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Original article

New bis(pyridyl)methane derivatives from 4-hydroxy-2-pyridones: synthesis and antitumoral activity

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Abstract

Bis(pyridyl)methane derivatives **5**–**40** were obtained from the reaction of 4-hydroxy-2-pyridones **3** and **4** with aldehydes. Compounds **5**–**40** were evaluated for cytotoxic activity against a panel of 60 human cancer cell lines by the National Cancer Institute and some of them demonstrated inhibitory effects on the growth of a wide range of cancer cell lines generally at 10^{-5} M level and in some case at 10^{-7} M concentrations.

Keywords: Bis(pyridyl)methanes; Antitumoral activity; Pyridones

1. Introduction

The development of potent and effective antineoplastic drugs has become one of the most intensely persuaded goals of contemporary medicinal chemistry. In this context we have been interested in exploiting the biological properties of pyridin-2(1*H*)one derivatives. In previous paper we have reported the synthesis of 4-hydroxy-6-oxopyridines and bis(pyridyl)methane derivatives [1]. These last are in vitro powerful inhibitors of the growth for many types of human tumour cells. These results prompted us to design new analogues with further structural modification to optimise structure–activity relationships and retain their cytotoxicity profile.

In this paper we describe the preparation and evaluation of activity of a new series of bis(pyridyl)methanes, designed taking in account the structure of 1-[bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl))methyl]-2,6-dichlorobenzene **40** that showed best growth inhibition values [1]. This compound lies outside the category of adequately studied classes of antitumour agents and was one of the small percentage which has been selected by the Biological Evaluation Committee of NCI for testing in a new in vivo Hollow Fiber Assay [2,3]. This new system consists of 12 selected human tumour cell lines encased in hollow fibers, which are implanted into athymic nude mice. Six to 8 days after administration of the test compound to mice, the fiber are collected, the cells removed, and growth inhibition is measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Although **28** showed a good activity in subcutaneous implants (Table 4) it does not meet the Biological Committee for Cancer Drugs criteria for further testing in subcutaneous human xenograft assays. With these results in mind and in order to investigate whether the new bis(pyridyl)methanes were endowed with a better antitumoral activity, structural modification were carried out on **40** principally by changing the substitution pattern of both 3- and 2-positions on pyridine rings and on the methyne aromatic ring.

2. Chemistry

The required bis(pyridyl)methanes were prepared according to our reported method [1]; the reaction of aminopropenenitriles **1** and aminopropenoates **2** with 1,3-bis(2,4,6-trichlorophenyl)malonate furnished the corresponding 4-hydroxy-6-oxopyridines **3** and **4** in

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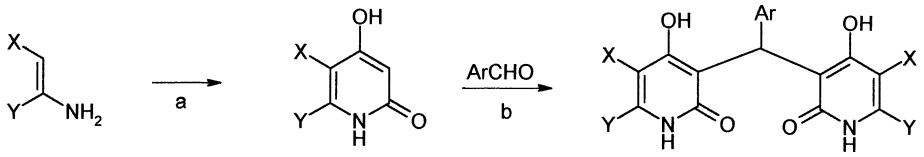


1- 4	Y
a	OEt
b	pyrrolidino
c	morpholino
d	4-MePiperazino
e	4-COOEtPiperazino
f	4-C_6H_5Piperazino
g	4-(4-$OMeC_6H_4$)Piperazino
h	4-(4-FC_6H_4)Piperazino
i	4-(3-ClC_6H_4)Piperazino
j	4-(3,4-$Cl_2C_6H_3$)Piperazino

Fig. 1. Conditions, (a) bis(2,4,6-trichlorophenyl)malonate, toluene, 95 °C; (b) ethanol, piperidine reflux.

good yields. These pyridones were used as synthons for the synthesis of title compounds (Fig. 1).

The condensation of aryl aldehydes with 2 mol of pyridone in presence of catalytic amounts of piperidine afforded the bis(pyridyl)methane derivatives **5**–**40** (Table 1) in moderate to good yields.

3. Pharmacology

Evaluation of anticancer activity on bis(pyridyl)methanes was performed at NCI. First bis(pyridyl)methanes **5**–**40** have been evaluated in primary anticancer assay at 10^{-4} M concentration against NCI-H460 (Lung), MCF7 (Breast) and SF-268 (CNS) cell lines (Table 2).

For NCI criteria, compounds which reduce the growth of any one of the cell lines to ca. 32% or less are passed on for evaluation in the full panel of cell lines over a 5 log dose range.

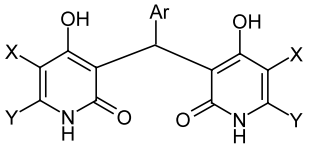
Bis(pyridyl)methanes **8**, **15**, **17** and **28**, that reached these criteria, were evaluated for their anticancer activity following the known in vitro disease-oriented antitumour screening program, which is based upon use of multiple panel of 60 human tumour cell lines [4,5]. Each compound is tested at minimum of five concentrations at 10-fold dilution against every cell line in the panel. A 48 h continuous drug exposure protocol is used and a sulforhodamine B (SRB) protein assay is used to estimate cell viability or growth [6]. The anticancer activity of each compound is deduced from dose–response curves and is presented in Tables 3 and 4 according to the data provided by NCI [7]. The response parameters GI_{50}, TGI and LC_{50} refer to the drug concentration that produced 50% inhibition, total growth inhibition and 50% cytotoxicity, respectively, and are expressed in micromolar concentrations (μM).

4. Results and discussion

From the analysis of data reported in Table 3, where the activity of compounds **8**, **15**, **17**, **21** and **28** is compared with compound **40** we can evince that all compounds maintained high growth inhibition activity at micromolar concentrations in all cell lines. In particular compound **17** exhibited high sensitivity against all leukaemia, melanoma and CNS cancer cell lines, the best results being against the CNS cancer SNB-75 cell line with GI_{50} 0.082 μM. The compound **15** displayed high activity against EKVX (GI_{50} 7.06 μM) cell line of non small cell cancer subpanel and against T-47D (GI_{50} 4.44 μM) cell line of breast cancer subpanel. The compound **8** shows GI_{50} values between 5.89 and 77.6 μM on all cell lines and TGI values of the same order of magnitude but on a minor number of cell lines (53 over 60). As far as regard cytotoxicity compound **8** shows activity on 41 cell lines while compounds **15**, **17** and **28** are cytotoxic on a limited number of cell lines.

