

Anticancer bisquaternary heterocyclic compounds: a *ras*-ional design

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Abstract

A new family of symmetrical bisquaternary compounds with semirigid linkers have shown to be highly specific for Choline Kinase (ChoK) inhibition and to exert antitumoural activity in cell lines and in mice. A three-parameter regression equation has been derived which satisfactorily describes the *ex vivo* inhibitory potency of ChoK of the title compounds. The electronic effect of the group at position 4 of the cationic head plays a critical role although the hydrophobic contribution, especially that of the linker, favors the ChoK inhibitory activity. The antiproliferative activity (*in vitro* assay) is correlated with the ChoK inhibition (*ex vivo* assay) through the electronic effect and a squared term of the overall lipophilicity of the molecules. We also provide *in vivo* evidence that ChoK is a novel target for the design of antitumoural drugs. All these results suggest that ChoK plays a crucial role in the onset of carcinogenesis.

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1. Introduction

Cancer is one of the most formidable afflictions in the world. Despite immense advances in the field of basic and clinical research, which have resulted in higher cure rates for a number of malignancies, cancer remains one of the leading causes of death in the Western world [1,2]. Although cancer mortality is second to heart disorders, the first is steadily increasing, while the latter is leveling off.

The ease and success of finding better drugs for any illness depend upon how best we can rationalize the design of the drugs. The rational design of an agent with specific activity towards a selected target requires this objective to be so precisely defined that it can be hit selectively in the presence of other identical or similar targets. Increasing knowledge of the biological complexities of cancer and the molecular genetic defects underlying tumorigenesis has provided new opportunities for rational anticancer drug discovery and development. By focusing on the primary genetic alterations in cancer

cells there is a greater likelihood that the biological effects of drugs against these targets will be confined to the tumor cells rather than affecting both tumor and normal cell processes (biological specificity) [3].

1.1. *Ras* signal transduction

The development of human cancer is thought to be the result of mutations in multiple genes that control normal cell proliferation, differentiation and apoptosis. Genes that are often found mutated in malignancies are referred to as proto-oncogenes. Activation of these proto-oncogenes to oncogenes and/or the loss of function of tumor suppressor genes may cause deranged intracellular signaling [2,4]. One of the most commonly altered gene products in human solid tumors include Ras proteins. The *ras* (an acronym for rat sarcoma, the source of the prototypic viral gene) gene encodes 21-kDa proteins, which play an important role in signal transduction. They pass on stimuli from external growth factors to the cell nucleus in a process, which is called signal transduction. Cells respond to extracellular mitogenic signals during the first (G_1) phase of the cell cycle [2,5,6]. Ras mutations, which are found in approxi-

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mately 25% of all human malignancies, lead to an increase in cell proliferation and tumor formation [7,8].

1.2. ChoK: a precursor of second messengers

Choline kinase (ChoK), a cytosolic enzyme present in various tissues, catalyses the phosphorylation of choline to form phosphorylcholine (PCho). ChoK has recently gained attention as a potential relevant component in signaling pathways involved in the regulation of cell growth. This latter function was noticed by the discovery of elevated levels of PCho in cells transformed by *ras* oncogenes [9]. It has been demonstrated that the generation of PCho from choline by ChoK is an essential process for growth factors, such as platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF), to exert their mitogenic action in mouse fibroblasts [10]. This conclusion is based on the fact that growth factors are unable to stimulate DNA synthesis if ChoK is inhibited. Furthermore, the exogenous addition of PCho, the product of ChoK activity, has mitogenic activity in murine fibroblasts [11].

1.3. The role of ChoK in the carcinogenesis process

The evidence that ChoK and PCho play a role in malignant transformation comes from the demonstration that *ras*-transformed cell lines show higher PCho levels than their normal counterparts as a consequence of an increased ChoK activity [10]. Evidence for a role of PCho in cell transformation and tumourigenesis comes from NMR studies performed in human tumors [12].

Hemicholinium-3 (HC-3, **1**), a choline homologue, has been described as a ChoK inhibitor in *in vitro* studies using immortalized cell lines ($IC_{50} = 600 \mu M$) in intact mammalian cells [10]. Nevertheless, HC-3 has a potent inhibitory effect on the high-affinity choline transport system with drastic toxic effects on cholinergic nerve terminals [13]. Since HC-3 has a potent paralyzing respiratory effect, it is a poor candidate for use in the clinic. Furthermore, HC-3 induces non-specific effects on signaling pathways that can be overcome with more specific molecules [10]. However, in spite of its negative effects, HC-3 has been used as a model structure for the further development of more active and specific ChoK inhibitors.

