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Short communication

Choline kinase inhibitory effect and antiproliferative activity of new 1,1′,1″-(benzene-1,3,5-triylmethylene)tris{4-[(disubstituted)amino]pyridinium} tribromides

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Abstract

Four derivatives of 1,1′-(benzene-1,3-diylmethylene)bis{4-[(disubstituted)amino]-pyridinium} dibromides (**2**–**5**) and six derivatives of 1,1′,1″-(benzene-1,3,5-triylmethylene)-tris{4-[(disubstituted)amino]pyridinium} tribromides (**6**–**11**) were synthesised and examined for their inhibition of human choline kinase (ChoK) and antiproliferative activities. The latter are more potent as ChoK inhibitors than the former, but the antiproliferative activities against the HT-29 cell line show the opposite tendency. The higher affinity of the trispyridinium compared with the bispyridinium ones may be due to direct binding of the third pyridinium group to ChoK or may arise from a reduction of the unfavourable entropy of binding via an increase of the 'local concentration' of pyridinium groups.

Keywords: Antiproliferative agents; HT-29; Human choline kinase; Bispyridium; Trispyridinium

1. Introduction

It is thought that the accumulation of certain mutated genes, including oncogenes, tumour suppressor genes, genes for DNA-repair enzymes, and invasion/metastasis-related genes, is necessary for the onset and progression of cancer. Mutation may cause further malignant changes in cellular proliferation, especially in enzymatic properties and activity [1–3]. Some of the changes in enzymatic properties and activity with proliferation may be advantageous to the cancer cells [4–7]. Studying the cellular properties of cancer cells improves our understanding of the mechanism of cellular growth control and sheds light on cancer prevention and treatment [8].

Choline kinase (ChoK) is the first enzyme in the cytidine 5′-diphosphate-choline pathway for the synthesis of phosphatidylcholine, and phosphorylates choline to phosphorylcholine (*P*Cho) using adenosine 5′-triphosphate (ATP) as the phosphate donor [9–11]. In vitro studies of oncogenic Ras proteins, products and growth factors have shown that *P*Cho contributes to cellular growth regulation and intracellular signal transduction. Ras proteins play a pivotal role in cellular signal transduction, and help regulate cellular proliferation and terminal differentiation [12–14]. Growth factors essential for cellular growth activate ChoK, elevating the intracellular *P*Cho level [15]. Furthermore, nuclear magnetic resonance (NMR) spectroscopy has demonstrated higher concentrations of *P*Cho in human tumour tissues and growth-promoted cells [16,17]. These results suggest that *P*Cho and ChoK may not only play a role in phospoholipid synthesis, but also in regulating cellular growth in cancer cells. Inhibition of ChoK has been demonstrated to be a novel efficient antitumour

Abbreviations: ChoK, choline kinase; IC_{50}, concentration resulting in 50% inhibition; *P*Cho, phosphorylcholine; QSAR, quantitative structure–activity relationship; SAR, structure–activity relationship.

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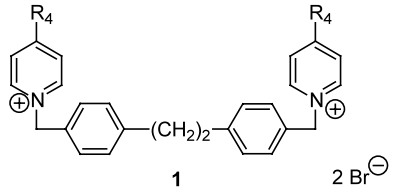
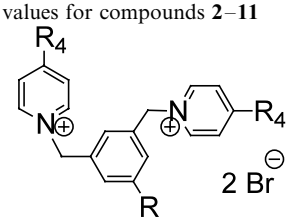


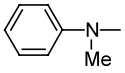
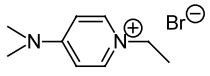
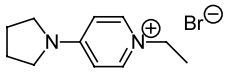
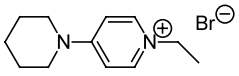
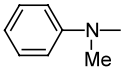
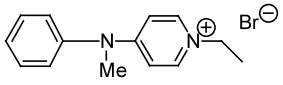
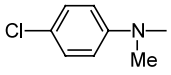
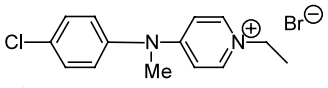
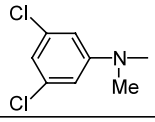
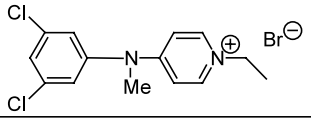
Fig. 1.

strategy in oncogene-transformed cells [8] and in vivo assays in the nude mice system [18].

Compound **1** being $R_4 = N$-methylanilino (Fig. 1) has recently been shown to be both the most potent ChoK inhibitor and antiproliferative agent against the HT-29 human colon cancer line ever reported [19]. We have previously studied the influence of the R_4 group on the ChoK inhibitory and the antiproliferative activities, using electron-withdrawing, -neutral and electron-donating groups [20]. The 4-(disubsituted)amino group contributes substantially and its role has been suggested to be electronic, probably via delocalisation of the positive charge of the ring [20]. Quantitative structure–activity relationship (QSAR) analysis on analogues of the general structure **1** has revealed that the ChoK inhibitory potency correlates well with the partial charge on the ring N atom (obtained from AM1 semiempirical molecular orbital calculations) and with

Table 1
Structure, biological results and calculated log *P* values for compounds **2**–**11**



Compound	R_4	R	IC_{50} (ChoK)	IC_{50} (HT-29)	clog *P*[a]
2		H	>100	>100	-5.22
3		H	84.2	50	-4.33
4		H	52.8	43	-3.31
5		H	37.5	5.1	-2.01
6			>100	>100	-8.83
7			7.8	>100	-7.48
8			1.5	>100	-5.97
9			5.1	79	-4.02
10			1.4	18.3	-1.86
11			3.4	21.5	1.04

[a]Predicted by using the Ghose-Crippen modified atomic contribution system [26] (ATOMIC5 option) of the PALLAS 2.0 programme [27].

the energy of the lowest unoccupied molecular orbital (E_{LUMO}) [21].

