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Enantioresolution, stereochemical characterization and biological activity of a chiral large-conductance calcium-activated potassium channel opener

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ABSTRACT

A number of large-conductance calcium-activated potassium (BK) channel openers based on the 2-aryl-1,4-benzothiazine scaffold were previously synthesized, and 2-(5-bromo-2-methoxyphenyl)-6-trifluoromethyl-2*H*-1,4-benzothiazin-3(4*H*)-one (**1**) was identified as the most active compound. Since a stereoselective activation of BK channels was demonstrated for arylindolone derivatives, the effect of the absolute configuration at the C-2 position on the vasorelaxing potency of 2-aryl-1,4-benzothiazines is investigated in this article. Compound **1** was initially evaluated as a racemate: subsequently, the "racemic approach" was used to isolate its enantiomers. The excellent enantioresolution obtained using the Sepapak-4 column (CSP **4**, cellulose *tris*(4-chloro-3-metylphenylcarbamate); $R_S = 8.36$; $\alpha = 2.03$) allowed to collect highly pure enantiomeric fractions, with enantiomeric excess (e.e.) values higher than 97% and 98% for the first- and second-eluted enantiomer, respectively. Electronic circular dichroism (ECD) studies on the two isolated enantiomers, combined with time-dependent density functional theory (TD-DFT) calculations allowed to characterize the configuration of the enantiomers and determine a (*R*), (*S*) elution order. Results from biological assays indicated that the racemate and the isolated enantiomers are endowed with comparable vasorelaxing potency.

1. Introduction

Recent efforts toward the identification of potent and selective activators of potassium channels led to the development of a structurally novel class of large-conductance calcium-activated potassium (BK) channel openers based on the 2-aryl-1,4-benzothiazine scaffold. As a result, the racemic 2-(5-bromo-2-methoxyphenyl)-6-trifluoromethyl-2*H*-1,4-benzothiazin-3(4*H*)-one (**1**, Fig. 1) was found to be the most active compound [1]. BK channels are present in virtually every cell type where they play a pivotal and specific role in a wide range of physiological processes, spanning from mediating fast after-hyperpolarizations following action potentials, to inhibition of neurotransmitter release, and relaxation of smooth muscle cells in bladder, arterioles and airways [2].

The functional versatility of BK channel proteins is conferred by a variety of means, including extensive alternative splicing [3] of the pore-forming α-subunit encoded by the single gene *slo1* [4] and co-assembly with auxiliary β-subunits [5]. Thus, a considerable diversity is generated within the BK family, which may be tissue and organ-specific [2,6]. Due to these properties and their central role in regulating cell activity, BK channels are particularly appealing as a therapeutic drug target [7,8]. In particular, BK channel openers, decreasing cell excitability and causing smooth muscle relaxation, could offer a novel therapeutic approach to several diseases associated with both the central nervous system and smooth muscles, such as stroke, epilepsy, bladder overactivity, asthma, and hypertension [9,10]. Several agents have been reported to activate BK channels [8,11]. Among the prototypical BK openers, the class of arylindolone derivatives [12,13], represented by the eutomer BMS-204352 (**2**, Fig. 1) [14], were studied in detail providing evidence for chiral discrimination by the BK protein.

This indication prompted the present study on the influence of the absolute configuration at the C-2 stereogenic center on

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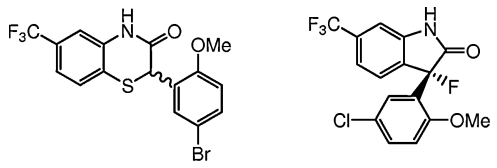

Fig. 1. Chemical structure of BK openers.

the biological activity of the 2-aryl-1,4-benzothiazine BK opener class, using compound **1** as the reference compound. The column screening procedure, the preparative enantioresolution of **1**, the stereochemical characterization of the absolute configuration at the C-2 position for the enantiomeric fractions, and the evaluation of the biological activity for the racemic mixture and the pure enantiomers are discussed in the following sections.

The "racemic approach" [15] was employed to obtain the enantiomers of **1**: the synthesis of the racemate, followed by the application of a chromatographic preparative enantioresolution method, is now largely recognized as the most convenient way for a rapid access to small amounts of highly pure enantiomers [15]. Among the benefits provided by the racemic approach, the reduced complexity of non-enantioselective synthesis protocols, and the possibility to simultaneously obtain all the stereoisomers are worth highlighting. The latter advantage is especially convenient in the case of preliminary comparative biological assays. Moreover, chromatographic preparative enantioresolutions can be profitably performed on analytical columns, when only limited amounts of each enantiomer are required. In most cases, the direct chromatographic preparative enantioresolution of a given racemate is carried out after a preliminary screening of the available chiral stationary phases (CSPs) which are suitable for the compound of interest [15]. Unfortunately, operational guidelines for the selection of the most appropriate CSP for a given application are rarely effective in practice, with some notable exceptions [16].