2. Results and discussion

2.1. Design considerations

The HC-3, **1** structure contains a central biphenyl system to which two choline-like chains containing the quaternary groups are attached. HC-3 was represented

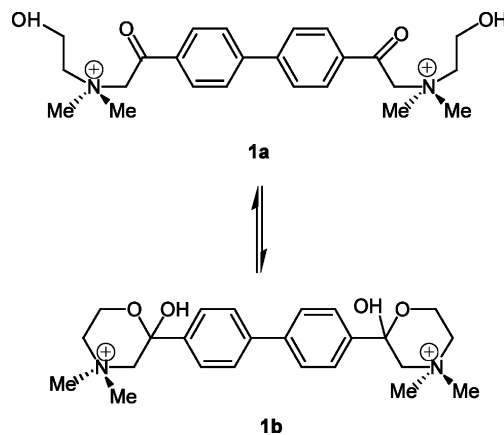


Fig. 1. Acyclic *bis*(hydroxycarbonyl) and cyclic *bis*(hemiketal) forms of hemicholinium-3 (HC-3).

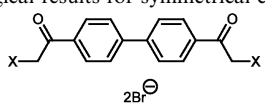
in early literature as structure **1a** (Fig. 1). However, interpretation of infrared spectral data led Schueler [14] to conclude that this compound exists as the *bis*-hemiketal **1b** (Fig. 1).

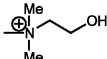
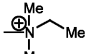
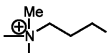
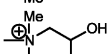
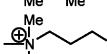
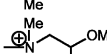
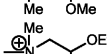
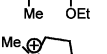
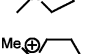
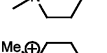
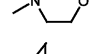
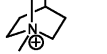
In the daily practice of medicinal chemistry, the analogical approach is currently used, in which the global complexity of the system is not affected and generally yields close analogues (or ‘me-too’ compounds) of the original active principle. Several modifications have been carried out on the cationic heads of the prototype HC-3, **1a** yielding symmetrical hemicholinium-like derivatives with the bisphenacyl moiety linking several ammonium groups (**2a–k**). Table 1 summarizes the structures of these open-chain-‘me-too’ compounds in order to test the significance of the tautomer **1a** in its inhibitory action against ChoK. All the modifications affect the hydroxyethyl moiety bearing the quaternary nitrogen atoms and are essentially the following:

- Oxidation level of the hydroxylated carbon (**2a**, **2b**, **2e** and **2f**).
- Its length and/or the presence of branching (**2c**, **2d**).
- Finally, compounds **2g–k** show the substitution of the lateral chain of the prototype **1a** by a five- or six-membered saturated heterocycle (**2g–i**) or by a bicyclic system (**2j–k**).

The analysis of the effect of the defined modifications of the HC-3, **1a** structure was tested in an *ex vivo* system using a purified ChoK preparation from yeast as a target. The deduced sequence comprises 582 aminoacid residues with a molecular mass of 66 316 Da and bears local sequence similarity to various protein kinases and bacterial antibiotic phosphotransferases [15]. The anti-proliferative activity has been determined with the HT-29 human cell line, a colon cancer that is resistant to chemotherapy and that expresses multidrug resistance (MDR). The MDR phenomenon has been shown to be

Table 1
Structure and biological results for symmetrical compounds **1** and **2**



Compd	X	(IC ₅₀) _{ex vivo} ^a (μM)	(IC ₅₀) _{antiprol} ^a (μM)
1a		500	2500
2a		>100	>100
2b		>100	>100
2c		3.5	>100
2d		>100	>100
2e		>100	>50
2f		>100	>50
2g		>100	>50
2h		7	>100
2i		100	>100
2j		30	>100
2k		0.5	>50

^a All values are the mean of two independent determinations performed in duplicate.

associated with reduced drug accumulation, which has been attributed to two different phenomena. One involves drug extrusion by an energy-dependent efflux pump that is more effective in resistance cells [16]. The other mode of action suggests the existence of an energy-dependent permeability barrier, which effectively restricts drug entry into the cells [17].

