European Journal of Pharmaceutical Sciences 39 (2010) 23–29

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Pharmacology of EAPB0203, a novel imidazo[1,2-*a*]quinoxaline derivative with anti-tumoral activity on melanoma

Sonia Khier[a], Carine Deleuze-Masquéfa[b,1], Georges Moarbess[b,1], Florence Gattacceca[a], Delphine Margout[a], Isabelle Solassol[c], Jean-François Cooper[d], Frédéric Pinguet[c], Pierre-Antoine Bonnet[b], Françoise M.M. Bressolle[a,*]

[a] *Clinical Pharmacokinetic Laboratory, EA4215, Faculty of Pharmacy, 15 Avenue Ch. Flahault, University Montpellier I, B.P. 14491, 34093 Montpellier Cedex 5, France*
[b] *Pharmacochemistry and Biomolecule Laboratory, EA4215, Faculty of Pharmacy, 15 Avenue Ch. Flahault, University Montpellier I, 34093 Montpellier Cedex 5, France*
[c] *Oncopharmacology Department, Pharmacy Service, Val d'Aurelle Anticancer Centre, Parc Euromédecine, 34298 Montpellier Cedex 5, France*
[d] *Biochemistry and Environment Laboratory, EA4215, Perpignan University, 66860 Perpignan Cedex 9, France*

ARTICLE INFO

Article history:
Received 27 July 2009
Received in revised form
30 September 2009
Accepted 13 October 2009
Available online 23 October 2009

Keywords:
Imidazo[1,2-*a*]quinoxaline
LC/ESI–MS
Melanoma
In vitro and *in vivo* cytotoxic activities
Pharmacokinetics
Sub-acute toxicity

ABSTRACT

In spite of the development of new anticancer drugs by the pharmaceutical industry, melanoma and T lymphomas are diseases for which medical advances remain limited. Thus, there was an urgent need of new therapeutics with an original mechanism of action. Since several years, our group develops quinoxalinic compounds. In this paper, the first preclinical results concerning one lead compound, **EAPB0203**, are presented. This compound exhibits *in vitro* cytotoxic activity on A375 and M4Be human melanoma cell lines superior to that of imiquimod and fotemustine. A liquid chromatography–mass spectrometry method was first validated to simultaneously quantify **EAPB0203** and its metabolite, **EAPB0202**, in rat plasma. Thereafter, the pharmacokinetic profiles of **EAPB0203** were studied in rat after intravenous and intraperitoneal administrations. After intraperitoneal administration the absolute bioavailability remains limited (22.7%). In xenografted mouse, after intraperitoneal administration of 5 and 20 mg/kg, **EAPB0203** is more potent than fotemustine. The survival time was increased up to 4 and 2 weeks compared to control mice and mice treated by fotemustine, respectively. The results of this study demonstrate the relationship between the dose of **EAPB0203** and its effects on tumor growth. Thus, promising efficacy, tolerance and pharmacokinetic data of **EAPB0203** encourage the development towards patient benefit.

1. Introduction

Melanoma is an increasingly common, potentially fatal form of skin cancer arising from the malignant transformation of melanocytes. Responsible of several thousand deaths each year, melanoma is the most frequent malignant tumor in the white human population worldwide (Greinert, 2009). Its frequency increases dramatically with age and chronic sun exposure. Only a few chemotherapeutic agents that are active in fighting cancer cells have been shown to be active in the treatment of melanomas, either one at a time or in combinations. Imidazoquinoline compounds have potent antiviral and antitumor properties in animals, and have been clinically approved for the topical treatment of genital and anal warts (Hengge and Cusini, 2003; Sauder, 2003). Imiquimod, the first member of the imidazoquinolone family is efficacious as a topical therapy for certain types of skin cancers: basal cell carcinoma, Bowen's disease, superficial squamous cell carcinoma, some superficial malignant melanomas and actinic keratosis (Steinmann et al., 2000; van Egmond et al., 2007). Extensive studies over the past years have indicated that imiquimod acts both: (i) indirectly, by activating the innate as well as the adaptive immune system via binding to cell surface receptors such as Toll-like receptors (TLR) 7 and 8, thereby inducing the activation of transcription factors like nuclear factor NF-κB and resulting in the secretion of pro-inflammatory cytokines predominantly interferon (IFN)-α, tumor necrosis factor (TNF)-α and interleukin (IL)-12 (Hemmi et al., 2002; Hengge and Cusini, 2003; Rudy, 2002; Sauder, 2003); and (ii) directly, by inducing direct *in vitro* and *in vivo* pro-apoptotic activities in a rather tumor selective manner, requiring the activation of the 'work horses' of apoptosis, the caspases' family of proteases (Schön et al., 2003, 2004). Imiquimod inhibits melanogenesis and proliferation of human melanocytes (Kang et al., 2009). Recently, its therapeutic spectrum was extended to cutaneous B-cell lymphomas (Spaner et al., 2005). Resiquimod, analog of imiquimod, has greater potency at inducing cytokine