From the comparison between bis(3-cyanopyridyl)methanes and bis(3-(ethoxycarbonyl)pyridyl)methanes, bearing the same substituents on all other positions, we can observe that replacement of ethoxycarbonyl group with a cyano group did not sensibly change the average sensitivity against all cell lines. As matter of fact compound **8** exhibited GI_{50} values comparable to those of the analog **40**. However, compound **40** showed LC_{50} values tenfold lower respect

Table 1
Bis(pyridyl)methane derivatives



Compounds	X	Y	Ar
5	CN	pyrrolidino	4-FC_6H_4
6	CN	pyrrolidino	2,6-$Cl_2C_6H_3$
7	CN	morpholino	4-FC_6H_4
8	CN	morpholino	2,6-$Cl_2C_6H_3$
9	CN	4-MePiperazino	4-FC_6H_4
10	CN	4-MePiperazino	2,6-$Cl_2C_6H_3$
11	CN	4-COOEtPiperazino	phenyl
12	CN	4-COOEtPiperazino	4-FC_6H_4
13	CN	4-COOEtPiperazino	2,6-$Cl_2C_6H_3$
14	COOEt	OEt	4-$CH_3C_6H_4$
15	COOEt	OEt	4-FC_6H_4
16	COOEt	OEt	2,6-$Cl_2C_6H_3$
17	COOEt	OEt	2-thienyl
18	COOEt	pyrrolidino	phenyl
19	COOEt	pyrrolidino	2,6-$Cl_2C_6H_3$
20	COOEt	pyrrolidino	2-NO_2-4-ClC_6H_3
21	COOEt	pyrrolidino	2-thienyl
22	COOEt	morpholino	phenyl
23	COOEt	morpholino	4-$CH_3C_6H_4$
24	COOEt	morpholino	4-FC_6H_4
25	COOEt	morpholino	2-ClC_6H_4
26	COOEt	morpholino	4-ClC_6H_4
27	COOEt	morpholino	2,4-$Cl_2C_6H_3$
28	COOEt	4-MePiperazino	2,6-$Cl_2C_6H_3$
29	COOEt	4-C_6H_5Piperazino	phenyl
30	COOEt	4-C_6H_5Piperazino	4-FC_6H_4
31	COOEt	4-C_6H_5Piperazino	2,6-$Cl_2C_6H_3$
32	COOEt	4-(4-$OMeC_6H_4$)Piperazino	4-FC_6H_4
33	COOEt	4-(4-$OMeC_6H_4$)Piperazino	2,6-$Cl_2C_6H_3$
34	COOEt	4-(4-FC_6H_4)Piperazino	4-FC_6H_4
35	COOEt	4-(4-FC_6H_4)Piperazino	2,6-$Cl_2C_6H_3$
36	COOEt	4-(3-ClC_6H_4)Piperazino	4-FC_6H_4
37	COOEt	4-(3-ClC_6H_4)Piperazino	2,6-$Cl_2C_6H_3$
38	COOEt	4-(3,4-$Cl_2C_6H_3$)Piperazino	4-FC_6H_4
39	COOEt	4-(3,4-$Cl_2C_6H_3$)Piperazino	2,6-$Cl_2C_6H_3$
40	COOEt	morpholino	2,6-$Cl_2C_6H_3$

Table 2
Results of primary assay for antitumoral activity of bis(pyridyl)methanes

Compound	Growth percentage at 10^{-4} M concentration		
	Cell line (cancer)		
	MCF7 (breast)	NCI–H460 (lung)	SF-268 (CNS)
5	96	99	102
6	47	90	89
7	90	102	106
8	3	92	18
9	95	98	104
10	94	100	100
11	98	99	110
12	91	102	102
13	89	96	93
14	104	102	100
15	31	15	14
16	91	98	104
17	24	78	29
18	89	100	103
19	100	105	91
20	104	97	108
22	96	99	102
23	100	96	103
24	99	98	74
25	94	101	107
26	98	100	93
27	88	101	108
28	0	2	0
29	93	93	103
30	95	94	92
31	81	95	85
32	88	102	85
33	74	87	95
34	64	77	105
35	70	72	103
36	89	100	104
37	96	100	99
38	88	100	106
39	92	102	108

to **8** on CCRF-CEM, HL 60(TB) and K 562 cell lines of leukaemia, NCI-H522 cell line of non small cell lung cancer, HCT-116 and SW-620 cell lines of colon cancer, M-14 and SK-MEL-28 cell lines of melanoma, CAKI-1, RXF-393 and UO-31 cell lines of renal cancer and MCF-7 cell line of breast cancer.

A good activity is correlated to the presence of electron-withdrawing substituents on phenyl ring. Highest inhibitory activities were obtained when the phenyl nucleus is 2,6-dichlorosubstituted. The shift of one of the two chlorine atoms in 4-position led to a drop in activity as well as removal of one chlorine atom. On the other hand, the introduction of fluorine on the phenyl ring seemed to not improve the biological properties of these molecules.

Furthermore, we can note that the presence of a 2-morpholino group on pyridine moiety is very important for the activity. Replacement of morpholino group with a pyrrolidine or a *N*-substituted piperazine led to loss of activity except for compounds **21** and **28** that retained a good activity. Examination of compounds with a 2-thienyl function on the methyne bridge evidentiated that the highest activity was observed for **17** in which the dialkylamino moiety is substituted with an ethoxy group. In addition bis(2-ethoxypyridyl)methanes retain their activity by replacement of 2-thienyl with 4-fluorophenyl. On the contrary, to our surprise, the replacement of 2-thienyl with an 2,6-dichlorophenyl was associated to poor activity.