Another structural feature of compounds **1** that merits investigation is the two positive charges. Since there are two pyridinium groups in **1**, one may question whether both are required for ChoK inhibition. To address this issue, the monopyridinium compounds were synthesised and tested ex vivo for ChoK inhibition. However, such compounds (half-molecule derivatives of **1**) proved to be less potent in their action than **1** (unpublished results). This low potency prevented the determination of reliable IC_{50} values for ChoK inhibition, leaving unanswered the question of the contribution of both pyridinium groups of **1** to ChoK inhibition. In the present work, the bispyridinium compounds **2**–**5** were synthesised and tested for inhibition of human ChoK and antiproliferative activities against the HT-29 cell line and their activities compared with those of the trispyridinium compounds **6**–**11** (Table 1) in order to study the influence of the third positive charge. Trispyridinium compounds are scarcely referred to in bibliography. The exception to the rule is given by Suckling [22] who demonstrated that benzene 1,3,5-tris(11-pyridiniumdecanyl)tricarboxylate trichloride forms complexes with phenol in methanol or acetonitrile. The benzene-1,3-diylmethylene linker was chosen as spacer for the bispyridinium compounds because it permits the introduction of a third branching (giving the benzene-1,3,5-triylmethylene spacer with the presence of a third reactive centre that could be quaternised further), maintaining basically the same scaffolding for both compounds **2**–**5** and **6**–**11**. A full discussion of the syntheses of the triscationic salts is included.

2. Chemistry

In this paper we have synthesised four symmetrical bispyridinium derivatives **2**–**5** and six symmetrical trispyridinium derivatives **6**–**11** (Table 1). The C-4 substituents of the pyridinium moiety are always strong electron-releasing groups (–NMe_2, pyrrolidino, piperidino and several *N*-methylanilino groups).

The compounds for this study were synthesised by reaction between the corresponding 4-substituted pyridines and α,α′-dibromo-*m*-xylene (in a 2/1 molar ratio) or 1,3,5-tris(bromomethyl)benzene (in a 3/1 molar ratio) in butanone at 100 °C in a sealed tube (Fig. 2). After filtration the products were recrystallised from EtOH or MeOH after adding Et_2O to turbidity. All new salts exhibit satisfactory ^{1}H-NMR, ^{13}C-NMR, and HR LSIMS in accord with their proposed structures.

An aspect of these polycationic organic salts, observed in previously reported syntheses [18–20], is that all salts exhibit significant hygroscopic characteristics, analysing for varying amounts of associated water in combustion analyses. The presence of two molecules of EtOH associated with **10** was confirmed by both elemental analysis and ^{1}H-NMR spectroscopy.

Most quaternisation reactions involving an annular nitrogen atom and an alkylating agent proceed by way of an S_N2 reaction [23]. The solvent plays a very important role in this type of reactions; for the choice of the most appropriate solvent for quaternisation reactions, one has to take into account that the species comprising the transition state of the reaction is more polar than the reagents and that the lone of the nitrogen of the pyridine must be free to attack the electrophile. Therefore, polar (because they stabilise the transition state), aprotic (because they do not interact with the *N*-lone pair by hydrogen bonding) solvents are the best to use. Butanone is the ideal solvent that leads to the title compounds. Nevertheless, in the case of the triscationic compound **6** (with the Me_2N– group at position 4 of the pyridinium moiety) ethanol was used due to the very scarce solubility of this compound in butanone (vide infra) as a consequence of its extreme polarity.

Another aspect that is worthy of pointing out is the reaction time that varies from 15 to 192 h. It has been observed that yields increase notably as timing augments. Moreover, purification of the final compounds is made easier since all the bromine atoms of the spacer are substituted thus avoiding the combined formation of the mono-, bis- and tris-salts. These compounds are very difficult to purify by recrystallisation due to the similarity of their physicochemical properties. Synthesis of **7** proves this statement: (a) on one hand, the monocationic salt **12** (20% yield) is obtained when the reaction time is 18 h, after recrystallysation from ethanol; (b) on the other, isolation of the pure target

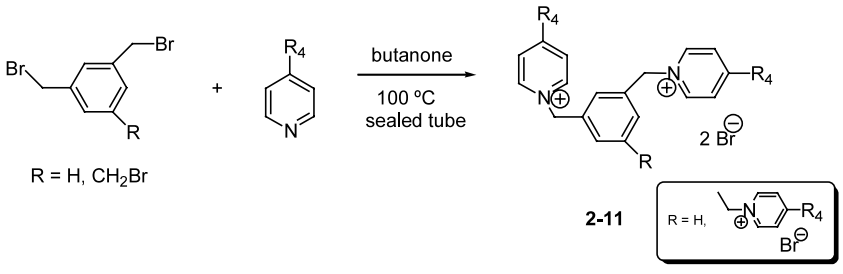


Fig. 2.

