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Original article

Synthesis and biological activity of new bispyridinium salts of 4,4′-bispyridyl-5,5′-perfluoroalkyl-2,2′-bisoxazoles

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ABSTRACT

A series of bispyridinium compounds were synthesized by a short sequence of reactions from symmetric diamides. All compounds were tested for their antiproliferative activity against HT-29, a cell line derived from a human colon adenocarcinoma, and their inhibitory activity against choline kinase (ChoK), a novel anticancer molecular target already in clinical trials. Most of the compounds analyzed showed good antiproliferative activities, in the micromolar range, with the identification of promising lead molecules as a new family of potential inhibitors of ChoK.

1. Introduction

One of the targets recently identified for antitumor drug discovery is choline kinase (ChoK) [1–3]. This enzyme plays a vital role in cell signaling pathways and regulation of cell growth, being involved in malignant transformation through ras oncogenes. ChoK is overexpressed in some of the most relevant human tumors such as breast, colon, lung, prostate, hepatic lymphomas and ovary [4]. Generation of ChoK inhibitors will provide new substances with potential anticancer activity [5–8].

As a part of our work related to the development of new antitumor agents, we have synthesized several families of 4,4′-bispyridyl-2,2′-bisoxazoles (**2**) and their salts (**3**) [9,10], molecules structurally related to the potent ChoK inhibitor hemicolinium (HC-3) and its bispyridinium derivatives [5] (Fig. 1).

Oxazoles and bisoxazoles represent an important class of five-membered heterocycles, found in a variety of biologically significant natural products [11]. Compounds wearing this structure have been reported to exhibit antiproliferative [12,13], anti-inflammatory [14], hypoglucemic [15], antibacterial [16,17] and antifungal activities [18]. Even if the proliferation of interesting structures within this family has inspired the development of methods to synthesize specific substitution patterns, most of them are focused, in the case of bisoxazoles [19], on the construction of a 2,4′ linkage by the stepwise formation of the rings [20] or by cross coupling of previously synthesized heterocycles [21,22]. Symmetrical and especially 2,2′-bisoxazoles are scarcely reported [23–25]. Usually their synthesis involve long stepwise procedures, instead of a simultaneous construction of both rings, and/or low reaction yields.

We have developed a new convergent synthesis of 4,4′-bispyridyl-2,2′-bisoxazoles 5,5′-methyl or perfluoralkyl disustituted **2** starting from symmetric diamides [9]. Quaternization of 4,4′-bispyridyl-5,5′-perfluoralkyl-2,2′-bisoxazoles with alkyl groups (methyl, allyl) have provided compounds **3** with antitumor activity in the micromolar range against HT-29 cells and other cell lines [10,26], even if unexpectedly, some of the generated structures showed very little effects on ChoK. We also described [10] oxazole **3b**, bearing a hydroxyethyl group, which displayed antiproliferative properties in tumor cell lines and prevented *in vivo* tumor growth in nude mice. Here we report the synthesis, antiproliferative and ChoK inhibitory activity of new bisoxazoles related to compound **3b**. We further explore the influence of the Z linker and perfluoroalkyl substituent of 2,2′ bisoxazoles in activity, extending the range of alkylating agents to those providing electron donating groups, and describing other counteranions besides iodine.

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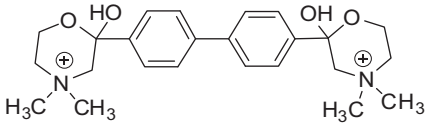
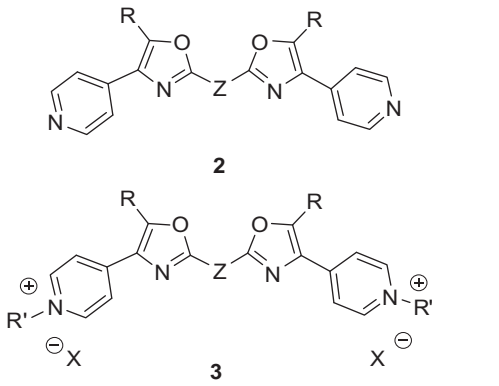


Hemicolinium



3b Z = *p*-biphenyl, R = CF_3, R' = CH_2-CH_2OH, X = I

Fig. 1. 4,4′-Bispiridyl-2,2′-bisoxazoles 5,5′-disustituted previously synthesized and hemicolinium.

2. Results and discussion

2.1. Chemistry

Sixteen final compounds **3** have been synthesized, using the one pot method described by our lab [9,10] to convert *N,N′*-bis(4-pyridylmethylen)diamides into bisoxazoles using perfluoroalkyl anhydrides as a fluorine source.

In this way (Scheme 1), the treatment of symmetric diamides **1** (easily prepared from the corresponding diacid chloride) with perfluoroalkyl anhydrides (bearing three, five, or seven fluorine) in dry toluene in the presence of pyridine provided 5,5′-bisperfluoroalkyl-2,2′-bisoxazoles **2** in low (41%) to excellent (94%) yields. Three different linkers (*p*-biphenyl, Ph–$C(CF_3)_2$–Ph, *p*-phenyl) were used. Quaternization of the pyridine nitrogen with functionalized alkylating agents afforded the target molecules **3**. The reaction was performed either in a dry solvent (acetone or alcohols) or without solvent, and a sealed tube was needed in some cases.

2.2. Biological activity

All the compounds were tested for antitumoral activity against the human adenocarcinoma HT-29 cells. They were also tested in an *ex vivo* system using recombinant human ChoK as target as described in the experimental section, in order to evaluate the inhibitory potency of the compounds toward ChoK without the possible interference due to permeability through membranes. Table 1 summarizes the activity of the pyridinium salts of bisoxazoles prepared, indicated as IC_{50}. The table is arranged in descending order of antiproliferative activity.

