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Toxicity of thienopyridines on human neutrophil granulocytes and lymphocytes

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ABSTRACT

Thienopyridines can cause neutropenia and agranulocytosis. The aim of the current investigations was to compare cytotoxicity of ticlopidine, clopidogrel, clopidogrel carboxylate and prasugrel for human neutrophil granulocytes with the toxicity for lymphocytes and to investigate underlying mechanisms. For granulocytes, clopidogrel, ticlopidine, clopidogrel carboxylate and prasugrel were concentration-dependently toxic starting at 10 μM. Cytotoxicity could be prevented by the myeloperoxidase inhibitor rutin, but not by the cytochrome P450 inhibitor ketoconazole. All compounds were also toxic for lymphocytes, but cytotoxicity started at 100 μM and could not be prevented by rutin or ketoconazole. Granulocytes metabolized ticlopidine, clopidogrel, clopidogrel carboxylate and prasugrel, and metabolization 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 neutrophils, all compounds investigated decreased the electrical potential across the inner mitochondrial membrane, were associated with cellular accumulation of ROS, mitochondrial loss of cytochrome c and induction of apoptosis starting at 10 μM. All of these effects could be inhibited by rutin, but not by ketoconazole. Similar findings were obtained in lymphocytes; but compared to neutrophils, the effects were detectable only at higher concentrations and were not inhibited by rutin. In conclusion, ticlopidine, clopidogrel, clopidogrel carboxylate and prasugrel are toxic for both granulocytes and lymphocytes. In granulocytes, cytotoxicity is more accentuated than in lymphocytes and depends on metabolization by myeloperoxidase. These findings suggest a mitochondrial mechanism for cytotoxicity for both myeloperoxidase-associated metabolites and, at higher concentrations, also for the parent compounds.

1. Introduction

The thienopyridines ticlopidine, clopidogrel and prasugrel block the ADP ($P2Y_{12}$) receptors on blood platelets and thereby efficiently inhibit platelet aggregation (Geiger et al., 1999; Savi et al., 2000, 2001). Thienopyridines are therefore used widely in patients with cardiovascular diseases, in particular in patients with acute coronary syndrome (Schomig et al., 1996; Wiviott et al., 2007; Yusuf et al., 2001), and also in patients with cerebrovascular or peripheral artery disease (Bhatt et al., 2006; CAPRIE-Steering-Committee, 1996; Hass et al., 1989).

The most important adverse reaction of these drugs is bleeding (Hansen et al., 2010; Sorensen et al., 2009). 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% (Hansen et al., 2010). Hematotoxicity is less frequent, occurring in up to 3% of the patients treated with ticlopidine (Gent et al., 1989; Hass et al., 1989) and approximately 1% of the patients treated with clopidogrel (Balamuthusamy and Arora, 2007). Important hematological adverse effects associated with these drugs include neutropenia and agranulocytosis (Akcay et al., 2004; Bedani et al., 1984; Farver and Hansen, 1994; Guerciolini et al., 1985; Gur et al., 1998), thrombocytopenia (Balamuthusamy and Arora, 2007; Koornstra et al., 1999; Szto et al., 1999), aplastic anemia (Symeonidis et al., 2002; Uz et al., 2010), thrombotic thrombocytopenic purpura (Kovacs et al., 1993; Nara et al., 2001), and the hemolytic uremic syndrome (Moy et al., 2000). For prasugrel, reports about neutropenia or agranulocytosis are so far lacking.

In patients with neutropenia or agranulocytosis associated with thienopyridines, bone marrow investigations have shown impaired myelopoiesis (Andres et al., 2001; Trivier et al., 2001), compatible with a toxic effect on the bone marrow. The association of the thienopyridines with aplastic anemia (Symeonidis et al., 2002; Uz et al., 2010) is also compatible with a toxic effect 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

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myeloperoxidase (MPO) in neutrophil granulocytes to a reactive thiophene-S-chloride derivative (Liu and Uetrecht, 2000), which may be associated with bone marrow toxicity. In line with this study, we have shown recently that metabolites formed from ticlopidine, clopidogrel or the clopidogrel metabolite clopidogrel carboxylate by MPO in neutrophil granulocytes are toxic for cultured human myelopoietic progenitor cells (Maseneni et al., 2012).

