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Short communication

Synthesis and antiproliferative activity of 2,6-Dibenzylamino-3,5-dicyanopyridines on human cancer cell lines

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Abstract

A new series of 2,6-dibenzylamino-3,5-dicyanopyridines were synthesized and evaluated for their in vitro anticancer activity toward cell lines of nine different types of human cancer. Some of newly prepared compounds demonstrated remarkable anticancer activity against most of the tested subpanel tumor cell lines.

Keywords: Pyridines; Dicyanopyridines; Anticancer activity; Cytostatic activity

1. Introduction

The pyridine scaffold is a widespread structural motif that can be found in many natural products and in several pharmacologically interesting compounds. Therefore the synthesis of pyridine derivatives, aiming to develop new drugs, is an active research area. Recently several researchers became interested to cyanopyridine derivatives. 2-Cyanopyridylureas derivatives have been claimed for their properties in treating hyper-proliferative and angiogenesis disorders [1]. The 3-cyano-2,6-dihydropyridine is a potent inhibitor of dihydrouracil dehydrogenase and its coadministration with 1-ethoxymethyl-5-fluorouracil enhances the antitumor effect [2].

3,5-Dicyanopyridines derivatives have been described as intermediates in the synthesis of pyrido[2,3-d]pyrimidines as antihistaminic agents [3], pyridothieno- and pyridodithienotriazines endowed with antihistaminic and cytotoxic activity [4], 3,4,6-triazabenz[d,e]anthracene and 3,4,6,9-tetrazabenz[d,e]anthracene that are DNA intercalating agents [5] and acyclo-3-deazapyrimidine S-nucleosides that are active toward HIV [6].

Besides their importance as synthetic intermediates 3,5-dicyanopyridines derivatives are endowed of biological activity. Thus 2-amino-6-[(2-aminophenyl)thio]-4-(2-furyl) pyridine-3,5-dicarbonitrile has been reported as lead compound that mimics the dominant negative host-encoded prion protein (PrP^{C}) mutants, which inhibit an abnormal isoform (PrP^{Sc}) formation [7]. 2-Guanadino-3,5-dicyanopyridines present moderate in vitro cytotoxic activity against P-388, A-549, HT-29 and MEL-28 tumoral cell lines as well as they are very potent stimulator of the release of histamine [3]. Some recent patents can be found on 2-thio-3,5-dicyano-4-aryl-6-aminopyrimidines as selective adenosine receptor ligands and for treatment of cancer [8], and on 3,5-dicyanopyridine derivatives exhibiting an excellent effect to open the maxi-K channel [9].

On the basis of above observations it is very attractive the development of 3,5-dicyanopyridine derivatives as potential antitumor agents. Continuing our interest in pyridine derivatives endowed with anticancer activity [10,11], we designed and synthesized a novel series 4-aryl or -heteroaryl-2,6-dibenzylamino-3,5-dicyanopyridines to evaluate their anticancer properties.

2. Chemistry

The target 2,6-dibenzylamino-3,5-dicyanopyridines **3-19** (Table 1) were synthesized as shown in Scheme 1. 4-Aryl-2,6-diamino-3,5-dicyanopyridines are generally prepared starting from the corresponding 2-alkoxy-6-amino-3,5-

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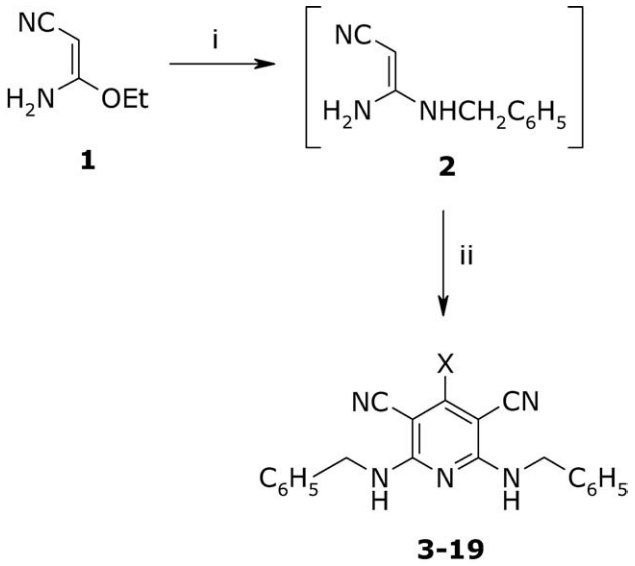
Table 1
Structure for 2,6-Dibenzylamino-3,5-dicyanopyridines **3-19**

Compd.	X
3	4-OMePh
4	3-OMePh
5	2,5-$(OMe)_2$Ph
6	3,4,5-$(OMe)_3$Ph
7	4-ClPh
8	2-ClPh
9	2,4-Cl_2Ph
10	2,6-Cl_2Ph
11	3-OHPh
12	4-OHPh
13	3-OH,4-OMePh
14	2-Pyridyl
15	3-Pyridyl
16	4-Pyridyl
17	2-Thienyl
18	2-Furyl
19	2-(5-(Me)furyl)



Scheme 1. Synthetic pathway to 3,5-dicyanopyridines **3-19**, i: Benzylamine, MeCN, r.t. 24 h; ii: X-CHO, reflux, 3 h.

dicyanopyridine or 2-chloro-6-amino-3,5-dicyanopyridine derivatives [12,13] or by reaction of arylidenemalononitrile with an imidate in the presence of ammonium acetate [14]. In this paper we developed an alternative and simple synthetic pathway to obtain 4-aryl-2,6-dialkylamino-3,5-dicyanopyridines.