* Corresponding author. Tel.: +33 4 67 54 80 75; fax: +33 4 67 54 80 75.
E-mail address: FBressolle@aol.com (F.M.M. Bressolle).
[1] Both these authors contributed equally to this work.

0928-0987/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2009.10.006

expression. Unfortunately, imiquimod and particularly resiquimod cannot be administered intravenously due to their toxicities. The interesting double mechanism of action of these compounds prompts us to synthesize analogs of imiquimod. Based on this knowledge, a new series of compounds has been synthesized, the imidazo[1,2-*a*]quinoxalines, in order to evaluate its potential anticancer properties, particularly in the treatment of melanoma and T lymphomas (Deleuze-Masquéfa et al., 2009a; Moarbess et al., 2008a). Among the synthesized compounds, two of them, **EAPB0203** and **EAPB0503**, have the highest *in vitro* cytotoxic activity on melanoma (Deleuze-Masquéfa et al., 2009a; Moarbess et al., 2008a). Moreover, **EAPB0203** at the dose of 20 mg/kg by intraperitoneal route was more potent than fotemustine in xenografted nude mice (Moarbess et al., 2008a). **EAPB0203** also had promising cytotoxic activity on T lymphomas (Moarbess et al., 2008b). Recently a liquid chromatography–mass spectrometry (LC–MS) method has been developed to quantify **EAPB0503** and its main metabolite in human and rat plasma (Khier et al., 2009). It was used to carry out a pharmacokinetic study in rat after administration of this compound intravenously.

The aim of the present paper was: (i) to develop a LC–MS method to quantify **EAPB0203** and its main metabolite, **EAPB0202**, in rat plasma; (ii) to determine pharmacokinetic parameters of these two compounds in rat after intravenous and intraperitoneal administrations; (iii) to estimate the sub-acute toxicity of **EAPB0203** in rat after repeated intravenous administrations, once a day for 5 consecutive days; and (iv) to evaluate the *in vivo* activity of **EAPB0203** in xenografted nude mice at a lower dose (5 mg/kg) in order to establish dose–response curve.

2. Materials and methods

2.1. Chemistry

EAPB0203 (*N*-methyl-1-(2-phenethyl)imidazo[1,2-*a*]quinoxalin-4-amine; molecular weight, 302; log*P*, 4.91; pK_a, 5.61), **EAPB0202** (1-(2-phenethyl)imidazo[1,2-*a*]quinoxalin-4-amine; molecular weight, 288; log*P*, 4.97; pK_a, 5.13) and the internal standard (IS, **EAPB0603**, 1-(3-hydroxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine; molecular weight, 290) used for method validation were synthesized according to a previously reported method from our group (Moarbess et al., 2008a; Deleuze-Masquéfa et al., 2009a, patent no. FR 2921927; patent N° WO 2009043934). Briefly, they were synthesized in good yields via a bimolecular condensation of 2-imidazole carboxylic acid, followed by a coupling with ortho-fluoroaniline and subsequent substitution on the imidazole ring by Suzuki Cross-Coupling reaction using microwave assistance. The purity of these standards was evaluated by elemental analysis, liquid chromatography–mass spectrometry (LC–MS) and NMR. They were stored at 20 °C protected from light.

