

5.5.2 Cytotoxicity of clopidogrel, ticlopidine and clopidogrel carboxylate for lymphocytes and neutrophil granulocytes

Cytotoxicity was assessed using PI staining and subsequent FACS analysis (Figure 2). For neutrophils exposed for 24 h, clopidogrel, ticlopidine or clopidogrel carboxylate showed a concentration-dependent toxicity starting at 10μM. This toxicity could at least partially be prevented by 20μM rutin, a MPO inhibitor [35], but not significantly by the CYP inhibitor ketoconazole [31]. Considering lymphocytes, clopidogrel and ticlopidine were cytotoxic starting at 10μM with a clear concentration-dependency, but this cytotoxicity could not be prevented by rutin or ketoconazole. Clopidogrel carboxylate was also cytotoxic for lymphocytes, starting at 10μM. This cytotoxicity was not clearly concentration-dependent, was less accentuated than the cytotoxicity associated with clopidogrel or ticlopidine and could not be prevented by rutin or ketoconazole.

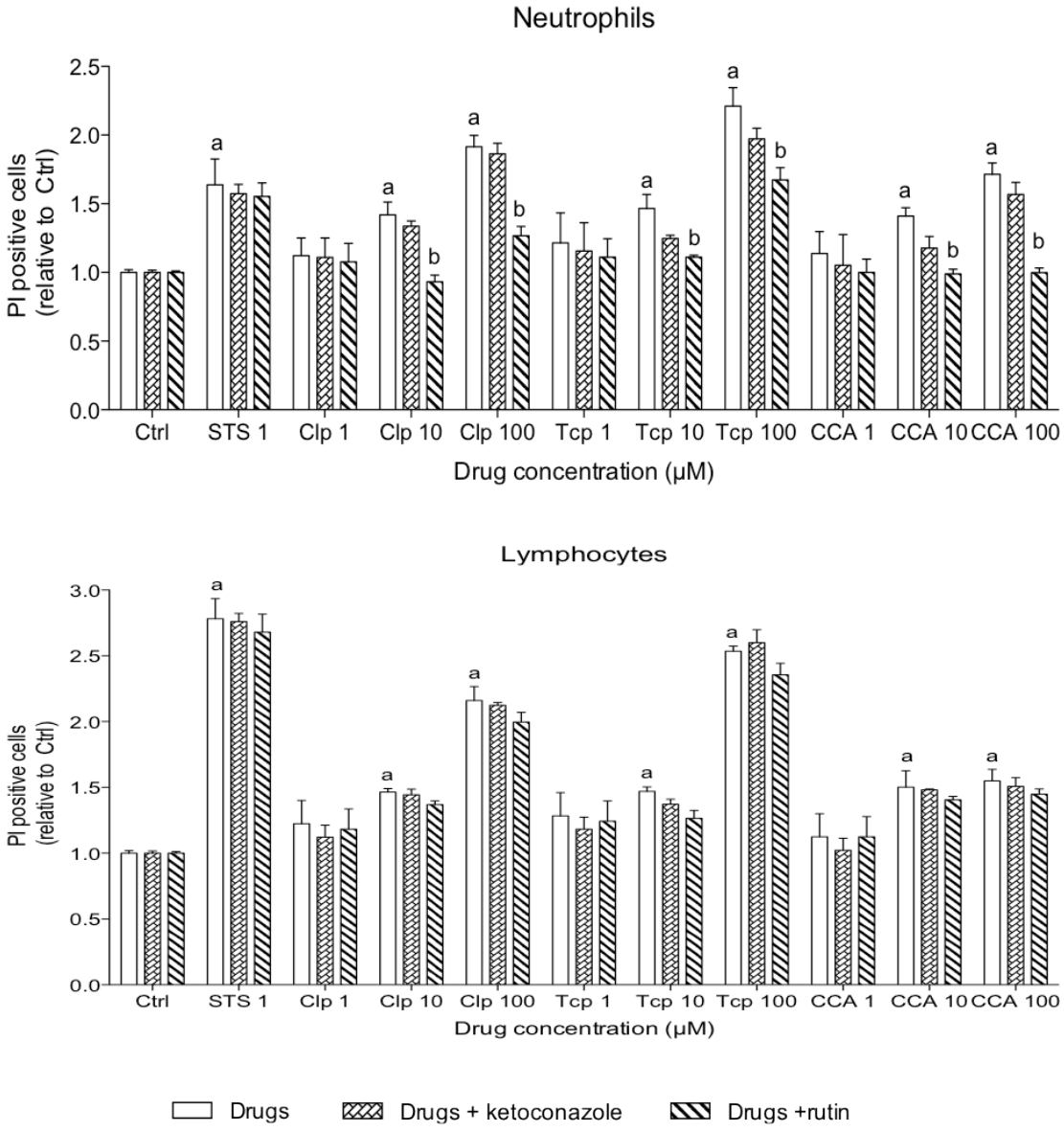

Figure 2 Cytotoxicity of clopidogrel, ticlopidine and clopidogrel carboxylate for neutrophil granulocytes and lymphocytes. Both neutrophil granulocytes and lymphocytes were exposed for 24h to the compounds mentioned above at the concentrations indicated in the Figure labels. Some incubations contained also 1μM ketoconazole (CYP3A4 inhibitor) or 20μM rutin (MPO inhibitor) as indicated in the figure labels. Cytotoxicity was measured using propidium iodine staining. DMSO-treated cells (0.1%) were used as control incubations (Ctrl) and cells treated with 1μM staurosporine as a positive control. The results were normalized to control incubations and are expressed as the means + SD of four independent experiments. (a) $p<0.05$ incubations containing drugs without pretreatment versus respective control incubations; (b) $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate.

5.5.3 Metabolism of clopidogrel, ticlopidine or clopidogrel carboxylate by neutrophil granulocytes and lymphocytes

The metabolism of the compounds mentioned above by freshly isolated human neutrophils or lymphocytes were studied using LC-MS/MS. After 24 h of incubation with neutrophils, the compounds were degraded by 20 to 60%, depending on the compound and on the initial concentration (Fig. 3). The metabolism of the compounds investigated was not significantly influenced by ketoconazole, which is compatible with the observation that neutrophils have no protein expression of CYP3A4 (Fig. 2). In the presence of rutin, the metabolism of the compounds was prevented almost completely, indicating that MPO present in neutrophils is the most important enzyme for metabolism of the thienopyridines investigated. In contrast, the incubations with lymphocytes showed no (clopidogrel carboxylate) or only a minor metabolization of the compounds tested (<20% for clopidogrel and ticlopidine), which could not be inhibited by ketoconazole or rutin (Fig. 4). This finding is compatible with the observation that lymphocytes have no protein expression of CYP3A4 and MPO (Fig. 2).

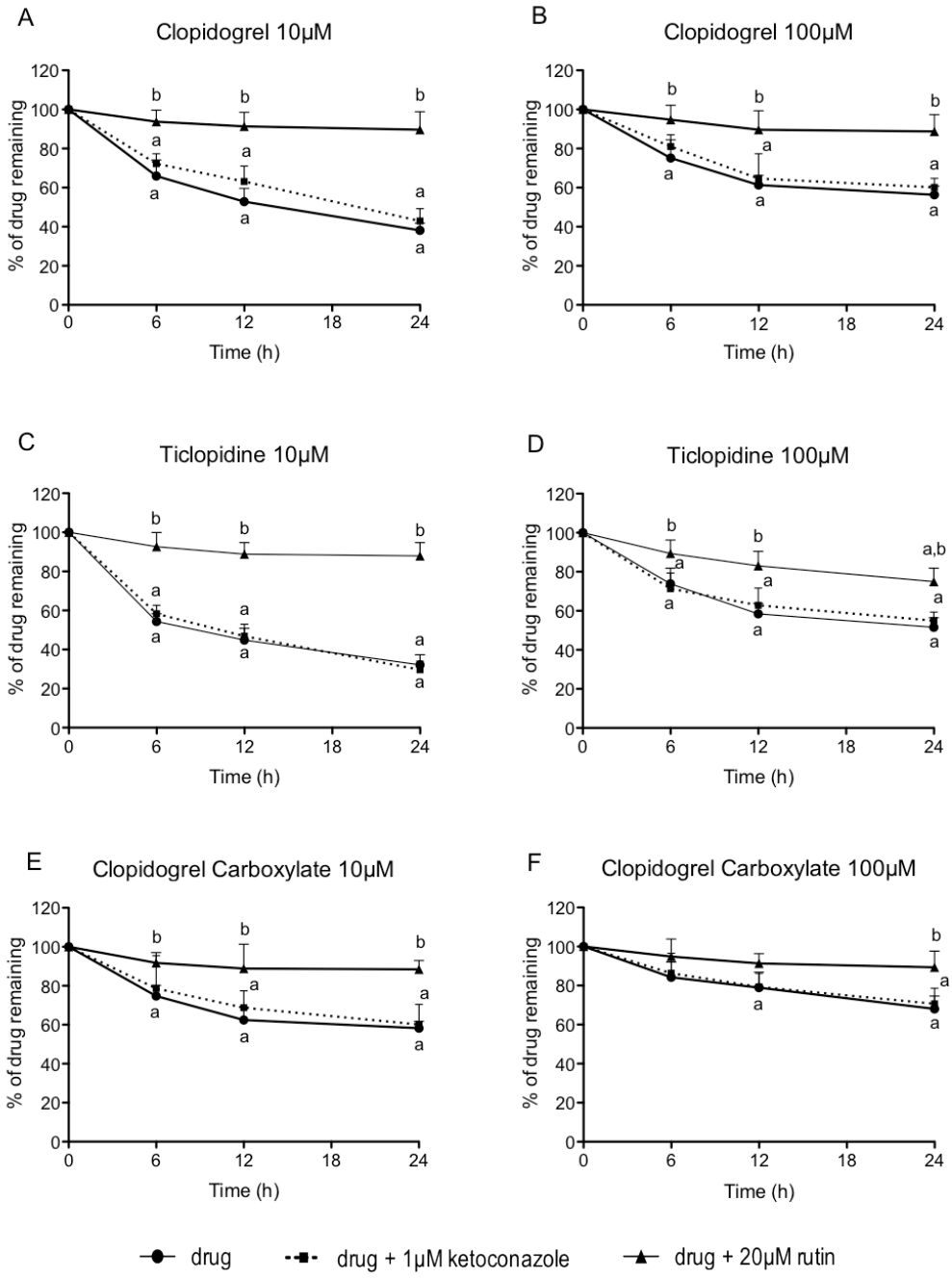

Figure 3 Metabolism of clopidogrel, ticlopidine and clopidogrel carboxylate by human neutrophil granulocytes. Clopidogrel 10μM (A), clopidogrel 100μM (B), ticlopidine10μM (C), ticlopidine100μM (D), clopidogrel carboxylate 10 μM (E) or clopidogrel carboxylate 100μM (F) were incubated with neutrophil granulocytes as described in Methods. Some incubations contained also the myeloperoxidase inhibitor rutin (20μM) or the CYP3A4 inhibitor ketoconazole (1μM) as indicated in the Figure labels. Data are expressed as the percentage of drug remaining during 24 h of incubation. The results are the means + SD of three independent determinations. (a) $p<0.05$ versus respective incubations at time 0 min; (b) $p<0.05$ incubations containing drug with 20μM rutin versus respective incubations containing drug only.

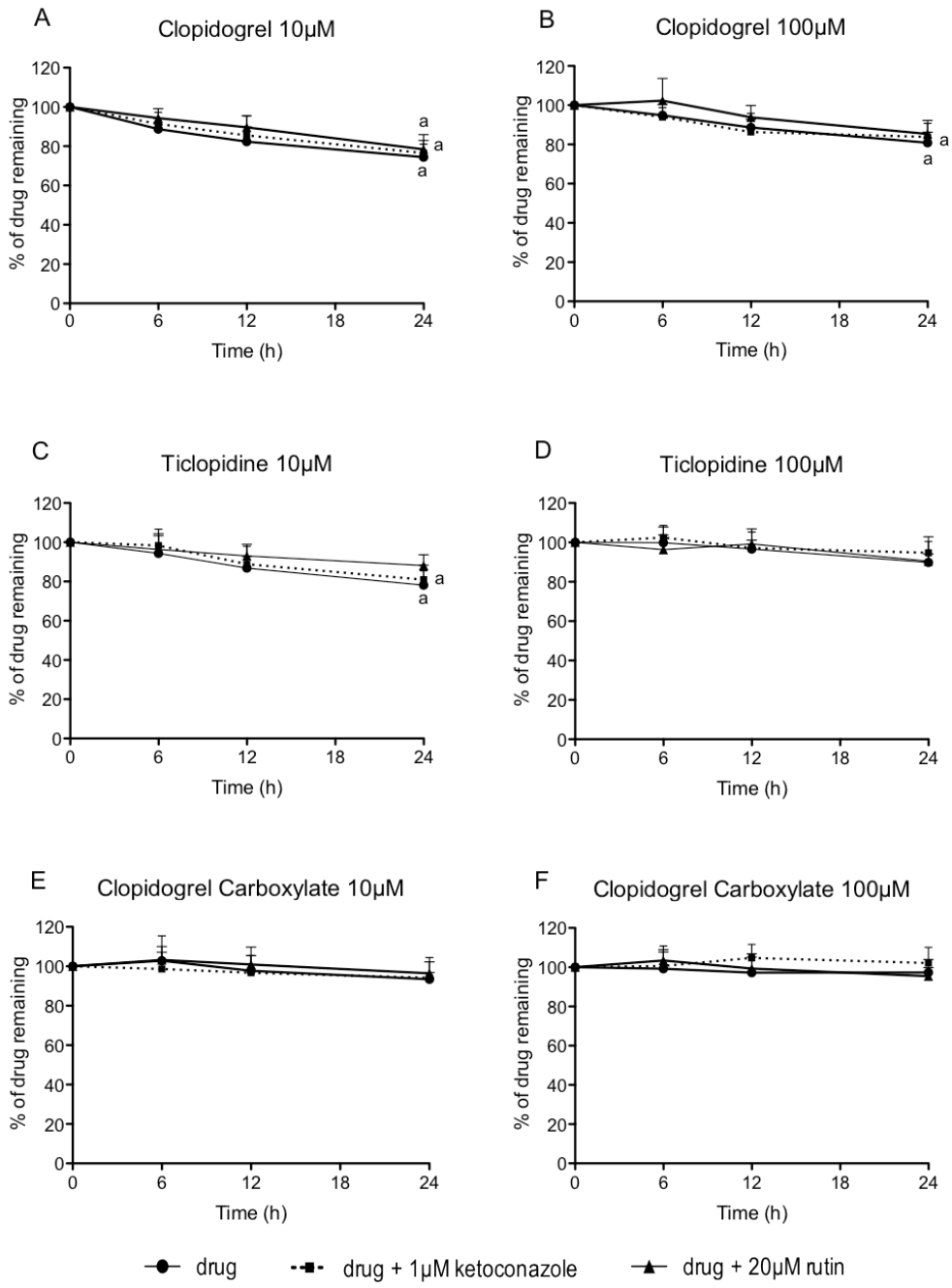

Figure 4 Metabolism of clopidogrel, ticlopidine and clopidogrel carboxylate by human lymphocytes. Clopidogrel 10μM (A), clopidogrel 100μM (B), ticlopidine10μM (C), ticlopidine100μM (D), clopidogrel carboxylate 10 μM (E) or clopidogrel carboxylate 100μM (F) were incubated with lymphocytes for 24 h. Some incubations contained also rutin (20μM) or ketoconazole (1μM) as indicated in the Figure. Data are expressed as the percentage of drug remaining during 24 h of incubation. The results are the means + SD of three independent determinations. (a) $p<0.05$ versus respective incubations at time 0 min; (b) $p<0.05$ incubations containing drug with 20μM rutin versus respective incubations containing drug only.

