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Synthesis and in vitro antitumoral activity of new hydrazinopyrimidine-5-carbonitrile derivatives

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Abstract—A new series of 2-amino-6-(2-alkyl or arylidenehydrazinyl)-4-(dialkylamino)pyrimidine-5-carbonitriles, **5**–**24**, were synthesized in satisfactory overall yield, using 2-amino-4-(dialkylamino)-6-hydrazino-5-pyrimidinecarbonitriles **3**, **4**, as key intermediates, by applying classical synthetic methods to construct the hydrazone moiety at C-6 of the pyrimidine ring. Hydrazinopyrimidine derivatives **5**–**24** were evaluated for their in vitro anticancer activity toward cell lines of nine different types of human cancers. Some of the newly prepared compounds demonstrated inhibitory effects on the growth of a wide range of cancer cell lines generally at 10^{-5} M level and in some cases at 10^{-7} M concentrations.

1. Introduction

A variety of anticancer drugs are currently in clinical use. Some of these compounds can be applied successfully for the treatment of several neoplastic diseases such as leukemias or testicular cancer. However, the effect of anticancer drugs on solid tumors has been poor. Because the response of solid tumors to available zanticancer chemotherapy has been reduced, new drugs with improved efficacy are desired.

Hydrazones are an important class of organic compounds, some of which show significant biological activities. For example, 2,6-di-*tert*-butyl-4-(2-azinylhydrazonomethyl) phenol derivatives possess potential as 5-lipoxygenase inhibitors.[1] 5-Lipoxygenase metabolites of arachidonic acid are implicated as mediators in a diversity of diseases, including asthma and many other inflammatory pathologies. Moreover, arylcarbaldehyde 4-(1-phenyl-3-methylpyrazolo-[3,4-*b*]pyridine) hydrazone derivatives were utilized as a new pharmacophoric tool for the development of more efficacious analgesics.[2] The search for anticancer drugs led to the discovery of several hydrazones having anticancer activity. An early report on arylidenehydrazinopyrimidines deals with the activity of these compounds as anticancer and immunomodulating agents.[3] Recently, Easmon et al.[4] reported on the cytotoxic effect of *N*-heteroaryl hydrazones that are particularly selective against colon carcinoma and that exhibit a novel mechanism of action. Hwu et al. have recently reported that photolytic cleavage of the nitrogen–nitrogen single bond in benzaldehyde phenylhydrazones produced aminyl ($R_2N^{\cdot}$) and iminyl ($R_2C{=}N^{\cdot}$) radicals. This photochemical property was utilized in the development of hydrazones as photo-induced DNA cleaving agents.[5]

On the basis of above observations, the development of novel heteroaromatic hydrazones as potential antitumor agents is very attractive. Continuing our interest in nitrogen heterocycles endowed with anticancer activity, we designed and synthesized a novel series of highly functionalized alkyl- and arylidenhydrazinopyrimidines to evaluate their anticancer properties.

2. Results and discussion

2.1. Chemistry

The synthetic approach to obtain 2-amino-6-(2-alkyl or arylidenehydrazinyl)-4-(dialkylamino)pyrimidine-5-carbonitriles **5**–**24** followed the reactions shown in Scheme 1. The required 2-amino-4-dialkylamino-6-methylthio-5-pyrimidinecarbonitriles **3** were prepared by treatment

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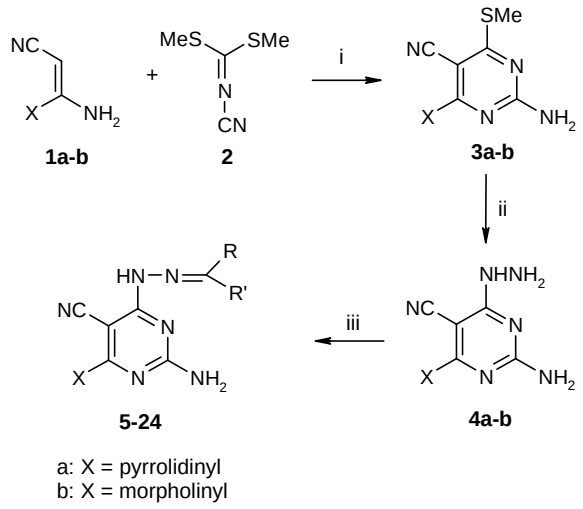

Scheme 1. Synthesis of hydrazinopyrimidines **5**–**24**. Reagents and conditions: (i) DMSO, 40% aq K_2CO_3, rt; (ii) EtOH, $NH_2NH_2{\cdot}H_2O$, reflux (iii) EtOH, AcOH, reflux.

of 3-amino-3-dialkylaminopropenenitriles **1** with *N*-[bis(methylthio)methylene] cyanamide **2** using readily available starting materials and simple experimental procedure according to the method described in our previous paper.[6] High yields of 6-hydrazinopyrimidines **4** were achieved upon refluxing for 3 h an ethanolic solution of **3** and hydrazine hydrate. These reaction conditions allow the selective displacement of the thiomethyl group by hydrazine.[7] Hydrazones **5**–**24** (Table 1) were obtained in good to excellent yield by coupling 6-hydrazinopyrimidines **4** with the appropriate aldehydes and ketones in ethanol using acetic acid as catalyst. All the newly synthesized compounds were purified by recrystallization and gave corrected analytical data. The IR and NMR spectral data are consistent with the assigned structure and exclusively account for formation of *E*-isomers.

