Brief Articles

Bispyridinium Cyclophanes: Novel Templates for Human Choline Kinase Inhibitors

Ana Conejo-García, Joaquín M. Campos, Rosario M. Sánchez-Martín, Miguel Á. Gallo, and Antonio Espinosa*

Departamento de Química Farmacéutica y Orgánica, Facultad de Farmacia, c/Campus de Cartuja s/n, 18071 Granada, Spain

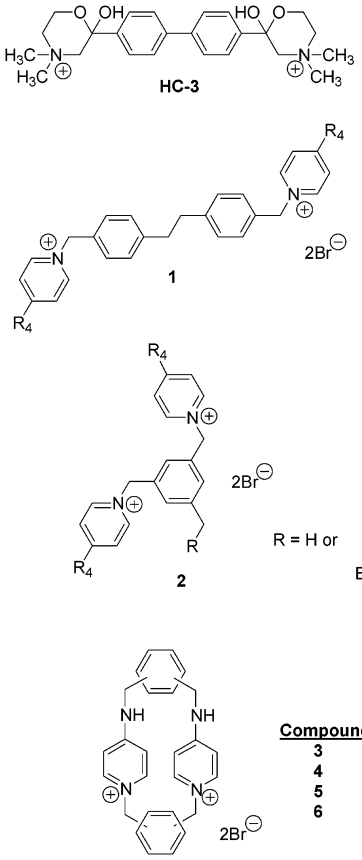
Received January 28, 2003

The synthesis and biological activities of four novel bispyridinium cyclophanes as choline kinase (ChoK) inhibitors are presented. Their synthetic methodology has been optimized according to dilution, temperature, and reaction time and provides pure bispyridinium cyclophanes in high yields very easily. One of these cyclophanes (**6**, 4,8-diaza-3(1,4),9(4,1)-dipyridina-1(1,4),6(1,3)-dibenzenacyclodecaphan-3^1,9^1-bis(ilium) dibromide) has an $IC_{50(ChoK)}$ of 0.3 μM and is the most potent human ChoK inhibitor described to date.

Introduction

Ras genes encode proteins that regulate key cellular signaling pathways.[1] Several lines of evidence have indicated that among other cellular changes the phospholipid metabolism was also altered as a result of oncogene-induced transformation.[2,3] Phospholipid molecules and their metabolites are presumed to participate in the processes of oncogene-induced transformation.[4] Phosphatidylcholine (PC), the major component of the plasma membrane, is hydrolyzed by phospholipase D (PLD) to yield phosphatidic acid (PA) and choline. PA is then hydrolyzed to generate diacylglycerol (DAG). On the other hand, choline is phosphorylated by choline kinase (ChoK) to generate phosphorylcholine (*P*Cho). Increased levels of ChoK activity and *P*Cho production in human cancers have been found,[5,6] in keeping with previous reports using nuclear magnetic resonance.[7,8] These observations have resulted in the development of an antitumoral strategy focused on ChoK. Research on ChoK inhibitors has identified hemicholinium-3 (HC-3, Chart 1) as a relatively potent and selective blocker.[9] This choline homologue with a biphenyl structure has been used for the design of new antitumoral drugs. Since HC-3 is a potent respiratory paralyzant, it is not a good candidate for use in the clinic. The synthesis of several derivatives was based on structural modifications of HC-3 that improve the ChoK inhibitory activity and omit the toxic effect. We have correlated the inhibitory effect on proliferation of symmetrical bisquaternary compounds[10] with their ability to inhibit the production of *P*Cho in whole cells. When the 1,2-ethylene-*p*-(bisbenzyldimethyldiyl) (**1**, Chart 1) moiety was used as a linker between the two 4-substituted pyridinium cationic heads, the structures were screened for their activity inhibiting isolated ChoK (under ex vivo conditions). The R_4 group, which is a tertiary amine, made a substantial contribution, and it was suggested[11] that its role was electronic, via delocalization of the positive charge. The importance of frontier orbital energies (LUMO) of model compounds has been emphasized and interpreted.[12] Fifty-six biscationic dibromides with distinct heads [bis(4-substituted)pyridinium, bis(4-aminoquinolinium), bisquinolinium, and bisisoquinolinium moieties] and several spacers between the two charged nitrogen atoms were synthesized. The electron characteristic of the substituent at position 4 of the heterocycle

Chart 1



Compound	Isomer
3	*p,p*
4	*m,m*
5	*p,m*
6	*m,p*

* To whom correspondence should be addressed. Phone: +34 958 243850. Fax: +34 958 243845. E-mail: aespinos@ugr.es.