5.5.4 Effect of clopidogrel, ticlopidine or clopidogrel carboxylate on the mitochondrial membrane potential

The mitochondrial membrane potential (Ψm) is maintained mainly by the proton gradient generated by the activity of complexes of the electron transport chain. The mitochondrial membrane potential is therefore a marker of mitochondrial function [36,37]. We investigated the changes in mitochondrial Ψm with the dye TMRE, which accumulates in mitochondria depending on Ψm (Fig. 5). In neutrophils, all compounds tested decreased Ψm in a concentration-dependent fashion, but without reaching significance for 10μM. This effect could almost completely be prevented by rutin, but not by ketoconazole. In contrast, in lymphocytes, only clopidogrel and ticlopidine were associated with a concentration-dependent decrease of Ψm, whereas clopidogrel carboxylate did not significantly affect Ψm. In contrast to neutrophils, neither rutin nor ketoconazole could prevent the toxicity of clopidogrel or ticlopidine on lymphocytes.

5.5.5 Effect of clopidogrel, ticlopidine or clopidogrel carboxylate on the production of reactive oxygen species (ROS)

Mitochondrial dysfunction can be associated with increased production of ROS [37]. As shown in Figure 6, ROS production by neutrophils increased dose-dependently and significantly in the presence of all compounds investigated, starting at 10μM. This increase could be prevented almost completely by the addition of rutin, whereas ketoconazole was clearly less effective. Concerning lymphocytes, there was a significant increase of cellular ROS levels only at high concentrations (100μM) of clopidogrel and ticlopidine and neither rutin nor ketoconazole could prevent this increase. Clopidogrel carboxylate had no effect on cellular ROS levels in lymphocytes.

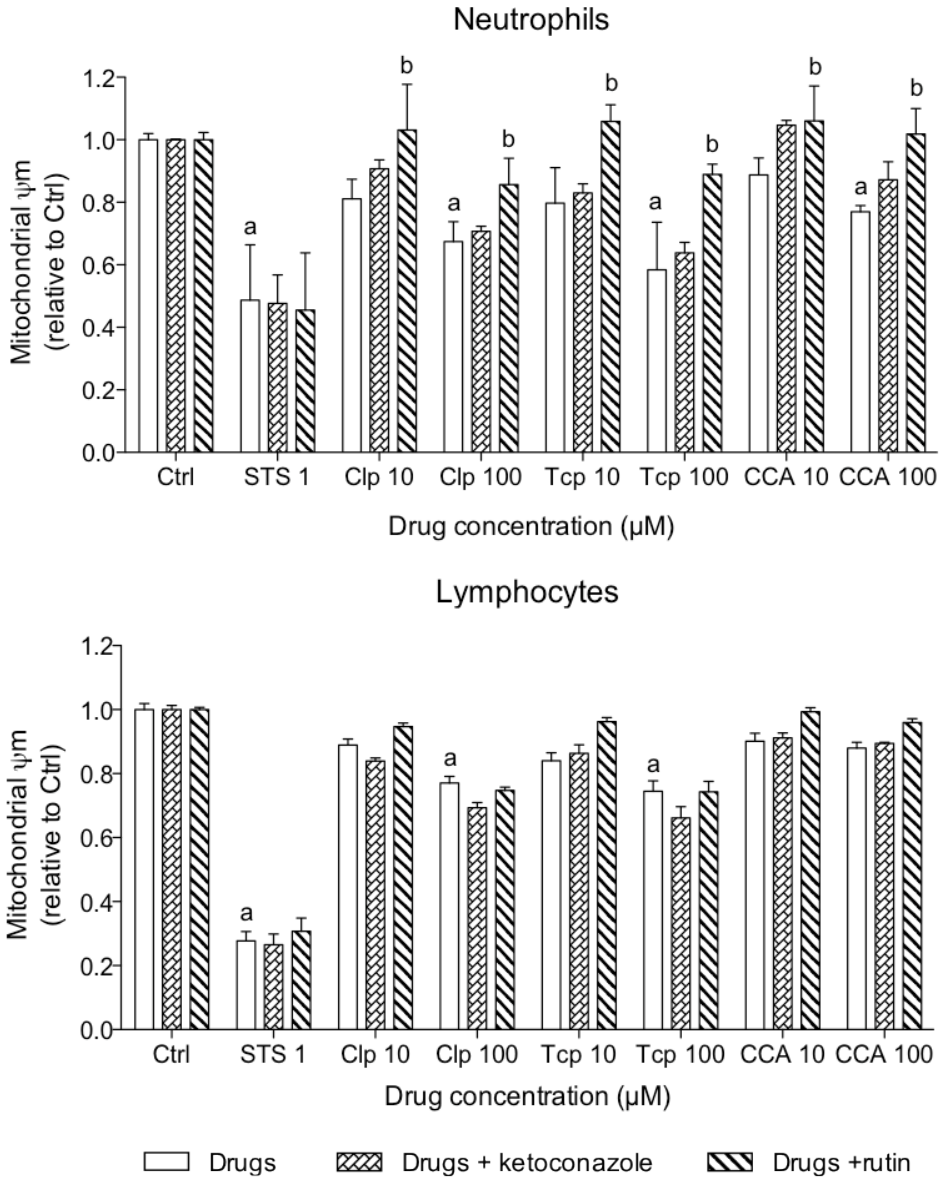

Figure 5 Effect of clopidogrel, ticlopidine and clopidogrel carboxylate on mitochondrial membrane potential in neutrophil granulocytes and lymphocytes. Cells were exposed for 24h to different concentrations of the compounds indicated above as indicated in the Figure labels. Some incubations contained ketoconazole (1μM) or rutin (20μM) as indicated in Figure labels. TMRE was used to determine the mitochondrial membrane potential (Ψm) as described in Methods. The results were normalized to control incubations containing 0.1% DMSO. Incubations containing 1μM staurosporine were used as a positive control. Results are presented as the mean + SD of four independent experiments. (a) $p<0.05$ incubations containing drugs alone versus respective control incubations (Ctrl); (b) $p<0.05$ Incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate.

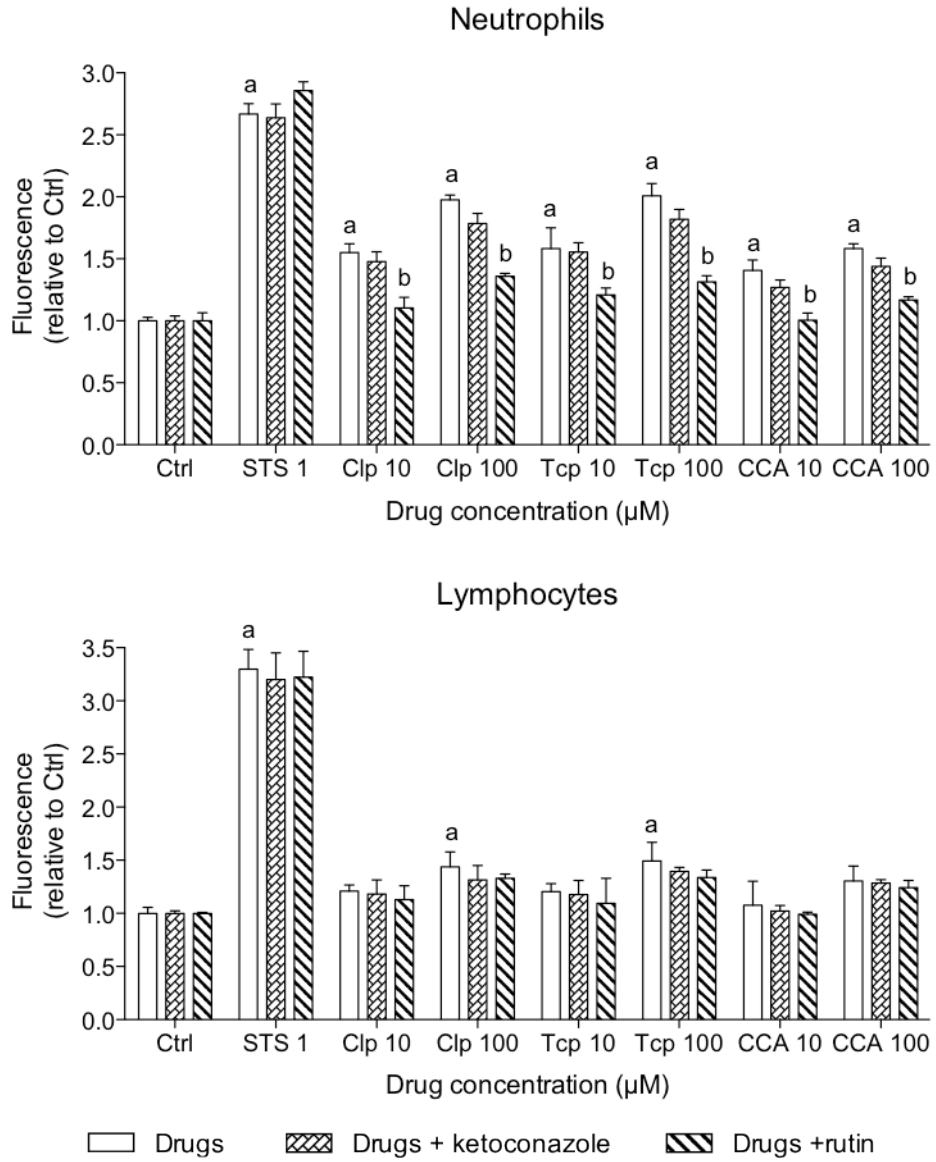

Figure 6 ROS formation by neutrophils granulocytes and lymphocytes. Cells were treated for 24h with clopidogrel, ticlopidine or clopidogrel carboxylate at the concentrations indicated in the Figure labels. Some incubations contained also 1μM ketoconazole or 20μM rutin as indicated in the Figure labels. Cellular levels of ROS were measured using 2′, 7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) as described in Methods. Cells treated with 0.1% DMSO were used as negative (Ctrl) and cells treated with 1μM staurosporine as a positive control. The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of four independent experiments. (a) $p<0.05$ incubations containing drugs alone versus respective control incubations (Ctrl); (b) $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate.

5.5.6 Effect of clopidogrel, ticlopidine or clopidogrel carboxylate on the mitochondrial cytochrome c content

Increased mitochondrial production of ROS can lead to mitochondrial membrane permeabilization and release of mitochondrial proteins (e.g. cytochrome c) into the cytoplasm. We therefore investigated the effect of the thienopyridines on the mitochondrial cytochrome c content (Figure 7). In neutrophils incubated for 24 h with clopidogrel, ticlopidine or clopidogrel carboxylate, the mitochondrial cytochrome c content showed a concentration-dependent decrease. This decrease could be prevented completely by rutin, but not by ketoconazole. In lymphocytes, only clopidogrel and ticlopidine were associated with a concentration-dependent decrease in the mitochondrial cytochrome c content, whereas clopidogrel carboxylate was without a significant effect. Rutin did not prevent the drop in mitochondrial cytochrome c for clopidogrel or ticlopidine and also ketoconazole was without a significant preventive effect.

5.5.7 Activation of caspase 9 by clopidogrel, ticlopidine or clopidogrel carboxylate

Release of cytochrome c from mitochondria into the cytoplasm can activate the apoptosome (apaf-1), which cleaves pro-caspase-9 to caspase-9 and eventually triggers apoptosis. We therefore determined the activity of caspase-9 as a surrogate of apoptosis (Figure 8). In neutrophils, all compounds investigated were associated with a concentration-dependent activation of caspase-9 starting at 10μM. The activation could at least partially be prevented by rutin, whereas ketoconazole was not associated with a significant prevention of caspase-9 activation. In lymphocytes, only the higher concentration (100μM) of clopidogrel and ticlopidine induced activation of caspase-9, whereas clopidogrel carboxylate had no effect on caspase-9.

In contrast to neutrophils, neither ketoconazole nor rutin could prevent acspase-9 activation.

