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In vitro approach to elucidate the relevance of carboxylesterase 2 and *N*-acetyltransferase 2 to flupirtine-induced liver injury

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ABSTRACT

The use of flupirtine, an analgesic, has been restricted in European countries because it causes liver injury in rare cases. Flupirtine is primarily metabolized to D-13223, an acetylamino form. In the process of D-13223 formation, it has been hypothesized that a reactive metabolite is formed which may be involved in flupirtine hepatotoxicity. The purpose of this study was to identify the potential reactive metabolite and the responsible enzymes in the human liver to get a clue to the mechanism of hepatotoxicity. Using recombinant enzymes, we found that D-13223 was formed from flupirtine via hydrolysis by carboxylesterase 2 (CES2) and subsequent acetylation by *N*-acetyltransferase (NAT) 2. A conjugate of *N*-acetyl-L-cysteine (NAC), a nucleophile, was detected by incubation of flupirtine with CES2, and the conjugate formation in human liver microsomes was inhibited by CES2 inhibitors, indicating that a reactive metabolite, which may be a quinone diimine, was produced in the process of CES2-mediated hydrolysis of flupirtine. The formation of the NAC conjugate in liver S9 samples from NAT2 slow acetylators was significantly higher than that from NAT2 rapid/intermediate acetylators, indicating that NAT2 could function as a detoxification enzyme for flupirtine. CES2-overexpressing HepG2 cells showed remarkable lactate dehydrogenase leakage under flupirtine treatment, while no cytotoxicity was observed in control cells, suggesting that the reactive metabolite formed by CES2-mediated hydrolysis of flupirtine would be a trigger of hepatotoxicity. NAT2 slow acetylators with high CES2 activity could be highly susceptible to flupirtine-induced liver injury.

1. Introduction

Flupirtine, an analgesic that is an antagonist of the *N*-methyl-D-aspartate receptor, is used for headaches, muscle cramps, and surgical pain in European countries [1]. Flupirtine is also used in the treatment of Parkinson's and Alzheimer's diseases, Creutzfeld-Jakob disease, and other neurodegenerative ailments because it has neuroprotective effects [2,3]. Until September 2007, 151 cases of flupirtine-associated liver injury (< 0.01% of patients) were reported in Germany [4]. Due to the liver injuries, the European Medicines Agency restricted the use of flupirtine [5]. It has been reported that flupirtine causes necrosis of hepatocytes in zone 3, where drug-metabolizing enzymes, such as cytochrome P450s and UDP-glucuronosyltransferases, are highly expressed [6–8]. In addition, infiltration of macrophages with ceroid pigment was observed in the livers injured by flupirtine treatment [4]. Thus, metabolism and allergic reaction are likely involved in hepatotoxicity of flupirtine. However, the mechanism of liver injury has not been thoroughly elucidated to date.

In humans, flupirtine is primarily metabolized to D-13223, an acetylamino form (Fig. 1). Within 8 h of the administration of flupirtine, D-13223 was found at 20% of the dose in human urine [9,10]. It has been proposed that D-13223 is formed via hydrolysis and acetylation [9,10]; however, this notion was not experimentally supported. As it is one of the causes of idiosyncratic drug-induced liver injury, reactive metabolite involvement has often been suspected [11]. Generally, quinones and their analogs are known to cause several toxicities,

Abbreviations: AADAC, arylacetamide deacetylase; AcetylCoA, acetyl coenzyme A; Ad, adenovirus; BNPP, bis-(*p*-nitrophenyl) phosphate; BSO, L-buthionine sulfoximine; CES, carboxylesterase; DFP, diisopropyl phosphorofluoride; DMSO, dimethyl sulfoxide; Eserine, sulfate physostigmine; GFP, green fluorescent protein; HEK293, human embryotic kidney 293; HIM, human intestine microsomes; HLM, human liver microsomes; HLS9, human liver supernatant 9000 g; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MOI, multiplicity of infection; MRM, multiple-reaction monitoring; NAC, *N*-acetyl-L-cysteine; NAT, *N*-acetyltransferase; PMSF, phenylmethylsulfonyl fluoride

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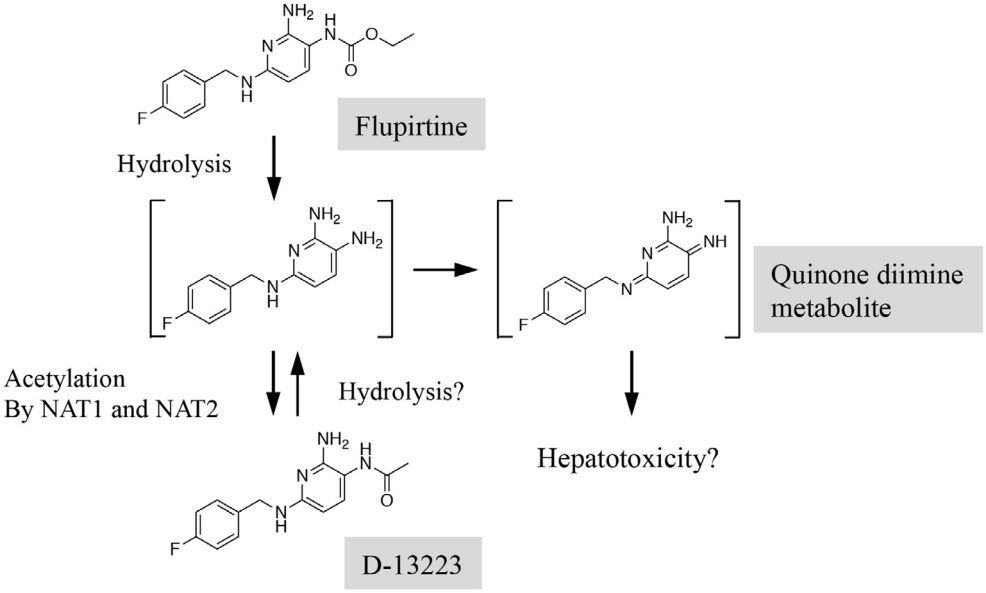

