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In vitro pharmacokinetic profile of a benzopyridooxathiazepine derivative using rat microsomes and hepatocytes: Identification of phases I and II metabolites

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ABSTRACT

In the present study, the in vitro metabolic behavior of a benzopyridooxathiazepine (BZN), a potent tubulin polymerization inhibitor, was investigated by liquid chromatography-UV detection (LC-UV). First, simple and fast LC-UV methods have been optimized and validated to evaluate the pharmacokinetic profile of BZN using rat liver microsomes or hepatocytes primary cultures suspensions. Whatever the medium investigated, baseline resolution between the internal standard and BZN was achieved in a run time less than 15 min using a Symmetry ODS column (150 mm × 4.6 mm i.d., 5 μm) and a mobile phase consisting of acetonitrile/water/formic acid 60:40:0.1 (v/v/v). Linearity was assessed in the 0.1–50 μM and in the 0.05–5 μM concentration ranges, respectively, in microsomal and hepatocyte matrix. According to the novel strategy based on the build of the accuracy profile, total error of the developed methods was included within the ±10% limits of acceptance. Then, from incubation of BZN with both liver microsomes and or hepatocytes, structural informations on phase I and phase II metabolites were acquired using liquid chromatography coupled to electrospray orbitrap mass spectrometer (LC–MS). Mass spectrum, double bond equivalent and elemental composition were useful data to access to the chemical structure of each metabolite. In microsomal suspension, four main metabolites were observed including monohydroxylation and dihydroxylation of the benzopyridooxathiazepine core, demethylation of the methoxyphenyl moiety, as well as their combinations. The phase II metabolites detected in hepatocytes suspension were the glucuronide adducts of both demethylated BZN and mono-oxygenated BZN. Based on the structural elucidation of the metabolites detected, we proposed an in vitro metabolic pathway of BZN, a new tubulin polymerization inhibitor.

1. Introduction

Benzopyridooxathiazepine derivatives are potent cytotoxic compounds [1]. They belong to the class of antimitotic agents and were reported to inhibit tubulin polymerization leading to cell cycle arrest and apoptosis. Among the series of synthesized products [2], 1-(4-methoxyphenyl-ethyl)-11H-benzo[*f*]-1,2-dihydro-pyrido[3,2,*c*][1,2,5]oxathiazepine 5,5 dioxide (BZN) is the best cytotoxic compound. Indeed, first results of in vitro cytotoxicity toward the L1210 leukemia cell line were very promising with IC_{50} value in the submicromolar range (IC_{50} = 9.5 nM). In our previous work, the chemical stability of BZN has been investigated under various stress conditions [3]. Although a slow degradation was noticed under oxidative (RT) and acidic (70 °C) conditions, BZN was found to be stable in most of stressed conditions. These results were encouraging for the development of BZN as a drug candidate.

Nevertheless, the commercial success of a new chemical entity depends on its pharmacological activity and several absorption, distribution, metabolism, excretion (ADME) parameters must be determined [4,5]. Hence, in a first approach, it is necessary to investigate the in vitro metabolism of a potent drug. Another interest of pharmacological studies is to optimize drug candidate synthesis. Indeed, the knowledge of metabolism or toxicological data is precious to redirect synthesis strategy and discover more active compounds [4,6].

Because most drugs are eliminated from the body predominantly by hepatic route, it is important to predict hepatic metabolism. On the one hand, liver microsomes were used extensively as an in vitro drug-metabolizing system since they may predict both routes and rate of metabolism [7]. On the other hand, pharmacokinetic study using hepatocytes is interesting to investigate reactions involved in both phases I and II metabolism [8,9]. Moreover, some authors [10,11] have shown that the in vitro

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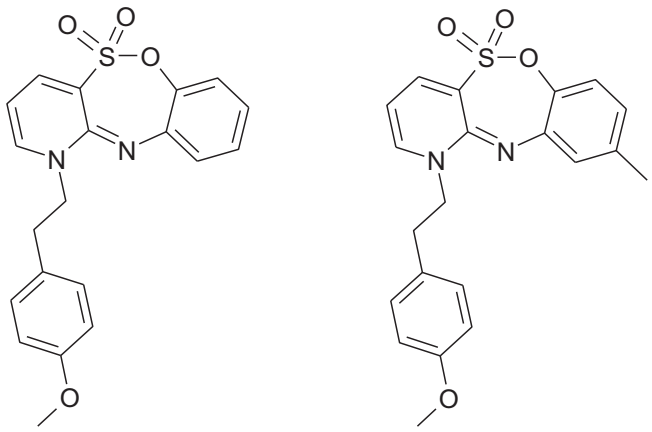

Fig. 1. Chemical structures of 1-(4-methoxyphenylethyl)-11*H*-benzo[*f*]-1,2-dihydro-pyrido[3,2,*c*][1,2,5]oxathiazepine 5,5 dioxide (**BZN**) and 1-(4-methoxyphenylethyl)-9-methyl-11*H*-benzo[*f*]-1,2-dihydro-pyrido[3,2,*c*][1,2,5]oxathiazepine 5,5 dioxide, the internal standard (**IS**).

study using freshly isolated hepatocytes was useful to predict in vivo intrinsic clearance of various drugs.

The aim of the present study was to determine the metabolic behavior of BZN in rat liver microsomes and hepatocytes. The first work consisted in the development and the validation of a rapid LC-UV method to evaluate the pharmacokinetic profile of BZN. Secondly, high resolution mass spectrometry was used to characterize the in vitro metabolite pathway from the incubation of BZN using rat microsomes or hepatocytes suspensions. The goal of this paper was to propose a metabolic pathway of a benzopyridooxathiazepine resulting in a new series of tubulin polymerization inhibitor.