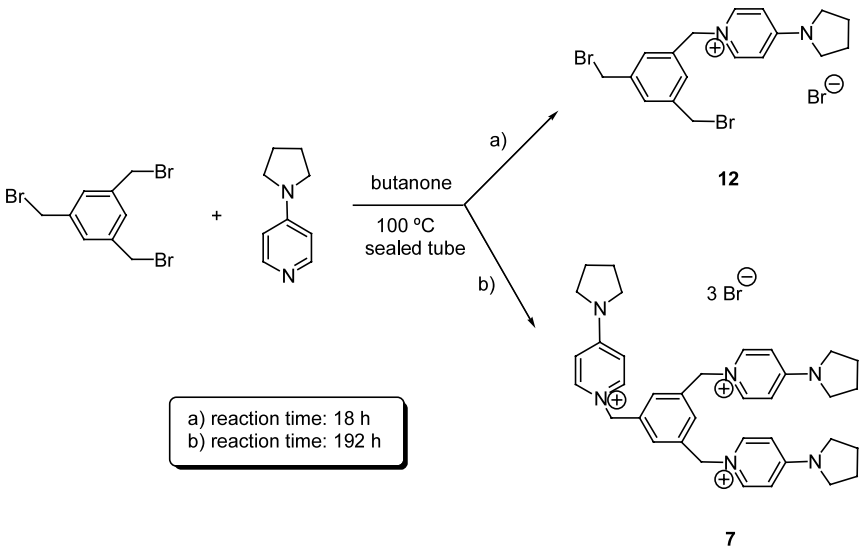


Fig. 3.

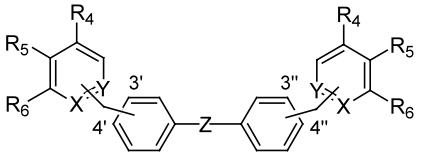
compound **7** (93% yield) is accomplished simply by filtering the solid and washing with the solvent when the reaction time is 192 h (Fig. 3).

Finally, the importance of the temperature in the quaternisation reactions must be highlighted due to its influence in the solubility of the mono- and di-quaternised intermediates. S_N2 takes place at room temperature, but the monocationic compound proceeding from the first bromine atom precipitates in butanone and must be dissolved again so that the quaternisation of the second pyridine moiety takes place. In the case of the final triscationic compounds the bisquaternised intermediates will have to dissolve themselves to complete the quaternisation reaction. Monocationic compound **13** was isolated and identified by ^{1}H-NMR and elemental analysis. Its preparation is shown in Fig. 4.

Thus, an increase of the temperature together with an elevated reaction time contribute to the solubility of intermediates, giving rise to an increase of yield of the reaction.

3. Pharmacology

Compounds **2**–**11** were tested in an ex vivo system using purified 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 [24], making these cells appropriate for the search of new antitumour drugs. The Hill equation was fitted to the data to obtain estimates of the IC_{50}. The activity in the in vitro assay



R_4 : electron-releasing, neutral or electron-withdrawing groups

R_5 + R_6: 2H
: $(CH=CH)_2$

X, Y: $N^{\oplus}$, CH
: CH, $N^{\oplus}$

Z: $(CH_2)_n$, being n = 0,1, 2, 3
: (CH=CH) *cis* or *trans*

Spacer isomer: 4',4'' or 3',3''

Fig. 5.

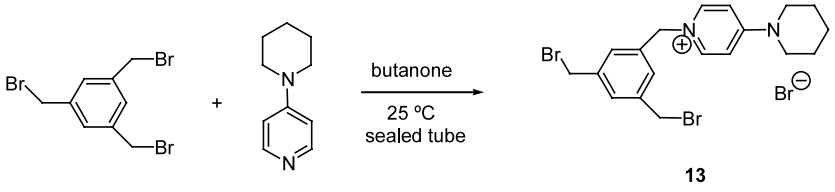


Fig. 4.

reflects the pharmacodynamic properties of the drugs rather than their affinity for the enzyme. In other words, it reflects how much of the drug can reach the enzyme rather than how well it binds to it.

4. Results and discussion

In a previous paper we have demonstrated that the electron characteristic of the substituent at position 4 of the heterocycle of the biscationic organic salts **14** (Fig. 5) and the theoretical lipophilic character of the whole molecule significantly affect the antitumoural activity against the HT-29 cancer cell line [19].

