Brief Articles

Enantioselectivity in Cardioprotection induced by (*S*)-(−)-2,2-Dimethyl-*N*-(4′-acetamido-benzyl)-4-spiromorpholone-chromane

Simona Rapposelli,*[,†] Vincenzo Calderone,[‡] Roberto Cirilli,[§] Maria Digiacomo,[†] Cristina Faggi,[||] Francesco La Torre,[§] Mariaelisa Manganaro,[†] Alma Martelli,[‡] and Lara Testai[‡]

Dipartimento di Scienze Farmaceutiche, Università di Pisa, Via Bonanno 6, I-56126 Pisa, Italy, Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy, Istituto Superiore di Sanità, Dipartimento del Farmaco, Viale Regina Elena 299, I-00161 Rome, Italy, CRIST, Centro Interdipartimentale di Cristallografia Strutturale, Università degli Studi di Firenze Polo Scientifico di Sesto Fiorentino, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Fi), Italy

Received November 19, 2008

This work aimed to determine the enantioselectivity in cardioprotection induced by the racemic mixture of the "archetype" **1a** of a new class of spirocyclic-benzopyran derivatives. The racemate was resolved by HPLC and the absolute configuration was accomplished by a combined strategy based on single-crystal X-ray diffraction and circular dichroism methods. The (*S*)-(−)-**1a** enantiomer, evaluated for its anti-ischemic activity, showed significant cardioprotective effects, whereas the (*R*)-(+)-**1a** enantiomer was completely lacking in anti-ischemic effects.

Introduction

In recent years, the incidence of myocardial ischemia has greatly increased. This pathological event may be limited by the activation of a protective mechanism that involves many mediators[1−3] such as adenosine, opioids, bradikynin, nitric oxide, calcium ions, and free radical species. This sort of "self-defence" mechanism, known as "ischemic preconditioning" (IPC[a]), gives myocardiocytes an increased resistance against ischemia-induced cell damage, resulting in a marked reduction of the infarct size.

Between the several triggers able to activate IPC, mitochondrial ATP-sensitive potassium (mito-K_{ATP}) channel is considered as a major effector in anti-ischemic cardiac protection.[4,5] As a consequence, the activation of this kind of channel seems to be a challenging target for increasing the resistance of the heart to tolerate a more prolonged ischemic insult and thus to confer a "pharmacological pre-conditioning". Unfortunately, almost all the potassium channel openers (KCOs) exhibit relevant undesired effects mainly due to their unsatisfactory selectivity toward the mitochondrial subtype of the K_{ATP} channel.

Recent papers of ours[6−8] reported the synthesis and pharmacological evaluation of original spiromorpholine- and spiromorpholone-benzopyran derivatives, designed in order to obtain selective activators of mito-K_{ATP} channels. Some compounds of this series, tested as racemic mixtures, showed appreciable cardioprotective effects on rat isolated and perfused hearts submitted to ischemia/reperfusion cycles; this activity was antagonized by the selective mito-K_{ATP} channel blocker 5-hydroxydecanoic acid (5-HD), thus indicating a clear implication of such a pharmacological target. Furthermore, the anti-ischemic effects were not associated with significant hypotensive and vasorelaxing properties, which represent one of the main limiting factors for the clinical use of nonselective K_{ATP}-openers against myocardial ischemia.

Bearing in mind these encouraging results, we carried out further investigations into this class of K_{ATP} channel openers. In particular, the objective of the present work was to verify if the anti-ischemic activity of this new class of 4-spiro-chromane derivatives might reside in only one or both enantiomers of the active compounds previously studied as racemic mixture. To this purpose, we selected the 2,2-dimethyl-*N*-(4′-acetamido-benzyl)-4-spiromorpholone-chromane **1a**, whose racemate showed a high degree of cardioprotection in a Langendorff perfused rat heart subjected to an ischemia/reperfusion cycle, associated with very modest direct vasorelaxing activity and with almost completely negligible hypotensive effects in vivo.[6,8]