Table 1. Hydrazinopyrimidines **5**–**24**

Compound	X	R	R′
5	Pyrrolidinyl	H	*n*-Pr
6	Pyrrolidinyl	H	*i*-Pr
7	Pyrrolidinyl	Me	Et
8	Pyrrolidinyl	H	4-$OMeC_6H_4$
9	Pyrrolidinyl	H	4-ClC_6H_4
10	Pyrrolidinyl	H	2,4-$Cl_2C_6H_3$
11	Pyrrolidinyl	H	2,6-$Cl_2C_6H_3$
12	Pyrrolidinyl	H	2-Furyl
13	Pyrrolidinyl	H	2-Thienyl
14	Pyrrolidinyl	H	3-Pyridyl
15	Morpholinyl	H	*n*-Pr
16	Morpholinyl	H	*i*-Pr
17	Morpholinyl	Me	Et
18	Morpholinyl	H	4-$OMeC_6H_4$
19	Morpholinyl	H	4-ClC_6H_4
20	Morpholinyl	H	2,4-$Cl_2C_6H_3$
21	Morpholinyl	H	2,6-$Cl_2C_6H_3$
22	Morpholinyl	H	2-Furyl
23	Morpholinyl	H	2-Thienyl
24	Morpholinyl	H	3-Pyridyl

2.2. Pharmacology

Evaluation of anticancer activity on hydrazones **5**–**24** was performed at the National Cancer Institute (NCI). First, all hydrazones were 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 approximately 32% or less, are passed on for evaluation in the full panel of cell lines over a 5-log dose range. Hydrazones **8**, **10**, **11**, **19**, and **21**, which 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 a multiple panels of 60 human tumor cell lines.[8,9] Each compound is tested at a 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.[10] 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.[11] The response parameters GI_{50}, TGI, and 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. In the tables we report only the activity of those compounds which exhibited GI_{50}, TGI, and LC_{50} less than 10×10^{-5} M.

The GI_{50} data reported in Table 3 indicate that all selected compounds **8**, **10**, **11**, **19**, and **21** showed a good level of cytostatic activity at 10^{-5} M and in some cases at 10^{-7} M concentrations against all subpanel cell lines. Compound **11** was the most active in the series showing

Table 2. Antiproliferative activity of hydrazinopyrimidines **5**–**24** at 10^{-4} M concentration expressed in growth percentage

Compound	Cell lines		
	MCF7	NCI-H460	SF-268
5	86	82	91
6	87	102	87
7	83	89	85
8	9	0	18
9	87	83	93
10	66	13	62
11	46	24	58
12	55	109	102
13	91	118	94
14	72	67	63
15	89	106	98
16	102	86	106
17	90	88	84
18	93	73	101
19	64	16	75
20	43	45	55
21	24	13	56
22	67	63	82
23	94	68	95
24	83	55	85