2,6-Dibenzylamino-3,5-dicyanopyridines **3-19** were obtained through an one-pot, two-step reaction starting from the easily available 3-amino-3-ethoxypropenenitrile **1**. This last was first treated with benzylamine in EtOH solution to give 3-amino-3-benzylaminopropenenitrile **2,** and then with the appropriate aryl or heteroarylaldehyde. The reaction mixture was heated at reflux and, after 3 h, 2,6-dibenzylamino-3,5-dicyanopyridines **3-19** were obtained in good to excellent yields. The pathway to formation of 3,5-dicyanopyridine ring was initiated by condensation of two molecules of 3-amino-3-benzylaminopropenenitrile **2** with the aldehyde, followed by intramolecular cyclization with loss of ammonia. Although 3-amino-3-benzylaminopropenenitrile **2** is isolable, as previously reported by us [15], recovering **2** from reaction mixture and reacting it with aldehydes in a separate vessel does not improve 3,5-dicyanopyridine **3-19** yields. All the newly synthesized compounds gave corrected analytical data. The IR and NMR spectral data were consistent with the assigned structure.

3. Pharmacology

Evaluation of anticancer activity on dicyanopyridines was performed at National Cancer Institute (NCI). First dicyanopyridines **3-19** have been evaluated for their cytotoxicity in primary 3-cell line test at 10^{-4} M concentration. The cell lines adopted in this screen were NCI-H460 (Lung), MCF7 (Breast) and SF-268 (CNS) due to their being good predictor of anticancer activity (Table 2).

For NCI criteria, compounds, which reduce the growth of any one of the cell lines to approximately 32% or less, are considered active and passed on for evaluation in the full panel of cell lines over a 5-log dose range. Dicyanopyridines **7, 8, 9, 11, 12**, that meet these criteria, were evaluated for their anticancer activity following the known in vitro disease-oriented antitumor screening program, which is based upon use of multiple panel of about 60 human tumor cell lines [16,17]. 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 [18]. The anticancer activity of each compound is deduced from dose–response curves and is presented in three different Tables according to the data provided by NCI [19]. The response parameters GI_{50}, TGI and

Table 2
Antiproliferative activity of compounds **3-19** at 10^{-4} M concentration expressed in growth percentage

Compound	Cell lines		
	MCF7	NCI-H460	SF-268
3	100	106	111
4	100	96	98
5	86	93	113
6	78	91	90
7	55	4	80
8	71	5	69
9	76	26	64
10	81	76	98
11	77	2	110
12	75	18	102
13	67	78	95
14	107	104	118
15	104	108	113
16	100	107	108
17	105	105	113
18	87	95	95
19	114	103	116

LC_{50} (Tables 3–5) refer to the drug concentration that produced 50% inhibition, total growth inhibition and 50% cytotoxicity, respectively, and are expressed in 10^{-5} molar concentrations. Moreover Table 3 shows full panel mean-graph midpoint values (MG-MID) that are the average of GI_{50} values of all cell lines.

4. Results and discussion

Evaluation of the data reported in Table 3 revealed that dicyanopyridines **7, 8, 9, 11, 12** showed appreciable anticancer activity against most of the tested subpanel tumor cell lines. From the analysis of GI_{50} data we can evince that all these dicyanopyridines exhibited cytostatic activity at 10^{-5} M and in some cases at 10^{-9} M concentration.

Dicyanopyridine **11** proved to be the most active member within the series, showing potent and broad spectrum of antitumoral activity (MG-MID value –5.12). Compound **11** showed an in vitro chemosentitive profile toward 33 different cancer cell lines with GI_{50} values lying in the micromolar concentration range (GI_{50} values between $0.168 \cdot 10^{-5}$ M and $0.779 \cdot 10^{-5}$ M). Compound **11** displayed some selectivity on renal cancer subpanel at GI_{50} (values between $0.201 \cdot 10^{-5}$ M and $1.38 \cdot 10^{-5}$ M), TGI (values between $0.577 \cdot 10^{-5}$ M and $8.76 \cdot 10^{-5}$ M) and LC_{50} levels (values between $2.54 \cdot 10^{-5}$ M and $8.83 \cdot 10^{-5}$ M). Furthermore the growth of all leukemia cell lines was inhibited by compound **11** at micromolar concentrations (GI_{50} values between $0.209 \cdot 10^{-5}$ M and $0.338 \cdot 10^{-5}$ M). Although less potent when compared to **11**, compounds **7**, **8** and **12** showed activity against most of the tested cell lines (MG-MID values 4.71, 4.75 and 4.62 respectively). In particular compound **12** displayed high antiproliferative activity against HOP-92 non-small cell lung cancer cell line (GI_{50} value $0.0315 \cdot 10^{-5}$ M and TGI value $0.798 \cdot 10^{-5}$ M). Compound **9** selectively exhibited high potency against leukemia HL-60(TB) cell line ($GI_{50} < 0.0001\ 10^{-5}$ M), HOP92 cell line (GI_{50} $0.112 \cdot 10^{-5}$ M), CNS cancer SF-268 cell line ($GI_{50} < 0.0001\ 10^{-5}$ M), leukemia SR cell line (GI_{50} $0.207 \cdot 10^{-6}$ M), and MCF7 of breast cancer (GI_{50} $0.120 \cdot 10^{-5}$ M). Furthermore compound **9** inhibited totally the growth of SF-268 and SR cell lines at $0.00175.10^{-5}$ M and $2.18.10^{-5}$ M concentration respectively.