From the present study it can be concluded that the antitumoral activity of bis(pyridyl)methanes appears to

Table 3
GI_{50}, TGI and LC_{50} values (μM) of compounds **5**–**40** against different tumour cell lines

Panel/cell line	Compounds																	
	8			**15**			**17**			**21**			**28**			**40**		
	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Leukaemia																		
CCRF-CEM	15.1	66.8	> 100	33.1	> 100	> 100	14.2	> 100	> 100	78.2	> 100	> 100	31.0	> 100	> 100	1.59	3.50	7.73
HL-60(TB)	12.9	29.3	66.7	17.2	54.3	> 100	13.7	40.7	> 100	n.t.	n.t.	n.t.	19.5	58.2	> 100	1.96	4.37	9.73
K-562	11.2	23.2	48.2	16.5	50.8	> 100	12.1	29.7	74.3	> 100	> 100	> 100	25.8	86.8	> 100	1.68	3.51	7.31
MOLT-4	16.1	36.2	81.2	20.1	71.0	>	6.48	22.5	70.2	> 100	> 100	> 100	23.5	62.2	> 100	1.87	5.23	> 100
RPMI-8226	21.4	60.8	> 100	16.1	58.3	> 100	12.1	> 100	> 100	56.4	> 100	> 100	39.2	> 100	> 100	1.98	4.87	> 100
SR	5.89	26.3	> 100	10.5	29.0	79.8	n.t.	n.t.	n.t.	> 100	> 100	> 100	21.9	67.6	> 100	2.83	8.22	> 100
Non small cell lung cancer																		
A549/ATCC	77.6	> 100	> 100	14.8	29.3	58.1	14.5	32.8	74.3	> 100	> 100	> 100	27.9	71.3	> 100	17.5	31.2	55.9
EKVX	18.3	39.0	83.2	7.06	33.2	> 100	11.5	32.7	93.3	> 100	> 100	> 100	14.5	34.2	80.7	18.6	32.6	57.1
HOP-62	22.1	79.3	> 100	13.0	36.1	> 100	10.4	35.8	> 100	> 100	> 100	> 100	30.3	> 100	> 100	15.0	29.8	55.6
HOP-92	18.0	37.8	79.3	1.46	18.1	48.8	9.42	48.2	> 100	> 100	> 100	> 100	19.1	37.6	74.1	1.15	4.51	22.6
NCI-H226	23.0	37.5	61.2	9.82	26.1	68.4	3.90	27.4	> 100	> 100	> 100	> 100	28.7	52.6	96.5	10.4	22.5	48.8
NCI-H23	16.2	32.8	66.6	19.5	43.6	97.5	21.4	82.3	> 100	> 100	> 100	> 100	40.3	> 100	> 100	6.86	20.7	50.1
NCI-H322M	19.3	> 100	> 100	16.9	60.8	> 100	14.4	52.1	> 100	> 100	> 100	> 100	25.9	48.4	90.7	18.1	32.0	56.5
NCI-H460	> 100	> 100	> 100	16.4	36.5	81.4	13.3	39.8	> 100	> 100	> 100	> 100	28.0	77.0	> 100	20.3	41.2	83.4
NCI-H522	13.4	28.2	59.1	18.6	42.0	94.9	13.8	27.6	55.2	> 100	> 100	> 100	21.9	42.0	80.6	1.83	3.72	7.58
Colon cancer																		
COLO 205	13.8	26.7	51.7	25.9	79.8	> 100	17.3	40.7	95.6	> 100	> 100	> 100	33.1	> 100	> 100	3.55	14.1	37.5
HCC-2998	12.5	27.5	60.3	18.1	39.0	84.0	13.9	35.0	88.1	> 100	> 100	> 100	36.8	78.0	> 100	18.2	32.3	57.2
HCT-116	28.3	> 100	> 100	25.7	> 100	> 100	17.5	45.0	> 100	> 100	> 100	> 100	38.1	> 100	> 100	1.68	3.21	6.13
HCT-15	15.0	28.2	53.1	18.4	40.4	88.9	14.5	37.5	96.8	> 100	> 100	> 100	30.7	97.7	> 100	3.40	12.3	57.9
HT29	15.4	28.9	54.3	16.1	31.7	62.3	16.2	35.3	77.0	> 100	> 100	> 100	19.8	39.1	77.2	3.56	12.9	36.0
KM12	16.1	32.7	66.7	16.9	37.7	84.1	18.0	42.9	> 100	> 100	> 100	> 100	23.5	49.7	> 100	14.5	27.6	52.6
SW-620	17.7	36.8	76.7	17.3	51.1	> 100	12.1	34.1	95.8	> 100	> 100	> 100	31.8	68.2	> 100	1.71	3.08	5.55
CNS cancer																		
SF-268	19.5	51.1	> 100	14.6	33.6	77.3	10.2	35.0	> 100	> 100	> 100	> 100	24.2	56.5	> 100	6.09	19.0	44.3
SF-295	30.6	> 100	> 100	17.7	49.8	> 100	12.0	49.6	> 100	> 100	> 100	> 100	28.1	63.3	> 100	16.5	30.4	56.1
SF-539	19.2	34.9	63.5	10.0	23.5	55.0	6.39	28.5	99.4	> 100	> 100	> 100	19.3	39.5	80.5	1.57	3.98	10.2
SNB-19	16.1	29.6	54.4	17.1	42.2	> 100	11.4	39.8	> 100	> 100	> 100	> 100	20.4	37.2	68.1	3.84	16.2	40.2
SNB-75	15.1	24.8	51.2	n.t.	n.t.	n.t.	0.082	9.72	64.9	> 100	> 100	> 100	n. t.	n. t.	n. t.	3.42	9.48	30.7
U251	16.8	30.4	55.1	15.2	36.3	86.8	12.5	30.3	73.9	> 100	> 100	> 100	23.1	50.0	> 100	4.72	15.6	40.0
Melanoma																		
LOX IMVI	13.0	25.6	50.6	16.6	33.1	66.3	12.6	30.7	75.0	> 100	> 100	> 100	34.5	> 100	> 100	2.37	6.29	26.3
MALME-3M	12.9	26.8	55.5	8.31	29.5	91.9	8.35	25.6	66.4	> 100	> 100	> 100	22.6	41.5	75.9	2.00	5.95	22.1
M14	18.0	42.9	> 100	24.6	63.3	> 100	16.1	35.6	78.4	> 100	> 100	> 100	30.0	68.9	> 100	1.74	3.18	5.83
SK-MEL-2	17.6	39.5	88.6	16.6	37.0	82.4	12.8	29.9	69.7	> 100	> 100	> 100	26.0	53.2	> 100	4.75	16.2	40.2
SK-MEL-28	17.9	32.9	60.3	11.8	27.0	61.7	13.1	43.6	> 100	> 100	> 100	> 100	18.0	34.1	64.8	1.69	3.37	6.69
SK-MEL-5	16.6	35.4	75.6	12.8	25.9	52.3	12.8	25.5	50.5	> 100	> 100	> 100	20.7	40.1	77.6	4.07	15.6	39.5
UACC-257	17.8	34.0	64.7	14.8	37.3	93.9	11.7	36.9	> 100	> 100	> 100	> 100	21.3	38.9	71.1	5.13	17.7	44.3

Table 3 (*Continued*)