Compounds **3a**–**3l** showed potent cytotoxicity against the human tumor-derived cell line tested, in the range described for compound **3b**. This confirmed our prediction that alkylating groups wearing electrondonating atoms would increase the potency of symmetric perfluoroalkyl-2,2′-bisoxazoles **3** as antiproliferative agents. Even if the initial design was focused on the generation of inhibitors against ChoK, the results show that in most cases antiproliferative and inhibition data correlates (**3a**, **3e**, **3f**, **3g**, **3h**, **3i, 3k**), but not always (**3b**, **3c** and **3o**, **3p**).

It is observed clearly that antiproliferative activity increases using *p*-biphenyl as linker. The distance between the cationic heads must have with this linker the optimum value. Once such a distance is exceeded (when spacer Ph–$C(CF_3)_2$–Ph is used) the antiproliferative activity decreases slightly, and when it is shortened (using a *p*-phenyl spacer) it drops completely. The relationship between the linker and ChoK inhibitory activity is not straightforward.

It is more difficult to see a clear relationship between the perfluoroalkyl sustituent and the biological activity. In most cases, a CF_3CF_2 chain seems to afford better antiproliferative activity than CF_3 or $CF_3CF_2CF_2$ (**3a** vs **3c** and **3d**, **3i** vs **3j**, **3l** vs **3m** and **3n**), but not always (**3e** and **3g** vs **3h**). Regarding the alkylating agent, both CH_2–CH_2OH and CH_2–$CON(CH_3)_2$ (which have in common the ability to form hydrogen bonds), provided good antiproliferative activity, but the best values for ChoK inhibition were obtained with the CH_2–$CON(CH_3)_2$ sustituent (**3g**, **3p**, **3h**). The introduction of a chloroethyl sustituent, unable to form hydrogen bonds, lowers the antiproliferative activity (**3l**, **3m, 3n**). The use of different counterions (chlorine, iodine, methylsulfonate) has not been studied thoroughly as this is not a key issue at this point of the development of the potential drugs.

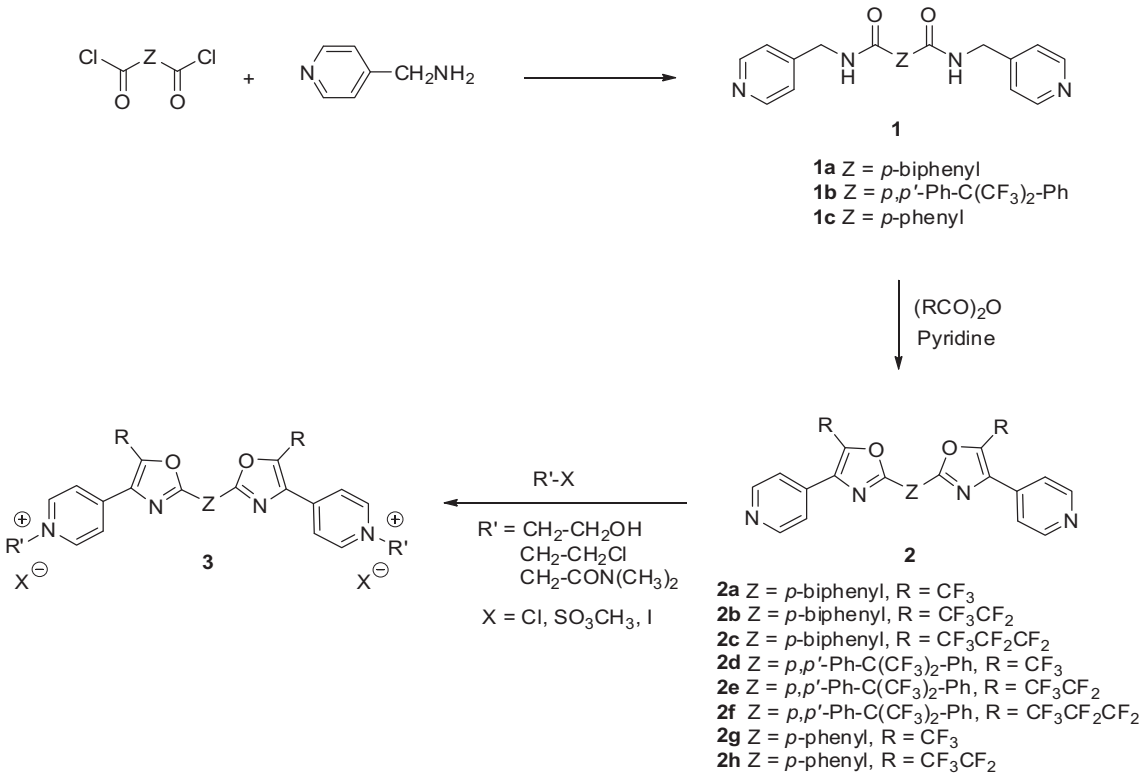
In summary, the synthesis and characterization of a new series of bispyridinium salts of 4,4′-bispyridyl-5,5′-perfluoroalkyl-2,2′-bisoxazol have been described. The tested compounds displayed antiproliferative activity against human adenocarcinoma HT-29 cells, and inhibitory activity towards ChoK. Compounds bearing a *p*-biphenyl linker and an alkylating agent able to form hydrogen bonds could serve as lead chemical entities for further modification to render them clinically useful drug agents.