We have tried to correlate the yeast ChoK inhibitory activity (ex vivo assay) of the whole set of compounds **14** [19] with the distance between the two quaternary N atoms and the attempts have been fruitless. The linkers are very flexible and it is not easy to decide which is the most stable conformation; moreover, the differences in energy between the lowest energy conformations are very small and, hence, the interconversion between them is very easy. This result raises a number of questions about the way in which these bisquaternary compounds interact with ChoK. Unpublished observations indicate that both positive-charged heterocycles are important for the ChoK inhibitory activity, and consequently the spacing of the two groups can be expected to be important for activity. However, there is a potential complication in that molecular orbital calculations show that the positive charge of the pyridinium group [21] is extensively delocalised over the H atoms, and the increase in potency associated with the presence of an amino substituent at position 4 of the pyridinium ring has been attributed to even greater delocalisation of the positive charge [20]. Consequently, there may be considerable tolerance in the spacing of the heterocycle groups, and it may be misleading in this situation to use interatomic distances as a variable in SAR studies.

Accordingly, we decided to use the benzene-1,3-diylmethylene moiety (with just one benzene ring) as a linker that connects the two positive nitrogen atoms of the pyridinium fragments; moreover, position 5 of this spacer could be further functionalised (through the benzene-1,3,5-tryilmetilene spacer) to study the influence of a third positive charge on the biological activities of our organic salts.

Biological results and the clog *P* values for compounds **2**–**11** are given in Table 1. Such clog *P* values were calculated by using the Ghose–Crippen modified atomic contribution system [25] (ATOMIC5 option) of the PALLAS 2.0 programme [26]. If compounds **2**–**5** are compared for their Chok inhibitory and antitumoural activities, we could argue that the lipophilicity of the molecule plays a leading role. It is clearly observed that as the lipophilicity increases (going from **2** to **5**) both biological activities augment also. The same trend is observed with the triscationic salts **6**–**9** in relation to the inhibitory ChoK activities; nevertheless, the antiproliferative activities of **6**–**9** are lower if they are compared with those of the corresponding biscationic analogues and they follow an opposite tendency. We could conclude that the introduction of a third positive charge leads to compounds that are between 7- and 35-fold more potent as Chok inhibitors (**3/7** > 10, **4/8** > 35, and **5/9** > 7) whereas the antiproliferative activity exhibits a > 15-fold drop in activity on passing from **5** to **9** (in the case of **6/2**, **7/3**, and **8/4** it is not possible to quantify how less active are the trispyridinium compounds compared with their corresponding biscationic analogues). Such a decrease in the antiproliferative response might be explained on the basis that the decrease in lipophilicity of compounds **6**–**9** unfavourably affects the passage through the cytoplasmic membrane. According to the fundamental role played by lipophilicity in relation to both biological activities of the trispyridinium derivatives **6**–**9**, we decided to introduce one chlorine atom in the *para* position or even two chlorine atoms in positions 3 and 5 of the *N*-methylanilino group (to give molecules **10** and **11**, respectively) and the results came up to our expectations. However, compounds **10** and **11** are nearly equipotent as both ChoK inhibitors and anticancer compounds. It is possible that one chlorine atom in the *N*-anilino fragment establishes the steric limit, over which the biological activity may start to decrease.

It could be questioned whether the increase in the ChoK inhibitory potency resulting from the presence of the third pyridinium group in the molecules **7**–**11** is large enough to be attributed to binding of this group to a third specific site in the enzyme. The possibility exists that the increase in affinity results from a statistical effect of three rather than two potential binding groups being present in the molecule. This would increase the 'local concentration' of the pyridinium groups in the vicinity of the enzyme, resulting in a less unfavourable entropy for the binding process. Alternatively, the third pyridinium group may be binding at a site, which is chemically different from the other two, resulting in a lower free energy gain.

5. Conclusions

Trispyridinium compounds **7**–**11** are more potent than the bispyridinium ones **3**–**5** as ChoK inhibitors. The contribution of the third pyridinium group of these compounds may arise from direct binding to the enzyme, or may be the consequence of reducing the unfavourable entropy of binding via increasing the 'local concentration' of pyridinium groups. Nevertheless, **7**–**11** are less active than **3**–**5** as antiproliferative agents

because the latter show better lipophilicity to cross the cytosolic membranes. Future work will concentrate on the synthesis and biological evaluation of more rigid analogues in the search of the 'active' conformation of these ChoK inhibitors.

6. Experimental protocols

6.1. Chemistry

For general procedures, see ref. [21]. DEPT experiments were carried out for the identification of the CH_3, CH_2, CH and C carbons. The following parameters were used for such experiments: PW (135°); 9.0 μs; recycle time 1 s; $1/2J(CH) = 4$ ms; 65 536 data points acquired and transformed from 1024 scans; spectral width, 15 kHz, and line broadening, 1.3 Hz.

6.1.1. General procedure for compounds 2–13

A butanone solution of α,α′-dibromo-*m*-xylene or 1,3,5-tris(bromomethyl)benzene and the corresponding 4-substituted pyridine (in a 1:2 molar ratio for the preparation of the biscationic organic salts and in a 1:3 molar ratio for the triscationic ones) was heated at 100 °C in a sealed tube for a period of time that goes from 15 to 192 h. In the case of **13**, the temperature was 25 °C. After filtration and thorough washing with butanone, ethyl acetate and diethyl ether, the solid was purified by recrystallisation from ethanol or methanol after adding diethyl ether to turbidity.