An aryl substituent at 4-positon of 3,5-dicyanopyridines appears to favourably modulate antiproliferative activity. Replacement of phenyl ring with an heterocyclic ring leads to a loss of activity. The presence of functionality as 4-chlorine atom or hydroxyl group in 3 or 4 position of phenyl ring has an enhancing effect on anticancer potency. Thus, 2,6-bis(benzylamino)-4-(3-hydroxyphenyl)pyridine-3,5-dicarbonitrile (**11**) is the most active member within the series. The displacement of hydroxy group from 3-position to 4-position on phenyl ring leads to **12** that retains antitumoral activity on the same cell lines but at higher concentrations. Methylation of hydroxy groups is detrimental for the activity, as well as the introduction on phenyl ring of two or more methoxy substituents. Furthermore the introduction of a 4-methoxy group on **11** leads to compound **13** that showed low activity. Significant antiproliferative activity is induced by a 2-chlorine atom on phenyl moiety (compound **8**). The displacement of chlorine from 2-position to 4-position (compound **7**) on phenyl ring seems do not affect activity. As matter of fact compounds **7** and **8** showed similar MG-MID values (4.71 and 4.75 respectively). The introduction of a second chlorine atom produces variable effects: the 2,4-dichloro derivative **9** shows potent and selective activity against some cell lines, in contrast the 2,6-dichloro derivative **10** is inactive.

A COMPARE [20] analysis was performed with the more active compounds to investigate whether it resemble anticancer drugs of the NCI standard agent database and to probably predict its mechanism of action. The COMPARE algorithm was developed to determine the degree of similarity of mean graph fingerprints obtained from the in vitro anticancer screen with patterns of activity of standard agents. The hypothesis is that, if the data pattern of a compound correlates well with the data pattern of compounds belonging to the standard agent database, the compound of interest may have the same mechanism of action as those agents with known mechanism. A correlation coefficient of 0.55– 0.6 is considered the lowest correlation that suggests a relationship with another compound [21]. Using GI_{50} values of dicyanopyridines **7, 8, 9, 11, 12** as seed, COMPARE analysis showed correlation coefficients with the standard agents below 0.55. These results illustrate that the mechanism of action of the novel dicyanopyridines may differ from that of the standard antitumor drugs. Therefore, their antitumoral activity may be caused by a new and unknown mechanism.

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, unless otherwise indicated, in Bruker Vector 22 spectrophotometer. ^{1}H-NMR spectra were recorded, in DMSO-d_6 solution, on a Varian Unity 300 spectrometer. The chemical shift are reported in part per million (δ, ppm) downfield from tetramethylsilane, which was used as internal standard Elemental analyses were carried out with a Carlo Erba model 1106 Elemental Analyzer and the values found were within ± 0.4% of theoretical values.

5.2. General Procedure for the Synthesis of 3,5-Dicyanopyridines (**3-19**)

Benzylamine (0.54 g, 5 mmol) was added to a solution of 3-amino-3-ethoxypropenenitrile **1** (0.55 g, 5 mmol) in anhydrous acetonitrile (10 mL). The resulting solution was kept at room temperature for 24 hours and then the appropriate alde-