Panel/cell line	Compounds																
	8			**15**			**17**			**21**			**28**			**40**		
	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
UACC-62	16.6	30.2	55.0	15.6	30.3	59.0	14.2	28.6	57.4	> 100	> 100	> 100	21.5	39.5	72.7	4.88	16.6	40.7
Ovarian cancer																		
IGROV1	18.1	35.3	68.8	14.9	30.3	61.6	13.9	28.6	58.7	n.t.	n.t.	n.t.	20.7	39.2	74.1	4.09	15.0	51.8
OVCAR-3	16.4	30.4	56.6	15.4	39.7	> 100	11.6	30.4	79.9	> 100	> 100	> 100	34.7	87.1	> 100	2.48	8.07	28.2
OVCAR-4	17.7	32.9	61.3	14.6	48.2	> 100	17.6	76.0	> 100	> 100	> 100	> 100	21.5	38.4	68.6	6.76	19.6	44.3
OVCAR-5	14.0	31.9	72.4	29.1	> 100	> 100	26.5	> 100	> 100	> 100	> 100	> 100	42.9	> 100	> 100	13.1	25.7	50.7
OVCAR-8	18.6	34.1	62.5	14.0	28.1	56.5	12.6	41.6	> 100	> 100	> 100	> 100	22.0	45.0	92.2	6.31	18.8	44.9
Renal cancer																		
786-0	32.0	> 100	> 100	16.4	39.7	96.3	12.1	59.3	> 100	> 100	> 100	> 100	27.6	87.0	> 100	1.81	3.20	5.66
A498	34.5	> 100	> 100	11.4	26.2	60.2	2.67	23.5	59.0	> 100	> 100	> 100	22.1	39.9	71.8	7.57	19.7	44.4
ACHN	16.1	29.6	54.4	16.3	31.7	61.5	12.8	25.4	50.4	> 100	> 100	> 100	20.7	42.8	88.2	2.97	8.32	30.6
CAKI-1	18.1	33.2	61.0	18.9	42.4	95.0	18.0	65.4	> 100	> 100	> 100	> 100	26.5	52.7	> 100	1.84	4.27	9.91
RXF 393	14.1	35.5	89.0	4.28	23.0	56.4	1.70	19.0	55.7	> 100	> 100	> 100	18.2	38.8	82.6	2.06	4.36	9.24
SN12C	20.1	34.5	59.1	14.7	30.2	62.0	15.4	34.6	77.7	> 100	> 100	> 100	26.3	48.0	87.5	1.58	4.54	15.8
TK-10	17.5	32.5	60.1	25.3	67.7	> 100	25.4	63.7	> 100	> 100	> 100	> 100	30.6	55.4	> 100	3.38	11.3	37.3
UO-31	18.2	33.5	61.7	12.8	25.7	51.5	10.9	26.7	65.4	> 100	> 100	> 100	21.5	39.1	70.8	1.88	3.73	7.39
Prostate cancer																		
PC-3	17.8	37.7	79.7	15.4	34.6	78.0	18.2	> 100	> 100	> 100	> 100	> 100	22.6	47.5	99.8	14.1	27.6	54.3
DU-145	23.9	69.2	> 100	11.1	24.3	53.2	13.9	26.9	51.8	> 100	> 100	> 100	17.8	38.3	82.5	9.96	21.5	46.4
Breast cancer																		
MCF7	16.8	34.2	69.9	20.4	60.7	> 100	14.9	53.9	> 100	> 100	> 100	> 100	29.5	> 100	> 100	2.07	3.86	7.19
NCI/ADR-RES	27.4	> 100	> 100	24.1	> 100	> 100	27.4	> 100	> 100	> 100	> 100	> 100	71.1	> 100	> 100	16.6	31.5	59.7
MDA-MB-231/ATCC	16.6	30.4	55.7	12.6	29.8	70.1	11.1	30.1	81.7	> 100	> 100	> 100	25.5	55.1	> 100	11.2	23.4	48.9
HS 578T	19.5	58.1	> 100	20.8	> 100	> 100	21.0	> 100	> 100	> 100	> 100	> > 100	33.8	75.0	> 100	3.47	15.9	63.9
MDA-MB-435	16.6	30.2	55.0	17.6	37.7	80.8	14.5	32.4	72.2	> 100	> 100	> 100	25.8	57.9	> 100	2.53	7.49	26.5
BT-549	17.6	32.8	61.1	9.71	23.6	56.4	5.01	22.7	64.5	> 100	> 100	> 100	20.6	40.8	80.8	4.95	15.7	39.6
T-47D	17.0	27.7	58.1	4.44	32.7	> 100	0.098	17.0	> 100	> 100	> 100	> 100	16.5	45.8	> 100	3.56	9.27	50.9

Table 4
Results of hollow fiber assay of compound **40**

Test	Score
IP	4
SC	8
Total	12
Cell kill	none

be related to some structural requirements and to the presence of particular substituents. As matter of fact morpholino and 2,6-dichlorophenyl moieties play an important role for antitumor activity in these type of compounds.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Stuart Scientific Melting point SMP1 and are uncorrected. IR spectra were recorded on Nujol mulls between salt plates in Bruker Vector 22 spectrophotometer. ^{1}H-NMR spectra were recorded on a varian Unity 300 spectrometer. It was not possible to register the ^{1}H-NMR spectra of some compounds since they are insoluble in all common deuterated solvents. Elemental analyses were carried out with a Fisons EA 1108 elemental analyser. Analytical TLC were carried out on Merck 0.2 mm precoated silica gel aluminium sheets (60 F-254). 3-Amino-3-dialkylaminopropenenitriles **1**, ethyl 3-aminopropenoates **2**, [8,9] 4-hydroxypyridones **3b–e** [10], **4b**, **4c** and bis(pyridyl)methanes **21** and **40** [1] were synthesised as previously described. Analyses of C, H, N were within ± 0.4% of the theoretical values.

5.2. Synthesis of 4-hydroxypiridones (*3*, *4*)

A mixture of the appropriate propenenitrile **1** or propenoate **2** (0.02 mol) and bis (2,4,6-trichlorophenyl)malonate (9.26 g, 0.02 mol) in toluene (20 mL) was heated in water bath at 95 °C for 2 h. After concentration in vacuo, the residue was treated with diethyl ether, filtered off and recrystallised from a suitable solvent.

5.2.1. 2-Ethoxy-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (*4a*)

Prepared in 60% yield starting from ethyl 3-amino-3-ethoxy-2-propenoate. M.p. 180 °C (from acetonitrile). Anal. ($C_{10}H_{13}NO_5$) C, H, N. IR (Nujol): 1661, 1645, 1611, 1557 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (m, 6H, CH_3), 4.17 (m, 4H, CH_2), 5.66 (s, 1H, H-5), 10.95, 11.42 (s, 2H, NH and OH).