*6.1.1.1. 1,1′-(Benzene-1,3-diylmethylene)bis[(4-dimethylamino)pyridinium)] dibromide (**2**).* Yield: 64.2%. Melting point (m.p.) 327 °C. ^{1}H-NMR (300.13 MHz, CD_3OD) δ 8.28 (d, $J = 7.7$, 4H, H-2,6$_{py}$), 7.57 (s, 1H, H-2$_{Ph}$), 7.52–7.39 (m, 3H, H-4,5,6$_{Ph}$), 7.04 (d, $J = 7.7$, 4H, H-3,5$_{py}$), 5.43 (s, 4H, CH_2N^+), 3.26 (s, 12H, CH_3). ^{13}C-NMR (75.57 MHz, CD_3OD) δ 157.88 (C-4$_{py}$), 143.08 (C-2,6$_{py}$), 137.39 (C-1,3$_{Ph}$), 131.31 (C-5$_{Ph}$), 129.84 (C-4,6$_{Ph}$), 129.36 (C-2$_{Ph}$), 109.11 (C-3,5$_{py}$), 61.05 (CH_2N^+), 40.30 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{22}H_{28}N_4Br_2Na$ $[M+Na]^+$ 529.0578. Found: *m/z* 529.0576. Anal. $C_{22}H_{28}N_4Br_2 \cdot 0.5H_2O$ (C, H, N).

*6.1.1.2. 1,1′-(Benzene-1,3-diylmethylene)bis[4-(pyrrolidino)pyridinium] dibromide (**3**).* Yield: 63.2%. M.p. 324 °C. ^{1}H-NMR (300.13 MHz, CD_3OD) δ 8.26 (d, $J = 7.6$, 4H, H-2,6$_{py}$), 7.57 (s, 1H, H-2$_{Ph}$), 7.51–7.38 (m, 3H, H-4,5,6$_{Ph}$), 6.87 (d, $J = 7.6$, 4H, H-3,5$_{py}$), 5.41 (s, 4H, CH_2N^+), 3.56 (t, $J = 6.8$, 8H, H-2,5$_{pyrr}$), 2.11 (q, $J = 6.8$, 8H, H-3,4$_{pyrr}$). ^{13}C-NMR (75.57 MHz, CD_3OD) δ 155.18 (C-4$_{py}$), 143.13 (C-2,6$_{py}$), 137.60 (C-1,3$_{Ph}$), 131.41 (C-5$_{Ph}$), 129.92 (C-4,6$_{Ph}$), 129.48 (C-2$_{Ph}$), 109.24 (C-3,5$_{py}$), 61.21 (CH_2N^+), 49.79 (C-2,5$_{pyrr}$), 26.14 (C-3,4$_{pyrr}$). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{26}H_{32}N_4Br$ $[M-Br]^+$ 479.1810. Found: *m/z* 479.1812. Anal. $C_{26}H_{32}N_4Br_2 \cdot 1.1H_2O$ (C, H, N).

*6.1.1.3. 1,1′-(Benzene-1,3-diylmethylene)bis[4-(piperidino)pyridinium] dibromide (**4**).* Yield: 67%. M.p. 316 °C. ^{1}H-NMR (300.13 MHz, CD_3OD) δ 8.25 (d, $J = 7.6$, 4H, H-2,6$_{py}$), 7.59 (s, 1H, H-2$_{Ph}$), 7.51–7.38 (m, 3H, H-4,5,6$_{Ph}$), 7.15 (d, $J = 7.6$, 4H, H-3,5$_{py}$), 5.39 (s, 4H, CH_2N^+), 3.72 (t, $J = 5.1$, 8H, H-2,6$_{pip}$), 1.71 (m, $J = 5.1$, 12H, H-3,4,5$_{pip}$). ^{13}C-NMR (75.57 MHz, CD_3OD) δ 156.85 (C-4$_{py}$), 143.67 (C-2,6$_{py}$), 137.50 (C-1,3$_{Ph}$), 131.45 (C-5$_{Ph}$), 130.05 (C-4,6$_{Ph}$), 129.64 (C-2$_{Ph}$), 109.42 (C-3,5$_{py}$), 61.04 (CH_2N^+), 49.15 (C-2,6$_{pip}$), 26.72 (C-3,5$_{pip}$), 24.95 (C-4$_{pip}$). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{28}H_{36}N_4Br$ $[M-Br]^+$ 507.2123. Found: *m/z* 507.2121. Anal. $C_{28}H_{36}N_4Br_2 \cdot 1H_2O$ (C, H, N).