This favorable profile was similar to that shown by diazoxide, a well-known cardioprotective K_{ATP}-opener exhibiting preferential activity toward mitochondrial channels (at the dose commonly used in this kind of experimental protocols) and quite different from that of cromakalim, a very potent K_{ATP}-channel activator of both sarcolemmal and mitochondrial channels, thus possessing anti-ischemic effects associated with strong vasorelaxing and hypotensive properties.[6,8]

On this basis, we decided to resolve the racemic mixture of **1a**, together with that of its methanesulphonamide analogue brominated in 6 position (**2b**) (Figure 1), selected for comparative purposes in order to strengthen the data deriving from their racemic resolution and to assign the absolute configurations to the single enantiomers. The direct enantioseparation of **1a** and **2b** was achieved by high performance liquid chromatography (HPLC) on a chiral stationary phase (CSP). Single enantiomers, isolated on a semipreparative scale, were submitted to comparative pharmacological and structural investigations. The absolute

* To whom correspondence should be addressed. Phone: +39-050-2219582. Fax: +39-050-2219605. E-mail: rappsi@farm.unipi.it.
† Dipartimento di Scienze Farmaceutiche, Università di Pisa.
‡ Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa.
§ Istituto Superiore di Sanità, Dipartimento del Farmaco.
|| CRIST, Centro Interdipartimentale di Cristallografia Strutturale, Università degli Studi di Firenze Polo Scientifico di Sesto Fiorentino.
[a] Abbreviations: IPC, ischemic preconditioning; RPP, rate pressure product; CD, circular dichroism; CSP, chiral stationary phase.

10.1021/jm801459f CCC: $40.75
Published on Web 02/12/2009

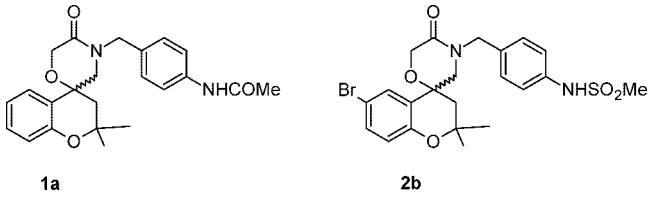

Figure 1. Structures of spirocyclic derivatives **1a** and **2b** endowed of anti-ischemic activity.

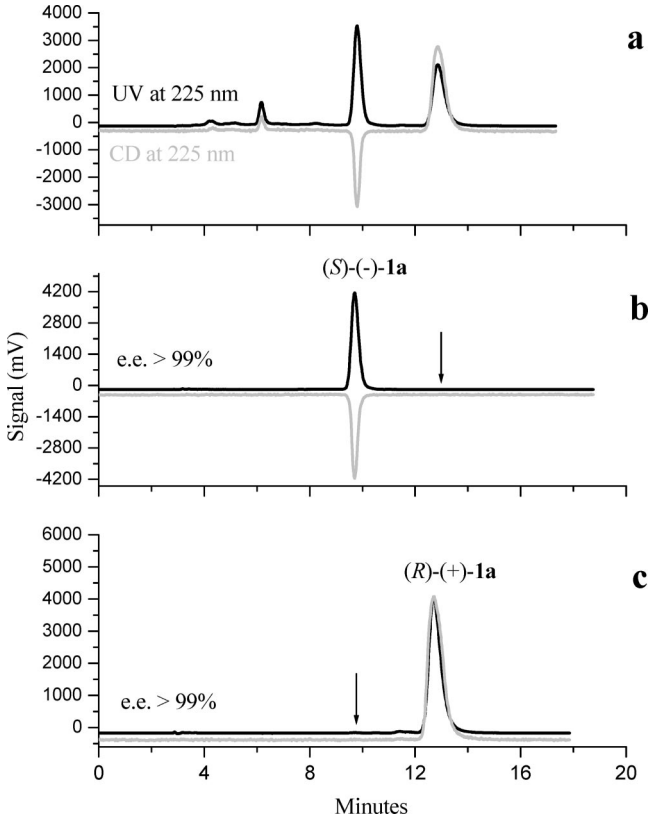

Figure 2. (a) Analytical chromatograms of **1a**; (b,c) chromatographic analysis of enantiomers isolated at semipreparative scale. Column, Chiralpak IA (250 mm × 4.6 mm I.D); eluent, *n*-hexane-ethanol 75/25 (v/v); flow-rate, 1.0 mL min^{-1}; detector, UV (black) and CD (gray) at 225 nm; temperature, 25 °C.

configuration assignment was accomplished by a combined strategy based on single-crystal X-ray diffraction and circular dichroism (CD) methods.

