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QSAR of 1,1′-(1,2-ethylenebisbenzyl)bis(4-substitutedpyridinium) Dibromides as Choline Kinase Inhibitors: a Different Approach for Antiproliferative Drug Design

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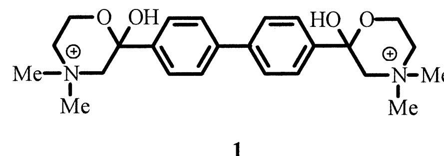
Abstract—Ten new structures of a series of the title compounds were synthesized and screened for their activity to inhibit choline kinase under ex vivo conditions. Their inhibitory potency correlates with the ^{13}C chemical shifts (in CD_3OD) of the methylene group bearing the positively charged nitrogen. The inhibitory effect on proliferation against the HT-29 cell line is strongly dependent on its ability to inhibit the production of phosphorylcholine. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction and Design Considerations

The rational design of novel antiproliferative and antitumour strategies is based on the recognition of novel intracellular targets for the modifications produced by the activity of different oncogenes and the changes in tumour suppressor genes.[1] Certain oncogenes have a high incidence in human tumours, for example, *ras* oncogenes, which are found in 30% of all human tumours. It has already been shown that when cells are transformed by these oncogenes, there is a constitutive activation of choline kinase (ChoK),[2–4] an enzyme responsible for the conversion of choline into phosphorylcholine (*P*Cho).[5] The increase in ChoK activity results in elevated levels of *P*Cho, a putative novel second messenger involved in proliferation.[6] It has been shown that hemicholinium-3 (**1**, HC-3), the most potent ChoK inhibitor,[7] blocks DNA synthesis stimulation by growth factors but has no effect on that of serum or insulin.[6] HC-3 is a competitive inhibitor of the high affinity choline transport system and has a high paralyzing respiratory effect.[8] This makes it impossible to use **1** as an in vivo antiproliferative agent. Thus, the first step for the development of these potential anticancer chemicals which affect ChoK is to improve the potency of **1** by modifying its specificity and antiproliferative activity.[9] We have reported symmetrical bis-quaternary derivatives with increased inhibitory activity towards ChoK,[10] and very recently, the in vivo antitumour activity of several ChoK inhibitors has been studied.[11]

The HC-3 structure contains a central biphenyl system to which two choline-like chains containing the quaternary groups are attached, forming oxazonium rings in solution.[8] We have synthesized ten bis-quaternary derivatives **2**–**11**, in which the modifications affect both the spacer and the two cationic heads of the prototype. The 1,4-oxazonium moieties of **1** were changed by pyridinium rings with different substituents on their position 4, with the aim of studying their electronic effects. Furthermore, in order to keep the number of atoms between the two positive charged nitrogen atoms of **1**, we used the 1,2-ethylene(bisbenzyl) moiety as a linker.



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Synthesis

The compounds for this study (Table 1, compounds **2**–**11**) were synthesized by reaction between the corresponding 4-substituted pyridines and bis-*p*-(bromomethyl)bibenzyl[12] in butanone at 100 °C in a sealed tube. After filtration the products were recrystallized from EtOH/Et_2O, except **6** (MeOH/Et_2O). 4-(*N,N*-diallylamino)-

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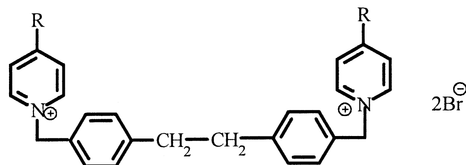
Table 1. Structure, biological results[a] and parameter values for the compounds **2**–**11**



Compound	R	$IC_{50ex\ vivo}$ (μM)	$IC_{50antiprol}$ (μM)	σ_P[b]	σ_I[c]	σ_R[d]	δCH_2N^+[e]	Σf[f]
2	$-NMe_2$	17[g]	2	−0.71	0.17	−0.88	61.541	0.473
3	$-NH_2$	23[g]	4	−0.63	0.17	−0.80	61.801	−0.842
4	$-CH_2OH$	100	>100	0.04	0.11	−0.07	64.794	−0.951
5	-Me	100	20	−0.17	−0.01	−0.16	64.628	0.701
6	-COOH	136.7	>1000	0.41	0.30	0.11	65.827	−0.071
7	-C≡N	>1000	200	0.65	0.57	0.08	66.526	−0.174
8	—N	17	0.55	—[h]	—[h]	—[h]	61.778	1.821
9	—N	20	1.0	—[h]	—[h]	—[h]	61.550	1.147
10	—N	9.6	0.40	—[h]	—[h]	—[h]	61.380	1.666
11	—N	15	0.40	—[h]	—[h]	—[h]	61.481	2.185

[a]All values are the mean of two independent determinations performed in duplicate.
[b]σ_P: Hammett constant for *para* substitution (ref 19).
[c]σ_I: Electronic parameter for inductive effects (ref 19).
[d]σ_R: Electronic parameter for resonance effects (ref 19).
[e]CH_2N^+: ^{13}C Chemical shift of the methylene bearing the positively charged nitrogen atom, in CD_3OD.
[f]Σf: sum of hydrophobic fragmental constants for substituent R (ref 22).
[g]Data from ref 10.
[h]Not available.

pyridine[13] and 4-piperidinopyridine[14] were prepared as previously reported. 4-Perhydroazepinopyridine (89%) is a new compound and was obtained from 4-chloropyridine and the corresponding secondary amine according to the method used for 4-(*N*,*N*-diallylamino)pyridine.[13] All new compounds gave satisfactory ^1H NMR, ^{13}C NMR, FABHRMS and combustion analyses.