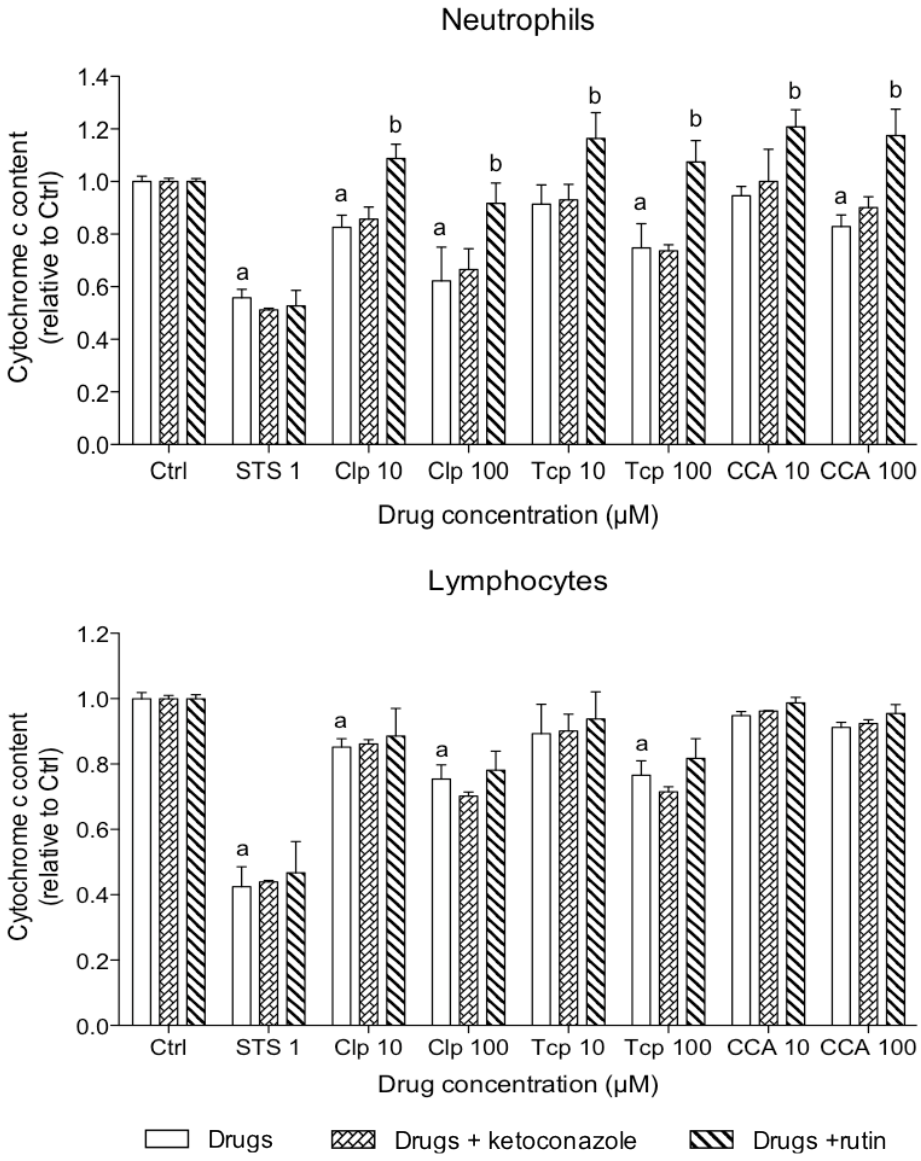

Figure 7 Mitochondrial cytochrome c content in neutrophil granulocytes and lymphocytes. Cells were exposed for 24h to the compounds mentioned above at the concentrations given in the Figure labels. Some incubations contained also ketoconazole (1μM) or rutin (20μM) as indicated in the Figure labels. Cells treated with 0.1% DMSO were used as a negative (Ctrl) and cells treated with 1μM staurosporine as a positive control. The mitochondrial cytochrome c content was determined after cell permeabilization using flow cytometry as described in Methods. The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of five independent experiments. (a) $p<0.05$ incubations containing drugs alone versus respective control incubations (Ctrl); (b) $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate.

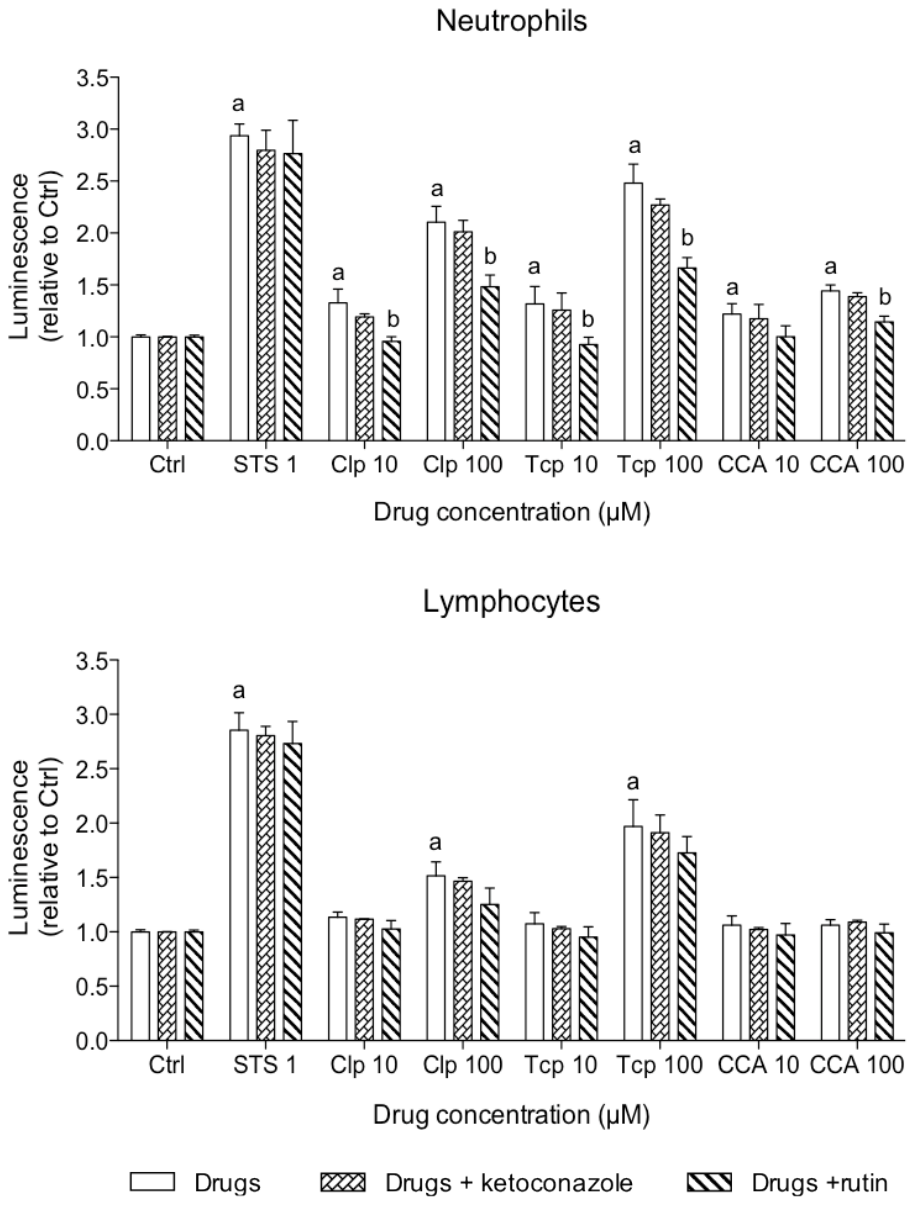

Figure 8 Caspase 9 activity in neutrophil granulocytes and lymphocytes. The cells were treated for 24h with clopidogrel, ticlopidine or clopidogrel carboxylate at the concentrations given in the Figure labels. Some incubations contained also 1μM ketoconazole or 20μM rutin as indicated in the Figure labels. Cells treated with 0.1% DMSO were used as negative (Ctrl) and cells treated with 1μM staurosporine as a positive control. The fluorometric assay was performed as described in Methods. The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of five independent experiments. (a) $p<0.05$ incubations containing drugs alone versus respective control incubations (Ctrl); (b) $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate.

5.6 Discussion

Our study showed that clopidogrel, the clopidogrel metabolite clopidogrel carboxylate as well as ticlopidine are toxic for neutrophil granulocytes in a concentration-dependent fashion. Cytotoxicity could almost completely be prevented by the addition of the MPO inhibitor rutin, but not by the CYP inhibitor ketoconazole. Clopidogrel, ticlopidine and clopidogrel carboxylate were also toxic for lymphocytes, but cytotoxicity for lymphocytes was less accentuated and could not be prevented by ketoconazole or rutin.

Both thienopyridines studied, clopidogrel and ticlopidine, have to be activated before they can bind to the $P2Y_{12}$-receptor and inhibit platelet aggregation. For clopidogrel, CYP1A2, 2B6, 2C9, 2C19 and 3A4 have been reported to be involved in the activation [31,39,40]. For ticlopidine, the CYPs involved in the activation are less clear, but CYP2B6 and 2C19 appear also to be involved [30]. Earlier studies assessing the expression of CYPs in human bone marrow have shown a high mRNA expression of CYP1A1, 1B1, 2C8, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1 [41], but not of CYPs involved in the activation of clopidogrel or ticlopidine. This is in line with the current study, showing a high expression of mainly CYP1B1 mRNA in granulocytes and lymphocytes, but not of CYPs involved in the activation of ticlopidine or clopidogrel. In line with mRNA expression, protein expression for CYP1A2, 2B6, 2C9, 2C19 and 3A4 was not detectable in granulocytes or lymphocytes. Accordingly, metabolism or cytotoxicity of clopidogrel or ticlopidine associated with CYPs in granulocytes or lymphocytes was negligible. For neutrophil granulocytes this is shown in Fig. 2 and Fig. 3, demonstrating that cytotoxicity and degradation of clopidogrel and ticlopidine can be prevented almost completely by blocking MPO, but not by the CYP inhibitor ketoconazole. Furthermore, in lymphocytes, where MPO is lacking

(Fig. 1), degradation was minimal for ticlopidine and clopidogrel and not detectable for clopidogrel carboxylate (Fig. 3).

As mentioned above and shown in Fig. 2, activation of clopidogrel or ticlopidine by MPO was also the cytotoxic principle of these compounds in neutrophil granulocytes. Accordingly, cytotoxicity could be prevented by the addition of rutin, but not of ketoconazole. Importantly, clopidogrel carboxylate could also be metabolized by MPO and the metabolites formed were toxic for neutrophil granulocytes. In line with these observations, in lymphocytes, where MPO is lacking (Fig. 1), cytotoxicity was less accentuated for clopidogrel or ticlopidine compared to neutrophils and could not be prevented by rutin or by ketoconazole. Similarly, clopidogrel carboxylate showed only a weak toxicity for lymphocytes which was not clearly concentration-dependent. This finding could be predicted from the observation that clopidogrel carboxylate was not metabolized by lymphocytes (Fig. 4). These results are in line with our observations in human hematopoietic stem cells [29]. In human hematopoietic stem cells, both ticlopidine and clopidogrel were cytotoxic, cytotoxicity was increased by metabolites formed by MPO and clopidogrel carboxylate was only cytotoxic after metabolization by MPO.

The current study was also designed to detect possible molecular mechanisms by which the investigated compounds exert cytotoxicity. The observed decrease in the mitochondrial membrane potential (Fig. 5) indicated that in particular metabolites formed by MPO from clopidogrel, ticlopidine or clopidogrel carboxylate impair mitochondrial function. The mitochondrial membrane potential can be decreased by various mechanisms, among them impairment of the mitochondrial electron transport chain [37,42]. Impairment of the electron transport chain, particularly at complex I or III, can be associated with increased production of ROS [43,44]. As shown in Fig. 6, cells exposed to clopidogrel or ticlopidine indeed showed increased ROS

accumulation, which could partially be blocked by rutin. Cellular accumulation of ROS can lead to mitochondrial membrane permeability transition, inducing mitochondrial swelling and eventually rupture of the outer mitochondrial membrane [45,46]. During this process, cytochrome c and other proteins located in the space between the inner and outer mitochondrial membrane are spilled into the cytoplasm, where they can become part of the apoptosome and initiate apoptosis [47,48]. The results of the current study are compatible with this mechanism, showing a loss of mitochondrial cytochrome c (Fig. 7) and activation of caspase 9 (Fig. 8) by the compounds investigated. The role of metabolites formed by MPO is highlighted by the fact that rutin (but not ketoconazole) could prevent these processes almost completely.

The median serum ticlopidine concentrations reached at steady state after the usual dose (250 mg every 12 h) were 990 $\mu g/L$ or approximately 4 μM [49], a concentration close to the concentration where we started to observe cytotoxicity for neutrophil granulocytes. Since cytotoxicity of ticlopidine could almost completely be prevented by rutin, metabolites formed by MPO appear to be the myelotoxic principle at low ticlopidine concentrations as proposed by Liu and Uetrecht [28]. The role of metabolites formed by MPO contained in neutrophil granulocytes concerning hematotoxicity of ticlopidine is also supported indirectly by the observation that lymphopenia has not been reported as an adverse reaction associated with ticlopidine. For clopidogrel, the serum concentration after the administration of a 600 mg loading dose is in the range of 38 $\mu g/L$ or 0.1 μM [50]. This concentration is much lower than the concentrations associated with cytotoxicity, indicating that non-metabolized clopidogrel is most probably not responsible for myelotoxicity. For clopidogrel carboxylate, serum concentrations in the range of 2780 $\mu g/L$ or approximately 10 μM have been reported in patients treated with the usual daily dose of 75 mg [51]. This concentration is close to

the concentration observed to be cytotoxic for neutrophil granulocytes and, to a lesser extent, also for lymphocytes. Considering clopidogrel, toxicity for neutrophil granulocytes can therefore be explained best by MPO-associated formation of toxic metabolites of clopidogrel carboxylate within neutrophil granulocytes. Such metabolites were not only toxic for mature neutrophil granulocytes, however, but also for hematopoietic progenitor cells [29].

We conclude that metabolite formation by MPO is essential for the hematotoxicity of ticlopidine and clopidogrel. For clopidogrel, the hematotoxic metabolites are most probably formed from the inactive metabolite clopidogrel carboxylate, and for ticlopidine from the parent substance. These metabolites are mitochondrial toxins leading to cellular ROS accumulation and induction of apoptosis by the endogenous pathway.

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6. Toxicity of clopidogrel, ticlopidine and prasugrel thiolactone on the human promyelocytic leukemia cell line

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6.1 Summary

We investigated the mechanism of cytotoxicity of ticlopidine, clopidogrel, clopidogrel carboxylate and prasugrel thiolactone on the promyelocytic leukemia cell line HL-60, highly passaged (spent) HL-60 cells stably transfected with cytochrome P450 (CYP) 3A4 (HL-60/CYP3A4). HL-60 cells had a high expression of myeloperoxidase (MPO) but no CYP3A4. In contrast, HL-60/3A4 cells had a high expression of CYP3A4 and no expression of MPO. All substances tested were cytotoxic for HL-60 cells, starting at 10μM. This toxicity could be prevented by the MPO inhibitor rutin, but not by the CYP inhibitor ketoconazole. All substances were metabolized by HL-60 cells, which could be blocked by rutin. Ticlopidine, clopidogrel and prasugrel thiolactone were also toxic for HL-60/CYP3A4 cells, again starting at 10μM. Cytotoxicity could be prevented by ketoconazole, but not by rutin. In contrast, clopidogrel carboxylate was not toxic for HL-60/CYP3A4 cells up to 100μM. Ticlopidine, clopidogrel and prasugrel thiolactone were metabolized by HL-60/CYP3A4 cells which could be blocked by ketoconazole. Metabolism of clopidogrel carboxylate by HL-60/CYP3A4 cells was negligible. Metabolites formed by MPO or by CYP3A4 decreased the mitochondrial membrane potential, increased ROS production, decreased the cellular GSH/GSSG ratio, were associated with a spill of cytochrome c into the cytosol and induced apoptosis. We conclude that all thienopyridines tested can form toxic metabolites following exposure to MPO, and, with the exception of clopidogrel carboxylate, also with CYP3A4. Cytotoxicity of the toxic metabolites formed can be explained by mitochondrial damage eventually leading to apoptosis. In vivo myelotoxicity of ticlopidine and clopidogrel can be explained by the formation of toxic metabolites by MPO within the bone marrow from the parent substance (ticlopidine) or from clopidogrel carboxylate (clopidogrel). Prasugrel is not myelotoxic in vivo, because the concentration of the metabolites reached in bone marrow is not high enough.