3. Experimental section

3.1. Chemistry

3.1.1. General

The ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded in a Bruker AC 300 (300 MHz) spectrometer. Chemical shifts were reported in ppm (δ) relative to the middle of solvent signals. Signals were designated as follows: s, singlet; bs, broad singlet; d, doublet; t, triplet; m, multiplet. IR spectra were taken on a Perkin–Elmer 1330 spectrometer. ESI mass spectra were obtained on Esquire-LC_00126 mass spectrometer from Bruker. Melting points were determined on a GallenKamp capillary melting point apparatus and were not corrected. For purification of crude reaction mixtures by flash chromatography, Merck silica gel (230–240 mesh) was used as the stationary phase. Identification of products was made by analytical TLC (Merck Kieselgel 60F-254) and UV light (λ = 254 mm). All the commercially available reagents were obtained from Aldrich and used without further purification. Reagents and solvents were handled by using standard syringe techniques. All solvents used were reagent grade.



Scheme 1. Synthesis of bispyridinium salts **3**.

*3.1.2. General procedure for the synthesis of symmetrical bisamides, **1***

Bisamides **1a, 1b y 1c**, were synthesized by acylation of 4-aminomethylpyridine (8.2 mmol, 2 eq) with different acid chlorides (4.1 mmol, 1 eq) using 5 mL of CH_2Cl_2 or AcOEt as solvent in Et_3N (13 mmol, 3 eq) using dimethylaminepyridine as catalyst (0.82 mmol, 0.2 eq) [6]. The bisamides were obtained in moderate to good yields (**1a**: 75%; **1b**: 42%; **1c**: 85%) after purification of crude by flash chromatography using as eluent a mixture of CH_2Cl_2:MeOH (95:5).

*3.1.3. General procedure for the synthesis of 4,4′-bispyridyl-5,5′-perfluoroalkyl-2,2′-bisoxazoles, **2***

To a stirred solution of the respective bisamide **1a,1b,1c**, (1 mmol) in anhydrous toluene (10 mL), kept under argon atmosphere and previously sonicated, pyridine was added (12 mmol). The mixture was cooled to 0 °C and the corresponding perfluoroalkyl anhydride (6 mmol) was added dropwise. The mixture was left to attain room temperature for 48 h. The solvent was evaporated, the obtained solid was dissolved in CH_2Cl_2, extracted with water and washed with 5% Na_2CO_3. The combined organics extracts were dried with $MgSO_4$. The solvent was evaporated and purification of crude was accomplished by flash column chromatography or by recrystallization in adequate solvent to obtain compounds **2c, 2e, 2f** and **2h**. Bisoxazoles **2a, 2b, 2d** and **2g** were described previously [6].

*3.1.3.1. 4,4-Bis[5-heptafluoropropyl-4-(4-pyridyl)oxazolyl]byphenyl, **2c**.* Obtained from **1a** (131.3 mg, 0.311 mmol) and heptafluorobutyric anhydride (0.46 mL, 3.732 mmol) as a white solid

Table 1
Antiproliferative and ChoK inhibitory activity of novel pyridinium salts **3**.

Compound	Z	R	R′	X	Yield	IC_{50}[b] (μM) antiproliferative	IC_{50}[b] (μM) *ex vivo*
3a	*p*-biphenyl	CF_3CF_2	CH_2—CH_2OH	Cl	70	0.84 ± 0.005	9.65 ± 0.04
3b[a]	*p*-biphenyl	CF_3	CH_2—CH_2OH	I	36	1.50 ± 0.03	23.80 ± 2.5
3c	*p*-biphenyl	CF_3	CH_2—CH_2OH	Cl	55	1.85 ± 0.01	>20
3d	*p*-biphenyl	$CF_3CF_2CF_2$	CH_2—CH_2OH	Cl	31	2.26 ± 0.03	17.30 ± 1.5
3e	*p*-biphenyl	$CF_3CF_2CF_2$	CH_2—$CON(CH_3)_2$	Cl	94	2.50 ± 0.02	4.25 ± 0.06
3f	*p*-biphenyl	CF_3CF_2	CH_2—CH_2OH	I	66	3.60 ± 0.02	3.10 ± 0.01
3g	*p*-biphenyl	CF_3	CH_2—$CON(CH_3)_2$	Cl	50	4.00 ± 0.06	0.30 ± 0.003
3h	*p*-biphenyl	CF_3CF_2	CH_2—$CON(CH_3)_2$	Cl	91	5.50 ± 0.01	2.00 ± 0.03
3i	*p,p′*-Ph—$C(CF_3)_2$—Ph	CF_3CF_2	CH_2—CH_2OH	Cl	65	6.00 ± 0.04	4.40 ± 0.02
3j	*p,p′*-Ph—$C(CF_3)_2$—Ph	$CF_3CF_2CF_2$	CH_2—CH_2OH	Cl	70	6.35 ± 0.01	13.50 ± 1.5
3k	*p,p′*-Ph—$C(CF_3)_2$—Ph	CF_3CF_2	CH_2—CH_2OH	I	58	7.70 ± 0.01	4.20 ± 0.01
3l	*p,p′*-Ph—$C(CF_3)_2$—Ph	CF_3CF_2	CH_2—CH_2Cl	SO_3CH_3	45	9.30 ± 0.01	14.20 ± 2.5
3m	*p,p′*-Ph—$C(CF_3)_2$—Ph	CF_3	CH_2—CH_2Cl	SO_3CH_3	42	13.75 ± 1.5	12.50 ± 1.7
3n	*p,p′*-Ph—$C(CF_3)_2$—Ph	$CF_3CF_2CF_2$	CH_2—CH_2Cl	SO_3CH_3	41	15.50 ± 1.5	11.60 ± 3.5
3o	*p,p′*-Ph—$C(CF_3)_2$—Ph	CF_3	CH_2—$CON(CH_3)_2$	Cl	91	>20	6.15 ± 0.02
3p	*p*-phenyl	CF_3	CH_2—$CON(CH_3)_2$	Cl	89	>50	1.35 ± 0.01
3q	*p*-phenyl	CF_3CF_2	CH_2—$CON(CH_3)_2$	Cl	65	>50	15.00 ± 4.5