Table 3
GI_{50} values of compounds **7-9, 11, 12**

	Compd				
	7	**8**	**9**	**11**	**12**
Panel/cell line					
Leukemia					
CCRF-CEM	2.82	1.52	1.02	0.338	2.74
HL-60(TB)	-	-	< 0.001	-	-
K-562	-	-	> 10	-	-
MOLT-4	3.43	2.68	3.46	0.330	2.04
RPMI-8226	1.89	4.86	3.35	0.311	2.19
SR	1.33	1.08	0.207	0.209	0.709
Non-Small Cell Lung Cancer					
A549/ATCC	2.83	2.13	> 10	1.33	3.41
EKVX	2.73	2.43	> 10	1.16	4.85
HOP-62	1.61	0.447	> 10	1.05	1.73
HOP-92	-	0.357	0.112	0.251	0.0315
NCI-H226	1.06	0.239	> 10	0.291	1.43
NCI-H23	2.28	1.75	> 10	1.10	4.53
NCI-H322 M	1.91	4.06	> 10	2.21	3.23
NCI-H460	1.02	0.499	> 10	1.49	2.12
NCI-H522	2.09	1.26	> 10	0.589	2.02
Colon Cancer					
COLO 205	4.69	7.40	> 10	3.67	3.63
HCC-2998	7.11	> 10	> 10	9.56	3.95
HCT-116	1.58	1.63	> 10	0.448	1.45
HCT-15	1.88	> 10	> 10	0.433	2.96
HT29	2.23	7.34	> 10	4.13	6.02
KM12	2.52	4.70	> 10	0.403	1.88
SW-620	1.62	1.89	7.44	0.449	3.61
CNS Cancer					
SF-268	1.43	1.26	< 0.001	1.95	2.73
SF-295	1.42	1.26	> 10	0.292	2.71
SF-539	2.59	1.79	> 10	0.518	1.31
SNB-19	3.16	2.34	> 10	1.71	5.05
SNB-75	2.21	1.03	2.61	3.94	6.78
U251	1.24	0.246	> 10	0.402	1.92
Melanoma					
LOX IMVI	1.24	0.475	> 10	0.296	1.67
MALME-3 M	1.44	2.10	> 10	1.01	2.95
M14	2.00	2.32	> 10	0.665	2.99
SK-MEL-2	1.20	2.29	> 10	0.623	4.11
SK-MEL-28	2.59	4.95	>10	6.10	6.67
SK-MEL-5	2.59	5.12	> 10	0.168	1.53
UACC-257	6.79	> 10	> 10	> 10	4.32
UACC-62	1.47	1.24	> 10	0.21	2.15
Ovarian Cancer					
IGROV1	1.35	0.508	3.56	1.97	2.35
OVCAR-3	1.47	1.84	> 10	0.331	2.96
OVCAR-4	0.957	0.503	3.47	2.36	2.77
OVCAR-5	2.42	2.68	9.38	2.13	6.03
OVCAR-8	1.90	1.14	> 10	1.92	5.81
SK-OV-3	3.32	6.27	> 10	0.779	3.45
Renal Cancer					
786-0	2.00	1.34	> 10	1.30	2.30
A498	1.04	1.92	> 10	0.201	2.49
ACHN	1.34	0.573	> 10	0.263	1.69
CAKI-1	2.01	1.46	> 10	0.277	1.07
RXF 393	2.66	2.46	7.07	1.38	3.91
SN12C	1.61	0.453	> 10	0.231	0.598
TK-10	1.44	2.23	> 10	0.411	2.26
UO-31	3.19	3.81	> 10	0.612	1.86
Prostate Cancer					
PC-3	1.79	1.20	5.58	0.466	2.56
DU-145	1.77	2.15	>10	1.28	3.24
Breast Cancer					
MCF7	1.38	2.00	0.120	1.27	3.97
NCI/ADR-RES	1.98	3.71	> 10	1.38	4.70
MDA-MB-231/ ATCC	1.42	0.576	> 10	0.223	0.268
HS 578T	1.56	1.13	8.57	0.376	2.29
MDA-MB-435	1.57	2.18	> 10	0.291	2.52
BT-549	1.96	1.87	-	1.01	3.28
T-47D	2.73	6.73	7.23	0.403	2.38
MG-MID[a]	**–4.71**	**–4.75**	**–4.28**	**–5.12**	**–4.62**

[a] Full panel mean-graph mid point (log GI_{50}).

Table 4
TGI values of compounds **7-9, 11, 12**

	Compd				
	7	**8**	**9**	**11**	**12**
Panel/cell line					
Leukemia					
CCRF-CEM	> 10	4.55	2.85	> 10	> 10
HL-60(TB)	-	-	> 10	-	-
K-562	-	-	> 10	-	-
MOLT-4	> 10	> 10	> 10	> 10	> 10
RPMI-8226	9.71	> 10	> 10	2.28	> 10
SR	5.28	3.28	2.18	0.748	9.17
Non-Small Cell Lung Cancer					
A549/ATCC	> 10	> 10	> 10	6.35	> 10
EKVX	7.90	8.53	> 10	4.50	> 10
HOP-62	3.12	1.80	> 10	2.31	9.30
HOP-92	-	2.55	> 10	1.89	0.798
NCI-H226	3.12	1.45	> 10	0.786	6.71
NCI-H23	8.00	> 10	> 10	3.74	> 10
NCI-H322 M	8.69	> 10	> 10	8.62	> 10
NCI-H460	2.87	3.01	> 10	3.14	5.38
NCI-H522	5.75	5.50	> 10	3.30	7.51
Colon Cancer					
COLO 205	> 10	> 10	> 10	> 10	> 10
HCC-2998	> 10	> 10	> 10	> 10	> 10
HCT-116	4.13	> 10	> 10	1.61	3.41
HCT-15	> 10	> 10	> 10	3.37	>10
HT29	> 10	> 10	> 10	> 10	> 10
KM12	> 10	> 10	> 10	> 10	5.97
SW-620	9.69	> 10	> 10	6.82	> 10
CNS Cancer					
SF-268	3.56	5.85	0.00175	4.65	> 10
SF-295	3.44	7.62	> 10	1.32	> 10
SF-539	6.81	4.05	> 10	2.21	3.74
SNB-19	> 10	> 10	> 10	> 10	> 10
SNB-75	7.23	4.88	> 10	> 10	> 10
U251	2.69	1.03	> 10	1.54	5.45
Melanoma					
LOX IMVI	2.93	3.59	> 10	1.15	4.14
MALME-3 M	5.45	9.02	> 10	3.25	6.85
M14	9.98	> 10	> 10	> 10	> 10
SK-MEL-2	1.89	9.34	> 10	2.30	9.07
SK-MEL-28	> 10	> 10	> 10	> 10	> 10
SK-MEL-5	> 10	> 10	> 10	0.432	3.43
UACC-257	> 10	> 10	> 10	> 10	> 10
UACC-62	3.35	6.02	> 10	0.704	> 10
Ovarian Cancer					
IGROV1	3.24	4.11	> 10	4.11	7.48
OVCAR-3	3.71	4.97	> 10	1.94	8.74
OVCAR-4	> 10	4.53	> 10	>10	>10
OVCAR-5	7.61	> 10	> 10	4.80	>10
OVCAR-8	5.72	> 10	> 10	5.22	>10
SK-OV-3	> 10	> 10	> 10	4.65	>10
Renal Cancer					
786-0	3.86	3.63	> 10	2.76	> 10
A498	2.64	4.69	> 10	0.577	6.38
ACHN	3.28	6.28	> 10	1.17	5.71
CAKI-1	4.67	> 10	> 10	1.94	4.48
RXF 393	7.41	> 10	> 10	3.49	> 10
SN12C	3.83	2.51	> 10	0.669	5.32
TK-10	3.28	6.60	> 10	1.74	5.94
UO-31	> 10	> 10	> 10	8.76	6.85
Prostate Cancer					
PC-3	4.87	> 10	> 10	2.88	> 10
DU-145	5.57	8.86	> 10	3.44	> 10
Breast Cancer					
MCF7	8.31	> 10	> 10	8.84	> 10
NCI/ADR-RES	5.99	> 10	> 10	4.80	> 10
MDA-MB-231/ ATCC	3.32	3.38	> 10	0.734	3.30
HS 578T	4.04	4.30	> 10	2.07	> 10
MDA-MB-435	4.36	> 10	> 10	0.997	6.98
BT-549	6.04	5.39	> 10	3.58	> 10
T-47D	> 10	> 10	> 10	> 10	> 10