Between the eleven compounds tested (Table 1), the 1,1'-[biphenyl-4,4'-bis(carbonylmethyl)]bis-(3-hydroxyquinuclidinium) dibromide **2k** was the most active compound so far described [10]. It is 1000-fold more active than HC-3, **1** (IC₅₀ = 0.5 μM against ChoK; IC₅₀ = 500 μM of HC-3, **1**). The presence of the hydroxy group is not necessary for the molecular recognition between the enzyme and its ligand, because 1,1'-[biphenyl-4,4'-bis(carbonylmethyl)]bis(quinuclidinium) dibromide **2j** shows an IC₅₀ = 30 μM, although its presence

notably increases the inhibitory activity against ChoK. Nevertheless **2k**, the most active compound against ChoK, shows a nearly null antiproliferative activity against the HT-29 human cell line (IC₅₀ > 50 μM). Possibly, the compound is not lipophilic enough to cross the cytosolic membranes.

We then decided to modify more drastically both the spacer and the two cationic heads of the prototype: maintaining the bisquaternary character of the newly synthesized molecules, the 1,4-oxazonium moieties of HC-3, **1** were changed for the more lipophilic pyridinium [18], quinolinium and isoquinolinium rings. Furthermore, in order to keep the number of atoms between the two positive-charged nitrogen atoms of the cyclocetalic form of HC-3, **1** we used the 1,2-ethylene(bisbenzyl) moiety as a linker. If the substituents of the pyridinium moieties are located at position 4, the aromatic system allows the study of the electronic influence between them and the positive charge (Fig. 2).

Afterwards, the modification of the spacer connecting the two cationic heads (by modifying the number of methylene groups between the two phenyl rings) was carried out (see Table 3).

The QSAR study tries to explain the variations observed in the biological activities of a group of congeners in terms of molecular variations caused by a change of the substituents. Two important applications of QSAR studies can be stated: the predictive aspect¹ and the diagnostic one. The former deals with the extrapolation and interpolation of a correlation study and the latter answers mechanistic aspects.

2.2. The predictive aspect

Table 2 shows the structure, biological results and log *P* values for compounds **3**. log *P* Values were calculated by using the Ghose–Crippen modified atomic contribution system [19] (ATOMIC5 option) of the PALLAS 2.0 programme.² In a recent publication [20] we have estimated by ¹³C NMR spectroscopy the previously unknown σ_R and σ_R^+ descriptors for the diallylamino, pyrrolidino, piperidino and perhydroazepino groups in a simple, fast and reproducible manner.

The substituents at position 4 of the pyridinium moiety were electron-releasing, neutral or electron-withdrawing groups (negative values of σ_R and σ_R^+ denote

¹ Although sometimes taken as a criterion, prediction is not the primary goal of QSAR analyses. It is results from interpolation, it is often trivial; if extrapolation goes too far outside the included parameter space, it usually fails. QSAR helps to understand structure–activity relationship in a quantitative manner to find the borders of certain properties.

² PALLAS FRAME MODULE, a prediction tool of physicochemical parameters, is supplied by CompuDrug Chemistry, Ltd, PO Box 23196, Rochester, NY 14692, USA.

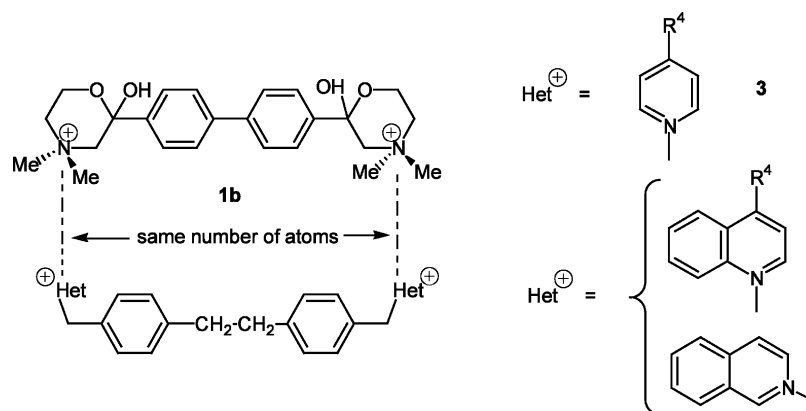


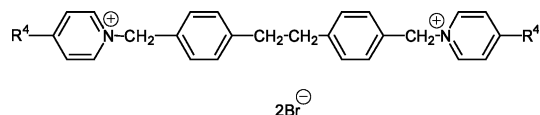
Fig. 2. Molecular variations carried out on the cyclic *bis*(hemiketal) form of hemicholinium-3 (HC-3).