6.2 Abbreviations

- MPO: myeloperoxidase
- CYP: cytochrome P450
- IMDM: Iscove's modified Dulbecco's medium
- PBS: phosphate-buffered saline
- FBS: fetal bovine serum
- PI: propidium iodide
- TMRE: tetramethylrhodamine ethyl ester
- DCFH-DA: dichlorodihydrofluorescein-diacetate

6.3 Introduction

The thienopyridines ticlopidine, clopidogrel and prasugrel are irreversible inhibitors of the ADP ($P2Y_{12}$)-receptors on blood platelets [1-3]. By blocking platelet activation by ADP, they inhibit platelet aggregation. Accordingly, they are used in patients where platelet activation plays an important role, in particular in patients with cardiovascular disease including acute coronary syndrome [4,5], stroke [6,7] or peripheral arterial disease [6].

All thienopyridines are prodrugs [8]. All of them form a thiolactone derivative, which is then converted to the respective active metabolite. The formation of the thiolactone derivative is CYP-dependent for ticlopidine and clopidogrel [8] and is achieved by carboxylesterases for prasugrel [9]. The formation of the active metabolite from the thiolactone derivative is also a CYP-dependent reaction. The most important CYPs involved in these reactions are CYP3A4, CYP2B6, CYP2C9 and CYP2C19 [8,10]. For prasugrel, the conversion of the thiolactone derivative (R-95913) to the active metabolite (R-138727) involves the formation of an S-oxide intermediate [11] achieved mainly by CYP3A4 and CYP2B6 [10]. The conversion of the S-oxide to the active metabolite R-138727 involves the formation of a glutathione conjugate [11]. Similar to prasugrel, clopidogrel is also an ester. Approximately 85% of clopidogrel is hydrolyzed during the absorption process, leading to the formation of the inactive metabolite clopidogrel carboxylate [8].

These agents are generally well tolerated. As expected, the most important adverse reaction associated with these drugs is bleeding. In large studies, major bleeding has been reported in 1 to 3.7% of the patients treated with clopidogrel [4-6] and in 2.4% of the patients treated with prasugrel [4]. Also hepatotoxicity has been described for both ticlopidine [12-14] and clopidogrel [15-17]. For prasugrel, patients with clinically relevant hepatotoxicity have so far not been reported. Myelotoxicity is an additional concern associated with both ticlopidine and clopidogrel. Both agents have been associated

with leucopenia and agranulocytosis as well as with aplastic anemia [18-20]. Similar to hepatotoxicity, myelotoxicity has so far not been described for prasugrel. Interestingly, a patient has recently reported who developed severe neutropenia two weeks after starting treatment with clopidogrel. Since the patient had been treated with bare metal stents due to an acute coronary syndrome, he needed aggressive platelet aggregation inhibition for preventing stent thrombosis. Therefore, clopidogrel was replaced by prasugrel. During this treatment, the neutrophil count gradually normalized, demonstrating that prasugrel was not myelotoxic in this patient [21].

In previous investigations, we have shown that both clopidogrel and ticlopidine show a concentration-dependent toxicity on human myeloid progenitor cells [22] and on mature human neutrophils and lymphocytes (Maseneni S et al. 2012;submitted). This toxicity was associated with both the respective parent compounds and with metabolites formed by myeloperoxidase (MPO) contained in neutrophil granulocytes [22]. Furthermore, metabolites formed by CYP3A4 can also contribute to cytotoxicity of these compounds, but most probably not in bone marrow [22].

HL-60 cells are a human promyelocytic leukemia cell line [23] which contain MPO. After several passages, HL-60 cells lose the capability to express MPO and are called spent cells [24]. Taking into account these properties, we reasoned that HL-60 cells could provide a suitable cell model to study the toxicity ticlopidine, clopidogrel, the clopidogrel metabolite clopidogrel carboxylate and prasugrel. Our aim was to study the molecular basis of the cytotoxicity of these compounds in HL-60 containing MPO and also in spent HL-60 cells stably transfected with human cytochrome P450 (CYP) 3A4. Since the same cells were used, cytotoxicity associated with metabolites produced by MPO or CYP3A4 could be compared directly.

6.4 Materials and methods

6.4.1 Materials

Clopidogrel hydrogen sulphate was extracted from commercially available tablets (brand name Plavix®) and clopidogrel carboxylate was obtained by alkaline hydrolysis of clopidogrel. These procedures were carried out by ReseaChem Life Sciences (Burgdorf, Switzerland). Both substances were >99% pure by NMR analysis. Ticlopidine was obtained from Sigma-Aldrich (Buchs, Switzerland). Prasugrel was obtained from Sirius Fine Chemicals SiChem GmbH (Bremen, Germany). Cell culture plates were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The ToxiLight® assay kit was purchased from Lonza (Basel, Switzerland). The FuGENE™ transfection kit was purchased from Promega (Dübendorf, Switzerland). All other chemicals and culture media used were purchased from Sigma (Buchs, Switzerland) and GIBCO (Lucerne, Switzerland), respectively.

6.4.2 Cell lines and cell culture

The human promyelocytic leukemia cells (HL-60) were purchased from the American Type Culture Collection. HL-60 cells and HL-60 overexpressing CYP3A4 (HL-60/CYP3A4 cells) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). Both cell lines were grown in a humidified incubator with 5% CO2 at 37ºC.

6.4.3 Expression of human CYP3A4 in HL-60 cells

The functional human CYP3A4 gene was excised from the pCR2.1-topo® vector (Zahno et .al 2010) and cloned into pCDNA 3.1 Hygro (+) plasmid (Invitrogen, Lucerne, Switzerland). Highly passaged HL-60 cells (Spent HL-60 cells, which have

lost the MPO gene) were transfected with the clone using FuGENETM transfection kit and allowed to grow in medium containing 150 μg/ml hygromycin B.

6.4.4 Cytotoxicity assays

Cytotoxicity was determined either by the determination of the activity of adenylate kinase in the supernatant of cell incubations or by propidium iodide staining. Adenylate kinase activity was determined using the ToxiLight assay kit as described previously [25]. Luminescence was measured using a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland). For propidium iodide staining, cells were centrifuged at 350 g for 5 min after treatment with the compounds indicated in the Result section and washed with PBS before being exposed to propidium iodide (final concentration 5 μg/ml). Flow cytometry analysis was carried out with FACS Calibur™ using CellQuest Pro software (BD Bioscience, Allschwil, Switzerland).

6.4.5 Protein expression of myeloperoxidase and CYP3A4

Protein expression in HL-60 cells and HL-60/CYP3A4 cells was checked by Western blotting using a monoclonal antibody against human CYP3A4 (Epitomics, Danvers, USA) and a polyclonal antibody against human myeloperoxidase (Cell Signaling Technology, Allschwil, Switzerland). The cells were lysed on ice for 15 min with 200 μl NET lysis buffer (50 mM NaCl, 5 mM EDTA, 0.05 M Tris-HCl pH 8.0, 1% NP-40 and a protease inhibitor tablet from Roche, Basel, Switzerland). The samples were vortexed and centrifuged for 10 min at 4°C at 11,000 g. The supernatants were collected and the protein concentration determined using the Pierce BCA protein assay kit (Darmstadt, Germany). Proteins (20 μg) were separated by electrophoresis on a denaturating SDS polyacrylamide gel (4%) and were then transferred onto a nitrocellulose membrane (BioradTransBlot, Hercules, CA). The membranes were

blocked with blocking buffer (PBS/Tween 20 containing 5% milk solution) for 1h at room temperature and washed twice with PBS/Tween 20. The blocked membranes were then incubated overnight at 4°C with blocking buffer containing primary antibodies against MPO (1:1000) or CYP3A4 (1:1000). Horseradish peroxidase-labeled anti-rabbit or anti-mouse antibodies (Jackson laboratories Inc, Suffolk, UK) were used in combination with a chemiluminescent substrate (ECL, Amersham, UK) for visualization of the proteins.

6.4.6 Quantification of clopidogrel, clopidogrel carboxylate, ticlopidine and prasugrel and prasugrel thiolactone (R-95913)

HL-60 cells and HL-60/CYP3A4 cells were incubated with clopidogrel, clopidogrel carboxylate, ticlopidine or prasugrel (10 and 100μM) at 37°C for different periods of time (0, 6, 12 and 24 h) in the presence or absence of the CYP3A4 inhibitor ketoconazole (1μM) [26] or the MPO inhibitor rutin (20μM) [27]. The reactions were stopped by addition of 300μl of methanol. After centrifugation at 3000 g for 30min, the samples were analyzed with the LC-MS/MS method described previously [22], which could also be used to detect and quantify prasugrel and prasugrel thiolactone. The specific transitions of prasugrel and prasugrel thiolactone were 374→206 and 332→109, respectively.

6.4.7 Experimental design

For the experiments, 2 x 10^5/ml HL-60 cells or HL-60/3A4 cells were seeded in 24-well plates. Stock solutions of test compounds (clopidogrel, clopidogrel carboxylic acid, ticlopidine and prasugrel) were prepared in DMSO. The test compounds were added at a concentration of 1, 10 or 100 μM in presence or absence of the CYP3A4 inhibitor ketoconazole (1μM) or the MPO inhibitor rutin (20μM). Control incubations contained DMSO; the DMSO concentration was identical in all incubations at 0.1%.

This DMSO concentration has been shown not to be cytotoxic [28]. Staurosporine 1 μM (STS), diethyl-maleate 200 μM (DEM) or Triton X were used as positive controls. The drug treatment was performed for 24 h at 37°C and 5% CO2.

6.4.8 Mitochondrial membrane potential

To detect the changes in mitochondrial membrane potential, tetramethylrhodamine ethyl ester (TMRE) was used. HL-60 cells or HL-60/CYP3A4 cells were incubated with drugs in the presence or absence of ketoconazole (1 μM) or rutin (20 μM) for 24h. The cells were washed twice with PBS and incubated with 100 nM TMRE in PBS for 30min at room temperature. The fluorescence signal of the cationic dye was monitored by flow cytometry.

6.4.9 Cytochrome c content in the cells

The cytochrome c content in the cells was analyzed using the method described by Waterhouse et al. [29] with some modifications. Briefly, after 24 h of treatment, the drug-treated cells were washed with PBS. The cells were then permeabilized on ice with 100 μl of digitonin (50 μg/ml in PBS containing 100 mM KCl) for 10 min. After permeabilization, the cells were fixed with paraformaldehyde (4% in PBS) at room temperature. After 30 min, the cells were washed twice with PBS and incubated for 1 h in blocking buffer (PBS containing 3% BSA and 0.05% saponin). After incubation with a monoclonal anti-cytochrome c antibody (BD Pharmingen, Allschwil, Switzerland) diluted 1:500 in blocking buffer at 4°C overnight, the cells were washed twice with PBS and incubated with an Alexa Fluor®-labeled secondary antibody diluted 1:500 in blocking buffer (Invitrogen, Basel, Switzerland) for 1 h at room temperature. After this procedure, the cells were analyzed by flow cytometry.

6.4.10 Reactive oxygen species (ROS)

Cellular ROS generation was measured using the 2′, 7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) assay [30]. DCFH-DA is a membrane-permeable, non-fluorescent compound accumulating within cells as DCFH upon deacetylation by esterases. In presence of ROS, DCFH is oxidized to the fluorescent dichlorofluorescein (DCF). Cells were co-incubated with drugs and 25 μM DCFH-DA in the presence or absence of ketoconazole (1 μM) or rutin (20 μM). After 24 h of incubation, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan Infinite pro 200 microplate reader (Tecan, Männedorf, Switzerland).

6.4.11 Determination of GSH and GSSG

Determination of glutathione (GSH) and oxidized glutathione (GSSG) was performed using the enzymatic recycling method of Rahman [31]. Briefly, cells were incubated with the test compounds with and without inhibitors for 24h. Diethyl-maleate (DEM), a glutathione depleting agent [32], served as a positive control. The cells were washed with PBS and 1ml of KPE buffer and sonicated for 30 sec. After centrifugation at 3000g for 4 min, the pellet was used for protein determination and the supernatant for the determination of total glutathione (GSH and GSSG). For the determination of total glutathione, a reaction mixture containing NADPH (0.8 mM), DTNB (1.6 mM) and glutathione reductase (4 U/ml) was added to samples or standards. The time-dependent formation of TNB was measured at 412 nm using the Tecan microplate reader. For the determination of GSSG, GSH was derivatized with 2-vinylpyridine and GSSG was determined using the recycling assay described above.

6.4.12 Detection of apoptosis

An *in situ* apoptosis detection kit, Vybrant TM Apoptosis kit #2 (Invitrogen, Basel, Switzerland) was used to measure the apoptotic cells. After 24 h of incubation of the cells with test compounds with and without inhibitors, cells were washed with PBS and stained with Annexin V-AlexaFluor 488 and propidium iodide as described in the kit protocol. The samples were incubated for 15 minutes and analyzed by flow cytometry.

6.4.13 Statistical analysis

Data are presented as means ± SD of at least three independent experiments, except where indicated. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between groups (e.g. incubations containing drugs vs. control incubations) were tested by one-way ANOVA followed by Bonferroni's post hoc test to localize significant results in the ANOVA. Differences between many groups at two levels were compared using two-way analysis of variance (ANOVA) followed by the protected Dunnett's post hoc test to localize significant results obtained in the ANOVA. A p-value <0.05 was considered as statistically significant.

6.5 Results

6.5.1 Characterization of cells used

The toxicity of the thienopyridines was assessed with two different cell types: non-modified HL-60 cells, a human promyelocytic leukemia cell line containing myeloperoxidase (MPO), and HL-60 overexpressing human CYP3A4 (HL-60/CYP3A4 cells). Western blot analysis revealed a high expression of MPO in HL-60 cells, whereas CYP3A4 expression was barely detectable in this cell type (fig. 1). In contrast, in HL-60/CYP3A4 cells, CYP3A4 was highly expressed and expression of MPO was almost absent.