Chemistry

The HPLC enantioseparation of compounds **1a** and **2b** was carried out on the amylose-based Chiralpak IA CSP using normal-phase conditions (for details see Supporting Information). Typical chromatograms of **1a** with simultaneous UV and CD detection are shown in Figure 2.

The stereochemical correspondences between the couples of the isolated enantiomers of **1a** and **2b** were established by CD analysis (Figure 3). The structures and absolute configurations of (+)-**2b** and (−)-**2b**, and therefore of (+)-**1a** and (−)-**1a**, were readily secured by X-ray crystallography (Figure 4). As illustrated in Figure 3, dextrorotatory and levorotatory enantiomers were shown to have (*R*) and (*S*) configuration, respectively.

Results

The potential cardioprotective effects of the two enantiomers of **1a** (preadministered ip at a dose of 40 mg/kg) were evaluated

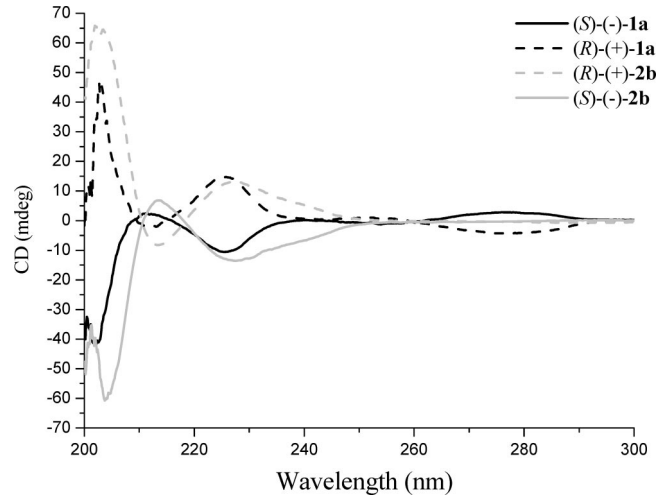

Figure 3. CD spectra of enantiomers of **1a** and **2b** in ethanol.

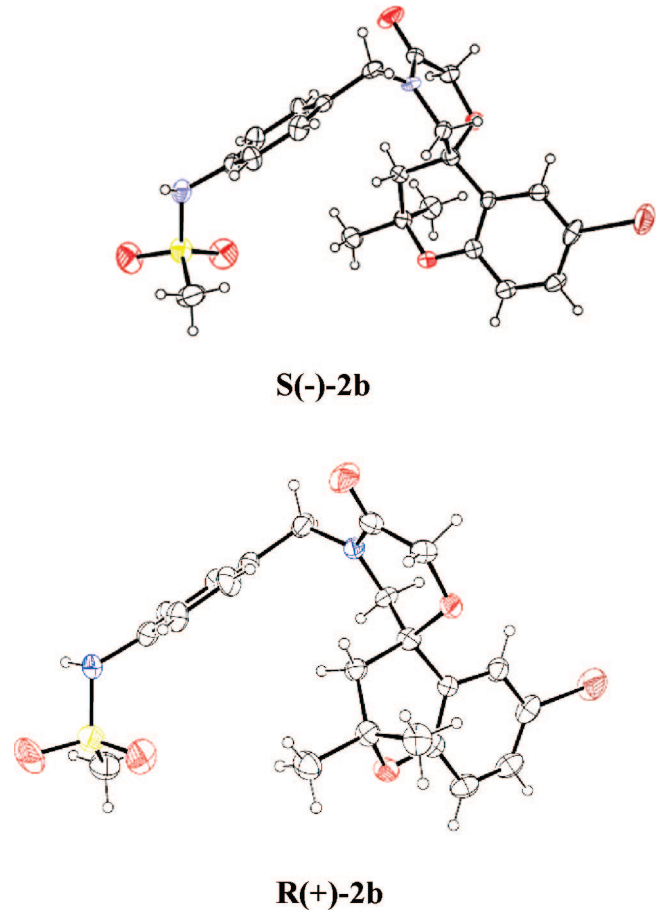

Figure 4. ORTEP drawing of (*S*)-(−)-**2b** and (*R*)-(+)-**2b**.

