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Original Contribution

Hepatocellular toxicity of clopidogrel: Mechanisms and risk factors

Anja Zahno [1], Jamal Bouitbir [1], Swarna Maseneni, Peter W. Lindinger, Karin Brecht, Stephan Krähenbühl *

Division of Clinical Pharmacology & Toxicology and Department of Biomedicine, University Hospital, CH-4031 Basel, Switzerland

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ABSTRACT

Clopidogrel is a prodrug used widely as a platelet aggregation inhibitor. After intestinal absorption, approximately 90% is converted to inactive clopidogrel carboxylate and 10% via a two-step procedure to the active metabolite containing a mercapto group. Hepatotoxicity is a rare but potentially serious adverse reaction associated with clopidogrel. The aim of this study was to find out the mechanisms and susceptibility factors for clopidogrel-associated hepatotoxicity. In primary human hepatocytes, clopidogrel (10 and 100 μM) was cytotoxic only after cytochrome P450 (CYP) induction by rifampicin. Clopidogrel (10 and 100 μM) was also toxic for HepG2 cells expressing human CYP3A4 (HepG2/CYP3A4) and HepG2 cells co-incubated with CYP3A4 supersomes (HepG2/CYP3A4 supersome), but not for wild-type HepG2 cells (HepG2/wt). Clopidogrel (100 μM) decreased the cellular glutathione content in HepG2/CYP3A4 supersome and triggered an oxidative stress reaction (10 and 100 μM) in HepG2/CYP3A4, but not in HepG2/wt. Glutathione depletion significantly increased the cytotoxicity of clopidogrel (10 and 100 μM) in HepG2/CYP3A4 supersome. Co-incubation with 1 μM ketoconazole or 10 mM glutathione almost completely prevented the cytotoxic effect of clopidogrel in HepG2/CYP3A4 and HepG2/CYP3A4 supersome. HepG2/CYP3A4 incubated with 100 μM clopidogrel showed mitochondrial damage and cytochrome *c* release, eventually promoting apoptosis and/or necrosis. In contrast to clopidogrel, clopidogrel carboxylate was not toxic for HepG2/wt or HepG2/CYP3A4 up to 100 μM. In conclusion, clopidogrel incubated with CYP3A4 is associated with the formation of metabolites that are toxic for hepatocytes and can be trapped by glutathione. High CYP3A4 activity and low cellular glutathione stores may be risk factors for clopidogrel-associated hepatocellular toxicity.

Introduction

In patients who are suffering from cardiovascular disease such as coronary heart disease, cerebrovascular disease, or peripheral arterial disease, clopidogrel, possibly in combination with aspirin, is an established antiplatelet therapy to reduce cardiovascular events [1,2]. Clopidogrel is a thienopyridine derivative that irreversibly inhibits platelet aggregation by selectively binding to the adenosine-5′-diphosphate $P2Y_{12}$ receptor on the platelet surface [3]. After oral administration, clopidogrel is rapidly absorbed and a small part is metabolically activated by the cytochrome P450 $(CYP)^2$ system [4–6]. The other part (approximately 90%) is converted to clopidogrel carboxylate by esterases [7] and is not active [8]. Clopidogrel has largely replaced ticlopidine, the first thienopyridine derivative in clinical use, because of its superior safety profile and more efficient antiplatelet effects. Common adverse effects associated with clopidogrel include gastrointestinal disorders (dyspepsia, nausea, vomiting), bleeding, rash, and diarrhea [9]. In addition, potentially more serious, idiosyncratic adverse reactions such as hypersensitivity syndrome [10], thrombotic thrombocytopenic purpura [11], neutropenia and agranulocytosis [12,13], as well as hepatotoxicity including liver failure [14–22], have been reported. In patients with clopidogrel-associated hepatotoxicity, the time between start of clopidogrel therapy and appearance of hepatic injury was variable, ranging from a few days to months. The pathomechanism of clopidogrel-associated liver injury is currently unclear; immunomediated mechanisms and “metabolic” toxicity have been suggested. Liver injury associated with clopidogrel is most often mixed hepatocellular and cholestatic and the clinical presentation and course appear to be similar to liver injury associated with ticlopidine. Interestingly, a patient with ticlopidine-associated liver injury could successfully be treated with clopidogrel, suggesting differences between these two thienopyridines in the mechanisms leading to liver injury [23].

As stated above, clopidogrel is a prodrug that is activated in the liver by several CYPs. Clopidogrel activation is conventionally regarded as a two-step process, first leading to the formation of

Abbreviations: CYP, cytochrome P450; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEM, diethyl maleate; GSH, reduced glutathione; ROS, reactive oxygen species.