*6.1.1.4. 1,1′-(Benzene-1,3-diylmetilene)bis[4-(N-methyanilino)pyridinium] dibromide (**5**).* Yield: 75.1%. M.p. 327 °C. ^{1}H-NMR (300.13 MHz, CD_3OD) δ 8.31 (bs, 4H, H-2,6$_{py}$), 7.60 (t, $J = 7.6$, 4H, H-3,5$_{anil}$), 7.59 (s, 1H, H-2$_{Ph}$), 7.51 (m, 4H, H-4$_{anil}$), 7.37 (d, $J = 7.6$, 4H, H-2,6$_{anil}$), 7.51–7.37 (m, 4H, H-4,5,6$_{Ph}$), 6.99 (bs, 4H, H-3,5$_{py}$), 5.44 (s, 4H, CH_2N^+), 3.55 (s, 6H, CH_3). ^{13}C-NMR (75.57 MHz, CD_3OD) δ 158.34 (C-4$_{py}$), 144.65 (C-1$_{anil}$), 143.64 (C-2,6$_{py}$), 137.19 (C-1,3$_{Ph}$), 131.89 (C-3,5$_{anil}$), 131.36 (C-5$_{Ph}$), 130.04 and 130.02 (C-4,6$_{Ph}$ and C-4$_{anil}$), 129.63 (C-2$_{Ph}$), 127.38 (C-2,6$_{anil}$), 110.31 (C-3,5$_{py}$), 61.39 (CH_2N^+), 41.34 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{32}H_{32}N_4Br$ $[M-Br]^+$ 551.1810. Found: *m/z* 551.1810. Anal. $C_{32}H_{32}N_4Br_2 \cdot 0.2H_2O$ (C, H, N).

*6.1.1.5. 1,1′,1″-(Benzene-1,3,5-tryilmetilene)tris[(4-dimethylamino)piridinium)] tribromide (**6**).* Yield. 60.1%. M.p. 332 °C. ^{1}H-NMR (400.12 MHz, CD_3OD) δ 8.28 (d, $J = 7.6$, 6H, H-2,6$_{py}$), 7.51 (s, 3H, H-2,4,6$_{Ph}$), 7.02 (d, $J = 7.6$, 6H, H-3,5$_{py}$), 5.42 (s, 6H, CH_2N^+), 3.25 (s, 18H, CH_3).^{13}C-NMR (100.62 MHz, CD_3OD) δ 158.56 (C-4$_{py}$), 143.32 (C-2,6$_{py}$), 138.66 (C-1,3,5$_{Ph}$), 130.01 (C-2,4,6$_{Ph}$), 109.35 (C-3,5$_{py}$), 60.74 (CH_2N^+), 40.46 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{30}H_{38}N_6Br$ $[M-BrH-Br]^+$ 561.2341. Found: *m/z* 561.2339. Anal. $C_{30}H_{39}N_6Br_3 \cdot 2.7H_2O$ (C, H, N).

*6.1.1.6. 1,1′,1″-(Benzene-1,3,5-triylmethylene)tris[4-(pyrrolidino)pyridinium] tribromide (**7**).* Yied: 93%. M.p. 109 °C (gel)—progressive fusion up to 275 °C. ^{1}H-NMR (300.13 MHz, CD_3OD) δ 8.28 (d, $J = 7.6$, 6H, H-2,6$_{py}$), 7.55 (s, 3H, H-2,4,6$_{Ph}$), 6.89 (d, $J = 7.6$, 6H, H-3,5$_{py}$), 5.43 (s, 6H, CH_2N^+), 3.57 (t, 12H, H-2,5$_{pyrr}$, $J = 6.7$), 2.13 (q, $J = 6.7$, 12H, H-3,4$_{pyrr}$). ^{13}C-NMR (75.57 MHz, CD_3OD) δ 155.19 (C-4$_{py}$), 143.19 (C-2,6$_{py}$), 138.73 (C-1,3,5$_{Ph}$), 129.98 (C-2,4,6$_{Ph}$), 109.93 (C-3,5$_{py}$), 60.76 (CH_2N^+), 49.82 (C-2,5$_{pyrr}$), 26.14 (C-3,4$_{pyrr}$). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{36}H_{45}N_6Br_2$

$[M-Br]^+$ 719.2072. Found: *m/z* 719.2075. Anal. $C_{36}H_{45}N_6Br_3 \cdot 3.8H_2O$ (C, H, N).

*6.1.1.7. 1,1′,1″-(Benzene-1,3,5-triylmethylene)tris[4-(piperidino)pyridinium] tribromide (**8**).* Yield: 98.1%. M.p. 248 °C (gel)—318 °C (fusion). ^{1}H-NMR (300.13 MHz, DMSO-d_6) δ 8.38 (d, $J = 6.9$, 6H, H-2,6$_{py}$), 7.49 (s, 3H, H-2,4,6$_{Ph}$), 7.25 (d, $J = 6.9$, 6H, H-3,5$_{py}$), 5.40 (s, 6H, CH_2N^+), 3.71 (bs, 12H, H-2,6$_{pip}$), 1.60 (m, 18H, H-3,4,5$_{pip}$). ^{13}C-NMR (75.57 MHz, DMSO-d_6) δ 154.74 (C-4$_{py}$), 142.46 (C-2,6$_{py}$), 137.20 (C-1,3,5$_{Ph}$), 127.78 (C-2,4,6$_{Ph}$), 108.04 (C-3,5$_{py}$), 58.36 (CH_2N^+), 47.36 (C-2,6$_{pip}$), 25.19 (C-3,5$_{pip}$), 23.31 (C-4$_{pip}$). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{39}H_{51}N_6Br_2$ $[M-Br]^+$ 761.2542. Found: *m/z* 761.2539. Anal. $C_{39}H_{51}N_6Br_3 \cdot 4.8H_2O$ (C, H, N).