the electron-donating character and positive value, the electron-withdrawing character of the substituent). To start with, the selection of the substituents to be introduced in position 4 of the pyridinium nucleus was dictated by criteria of minimum effort and maximum informative content. Following this line, we chose the smallest number of easily synthetically accessible compounds which could give the maximum spread and orthogonality of the main physicochemical properties, i.e. the electronic and hydrophobic ones. From the results obtained it is clear that the presence of electron-

withdrawing groups (–COOH, **3e** and –CN, **3f**) leads to inactive compounds as ChoK inhibitors. Although the exact $(IC_{50})_{ex\ vivo}$ value of **3f** is not available, follows the general trend from a qualitative point of view. On the other hand, the presence of a strong electron-releasing group seems to be important, since the electron-neutral bearing compounds (**3c** and **3d**) are less potent than **3a**. The amino (–NH₂) group participates in the delocalization of the ring charge, and makes a substantial contribution for ChoK inhibitory activity. Its replacement by other groups and QSAR on the resultant

Table 2

Structure, biological results and parameter values for compounds **3**



Comp	R ⁴	(IC ₅₀) _{ex vivo} (μM) ^a	(IC ₅₀) _{antiprol} (μM) ^a	σ _R ^b	σ _R ⁺ ^c	clog P ^d	π _{R4} ^e
3a	–NMe ₂	17.0	2.00	–0.88	–1.22	–2.83	0.18 ^f
3b	–NH ₂	23.0	4.00	–0.80	–1.10	–4.67	–1.23 ^f
3c	–CH ₂ OH	100	> 100	–0.07	–0.15	–4.25	–1.03 ^f
3d	–CH ₃	100	20.0	–0.16	–0.25	–1.82	0.56 ^f
3e	–COOH	136.7	> 1000	0.11	^g	–3.50	–0.32 ^f
3f	–C≡N	> 1000	200	0.08	0.13	–3.15	–0.57 ^f
3g	–N(Allyl) ₂	17.0	0.55	–0.80 ^h	–1.09 ^h	–0.44	1.34 ^e
3h	–NC ₄ H ₈ ⁱ	20.0	1.00	–0.85 ^h	–1.16 ^h	–1.94	0.59 ^e
3i	–NC ₅ H ₁₀ ^j	9.60	0.40	–0.89 ^h	–1.21 ^h	–0.93	0.85 ^e
3j	–NC ₆ H ₁₂ ^k	15.0	0.40	–0.86 ^h	–1.18 ^h	0.09	1.60 ^e
3k	–NmePh	6.4	0.34	–0.78 ^h	–1.07 ^h	0.37	1.67 ^e

^a All values are the mean of two independent determinations performed in duplicate.

^b σ_R: Electronic parameter for resonance effects (ref. [21]).

^c σ_R⁺: Electronic parameter where a positive charge is delocalized between substituent and reaction center via ‘through resonance’ (ref. [21]).

^d Predicted by using the Ghose–Crippen modified atomic contribution system (ATOMIC5 option, ref. [19]) of the PALLAS 2.0 programme.

^e π_{R4} = clog P_{R4} – clog P_H; clog P values have been calculated using the CDR option of PALLAS 2.0 programme.

^f see ref. [22].

^g Not available.

^h These values were estimated by ¹³C NMR spectroscopy (see ref [20]).

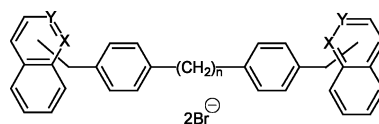
ⁱ Pyrrolidino.

^j Piperidino.

^k Perhydroazepino.