2. Experimental

2.1. Chemicals

1-(4-Methoxyphenylethyl)-11*H*-benzo[*f*]-1,2-dihydro-pyrido [3,2,*c*][1,2,5]oxathiazepine 5,5 dioxide (BZN) and 1-(4-methoxyphenylethyl)-9-methyl-11*H*-benzo[*f*]-1,2-dihydro-pyrido[3,2,*c*] [1,2,5]oxathiazepine 5,5 dioxide (IS) were synthesized in our laboratory according to the procedure described by Lebegue *et al.* [1] and Gallet *et al.* [2] (Fig. 1). Rat liver microsomes (RLM, 20 mg mL^{-1}), NADPH regenerating system and phosphate buffer (500 mM, pH 7.4) were purchased from BD biosciences (Interchim, Montluçon, France). All solvents were of HPLC grade. Methanol (MeOH) and acetonitrile (ACN) were purchased from VWR (Val de Fontenay, France). Dimethylsulfoxide (DMSO), NaCl, KCl, $CaCl_2$, $NaHCO_3$, Na_2HPO_4, penicillin G 100 UI, streptomycin, collagenase type IV, trypan blue, William's medium were purchased from Sigma Aldrich (Steinheim, Germany) and HCOOH and NH_4OH (28 wt%) from Prolabo (Val de Fontenay, France). HEPES was obtained from Flandre Chimie (Villeneuve d'Ascq, France). Xylamine, ketamine were purchased from veterinary laboratories Merial (Lyon, France). Ultra-pure 18 MΩ water was supplied from a Milli-Q system (Millipore, Saint-Quentin en Yvelines, France).

2.2. Chromatographic apparatus and conditions

2.2.1. LC-UV conditions

Chromatographic analyses were performed on a Waters system equipped with a Waters gradient quaternary 600E pump model, a Waters on-line degasser apparatus, a 7125 Rheodyne injector (20 μL loop) and a Waters 996 photodiode array detector (Milford, MA, USA). Data were collected and processed on a computer running with Empower software (version 2) from Waters. Separations were carried out on a reversed-phase Symmetry C18 (150 mm × 4.6 mm i.d., 5 μm) column from Waters preceded with a Lichrospher C18 (4 mm × 4 mm i.d., 5 μm) guard column from Merck (Fontenay-sous-bois, France), both kept at 25 °C. The analytes were eluted under isocratic conditions using a mobile phase composed of ACN/H_2O/HCOOH (60:40:0.1, v/v/v) delivered at a flow rate of 1 mL min^{-1}. All compounds were detected at 318 nm (λ_{max} for all compounds).

2.2.2. LC–MS conditions

An UPLC-ESI-MS system including an Accela Autosampler, an Accela LC pump and an Exactive FTMS mass spectrometer (Thermo Fisher Scientific, San Jose, USA) was used for the characterization of metabolites of BZN. Chromatographic separations were performed on a Hypersil Gold C18 (150 mm × 2.1 mm i.d., 3 μm) from Thermo. The mobile phase was a mixture of ACN/H_2O/HCOOH (60:40:0.1, v/v/v). The analytes were eluted under isocratic conditions at a flow rate of 0.2 μL min^{-1}. The temperature was set at 25 °C and the volume of injection was 5 μL. All ESI-MS experiments were acquired in the positive mode and the ESI-source parameters were as follows: sheath and auxiliary gas flow rate were 60 and 10 (nitrogen, arbitrary units), respectively; 4.5 kV spray voltage, 350 °C capillary temperature, 42.5 V capillary voltage, 95 V tube lens voltage and 20 V skimmer voltage. The scan range was m/z 50–600. Prior analysis, the orbitrap mass analyzer was externally calibrated, in the scan range m/z 70–650, to obtain mass accuracy with ±5 ppm. UPLC-ESI/MS system was controlled with Xcalibur software version 2.1. All data were processed using the same software which provides accurate mass from raw data. In addition, chemical formula calculator was used to provide chemical formula and saturation values (double bond equivalent, DBE).

2.3. Method validation

In general manner, stock solutions of BZN (5 mM) and IS (2 mM) were prepared in ACN. Working solutions of BZN were prepared by serial dilutions in the mobile phase whereas IS working solution (200 μM) was prepared in ACN. All solutions were stored at 4 °C for one month without any degradation.

2.3.1. Preparation of calibration and validation standards for quantitation of BZN in RLM

Previous BZN working solution was incubated with RLM (0.125 mg) in a 100 mM phosphate buffer (pH 7.4) without NADPH to avoid any metabolic activity. Nine calibration standards were prepared in the 0.2–100 μM concentration range. Four levels of validation standards, corresponding to low (0.1 μM), medium (2.5 and 15 μM) and high (50 μM) concentrations were also prepared. Each solution, containing 20 μM IS, was vortex for 20 s, centrifugated (13,000 × *g* for 3 min) and a 20 μL aliquot was injected directly onto the analytical system.

2.3.2. Preparation of calibration and validation standards for quantitation of BZN in rat hepatocytes

A series of six calibration standards were incubated with thermal inactivated hepatocytes (800 μL), in the 0.05–5 μM concentration range. Three levels of validation standards were also prepared at 0.09, 2 and 4 μM. All solution contained 1 μM IS. Before LC-UV analysis, a solid phase extraction was performed.