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Physicochemical characterization and *in vitro* permeation of an indirubin derivative

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ABSTRACT

The active component of the traditional Chinese medicine, indirubin, exerts anticancer effect on different cancer cell lines. E804, a potent derivative of indirubin inhibits the activation of Stat3 and Stat5 in chronic myelocytic leukaemia (CML) cells. However, physicochemical properties and permeation rate of the compound relevant to the drug formulation have never been reported. Therefore, the ionization constant (pK_a), lipophilicity (log D/P), aqueous and organic solubility of E804 and its permeation across Caco-2 cells were investigated. Both high throughput and traditional determinations were used in this study. The Caco-2 cell permeation assay was carried out in Poloxamer 188/HBSS++ solution in order to maintain the solubility of drug. The potential P-gp (P-glycoprotein) interaction for E804 was determined through Calcein-AM uptake assay. The results showed that E804 did not have a detectable pK_a in the range of pH 2–11. Log D (distribution coefficient) and Log P (partition coefficient) were determined to be 3.54 ± 0.03. Aqueous solubility test revealed that E804 is practically insoluble in water. Among organic solvents E804 showed the highest solubility in DMSO. The $P_{app\ A\to B}$ and $P_{app\ B\to A}$ across Caco-2 cell monolayer were $2.0 \pm 0.25 \times 10^{-6}$ cm/s and $1.14 \pm 0.12 \times 10^{-6}$ cm/s respectively, and the calculated efflux ratio (ER) was 0.57. Calcein-AM uptake assay showed that E804 was not a strong substrate for P-gp. The results indicate that solubility is the major rate limiting step for the drug permeation. The high membrane permeability makes E804 promising for the oral delivery. Therefore, further investigation on solubility of E804 in lipid vehicles is needed to determine an appropriate formulation for the drug.

1. Introduction

Indirubin has been identified as the active constituent of the traditional Chinese medicine *Danggui Longhui Wan* which has been used for treatment of chronic diseases along the centuries, especially chronic myelocytic leukaemia (CML) (Tang and Eisenbrand, 1992). Indirubin and its derivatives have been identified as potent inhibitors of cyclin-dependent kinases (CDKs) (Damiens et al., 2001; Marko et al., 2001; Miller et al., 2011; Perabo et al., 2006; Yano et al., 2010) and have been shown to inhibit the cell growth in different cancer cell lines. In a 2 years clinical study on CML patients, orally administered indirubin showed hematologic complete or partial remission in about 60% of the patients (Cooperative Group of Clinical Therapy of Indirubin, 1980). In addition to CKDs, molecular targets of indirubins have been further unveiled, which include glycogen synthase kinase-3 (Leclerc et al., 2001; Meijer et al., 2003; Vougogiannopoulou et al., 2008), aryl hydrocarbon receptor (Adachi et al., 2001), c-Jun NH2-terminal kinase (JNK) (Xie et al., 2004), glycogen phosphorylase b (Kosmopoulou et al., 2004). Different analogues of indirubin have been synthetized for improving its potency, selectivity and enhancing solubility (Meijer et al., 2007; Sridhar et al., 2006; Xiao et al., 2002). An indirubin derivative, indirubin-3′-oxime 2,3-dihydroxypropyl ether (E804; Fig. 1) has been shown to potently inactivate the Signal Transducer and Activator of Transcription 3 and 5 (Stat3&Stat5) proteins in human cancer cells due to inhibition of Src tyrosine kinase (Nam et al., 2005, 2012). Very recently, we also revealed the novel inhibitory function of E804 in TGFß/BMP signaling through ubiquitination of total regulator-Smad (Cheng et al., 2012).

These *in vitro* inhibitory findings however, need to be further examined *in vivo* in order to have a better understanding of E804 function in the body. But a major drawback of all indirubins is their extremely low solubility in aqueous solution, making the *in vivo* studies at adequate doses difficult. It is known that improper

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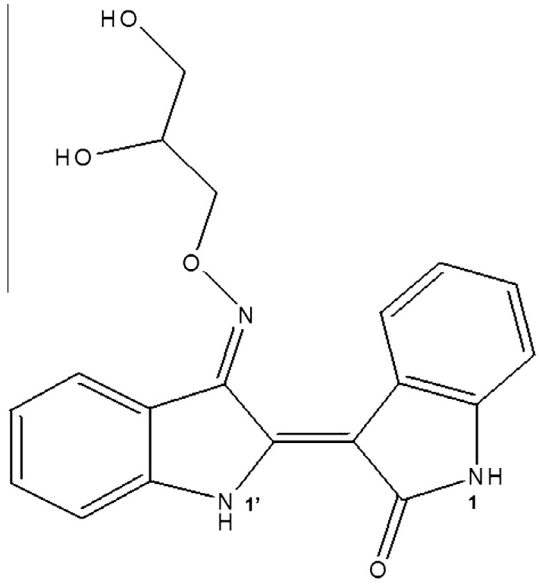

Fig. 1. Chemical structure of indirubin-3′-oxime 2,3-dihydroxypropyl ether (E804).

pharmacokinetic properties of an active pharmaceutical compound could discard the compound through drug development process (Kennedy, 1997). Determination of physicochemical properties (pK_a, solubility, and log P) facilitates the prediction of pharmacokinetic parameters (absorption, distribution, metabolism and excretion) of a compound (Henchoz et al., 2009). Despite the number of reports about potential effect of E804 on cancer cell lines and its promising effect in CML treatment, physicochemical properties of this compound such as ionization constant, lipophilicity, and solubility profile have not been documented. The permeation of the compound across the intestinal cell monolayer and its potential interaction with important efflux transporter P-glycoprotein (P-gp) has also not been reported. Determination of these parameters is absolutely necessary to ensure a better understanding of ADME behavior of the drug and helps to choose an appropriate dosage form in the process of formulation.