Table 3. GI_{50} values of compounds **8**, **10**, **11**, **19**, and **21**

Panel/cell line	Compound				
	8	**10**	**11**	**19**	**21**
Leukemia					
CCRF-CEM	1.72	2.35	0.352	2.58	0.627
HL-60(TB)	2.82	2.77	0.341	0.984	n.t.
K-562	1.30	1.67	0.287	1.30	0.234
MOLT-4	1.78	2.50	0.288	1.36	0.408
RPMI-8226	1.41	3.09	0.256	1.64	0.386
SR	0.772	1.07	0.0795	1.17	0.110
Non-small cell lung cancer					
A549/ATCC	1.34	2.96	0.365	2.96	1.91
EKVX	2.59	—	3.97	9.39	5.41
HOP-62	2.02	1.52	0.17	8.66	1.39
HOP-92	1.78	1.76	0.941	3.63	1.24
NCI-H226	1.24	1.57	1.18	5.36	2.45
NCI-H23	2.05	3.85	0.116	8.78	4.45
NCI-H322M	2.11	6.04	0.818	3.98	5.69
NCI-H460	1.64	6.39	0.820	8.77	0.532
NCI-H522	2.19	2.89	0.263	3.25	0.435
Colon cancer					
COLO 205	3.71	8.81	0.529	6.62	1.49
HCC-2998	0.859	—	0.134	2.20	0.396
HCT-116	0.589	2.09	0.101	2.97	0.472
HCT-15	1.72	1.93	0.0645	2.67	0.452
HT29	3.47	3.71	0.196	2.67	1.94
KM12	2.48	—	0.302	3.61	0.406
SW-620	3.04	1.95	0.138	4.71	0.611
CNS cancer					
SF-268	1.77	6.83	—	—	1.85
SF-295	1.68	1.88	0.102	3.32	0.469
SF-539	0.650	2.28	0.177	7.51	0.724
SNB-19	2.57	4.99	0.714	—	3.45
SNB-75	2.36	0.626	0.201	5.73	3.55
U251	1.76	0.902	0.251	4.04	1.56
Melanoma					
LOX IMVI	0.739	3.18	0.216	2.16	0.897
M14	1.70	4.42	0.136	6.74	0.679
SK-MEL-2	2.13	7.23	0.555	9.93	0.830
SK-MEL-28	3.03	3.76	1.64	—	8.21
SK-MEL-5	1.93	2.15	0.0722	1.90	0.371
UACC-257	2.16	—	0.252	3.22	0.677
UACC-62	1.89	2.56	0.147	2.54	0.370
Ovarian cancer					
IGROV1	2.10	1.38	0.0783	2.60	0.395
OVCAR-3	2.15	2.56	0.0878	2.25	0.360
OVCAR-4	2.33	1.48	—	9.44	—
OVCAR-5	2.43	2.46	0.831	—	4.34
OVCAR-8	0.782	2.75	0.353	3.14	6.06
SK-OV-3	2.71	4.78	0.512	—	2.02
Renal cancer					
786-0	1.62	1.95	0.393	6.71	0.582
A498	1.29	1.95	0.0502	2.55	0.267
ACHN	1.74	2.45	0.318	2.31	0.548
CAKI-1	2.09	2.17	0.129	4.33	1.21
RXF 393	1.02	2.15	0.234	1.50	1.66
SN12C	1.86	5.19	0.468	5.51	1.68
TK-10	1.94	5.43	0.590	4.01	2.08
UO-31	2.06	3.20	0.536	3.96	1.45
Prostate cancer					
PC-3	1.47	1.22	0.289	2.46	0.392
DU-145	1.18	1.07	0.522	2.88	1.60
Breast cancer					
MCF7	1.53	—	0.580	7.73	0.318
NCI/ADR-RES	1.41	1.94	0.150	4.31	0.998
MDA-MB-231/ATCC	1.22	2.48	0.325	2.53	1.17
HS578T	1.30	0.240	0.305	4.11	1.33
MDA-MB-435	2.41	6.22	0.0315	3.50	0.326
BT-549	1.91	—	1.13	—	1.80
T-47D	3.12	8.84	—	—	4.80

n.t., means not tested; (—) values $>10 \times 10^{-5}$ M.

high cytostatic activity on leukemia SR, colon cancer HCT-15, ovarian cancer IGROV1 and OVCAR-3, melanoma SK-MEL-5, renal cancer A498, and breast cancer MDA-MB-435 cell lines (GI_{50} values between 3.15×10^{-7} and 8.78×10^{-7} M). The same compound displayed a significant inhibitory activity on almost all cell lines of liquid and solid tumors at micromolar concentrations. Hydrazone **11** totally inhibited the growth of 20 cell lines at 0.318–9.73×10^{-5} M concentrations, showing a good selectivity on the leukemia panel. In addition, it was not cytotoxic, except for three cell lines of this panel (HL-60 TB, K-562, and SR). Hydrazone **21** produced 50% growth inhibition of 26 cell lines at micromolar concentrations. The same compound inhibited totally the growth of 24 cell lines at 10^{-5} M concentrations exhibiting high potency against leukemia SR cell line (TGI value 3.18×10^{-6} M and LC_{50} value 9.20×10^{-6} M). Compound **21** is cytotoxic against HCC-298 cell line of colon cancer only (LC_{50} value 6.48×10^{-5} M). Hydrazone **8** displayed a significant growth inhibitory activity on all the 60 cell lines showing GI_{50} values between 5.89 and 34.7×10^{-6} M, and total growth inhibitory activity on 56 cell lines at 2.1–8.94×10^{-5} M concentrations. Compound **8** showed cytotoxic activity against 31 cell lines with particular selectivity against all renal cancer and prostate cancer cell lines. Compound **10** demonstrated cytostatic activity against 54 cell lines showing the best results for CNS cancer SNB75 and U251 cell lines (GI_{50} values 6.26×10^{-6} and 9.02×10^{-6} M, respectively), and breast cancer HS578T cell line (GI_{50} value 2.40×10^{-6} M). Hydrazone **10** totally inhibited the growth of 20 cell lines, once more showing the best results against breast cancer HS578T cell line (TGI value 7.01×10^{-6} M). Interestingly, **10** was not cytotoxic on this cell line (LC_{50} value $>10 \times 10^{-5}$ M), while it showed cytotoxic activity on three lung, CNS, and renal cancer cell lines with LC_{50} values between 5.74 and 6.89×10^{-5} M. Compound **19** displayed inhibitory activity at 10^{-5} M concentration on 52 cell lines with particular selectivity against HL-60 (TB) leukemia cell line (GI_{50} 9.84×10^{-6} M). At 10^{-5} M concentrations, hydrazone **19** totally inhibited the growth of HCT-15, LOX IMVI, IGROV1, OVCAR-3, ACHN, A498 cell lines as well as all leukemia cell lines. Furthermore, **19** showed cytotoxic activity on four leukemia cell lines with LC_{50} values between 7.15 and 8.68×10^{-5} M.