on Langendorff perfused rat hearts subjected to ischemia−reperfusion cycles. Diazoxide and cromakalim, at doses of 40 and 1 mg/kg, respectively, were also tested as reference drugs. The ischemic damage was determined through the recording of the functional postischemic parameter of rate−pressure product (RPP%) recorded during reperfusion, expressed as a percentage of the RPP value recorded at the last minute of the preischemic period. At the end of reperfusion, the treatment of the heart with triphenyltetrazolium chloride (TTC) made it possible to carry out a morphological comparison of the necrotic and healthy areas of the left ventricular tissue, colored white (or pale pink) and red, respectively, and then to calculate the

Table 1. Functional (Rate Pressure Product RPP-120′) and Morphological (% Ischemic Area vs Total Area) Parameters of the Two Isolated Enantiomeric Forms of **1a** (Data are Expressed as Mean ± Standard Error for $n = 4-6$ different experiments)

compd	RPP 120′ (%)[a]	A_i/A_{tot} (%)[a]
vehicle	23 ± 4	38 ± 6
cromakalim	86 ± 12	23 ± 3
diazoxide	45 ± 7	25 ± 4
(±)-**1a**	59 ± 13	22 ± 5
(*R*)-(+)-**1a**	27 ± 5	35 ± 2
(*S*)-(-)-**1a**	64 ± 20	16 ± 6

[a] Data are expressed as mean ± standard error for $n = 4-6$ different experiments.

morphological parameter ischemia-injured area as a % of the total area (A_i/A_{tot}%). As shown in Table 1, the ischemia/reperfusion cycle induced a dramatic reduction of the postischemic functional parameter (RPP-120′ = 23 ± 4%) and a wide extension of the myocardial injured areas (A_i/A_{tot} = 38 ± 6%) in hearts isolated from vehicle-treated rats. As clearly observed in previous works, the reference drugs diazoxide and cromakalim gave myocardium an increased resistance against ischemia−reperfusion, resulting in an improved recovery of postischemic cardiac functionality (RPP-120′ = 45 ± 7% and 86 ± 12%, respectively) and a marked reduction of the morphological evidence of tissue damage (A_i/A_{tot} = 25 ± 4% and 23 ± 2%, respectively). When administered as a racemic mixture, compound **1a** demonstrated its cardioprotective properties as described in previous studies, with an overall amelioration of both the postischemic indicators (RPP 120′ = 59 ± 13% and A_i/A_{tot} = 22 ± 5%).

As concerns the two enantiomers of **1a**, the (*S*)-(−)-**1a** enantiomer showed significant cardioprotective effect, with a clear improvement of both the functional and the morphological markers (RPP-120′ = 64 ± 20%; A_i/A_{tot} = 16 ± 6%). Such an anti-ischemic activity was almost comparable to that exhibited by the racemic mixture. On the contrary, the (*R*)-(+)-**1a** enantiomer was completely devoid of anti-ischemic effects because the postischemic functional and morphological parameters recorded in hearts isolated from animals pretreated with (*R*)-(+)-**1a** (RPP-120′ = 27 ± 5%; A_i/A_{tot} = 35 ± 2%) were almost superimposable on those observed in the vehicle-treated group.

Conclusions

This work demonstrated that the interaction of the new class of anti-ischemic spirocyclic-benzopyran derivatives with their intracellular target is enantioselective and that the cardioprotective properties of the selected compound **1a** resides in the levorotatory enantiomer while the dextrorotatory one is devoid of any activity. In addition, this result appears to be of particular interest because the peculiarity of such mito-K_{ATP} activators is the limited degree of conformational freedom that could let us to hypothesize a steric model for the interaction with the active site of the mito-KATP channel and therefore it could contribute to the rational design and development of a new innovative class of anti-ischemic drugs with improved potency and selectivity for the cardiac target. This enantioselectivity in cardioprotection showed by this spirocyclic-benzopyran derivative is in accordance with the results obtained for the benzopyran cyanoguanidine BMS180447,[9] another well-characterized activator of mito-K_{ATP} channels.