* Corresponding author. Fax: +41 61 265 4560.
E-mail address: kraehenbuehl@uhbs.ch (S. Krähenbühl).

[1] These authors contributed equally to this study.

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2-oxo-clopdigrel by oxidation of the thiophene ring and then to the active metabolite by oxidative opening of the thiolactone structure of 2-oxo-clopidogrel [7]. CYPs involved in the first step are mainly CYP1A2, 2C19, and 2B6 [24] and, as shown recently, also CYP3A4 [25]. CYPs involved in the second step are mainly 2C19, 3A4, 2C9, and 2B6 [24]. The active metabolite of clopidogrel carries a mercapto group [25,26], which can bind covalently to the platelet $P2Y_{12}$ receptor but also to other proteins such as glutathione and CYPs [27,28]. Recent studies show, however, that both steps of the activation process of clopidogrel are more complicated, leading to many other possibly reactive intermediates [25,29,30]. Regarding the potential to form off-target adducts [27,28], the active metabolite (and possibly also other metabolites formed) could be responsible not only for the pharmacological action of clopidogrel, but also for toxic effects, including hepatotoxicity. In support of a toxic mechanism involving clopidogrel metabolites, we have shown recently that incubation of clopidogrel with CYP3A4 is associated with the formation of metabolites that are toxic for granulocyte precursors [31]. Patients with increased production of such metabolites and/or decreased defense systems may therefore be at risk for clopidogrel-associated toxicity.

Taking into account these considerations, this study was performed to investigate possible mechanisms and risk factors associated with hepatocellular toxicity of clopidogrel and the most abundant clopidogrel metabolite clopidogrel carboxylate. We used three different cell systems to assess the toxicity of these compounds: primary human hepatocytes with or without induction with rifampicin, HepG2 cells overexpressing human CYP3A4, and HepG2 cells in combination with CYP3A4 supersomes.

Material and methods

Material

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

Cell lines and cell culture

Cryopreserved primary human hepatocytes (BD Gentest) were recovered using the manufacturer's protocol and cultured for 24 h before the addition of drugs.

The hepatoma cell line HepG2 was provided by Professor Dietrich von Schweinitz (University Hospital, Basel, Switzerland). HepG2 cells stably transduced with human CYP3A4 were prepared as described [32]. Wild-type HepG2 cells (HepG2/wt) and HepG2 cells stably transduced with CYP3A4 (HepG2/CYP3A4) were cultured in Dulbecco's modified Eagle's medium (with 2 mmol/L GlutaMAX, 1.0 g/L glucose, and sodium bicarbonate) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 10 mM Hepes buffer, pH 7.2, and nonessential amino acids. The culture conditions were 5% CO_2 and 95% air atmosphere at 37 °C.

Treatment of primary human hepatocytes, HepG2/wt, and HepG2/CYP3A4

Primary human hepatocytes were cultured for 24 h before the addition of 20 μM rifampicin for 72 h. This rifampicin concentration is associated with a significant induction of CYP3A4 expression and function [33]. We then treated the cells with clopidogrel (1, 10, or 100 μM) for 24 h and determined cytotoxicity as described below.

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

Co-incubations of HepG2 cells with CYP3A4 supersomes and control supersomes

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

Cytotoxicity assays

Cytotoxicity was determined with two assays, which gave similar results when both were used for the same sample (see Supplementary Table and Figures). In one assay, we determined the release of adenylate kinase using the firefly luciferase system (ToxiLight BioAssay Kit; Cambrex Bio Science). After 24 h of incubation, 100 μl assay buffer was supplied to 20 μl supernatant from drug-treated cells in the presence or absence of ketoconazole (concentrations indicated in the figures) and luminescence was measured after 5 min.

In the other assay, we determined the intracellular ATP content using the CellTiterGlo luminescent cell viability assay (Lonza). In accordance with the manufacturer's manual, 100 μl assay buffer was added to each well of a 96-well plate containing 100 μl culture medium. After incubation in the dark for 30 min, luminescence was measured using a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland).

Quantification of clopidogrel metabolism using HPLC

HepG2/wt or HepG2/CYP3A4 cells (450,000 per well) were seeded in 24-well plates and allowed to adhere overnight. Supersomes were used as mentioned above. After the incubation with clopidogrel (10, 50, and 100 μM) for various periods of time (6, 12, and 24 h), 120 μl acetonitrile containing the internal standard (6.5 μM naproxen) was added and cells were detached with a cell scraper. Cells were lysed by freeze–thaw cycles and the cleared lysate was subjected to HPLC analysis. The analysis was performed as described previously [8].