Table 4. TGI values of compounds **8**, **10**, **11**, **19**, and **21**

Panel/cell line	Compound				
	8	**10**	**11**	**19**	**21**
Leukemia					
CCRF-CEM	4.92	5.83	1.61	7.89	—
HL-60(TB)	8.68	5.94	0.704	2.72	n.t.
K-562	2.87	4.95	0.675	3.23	1.13
MOLT-4	5.89	—	1.83	3.44	4.06
RPMI-8226	3.90	—	0.821	4.43	2.62
SR	2.75	4.29	0.303	2.89	0.318
Non-small cell lung cancer					
A549/ATCC	5.08	6.50	—	—	—
EKVX	6.03	—	—	—	—
HOP-62	4.09	3.24	—	—	—
HOP-92	3.64	4.37	—	—	—
NCI-H226	3.06	—	—	—	—
NCI-H23	4.81	—	0.911	—	—
NCI-H322M	5.11	—	—	—	—
NCI-H460	4.67	—	—	—	—
NCI-H522	4.77	—	1.33	—	2.73
Colon cancer					
HCC-2998	2.61	—	2.10	—	1.28
HCT-116	2.25	—	1.68	—	3.13
HCT-15	4.72	—	2.00	9.73	5.74
KM12	7.08	—	—	—	7.17
CNS cancer					
SF-268	4.20	—	—	—	—
SF-295	3.59	—	—	—	—
SF-539	2.18	—	0.558	—	3.04
SNB-19	7.25	—	—	—	—
SNB-75	5.18	4.98	—	—	—
U251	4.52	2.34	—	—	—
Melanoma					
LOX IMVI	—	—	—	6.24	—
M14	3.65	—	—	—	3.87
SK-MEL-2	4.61	—	—	—	8.19
SK-MEL-28	7.82	—	—	—	—
SK-MEL-5	3.90	—	0.711	—	2.40
UACC-257	4.47	—	—	—	—
UACC-62	3.84	8.42	—	—	3.14
Ovarian cancer					
IGROV1	4.44	3.95	9.73	9.74	3.48
OVCAR-3	4.02	—	—	6.05	—
OVCAR-4	4.74	—	—	—	—
OVCAR-5	4.70	9.97	—	—	—
OVCAR-8	4.72	—	4.05	—	—
SK-OV-3	7.33	—	—	—	—
Renal cancer					
786-0	3.12	4.76	—	—	—
A498	2.81	3.61	0.318	6.92	1.55
ACHN	3.45	9.02	—	9.26	—
CAKI-1	4.03	6.32	—	—	—
RXF 393	3.11	6.62	—	—	9.42
SN12C	3.58	—	—	—	8.57
TK-10	3.99	—	—	—	9.20
UO-31	3.81	—	—	—	8.64
Prostate cancer					
PC-3	3.22	3.84	4.00	—	9.58
DU-145	3.20	—	—	—	—
Breast cancer					
MCF7	4.78	—	—	—	—
NCI/ADR-RES	4.02	8.24	0.698	—	6.48
MDA-MB-231/ATCC	3.29	—	4.22	—	5.61
HS578T	3.40	0.701	—	—	8.21
MDA-MB-435	6.46	—	0.515	—	3.60
BT-549	4.63	—	—	—	—
T-47D	8.94	—	—	—	—

n.t., means not tested; (—) values $>10 \times 10^{-5}$ M.

Table 5. LC_{50} values of compounds **8**, **10**, **11**, **19**, and **21**

Panel/cell line	Compound				
	8	**10**	**11**	**19**	**21**
Leukemia					
HL-60(TB)	—	—	3.13	7.43	n.t.
K-562	6.33	—	6.82	8.05	—
MOLT-4	—	—	—	8.68	—
RPMI-8226	—	—	—	—	—
SR	8.49	—	1.31	7.15	0.920
Non-small cell lung cancer					
HOP-62	8.26	6.89	—	—	—
HOP-92	7.42	—	—	—	—
NCI-H226	7.57	—	—	—	—
Colon cancer					
HCC-2998	7.19	—	—	—	6.48
HCT-116	7.15	—	—	—	—
CNS cancer					
SF-268	9.96	—	—	—	—
SF-295	7.68	—	—	—	—
SF-539	6.12	—	—	—	—
U251	—	5.74	—	—	—
Melanoma					
M14	7.80	—	—	—	—
SK-MEL-2	9.94	—	—	—	—
SK-MEL-5	7.85	—	—	—	—
UACC-257	9.23	—	—	—	—
UACC-62	7.78	—	—	—	—
Ovarian cancer					
IGROV1	9.39	—	—	—	—
OVCAR-3	7.53	—	—	—	—
OVCAR-4	9.65	—	—	—	—
OVCAR-5	9.07	—	—	—	—
Renal cancer					
786-0	5.99	—	—	—	—
A498	6.09	6.70	—	—	—
ACHN	6.84	—	—	—	—
CAKI-1	7.78	—	—	—	—
RXF 393	9.51	—	—	—	—
SN12C	6.89	—	—	—	—
TK-10	8.21	—	—	—	—
UO-31	7.05	—	—	—	—
Prostate cancer					
PC-3	7.02	—	—	—	—
DU-145	8.66	—	—	—	—
Breast cancer					
MDA-MB-231/ ATCC	8.86	—	—	—	—
HS578T	8.87	—	—	—	—