An additional explanation for hematotoxicity of these compounds could be a direct toxic effect on mature neutrophil granulocytes in bone marrow and/or in the peripheral blood. Since neutrophil granulocytes contain MPO (Liu and Uetrecht, 2000), they should be susceptible at least to ticlopidine, clopidogrel and clopidogrel carboxylate (Maseneni et al., 2012), and possibly also to prasugrel. In addition, all thienopyridines are prodrugs needing activation to be active. Activation of the thienopyridines involves different cytochrome P450 enzymes (CYPs), resulting in the generation of compounds carrying a free mercapto-group which can covalently bind a cysteine residue of the $P2Y_{12}$-receptor on platelets (Farid et al., 2010). This free mercapto-group is reactive and can possibly also bind other proteins or peptides carrying mercapto-groups such as for instance glutathione (Zahno et al., 2010). This possibility is also supported by the observation that both ticlopidine and clopidogrel are mechanism-based inhibitors of CYP2B6 and CYP2C19 (Nishiya et al., 2009; Richter et al., 2004). Since hematopoietic cells express CYPs (Bieche et al., 2007), CYP-associated production of cytotoxic thienopyridine metabolites by hematopoietic cells in bone marrow and by mature peripheral leukocytes appears to be possible.

In order to study these questions, we isolated mature neutrophils from peripheral blood, analyzed the expression of their CYPs and investigated the mechanisms of cytotoxicity associated with the thienopyridines. We compared the findings with lymphocytes isolated from the same blood samples. Lymphocytes do not contain MPO and could therefore be used as a negative control to study the effect of MPO on the toxicity of the thienopyridines investigated.

2. Materials and methods

2.1. Materials

Clopidogrel hydrogen sulphate and prasugrel hydrochloride were extracted from commercially available tablets (brand names Plavix® and Efient®, respectively). And clopidogrel carboxylate was obtained by alkaline hydrolysis of clopidogrel. These procedures were carried out by ReseaChem Life Sciences (Burgdorf, Switzerland). All 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).

2.2. Cell lines and cell culture

Normal donor buffy coats were obtained from the local blood donation center, Basel (Switzerland). The sex of the donors could not be determined, since the donors remained anonymous. Under these conditions, the study had not to be approved by the Ethics Committee. Blood cells were isolated from the buffy coat by a modification of the method described by Klebanoff et al. (1992). Briefly, the buffy coat was diluted 1:2 with Iscove's modified Dulbecco's medium (IMDM) and mixed with 4% dextran (MW 500 kDa) 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. The purity of the isolates was >90% for both lymphocytes and neutrophil granulocytes. 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 (ticlopidine, clopidogrel, clopidogrel carboxylic acid and prasugrel) were prepared in DMSO. The test compounds were added at a concentration of 1–100 μM in presence or absence of CYP3A4 inhibitor ketoconazole (1 μM) (Zahno et al., 2010) or the MPO inhibitor rutin (20 μM) (Pincemail et al., 1988). The DMSO concentration was 0.1% in all incubations, including control incubations. This DMSO concentration is not cytotoxic (Waldhauser et al., 2006). Staurosporine (STS, final concentration 1 μM) was used as a positive control for cytotoxicity. Individual experiments were always performed in triplicates and were repeated at least three times using different cell isolations.

2.3. 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 350 μL 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 10 ng cDNA was used. Forward and reverse primers for all CYPs tested, MPO and GAPDH (see supplementary 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).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2013.03.002.

2.4. Protein expression of myeloperoxidase and CYPs

Neutrophils or lymphocytes were lysed on ice for 15 min with 200 μL NET lysis buffer (50 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 8.0, 1% NP-40 and a protease inhibitor tablet from Roche [Basel, Switzerland]). The samples were centrifuged for 10 min 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 (20 μg) 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 1 h 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.

2.5. 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 24 h with the test compounds in the presence or absence of the CYP3A4 inhibitor ketoconazole (1 μM) or the MPO inhibitor rutin (20 μM). After the incubation, cells were centrifuged at 350 g for 5 min and washed with PBS before PI staining (final concentration 5 μg/mL). Flow cytometry analysis was carried out with a FACS Calibur™ using CellQuest Pro software (BD Bioscience, Allschwil, Switzerland).

2.6. Metabolism of thienopyridines by neutrophil granulocytes and lymphocytes

Neutrophils or lymphocytes (4.5×10^6/mL in 24-well plates) were incubated with clopidogrel, ticlopidine, clopidogrel carboxylate or prasugrel (10 or 100 μM) at 37 °C for different periods of time (0, 6, 12 and 24 h) in the presence or absence of the CYP3A4 inhibitor ketoconazole (1 μM) or the MPO inhibitor rutin (20 μM). Reactions were stopped by the addition of 300 μL of methanol and the precipitated proteins were removed by centrifugation at 3000 × *g* for 30 min. The samples were analyzed by LC–MS as described previously (Maseneni et al., 2012).

2.7. Electrical potential across the inner mitochondrial membrane ($\Delta\Psi$)

To detect the changes in the mitochondrial membrane potential ($\Delta\Psi$), cells were exposed to tetramethylrhodamine ethyl ester (TMRE). Cells were incubated with drugs in presence or absence of ketoconazole (1 μM) or rutin (20 μM) for 24 h. After the incubation, cells were washed twice with PBS and incubated with 100 nM TMRE in PBS for 30 min at room temperature. The fluorescence signal of the cationic dye