Experimental Section

Compounds **1a** and **2b** were synthesized and pharmacologically tested as previously reported.[6] HPLC enantioseparations were performed by using stainless-steel Chiralpak IA (250 mm × 4.6 mm ID and 250 mm × 10 mm ID) (Daicel, Chemical Industries, Tokyo, Japan) columns. HPLC-grade solvents were supplied by Carlo Erba (Milan, Italy). HPLC apparatus consisted in a Perkin-Elmer (Norwalk, CT) 200 lc pump equipped with a Rheodyne (Cotati, CA) injector, a HPLC Dionex (CA) model TCC-100 oven and a Jasco (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan) model 2095 Plus UV/CD detector.

The mobile phases were filtered and degassed by sonication immediately before using. In analytical enantioseparations, standard solutions were prepared by dissolving about 2 mg of sample into 10 mL of mobile phase. The injection volume was 10−20 μL. In semipreparative enantioseparation, a 1 mL sample loop was used. After semipreparative separation, the collected fractions were analyzed by chiral analytical columns to determine their enantiomeric excess (ee).

The column hold-up time (t_0 = 3.0 min for 250 mm × 4.6 mm ID column) was determined from the elution of an unretained marker (toluene), using ethanol as eluent, delivered at a flow-rate of 1.0 mL/min.

The eluent composition and the corresponding analytical chromatographic data for each resolved compound are summarized as follows. **1a**: *n*-hexane-ethanol 75/25 (v/v), $k_1 = 2.27$, $\alpha = 1.45$, $R_s = 4.55$. **2b**: *n*-hexane-ethyl acetate-trifluoroacetic acid 40/60/0.1 (v/v/v), $k_1 = 0.88$, $\alpha = 1.36$, $R_s = 2.92$. k_1: retention factor of the first eluted enantiomer, defined as $(t_1 - t_0)/t_0$, where t_0 is the void time of the column; α: enantioselectivity factor defined as k_2/k_1; R_s: resolution factor defined as $2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are retention times and w_1 and w_2 are band widths at the baseline in time units. Other analytical chromatographic conditions: flow-rate, 1.0 mL/min; temperature, 25 °C; detector, UV and CD at 225 nm (for **1a**) and 280 nm (for **2b**).

Specific rotations of enantiomers of **1a** and **2b**, dissolved in ethanol, were measured at 589 nm by a Perkin-Elmer polarimeter model 241 equipped with a Na lamp. The volume of the cell was 1 mL and the optical path was 10 cm. The system was at a temperature of 20 °C by a Neslab RTE 740 cryostat. The circular dichroism (CD) spectra of stereoisomers of **1a** and **2b**, dissolved in ethanol (concentration about 0.2 mg/mL) in a quartz cell (0.1 cm path length) at 25 °C, were measured by using a Jasco model J-700 spectropolarimeter. The spectra are averages computed over three instrumental scans, and the intensities are presented in terms of ellipticity values (mdeg).

RX-analysis was carried out with a Oxford Diffraction KM4 Xcalibur2 goniometer at room temperature.

Graphite-monochromated Mo Kα radiation (40 mA/−40 kV) and a KM4 CCD/SAPPHIRE detector were used for cell parameter determination and data collection.

The integrated intensities, measured using the ω scan mode, were corrected for Lorentz and polarization effects.[10] The substantial redundancy in data allows empirical absorption corrections (SADABS)[11] to be applied using multiple measurements of symmetry-equivalent reflections.

The structure was solved by direct methods of SIR2004[12] and refined using the full-matrix least-squares on F^2 provided by SHELXL97.[13]

The X-ray CIF files for these structures have been deposited at the Cambridge Crystallographic Data Center with the deposition numbers CCDC 710382 and CCDC 710383. Copies of the data can be obtained, free of charge, from CCDC, 12 Union Road, Cambridge, CB2 1EZ UK (e-mail: deposit@ccdc.cam.ac.uk; Internet: //www.ccdc.cam.ac.uk).