n.t., means not tested; (—) values $>10 \times 10^{-5}$ M.

The activity appears to be related to some structural requirements, as the 6-substituent on the pyrimidine ring. In fact, the presence of an arylidenehydrazinyl on 6-position favorably affects the activity. The introduction in the same position of an heteroarylidenehydrazinyl group (compounds **12**–**14** and **22**–**24**) or alkylideneidrazinyl group (compounds **5**–**7** and **15**–**17**) is detrimental. Among 6-arylidenehydrazinyl derivatives chlorine atoms on arylidene moiety were effective in inducing strong antitumoral activity. The best activity is shown by 2,4-dichloro- and 2,6-dichlorosubstituted derivatives, whereas the presence of 4-chlorophenyl or 4-methoxyphenyl produces variable effects: in compounds **9** and **18** led to clear reduction in inhibitory activity, while in compounds **8** and **19** the activity is maintained.

Furthermore, we can note that the presence of a 4-pyrrolidino group on pyrimidine ring is very important for the activity. Replacement of pyrrolidino group with a morpholino produces negative effect on activity, except for compounds **19** and **21**, which retained a good activity.

A COMPARE[12] analysis was performed with compound **11** to investigate whether it resembled 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. Using GI_{50} values of compound **11** (NSC 733012) as seed, COMPARE analysis showed that the highest correlation coefficients with standard agents were 0.584 with maysanine, 0.579 with rhizoxin, and 0.57 with vinblastine.[13] Furthermore, the COMPARE analysis run for the same compound utilizing the database of all public compounds[14] revealed correlation coefficients of 0.771–0.722 with same combretastatin analogs.[15,16] Since a correlation coefficient of 0.55–0.6 is considered the lowest correlation that suggests a relationship with another compound, [17] it is probable that this compound may exert its antitumor activity partly through affecting mitosis.

3. Experimental

3.1. Chemistry

Melting points were determined on a Stuart Scientific Melting point SMP1 and are uncorrected. Proton NMR spectra were recorded on a Varian Unity 300 spectrometer. The chemical shifts are reported in parts per million (δ, ppm) downfield from tetramethylsilane (TMS), which was used as internal standard. Infrared spectra were obtained with a Bruker Vector 22 spectrophotometer. Elemental analyses were carried out with a Carlo Erba model 1106 Elemental Analyzer and the values found were within 0.4% of theoretical values. The 3-amino-3-(pyrrolidino) propenenitrile **1a**, 3-amino-3-(morfolino)propenenitrile **1b**, 2-amino-6-methylthio-4-pyrrolidino-5-pyrimidinecarbonitrile **3a**, 2-amino-6-methylthio-4-morpholino-5-pyrimidinecarbonitrile **3b**, 2-amino-6-hydrazino-4-pyrrolidino-5-pyrimidinecarbonitrile **4a**, and 2-amino-6-hydrazino-4-morpholino-5-pyrimidinecarbonitrile **4b** were prepared by previously described procedures.[6,7]

3.2. General procedure for the synthesis of hydrazones 5–24

To a solution of 6-hydrazinopyrimidines **4** (2.5 mmol), the appropriate aldehyde or ketone (2.5 mmol) in ethanol (30 mL) was added with a few drops of acetic acid and refluxed for 1 h. After cooling, the resulting precipitate was filtered and recrystallized from the appropriate solvent to give target hydrazones.

3.2.1. 2-Amino-6-(2-(butylidene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 5. Yield 84%. Mp 235–236 °C (benzene). ^{1}H NMR (DMSO-d_6) δ 0.87 (t, J = 7.3 Hz, 3H, CH_3), 1.45 (m, 2H, CH_2), 1.81, 3.54 (m, 8H, pyrrolidinyl), 2.12 (q, J = 5.8 Hz, 2H, CH_2), 6.48 (s, 2H, NH_2), 7.43 (t, J = 5.4 Hz, 1H, CH), 10.13 (s, 1H, NH). IR (Nujol) 3480, 3240, 3140, 2170, 1610, 1580 cm^{-1}. Anal. Calcd for $C_{13}H_{19}N_7$: C, 57.12; H, 7.01; N, 35.87. Found: C, 57.15; H, 6.99; N, 35.90.