5.2.2. 2-(4-Methylpiperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (*4d*)

Prepared in 57% yield starting from ethyl 3-amino-3-(4-methylpiperazino)-2-propenoate. M.p. 158–160 °C (from acetonitrile). Anal. ($C_{13}H_{19}N_3O_4$) C, H, N. IR (Nujol): 1650, 1591, 1562 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, $J = 7.2$ Hz, 3H, CH_3), 2.20 (s, 3H, CH_3), 2.39, 3.20 (m, 8H, piperazinyl), 4.17 (q, $J = 7.2$ Hz, 2H, CH_2), 5.38 (s, 1H, H-5), 7.40 (s, 2H, NH and OH).

5.2.3. 2-(4-(Ethoxycarbonyl)piperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (*4e*)

Prepared in 77% yield starting from ethyl 3-amino-3-(4-(ethoxycarbonyl)piperazino)-2-propenoate. M.p. 178–180 °C (from ethanol). Anal. ($C_{15}H_{21}N_3O_6$) C, H, N. IR (Nujol): 1660, 1585, 1560 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.20 (m, 6H, CH_3), 2.38, 3.19 (m, 8H, piperazinyl), 4.19 (m, 4H, CH_2), 5.40 (s, 1H, H-5), 8.05 (s, 2H, NH and OH).

5.2.4. 2-(4-Phenylpiperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (*4f*)

Prepared in 82% yield starting from ethyl 3-amino-3-(4-phenylpiperazino)-2-propenoate. M.p. 232–233 °C (from 1-propanol). Anal. ($C_{18}H_{21}N_3O_4$). C, H, N. IR (Nujol): 1642, 1598, 1579, 1553 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, $J = 7.2$ Hz, 3H, CH_3), 3.13, 3.30 (m, 8H, piperazinyl), 4.20 (q, $J = 7.2$ Hz, 2H, CH_2), 5.42 (s, 1H, H-5), 6.74, 6.90. 7.17 (m, 5H, Ar), 10.63, 10.88 (s, 2H, NH and OH).

5.2.5. 2-(4-(4-Methoxyphenyl)piperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (*4g*)

Prepared in 78% yield starting from ethyl 3-amino-3-(4-(4-methoxyphenyl)piperazino)-2-propenoate. M.p. 205 °C (from). Anal. ($C_{19}H_{23}N_3O_5$). C, H, N. IR (Nujol): 1671, 1648, 1567 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.22 (t, $J = 7.0$ Hz, 3H, CH_3), 2.99, 3.31 (m, 8H, piperazinyl), 4.19 (q, $J = 7.0$ Hz, 2H, CH_2), 5.61 (s, 1H, H-5), 6.78, 6.86 (m, 4H, Ar), 10.62, 10.88 (s, 2H, NH and OH).

5.2.6. 2-(4-(4-Fluorophenyl)piperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (*4h*)

Prepared in 80% yield starting from ethyl 3-amino-3-(4-(4-fluorophenyl)piperazino)-2-propenoate. M.p. 218–220 °C (from 1-propanol). Anal. ($C_{18}H_{20}FN_3O_4$) C, H, N. IR (Nujol): 1645, 1591, 1552 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, $J = 7.2$ Hz, 3H, CH_3), 3.07, 3.31 (m, 8H, piperazinyl), 4.19 (q, $J = 7.2$ Hz, 2H, CH_2), 5.42 (s, 1H, H-5), 6.97 (m, 4H, Ar), 10.63, 10.88 (s, 2H, NH and OH).

*5.2.7. 2-(4-(3-Chlorophenyl)piperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (**4i**)*

Prepared in 68% yield starting from ethyl 3-amino-3-(4-(3-chlorophenyl)piperazino)-2-propenoate. M.p. 174–175 °C (from 1-propanol). Anal. ($C_{18}H_{20}ClN_3O_4$) C, H, N. IR (Nujol): 1647, 1594, 1550 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.22 (t, $J = 7.2$ Hz, 3H, CH_3), 3.18, 3.30 (m, 8H, piperazinyl), 4.20 (q, $J = 7.2$ Hz, 2H, CH_2), 5.44 (s, 1H, H-5), 6.74, 7.16 (m, 4H, Ar), 10.64, 10.88 (s, 2H, NH and OH).

*5.2.8. 2-(4-(3,4-Dichlorophenyl)piperazino)-4-hydroxy-1,6-dihydro-6-oxopyridine-3-carboxylic acid ethyl ester (**4j**)*

Prepared in 75% yield starting from ethyl 3-amino-3-(4-(3,4-dichlorophenyl)piperazino)-2-propenoate. M.p. 198–200 °C (from 1-propanol). Anal. ($C_{18}H_{19}Cl_2N_3O_4$) C, H, N. IR (Nujol): 1668, 1648, 1582 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (t, $J = 7.2$ Hz, 3H, CH_3), 3.08, 3.24 (m, 8H, piperazinyl), 4.18 (q, $J = 7.2$ Hz, 2H, CH_2), 5.44 (s, 1H, H-5), 6.89, 7.10, 7.36 (m, 4H, Ar), 10.61, 10.87 (s, 2H, NH and OH).

*5.3. Synthesis of bis(pyridyl)methanes (**5**–**40**)*

A mixture of the appropriate 4-hydroxypyridone **3**, **4** (0.0025 mol) and aldehyde (0.00125 mol) in ethanol (20 mL) was added a few drops of piperidine and heated at reflux until disappearance of starting material. The formed precipitate was filtered off and washed with diethyl ether.

*5.3.1. 1-[Bis(3-cyano-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**5**)*

Prepared in 40% yield starting from **3b** and 4-fluorobenzaldehyde, refluxing for 12 h. M.p. 285 °C dec. Anal. ($C_{27}H_{25}FN_6O_4$) C, H, N. IR (Nujol): 3232, 3139, 3054, 2218, 1614, 1598 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.23 (m, 12H, CH_3), 4.21 (m, 8H, CH_2), 5.93 (s, 1H, CH), 6.92 (m, 4H, Ar), 11.42, 12.42 (br s, 4H, NH and OH).

*5.3.2. 1-[Bis(3-cyano-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**6**)*

Prepared in 53% yield starting from **3b** and 2,6-dichlorobenzaldehyde, refluxing for 5 h. M.p. 290 °C dec. Anal. ($C_{27}H_{24}Cl_2N_6O_4$) C, H, N. IR (Nujol): 3247, 3164, 2209, 1620, 1589 cm^{-1}.