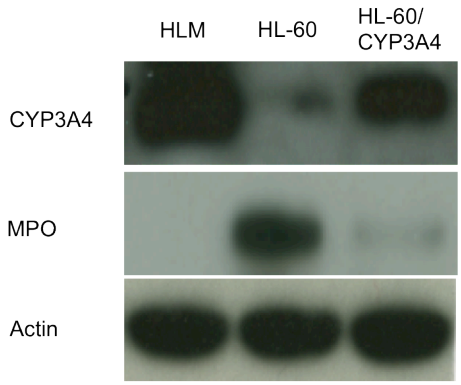

Figure 1 Protein expression of myeloperoxidase (MPO) and CYP3A4 by Western blotting. Human liver microsomes (HLM) were used as a positive control for CYP3A4 enzyme expression and actin as a control to confirm equal loading.

6.5.2 Cytotoxicity of thienopyridines

HL-60 cells were incubated with different concentrations (1, 10 and 100 μM) of clopidogrel, ticlopidine, prasugrel, and clopidogrel carboxylate for 24 h and the cellular release of adenylate kinase was determined as a surrogate of cytotoxicity (Fig. 2). All drugs showed a dose-dependent cytotoxicity starting at 10 μM.

An almost identical picture was obtained for HL-60/CYP3A4 cells (Fig. 2). For clopidogrel, ticlopidine and prasugrel, cytotoxicity was dose-dependent, starting at

10 μM. In contrast to HL-60 cells, clopidogrel carboxylate was not toxic for HL-60/CYP3A4 cells up to 100 μM. Concerning prasugrel, it is important to realize that the parent compound was rapidly (<5 min) and completely hydrolyzed to prasugrel thiolactone (Fig. 3). The cells were therefore exposed to prasugrel thiolactone and not to prasugrel.

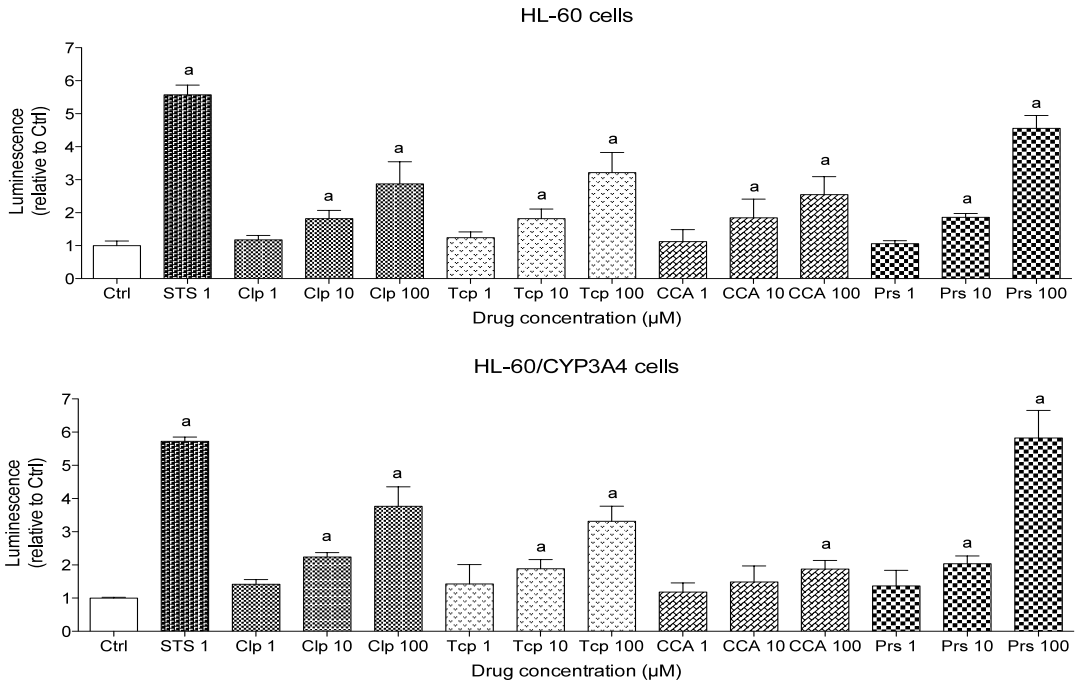

Figure 2 Cytotoxicity of clopidogrel, ticlopidine, prasugrel thiolactone, and clopidogrel carboxylate for HL-60 and HL-60/CYP3A4 cells. Cells were exposed for 24h to the compounds mentioned above at the concentrations indicated in the Figure labels. Adenylate kinase was measured as a surrogate for cytotoxicity using the ToxiLight® assay. Control (Ctrl) incubations contained 0.1% DMSO and incubations containing 1μM staurosporine served as positive controls. The results are expressed as the means + SD of four independent experiments. **a** $p<0.05$ incubations containing drugs only (no inhibitor) versus respective control incubations (Ctrl). **b** $p<0.05$ incubations containing drugs and inhibitor (ketoconazole or rutin) versus respective incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

6.5.3 Metabolism of clopidogrel, ticlopidine, prasugrel or clopidogrel carboxylate by HL-60 and HL-60/CYP3A4 cells

To further characterize the two cell systems established, metabolism of the test compounds mentioned above was studied using LC-MS/MS. The cells were incubated for 24h with the test compounds in the presence or absence of the CYP3A4 inhibitor ketoconazole [26] or the MPO inhibitor rutin [27]. Two different initial concentrations were chosen, 10 μM (Fig. 3) and 100 μM (Fig. 4). As mentioned above prasugrel is rapidly and quantitatively hydrolyzed under the conditions used (data not shown); therefore prasugrel thiolactone is given in Fig. 3 and Fig. 4. As shown in fig. 3, in the presence of HL-60 cells, all compounds were degraded time-dependently by 30 to 70% over 24 hours. Degradation could be blocked almost completely by rutin, but not by ketoconazole. Similar results were obtained with an initial concentration of 100 μM (Fig. 4). In the presence of HL-60/CYP3A4 cells, clopidogrel, ticlopidine and prasugrel thiolactone at an initial concentration of 10 μM were degraded by 50 to 95% over 24 hours (Fig. 3). The degradation could be blocked by ketoconazole, but not by rutin. In contrast to HL-60 cells, clopidogrel carboxylate was not degraded by HL-60/CYP3A4 cells. Similar results were obtained for an initial concentration of 100 μM (Fig. 4).

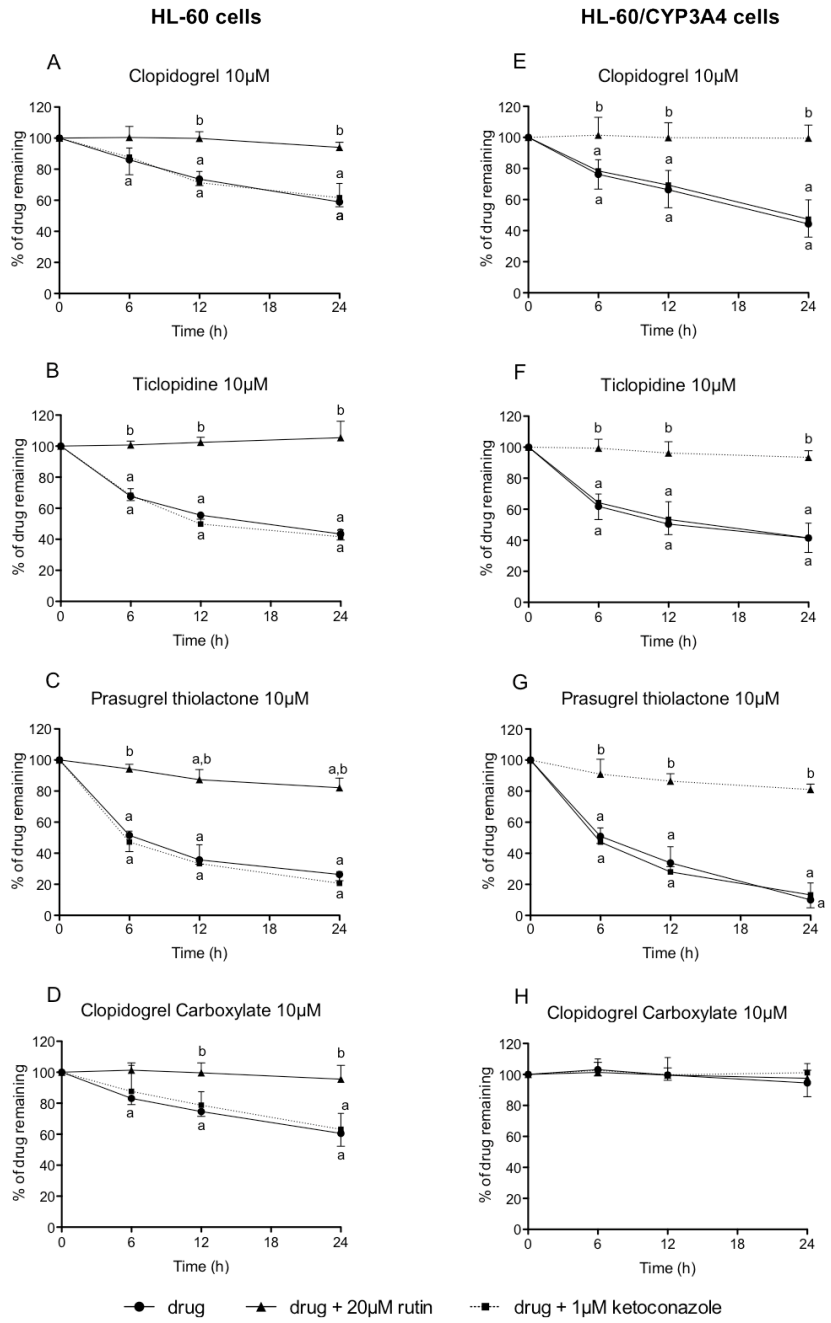

Figure 3 Metabolism of clopidogrel, ticlopidine, prasugrel thiolactone and clopidogrel carboxylate by HL-60 cells (Fig.3A-D) or HL-60/CYP3A4 cells (Fig. 3E-G). The test compounds were added to the cells at an initial concentration of 10μM and the concentration of the compounds was determined for 24 hours. Some incubations contained also the myeloperoxidase inhibitor rutin (20μM) or the CYP3A4 inhibitor ketoconazole (1μM) as indicated in the Figure label. Data are expressed as the percentage of parent drug remaining during 24h of incubation. The results are the means + SD of three independent determinations. **a** $p<0.05$ versus respective incubations at time 0 min; **b** $p<0.05$ incubations containing drug with 20μM rutin or ketoconazole 1μM versus respective incubations containing drug only.

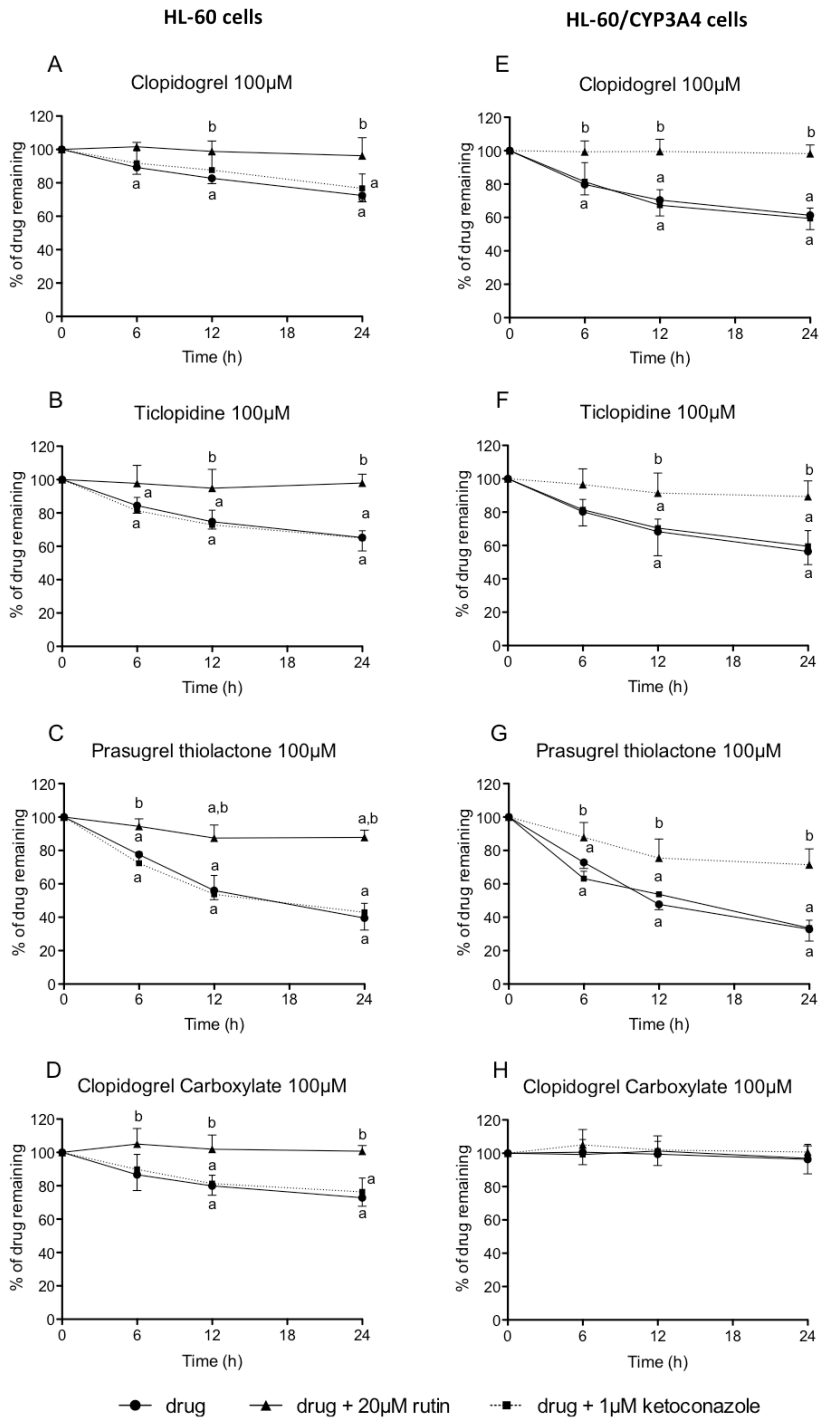

Figure 4 Metabolism of clopidogrel, ticlopidine, prasugrel thiolactone and clopidogrel carboxylate by HL-60 cells (Fig.3A-D) or HL-60/CYP3A4 cells (Fig. 3E-G). The test compounds were added to the cells at an initial concentration of 100μM and the concentration of the compounds was determined for 24 hours. Some incubations contained also the myeloperoxidase inhibitor rutin (20μM) or the CYP3A4 inhibitor ketoconazole (1μM) as indicated in the Figure label. Data are expressed as the percentage of parent drug remaining during 24h of incubation. The results are the means + SD of three independent determinations. **a** $p<0.05$ versus respective incubations at time 0 min; **b** $p<0.05$ incubations containing drug with 20μM rutin or ketoconazole 1μM versus respective incubations containing drug only.