2. Experimental

2.1. Materials

Racemic **1** was synthesized and characterized according to a previously reported procedure [1]. Analytical grade 2-propanol (IPA), *n*-hexane, chloroform, ethyl acetate (EtOAc), and 1,3,5-tri-*tert*-butylbenzene (used as the unretained marker for the calculation of the chromatographic performance) were purchased from Sigma-Aldrich (Milano, Italy). For biological studies, dimethyl sulfoxide (DMSO), tyrode salt solution, acetylcholine chloride, and potassium chloride (KCl) were purchased from Sigma-Aldrich. HPLC-grade water was obtained from a New Human Power I Scholar water purification system (Human Corporation, Seoul, Korea). All the employed mobile phases were degassed by sonication for 10 min before use. Samples for HPLC analysis were dissolved in the selected mobile phase and injected at the approximate concentration of 0.5–1.0 mg mL^{-1}. Samples for electronic circular dichroism (ECD) and UV spectroscopic analysis were prepared at a 100 μM concentration in IPA.

2.2. Instrumentations

For the quantitative enantioresolution analyses, the following six columns (Fig. 2) were preliminary screened with the same eluent system: Lux Amylose-2 (CSP **1**; amylose *tris*(5-chloro-2-methylphenylcarbamate)), Chiralpak AD-H (CSP **2**; amylose *tris*(3,5-dimethylphenylcarbamate)), Lux Cellulose-2 (CSP **3**; cellulose *tris*(3-chloro-4-methylphenylcarbamate)), Sepapak-4 (CSP **4**; cellulose *tris*(4-chloro-3-methylphenylcarbamate)), Chiralcel OD-H (CSP **5**; cellulose *tris*(3,5-dimethylphenylcarbamate)), and Chiralpak IB (CSP **6**; cellulose *tris*(3,5-dimethylphenylcarbamate)). CSP **1** and CSP **3** were purchased from Phenomenex (Torrance, CA, USA); CSP **2**, CSP **5** and CSP **6** were purchased from Chiral Technologies (West Chester, PA, USA); CSP **4** was kindly provided by Sepaserve GmbH (Münster, Germany).

In CSPs **1**–**5**, the chiral selector is adsorbed onto a 5 μm silica gel. In CSP **6**, the chiral selector is immobilized onto a 5 μm silica gel. All the columns were characterized by the same 250 mm × 4.6 mm I.D. dimensions.

Columns were conditioned with the selected mobile phase at a 1.0 mL min^{-1} flow rate for at least 40 min before use. All the analyses were carried out at a 1.0 mL min^{-1} flow rate and with a 25 °C column temperature.

The HPLC analyses were performed on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a 100 μL stainless steel loop. Column temperature was controlled through a Grace (Sedriano, Italy) heater/chiller (Model 7956R) thermostat. The same HPLC system was used for the preparative enantioresolution of **1** with CSP **4**.

Electronic circular dichroism (ECD) and UV spectroscopic analysis was carried out at 25 °C on a Jasco (Tokyo, Japan) J-810 spectropolarimeter equipped with a PTC-423S Peltier-type temperature control system, using a 2 nm spectral bandwidth, a 50 nm min^{-1} scanning speed and a 2 s data integration time; spectra were averaged over 3 accumulation cycles. Quartz cells (Hellma, Milan, Italy) with a 1 mm path length were used to measure spectra in the 350–200 nm spectral range.

2.3. Theoretical chiroptical spectroscopy

The theoretical chiroptical properties of (*R*)-**1** were determined according to the standard protocol for stereochemical characterization by time-dependent density functional theory (TD-DFT) calculations [17,18]. A preliminary conformational search was performed by molecular mechanics (MM) calculations using the MMFF94s force field [19] and the Spartan'02 [20] software. DFT geometry optimization and frequency calculations were carried out using the Gaussian 09 software [21] (for the full citation, see the Supporting information), the B97D functional [22] with the resolution of identity (RI) approximation [23,24], the TZVP Ahlrichs-type triple-ζ valence plus polarization basis set [25] and the IEFPCM solvation model [26,27] for 2-propanol. Conformational clustering was performed with a RMSD threshold value of 0.01 Å for heavy atoms.

TD-DFT calculations were also carried out using the Gaussian 09 software. The PBE0 functional [28,29] was used in combination with the TZVP basis set and the IEFPCM solvation model for 2-propanol; calculations were performed on all optimized conformers. Theoretical values of oscillator strength (f_j), rotational strength in dipole velocity formalism (R_j) and excitation energy (expressed as wavelength, λ_j) were calculated for the 50 lowest-energy electronic transitions of each optimized conformer. The theoretical spectra of optimized conformers were then derived by approximation of f_j and R_j values to Gaussian bands with a $\Delta\sigma$ value of 0.25 eV [30]. The theoretical UV and ECD spectra of (*R*)-**1** were finally derived as the weighted average of the contribution of all conformers according to their Boltzmann equilibrium populations at 298.15 K and 1 atm, based on free energy values (χ_G), and compared to the experimental spectra.