Table 3

Structures, activity data and parameter used for the derivation of the QSAR Eq. (8)



Comp	X	Y	n	(IC ₅₀) _{ex vivo} (μM) ^a	(IC ₅₀) _{antiprol} (μM) ^a	clog P ^b
4a	=N ⁺ -	=CH-	0	50	10	-0.71
4b	=N ⁺ -	=CH-	1	100	6.02	-0.86
4c	=N ⁺ -	=CH-	2	34	4	-0.43
4d	=N ⁺ -	=CH-	3	9	2.5	0.08
4e	=CH-	=N ⁺ -	0	> 100	ND ^c	-0.85
4f	=CH-	=N ⁺ -	1	60	20	-1.00
4g	=CH-	=N ⁺ -	2	60	20	-0.57
4h	=CH-	=N ⁺ -	3	20	2	-0.06

^a All values are the mean of two independent determinations performed in duplicate.^b Predicted by using the Ghose–Crippen modified atomic contribution system (ATOMIC5 option, ref. [19]) of the PALLAS 2.0 programme.^c ND: Not determined.

compounds suggests that an electronic effect remains the prime factor, probably via delocalization of the positive charge of the pyridinium ring. When replaced by the dimethylamino (-NMe₂) group (compound **3a**) it does not lose potency. It is clear that the more electron-releasing that R⁴ is, the more potent is the compound. In principle this result seems contradictory: the essential positive charge on the nitrogen will be increased by the electron-withdrawal by the substituents, and thus, the interaction of nitrogen with a putative anionic site of the enzyme will be increased. We will explain this apparent inconsistency in the ‘Section 2.3. The diagnostic aspect’. The inhibitory potency of the compounds does correlate with the resonance effect of R⁴. This is consistent with conventional chemical concepts. R⁴ is in direct conjugation with the positively charged ring nitrogen. The greater resonance effect of R⁴ would obviously cause better delocalization of the positive charge. This trend can be quantified using the resonance electronic effect of R⁴, σ_R [21,22]:

$$p(\text{IC}_{50})_{\text{ex vivo}} = 3.92(\pm 0.00) - 0.92(\pm 0.06)\sigma_{\text{R}}$$

$$n = 5, r = 0.994, s = 0.052, F_{1,3} \\ = 250.13 \text{ (significance at } \alpha < 0.001 \text{)} \quad (1)$$

where, $p\text{IC}_{50} = -\log \text{IC}_{50}$, bearing in mind that the higher the value of $p\text{IC}_{50}$ the more potent is the compound, n is the number of compounds, r is the correlation coefficient, s is the standard deviation, F is the F ratio between the variances of observed and calculated activities, and data within parentheses are standard errors of estimate.

On the other hand, the correlation between the inhibitory potency and the descriptor σ_R⁺ [21,22] is to be studied (eq 2):

$$p(\text{IC}_{50})_{\text{ex vivo}} = 3.85(\pm 0.04) - 0.73(\pm 0.04)\sigma_{\text{R}}^+ \\ n = 4, r = 0.996, s = 0.042, F_{1,2} \\ = 281.06 \text{ (significance at } \alpha < 0.001 \text{)} \quad (2)$$

The parameter σ_R⁺ was defined for systems where a positive charge is delocalized between substituent and reaction center via ‘through resonance’. Eqs 1 and 2 represent highly significant correlations. Though the number of data points is small, the high correlation coefficients establish the importance of electronic characters (σ_R and σ_R⁺) of the substituents. Accordingly, substituents with higher or similar electron-releasing effects than the -NH₂ and -NMe₂ groups were sought. Therefore, an acyclic amino group such as the diallylamino one, and endocyclic amino groups such as the pyrrolidino, piperidino, and perhydroazepino moieties, could exert a favorable electron-releasing effect [20]. Even the N-methylanilino group was tried [20]. Moreover their higher lipophilicities compared with those of the -NH₂ and -NMe₂ groups will help in the anti-proliferative activities of even more potent compounds [20]. All the compounds showed in Table 2 give rise to eqs 3 and 4:

$$p(\text{IC}_{50})_{\text{ex vivo}} = 3.92(\pm 0.11) - 1.09(\pm 0.15)\sigma_{\text{R}} \\ n = 10, r = 0.928, s = 0.181, F_{1,8} \\ = 49.78 \text{ (significance at } \alpha < 0.001 \text{)} \quad (3)$$

$$p(\text{IC}_{50})_{\text{ex vivo}} = 3.84(\pm 0.16) - 0.87(\pm 0.16)\sigma_{\text{R}}^+ \\ n = 9, r = 0.896, s = 0.194, F_{1,7} \\ = 28.53 \text{ (significance at } \alpha < 0.001 \text{)} \quad (4)$$

The quality of the two eqs 3 and 4 is similar and this is largely due to the high collinearity between σ_R and σ_R⁺ [$r = 0.999$ for the groups shown in Table 2, $n = 9$].