Table 5
LC_{50} values of compounds **7-9, 11, 12**

	Compd				
	7	**8**	**9**	**11**	**12**
Panel/cell line					
Leukemia					
CCRF-CEM	> 10	> 10	8.04	> 10	> 10
HL-60(TB)	-	-	> 10	-	-
K-562	-	-	> 10	-	-
MOLT-4	> 10	> 10	> 10	> 10	> 10
RPMI-8226	> 10	> 10	> 10	> 10	> 10
SR	> 10	9.96	> 10	> 10	> 10
Non-Small Cell Lung Cancer					
A549/ATCC	> 10	> 10	> 10	> 10	> 10
EKVX	> 10	> 10	> 10	> 10	> 10
HOP-62	6.04	5.33	> 10	506	> 10
HOP-92	-	> 10	> 10	7.73	> 10
NCI-H226	9.21	> 10	> 10	5.84	> 10
NCI-H23	> 10	> 10	> 10	> 10	> 10
NCI-H322 M	> 10	> 10	> 10	> 10	> 10
NCI-H460	8.11	> 10	> 10	6.61	> 10
NCI-H522	> 10	> 10	> 10	> 10	> 10
Colon Cancer					
COLO 205	> 10	> 10	> 10	> 10	> 10
HCC-2998	> 10	> 10	> 10	> 10	> 10
HCT-116	> 10	> 10	> 10	4.01	> 10
HCT-15	> 10	> 10	> 10	> 10	> 10
HT29	> 10	> 10	> 10	> 10	> 10
KM12	> 10	> 10	> 10	> 10	> 10
SW-620	> 10	> 10	> 10	> 10	> 10
CNS Cancer					
SF-268	8.91	> 10	> 10	> 10	> 10
SF-295	8.34	> 10	> 10	6.04	> 10
SF-539	> 10	9.14	> 10	8.30	> 10
SNB-19	> 10	> 10	> 10	> 10	> 10
SNB-75	> 10	> 10	> 10	> 10	> 10
U251	5.82	6.98	> 10	4.89	> 10
Melanoma					
LOX IMVI	6.88	> 10	> 10	4.03	> 10
MALME-3 M	> 10	> 10	> 10	> 10	> 10
M14	> 10	> 10	> 10	> 10	> 10
SK-MEL-2	6.06	> 10	> 10	7.76	> 10
SK-MEL-28	> 10	> 10	> 10	> 10	> 10
SK-MEL-5	> 10	> 10	> 10	> 10	7.70
UACC-257	> 10	> 10	> 10	> 10	> 10
UACC-62	7.62	> 10	> 10	> 10	> 10
Ovarian Cancer					
IGROV1	7.80	> 10	> 10	8.58	> 10
OVCAR-3	9.33	> 10	> 10	> 10	> 10
OVCAR-4	> 10	> 10	> 10	> 10	> 10
OVCAR-5	> 10	> 10	> 10	> 10	> 10
OVCAR-8	> 10	> 10	> 10	> 10	> 10
SK-OV-3	> 10	> 10	> 10	> 10	> 10
Renal Cancer					
786-0	7.47	9.82	> 10	5.83	> 10
A498	6.75	> 10	> 10	2.54	> 10
ACHN	8.05	> 10	> 10	4.34	> 10
CAKI-1	> 10	> 10	> 10	> 10	> 10
RXF 393	> 10	> 10	> 10	8.83	> 10
SN12C	9.12	> 10	> 10	2.89	> 10
TK-10	7.49	> 10	> 10	6.18	> 10
UO-31	> 10	> 10	> 10	> 10	> 10
Prostate Cancer					
PC-3	> 10	> 10	> 10	> 10	> 0
DU-145	> 10	> 10	> 10	9.20	> 10
Breast Cancer					
MCF7	> 10	> 10	> 10	> 10	> 10
NCI/ADR-RES	> 10	> 10	> 10	> 10	> 10
MDA-MB-231/ ATCC	7.76	> 10	> 10	5.03	> 10
HS 578T	> 10	> 10	> 10	> 10	> 10
MDA-MB-435	> 10	> 10	> 10	> 10	> 10
BT-549	> 10	> 10	> 10	> 10	> 10
T-47D	> 10	> 10	> 10	> 10	> 10

hyde (2.5 mmol) was added. The resulting mixture was refluxed for 3 h. After cooling, the formed precipitate was filtered off and washed with diethyl ether.