3.2.2. 2-Amino-4-(2-(2-methylpropylidene)hydrazinyl)-6-(pyrrolidin-1-yl)pyrimidine-5-carbonitrile 6. Yield 87%. Mp 220–221 °C (benzene). ^{1}H NMR (DMSO-d_6) δ 1.01 (d, J = 6.8 Hz, 6H, CH_3), 1.82, 3.55 (m, 8H, pyrrolidinyl), 2.43 (m, 1H, CH), 6.44 (s, 2H, NH_2), 7.35 (m, 1H, CH), 10.19 (m, 1H, NH). IR (Nujol) 3500, 3410, 3340, 3260, 3150, 2190, 1620, 1590 cm^{-1}. Anal. Calcd for $C_{13}H_{19}N_7$: C, 57.12; H, 7.01; N, 35.87. Found: C, 57.08; H, 7.00; N, 35.85.

3.2.3. 2-Amino-6-(2-(butan-2-ylidene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 7. Yield 85%. Mp 205–206 °C (ethanol). ^{1}H NMR (DMSO-d_6) δ 1.03 (t, J = 7.6 Hz, 3H, CH_3), 1.82 (s, 3H, CH_3), 1.81, 3.54 (m, 8H, pyrrolidinyl), 2.19 (q, J = 7.6 Hz, 2H, CH_2), 6.43 (s, 2H, NH_2), 8.90 (s, 1H, NH). IR (Nujol) 3380, 3310, 3230, 2190, 1645, 1565 cm^{-1}. Anal. Calcd for $C_{13}H_{19}N_7$: C, 57.12; H, 7.01; N, 35.87. Found: C, 57.08; H, 7.00; N, 35.85.

3.2.4. 2-Amino-6-(2-(4-methoxybenzylidene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 8. Yield 98%. Mp 278–279 °C (1-propanol). ^{1}H NMR (DMSO-d_6) δ 1.82, 3.56 (m, 8H, pyrrolidinyl), 3.74 (s, 3H, OCH_3), 6.45 (s, 2H, NH_2), 6.92, 7.62 (m, 4H, Ar), 7.98 (s, 1H, CH), 10.76 (s, 1H, NH). IR (Nujol) 3320, 3190, 2180, 1650, 1600, 1580 cm^{-1}. Anal. Calcd for $C_{17}H_{19}N_7O$: C, 60.52; H, 5.68; N, 29.06. Found: C, 70.55; H, 5.67; N, 29.10.

3.2.5. 2-Amino-6-(2-(4-chlorobenzylidene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 9. Yield 85%. Mp 274–275 °C (1-propanol). ^{1}H NMR (DMSO-d_6) δ 1.84, 3.56 (m, 8H, pyrrolidinyl), 6.51 (s, 2H, NH_2),

7.35, 7.58 (m, 4H, Ar), 8.12 (s, 1H, CH), 10.52 (s, 1H, NH). IR (Nujol) 3340, 3240, 3150, 2160, 1640, 1570 cm^{-1}. Anal. Calcd for $C_{16}H_{16}ClN_7$: C, 56.22; H, 4.72; N, 28.69. Found: C, 56.26; H, 4.70; N, 28.73.

3.2.6. 2-Amino-6-(2-(2,4-dichlorobenzylidene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 10. Yield 80%. Mp 272–273 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 1.83, 3.60 (m, 8H, pyrrolidinyl), 6.68 (s, 2H, NH_2), 7.40, 7.62, 8.15 (m, 3H, Ar), 8.42 (s, 1H, CH), 11.28 (s, 1H, NH). IR (Nujol) 3320, 3270, 3160, 2160, 1650, 1580, 1565 cm^{-1}. Anal. Calcd for $C_{16}H_{15}Cl_2N_7$: C, 51.08; H, 4.02; N, 26.06. Found: C, 51.03; H, 4.04; N, 26.10.

3.2.7. 2-Amino-6-(2-(2,6-dichlorobenzylidene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 11. Yield 98%. Mp 240–241 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 1.82, 3.56 (m, 8H, pyrrolidinyl), 6.62 (s, 2H, NH_2), 7.37, 7.48 (m, 3H, Ar), 8.35 (s, 1H, CH), 10.97 (s, 1H, NH). IR (Nujol) 3320, 3260, 3200, 2180, 1655, 1560 cm^{-1}. Anal. Calcd for $C_{16}H_{15}Cl_2N_7$: C, 51.08; H, 4.02; N, 26.06. Found: C, 51.13; H, 4.01; N, 26.04.