5.3.3. 1-[Bis(3-cyano-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (7)

Prepared in 80% yield starting from **3c** and 4-fluorobenzaldehyde, refluxing for 12 h. M.p. 285 °C dec. Anal. ($C_{27}H_{25}FN_6O_6$) C, H, N. IR (Nujol): 2220, 1614, 1589 cm^{-1}.

*5.3.4. 1-[Bis(3-cyano-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**8**)*

Prepared in 40% yield starting from **3c** and 2,6-dichlorobenzaldehyde, refluxing for 4 h. M.p. 280 °C dec. Anal. ($C_{27}H_{24}Cl_2N_6O_6$) C, H, N. IR (Nujol): 2212, 1637, 1618, 1590 cm^{-1}.

*5.3.5. 1-[Bis(3-cyano-4-hydroxy-2-(4-methylpiperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**9**)*

Prepared in 60% yield starting from **3d** and 4-fluorobenzaldehyde, refluxing for 3 h. M.p. 250 °C. Anal. ($C_{29}H_{31}FN_6O_4$) C, H, N. IR (Nujol): 2210, 1610 cm^{-1}.

*5.3.6. 1-[Bis(3-cyano-4-hydroxy-2-(4-methylpiperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**10**)*

Prepared in 94% yield starting from **3d** and 2,6-dichlorobenzaldehyde, refluxing for 5 h. M.p. 290 °C dec. Anal. ($C_{29}H_{30}Cl_2N_8O_4$) C, H, N. IR (Nujol): 3405, 3386, 3246, 3139, 3118, 3069, 2207, 1607, 1583 cm^{-1}.

*5.3.7. Bis(3-cyano-4-hydroxy-2-(4-(ethoxycarbonyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]benzene (**11**)*

Prepared in 86% yield starting from **3e** and benzaldehyde, refluxing for 3 h. M.p. 238–240 °C. Anal. ($C_{33}H_{36}N_8O_8$) C, H, N. IR (Nujol): 2200, 1655, 1610, 1560 cm^{-1}.

*5.3.8. 1-[Bis(3-cyano-4-hydroxy-2-(4-(ethoxycarbonyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**12**)*

Prepared in 80% yield starting from **3e** and 4-fluorobenzaldehyde, refluxing for 5 h. M.p. 230–235 °C. Anal. ($C_{33}H_{35}FN_8O_8$) C, H, N. IR (Nujol): 2215, 1665, 1622, 1581 cm^{-1}.

*5.3.9. 1-[Bis(3-cyano-4-hydroxy-2-(4-(ethoxycarbonyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**13**)*

Prepared in 45% yield starting from **3e** and 2,6-dichlorobenzaldehyde, refluxing for 5 h. M.p. 280 °C dec. Anal. ($C_{33}H_{34}Cl_2N_8O_8$) C, H, N. IR (Nujol): 3061, 2214, 1693, 1641, 1588 cm^{-1}.

*5.3.10. 1-[Bis(2-ethoxy-3-(ethoxycarbonyl)-4-hydroxy-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-methylbenzene (**14**)*

Prepared in 70% yield starting from **4a** and 4-methylbenzaldehyde, refluxing for 2 h. M.p. 254–255 °C. Anal. ($C_{28}H_{32}N_2O_{10}$) C, H, N. IR (Nujol): 1645, 1610, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.24 (m, 12H, CH_3), 4.21 (m, 8H, CH_2), 2.19 (s, 3H, CH_3),

5.97 (s, 1H, CH), 6.85 (m, 4H, Ar), 11.34, 12.30 (s, 4H, NH and OH).

*5.3.11. 1-[Bis(2-ethoxy-(3-ethoxycarbonyl)-4-hydroxy-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**15**)*

Prepared in 72% yield starting from **4a** and 4-fluorobenzaldehyde, refluxing for 2 h. M.p. 225–227 °C. Anal. ($C_{27}H_{29}FN_2O_{10}$) C, H, N. IR (Nujol): 1649, 1602, 1559 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.23 (m, 12H, CH_3), 4.21 (m, 8H, CH_2), 5.93 (s, 1H, CH), 6.92 (m, 4H, Ar), 11.42, 12.42 (s, 4H, NH and OH).

*5.3.12. 1-[Bis(2-ethoxy-(3-ethoxycarbonyl)-4-hydroxy-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**16**)*

Prepared in 78% yield starting from **4a** and 2,6-dichlorobenzaldehyde, refluxing for 6 h. M.p. 168–170 °C (from benzene). Anal. ($C_{27}H_{28}Cl_2N_2O_{10}$) C, H, N. IR (Nujol): 1661, 1645, 1611, 1558 cm^{-1}.

*5.3.13. 2-[Bis(2-ethoxy-(3-ethoxycarbonyl)-4-hydroxy-1,6-dihydro-6-oxopyridin-5-yl)methyl]-thiophene (**17**)*

Prepared in 70% yield starting from **4a** and 2-thiophenecarboxyaldehyde, refluxing for 2 h. M.p. 204–205 °C (from EtOH). Anal. ($C_{25}H_{28}N_2O_{10}S$) C, H, N. IR (Nujol): 1700, 1645, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (m, 12H, CH_3), 4.16 (m, 8H, CH_2), 6.22 (s, 1H, CH), 6.40, 6.74, 7.09 (m, 3H, thienyl), 8.25 (s, 4H, NH and OH).

*5.3.14. [Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]benzene (**18**)*

Prepared in yield starting from **4b** and benzaldehyde, refluxing for 2 h. M.p. 230 °C dec. Anal. ($C_{31}H_{34}ClN_5O_{10}$) C, H, N. IR (Nujol): 3220, 3100, 1710, 1570 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.18 (m, 6H, CH_3), 1.83, 3.20 (m, 16H, pyrrolidinyl), 4.12 (m, 4H, CH_2), 5.63 (s, 1H, CH), 7.13 (m, 5H, Ar), 10.47, 12.40 (s, 4H, NH and OH).

*5.3.15. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**19**)*

Prepared in 40% yield starting from **4b** and 2,6-dichlorobenzaldehyde, refluxing for 4 h. M.p. 220 °C dec. Anal. ($C_{31}H_{34}Cl_2N_4O_8$) C, H, N. IR (Nujol): 3400, 3080, 2730, 2640, 1650, 1630, 1585 cm^{-1}.