*5.2.1. 2,6-Bis(benzylamino)-4-(4-methoxyophenyl)pyridine-3,5-dicarbonitrile (**3**)*

Yield 88%. M.p. 245 °C. Anal. ($C_{28}H_{23}N_5O$) C, H, N. IR (Nujol): 3376, 3334, 2195, 1588 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 3.78 (s, 3H, OCH_3), 4.47 (d, J = 5.8 Hz, 4H, CH_2), 7.03 (d, J = 8.8 Hz, 2H, aryl), 7.40 (d, J = 8.4 Hz, 2H, Aryl), 7.17 (m, 10H, benzyl), 8.08 (t, J = 5.8 Hz, 2H, NH).

*5.2.2. 2,6-Bis(benzylamino)-4-(3-methoxyphenyl)pyridine-3,5-dicarbonitrile (**4**)*

Yield 82%. M.p. 210 °C. Anal. ($C_{28}H_{23}N_5O$) C, H, N. IR (Nujol): 3347, 3302, 2219, 2202, 1595 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 3.75 (s, 3H, OCH_3), 4.48 (d, J = 5.8 Hz, 4H, CH_2), 7.02, 7.19, 7.34 (m, 14H, aryl and benzyl), 8.13 (t, J = 5.8 Hz, 2H, NH).

*5.2.3. 2,6-Bis(benzylamino)-4-(2,5-dimethoxyphenyl)pyridine-3,5-dicarbonitrile (**5**)*

Yield 75%. M.p. 235 °C. Anal. ($C_{29}H_{25}N_5O_2$) C, H, N. IR (Nujol): 3370, 3338, 2202, 1578 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.68 (s, 6H, $2OCH_3$), 4.47 (d, J = 6.1 Hz, 4H, CH_2), 6.84-7.18 (m, 13H, aryl and benzyl), 8.08 (t, J = 6.1 Hz, 2H, NH).

*5.2.4. 2,6-Bis(benzylamino)-4-(3,4,5-trimethoxyphenyl)pyridine-3,5-dicarbonitrile (**6**)*

Yield 80%. M.p. 200 °C. Anal. ($C_{30}H_{27}N_5O_3$) C, H, N. IR (Nujol): 3320, 2203, 1573 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.69 (s, 3H, OCH_3), 3.75 (s, 6H, OCH_3), 4.48 (d, J 5.8 Hz, 4H, CH_2), 6.80 (s, 2H, aryl), 7.20 (s, 10H, benzyl), 8.11 (t, J = 5.8 Hz, 2H, NH).

*5.2.5. 2,6-Bis(benzylamino)-4-(4-chlorophenyl)pyridine-3,5-dicarbonitrile (**7**)*

Yield 93%. M.p. 220 °C. Anal. ($C_{27}H_{20}N_5Cl$) C, H, N. IR (Nujol): 3317, 3085, 3030, 2200, 2159, 1592, 1578 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.54 (d, J = 5.8 Hz, 4H, CH_2), 7.16-7.58 (m, 14H, aryl and benzyl), 8.05 (t, J = 5.8 Hz, 2H, NH).

*5.2.6. 2,6-Bis(benzylamino)-4-(2-chlorophenyl)pyridine-3,5-dicarbonitrile (**8**)*

Yield 90%. M.p. 210 °C. Anal. ($C_{27}H_{20}N_5Cl$) C, H, N. IR (Nujol): 3503, 3464, 3356, 2204, 1624 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.50 (d, J = 5.8 Hz, 4H, CH_2), 7.18-7.61 (m, 14H, aryl and benzyl), 8.12 (t, J = 5.8 Hz, 2H, 2NH).

*5.2.7. 2,6-Bis(benzylamino)-4-(2,4-dichlorophenyl)pyridine-3,5-dicarbonitrile (**9**)*

Yield 82%. M.p. 225 °C. Anal. ($C_{27}H_{19}N_5Cl_2$) C, H, N. IR (Nujol): 3335, 3285, 2210, 1657, 1580 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.62 (d, J = 5.8 Hz, 4H, CH_2), 7.18 (s, 10H, benzyl), 7.54 (m, 2H, aryl), 7.83 (s, 1H, aryl), 8.29 (d, J = 5.8 Hz, 2H, NH).

*5.2.8. 2,6-Bis(benzylamino)-4-(2,6-dichlorophenyl)pyridine-3,5-dicarbonitrile (**10**)*

Yield 87%. M.p. 220 °C. Anal. ($C_{27}H_{19}N_5Cl_2$) C, H, N. IR (Nujol): 3373, 2204, 1626, 1581 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.54 (d, J = 5.8 Hz, 4H, CH_2), 7.10 (m, 10H, benzyl), 7.26-7.66 (m, 3H, aryl), 8.40 (t, J = 5.8 Hz, 2H, NH).