10.1021/jm030792i CCC: $25.00
Published on Web 06/27/2003

and the theoretical lipophilic character of the whole molecule were found to significantly affect the antitumor activity.[13] We have lately studied the role played by a third positive charge on both ChoK inhibitory and antiproliferative activities. A benzene ring was used as a linker in order to carry out such a study because it allows us to obtain symmetrical bis- and triscationic compounds (**2**, Chart 1). Trispyridinium compounds are more potent than the bispyridinium ones as inhibitors of human ChoK. Actually, the triscationic compound **2** (Chart 1, R_4 = 4-chloro-*N*-methylanilino) was the most potent ChoK inhibitor so far described ($IC_{50} = 1.4\ \mu M$).[14] Nevertheless, the trispyridinium compounds are less active than the bispyridinium ones as antiproliferative agents because the latter show better lipophilicity to cross the cytosolic membranes. We have very recently published a review on ChoK inhibitors.[15]

Up to now, all the structures synthesized by our group are open compounds and accordingly are highly flexible molecules. However, rigidification has been a habitual tactic used to increase the activity of a drug or to reduce its side effects. Incorporating the skeleton of a flexible drug into a ring is the usual way of "locking" a conformation that, moreover, can give information on the active conformation of the compound. As a first stage in the design of this new class of ChoK inhibitors, we have embarked upon the synthesis of the most simple model of cyclic compounds that have only one benzene ring as a linker. The purpose is to study how the rigidity increase affects the ChoK inhibitory and/or antiproliferative activities, and hence, the following bispyridinium cyclophane family (**3**−**6**, Chart 1) has been prepared. These compounds differ from each other by the substitution pattern shown by the benzene rings. The first prefix takes into account the upper linker that connects the amino groups, whereas the second one is related to the disubstitution pattern of the lower benzene ring that links the N^+ atoms. In the present study, we report the synthesis and biological activities of several bispyridinium cyclophanes as human ChoK inhibitors that are antiproliferative agents.

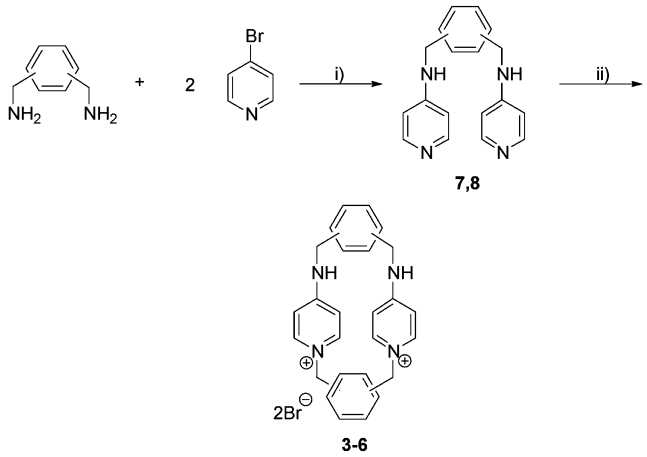
As can be seen in Chart 1 (**3**−**6**), compounds derivated from both ortho benzenes have not been obtained because of the lack of solubility of the intermediates that makes the final cyclization reaction impossible (data not shown).

Chemistry

The cyclophanes were synthesized according to Scheme 1. The dipyridines **7** and **8** (para and meta isomers, respectively) are novel and were prepared from the commercially available diamines and 4-bromopyridine in the presence of phenol, which is known to catalyze the reaction in the case of 2- and 4-haloquinolines.[16] As a reaction medium, phenol reduces both the reaction time and temperature of halogen-replacement reactions. Dipyridines **7** and **8** were characterized as the bis-hydrobromide compounds after recrystallization from methanol. The conversion of **7** and **8** to the desired cyclophanes was carried out under high-dilution conditions in acetonitrile at the reflux temperature of the mixture.

To start with, the initial concentration was 0.04 M and the reaction was carried out by adding a solution

Scheme 1[a]



[a] (i) Phenol; (ii) 1,3- or 1,4-bis(bromomethyl)benzene, refluxing acetronitrile.

of the dibromide drop by drop to the dipyridine structure; in this way the obtention of the cyclophanes is favored and, accordingly, polymer formation is kept to a minimum. Nevertheless, the ^1H NMR spectra of the structures obtained show the presence of a second compound very similar to the desired compounds. High-resolution liquid secondary ion mass spectrometry (LSIMS) data and elemental analyses demonstrated that the impurity should have the same molecular formula as our targets. This led us to think that the substance obtained was the open monocationic compound (see Supporting Information) as a consequence of an incomplete cyclization process.