Accordingly, eq 3 is preferred since it has a slightly better standard deviation (s) and in all the following reasoning we will quantify the electronic properties of the R^4 group with the σ_R parameter.

2.3. The diagnostic aspect

Details of the chemistry of drug–enzyme interactions are usually not known; hence, the type of parameter needed to model this interaction is not properly defined. The electronic influence of R^4 on **3** alters its charge distribution (data not shown) and molecular orbital (MO) energies. During the past three decades most of the QSARs derived for biological systems have relied on the use of Hammett-type σ constants to account for electronic variation associated with changes in molecular structure. As a result, these studies have often been limited to sets of congeners that could be treated by using Hammett-type substituent constants. Moreover, the Hammett constants do not reflect which portion of the drug molecule would actually be involved in the interaction with the enzyme. A quantum chemical treatment of electronic effects does provide some help in this direction and is potentially more powerful than the Hammett-type approach since it allows greater flexibility in the construction of the data set. Thus, MO calculations were made [23]. To simplify the computational problem, the calculations were made on model compounds consisting of only one of the two substituted pyridinium moieties and in which the spacer between the two charged nitrogen atoms was replaced by a methyl group. When we related $p(\text{IC}_{50})_{\text{ex vivo}}$ with the energy of the frontier orbitals, it was found that the higher the energies of the HOMO or the LUMO are (i.e. get closer to zero), the more potent the compound. If HOMO is considered to contribute at the level of interaction of the compound with ChoK, it follows that the compound acts as an electron donor, with the enzyme acting as an ‘electron sink’. Fundamental chemical concepts are in discordance with this because these compounds are electron deficient and could not act as electron donors. It is, therefore, difficult to attribute a chemical meaning to the E_{HOMO} correlation.

2.4. Solvation of the model compounds

However, if LUMO is assumed to contribute at the level of interaction of the compound with ChoK, it is necessary to consider the possibility of the formation of a charge transfer interaction of the HOMO of ChoK with the LUMO of the compound. With the solvent (H_2O) molecules acting as electron donors, the bispyridinium compounds **2** act as electron acceptors. The increase of the E_{LUMO} of the molecule would result in its weaker solvation, and thus, a stronger interaction with ChoK. A possible chemical meaning for the E_{LUMO}

correlation is, therefore, offered by the hypothesis that a higher value for E_{LUMO} indicates a weaker solvation of the compound [23].

2.5. QSAR studies between ex vivo ChoK inhibition and clog P

In order to study the possible influence of lipophilicity on the inhibition of ChoK under ex vivo conditions, we selected structures with the following characteristics:

- 1) Cationic heads that allow the dispersion to a great extent of the positive charge with a ‘zero electronic effect’, i.e. with no substituents at position 4. To this end we have used unsubstituted quinolinium and isoquinolinium rings.
- 2) Alkyl spacers with different number of methylene groups.

Table 3 shows the structure, clog P values and biological values of bisalts **4**. Very recently we have unequivocally assigned all the ^1H and ^{13}C NMR resonances of **4a–h** by the concerted application of one- and two-dimensional NMR techniques [24].

For these compounds, the ChoK inhibition activity is found to be correlated with lipophilicity as shown in Eq. (5):

$$p(\text{IC}_{50})_{\text{ex vivo}} = 4.83(\pm 0.09) + 0.81(\pm 0.15)\text{clog } P$$