Acknowledgment. We thank the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) for partial support of this project through a grant.

Supporting Information Available: Experimental methods, crystallographic information, X-ray structural analysis, pharmacological procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Lasley, R. D.; Konyn, P. J.; Hegge, J. O.; Mentzer, R. M. Effects of ischemic and adenosine preconditioning on interstitial fluid adenosine and myocardial infarct size. *Am. J. Physiol. Heart Circ. Physiol.* **1995**, *38*, H1460–H1466.

(2) Zhang, W. M.; Jin, W. Q.; Wong, T. M. Multiplicity of kappa opioid receptor binding in the rat cardiac sarcolemma. *J. Mol. Cell Cardiol.* **1996**, *28*, 1547–1554.

(3) Schulz, R.; Post, H.; Vahlhaus, C.; Heusch, G. Ischemic preconditioning in pigs: a graded phenomenon: its relation to adenosine and bradykinin. *Circulation* **1998**, *98*, 1022–1029.

(4) Sato, T.; Sasaki, N.; O'Rourke, B.; Marbán, E. Nicorandil, a potent cardioprotective agent, acts by opening mitochondrial ATP-dependent potassium channels. *J. Am. Coll. Cardiol.* **2000**, *35*, 514–518.

(5) Sato, T.; Sasaki, N.; Seharaseyon, J.; O'Rourke, B.; Marbán, E. Selective pharmacological agents implicate mitochondrial but not sarcolemmal K(ATP) channels in ischemic cardioprotection. *Circulation* **2000**, *101*, 2418–2423.

(6) Breschi, M. C.; Calderone, V.; Martelli, A.; Minutolo, F.; Rapposelli, S.; Testai, L.; Tonelli, F.; Balsamo, A. New benzopyran-based openers of the mitochondrial ATP-sensitive potassium channel with potent anti-ischemic properties. *J. Med. Chem.* **2006**, *49*, 7600–7602.

(7) Balsamo, A.; Calderone, V.; Rapposelli, S. Preparation of 4-spiro-heterocyclic 2,2-dimethylchromanes as activators of ATP-sensitive potassium (KATP) channels. PCT Int. Appl. WO2008007210, 2008, p 61.

(8) Breschi, M. C.; Calderone, V.; Digiacomo, M.; Manganaro, M.; Martelli, A.; Minutolo, F.; Rapposelli, S.; Testai, L.; Tonelli, F.; Balsamo, A. Spirocyclic benzopyran-based derivatives as new anti-ischemic activators of mitochondrial ATP-sensitive potassium channel. *J. Med. Chem.* **2008**, *51*, 6945–6954.

(9) Atwal, K. S.; Grover, G. J.; Ahmed, S. Z.; Ferrara, F. N.; Harper, T. W.; Kim, K. S.; Sleph, P. G.; Dzwonczyk, S.; Russell, A. D.; Moreland, S.; McCullough, J. R.; Normandin, D. E. Cardioselective anti-ischemic ATP-sensitive potassium channel openers. *J. Med. Chem.* **1993**, *36*, 3971–3974.

(10) Walker, N.; Stuart, D. An empirical method for correcting diffractometer data for absorption effects. *Acta Crystallogr., Sect. A: Found. Crystallogr.* **1983**, *39*, 158–166.

(11) Sheldrick, G. M. SADABS version 2.03, a Program for Empirical Absorption Correction; Universität Göttingen: Göttingen, Germany, 1997−2001.

(12) Burla, M. C.; Caliandro, R.; Camalli, M.; Carrozzini, B.; Cascarano, G. L.; De Caro, L.; Giacovazzo, C.; Polidori, G.; Spagna, R. SIR2004: an improved tool for crystal structure determination and refinement. *J. Appl. Crystallogr.* **2005**, *38*, 381–388.

(13) Sheldrick, G. M. *SHELXL97: Program for Crystal Structure Refinement*; Institut für Anorganische Chemie de Universität Göttingen: Göttingen, Germany, 1997.

JM801459F