*5.3.16. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-pyrrolidino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-chloro-2-nitrobenzene (**20**)*

Prepared in 75% yield starting from **4b** and 4-chloro-2-nitrobenzaldehyde, refluxing for 2 h. M.p. 230–233 °C. Anal. ($C_{31}H_{34}ClN_5O_{10}$) C, H, N. IR (Nujol): 3232, 3139, 1711, 1590, 1533 cm^{-1}.

*5.3.17. [Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]benzene (**22**)*

Prepared in quantitative yield starting from **4c** and benzaldehyde, refluxing for 3 h. M.p. 246–248 °C dec. Anal. ($C_{31}H_{36}N_4O_{10}$) C, H, N. IR (Nujol): 1710, 1640, 1610, 1580 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.21 (m, 6H, CH_3), 3.13, 3.59 (m, 16H, morpholinyl), 4.16 (m, 4H, CH_2), 5.82 (s, 1H, CH), 7.01–7.23 (m, 5H, Ar), 11.50, 12.27 (s, 4H, NH and OH).

*5.3.18. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-methylbenzene (**23**)*

Prepared in quantitative yield starting from **4c** and 4-methylbenzaldehyde, refluxing for 3 h. M.p. 245–247 °C dec. Anal. ($C_{32}H_{35}N_4O_{10}$) C, H, N. IR (Nujol): 3220, 1710, 1640, 1600, 1560 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.19 (m, 6H, CH_3), 1.58 (s, 3H, CH_3), 3.01, 3.54 (m, 16H, morpholinyl), 4.09 (m, 4H, CH_2), 5.64 (s, 1H, CH), 6.91 (m, 4H, Ar), 9.46 (brs, 4H, NH and OH).

*5.3.19. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**24**)*

Prepared in 80% yield starting from **4c** and 4-fluorobenzaldehyde, refluxing for 2 h. M.p. 260 °C dec. Anal. ($C_{31}H_{35}FN_4O_{10}$) C, H, N. IR (Nujol): 1720, 1645, 1622, 1589 cm^{-1}.

*5.3.20. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2-chlorobenzene (**25**)*

Prepared in 94% yield starting from **4c** and 2-chlorobenzaldehyde, refluxing for 1 h. M.p. 243 °C. Anal. ($C_{31}H_{35}ClN_4O_{10}$) C, H, N. IR (Nujol): 1716, 1649, 1613, 1600, 1563 cm^{-1}.

*5.3.21. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-chlorobenzene (**26**)*

Prepared in 96% yield starting from **4c** and 4-chlorobenzaldehyde, refluxing for 1 h. M.p. 263 °C dec. Anal. ($C_{31}H_{35}ClN_4O_{10}$) C, H, N. IR (Nujol): 1643, 1611, 1533 cm^{-1}.

*5.3.22. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-morpholino-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,4-dichlorobenzene (**27**)*

Prepared in 85% yield starting from **4c** and 2,4-dichlorobenzaldehyde, refluxing for 1 h. M.p. 240 °C dec. Anal. ($C_{31}H_{34}Cl_2N_4O_{10}$) C, H, N. IR (Nujol): 1722, 1616, 1580 cm^{-1}.

*5.3.23. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-methylpiperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**28**)*

Prepared in 45% yield starting from **4d** and 2,6-dichlorobenzaldehyde, refluxing for 2 h. M.p. 258–260 °C. Anal. ($C_{36}H_{40}Cl_2N_6O_8$) C, H, N. IR (Nujol): 1718, 1620, 1585 cm^{-1}.

*5.3.24. [Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-phenylpiperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]benzene (**29**)*

Prepared in quantitative yield starting from **4f** and benzaldehyde, refluxing for 3 h. M.p. 228–230 °C. Anal. ($C_{43}H_{46}N_6O_8$) C, H, N. IR (Nujol): 1721, 1645, 1616, 1599 cm^{-1}.

*5.3.25. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-phenylpiperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**30**)*

Prepared in 86% yield starting from **4f** and 4-fluorobenzaldehyde, refluxing for 2 h. M.p. 208–210 °C. Anal. ($C_{43}H_{45}FN_6O_8$) C, H, N. IR (Nujol): 1721, 1620, 1597 cm^{-1}.

*5.3.26. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-phenylpiperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**31**)*

Prepared in 70% yield starting from **4f** and 2,6-dichlorobenzaldehyde, refluxing for 2 h. M.p. 204–205 °C (From ethanol). Anal. ($C_{43}H_{44}Cl_2N_6O_8$) C, H, N. IR (Nujol): 3094, 3059, 1693, 1625, 1597 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.19 (m, 6H, CH_3), 3.13, 3.29 (m, 16H, piperazinyl), 4.17 (m, 4H, CH_2), 6.72–7.19 (m, 14H, Ar and CH), 11.58, 12.63 (s, 4H, NH and OH).

*5.3.27. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(4-methoxyphenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**32**)*

Prepared in 98% yield starting from **4g** and 4-fluorobenzaldehyde, refluxing for 3 h. M.p. 240 °C dec. Anal. ($C_{45}H_{49}FN_6O_{10}$) C, H, N. IR (Nujol): 1718, 1612 cm^{-1}.

*5.3.28. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(4-methoxyphenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**33**)*

Prepared in 76% yield starting from **4g** and 2,6-dichlorobenzaldehyde, refluxing for 10 h. M.p. 178–180 °C. Anal. ($C_{45}H_{48}Cl_2N_6O_{10}$) C, H, N. IR (Nujol): 1720, 1630, 1572 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.20 (m, 6H, CH_3), 3.28 (m, 16H, piperazinyl), 3.63 (s, 6H, CH_3), 4.22 (m, 4H, CH_2), 6.92 (m, 12H, Ar and CH).

*5.3.29. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(4-fluorophenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**34**)*

Prepared in 90% yield starting from **4h** and 4-fluorobenzaldehyde, refluxing for 1 h. M.p. 242–244 °C. Anal. ($C_{43}H_{43}F_3N_6O_8$) C, H, N. IR (Nujol): 1715, 1653, 1623, 1592 cm^{-1}.

*5.3.30. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(4-fluorophenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**35**)*

Prepared in 65% yield starting from **4h** and 2,6-dichlorobenzaldehyde, refluxing for 6 h. M.p. 223–225 °C. Anal. ($C_{43}H_{42}Cl_2F_2N_6O_8$) C, H, N. IR (Nujol): 1718, 1625, 1578 cm^{-1}.