$$n = 7, r = 0.922, s = 0.151, F_{1,5}$$

$$= 28.57 \text{ (significance at } \alpha < 0.005) \quad (5)$$

We suggest, on the basis of this equation 5, that hydrophobic interactions may occur between the bisalts **4** and ChoK. The greatest contribution of QSAR study is that it has provided a systematic and fairly complete understanding in quantitative terms of the role of hydrophobicity in drug action [25]. Hydrophobicity is not only related to absorption and distribution phenomena but also to the interactions with the active site of ChoK. It seems that besides the electrostatic interactions, the cationic heads and the spacers between the two positive nitrogen atoms appear to hydrophobically bind more strongly to the enzyme. Hydrophobic interactions are most important for non-covalent interactions in aqueous solution. Loosely associated water molecules at hydrophobic surfaces have a degree order and are, thus, in an unfavorable entropic state. The association of the hydrophobic areas of a drug and its binding site releases the ordered water molecules, which leads to a gain in entropy. It has been reported that the planar quinolinium and isoquinolinium cations bind to artificial receptors having aromatic rings more strongly than the alkylammonium cations do [26]. It must be kept in mind that we have tested all compounds in an ex vivo system using purified ChoK from yeast (in a test tube

without cells) as a target. This assay allows us to evaluate the effect on **1–4** activities without considering the possible effects on other properties such as permeability into intact cells, specific cellular environments, putative intracellular modifications of the synthesized compounds or enzyme compartmentalization.

The electrostatic interaction occurs between ChoK and the bisalts **1–4** and the immediate environment (water structure) can dramatically affect the occurrence of this phenomenon. What is also apparent is the fact that the biological effect cannot be solely attributed to the electrostatic interaction. This does not diminish the importance of the phenomenon, but rather reaffirms the need for investigators to visualize the drug as a composite of several physicochemical properties which contribute to its overall character.

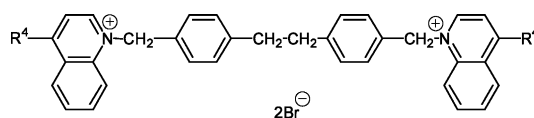
Due to the fact that both the electron-donating ability of the substituent at position 4 of the heteroaromatic cationic head (Table 2) and the lipophilicity of the bisalts (Table 3) play a very significant role in the inhibition of ChoK, we tried to put together these two features in the same molecule, and prepared compounds **4i** and **4j** (Table 4) with the idea of obtaining even more potent ChoK inhibitors. It must be pointed out that the $-\text{NH}_2$ group is a strong electron-donating group while the pivaloylamino moiety should be a weak one; for the latter its σ_R value is not available and we used the corresponding value of the very similar acetamido group ($\sigma_R = -0.35$) [21]. The results came up to our expectations and we next attempted to correlate the ChoK inhibitory potency with the two descriptors, obtaining eq 6, including all the compounds of Tables 2–4:

$$p(\text{IC}_{50})_{\text{ex vivo}} = 4.44 - 0.73(\pm 0.14)\sigma_R + 0.12(\pm 0.04)\text{clog } P$$

$$n = 19, r = 0.836, s = 0.241, F_{2,16}$$

$$= 18.61 \text{ (significance at } \alpha < 0.001 \text{)} \quad (6)$$

Table 4
Structure, biological results and parameter values for compounds **4i** and **4j**



Comp	R ⁴	(IC ₅₀) _{ex vivo} (μM) ^a	(IC ₅₀) _{antiprol} (μM) ^a	σ _R ^b	clog P ^c	π _{R4} ^d
4i	-NH ₂	10.0	2.00	-0.80	-2.13	-1.23
4j	-NHC(=O)Bu ^t	10.5	4.74	-0.35 ^e	1.40	2.18

^a All values are the mean of two independent determinations performed in duplicate.

^b σ_R: Electronic parameter for resonance effects (ref. [22]).

^c Predicted by using the Ghose–Crippen modified atomic contribution system (ATOMICS option, ref. [19]) of the PALLAS 2.0 programme.

^d π_{R4} = clog P_{R4} - clog P_H; clog P values have been calculated using the CDR option of PALLAS 2.0 programme.

^e Since the σ_R value of the pivaloylamino group is unavailable, we have used the acetamido one instead (see ref. [21]).

Interestingly, our QSAR studies showed that the potency of inhibitors does not necessarily depend on overall log P but on π at a specific site on the molecules.