[a] Described in reference [10].
[b] IC_{50} values are means ± SEM of at least three independent measurements.

with a yield of 30% after recrystallization from $CHCl_3$/MeOH/Et_2O. Mp: 192–194 °C. ^{1}H NMR ($CDCl_3$) δ: 8.79 (d, J = 5.5 Hz, 4H, Py), 8.26 (d, J = 8.5 Hz, 4H, Ph), 7.82 (d, J = 7.9 Hz, 4H, Ph), 7.76 (d, J = 5.5 Hz, 4H, Py); ^{13}C NMR ($CDCl_3$) δ: 162.9, 148.8, 143.2, 142.3, 138.2, 135.3, 136.7, 127.8, 127.7, 124.9, 123.3, 109.3; IR (cm^{-1}) (KBr): 1614, 1589, 1545, 1378, 1232, 1199, 1116, 877.

3.1.3.2. 2,2-Bis[5-pentafluoroethyl-4-(4-pyridyl)oxazolyl]hexafluoropropane, ***2e****.* Obtained from **1b** (300 mg, 0.52 mmol) and pentafluoropropionic anhydride (0.61 mL, 3.14 mmol) as a white solid with a yield of 50% after flash chromatography with a mixture CH_2Cl_2/MeOH 3%. Mp: 193–195 °C. ^{1}H NMR ($CDCl_3$) δ: 8.8 (bs, 4H, Py), 8.19 (d, 4H, J = 8.7 Hz, Ph), 7.8 (d, 4H, J = 5.0 Hz, Py), 7.6 (d, 4H, J = 8.2 Hz, Ph); ^{13}C NMR ($CDCl_3$) δ: 161.2, 147.11, 140.8, 137.9, 135.8, 134.5, 134.1, 129.9, 126.3, 125.2, 122.6; IR (cm^{-1}) (KBr): 1619, 1592, 1550, 1505, 1375, 1341, 1170, 901.

3.1.3.3. 2,2-Bis[5-heptafluoropropyl-4-(4-pyridyl)oxazolyl]hexafluoropropane, ***2f****.* Obtained from **1b** (678 mg, 1.185 mmol) and heptafluorobutyric anhydride (1.74 mL, 7.11 mmol) as a foaming solid with a yield of 37% after flash chromatography with a mixture CH_2Cl_2/MeOH 3%. Mp: 195–197 °C. ^{1}H NMR ($CDCl_3$) δ: 8.78 (d, 4H, J = 5.49 Hz, Py), 8.19 (d, 4H, J = 8.5 Hz, Ph), 7.77 (d, 4H, J = 5.49 Hz, Py), 7.59 (d, 4H, J = 7.9 Hz, Ph); ^{13}C NMR ($CDCl_3$) δ: 162.0, 150.0, 142.9, 136.9, 136.6, 134.2, 130.9, 127.1, 126.3, 122.9, 111.3.

3.1.3.4. 1,4-Bis[5-pentafluoropropyl-4-(4-pyridyl)oxazolyl]benzene, ***2h****.* Obtained from **1c** (226 mg, 0.65 mmol) and pentafluoropropionic anhydride (0.8 mL, 4.1 mmol) as a beige solid with a yield of 21% after flash chromatography with a mixture CH_2Cl_2/MeOH 3%. Mp: 189–191 °C. ^{1}H NMR ($CDCl_3$) δ: 8.77 (d, 4H, Py, J = 5.5 Hz, Py), 8.3 (s, 4H, J = 8.2 Hz, Ph), 7.69 (d, 4H, J = 5.5 Hz, Py); ^{13}C NMR ($CDCl_3$) δ: 161.9, 151.2, 142.8, 136.7, 134.5, 128.3, 127.8, 122.9, 118.0. 109.3; IR (cm^{-1}) (KBr): 1702, 1590, 1565, 1496, 1340, 1332, 1311, 993.

3.1.4. General procedure for the synthesis of pyridinium salts of 4,4′-bispyridyl-5,5′-perfluoroalkyl-2,2′-bisoxazoles, ***3***

Excess of the alkylating agent (10 mmol) (2-hydroxyethyl iodide, 2-hydroxyethyl chloride, 2-chloroethyl methanosulphonate, 2-chloro-N,N′-dimethylacetamide) was added to a stirred solution of bisoxazol (1 mmol) in a dry solvent (50 mL) (acetone or isopropanol) or without solvent and kept under argon atmosphere. After refluxing until total consumption of the starting material (in some cases in a sealed tube), the reaction mixture was cooled and then the resulting solid was filtered, washed with dry solvent and dried. Crude was purified by recrystallization when it was necessary.

