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Role of benoxaprofen and flunoxaprofen acyl glucuronides in covalent binding to rat plasma and liver proteins in vivo

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Abstract

Benoxaprofen (BNX) has been implicated in rare but serious hepatotoxicity which led to its withdrawal from the world market. Flunoxaprofen (FLX), a structural analog, appears to be less toxic. It has been postulated that the nonsteroidal antiinflammatory drugs associated toxicity may be related to covalent modification of proteins by their reactive acyl glucuronides, and the extent of covalent protein binding depends on both reactivity of the acyl glucuronide and the exposure to the reactive metabolite. The disposition of BNX and FLX in rats were compared upon intravenous administration of 20 mg/kg of BNX, FLX or their metabolites. Covalent binding of BNX and FLX to plasma and liver proteins were also determined, and an immunochemical approach was used to detect their hepatic targets. Similar concentrations of plasma protein adducts for BNX and FLX were detected even though the AUC of BNX-glucuronide (BNX-G) was almost twice that of FLX-glucuronide (FLX-G). Similar concentrations of liver protein adducts for BNX and FLX were also detected at 8 h, however, the hepatobiliary exposure of BNX-G was only 1/3rd that of FLX-G indicating that BNX-G was more reactive than FLX-G, which was in agreement with in vitro data. Proteins of 110 and 70 kDa were the major liver protein targets modified by covalent attachment of BNX and FLX. In conclusion, measuring covalent binding to tissue proteins in animals in addition to plasma adducts should be considered when evaluating and comparing carboxylic acid analogs that form reactive acyl glucuronides.

Keywords: Reactive acyl glucuronide; Covalent protein binding; NSAIDs; Reversible metabolism; Immunoblot; Liver protein targets

1. Introduction

A major metabolic pathway for drugs and endobiotics bearing carboxylic acid groups is coupling with glucuronic acid to yield acyl glucuronide conjugates. Unlike other types of glucuronides, acyl glucuronides are chemically unstable in vitro and in vivo [1,2]. This reactivity stems from the inherent susceptibility of the acyl (ester) group to nucleophilic substitution, and manifests itself along three related pathways: (1) hydrolysis, (2) intramolecular rearrangement, and (3) covalent binding to proteins via nucleophilic displacement and/or imine mechanisms. It has been suggested that acyl glucuronides of a number of carboxylic acids drugs are implicated in adverse reactions due to the observation that they are able to bind irreversibly to cellular proteins [3]. Drug plasma protein-adducts have been detected in humans and animals after the administration of diclofenac [4], diflunisal [5–7], suprofen [8], zomepirac [9], valproic acid [10], tolmetin [11,12], clofibric acid [13], salicylate [14], and ibuprofen [15]; all of these drugs form acyl glucuronides by metabolism and hypersensitivity reactions have been reported related to their use in some patients [16,17].

Abbreviations: $A(m)_{e,bile,0\rightarrow 8\ h}$, cumulative biliary excretion of metabolite up to 8 h; AUC, area under the plasma concentration curve; BNX, benoxaprofen; BNX-G, benoxaprofen glucuronide; CL, systemic clearance; $Cl_{f,bile}$, biliary formation clearance; $Cl_{e,bile}$, biliary excretion clearance; DMF, *N*,*N*-dimethylformamide; ECL, enhanced chemiluminescence; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunoabsorbance assay; FCS, fetal calf serum; FLX, flunoxaprofen; FLX-G, flunoxaprofen glucuronide; HPLC, high performance liquid chromatography; HSA, human serum albumin; IBP, ibuprofen; KLH, keyhole limpet hemocyanine; NSAIDs, nonsteroidal antiinflammatory drugs; PBS, phosphate buffered saline; RSA, rat serum albumin; SDS-PAGE, sodiumdodecyl sulfate polyacrylamide gel electrophoresis; $t_{1/2}$, half life