In definition, the ionization constant (pK_a) of a drug indicates the ionized or unionized forms of a molecule over a pH range of a solution (Box and Comer, 2008).The pK_a value represents the pH that the drug is 50% ionized and 50% unionized. Lipophilicity is the ratio of the drug's equilibrium concentration in 1-octanol and water; and describes its partitioning between a lipidic and an aqueous environment (Henchoz et al., 2009). Distribution coefficient (D, usually shown in its logarithm as log D) represents the ratio of ionized and unionized species of the drug at a specific pH; however partition coefficient (P, usually log P) shows the partitioning of the drug at a pH that the drug is in its natural form (unionized species). This parameter could be correlated to solubility, distribution (absorption, membrane permeation, plasma protein binding), metabolism and toxicity (Box and Comer, 2008; Henchoz et al., 2009; Kerns and Di, 2004).

Appropriate solubility of a compound is crucial for the drug absorption after oral administration. In fact the drug solubility and efficient permeability are considered as prominent factors for drug absorption. Insufficient aqueous solubility or membrane permeability results in low bioavailability. The term "intrinsic solubility" refers to the solubility of a compound in its natural form (free acid or base) over the pH that it is unionized. The "thermodynamic solubility" is the solubility of both ionized and unionized species of the molecule in an equilibrium solution and at a specific pH (Box and Comer, 2008; Guo and Shen, 2004).

A further hurdle for successful drug development may be involvement of membrane efflux transporters, especially P-glycoprotein (P-gp) efflux transporters, which are located on the apical side of intestinal cells, and limit the bioavailability of their substrates (del Amo et al., 2009).

In this study, we determined the pK_a, log D/log P, and solubility of E804 in aqueous and organic solvents. Both high throughput and traditional determinations were used. For pK_a determination, traditional potentiometric, capillary electrophoresis (CE), and photometric titration were applied. For lipophilicity measurement, microscale shake flask and Carrier Mediated Distribution System (CAMDIS) were performed. Solubility profile was determined by classical saturation shake flask method. Moreover, the permeability of E804 was investigated across the Caco-2 cells monolayer and its active apical efflux was determined by calculation of the efflux ratio. The P-gp interaction of E804 was also studied on CEM/ADR5000 P-pg overexpressing cells.

2. Materials and methods

2.1. Materials

Tris-(hydroxymethyl)-aminomethan (TRIS) was obtained from Carl Roth (Germany). Dimethylsulfoxide (DMSO) was purchased from J.T. Baker (Germany). Bovine serum albumin (BSA), formic acid and Hank's Balanced Salt Solution supplemented with $MgSo_4$ and $CaCl_2$ ($HBSS^{++}$) were purchased from Sigma–Aldrich (Munich, Germany). 1-Octanol was obtained from Merck (Darmstadt, Germany). Kolliphor® P188 (Poloxamer 188) was kindly received as a gift from BASF (Ludwigshafen, Germany). Verapamil was purchased from Abbott Laboratories (Ludwigshafen, Germany). Ethanol, methanol, acetonitrile and water HPLC grade were purchased from VWR (Germany). Rat tail collagen was purchased from Roche (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and supplements were purchased from Biochrom (Berlin, Germany). Argon gas was used to protect the mixed-solvent solution from absorbing the atmospheric CO_2. E804 was received from the department of Food/Chemistry and Environmental Toxicology, University of Kaiserslautern at analytical grade. Caco-2 cells and CEM/ADR5000 leukemia cells were obtained from German Cancer Research Center (DKFZ, Heidelberg, Germany).

2.2. Methods

2.2.1. pK_a Determination

2.2.1.1. Potentiometric titration. Potentiometric titration is a widely used method for pK_a determination of a compound. The titration method used in this study was based on a method described before (Avdeef, 2001; Avdeef et al., 1993). First, a blank titration is carried out for the electrode calibration. Then, the solution of the protogenic substance is titrated with exact volumes of a standardized acid or base while stirring, and the pH is constantly measured by a precise pH electrode, between the pH 2–12. The result produces two potentiometric titration curves which show the determined pH versus the added volume of titrant. The first curve shows the blank and the second one the sample curve. The titration curve is then transformed to the Bjerrum plot by subtraction of blank titration curve from sample titration curve at fixed pH values. It helps to reveal all the pK_a values of the molecule that might be overlapping.

In the mixed-solvent solutions, the p_sK_a (the apparent ionization constant) is determined in each methanol–water mixture from the difference between the titration curve of the sample and the blank. The aqueous pK_a is then obtained by extrapolation of the p_s-