6.5.4 Influence of enzyme inhibitors on cytotoxicity clopidogrel, ticlopidine, prasugrel and clopidogrel carboxylate

Cytotoxicity was assessed using PI staining and subsequent FACS analysis (Fig. 5). For HL-60 cells exposed for 24h, clopidogrel, ticlopidine, prasugrel thiolactone and clopidogrel carboxylate showed a concentration-dependent toxicity. Cytotoxicity could at least partially be prevented by the MPO inhibitor rutin [27]. In comparison, for HL-60/CYP3A4 cells only clopidogrel, ticlopidine and prasugrel thiolactone were cytotoxic in a concentration-dependent fashion, whereas clopidogrel carboxylate was not toxic. Cytotoxicity of clopidogrel, ticlopidine and prasugrel could be prevented by the CYP3A4 inhibitor ketoconazole

6.5.5 Measurement of mitochondrial membrane potential Ψm

Since mitochondria are important mediators of cell death, we investigated the effect of the thienopyridine compounds studied on Ψm. As shown in Fig. 6, in HL-60 cells, all compounds tested were associated with a concentration-dependent decrease in Ψm. With the exception of 100 μM prasugrel thioalctone, where rutin was only partially preventive, rutin was able to prevent this decrease completely. A similar picture could be observed in HL-60/CYP3A4 cells, where ketoconazole could prevent almost completely the drop in Ψm. In contrast to HL-60 cells, clopidogrel carboxylate was not reducing Ψm in HL-60/CYP3A4 cells.

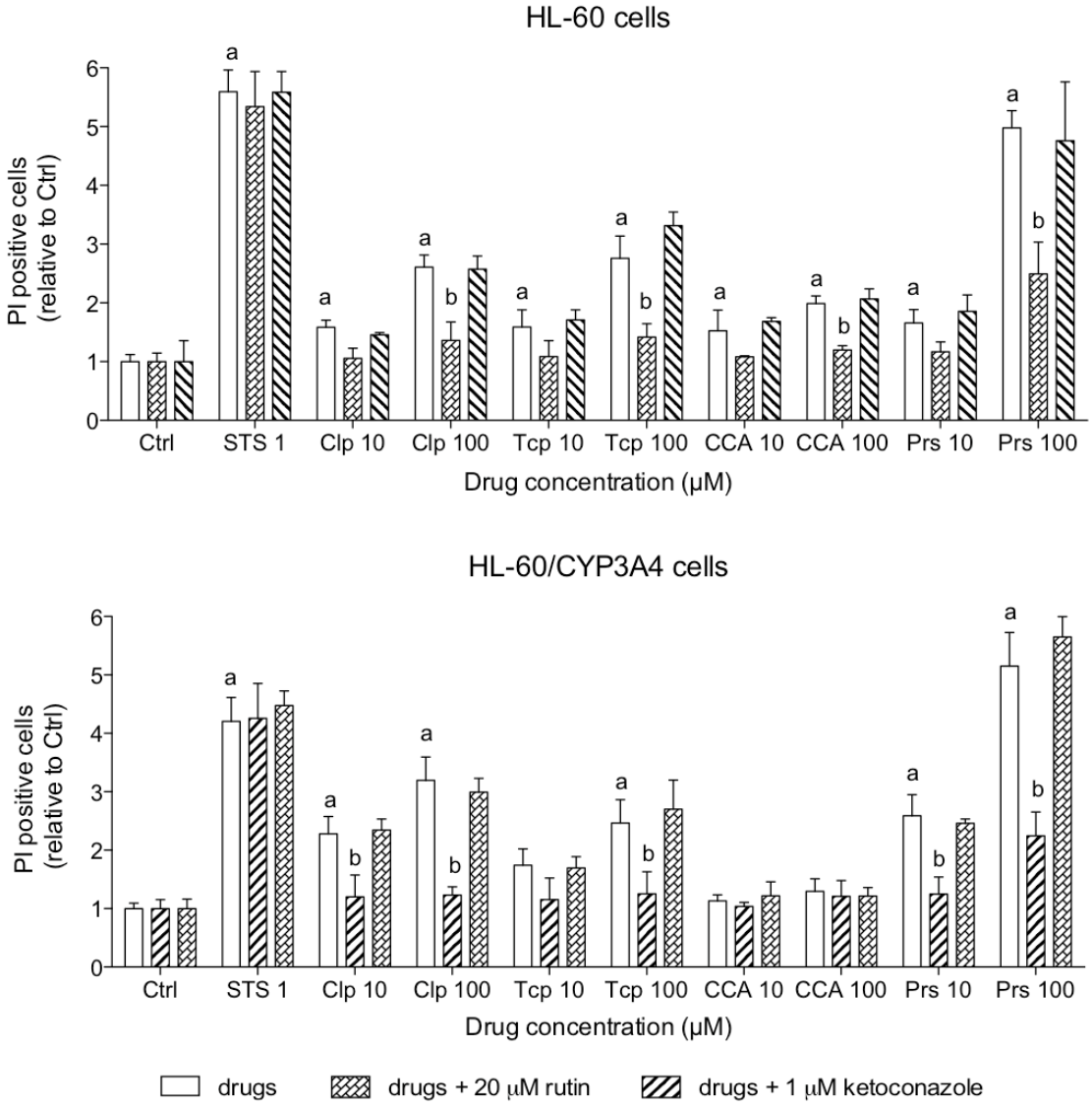

Figure 5 Effect of enzyme inhibitors on the cytotoxicity of clopidogrel, ticlopidine, prasugrel thiolactone, and clopidogrel carboxylate for HL-60 and HL-60/3A4 cells. Cells were exposed for 24h to the compounds mentioned above at the concentrations indicated in the Figure labels. Some incubations contained also 1μM ketoconazole (CYP3A4 inhibitor) or 20μM rutin (MPO inhibitor) as indicated in the figure labels. Cytotoxicity was measured using propidium iodine staining. Control incubations (Ctrl) contained 0.1% DMSO (Ctrl). Positive control incubations contained 1μM staurosporine. The results were normalized to control incubations (Ctrl) and are expressed as the means + SD of five independent experiments. **a** $p<0.05$ incubations containing drugs only (no enzyme inhibitor) versus respective control incubations (Ctrl); **b** $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

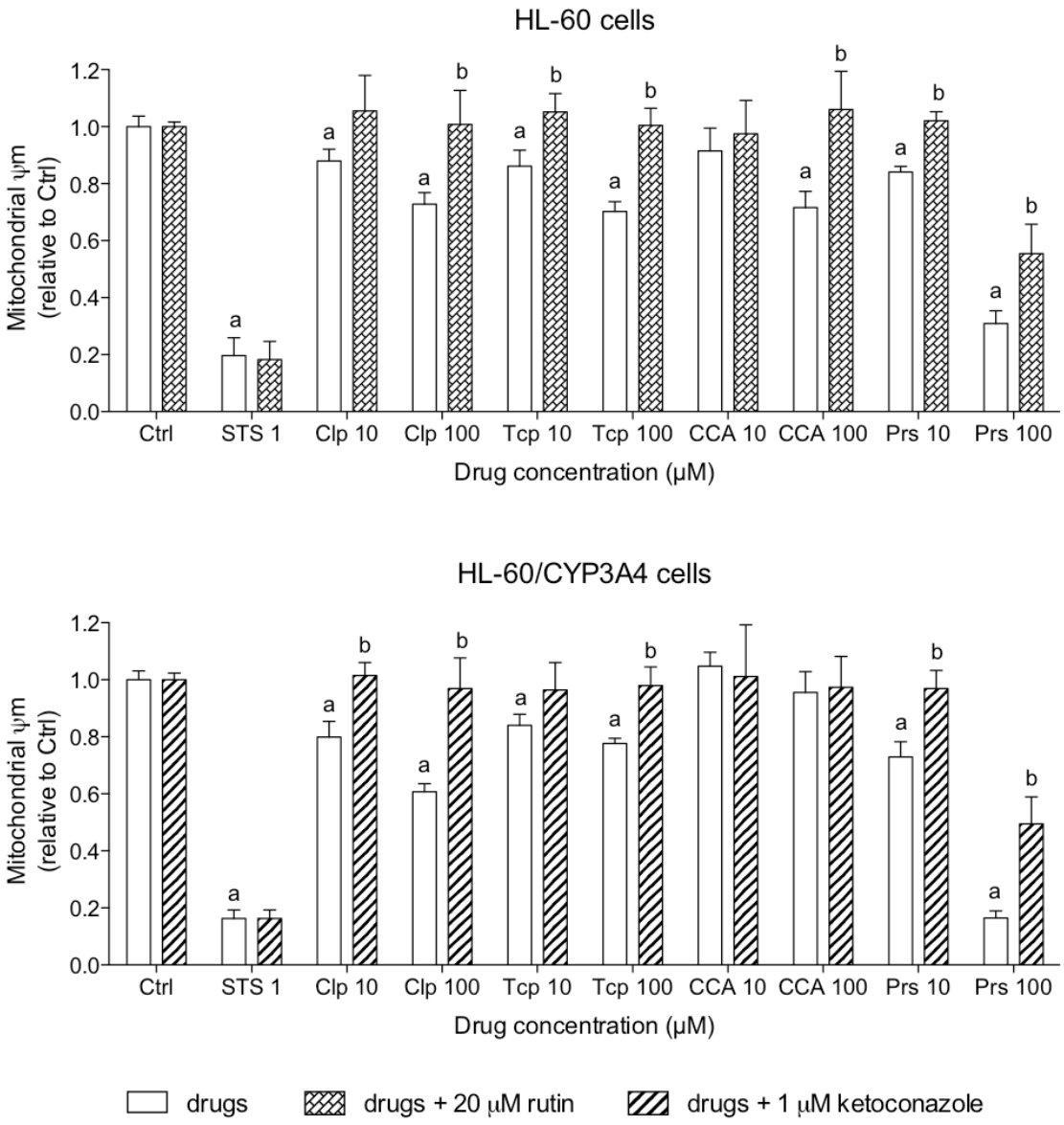

Figure 6 Effect of clopidogrel, ticlopidine, prasugrel thiolactone, and clopidogrel carboxylate on the mitochondrial membrane potential in HL-60 and HL-60/CYP3A4 cells. Cells were incubated for 24 h with different concentrations of the compounds mentioned above as indicated in the Figure labels. Some incubations contained ketoconazole (1μM) or rutin (20μM) as indicated. The decrease of mitochondrial membrane potential was measured using the fluorescent dye TMRE. The results were normalized to control incubations containing 0.1% DMSO (Ctrl). Incubations containing 1μM staurosporine served as positive controls. Results are presented as the mean + SD of three independent experiments. **a** $p<0.05$ incubations containing drugs alone (no enzyme inhibitors) versus respective control incubations (Ctrl); **b** $p<0.05$ Incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

6.5.6 Effect of thienopyridines on cellular ROS production

Mitochondrial dysfunction can be associated with increased ROS production [30]. As shown in supplementary fig. 3, the cellular ROS content of HL-60 cells increased in concentration-dependent fashion in the presence of clopidogrel, ticlopidine, prasugrel thiolactone and clopidogrel carboxylate. Accumulation of ROS could be prevented by the addition of rutin, indicating that metabolites formed by MPO were responsible for this finding. Similarly, the cellular ROS content of HL-60/CYP3A4 cells increased in a concentration-dependent fashion in the presence of clopidogrel, ticlopidine or prasugrel thiolactone, but not in the presence of clopidogrel carboxylate. The CYP3A4 inhibitor ketoconazole prevented ROS accumulation at least partially.

6.5.7 Effect of thienopyridines on the cellular GSH/GSSG ratio

The GSH/GSSG ratio can be regarded as a marker of oxidative stress, since GSH is oxidized to GSSG during the degradation of ROS such as hydrogen peroxide. As shown in fig. 5, all thienopyridines studied decreased the GSH/GSSG ratio in HL-60 cells. This decrease was concentration-dependent and could be prevented by the addition of rutin. In HL-60/CYP3A4 cells, a similar decrease was observed for clopidogrel, ticlopidine and prasugrel thiolactone, but not for clopidogrel carboxylate. This decrease could at least partially be prevented by the addition of the CYP3A4 inhibitor ketoconazole.