*5.2.9. 2,6-Bis(benzylamino)-4-(3-hydroxyphenyl)pyridine-3,5-dicarbonitrile(**11**)*

Yield 63%. M.p. 215 °C. Anal. ($C_{27}H_{21}N_5O$) C, H, N. IR (Nujol): 3348, 2200, 1566 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.47 (d, J = 5.8 Hz, 4H, CH_2), 6.82, 7.25 (m, 4H, aryl), 7.16 (s, 10H, benzyl), 8.10 (t, J = 5.8 Hz, 2H, NH), 9.73 (s, 1H, OH).

*5.2.10. 2,6-Bis(benzylamino)-4-(4-hydroxyphenyl)pyridine-3,5-dicarbonitrile (**12**)*

Yield 68%. M.p. 260 °C. Anal. ($C_{27}H_{21}N_5O$) C, H, N. IR (Nujol): 3496, 3318, 2201, 1628, 1592 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.47 (d, J = 5.8 Hz, 4H, CH_2), 6.82 (d, J = 8.4 Hz, 2H, aryl), 7.16 (s, 10H, benzyl), 7.28 (d, J = 8.4 Hz, 2H, Aryl), 8.05 (t, J = 5.8 Hz, 2H, NH), 10.04 (s, 1H, OH).

*5.2.11. 2,6-Bis(benzylamino)-4-(4-hydroxy-3-methoxyphenyl)pyridine-3,5-dicarbonitrile(**13**)*

Yield 60%. M.p. 220 °C. Anal. ($C_{28}H_{23}N_5O_2$) C, H, N. IR (Nujol): 3403, 3309, 2211, 1587 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.75 (s, 3H, OCH_3), 4.47 (d, J = 5.8 Hz, 4H, CH_2), 6.86, 7.05 (m, 3H, aryl), 7.16 (s, 10H, benzyl), 8.05 (t, J = 5.8 Hz, 2H, NH), 9.48 (s, 1H, OH).

*5.2.12. 2,6-Bis(benzylamino)-4-(2-pyridinyl)pyridine-3,5-dicarbonitrile(**14**)*

Yield 57% M.p. 220 °C. Anal. ($C_{26}H_{20}N_6$) C, H, N. IR (Nujol) 3326, 2200, 1597, 1577 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.49 (d, J = 5.8 Hz, 4H, CH_2), 7.18 (s, 10H, Benzyl), 7.51, 7.65, 7.96, 8.70 (m, 4H, pyridyl), 8.20 (t, J = 5.8 Hz, 2H, NH).

*5.2.13. 2,6-Bis(benzylamino)-4-(3-pyridinyl)pyridine-3,5-dicarbonitrile (**15**)*

Yield 68%. M.p. 250 °C. Anal. ($C_{26}H_{20}N_6$) C, H, N. IR (Nujol): 3350, 3311, 2215, 2198, 1581 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.49 (m, 4H, CH_2), 7.18 (m, 10H, benzyl), 7.54, 7.94, 8.68 (m, 4H, pyridyl), 8.24 (m, 2H, NH).

*5.2.14. 2,6-Bis(benzylamino)-4-(4-pyridinyl)pyridine-3,5-dicarbonitrile (**16**)*

Yield 70%. M.p. 235 °C. Anal. ($C_{26}H_{20}N_6$) C, H, N. IR (Nujol) 3475, 3328, 3032, 2202, 1632, 1596 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.48 (d, J = 5.8 Hz, 4H, CH_2), 7.17 (m, 10H, benzyl), 7.49, 8.71 (m, 4H, pyridyl), 8.27 (t, J = 5.8 Hz, 2H, NH).

*5.2.15. 2,6-Bis-benzylamino-4-thiophen-2-yl-pyridine-3,5-dicarbonitrile (**17**)*

Yield 57%. M.p. 215 °C. Anal. ($C_{25}H_{19}N_5S$) C, H, N. IR (Nujol): 3345, 2198, 1591 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.47

(d, J = 5.8 Hz, 4H, CH_2), 7.06 (s, 10H, benzyl), 7.09, 7.44, 7.82 (m, 3H, thienyl), 8.17 (t, J = 5.8 Hz, 2H, NH).

*5.2.16. 2,6-Bis-benzylamino-4-furan-2-yl-pyridine-3,5-dicarbonitrile(**18**)*

Yield 61%. M.p. 240 °C. Anal. ($C_{25}H_{19}N_5O$) C, H, N. IR (Nujol) 3342, 3030, 2202, 1589 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 4.46 (d, J = 4.5 Hz, 4H, CH_2), 6.73, 7.20, 7.98 (m, 3H, furyl), 7.15 (s, 10H, benzyl), 8.14 (t, J = 4.5 Hz, 2H, NH).

*5.2.17. 2,6-Bis-benzylamino-4-(5-methyl-furan-2-yl)-pyridine-3,5-dicarbonitrile (**19**)*

Yield 57%. M.p. 245 °C. Anal. ($C_{26}H_{21}N_5O$) C, H, N. IR (Nujol) 3348, 2210, 1592, 1569 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.33 (s, 3H, CH_3), 4.46 (d, J = 5.8 Hz, 4H, CH_2), 6.36 (m, 1H, furyl), 7.16 (m, 11H, benzyl and furyl), 8.06 (t, J = 5.8 Hz, 2H, NH).