Thus, a 50% mixture was obtained, which made the separation of both by recrystallization impossible because of the similarity of their physicochemical properties and forced us to improve the synthesis method in order to oblige the monocationic salt to cyclize. The monoquaternized salt has to be dissolved to undergo the cyclization step with the participation of the other pyridine nitrogen atom. Therefore, increment of dilution, the use of a solvent more polar than acetonitrile and/or extending the reaction time facilitated the macrocycle formation.

Similar bisquinolinium cyclophanes[17,18] needed to be purified by tedious reverse-phase preparative HPLC because conventional purification methods failed to give analytically pure samples for biological testing, despite having been obtained under high-dilution conditions (0.001−0.002 M). In our case, this represents a great advantage for the accessibility of such an interesting class of compounds.

Biological Testing

Compounds **3**−**6** were tested in an ex vivo system using human ChoK as a target. This assay allowed us to evaluate the affinity of the compounds for ChoK, without considering the possible passage through membranes. The effects on cell proliferation by the ChoK inhibitors in ras-transformed cells were next investigated on the HT-29 cell line (in vitro assay). This cell line was established from a colon adenocarcinoma, one of the most frequent solid cancers in humans that are mainly resistant to chemotherapy,[19] making these cells appropriate for the search of new antitumor drugs. IC_{50}

Table 1. ChoK Inhibitory and Antiproliferative Activities of Cyclophanes **3**−**6**

compd	isomer[a]	$IC_{50(ChoK)}$ (μM)	$IC_{50(HT-29)}$ (μM)
6	*m*,*p*	0.3	28.8
3	*p*,*p*	2.1	36.9
4	*m*,*m*	13.2	>100
5	*p*,*m*	24.8	58.6

[a] The first prefix takes into account the upper linker that connects the amino groups, whereas the second one is related to the disubstitution pattern of the lower benzene ring that links the N^+ atoms.

values were obtained from a nonlinear least-squares fit of the Hill equation to the data. The activity in the in vitro assay reflects the pharmacodynamic properties of the compounds rather than their affinity for the enzyme.

Results and Discussion

The biological results of the four bispyridinium cyclophanes are shown in Table 1. Compound **6** is approximately 5-fold more potent than the most effective inhibitor of human ChoK previously reported (see ref 14 and Chart 1, triscationic compound **2**, R_4 = 4-chloro-*N*-methylanilino).

The first thing that draws attention is the fact that four structurally isomeric compounds present biological activities that are so different. This indicates that the substitution model of the cyclophane linkers is very significant for biological activity.

On one hand, regarding the ChoK inhibitory activity, it can be accepted that the lower benzene ring must be para because the two most active compounds (**6** and **3**) correspond to this pattern. If the $IC_{50(ChoK)}$ values of the two compounds with the meta pattern for the lower linker (**4** and **5**) are compared between them and if the two compounds with the para pattern for the lower one (**6** and **3**) are compared too, it can be inferred that the inhibitory activity notably increases when the upper benzene ring is meta.

On the other hand, the influence of the isomerism of the lower linker on the antiproliferative activity follows the same trends shown in the ChoK activity. The fact that the enzymatic inhibition and the antiproliferative activity do not display an exact relationship may be explained on the basis that $IC_{50(ChoK)}$ only assesses the affinity of the ligand by the enzyme whereas in the $IC_{50(HT-29)}$, other biological factors intervene such as the passage through the cellular membranes. Although the anticancer activities of the cyclophanes are modest, the increase in lipophilicity should favorably affect the passage through the cytoplasmic membrane.

Conclusion

The synthesis and biological activities of novel bispyridinium cyclophanes as ChoK inhibitors have been described. The ChoK inhibition activities of the cyclophanes (**3**−**6**) strongly depend on the disubstitution model of the upper and lower benzene rings. Compound **6** is the most potent human ChoK inhibitory agent reported to date, showing activity in the low micromolar range. Clearly the bispyridinium cyclophane derivatives that we have described here are useful pharmacological tools for the further investigation of ChoK inhibitors as antiproliferative agents.