Biological Testing

Compounds **2**–**11** were tested in an ex vivo system using purified ChoK from yeast as a target.[15] This assay allowed us to evaluate the effect on **2**–**11** 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 effects on cell proliferation by the ChoK inhibitors in *ras*-transformed cells were next investigated.[16,17] The Hill equation was fitted to the data to obtain estimates of the IC_{50}.

Discussion of Results

It should be noted that all the substituents R in Table 1 are electron-releasing, neutral or electron-withdrawing. From the results obtained it is clear that the presence of electron-withdrawing groups (-COOH, compound **6** and -C≡N, compound **7**) leads to inactive compounds as ChoK inhibitors. On the other hand, the presence of a strong electron-releasing group seems to be important, since the electron-neutral bearing compounds (**4** and **5**) are less potent than **2**. Since the $-NH_2$ group participates in the delocalization of the ring charge, and carries a fractional positive charge which makes the hydrogens more acidic,[18] it is an excellent H-bond donor. When replaced by the $-NMe_2$ group (compound **2**) it does not lose potency, and evidently does not act as an H-bond donor. Most probably neither does it act as an H-bond acceptor, since its lone pair of electrons, involved in the delocalization of the ring charge, are not readily available for H-bonding. It is clear that the more electron-releasing that R is, the more potent the compound is. The null importance of H-bonding is reaffirmed, as the

compounds bearing the -CH_2OH and -Me fragments (compounds **4** and **5**, respectively) have the same potency. This trend can be quantified using the Hammett constant for *para* substitution,[19] σ_P, and the correlation equation that results is 1:

$$p(IC_{50})_{ex\ vivo} = 4.08\,(\pm 0.08) - 0.84\,(\pm 0.17)\,\sigma_P$$
$$n = 5, r = 0.943, s = 0.160\ (\text{significance at } P < 0.025) \quad (1)$$

where $pIC_{50} = -\log IC_{50}$, n is the number of compounds, r is the correlation coefficient, s is the standard deviation, and data within parentheses are standard errors of estimate.

σ_P is a descriptor of the overall electronic effect of R. When this is separated into its inductive and resonance components as represented by σ_I[19] and σ_R[19] respectively, the following correlations result:

$$p(IC_{50})_{ex\ vivo} = 4.22\,(\pm 0.30) + 0.19\,(\pm 2.15)\,\sigma_I$$
$$n = 5, r = 0.052, s = 0.482 \quad (2)$$

$$p(IC_{50})_{ex\ vivo} = 3.92\,(\pm 0.00) - 0.92\,(\pm 0.06)\,\sigma_R$$
$$n = 5, r = 0.994, s = 0.052\ (\text{significance at } P < 0.001) \quad (3)$$

The inhibitory potency of the compounds does not correlate with the inductive effect of R but correlates well with its resonance effect. This is consistent with conventional chemical concepts. R is in direct conjugation with the positively charged ring nitrogen. It is, therefore, probable that R has little inductive effect from such a distance, while its resonance effect would dominate since it does not depend on distance. The greater resonance effect of R would obviously cause better delocalization of the positive charge. Following these findings, a substituent of higher electron-releasing effect than the -NH_2 or -NMe_2 groups was sought. Therefore, an endocyclic amino group, such as the pyrrolidino, piperidino and the perhydroazepino moieties, could exert a greater electron-releasing effect. Note that the corresponding Hammett substituent constants (σ_P, σ_R, σ_I) are available only for compounds **2**–**7**, and hence attempts were made to correlate the inhibitory effects with other electronic parameters, trying to obtain a larger set of data. The NMR chemical shift serves as a sensitive probe for the estimation of the electronic effects of substituents. In fact, the electron distribution of **2**–**11**, according to the nature of the R group, is responsible for the ^{13}C-NMR chemical shifts of all the carbon atoms of the pyridinium fragment and particularly of the methylene moiety bearing the positively charged nitrogen atom.[20] The ^{13}C-NMR spectra (in CD_3OD) were, therefore, determined for compounds **2**–**11**, and the existence of any correlation between the $p(IC_{50})_{ex\ vivo}$ and the chemical shift was investigated. The substituent-produced chemical shifts show a clear trend in their dependence on the nature of the substituents. In fact, the data for compounds in Table 1 demonstrate that the donors shift the methylene resonance upfield and strong electron donors produce the maximum displacement. Obviously, the opposite holds for electron-withdrawing groups. Hydrogen bonding interaction between a substituent and solvent can lead to substantial modifications in the electronic properties of the substituents.[21] It has to be noted, however, that an excellent correlation is obtained between the ex vivo inhibitory potency and the δCH_2N^+ (eq. (4)):

$$p(IC_{50})_{ex\ vivo} = 19.35\,(\pm 1.20) - 0.24\,(\pm 0.04)\,\delta CH_2N^+$$
$$n = 9, r = 0.978, s = 0.097\ (\text{significance at } P < 0.001) \quad (4)$$