2.2. Chemicals and reagents

All reagents were of analytical grade. Purified water (Milli-Q purification system, Millipore, Bedford, MA, USA), trifluoroacetic acid (TFA, Sigma, St. Louis, MO, USA), acetonitrile and dichloromethane (Carlo Erba, Val de Reuil, France), ammonium formate (Fluka, Vandoeuvre, France), formic acid (Prolabo, Paris, France) and Oasis HLB cartridges (30 mg sorbent, Waters, Saint Quentin, France) were used. Drug-free rat plasma provided from Charles River (L'Arbresle Cedex, France). Ammonium formate buffer was prepared by mixing ammonium formate and water to a concentration of 2 mM; the pH was adjusted to pH 3 with formic acid. Stock solutions of **EAPB0203**, **EAPB0202** and IS (40 mg/L) were prepared in a mixture of acetonitrile–water–formic acid (49.5:49.5:1, v/v/v). These solutions were further diluted extemporaneously with the same mixture as appropriate to prepare working solutions (**EAPB0203** and **EAPB0202**: 0.125–25 mg/L; IS: 1.6 and 8 mg/L). These stock solutions were stored at +4 °C for 1 month.

2.3. LC–MS analysis

LC separation and MS detection were performed using a Hewlett Packard Agilent 1100 quadrupole mass spectrometer working with an electrospray ionization source (ESI) (Agilent Technologies, Les Ulis, France). The LC–MS conditions were close to those previously published for two other compounds of the same chemical series (Khier et al., 2009). The chromatographic separation was achieved on a C8 Zorbax eclipse XDB analytical column (150 mm × 4.6 mm, 5 μm, Agilent Technologies). The optimum mobile phase consisted of, acetonitrile as solvent A and 2 mM ammonium formate as buffer B. The gradient elution was employed according to the following linear programme: time zero, 30% solvent A; 9 min, 100% solvent A; 10 min, 100% solvent A; 11 min, 30% solvent A; and 14 min, 30% solvent A. The flow rate was 0.8 mL/min and the injection volume was 10 μL; the column was maintained at 20 °C and the autosampler temperature was 4 °C. The data acquisition was under the control of HPChem Software (version 08.04, Agilent Technologies). The mass spectrometer was operated in positive ion mode. Heated N_2 gas (350 °C and 10 L/min) was used to evaporate solvent from the electrospray chamber and compressed N_2 gas (35 psi) was used for nebulisation. Voltages were set at +3.0 kV for the capillary and +0.5 kV for the skimmer lens. The sampling cone voltages were set at 100 V for **EAPB0203** and IS, and at 80 V for **EAPB0202**. For quantitative measurement of **EAPB0203** and **EAPB0202**, selective ion monitoring (SIM) was employed.

2.4. Quantitation of *EAPB0203* and *EAPB0202* in rat plasma

2.4.1. Sample preparation

A sample pre-treatment procedure close to that previously described to analyse other compounds of the same chemical series, in rat and human plasma (Khier et al., 2009), was used with success to quantify these two new compounds. Briefly, rat plasma samples were pretreated to remove proteins. For this reason, 0.5 mL of water containing 10 mL/L TFA was added to 0.5 mL of plasma samples (calibrators, QC or unknown samples) containing the internal standard solution (20 μL, 1.6 mg/L for low calibration curves, 8 mg/L for high calibration curves). After centrifugation (4 °C) for 10 min at 4000 × *g*, the supernatant was transferred onto an Oasis HLB cartridge preconditioned with 1 mL of methanol and 1 mL of water. The cartridge was then washed with 1 mL of water and the analytes were eluted with 2 mL of dichloromethane. The eluates were evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted with 100 μL of a mixture containing acetonitrile–water–formic acid (49.5:49.5:1, v/v/v).

2.4.2. Calibration curves and QC samples

To 0.5 mL of blank rat plasma, 20 μL of working solutions of the two analytes were added in order to obtain calibration curves in the 5–300 μg/L (low calibration curves), and 100–1000 μg/L (high calibration curves) ranges.

Six levels of QC samples (15, 75, 200, 300, 500 and 750 μg/L) were prepared by adding to 0.5 mL of drug-free matrix 20 μL of the QC working solutions.