3.2.8. 2-Amino-6-(2-(furan-2-ylmethylene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 12. Yield 78%. Mp 275–276 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 1.82, 3.56 (m, 8H, pyrrolidinyl), 6.55 (s, 2H, NH_2), 6.85, 6.90, 7.48 (m, 3H, furyl), 7.88 (s, 1H, CH), 10.82 (s, 1H, NH). IR (Nujol) 3440, 3280, 3240, 3160, 2160, 1655, 1630 cm^{-1}. Anal. Calcd for $C_{14}H_{15}N_7O$: C, 56.56; H, 5.09; N, 32.98. Found: C, 56.60; H, 5.10; N, 33.00.

3.2.9. 2-Amino-4-(pyrrolidino-1-yl)-6-(2-(thiophen-2-ylmethylene)hydrazinyl)pyrimidine-5-carbonitrile 13. Yield 98%. Mp 260–261 °C (ethanol). ^1H NMR (DMSO-d_6) δ 1.83, 3.58 (m, 8H, pyrrolidinyl), 6.54 (s, 2H, NH_2), 7.05, 7.30, 7.53 (m, 3H, thienyl), 8.29 (s, 1H, CH), 10.72 (s, 1H, NH). IR (Nujol) 3320, 3240, 3180, 2180, 1650, 1590 cm^{-1}. Anal. Calcd for $C_{14}H_{15}N_7S$: C, 53.66; H, 4.82; N, 31.29. Found: C, 53.60; H, 4.80; N, 31.33.

3.2.10. 2-Amino-6-(2-(pyridin-3-ylmethylene)hydrazinyl)-4-(pyrrolidino-1-yl)pyrimidine-5-carbonitrile 14. Yield 98%. Mp 287–288 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 1.84, 3.60 (m, 8H, pyrrolidinyl), 6.55 (s, 2H, NH_2), 7.39, 8.06, 8.12, 8.50 (m, 4H, pyridyl), 8.83 (s, 1H, CH), 11.14 (m, 1H, NH). IR (Nujol) 3500, 3410, 3340, 3260, 3150, 2190, 1620, 1590 cm^{-1}. Anal. Calcd for $C_{16}H_{16}N_8$: C, 58.43; H, 5.23; N, 36.34. Found: C, 58.55; H, 5.21; N, 36.30.

3.2.11. 2-Amino-6-(2-(butylidene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 15. Yield 98%. Mp 198–200 °C (benzene). ^1H NMR (DMSO-d_6) δ 0.86 (m, 3H, CH_3), 1.47 (m, 2H, CH_2), 2.14 (m, 2H, CH_2), 3.59 (m, 8H, morpholinyl), 6.69 (s, 2H, NH_2), 7.41 (t, J = 6.3 Hz, 1H, CH), 10.45 (s, 1H, NH). IR (Nujol) 3330, 3210, 3140, 2180, 1650, 1580 cm^{-1} Anal. Calcd for $C_{13}H_{19}N_7O$: C, 53.96; H, 6.62; N, 33.89. Found: C, 54.01; H, 6.64; N, 33.93.

3.2.12. 2-Amino-4-(2-(2-methylpropylidene)hydrazinyl)-6-(morpholino-1-yl)pyrimidine-5-carbonitrile 16. Yield 90%. Mp 194–195 °C (benzene). ^1H NMR (DMSO-d_6) δ 1.02 (m, 6H, CH_3), 3.60 (m, 8H, morpholinyl), 2.42 (m, 1H, CH), 6.65 (s, 2H, NH_2), 7.32 (m, 1H, CH), 10.50 (m, 1H, NH). IR (Nujol) 3300, 3240, 3190, 2170, 1630, 1570 cm^{-1}. Anal. Calcd for $C_{13}H_{19}N_7O$: C, 53.96; H, 6.62; N, 33.89. Found: C, 54.01; H, 6.60; N, 33.92.

3.2.13. 2-Amino-6-(2-(butan-2-ylidene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 17. Yield 70%. Mp 145–146 °C (2-Propanol). ^1H NMR (DMSO-d_6) δ 1.03 (t, J = 7.3 Hz, 3H, CH_3), 1.84 (s, 3H, CH_3), 3.60 (m, 8H, morpholinyl), 2.21 (q, J = 7.3 Hz, 2H, CH_2), 6.61 (s, 2H, NH_2), 9.34 (s, 1H, NH). IR (Nujol) 3390, 3290, 3220, 2190, 1640, 1565 cm^{-1}. Anal. Calcd for $C_{13}H_{19}N_7O$: C, 53.96; H, 6.62; N, 33.89. Found: C, 53.92; H, 6.61; N, 33.85.

3.2.14. 2-Amino-6-(2-(4-methoxybenzylidene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 18. Yield 72%. Mp 245–246 °C (benzene). ^1H NMR (DMSO-d_6) δ 3.60 (m, 8H, morpholinyl), 3.75 (s, 1H, OCH_3), 6.70 (s, 2H, NH_2), 6.91, 7.64 (m, 4H, aryl), 7.96 (s, 1H, CH), 11.34 (s, 1H, NH). IR (Nujol) 3340, 3200, 2190, 1640, 1610, 1580 cm^{-1}. Anal. Calcd for $C_{17}H_{19}N_7O_2$: C, 57.78; H, 5.42; N, 27.75. Found: C, 57.73; H, 5.41; N, 27.79.