3.1.4.1. 4,4′-Bis[(5-pentafluoroethyl-4-(hydroxiethylpyridinium-4-yl) oxazolyl)]biphenyldichloride, ***3a****.* Obtained from bisoxazol **2b** (60 mg, 0.09 mmol) and 2-chloroethanol (1 mL, 14.9 mmol) after refluxing under argon atmosphere for two hours. After cooling, acetone was added and a solid was precipitated. It was filtered and washed with acetone to obtain 51 mg of compound **3a** as a white solid. Yield: 70%. Mp: >250 °C. ^{1}H NMR (DMSO-d_6) δ: 9.17 (d, 4H, Py, J = 6.72 Hz); 8.43 (d, 4H, Py, J = 6.72 Hz); 8.25 (d, 4H, Ph, J = 8.52 Hz); 8.10 (d, 4H, Ph, J = 8.55 Hz); 5.44 (t, OH, 2H, J = 5.49 Hz); 4.74 (bs, 4H, CH_2N); 3.90 (bs, 4H, CH_2OH); ^{13}C NMR (MeOH-d_4): 165.3, 147.1, 146.7, 145.0, 141.0, 138.1, 137.6, 129.6, 128.0, 125.8, 119.8, 110.8, 65.1, 61.6; IR (cm^{-1}) (KBr): 3392.6, 1645.6, 1615.2, 1224.7, 1115.4, 940; MS m/e (electrospray): 383.9 ($C_{36}H_{26}F_{10}Cl_2N_4O_4$, $[M^+ - (2 \times Cl^-)]/2$).

3.1.4.2. 4,4′-Bis[(5-trifluoromethyl-4-(hydroxiethylpyridinium-4-yl) oxazolyl)]biphenyldichloride, ***3c****.* Obtained from bisoxazol **2a** (50 mg, 0.09 mmol) and 2-chloroethanol (1 mL, 15 mmol) after refluxing under argon atmosphere for four hours. After cooling, acetone was added until a solid precipitated. It was filtered and washed with acetone to obtain 35 mg of compound **3c** as a beige solid. Yield: 55%. Mp: >250 °C. ^{1}H NMR (DMSO-d_6) δ: 9.17(d, 4H, Pyr, J = 6.27 Hz); 8.47 (d, 4H, Pyr, 6.09 Hz); 8.31(d, 4H, Ph, J = 7.92 Hz); 8.12 (d, 4H, Ph, J = 8.55 Hz); 5.45 (bs, 2H, OH); 4.76 (bs, 4H, CH_2N); 3.92 (bs, 4H, CH_2OH); ^{13}C NMR (DMSO-d_6) δ: 164.2, 147.0, 146.9, 144.9, 138.3, 129.3, 129.0, 127.51, 125.9, 120.2, 66.9, 65.1, 61.6.

3.1.4.3. 4,4′-Bis[(5-heptafluoropropyl-4-(hydroxiethylpyridinium-4-yl)oxazolyl)]biphenyldichloride, ***3d****.* Obtained from bisoxazol **2c** (50 mg, 0.064 mmol) and 2-chloroethanol (1 mL, 15 mmol) after heating at 110 °C in a sealed tube under argon atmosphere for three days. After cooling, acetone (2 mL) was added and then diethyl ether until a yellow solid was precipitated. The solid was filtered and purified by recristallization from MeOH/Et_2O/acetone to obtain 40.1 mg of compound **3d** as a pale yellow solid. Yield: 31%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.15 (d, J = 6.7 Hz, 4H, Py), 8.51 (d, J = 6.12 Hz, 4H, Py), 8.34 (d, J = 7.9 Hz, 4H, Ph), 8.04 (d, J = 7.9 Hz, 4H, Ph), 4.80 (t, J = 4.3 Hz, 4H, CH_2Py), 4.06 (t, J = 4.3 Hz, 4H, CH_2Py); ^{13}C NMR (MeOH-d_4) δ: 165.3, 147.1, 146.8, 145.1, 141.0, 138.1, 137.6, 129.3, 128.1, 125.8, 119.8, 110.8, 65.1, 61.6.

3.1.4.4. 4,4′-Bis[(5-heptafluoropropyl-4-(4-N,N′-dimethylacetamide pyridinium-4-yl)oxazolyl)]biphenyl dichloride, ***3e****.* Obtained from bisoxazol **2c** (41 mg, 0.053 mmol) and 2-chloro-*N,N′*-dimethylacetamide (0.9 mL, 8.75 mmol) after heating at 110 °C in a sealed tube under argon atmosphere for 48 h. After cooling, acetone was added until a solid precipitated. It was filtered to obtain 62.6 mg of compound **3e** as a white solid. Yield: 94%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.03 (d, 4H, J = 6.7 Hz, Py), 8.54 (d, 4H, J = 6.1 Hz, Py), 8.34 (d, 4H, J = 8.5 Hz, Ph), 8.04 (d, 4H, J = 8.5 Hz, Ph), 5.85 (s, 4H, CH_2), 3.19 (s, 6H, CH_3), 3.05 (s, 6H, CH_3); ^{13}C NMR (MeOH-d_4) δ: 165.8, 165.4, 148.3, 147.1, 145.0, 141.3, 137.7, 129.3, 129.2, 127.7, 125.8, 119.8, 114.1, 110.8, 62.8, 36.6, 36.3.

3.1.4.5. 4,4′-Bis[(5-pentafluoroethyl-4-(hydroxiethylpyridinium-4-yl) oxazolyl)]biphenyldiiodide, ***3f****.* Obtained from bisoxazol **2b** (45 mg, 0.04 mmol) and 2-hydroxyethyl iodide (0.45 mL, 6.4 mmol) after refluxing for 72 h. The precipitated solid was filtered and washed with acetone to obtain 92 mg of compound **3f** as a beige solid. Yield: 90%. Mp: >250 °C. ^{1}H NMR (DMSO-d_6) δ: 9.16 (d, 4H, J = 6.7 Hz, Py), 8.44 (d, 4H, J = 6.1 Hz, Py), 8.26 (d, 4H, J = 8.5 Hz, Ph), 8.10 (d, 4H, J = 8.5 Hz, Ph), 5.33 (t, 2H, J = 5.5 Hz, OH), 4.74 (bs, 4H, CH_2–N), 3.91 (bs, 4H, CH_2OH); ^{13}C NMR (DMSO-d_6) δ: 162.8, 145.9, 143.8, 142.4, 139.7, 134.7, 127.9, 126.4, 124.1, 124.0, 110.0, 63.3, 59.9; IR (cm^{-1}) (KBr): 3422, 1639, 1613, 1458, 1403, 1373, 1328, 1212, 1119. MS: 384 ($C_{36}H_{26}F_{10}I_2N_4O_4$, $[M^+ - 2I^-/2]$).