*6.1.1.8. 1,1′,1″-(Benzene-1,3,5-triylmethylene)tris[4-(N-methylanilino)pyridinium] tribromide (**9**).* Yield: 38.3%. M.p. 90 °C (gel)—314 °C (melting). ^{1}H-NMR (400.12 MHz, DMSO-d_6) δ 8.44 (bs, 6H, H-2,6$_{py}$), 7.57 (t, $J = 7.6$, 6H, H-3,5$_{anil}$), 7.48 (s, 3H, H-2,4,6$_{Ph}$), 7.46 (m, $J = 7.6$, 3H, H-4$_{anil}$), 7.38 (d, $J = 7.6$, 6H, H-2,6$_{anil}$), 6.91 (bs, 6H, H-3,5$_{py}$), 5.47 (s, 6H, CH_2N^+), 3.48 (s, 9H, CH_3). ^{13}C-NMR (100.62 MHz, DMSO-d_6) δ 156.26 (C-4$_{py}$), 143.19 (C-1$_{anil}$), 142.87 (C-2,6$_{py}$), 137.11 (C-1,3,5$_{Ph}$), 130.68 (C-3,5$_{anil}$), 128.67 and 128.20 (C-2,4,6$_{Ph}$ and C-4$_{anil}$), 126.33 (C-2,6$_{anil}$), 109.03 (C-3,5$_{py}$), 58.89 (CH_2N^+), 40.97 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{45}H_{44}N_6Br$ $[M-BrH-Br]^+$ 747.2811. Found: *m/z* 747.2815. Anal. $C_{45}H_{45}N_6Br_3 \cdot 5.1H_2O$ (C, H, N).

*6.1.1.9. 1,1′,1″-(Benzene-1,3,5-triylmethylene)tris[4-(4-chloro-N-methylanilino)pyridinium] tribromide (**10**).* Yield: 78.8%. M.p. 136.4 °C (gel)—189 °C (foam)—231 °C (melting). ^{1}H-NMR (300.13 MHz, DMSO-d_6) δ 8.50 (bs, 6H, H-2,6$_{py}$), 7.65 (d, $J = 8.5$, 6H, H-3,5$_{anil}$), 7.48 (m, 9H, H-2,4,6$_{Ph}$ and H-2,6$_{anil}$), 6.95 (bs, 6H, H-3,5$_{py}$), 5.51 (s, 6H, CH_2N^+), 3.46 (s, 9H, CH_3). ^{13}C-NMR (75.57 MHz, DMSO-d_6) δ 156.22 (C-4$_{py}$), 142.84 (C-2,6$_{py}$), 141.97 (C-1$_{anil}$), 137.02 (C-1,3,5$_{Ph}$), 132.80 (C-4$_{anil}$), 130.53 (C-3,5$_{anil}$), 128.52 (C-2,4,6$_{Ph}$), 128.44 (C-2,6$_{anil}$), 109.19 (C-3,5$_{py}$), 58.74 (CH_2N^+), 40.85 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{45}H_{42}N_6Cl_3Br_2$ $[M-Br]^+$ 929.0903. Found: *m/z* 929.0909. Anal. $C_{45}H_{42}N_6Cl_3Br_3 \cdot 2CH_3CH_2OH \cdot H_2O$ (C, H, N).

*6.1.1.10. 1,1′,1″-(Benzene-1,3,5-triylmethylene)tris[4-(3,5-dichloro-N-methylanilino)pyridi-nium] tribromide (**11**).* Yield: 45%. M.p. 103 °C (gel)—progressive melting up to 275 °C. ^{1}H-NMR (300.13 MHz, DMSO-d_6) δ 8.49 (d, $J = 7.1$, 6H, H-2,6$_{py}$), 7.76 (s, 3H, H-4$_{anil}$), 7.62 (d, $J = 1.7$, 6H, H-2,6$_{anil}$), 7.49 (s, 3H, H-2,4,6$_{Ph}$), 7.04 (d, $J = 7.1$, 6H, H-3,5$_{py}$), 5.50 (s, 6H, CH_2N^+), 3.46 (s, 9H, CH_3). ^{13}C-NMR (75.57 MHz, DMSO-d_6) δ 156.30 (C-4$_{py}$), 145.25 (C-1$_{anil}$), 142.98 (C-2,6$_{py}$), 137.01 (C-1,3,5$_{Ph}$), 135.38 (C-3,5$_{anil}$), 128.39 (C-2,4,6$_{Ph}$), 128.09 (C-4$_{anil}$), 125.95 (C-2,6$_{anil}$), 109.55 (C-3,5$_{py}$), 58.89 (CH_2N^+), 40.71 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{45}H_{39}N_6Cl_6Br_2$ $[M-Br]^+$ 1030.9734. Found: *m/z* 1030.9739. Anal. $C_{45}H_{39}N_6Cl_6Br_3 \cdot 4.5H_2O$ (C, H, N).