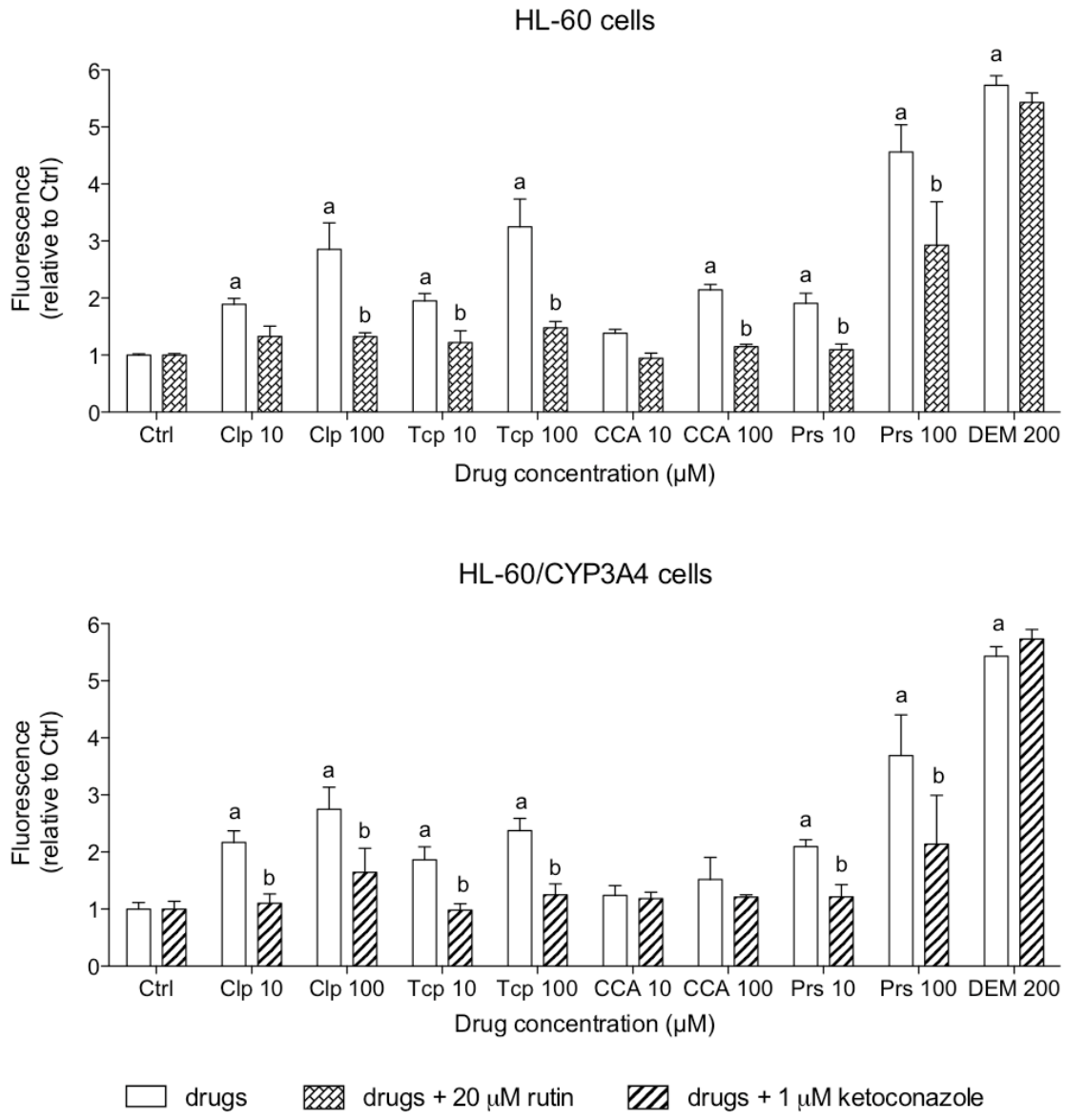

Figure 7 ROS accumulation in HL-60 and HL-60/CYP3A4 cells in the presence of clopidogrel, ticlopidine, prasugrel thiolactone or clopidogrel carboxylate. Cells were exposed for 24h to the above-mentioned compounds and to enzyme inhibitors as indicated in the figure labels. Cellular levels of ROS were measured using 2′, 7′-dichlorodihydrofluorescein-diacetate. Incubations containing 0.1% DMSO were used as control (Ctrl). Incubations with depletion of the cellular glutathione by 200μM diethylmaleate were used as positive controls (DEM). The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of three independent experiments. **a** $p<0.05$ incubations containing drugs alone (no enzyme inhibitors) versus respective control incubations (Ctrl); **b** $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

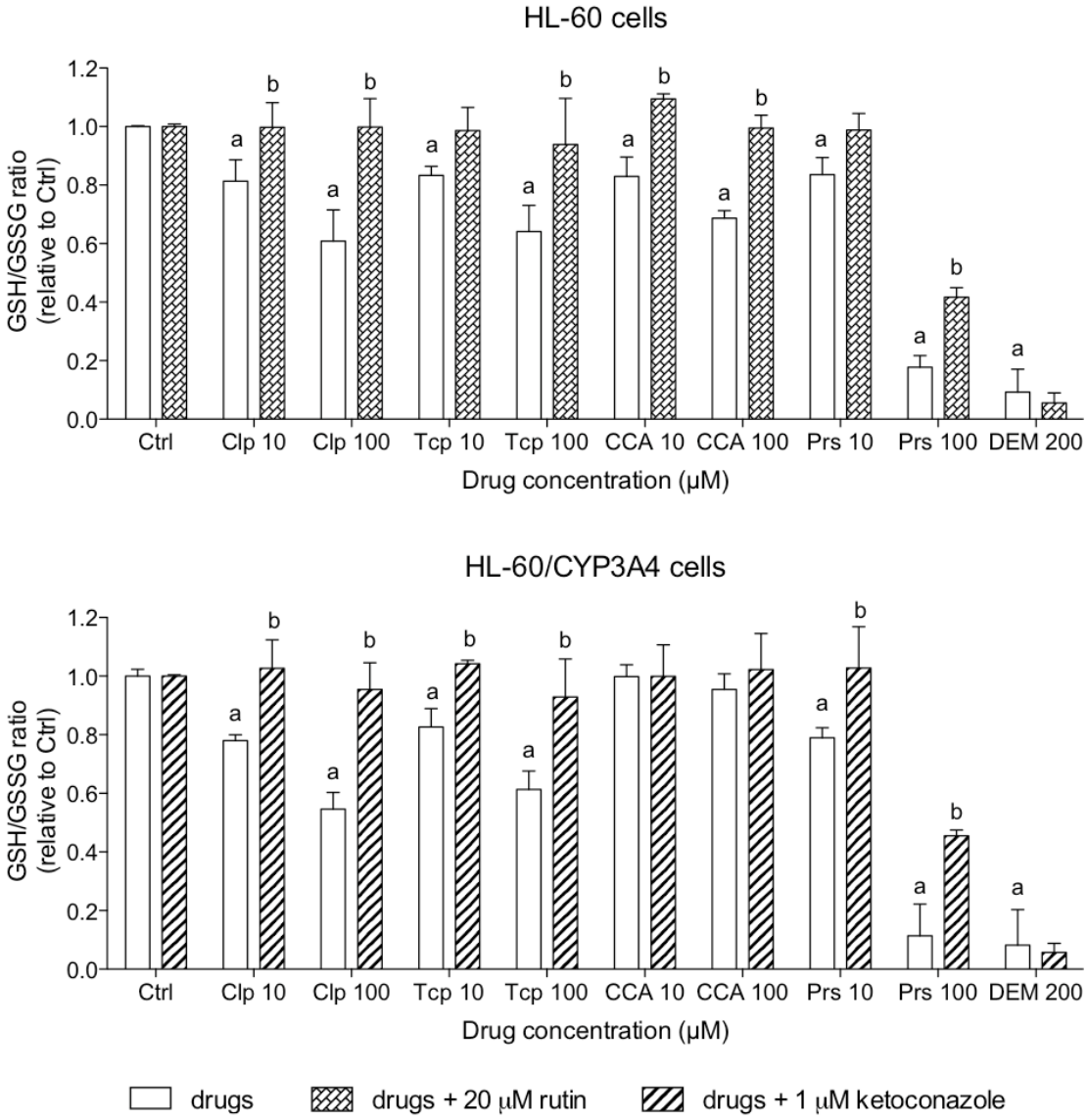

Figure 8 Effect of clopidogrel, ticlopidine, prasugrel thiolactone, and clopidogrel carboxylate on the GSH/GSSG ratio in HL-60 and HL-60/CYP3A4 cells. Cells were incubated for 24h with different concentrations (indicated in the figure labels) of the compounds mentioned above. Some incubations contained ketoconazole (1μM) or rutin (20μM) as indicated in Figure labels. Incubations containing 0.1% DMSO served as controls (Ctrl). Incubations containing the GSH depleting agent diethylmaleate (200 μM) served as a second control (DEM). The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of three independent experiments. **a** $p<0.05$ incubations containing drugs alone (no enzyme inhibitors) versus respective control incubations (Ctrl); **b** $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

6.5.8 Effect of thienopyridines on the cellular cytochrome c content

Increased mitochondrial ROS production can lead to the opening of the mitochondrial permeability transition pore with subsequent release of cytochrome c into the cytosol and initiation of apoptosis [30]. In HL-60 cells, all thienopyridines tested were associated with a concentration-dependent in mitochondrial cytochrome c (Fig. 6). This decrease could be prevented by the addition of rutin. In comparison, in HL-60/CYP3A4 cells, such a decrease could only be observed for clopidogrel, ticlopidine and prasugrel thiolactone, but not for clopidogrel carboxylate. The addition of ketoconazole could partially prevent this decrease.

6.5.9 Determination of apoptosis

Since the release of cytochrome c is associated with apoptosis, we determined the proportion of apoptotic cells using annexin V/PI staining. Parallel to the findings for cytochrome c release presented above, all thienopyridines induced apoptosis in a concentration-dependent fashion in HL-60 cells (Fig. 7). As expected, the addition of rutin could prevent apoptosis. In comparison, only clopidogrel, ticlopidine and prasugrel thioalctone, but not clopidogrel carboxylate, induced apoptosis in HL-60/CYP3A4 cells. Similar to the observation regarding cytochrome c release, ketoconazole could partially prevent induction of apoptosis.

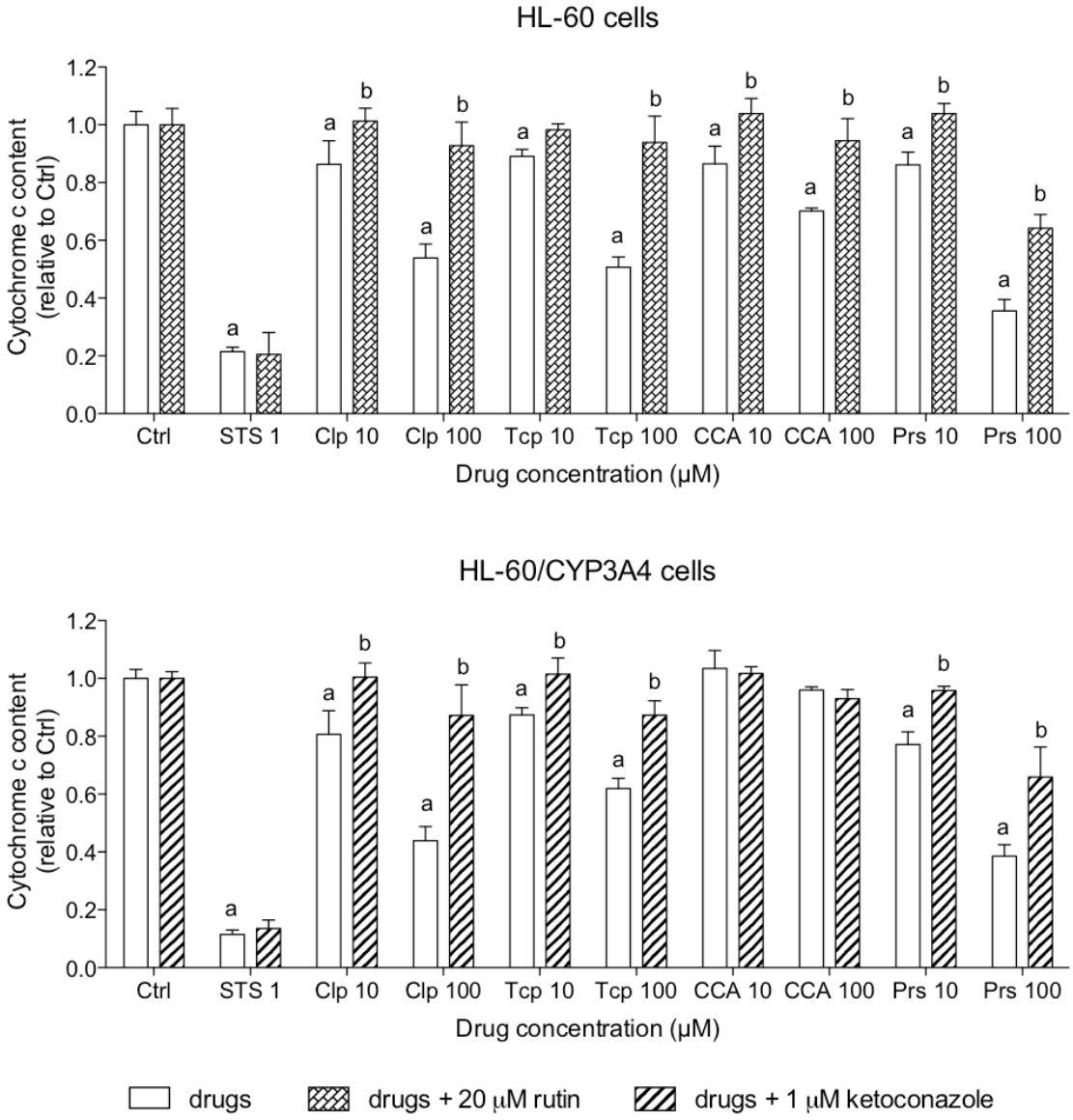

Figure 9 Effect of clopidogrel, ticlopidine, prasugrel thiolactone and clopidogrel carboxylate on the mitochondrial cytochrome c content in HL-60 and HL-60/CYP3A4 cells. Cells were incubated with the test compounds for 24h at the concentrations given in the Figure labels. Some incubations contained also ketoconazole or rutin as indicated in the Figure labels. Control incubations contained 0.1% DMSO (Ctrl). Positive control incubations contained 1μM staurosporine. The mitochondrial cytochrome c content was determined by flow cytometry after cell permeabilization as described in Methods. The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of four independent experiments. **a** $p<0.05$ incubations containing drugs alone (no enzyme inhibitors) versus respective control incubations (Ctrl); **b** $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