*5.3.31. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(3-chlorophenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**36**)*

Prepared in 85% yield starting from **4i** and 4-fluorobenzaldehyde, refluxing for 3 h. M.p. 245 °C dec. Anal. ($C_{43}H_{43}Cl_2FN_6O_8$) C, H, N. IR (Nujol): 1710, 1652, 1619, 1591 cm^{-1}.

*5.3.32. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(3-chlorophenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**37**)*

Prepared in 75% yield starting from **4i** and 2,6-dichlorobenzaldehyde, refluxing for 10 h. M.p. 204–205 °C (From 1-propanol). Anal. ($C_{43}H_{42}Cl_4N_6O_8$) C, H, N. IR (Nujol): 1711, 1620, 1593 cm^{-1}.

*5.3.33. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(3,4-dichlorophenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-4-fluorobenzene (**38**)*

Prepared in quantitative yield starting from **4j** and 4-fluorobenzaldehyde, refluxing for 3 h. M.p. 238 °C dec. Anal. ($C_{43}H_{41}Cl_4FN_6O_8$) C, H, N. IR (Nujol): 1711, 1612, 1587 cm^{-1}.

*5.3.34. 1-[Bis(3-(ethoxycarbonyl)-4-hydroxy-2-(4-(3,4-dichlorophenyl)piperazino)-1,6-dihydro-6-oxopyridin-5-yl)methyl]-2,6-dichlorobenzene (**39**)*

Prepared in 80% yield starting from **4j** and 2,6-dichlorobenzaldehyde, refluxing for 8 h. M.p. 235 °C dec. Anal. ($C_{43}H_{40}Cl_6N_6O_8$) C, H, N. IR (Nujol): 1709, 1639, 1601 cm^{-1}.

5.4. Pharmacology

The compounds were tested by NCI in an in vitro 3-cell line, one dose primary anticancer assay as a primary cancer screen. The 3-cell line panel consists of the MCF7 (breast), NCI-H460 (lung) and SF-268 (CNS). Each cell line is inoculated and preincubated on a microtriter plate. Test agents are then added at single 10^{-4} M

concentration and the culture incubated for 48 h. End-point determinations are made with alamar blue [11]. Results for each test agent are reported as the percent of growth of the treated cells when compared with untreated control cells. Compounds which reduce the growth of any one of the cell lines to ca. 32% or less are passed on for evaluation in the full panel of 60 cell lines over a 5 log dose range.

A total of 60 human tumour cell lines, derived from nine cancer types (leukaemia, lung, colon, brain, melanoma, ovarian, renal, prostate and breast) formed the basis of this test. The tumour cells were cultured in RPMI1640 medium supplemented with 5% foetal calf serum and 2mM L-glutamine. The tumour cells are inoculated over a series of standard 96-well microtrite plates in 100 μL of medium [4,5]. Density of inoculum depends on the type of tumour cell and from its growth characteristics [12]. These cells are then preincubated on the microtrite plate for 24 h before adding the compounds. These were tested in DMSO solution at five different concentration (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M). After an incubation of the chemical agent for 48 h with the tumour cell lines a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The cytotoxic effects are evaluated and the assay results and dose response parameters were calculated as previously described [7].

5.4.1. Hollow fiber assay for preliminary in vivo testing

Each compound is tested against a standard panel of 12 human tumour cell lines including NCI-H23, NCI-H522, MDA-MB-231, MDA-MB-435, SW-620, COLO 205, LOX IMVI, UACC-62, OVCAR-3, OVACAR-5, U251 and SF-295. The cell lines are cultivated in RPMI-1640 containing 10% FBS and 2mM L-glutamine. On the day preceding hollow fiber preparation, the cells are harvested by standard trypsinization technique and resuspended at the desired cell density (varies by cell line between 2 and 10×10^6 cells per mL). The cell suspension is flushed into 1 mm I.D. polyvinylidene hollow fibers with a molecular weight exclusion of 500 000 Da. The hollow fiber are heat-sealed at 2 cm intervals and the samples generated from these seals are placed into tissue culture medium and incubated at 37 °C in 5% CO_2 for 24–48 h prior to implantation. A total of three different tumour lines are prepared for each experiment so that each mouse receives three intraperitoneal implants (one of each tumour line) and three subcutaneous implants (one of each tumour line). On the day of implantation, samples of each tumour cell line are quantitated for viable cell mass by a stable MTT assays so that at time 0 cell mass is known. Thus, the cytostatic and cytocidal capacities of the test compound can be assayed. Mice are treated with experimental agents starting on day 3 or 4 following fiber implantion and continuing once daily for total four doses. Each agent is assessed by intraperitoneal injection at two dose levels with three mice per dose per experiment. Vehicle controls consist of six mice receiving the compound diluent only. The fibers are collected from mice on the day following the fourth compound treatment and subjected to stable endpoint MTT assay. The optical density of each sample is determined spectrophotometrically at 540 nm and the mean of each treatment group is calculated. The percent net growth in each treatment group is calculated and compared with the percent net cell growth in the vehicle treated controls. Each compound is assessed in a total four experiments (3 cell lines per experiment $\times$ 4 experiments = 12 cell lines). Compounds are selected for further testing on the basis of several hollow fiber assay criteria. These include, (1) a reduction in net cell growth of 50% or greater in ten of the 48 possible test combination (12 cell lines $\times$ 2 sites $\times$ 2 compound doses); (2) a reduction in net cell growth of 50% or greater in a minimum of the 24 distant site combinations (intraperitoneal drug per subcutaneous culture); and/or (3) cell kill of one or more cell lines in either implant site (reduction in the viable cell mass below the level present at the start of the experiment). To simplify evaluation, a point system has been adopted which allows rapid viewing of the activity of a given compound. For this, a value of 2 is assigned for each compound dose which results in a 50% or greater reduction in viable cell mass. The intraperitoneal and subcutaneous samples are scored separately so that criteria (1) and (2) can be evaluated. Compounds with a combined IP + SC score 20, a SC score 8 or a net cell kill of one or more cell lines are referred for further studies. The maximum possible score for an agent is 96 (12 cell lines $\times$ 2 sites $\times$ 2 dose levels $\times$ 2 [score]). These criteria were statistically validated by comparing the activity outcomes of >80 randomly selected compounds in the hollow fiber assay and in xenograft testing. This comparison indicated that there was a very low probability of missing a xenograft active compound if the hollow fiber assay were used as the initial in vivo screening tool. Due to the design of the hollow fiber assay, the results of individual cell lines are not reported since the statistical power of the assay is based on the impact of a compound against the entire panel of cells [2,3].

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