Experimental Section

Chemistry. Melting points were taken in open capillaries on an Electrothermal melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on a 400 MHz ^{1}H and 100 MHz ^{13}C NMR Bruker ARX 400 or 300 MHz ^{1}H and 75 MHz ^{13}C NMR Bruker AMX-300 spectrometers, and chemical shifts (ppm) are reported relative to the solvent peak. Signals are designated as follows: s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; pst, pseudotriplet; m, multiplet. All the final products had satisfactory (within ±0.4%) C, H, and N analyses. The compounds gave accurate mass spectra, having the correct isotope abundance and no extraneous peaks. High-resolution liquid secondary ion mass spectra (HR LSIMS) were carried out on a VG AutoSpec Q high-resolution mass spectrometer (Fisons Instruments). All compounds were dried at 40 °C and 0.1 mmHg for 15 h, but many held on tenaciously to water, which appears to be a solvate. The final compounds (**3**−**6**) have been named according to IUPAC Recommendations for phane structures.[20,21]

General Procedure for the Preparation of 1,4-Bis-[(pyridin-4-yl)aminomethyl]benzene Hydrobromide (7) and 1,3-Bis[(pyridin-4-yl)aminomethyl]benzene Hydrobromide (8). 4-Bromopyridine and the respective diamine (molar ratio 1:2) in a high excess of phenol were heated at 180 °C for 7 h. After cooling to room temperature and being diluted with methanol, the reaction mixture was acidified up to pH 1−2 with HBr in 33% glacial AcOH. On addition of diethyl ether, a solid precipitated and was collected by vacuum filtration and washed thoroughly with the same solvent to remove phenol. Compounds were purified by recrystallization from methanol, obtaining the target molecules **7** and **8** as white solids (yields 65%).

General Procedure for the Preparation of the Bispyridinium Cyclophanes (3−6). Over a suspension of the dipyridine (**7** and **8**) in refluxing acetonitrile a solution of the appropriate dibromide in acetonitrile was added dropwise (molar ratio 1:1). The minimun dilution for cyclization to take place was 0.01 M. The solution was heated under reflux for 24−72 h. During the course of the reaction, a white precipitate formed. The solvent was removed in vacuo, and the precipitate was washed with ethyl acetate and diethyl ether. The compounds were purified by recrystallization from methanol. In the Supporting Information, a comparative analysis of dilutions and reaction times is undertaken to obtain each of the four cyclophanes.

Pharmacology. The ex vivo ChoK inhibition[22,23] and antiproliferative assays against HT-29 cells[9] were followed in accordance with the protocols previously reported. The results are recorded in the table.

Acknowledgment. We thank the Spanish CICYT (Project SAF98-0112-C02-01) for financial support. The award of grants from the Ministerio de Educación, Cultura y Deporte to A.C.G. and from the Junta de Andalucía to R.M.S. is gratefully acknowledged. We are deeply indebted to J. C. Lacal (Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain) and his group for the biological testing of compounds described in this study.

Supporting Information Available: Detailed methods for the cyclization process and ^{1}H and ^{13}C NMR assignments of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Barbacid, M. Ras genes. *Annu. Rev. Biochem.* **1987**, *56*, 779−827.
(2) Tsai, M.; Yu, C.; Stacey, D. The effect of GTPase activating protein upon ras is inhibited by mitogenically responsive lipids. *Science* **1989**, *243*, 522−526.