3.1.4.6. 4,4′-Bis[(5-trifluoromethyl-4-(N,N′-dimethylacetamide-pyridinium-4-yl)oxazolyl)]biphenyl dichloride, ***3g****.* Obtained from bisoxazol **2a** (50 mg, 0.086 mmol) and 2-chloro-*N,N′*-dimethylacetamide (2.5 mL, 40 mmol) after heating at 110 °C in a sealed tube for five days. After cooling, acetone was added until a solid was obtained. The solid was filtered and purified by recristallization from MeOH/Et_2O to obtain 35 mg of compound **3g** as a beige solid. Yield: 50%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.02 (d, 4H, J = 5.5 Hz, Py), 8.56 (d, 4H, J = 5.4 Hz, Py), 8.34 (d, 4H, J = 6.8 Hz, Ph), 8.02 (d, 4H, J = 6.6 Hz, Ph), 5.84 (s, 4H, CH_2), 3.2 (s, 6H, CH_3), 3.06 (s, 6H, CH_3); ^{13}C NMR (MeOH-d_4) δ: 165.9, 164.2, 148.3, 146.9, 144.8, 139.6, 138.2, 129.2, 129.1, 127.2, 125.8, 120.5, 62.7, 36.4, 36.6.

3.1.4.7. 4,4′-Bis[(5-pentafluoroethyl-4-(N,N′-dimethylacetamidepyridinium-4-yl)oxazolyl)]biphenyl dichloride, ***3h****.* Obtained from

bisoxazol **2b** (50 mg, 0.074 mmol) and 2-chloro-*N,N′*-dimethylacetamide (0.9 mL, 8.75 mmol) after heating at 110 °C in a sealed tube for 48 h. After cooling, acetone was added until a solid was obtained. The solid was filtered and washed with Et_2O to give 62.6 mg of compound **3h** as a white solid. Yield: 91%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.02 (d, 4H, J = 7.3 Hz, Py), 8.54 (d, 4H, J = 6.1 Hz, Py), 8.34 (d, 4H, J = 8.5 Hz, Ph), 8.03 (d, 4H, J = 8.5 Hz, Ph), 5.84 (s, 4H, CH_2), 3.19 (s, 6H, CH_3), 3.05 (s, 6H, CH_3); ^{13}C NMR (MeOH-d_4) δ: 165.8, 165.2, 148.3, 147.1, 145.0, 140.9, 137.7, 129.3, 127.6, 125.8, 119.8, 117.9, 110.8, 62.8, 36.6, 36.3. MS: 849.3 ($C_{40}H_{32}Cl_2F_{10}N_6O_4$, [$M^+$]); 764 ($C_{40}H_{32}Cl_2F_{10}N_6O_4$, [$M^+ - 85^+$]).

3.1.4.8. 2,2-Bis[5-pentafluoroethyl-4-(4-hydroxiethyl-pyridinium-4-yl)oxazolyl]hexafluoropropane dichloride, ***3i***. Obtained from bisoxazol **2e** (73 mg, 0.09 mmol) and 2-chloroethanol (1 mL, 15 mmol) after refluxing under argon atmosphere for five and a half hours. After cooling, Et_2O was added. The precipitated solid was filtered and washed with Et_2O and $CHCl_3$ to obtain 57 mg of compound **3i** as a white solid. Yield: 65%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.13 (d, 4H, Py, J = 6.72 Hz); 8.50 (d, 4H, Py, J = 6.72 Hz); 8.30 (d, 4H, Ph, J = 9.15 Hz); 7.71 (d, 4H, Ph, J = 8.55 Hz); 4.79 (t, 4H, CH_2N, J = 4.89 Hz); 4.05 (t, 4H, CH_2OH, J = 4.89 Hz); ^{13}C NMR (MeOH-d_4) δ: 164.4, 147.1, 146.5, 141.0, 138.3, 138.0, 137.5, 132.4, 128.7, 128.0, 127.3, 120.0, 110.5, 65.1, 61.6; IR (cm^{-1}) (KBr): 3417, 1647.1, 1339.9, 1212.0, 939.5, 752.4, 722.7.

3.1.4.9. 2,2-Bis[5-heptafluoroprophyl-4-(4-hydroxyethyl-pyridinium-4-yl)oxazolyl]hexafluoropropane dichloride, ***3j***. Obtained from bisoxazol **2f** (50 mg, 0.054 mmol) and 2-chloroethanol (1 mL, 15 mmol) after heating at 100 °C under argon atmosphere for 32 h. After cooling it was added Et_2O and acetone (20:1). The precipitated solid was filtered and washed with Et_2O. The resulting solid was purified by recristallization from MeOH/$CHCl_3$/Et_2O to obtain 15 mg of compound **3j** as a white solid. Yield: 70%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.13 (d, 4H, J = 5.5 Hz, Py), 8.49 (d, 4H, J = 5.5 Hz, Py), 8.31 (d, 4H, J = 7.3 Hz, Ph), 7.72 (d, 4H, J = 7.9 Hz, Ph), 4.79 (t, 4H, J = 4.3 Hz, CH_2Py), 4.05 (t, 4H, J = 4.8 Hz, CH_2OH); ^{13}C NMR (MeOH-d_4) δ: 168.4, 163.4, 148.1, 146.9, 140.0, 138.2, 132.4, 131.6, 128.8, 128.7, 127.4, 126.8, 120.4, 110.6, 62.0.