5.3. Pharmacology

5.3.1. Primary anticancer assay

The compounds were tested by NCI in an in vitro three cell line, one dose primary anticancer assay as a primary cancer screen. The three-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 [22]. Results for each test agent are reported as the percent of growth of the treated cells when compared to untreated control cells. Compounds, which reduce the growth of any one of the cell lines to approximately 32% or less, are passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range.

5.3.2. Determination of GI_{50}, TGI and LC_{50} values

A total of 60 human tumor cell lines, derived from nine cancer types (leukemia, lung, colon, brain, melanoma, ovarian, renal, prostate and breast) formed the basis of this test. The tumor cells were cultured in RPMI1640 medium supplemented with 5% foetal calf serum and 2 mM L-glutamine. The tumor cells are inoculated over a series of standard 96-well microtrite plates in 100 mL of medium [16,17]. Density of inoculum depends on the type of tumor cell and from its growth characteristics [23]. 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 concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M). After an incubation of the chemical agent for 48 h with the tumor 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 [19].

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References

[1] W.J. Scott, J. Dumas, S. Boyer, W. Lee, Y. Chen, B. Phillips, s. verna, J. Chen, J. Fan, B. Raudenbush, L. Yi, Q. Zhu, L. Adnane, US Patent 0235829A1 (2004).
[2] K. Tatsumi, T. Yamanuchi, K. Kiyono, K. Kishi, Y. Yanagihara, T. Imaoka, T. Kawaguchi, J. Biochem. (Tokyo) 114 (1993) 912–918.
[3] J.M. Quintela, C. Peinador, L.M. Botana, M. Estèvez, R. Riguera, Bioorg. Med. Chem. 5 (1997) 1543–1553.
[4] J.M. Quintela, C. Peinador, M.C. Veiga, L.M. Botana, A. Alfonso, R. Riguera, Eur. J. Med. Chem. 33 (1998) 887–897.
[5] J.M. Quintela, C. Peinador, Tetrahedron 52 (1996) 10497–10506.
[6] A.M.E. Attia, A.E.-H.A.A. Ismail, Tetrahedron 59 (2003) 1749–1752.
[7] V. Perrier, A.C. Wallace, K. Kaneko, J. Safar, S.B. Prusiner, F.E. Cohen, Proc. Natl. Acad. Sci. USA 97 (2000) 6073–6078.
[8] U. Rosentreter, R. Henning, M. Bauser, T. Kramer, A. Vaupel, W. Hubsch, K. Dembowsky, O. Salcher-Schrauf-Statter, J.-P. Stasch, T. Krahn, E. Perzborn, P.C.T. Int, Appl. WO (2001) A2 (01/25210).
[9] H. Harada, W.S. Watanuki, K. Kawaguchi, T. Okazaki, Y. hirano, C. Saitoh, US Patent 0232860 A1 (2003).
[10] M.T. Cocco, C. Congiu, V. Onnis, Eur. J. Med. Chem. 35 (2000) 545–552.
[11] M.T. Cocco, C. Congiu, V. Onnis, Eur. J. Med. Chem. 38 (2003) 37–47.
[12] L. Castedo, J.M. Quintela, R. Riguera, Eur. J. Med. Chem. 19 (1984) 555–558.
[13] T.J. Murray, S.C. Zimmermann, S.V. Kolotuchin, Tetrahedron 51 (1995) 635–648.
[14] M.M. Mashaly, M. Hammouda, Naturforsch. 54 (1999) 1205–1209.
[15] M.T. Cocco, C. Congiu, V. Onnis, M.L. Schivo, A. De Logu, Alessandro, Farmaco 50 (1995) 73–76.
[16] M.R. Grever, S.A. Schepartz, B.A. Chabner, Semin. Oncol. 19 (1992) 622–638.
[17] M.R. Boyd, K.D. Paull, Drug Dev. Res. 34 (1995) 91–109.
[18] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. Mc Mahon, D. Vistica, J.T. Warren, H. Bokesh, S. Kenney, M.R. Boyd, J. Natl. Cancer Inst. 82 (1990) 1107–1112.
[19] M.R. Boyd, Princ. Pract. Oncol. 3 (1989) 1.
[20] K.D. Paull, R.H. Shoemaker, L. Hodes, A. Monks, D.A. Scudiero, L. Rubinstein, J. Plowman, M.R. Boyd, J. Natl. Cancer Inst. 81 (1989) 1088–1092.
[21] J.N. Weinstein, T.G. Myers, P.M. O'Connor, S.H. Friend, A.J. Jr Fornace, K.W. Kohn, T. Fojo, S.E. Bates, L.V. Rubinstein, N.L. Anderson, J.K. Buolamwini, W.W. van Osdol, A.P. Monks, D.A. Scudiero, E.A. Sausville, D.W. Zaharevitz, B. Bunow, V.N. Viswanadhan, G.S. Johnson, R.E. Wittes, K.D. Paull, Science 275 (1997) 343–349.
[22] G.D. Gray, E. Wickstrom, Biotechniques 21 (1996) 780–782.
[23] A. Monks, D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A. Vaigro-Wolff, M. Gray-Goodrich, H. Campbell, J. Mayo, M. Boyd, J. Natl. Cancer Inst. 83 (1991) 757–766.