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Benoxaprofen (BNX) and flunoxaprofen (FLX) are a pair of structural analogs but with apparent divergent hepatotoxicity in humans. BNX was withdrawn from U.S. and British markets due to several fatal incidences of cholestatic jaundice in elderly patients [17]. FLX, which appeared to be less toxic, though data is limited, was only marketed in Italy and no fatal adverse events related to FLX have been reported in available sources. Formation of acyl glucuronide represents a major metabolic pathway for both BNX and FLX in humans [18,19]. Similar concentrations of BNX- and FLX-plasma protein adducts have been detected in the systemic circulation in human [20]. The major site of conjugation for BNX and FLX in humans is believed to be the liver. Experimental studies in mice and rats to examine organ tolerance to NSAIDs suggested that BNX was more toxic than FLX [21]. BNX was also shown to be more toxic than ibuprofen (IBP), a well-tolerated NSAID, in primary cultures of rat hepatocytes in vitro [22]. Disposition and covalent protein binding of BNX and FLX, however, have not been well characterized in rat. It has been suggested that the extent of irreversible tissue binding at a particular time depends on both the stability/reactivity of the reactive acyl glucuronide and the exposure of the organ to the reactive metabolite [6,8]. Differences in the disposition of these two NSAIDs may in part influence their protein adducts formations in vivo. In the present study, we first compared the reversible metabolism of BNX and FLX in rats where dosing of both parent compound and its respective acyl glucuronide are possible. In addition, covalent adduct formation of BNX and FLX in systemic circulation and in liver tissue was characterized to determine if protein adduct formation in plasma and liver tissue correlates with systemic or hepatobiliary exposure to labile and reactive acyl glucuronides. Furthermore, studies directed at identification of hepatic protein targets of BNX and FLX in rats, which could be causative factors or correlated with hepatotoxic responses, have been lacking. It has been demonstrated previously by Bailey and Dickinson [23] that the pattern of protein modification in liver varied from drug to drug. An immunochemical approach was therefore taken to compare the protein targets in livers from rats treated with BNX and FLX, and compared to livers from rats treated with IBP, the relatively nontoxic NSAID. Finally, the stability and reactivity of BNX acyl glucuronide (BNX-G) and FLX acyl glucuronide (FLX-G) were also evaluated in vitro.

2. Materials and methods

2.1. Materials

Rac-BNX was extracted and purified from Tablets of Oraflex® previously marketed by Eli Lilly (Indianapolis, IL). Anal. Calcd for BNX ($C_{16}H_{12}ClNO_3$): C, 63.69; H, 4.01; N, 4.64; Cl, 11.75; O, 15.91. Found: C, 63.05; H, 4.31; N, 4.71; Cl, 11.68; O, 16.25. The purity of BNX was confirmed based on elemental analysis and analytical HPLC using UV detection at wavelength of 210 nm. *S*-FLX, the marketed form, was obtained by a generous contribution from Dr. A. Forgione (Ravizza Laboratories, Milan, Italy), and was determined to be pure based on HPLC with UV detection. BNX-G and FLX-G were extracted and purified from urine using preparative HPLC, as described previously [24]. β-Glucuronidase (type B-10 from bovine liver), human serum albumin (HSA, fraction V), IBP, rat serum albumin (RSA), Triton X-100, EDTA, and thimerosal were obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membrane (0.45 μm), and Bradford reagent used for protein analysis were purchased from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence (ECL) reagents, goat anti-rabbit IgG (peroxidase conjugate), and hyperfilm ECL were obtained from Amersham (Arlington Heights, IL). Methanol and acetonitrile were HPLC grade from J.T. Baker (Pillipsburg, NJ). All other chemicals used were of reagent grade. Human plasma was provided by UNC Hospital Blood Center (Chapel Hill, NC). Male Sprague-Dawley rats (200–300 g) were supplied by Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Experiments were approved by the University's Institutional Animal Care and Use Committee.

2.2. Pharmacokinetic studies

Adult, male Sprague-Dawley rats (200–300 g) were maintained under a 12 h light/dark cycle in a temperature-controlled environment, with free access to food and water. All animals were anesthetized with intramuscular injection of ketamine:acepromazine (75:2 mg/kg) into the thigh. About 20 mg/kg of each parent drug was administered intravenously through cannulated jugular vein to six control, bile duct-intact animals and six bile duct-cannulated animals. Serial blood samples of 0.2 ml were collected through cannulated carotid artery over 8 h for analysis of plasma concentrations of both parent drugs and their glucuronide metabolites. At 1, 4, and 8 h after the administration of BNX and FLX, additional blood samples were drawn for measuring drug–protein adducts in plasma. For bile duct-cannulated animals, bile samples were also collected at hour intervals. The pH of both plasma and bile samples were adjusted with 43% phosphoric acid to between 2 and 4 to prevent degradation of acyl glucuronides. For the determination of the drugs covalently bound to liver tissues, the animals were euthanized 8 h after the drug administration, then livers were harvested. All biological samples were stored at −20 °C until analyzed. In addition, six bile duct-cannulated animals were each administered a bolus dose of 20 mg/kg (parent equivalents) of BNX-G or FLX-G as solutions in 0.15 M phosphate buffer at pH 5. Blood samples were collected as frequently as possible during the first 30 min to