The use of such site-specific π factors fits very well in Eq. (7):

$$p(\text{IC}_{50})_{\text{ex vivo}} = 0.55 - 1.04(\pm 0.13)\sigma_R + 0.63(\pm 0.15)\pi_{\text{spacer}}$$

$$+ 0.30(\pm 0.08)\pi_{\text{cat head}}$$

$$n = 19, r = 0.917, s = 0.181, F_{3,15}$$

$$= 26.46 \text{ (significance at } \alpha < 0.001 \text{)} \quad (7)$$

In deriving eq 7 we must know that π_{cat head} is zero (π_{2H} due to the H-2 and H-3 protons of the pyridinium moiety) for compounds **3a–j**, while is 1.27 (π_{CH₂=CH-CH-CH₂} is the contribution of the benzene ring fused to the pyridinium one) for the bisquinolinium and bisisoquinolinium structures **4**. π_{spacer} is the substituent constant of the $-\text{CH}_2-\text{C}_6\text{H}_4-(\text{CH}_2)_n-\text{C}_6\text{H}_4-\text{CH}_2-$ grouping, ranging from 5.14 ($n = 0$ of the spacer) and 6.28 ($n = 3$ of the spacer). Both the higher coefficient and values of π_{spacer} compared with those of π_{cat head} lead to the conclusion that the former hydrophobicity component is far more important than the latter. In conclusion, the positive coefficients of the π terms means that hydrophobic moieties and electron-donating groups favor the ChoK inhibitory activity, at least within the spanned range of spacers and heteroaromatic rings used.

2.6. Relationship between ChoK inhibition and antiproliferative activity

As our ultimate goal is to find a good antiproliferative drug, we next evaluated the parameters that influence the anticancer activity of the symmetrical compounds **2–4** (Tables 2–4) and the relationship between the antiproliferative potency in vitro and the inhibitory

potency *ex vivo* of ChoK was obtained by means of Eq. (8):

$$p(\text{IC}_{50})_{\text{antiprol}} = 2.14 + 0.68(\pm 0.25)p(\text{IC}_{50})_{\text{ex vivo}} - 0.04(\pm 0.02) \times (\text{clog } P)^2 - 0.90(\pm 0.25)\sigma_R$$

$$n = 17, r = 0.909, s = 0.276, F_{3,13} = 20.65 \text{ (significance at } \alpha < 0.001 \text{)} \quad (8)$$

The presence of the hydrophobicity term was to be expected because eq (8) correlates an *in vitro* activity (against the HT-29 cell line) with an *ex vivo* one (against ChoK).

Our recent findings suggest that these drugs not only induce cytostatic effects, but also induce an apoptotic response in tumoral cells without toxic effects on non tumoral cell lines (unpublished results). Half-molecule derivatives of **1**, **2**, **3**, **4c**, **4g**, **4i** and **4j** were highly toxic and much less potent as ChoK inhibitors (unpublished results). These observations correlate with the postulated multimeric form of the enzyme [27], suggesting that the bisphenyl structure of these inhibitors simultaneously interacts with two subunits of the enzyme, and thus, is more efficient for complete inhibition of ChoK.

2.7. *In vivo* antiproliferative activity

Some of the new ChoK inhibitors including **4j**³ were well tolerated at 5–10 mg/kg in mice, injected for five consecutive days [28]. Subsequent assays determined that the maximum tolerated dose for **4j** was 35 mg/kg. This compound was selected to be tested as antitumoural agent *in vivo*, due to its high antiproliferative activity and relative low toxicity. The effect of **4j** on HT-29 tumor xenografts (tumors were induced in nude mice by *s.c.* inoculation of HT-29 cells) using a schedule consisting of six daily doses of 35 mg/kg, separated by 1 week. Such a schedule caused an inhibition of 50% in the tumor growth [28].

3. Concluding remarks

In conclusion, it was particularly interesting to find that the enhanced antiproliferative activity of the new compounds was not accompanied by a potentiation of the neurological toxicity of HC-3. We successfully increased both the ChoK inhibitory and antiproliferative activities by substituting the oxazonium moiety of HC-3 by pyridinium, and quinolinium rings with different groups on their position 4, and using the 1,2-ethylene(bisbenzyl) moiety instead of the 4,4'-biphenyl

moiety of HC-3. In an attempt to understand the ChoK inhibitory activity, a quantitative structure–activity relationship was developed. The QSAR equations have described the forces involved in quantitative terms. Here we provide *in vivo* evidence that ChoK is a novel target for the design of antitumoural drugs.

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³ Compound **4j** corresponds to MN168B of ref. [28].

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