3.1.4.10. 2,2-Bis[5-pentafluoroethyl-4-(4-hydroxiethyl-pyridinium-4yl)oxazolyl]hexafluoropropane diiodide, ***3k***. Obtained from bisoxazol **2e** (69 mg, 0.088 mmol) and 2-chloroethanol (0.13 mL, 1.41 mmol) after heating in isopropanol at 110 °C for five days. After cooling the precipitated solid was filtered and washed with acetone to obtain 60 mg of compound **3k** as a beige solid. Yield: 58%. Mp: >250 °C. ^{1}H NMR (DMSO-d_6) δ: 9.17 (d, 4H, J = 6.7 Hz, Py), 8.42 (d, 4H, J = 6.7 Hz, Py), 8.28 (d, 4H, J = 8.5 Hz, Ph), 7.70 (d, 4H, J = 8.5 Hz, Ph), 4.74 (t, 4H, CH_2–N, J = 4.89 Hz), 3.91 (t, 4H, CH_2OH, J = 4.89 Hz); ^{13}C NMR (DMSO-d_6) δ: 162.3, 146.3, 146.1, 143.8, 139.9, 135.9, 135.2, 131.1, 127.8, 126.6, 125.7, 110.5, 63.4, 60.1; IR (cm^{-1}) (KBr): 3446, 2360, 1643, 1560, 1507, 1460, 1420, 1212; MS: 827 ($C_{39}H_{26}F_{16}I_2N_4O_4$, [$M^+ - 2 \times CF_3$]).

3.1.4.11. 2,2-Bis[5-pentafluoroethyl-4-(4-chloroethyl-pyridinium-4-yl)oxazolyl]hexafluoropropane dimethylsulfonide, ***3l***. Obtained from bisoxazol **2e** (73 mg, 0.09 mmol) and 2-chloroethyl-methanesulfonate (1 mL, 8.76 mmol) after heating at 100 °C under argon atmosphere for four hours. After cooling Et_2O and acetone were added. The precipitated solid was filtered and washed with Et_2O to obtain 39 mg of compound **3l** as a white solid. Yield: 45%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.21 (d, 4H, Py, J = 6.69 Hz); 8.54 (d, 4H, Py, J = 7.32 Hz); 8.30 (d, 4H, Ph, J = 8.52 Hz); 7.31 (d, 4H, Ph, J = 8.55 Hz); 5.08 (t, 4H, CH_2N, J = 5.49 Hz); 4.24 (t, 4H, CH_2Cl, J = 5.49 Hz); 2.69 (s, 6H, CH_3SO_3); ^{13}C NMR (MeOH-d_4) δ: 165.3, 147.3, 145.0, 140.8, 137.8, 137.7, 128.2, 128.1, 127.3, 126.9, 110.7, 119.5, 63.0, 43.4, 39.5; IR (cm^{-1}) (KBr): 3422.9, 1647.8, 1177.4, 1058.7, 939.9.

3.1.4.12. 2,2-Bis[5-pentafluoroethyl-4-(4-chloroethyl-pyridinium-4-yl)oxazolyl]hexafluoropropane dimethylsulfonide, ***3m***. Obtained from bisoxazol **2d** (65 mg, 0.09 mmol) and 2-chloroethyl-methanesulfonate (1 mL, 8.76 mmol) after heating at 100 °C under argon atmosphere for nine hours. After cooling Et_2O and acetone were added. The precipitated solid was filtered and washed with Et_2O to obtain 38.5 mg of compound **3m** as a white solid. Yield: 42%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.20 (d, 4H, Py, J = 6.72 Hz); 8.56 (d, 4H, Py, J = 6.72 Hz); 8.33 (d, 4H, Ph, J = 8.55 Hz); 7.71 (d, 4H, Ph, J = 8.55 Hz); 5.09 (t, 4H, CH_2N, J = 4.89 Hz); 4.24 (t, 4H, CH_2Cl, J = 4.89 Hz); 2.69 (s, 6H, CH_3SO_3); ^{13}C NMR (MeOH-d_4) δ: 162.9, 146.6, 139.5, 138.9, 137.7, 131.8, 128.2, 127.3, 126.9, 119.5, 110.6, 66.4, 63.0, 43.4, 38.9. IR (cm^{-1}) (KBr): 3433.3, 1647.2, 1387.4, 1209.0, 1111.0, 1059.0, 993.1.

3.1.4.13. 2,2-Bis[5-heptafluoroprophyl-4-(4-chloroethyl-pyridinium-4-yl)oxazolyl]hexafluoropropane dimethylsulfonide, ***3n***. Obtained from bisoxazol **2f** (50 mg, 0.054 mmol) and 2-chloroethyl-methanesulfonate (1 mL, 9 mmol) after heating at 100 °C under argon atmosphere for 40 h. After cooling 4 mL of mixture Et_2O/acetone (20:1) was added. The precipitated solid was filtered and purified by trituration with Et_2O to obtain 27.6 mg of compound **3n** as a white solid. Yield: 41%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.21 (d, 4H, J = 6.7 Hz, Py), 8.54 (d, 4H, J = 6.7 Hz, Py), 8.31 (d, 4H, J = 9.1 Hz, Ph), 7.72 (dd, 4H, J = 8.5 Hz, Ph), 5.1 (t, 4H, J = 5.5 Hz, CH_2Py), 4.24 (t, 4H, J = 5.5 Hz, CH_2Cl), 2.69 (s, 12H, CH_3SO_3).