Fig. 1. Proposed metabolic pathways and related liver injury of flupirtine in humans.

such as hepatotoxicity and cardiotoxicity, due to their high reactivity [12,13]. In the case of flupirtine, a quinone diimine has been thought to be a reactive metabolite and may be formed from the hydrolyzed metabolite of flupirtine through non-enzymatic conversion. However, it remains unclear whether the reactive metabolite is involved in flupirtine-induced toxicity. Given that the quinone diimine formed from flupirtine may cause hepatotoxicity, the inter-individual differences in activities of the enzymes involved in the flupirtine metabolism could affect the susceptibility to toxicity. In this study, we sought to clarify the metabolic pathways of flupirtine and the responsible enzymes, and investigated whether the presumed reactive metabolite(s) are associated with hepatotoxicity by *in vitro* assay.

2. Materials and methods

2.1. Materials

Human liver microsomes (HLM) (pooled from 50 individuals), human intestine microsomes (HIM) (pooled from 7 individuals), recombinant human *N*-acetyltransferase (NAT) 1 and NAT2 were purchased from Corning (Corning, NY). Recombinant carboxylesterase (CES) 1, CES2, and arylacetamide deacetylase (AADAC) were previously prepared [14]. Flupirtine and D-13223 were purchased from ChemScene (Monmouth Junction, NJ) and Santa Cruz Biotechnology (Dallas, TX), respectively. Acetyl coenzyme A (acetylCoA), diisopropyl fluorophosphate (DFP), *N*-acetyl-L-cysteine (NAC), phenylmethylsulfonyl fluoride (PMSF), and sulfate physostigmine (eserine) were purchased from Wako Pure Chemicals (Osaka, Japan). Bis(*p*-nitrophenyl) phosphate (BNPP) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of the highest grade commercially available.

2.2. Preparation of human liver S9

Human liver samples were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan). The donor information is shown in Table 1. Human liver S9 (HLS9) was prepared as described previously [15].

2.3. Measurement of D-13223 formation from flupirtine

D-13223 formation from flupirtine was measured as follows: a typical incubation mixture (final volume of 200 µL) contained 100 mM potassium phosphate buffer (pH 7.4), enzyme sources (HLS9, 0.8 mg/mL; HLM, HIM, CES1, CES2, and AADAC, 0.4 mg/mL; NAT1, 0.5 µg/mL; NAT2, 0.25 µg/mL), 1 mM acetylCoA, 100 µM dithiothreitol, and 100 µM ethylenediaminetetraacetic acid. We confirmed that the D-13223 formation rates were linear up to 1 mg/mL for HLM, HIM, HLS9, CES2, and AADAC concentrations, 1 µg/mL for NAT1, and 0.5 µg/mL for NAT2 and were linear up to the 6 h incubation time. Flupirtine was dissolved with dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubation mixture was 1%. The reactions were initiated by the addition of flupirtine at a final concentration of 60 µM after a 2-min preincubation at 37 °C. After the 3 h (in the case of HLM or recombinant hydrolases in the presence of NATs) or 6 h incubation (in the case of HLS9), the reactions were terminated by the addition of 200 or 100 µL of ice-cold acetonitrile. After removal of the protein by centrifugation at 20,400*g* for 5 min, a portion of the supernatant was subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS). The LC equipment was comprised of a CBM-20A controller (Shimadzu, Kyoto, Japan), LC-20AD pumps (Shimadzu), a SIL-20AC HT autosampler (Shimadzu), a CTO-20AC column oven (Shimadzu), and an SPD-20A UV detector (Shimadzu) equipped with a Develosil ODS-UG-3 (3 µm particle size, 2.0 mm i.d. × 150 mm; Nomura Chemical, Seto, Japan). The column temperature was set at 40 °C, and the flow rate was 0.2 mL/min. The mobile phase was 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The conditions for elution were as follows: 10–90% B (0–2 min), 90% B (2–5 min), and 10% B (5–8 min). The LC was connected to LCMS-8040 (Shimadzu) in the positive electrospray ionization mode. Nitrogen was used as the nebulizing and drying gas at 3 L/min and 15 L/min, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using argon as the collision gas at 230 kPa. D-13223 was monitored in the multiple reaction monitoring (MRM) mode at *m/z* 275.30 and 109.10 (collision energy: −23 V). The analytical data were processed using LabSolutions (version 5.82 SP1, Shimadzu).

2.4. Detection of NAC conjugate(s)

The hydrolyzed metabolite of flupirtine has been reported to be