(3) Yu, C.; Tsay, M.; Stacey, D. Serum stimulation of NIH 3T3 cells induces the production of lipids able to inhibit GTPase activating protein activity. *Mol. Cell. Biol.* **1990**, *10*, 6683−6689.
(4) Lacal, J. C.; Carnero, A. Regulation of ras proteins and their involvement in signal transduction pathways. *Oncol. Rep.* **1994**, *1*, 677−693.
(5) Nakagami, K.; Uchida, T.; Ohwada, S.; Koibuchi, Y.; Suda, Y.; Sekine, T.; Morishita, Y. Increased choline kinase activity and elevated phosphocholine levels in human colon cancer. *Jpn. J. Cancer Res.* **1999**, *90*, 419−424.
(6) Ruiz-Cabello, J.; Cohen, J. S. Phospholipid metabolites as indicators of cancer cell function. *NMR Biomed.* **1992**, *5*, 226−233.
(7) de Certaines, J. D.; Larsen, V. A.; Podo, F.; Carinelli, G.; Briot, O.; Henriksen, O. In vivo 31P MRS of experimental tumours. *NMR Biomed.* **1993**, *6*, 345−365.
(8) Smith, T. A. D.; Bush, C.; Jameson, C.; Titley, J. C.; Leach, M. O.; Wilman, D. E. V.; McCready, V. R. Phospholipid metabolites, prognosis and proliferation in human breast carcinoma. *NMR Biomed.* **1993**, *6*, 318−323.
(9) Hernández-Alcoceba, R.; Fernández, F.; Lacal, J. C. In vivo antitumor activity of choline kinase inhibitors: a novel target for anticancer drug discovery. *Cancer Res.* **1999**, *59*, 3112−3118.
(10) Hernández-Alcoceba, R.; Saniger, L.; Campos, J.; Núñez, M. C.; Khaless, F.; Gallo, M. Á.; Espinosa, A.; Lacal, J. C. Choline kinase inhibitors as a novel approach for antiproliferative drug design. *Oncogene* **1997**, *15*, 2289−2301.
(11) Campos, J.; Núñez, M. C.; Rodríguez, V.; Gallo, M. Á.; Espinosa, A. QSAR of 1,1′-(1,2-ethylenebisbenzyl)bis(4-substitutedpyridinium) dibromides as choline kinase inhibitors: a different approach for antiproliferative drug design. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 767−770.
(12) Campos, J.; Núñez, M. C.; Rodríguez, V.; Entrena, A.; Hernández-Alcoceba, R.; Fernández, F.; Lacal, J. C.; Gallo, M. Á.; Espinosa, A. LUMO energy of model compounds of bispyridinium compounds as an index for the inhibition of choline kinase. *Eur. J. Med. Chem.* **2001**, *36*, 215−225.
(13) Campos, J.; Núñez, M. C.; Sánchez, R. M.; Gómez-Vidal, J. A.; Rodríguez-González, A.; Báñez, M.; Gallo, M. Á.; Lacal, J. C.; Espinosa, A. Quantitative structure−activity relationships for a series of symmetrical bisquaternary anticancer compounds. *Bioorg. Med. Chem.* **2002**, *10*, 2215−2231.
(14) Conejo-García, A.; Campos, J.; Sánchez, R. M.; Rodríguez-González, A.; Lacal, J. C.; Gallo, M. Á.; Espinosa, A. Choline kinase inhibitory effect and antiproliferative activity of new 1,1′,1″-(benzene-1,3,5-triylmethylene)tris{4-[(disubstituted)amino]pyridinium} tribromides. *Eur. J. Med. Chem.* **2003**, *38*, 109−116.
(15) Campos, J.; Núñez, M. C.; Conejo-García, A.; Sánchez-Martín, R. M.; Hernández-Alcoceba, R.; Rodríguez-González, A.; Lacal, J. C.; Gallo, M. Á.; Espinosa, A. QSAR-derived choline kinase inhibitors: How rational can antiproliferative drug design be? *Curr. Med. Chem.* **2003**, *10*, 1241−1253.
(16) Surrey, A. R.; Cutler, R. K. The role of phenol in the reaction of 4,7-dichloroquinoline with novol diamine. *J. Am. Chem. Soc.* **1951**, *73*, 2623−2626.
(17) Campos Rosa, J.; Galanakis, D.; Piergentili, A.; Bhandari, K.; Ganellin, C. R.; Dunn, P. M.; Jenkinson, D. H. Synthesis, molecular modeling, and pharmacological testing of bis-quinolinium cyclophanes: Potent, non-peptidic blockers of the apamin-sensitive Ca^{2+}-activated K^+ channel. *J. Med. Chem.* **2000**, *43*, 420−431.
(18) Chen, J.-Q.; Galanakis, D.; Ganellin, C. R.; Dunn, P. M.; Jenkinson, D. H. Bis-quinolinium cyclophanes: 8,14-diaza-1,7-(1,4)-diquinolinacyclotetradeca-phane (UCL 1848), a highly potent and selective, nonpeptidic blocker of the apamin-sensitive Ca^{2+}-activated K^+ channel. *J. Med. Chem.* **2000**, *43*, 3478−3481.
(19) Schnall, S.; Macdonald, J. S. In *Manual of Oncologic Therapeutics*; Macdonald, J. S., Haller, D. G., Mayer, R. J., Eds.; J. B. Lippincott Co.: Philadelphia, PA, 1995; pp 170−184.
(20) Phane Nomenclature. Part I: Phane Parent Names. IUPAC Recommendations 1998. http://www.chem.qmw.ac.uk/iupac/phane.
(21) Phane Nomenclature. Part II: Substitution Derivatives of Phane Parent Hydrides. IUPAC Recommendations 2001. http://www.chem.qmw.ac.uk/iupac/phane.
(22) Lucas, L.; Hernández-Alcoceba, R.; Rodríguez, P.; Lacal, J. C. Modulation of phospholipase D by hexadecylphosphorylcholine: a putative novel mechanism for its antitumoral activity. *Oncogene* **2001**, *20*, 1110−1117.
(23) Ramírez de Molina, A.; Peñalva, V.; Lucas, L.; Lacal, J. C. Regulation of choline kinase activity by Ras proteins involves RaI-GDS and PI3K. *Oncogene* **2002**, *21*, 937−946.

JM030792I