3.1.4.14. 2,2-Bis[5-trifluoromethyl-4-(4-N,N′-dimethylacetamide-pyridinium-4-yl)oxazolyl]hexafluoro propanedichloride, ***3o***. Obtained from bisoxazol **2d** (88 mg, 0.1208 mmol) and 2-chloro-*N,N′*-dimethylacetamide (0.9 mL, 8.75 mmol) after heating at 110 °C in a sealed tube for four days. After cooling, 2 mL of Et_2O/CH_2Cl_2 (20:1) was added until a solid precipitated. The solid was filtered and purified by recristallization from MeOH/Et_2O to give 71.4 mg of compound **3o** as a white solid. Yield: 61%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.01 (d, 4H, J = 5.5 Hz, Py), 8.54 (d, 4H, J = 6.7 Hz, Py), 8.32 (d, 4H, J = 8.5 Hz, Ph), 7.72 (d, 4H, J = 7.95 Hz, Ph), 5.83 (s, 4H, CH_2), 3.19 (s, 6H, CH_3), 3.04 (s, 6H, CH_3); ^{13}C NMR (MeOH-d_4) δ: 165.8, 163.4, 148.3, 146.8, 140.0, 139.3, 138.3, 138.1, 132.4, 128.7, 127.4, 127.2, 126.6, 120.4, 62.7, 36.5.

3.1.4.15. 1,4-Bis[(trifluoromethyl-4-(4-N,N′-dimethylacetamide-pyridinium-4-yl)oxazolyl)]benzene dichloride, ***3p***. Obtained from bisoxazol **2g** (45.2 mg, 0.089 mmol) and 2-chloro-*N,N′*-dimethylacetamide (1 mL, 9.7 mmol) after heating at 100 °C in a sealed tube for ten days. After cooling, acetone was added until a solid was obtained. The solid was filtered and washed with Et_2O to obtain 59.8 mg of compound **3p** as a white solid. Yield: 89%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.01 (d, 4H, J = 5.3 Hz, Py), 8.54 (d, 4H, J = 5.7 Hz, Py), 8.45 (s, 4H, Ph), 5.82 (s, 4H, CH_2), 3.18 (s, 6H, CH_3), 3.04 (s, 6H, CH_3); ^{13}C NMR (MeOH-d_4) δ: 165.8, 163.3, 149.6, 147.1, 139.8, 130.1, 128.6, 127.2, 120.4, 114.9, 62.8, 36.6, 36.3.

3.1.4.16. 1,4-Bis[(pentafluoropropyl-4-(4-N,N′-dimethylacetamide-pyridinium-4-yl)oxazolyl)]benzene dichloride, ***3q***. Obtained from bisoxazol **2h** (52.9 mg, 0.087 mmol) and 2-chloro-*N,N′*-dimethylacetamide (1 mL, 9.7 mmol) after heating at 100 °C in a sealed tube for twelve days. After cooling, acetone was added until a solid was obtained. The solid was filtered and purified by recrystallization from MeOH/Et_2O to obtain 48.2 mg of compound **3q** as a white solid. Yield: 65%. Mp: >250 °C. ^{1}H NMR (MeOH-d_4) δ: 9.01 (d, 4H, J = 5.3 Hz, Py), 8.55 (d, 4H, J = 5.7 Hz, Py), 8.45 (s, 4H, Ph), 5.82 (s,

4H, CH_2), 3.19 (s, 6H, CH_3), 3.05 (s, 6H, CH_3); ^{13}C NMR (MeOH-d_4) δ: 165.8, 164.3, 148.4, 146.9, 141.1, 138.3, 129.6, 129.4, 127.7, 118.8, 110.9, 62.8, 36.6, 36.3.

3.2. In vitro inhibitory activity and cell proliferation inhibitory activity

3.2.1. Choline kinase ex vivo activity assays

All the compounds were tested in an *ex vivo* system in order to evaluate their inhibitory effect towards choline kinase as previously reported [5]. The inhibitory effect against the choline kinase activity is achieved expressing the human ChoKα isoform in *Escherichia coli* bacteria lacking such enzyme activity; therefore the activity that could be observed is exclusively from the human recombinant expressed isoform. Cell extracts were incubated for 30 min at 37 °C in a buffer containing 50 μl of reaction buffer [100 mM Tris pH 8, 10 mM $MgCl_2$, 10 mM ATP, and 200 μM [methyl-^{14}C] choline chloride (55 mCi/nmol, 2 mCi/ml; Amersham Biosciences, UK)] for ChoKα assay. The reaction was stopped in ice by adding 10 μl of 500 mM EDTA. Thirty μl of every sample were resolved by thin layer chromatography plates (LK6D Silica gel 60 A, Whatman Inc. New Jersey) using as liquid phase 0.9% NaCl:methanol; ammonium hydroxide 50:70:5; V:V:V. Radioactive metabolites (Choline and Phosphocholine) were automatically quantifed by an electronic auto radiography system. The inhibitory concentrations at which 50% was reached (IC_{50}) were calculated using the ratio PCho/(PCho + Cho) versus concentration of the inhibitors. IC_{50} values were obtained from nonlinear least-squares fit of the Hill equation to the data.

3.2.2. Cell proliferation assays

Human HT-29 (colorectal adenocarcinoma) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) under standard conditions of temperature (37 °C), humidity (95%) and CO_2 (5%). Cells were seeded on 24-well plates and incubated for 24 h in growth medium. Then, cells were incubated in fresh medium containing increasing concentrations (quadruplicates for each concentration) of the compounds for 72 h. The colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to asses cell viability. The reaction correlates with absorbance at 595 nm in a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The IC_{50} (50% inhibitory concentration of a substance) is quantified by plotting the logOD (optical density) versus log drug concentration.

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