3.2.15. 2-Amino-6-(2-(4-chlorobenzylidene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 19. Yield 98%. Mp 265–276 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 3.62 (m, 8H, morpholinyl), 6.78 (s, 2H, NH_2), 7.42, 7.73 (m, 4H, Ar), 8.01 (s, 1H, CH), 11.22 (s, 1H, NH). IR (Nujol) 3350, 3240, 3140, 2180, 1655, 1575 cm^{-1}. Anal. Calcd for $C_{16}H_{16}ClN_7O$: C, 53.71; H, 4.51; N, 27.40. Found: C, 53.76; H, 4.50; N, 27.37.

3.2.16. 2-Amino-6-(2-(2,4-dichlorobenzylidene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 20. Yield 98%. Mp 268–269 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 3.62 (m, 8H, morpholinyl), 6.84 (s, 2H, NH_2), 7.44, 7.64, 8.15 (m, 3H, aryl), 8.41 (s, 1H, CH), 11.46 (s, 1H, NH). IR (Nujol) 3470, 3240, 3150, 2180, 1625, 1580 cm^{-1}. Anal. Calcd for $C_{16}H_{15}Cl_2N_7O$: C, 48.99; H, 3.85; N, 25.00. Found: C, 48.95; H, 3.86; N, 25.04.

3.2.17. 2-Amino-6-(2-(2,6-dichlorobenzylidene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 21. Yield 65%. Mp 235–236 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 3.61 (m, 8H, morpholinyl), 6.87 (s, 2H, NH_2), 7.38, 7.48 (m, 3H, aryl), 8.34 (s, 1H, CH), 11.23 (s, 1H, NH). IR (Nujol) 3380, 3300, 3200, 2160, 1630, 1570 cm^{-1}. Anal. Calcd for $C_{16}H_{15}Cl_2N_7O$: C, 48.99; H, 3.85; N, 25.00. Found: C, 49.04; H, 3.84; N, 25.04.

3.2.18. 2-Amino-6-(2-(furan-2-ylmethylene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 22. Yield 74%. Mp 240–241 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 3.61 (m, 8H, morpholinyl), 6.56 (s, 2H, NH_2), 6.78, 7.42 (m, 3H, furyl), 8.02 (s, 1H, CH), 10.95 (s,

1H, NH). IR (Nujol) 3460, 3250, 3110, 2200, 1645, 1580 cm^{-1}. Anal. Calcd for $C_{14}H_{15}N_7O_2$: C, 53.67; H, 4.83; N, 31.29. Found: C, 53.71; H, 4.84; N, 31.25.

3.2.19. 2-Amino-4-(morpholino-1-yl)-6-(2-(thiophen-2-ylmethylene)hydrazinyl)pyrimidine-5-carbonitrile 23. Yield 70%. Mp 242–243 °C (ethanol). ^1H NMR (DMSO-d_6) δ 3.60 (m, 8H, morpholinyl), 6.74 (s, 2H, NH_2), 7.05, 7.31, 7.54 (m, 3H, thienyl), 8.25 (s, 1H, CH), 10.99 (s, 1H, NH). IR (Nujol) 3450, 3250, 3080, 2180, 1625, 1590 cm^{-1}. Anal. Calcd for $C_{14}H_{15}N_7OS$: C, 51.05; H, 4.59; N, 29.77. Found: C, 51.00; H, 4.60; N, 29.80.

3.2.20. 2-Amino-6-(2-(pyridin-3-ylmethylene)hydrazinyl)-4-(morpholino-1-yl)pyrimidine-5-carbonitrile 24. Yield 98%. Mp 270–271 °C (1-propanol). ^1H NMR (DMSO-d_6) δ 3.62 (m, 8H, morpholinyl), 6.79 (s, 2H, NH_2), 7.40, 8.13, 8.50 (m, 4H, pyridinyl), 8.84 (s, 1H, CH), 11.36 (s, 1H, NH). IR (Nujol) 3480, 3340, 2160, 1600, 1570 cm^{-1}. Anal. Calcd for $C_{16}H_{16}N_8O$: C, 55.55; H, 4.97; N, 34.55. Found: C, 55.60; H, 4.96; N, 34.52.

3.3. 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 microtiter plate. Test agents are then added at single 10^{-4} M concentration and the culture is incubated for 48 h. End-point determinations are made with alamar blue.[18] 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.

3.4. 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% fetal calf serum and 2 mM L-glutamine. The tumor cells are inoculated over a series of standard 96-well microtiter plates in 100 mL of medium.[8,9] Density of inoculum depends on the type of tumor cell and on its growth characteristics.[19] These cells are then preincubated on the microtiter plate for 24 h before adding the compounds. They 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 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.[11]

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