*6.1.1.11. 1-[3,5-Bis(bromomethyl)benzyl]-{4-[(pyrrolidino)pyridinium]} bromide (**12**).* Reaction time: 18 h. ^{1}H-NMR (300.13 MHz, CD_3OD) δ 8.21 (d, $J = 7.6$, 2H, H-2,6$_{py}$); 7.53 (s, 1H, H-4$_{Ph}$), 7.38 (s, 3H, H-2,6$_{Ph}$), 6.88 (d, $J = 7.6$, 2H, H-3,5$_{py}$), 5.36 (s, 2H, CH_2N^+), 4.56 (s, 4H, CH_2Br), 3.56 (t, $J = 6.7$, 4H, H-2,5$_{pyrr}$), 2.11 (m, 4H, H-3,4$_{pyrr}$).

*6.1.1.12. 1-[3,5-Bis(bromomethyl)benzyl]-{4-[(piperidino)pyridinium]} bromide (**13**).* Temperature of the reaction: 25 °C. ^{1}H-NMR (400.12 MHz, DMSO-d_6) δ 8.34 (d, $J = 7.5$, 2H, H-2,6$_{py}$), 7.52 (s, 1H, H-4$_{Ph}$), 7.41 (s, 3H, H-2,6$_{Ph}$), 7.24 (d, $J = 7.5$, 2H, H-3,5$_{py}$), 5.35 (s, 2H, CH_2N^+), 4.67 (s, 4H, CH_2Br), 3.65 (bs, 4H, H-2,6$_{pip}$), 1.57 (m, 6H, H-3,4,5$_{pip}$). Anal. $C_{19}H_{23}N_2Br_3$ (C, H, N).

6.1.2. General procedure for the synthesis of 4-(N-methylanilino)pyridine hydrochlorides

4-Pyrrolidinopyridine was obtained from Aldrich. 4-Piperidinopyridine [21] and 4-(*N*-methylanilino)pyridine [19] were synthesised according to literature procedures. 4-Chloropyridine hydrochloride (19.9 mmol) was refluxed with excess of the amine (59.9 mmol) in *n*-pentanol (40 mL) under an argon atmosphere for 1 day. The mixture was rotaevaporated off and the residue was purified by flash chromatography using the mixture Cl_3CH–MeOH, 10:0.5 as eluant or by recrystallisation from acetone, giving the title compound as a colourless solid.

6.1.2.1. 4-(4-Chloro-N-methylanilino)pyridine hydrochoride. Yield: 48.3%. M.p. 252 °C; ^{1}H-NMR (300.13 MHz, CD_3OD): δ 8.19 (d, $J = 7.3$, 2H, H-2,6$_{py}$), 7.62 (dd, $J = 2.7$, 8.7, 2H, H-3,5$_{anil}$), 7.40 (dd, $J = 2.7$, 8.7, 2H, H-2,6$_{anil}$), 6.95 (bs, 2H, H-3,5$_{py}$), 3.52 (s, 3H, CH_3). ^{13}C-NMR (75.57 MHz, CD_3OD) δ 159.62 (C-4$_{py}$), 143.58 (C-1$_{anil}$), 140.76 (C-2,6$_{py}$), 135.63 (C-4$_{anil}$), 132.11 (C-3,5$_{anil}$), 129.51 (C-2,6$_{anil}$), 109.63 (C-3,5$_{py}$), 41.23 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{12}H_{12}N_2Cl$ $[M-Cl]^+$ 219.0689. Found: *m/z* 219.0693. Anal. $C_{12}H_{12}N_2Cl_2 \cdot 0.4H_2O$ (C, H, N).

6.1.2.2. 4-(3,5-Dichloro-N-methylanilino)piridine hydrochloride. Yield: 53.6%. M.p. 184 °C (gel)—213 °C (melting). ^{1}H-NMR (300.13 MHz, $CDCl_3$) δ 8.25 (d, $J = 6.9$, 2H, H-2,6$_{py}$), 7.34 (t, $J = 1.8$, 1H, H-4$_{anil}$), 7.15

(d, $J = 1.8$, 2H, H-2,6$_{anil}$), 6.73 (d, $J = 6.9$, 2H, H-3,5$_{py}$), 6.13 (bs, 1H, NH), 3.41 (s, 3H, CH_3). ^{13}C-NMR (75.57 MHz, $CDCl_3$) δ 155.31 (C-4$_{py}$), 146.39 (C-1$_{anil}$), 144.85 (C-2,6$_{py}$), 136.53 (C-3,5$_{anil}$), 127.65 (C-4$_{anil}$), 124.82 (C-2,6$_{anil}$), 109.11 (C-3,5$_{py}$), 40.25 (CH_3). HR LSIMS (thioglycerol) Calc. *m/z* for $C_{12}H_{11}N_2Cl_2$ $[M-Cl]^+$ 253.0299. Found: *m/z* 253.0297. Anal. $C_{12}H_{11}N_2Cl_3 \cdot 0.5H_2O$ (C, H, N).

In the case of Sections 6.1.2.1 and 6.1.2.2 the corresponding hydrochlorides were converted into the neutral bases after neutralising the salts with 1 N NaOH, extracting with diethyl ether, drying ($MgSO_4$), filtering and rotaevaporating off. The residues were purified by flash chromatography using the mixture Cl_3CH–MeOH, 9:1 as eluant, giving the title compounds.

6.2. Pharmacology

The ex vivo ChoK inhibition [27,28] and antiproliferative assays against HT-29 cells [18] were followed in accordance with the protocols previously reported.

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