Again, it may be suggested that this is because there is no hydrogen bonding between the nitrogen lone pair of -NMe_2 or the hydrogens of -NH_2, and between the active site of the enzyme or the NMR solvent (CD_3OD).

The correlation observed between the inhibitory potency under ex vivo conditions and the ^{13}C-NMR chemical shifts of the symmetrical bis-pyridinium compounds, confirms that it is a suitable and straightforward technique for the determination of their ChoK inhibition. In addition, as NMR is a nondestructive technique, the compounds may always be recovered.

As the main aim of this communication is to study compounds with selective antitumour activity, the relationship between the antiproliferative potency in vitro and the inhibitory potency ex vivo of ChoK was obtained by means of eq (5), in which Σf is the sum of the Rekker hydrophobic fragmental constants[22] for R:

$$p(IC_{50})_{antiprolif.} = -1.35 + 1.48\,(\pm 0.17)\,p(IC_{50})_{ex\ vivo} + 0.25\,(\pm 0.05)\,\Sigma f$$
$$n = 7, r = 0.987, s = 0.123\ (\text{significance at } P < 0.001) \quad (5)$$

The presence of the hydrophobicity term was to be expected as eq. (5) correlates an in vitro activity (against the HT-29 cell line) with an ex vivo one (against ChoK). Nevertheless, the small value of the coefficient of Σf draws attention. Hydrophobicity is not only related to absorption and distribution phenomena but also to the interactions with the receptor sites. It has been argued that small coefficients affecting Σf (up to 0.5) reflect partial desolvation and binding along the surface of the enzyme, although this view is under debate.[23]

The correlation between the antiproliferative activity, the electronic and the hydrophobicity descriptors is excellent (eq. (6)). In general, it is preferable to scale descriptors to approximately the same numerical values. This has the advantage that coefficients can be readily compared and the stability of the statistical analyses is also improved.[24] If the δCH_2N^+ descriptor is scaled by 0.1, $\delta^* CH_2N^+$ and Σf became roughly equiscalar. In this light, it is easily shown that the electronic effect is more important than the hydrophobicity term.

$$p(IC_{50})_{antiprolif.} = 29.68 - 3.90\,(\pm 0.22)\,\delta^{*}CH_2N^{+} + 0.33\,(\pm 0.04)\,\Sigma f$$
$$n = 8,\ r = 0.996,\ s = 0.098\ \text{(significance at } P < 0.001) \quad (6)$$

where $\delta^{*}CH_2N^{+} = 0.1 \times \delta CH_2N^{+}$

Conclusion

The 4-amino group makes a substantial contribution for ChoK inhibitory activity, acting neither as a H-bond donor nor as a H-bond acceptor. Its replacement by other groups and QSAR on the resultant compounds suggests that an electronic effect (expressed as the ^{13}C-NMR chemical shifts in CD_3OD) remains the prime factor, probably via delocalization of the positive charge of the pyridinium ring. These compounds have antiproliferative activity against the human HT-29 cell line, which is more efficient towards oncogene-transformed cells where a constitutive increase in ChoK is observed.[2–4] These compounds have IC_{50} values in the submicromolar range and may constitute leads for the development of more potent and selective inhibitors of ChoK.

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16. Typical procedure: HT-29 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% new-born calf serum (NCS) under standard conditions of temperature (37 °C), humidity (95 °C), and carbon dioxide (5%). The cells were seeded on 24-well plates and incubated for 24 h in DMEM supplemented with 10% NCS. Then the cells were washed with TD buffer (137 mM NaCl, 5 mM KCl, 20 mM Tris, pH 7.4) and incubated in DMEM containing the indicated amounts of serum and different concentrations of 5-FU derivatives. Three days later, the wells were aspirated, fresh medium added, and the cells maintained for three additional days. Quantification of the cells remaining in each well was carried out using the Crystal Violet method (See ref 17), with some modifications. Briefly, the cells were washed with TD buffer and fixed with 1% glutaraldehyde for 15 min. After having been washed again with TD, cell nuclei were stained with 0.1% Crystal Violet for at least 30 min, and washed three times with distilled water. Adsorbed dye was resuspended in 10% acetic acid and absorbance at 595 nm was determined in a spectrometer.
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