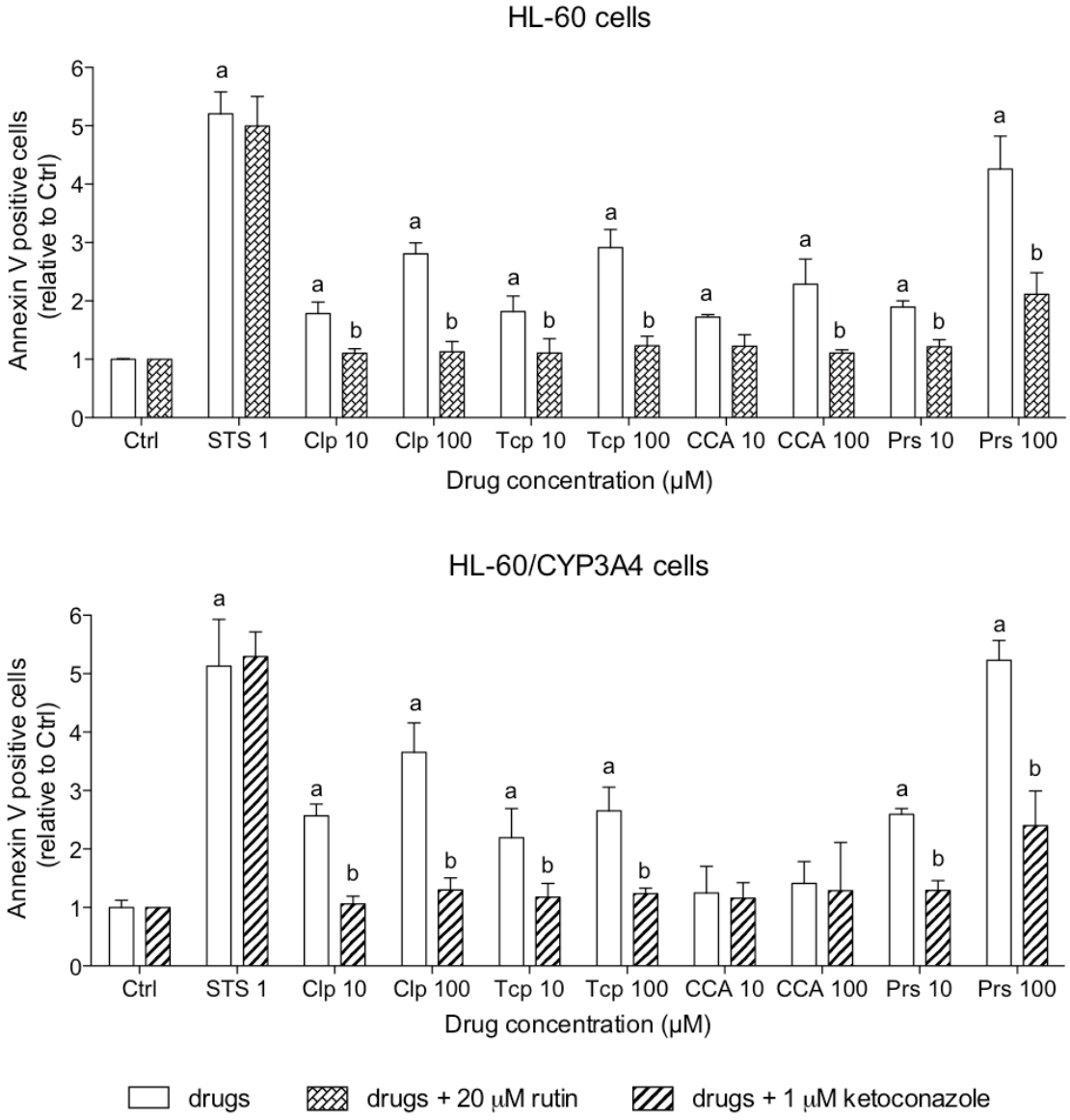

Figure 10 Induction of apoptosis in HL-60 and HL-60/CYP3A4 cells by clopidogrel, ticlopidine, prasugrel thiolactone and clopidogrel carboxylate. Cells were treated with the compounds mentioned above for 24h at the concentrations indicated in the figure labels. Apoptosis was determined by staining with annexin V/propidium iodine followed by flow cytometry. Control incubations contained 0.1% DMSO (Ctrl). Positive control incubations contained 1μM staurosporine. The results were normalized to control incubations (Ctrl) and are expressed as mean + SD of four independent experiments. **a** $p<0.05$ incubations containing drugs alone (no enzyme inhibitors) versus respective control incubations (Ctrl); **b** $p<0.05$ incubations containing drugs and rutin or ketoconazole versus corresponding incubations containing drugs only. STS: staurosporine, Clp: clopidogrel, Tcp: ticlopidine, CCA: clopidogrel carboxylate, Prs: prasugrel thiolactone.

6.6 Discussion

Our study shows that all compounds tested were metabolized by MPO and were toxic for HL-60 cells. Cytotoxicity was clearly concentration-dependent and started at 10 μM. Prevention of the cytotoxicity by rutin confirmed that the toxicity was associated with metabolites formed by MPO. With the exception of clopidogrel carboxylate, all compounds tested were also metabolized by CYP3A4. They showed a concentration-dependent toxicity on HL-60/CYP3A4 cells which could be prevented by ketoconazole. Clopidogrel carboxylate was not cytotoxic in this cell model.

Regarding the role of MPO in metabolism and cytotoxicity of clopidogrel, clopidogrel carboxylate and ticlopidine, the current study confirms our observations in myeloid progenitor cells [22] and in mature neutrophil granulocytes and lymphocytes (Maseneni S. et al. 2012;submitted). As described by Liu and Uetrecht in detail for ticlopidine [33], these compounds are metabolized by MPO and the metabolites formed by MPO increase their cytotoxicity. Importantly, this is also true for the inactive clopidogrel metabolite clopidogrel carboxylate, which may explain myelotoxicity of clopidogrel [22].

Our study shows that also prasugrel thiolactone is metabolized by MPO (Fig. 2 and supplementary Fig. 2) and that the metabolites produced are toxic for HL-60 cells (Fig. 3). In contrast to ticlopidine and clopidogrel, myelotoxicity has so far not been described for prasugrel. After ingestion of 15 mg prasugrel (close to the normal maintenance dose of 10 mg per day), the C_{max} of prasugrel thiolactone (R-95913) is in the range of 70 μg/L, corresponding to approximately 0.2 μM [34]. Prasugrel thiolactone has a half-life in the range of 4 h and can therefore be expected to reach the bone marrow. In the current study, prasugrel thiolactone was not toxic at 1 μM and started to be toxic at 10 μM. Our data are therefore in agreement with the clinical

observation that prasugrel is not myelotoxic; the concentrations reached in the bone marrow are too low, even after having been metabolized by MPO.

In addition to MPO, ticlopidine, clopidogrel and prasugrel thiolactone were also metabolized by CYP3A4. This was not the case for clopidogrel carboxylate, a finding, which is in agreement with previous results obtained human recombinant CYP3A4 (Maseneni et al., 2012). The finding that clopidogrel carboxylate cannot be metabolized by CYP3A4 can be explained by inability of the polar carboxyl moiety to access the active site of CYP3A4 (Zahno et al., 2010). Studies from our laboratory [22] and from others [35,36] have shown that expression of the CYPs, which are important for the metabolism and activation of the thienopyridines, among them CYP3A4, CYP2B6, CYP2C9 and CYP2C19, is virtually absent in bone marrow. Potentially toxic metabolites formed from thienopyridines formed by CYPs have therefore to be transported (mainly from liver) to the bone marrow to exert their toxicity. This may be possible for ticlopidine, but not for clopidogrel, for which the concentrations reached are not high enough and the intermediates formed are to reactive [22]. Assuming that CYP3A4 converted prasugrel thiolactone into the active metabolite R-138727, the same conclusion can be drawn also for prasugrel. After oral ingestion of 15 mg prasugrel, the plasma C_{max} of R-138727 reached 80 μg/ml, corresponding to approximately 0.2 μM. The half-life of R-138727 is also in the range of 4 h, this metabolite reaches therefore the bone marrow. In the presence of CYP3A4, prasugrel thiolactone was not toxic at 1 μM and started to be toxic at 10 μM. Again, the concentration reached in vivo is too low to be myelotoxic, again explaining the absence of myelotoxicity for prasugrel. On the other hand, the liver may be exposed to higher concentrations of toxic metabolites produced by CYPs, which may explain the hepatotoxicity associated with ticlopidine and clopidogrel [12-17]. This hypothesis is currently being investigated in human liver cell models.

The mechanism of cytotoxicity is compatible with a primary effect on mitochondria for both MPO- and CYP3A4-associated metabolites, as suggested by the decrease in the mitochondrial membrane potential. This finding is in agreement with our previous studies [22] (Maseneni et al. 2012; submitted). Increased production of ROS, cytochrome c spillage into the cytosol and apoptosis are consequences of the initial mitochondrial damage [29,30]. Interestingly, both MPO- and CYP3A4-associated metabolite formations were associated with an decrease in the cellular GSH/GSSG ratio. This can be explained by the formation and detoxification of ROS, but may also be, in the case of CYP3A4, a consequence of the formation of the respective active metabolites, which is dependent on GSH [11].

In conclusion, myelotoxicity of ticlopidine and clopidogrel is mainly explained by the formation of toxic metabolites by MPO within the bone marrow from the parent compound (ticlopidine) or from clopidogrel carboxylate (clopidogrel). Prasugrel is not myelotoxic, because the concentration of the metabolites reached in bone marrow is not high enough. Cytotoxicity of toxic metabolites formed by MPO or CYP3A4 can be explained by mitochondrial damage eventually leading to apoptosis.

6.7 Funding source

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7. Discussion

Thienopyridines are prodrugs that requires hepatic conversion mainly by cytochrome P450 enzymes to an active metabolite in order to exert their antiplatelet effect.[24,113,114] Thienopyridine therapy although increased inhibition of $P2Y_{12}$ receptor and improved platelet function efficacy when compared to aspirin, adverse drug reaction was also increased. Besides hepatotoxicity [84,87], also bone marrow toxicity [78,106,115-117] may limit their use. The mechanisms associated with myelotoxicity of thienopyridines are currently unclear. Therefore this thesis investigates the mechanism by which thienopyridines induce myelotoxicity.

The first part of this thesis was designed to study an *in vitro* model of cytotoxicity of clopidogrel, ticlopidine and clopidogrel carboxylate in human hematopoietic progenitor cells. Using LC-MS, CYP3A4 supersomes and human neutrophils derived from peripheral blood were utilised to assess the metabolism of clopidogrel, ticlopidine and clopidogrel carboxylate in presence of CYP3A4 inhibitor (ketoconazole) or MPO inhibitor (rutin). In agreement with other findings [23,111,112], clopidogrel and ticlopidine was readily metabolised by CYP3A4 supersomes. Clopidogrel carboxylate, the major inactive metabolite of clopidogrel produced by hydrolysis with esterases [23] was not metabolized by CYP3A4. In contrast, human neutrophils metabolized ticlopidine, clopidogrel and clopidogrel carboxylate significantly. The results revealed that preincubation of clopidogrel with CYP3A4 were associated with increased toxicity, suggesting that the toxic metabolite could be important for myelotoxicity. As the CYP-mediated metabolites are not produced in bonemarrow[118,119], they would have to be transported into bone marrow to exert myelotoxicity. Since the plasma concentration of active metabolite of clopidogrel was very low [113], the possibility that MPO and or CYP3A4 associated with active

metabolite of clopidogrel appears therefore to be unlikely. Most likely the myelotoxicity of clopidogrel is likely to be MPO-associated formation of toxic metabolites form clopidogrel carboxylate. Similar to clopidogrel, ticlopidine was also metabolized by CYP3A4.[120] Due to their high plasma concentration, it appears that the metabolites of ticlopidine could reach bone marrow to contribute to the myelotoxicity of ticlopidine.[121,122] for ticlopidine, the myelotoxicity could be explained by ticlopidine itself and MPO-associated formation of metabolites.

The second part of the thesis investigates the molecular mechanism of cell death due to clopidogrel, ticlopidine and clopidogrel carboxylate in human neutrophils and lymphocytes. The metabolism or cytotoxicity of clopidogrel or ticlopidine associated with CYPs in neutrophils or lymphocytes are negligible due to low expression of CYPs.[118] Our results showed metabolism or cytotoxicity of clopidogrel, ticlopidine and clopidogrel carboxylate for neutrophils. This metabolism and cytotoxicity was inhibited by MPO inhibitor, rutin but not by the CYP3A4 inhibitor, ketoconazole. Further more in lymphocytes, where MPO is lacking, metabolism of clopidogrel and ticlopidine were minimum and not detectable in clopidogrel carboxylate. There results support our previous findings in haematopoietic progenitor cells. Further more, molecular mechanism by which compounds exert toxicity was studies. The results revealed a decrease in mitochondrial membrane indicating by metabolites formed by MPO from clopidogrel, ticlopidine and clopidogrel carboxylate could impair mitochondrial function. The impairment of electron transport could be associated with in cellular accumulation of ROS.[123,124] An increase in ROS production was observed in metabolites of clopidogrel, ticlopidine and clopidogrel carboxylate formed by MPO, which could be completely inhibited by blocker of MPO and not by blocker of CYP3A4. Increase in ROS production can eventually lead to release or

mitochondrial proteins (cytochrome c) and triggers caspases to induce apoptosis [125-127], which is compatible with this thesis results by compounds investigated. The metabolites formed by MPO of clopidogrel, ticlopidine and clopidogrel carboxylate are important for hematotoxicity. Since the plasma concentration of clopidogrel in vivo is very low, the hematotoxicity associated with clopidogrel is mainly due to the inactive metabolite of clopidogrel. In ticlopidine, the toxicity could be explained by ticlopidine itself and the metabolites formed by MPO. These metabolites formed are mitochondrial toxins leading to accumulation of the cellular ROS triggering to the induction of apoptosis.

The final part of thesis specifically investigates the myelotoxicity of clopidogrel, ticlopidine, prasugrel thiolactone, and clopidogrel carboxylate in both CYP3A4 (HL-60/CYP3A4 cells) and MPO (HL-60 cells) cell models. HL-60 cells had high amount of MPO expression but no CYP3A4. In contrast HL-60/CYP3A4 cells had high expression of CYP3A4 but no MPO. HL-60 cells showed degradation of clopidogrel, ticlopidine, prasugrel thiolactone, and clopidogrel carboxylate, supporting the data of Liu and Uetrecht [111]. The degradation of the compounds in HL-60 cells was associated with cytotoxicity that could be inhibited by MPO blocker (rutin) and not by CYP3A4 inhibitor (ketoconazole), indicating the role of MPO metabolism and supporting our previous data with myeloid progenitor cells and granulocytes. In contrast, HL-60/CYP3A4 cells metabolize clopidogrel, ticlopidine and prasugrel thiolactone but not clopidogrel carboxylate. The toxicity of HL-60/CYP3A4 associated with clopidogrel, ticlopidine and prasugrel thiolactone were inhibited by ketoconazole completely. Clopidogrel carboxylate showed no toxicity in HL-60/CYP3A4, suggesting the role of CYP3A4 metabolism of thienopyridines. The mechanism of cytotoxicity is compatible with mitochondrial dependent pathway

leading to apoptosis in both the cell models. An oxidative stress reaction (as evidenced by decrease in glutathione levels) promoted elevated ROS level, damaged mitochondrial, decrease of cellular cytochrome c content, and triggering of caspases for apoptosis.

In Summary, the myelotoxicity of clopidogrel and ticlopidine is mainly by the formation of toxic metabolites by MPO within the bone marrow from the parent compound (ticlopidine) or from clopidogrel carboxylate (clopidogrel). Prasugrel is not myelotoxic, because the concentration of the metabolites reached in bone marrow is not high enough. Cytotoxicity of toxic metabolites formed by MPO or CYP3A4 can be explained by mitochondrial damage eventually leading to apoptosis.

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