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Synthesis and in vitro antitumoral activity of new 3,5-dicyanopyridine derivatives

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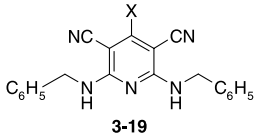
Abstract—A new series of 2-amino-4-aryl-6-dialkylamino-3,5-dicyanopyridines, **20**–**47**, were synthesized in satisfactory overall yield, through a simple synthetic strategy using 3-amino-3-(dialkylamino)-propenenitriles **1** and **2** as key intermediates. 3,5-Dicyanopyridine derivatives **20**–**47** 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^{-6} M level and in some cases at 10^{-8} M concentration.

1. Introduction

Polysubstituted pyridines represent molecular frameworks that serve as a platform for developing pharmaceutical agents for various applications. Among these compounds pyridine-3,5-dicarbonitrile derivatives have attracted great interest in recent years because of their noteworthy utility in different medicinal fields. Thus, 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.[1] Numerous patents have revealed significant and diverse medicinal utility of various compounds with 6-alkyl-(or -arylthio)-2-amino-4-aryl-(or -heteroaryl)-3,5-dicyanopyridine structural motif. Thus, these compounds inhibit MK-2 activity and they could be useful for the prevention and treatment of diseases and disorders that are mediated by TNFα, for example they can be used for the prevention or treatment of arthritis and cancers such as colorectal cancer,[2] and modulate androgen receptor function.[3] In addition, they serve as maxi-K channel potassium channel openers with applications in treating urinary incontinence,[4] inhibit IKK2 with a potential for treating HBV infection,[5] and exhibit anti-bacterial, anti-biofilm, and anti-infective properties.[6] Other remarkable recent findings take account of the identification of 2-amino-6-[(2-aminophenyl)thio]-4-(2-furyl)pyridine-3,5-dicarbonitrile as lead in developing therapeutic agents for the treatment of Creutzfeldt-Jacob disease[7] as well as 6-alkylthio-2-amino-4-aryl-(or -heteroaryl)-3,5-dicyano-pyridines as selective ligands of adenosine receptor implicated in Parkinson's disease, hypoxia/ischemia, asthma, kidney disease, epilepsy, and cancer.[8–10]

Because of our ongoing interest in the search for novel antitumor pyridine derivatives, we started a study aimed to evaluate new derivatives bearing the 3,5-dicyanopyridine motif. Recently we showed that certain 4-(substituted)-aryl or -heteroaryl-2,6-dibenzylamino-3,5-dicyanopyridines **3**–**19** (Fig. 1) had varying degrees of efficacy as antiproliferative agents.[11] In this study, we have found that the presence of functionality such as 4-chlorine atom or hydroxyl group in 3 or 4 position of phenyl ring has an enhancing effect on anticancer



3-19

3 X = 4-OMePh, **4** X = 3-OMePh, **5** X = 2,5- $(OMe)_2$Ph,
6 X = 3,4,5-$(OMe)_3$Ph, **7** X = 4-ClPh, **8** X = 2-ClPh, **9** X = 2,4-Cl_2Ph,
10 X = 2,6-Cl_2Ph, **11** X = 3-OHPh, **12** X = 4-OHPh, **13** X = 3-OH-4-OMePh,
14 X = 2-pyridyl, **15** X = 3-pyridyl, **16** X = 4-pyridyl, **17** X = 2-thienyl,
18 X = 2-furyl, **19** X = 5-Me-2-furyl

Figure 1. 2,6-Dibenzylamino-3,5-dicyanopyridines **3**–**19**.

Keywords: Pyridines; Dicyanopyridines; Anticancer activity; Cytostatic activity.
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potency. Thus, the synthesized compounds bearing these features showed high antiproliferative activity, and among them, 2,6-bis(benzylamino)-4-(3-hydroxyphenyl)pyridine-3,5-dicarbonitrile **11** exhibited the best activity.[11] These results prompted us to begin a program to modify these molecules to obtain new derivatives endowed with better anticancer activity. With the aim to study the influence of amino groups bound to C-2 and C-6 of the pyridine ring on activity, structural modifications at these positions are now examined. In this paper, the synthesis of a new series of derivatives bearing a morpholine or thiomorpholine at C-6 and a primary amino group at C-2 of the pyridine ring, and results of their antiproliferative activity are reported.

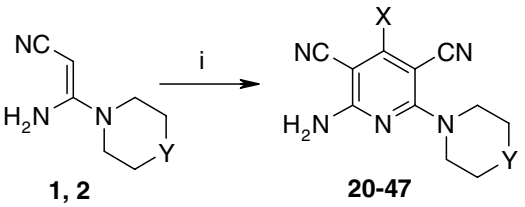
2. Results and discussion

2.1. Chemistry

The target 3,5-dicyanopyridines **20–47** (Table 1) were synthesized as shown in Scheme 1. Although various methods to prepare 2-amino-4-aryl-3,5-dicyanopyridines have been reported in recent years, analysis of this literature reveals that all published approaches involve multistep sequences,[8,9] and their usefulness is limited by the lack of generality and the low yields. A recent paper reported on the one-step three-component synthesis of 2-amino-4-aryl-3,5-dicyano-6-sulfanylpyridines, however the better yields obtained with this method do not reach 50%.[12] Furthermore, 6-amino-4-aryl-2-(pyrrolidin-1-yl)-pyridine-3,5-dicarbonitriles were obtained as by-products in the synthesis of 4,6-diaryl-2-(pyrrolidin-1-yl)-nicotinonitriles and 3-amino-2,4-dicyano-5-aryl-biphenyls when a large amount of pyrrolidine was used as catalyst.[13]

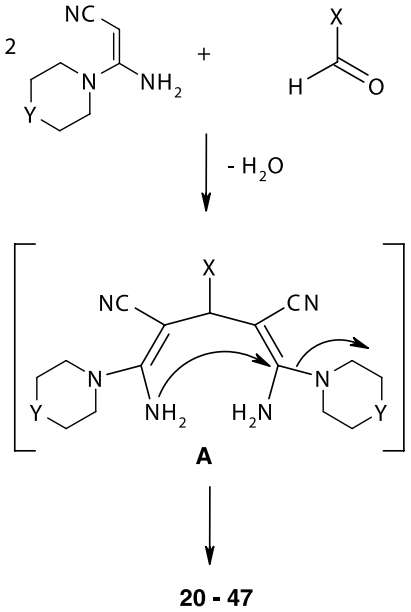
Table 1. 3,5-Dicyanopyridines **20–47**

Compound	X	Y
20	4-OMePh	O
21	2,5-$(OMe)_2$Ph	O
22	3,4,5-$(OMe)_3$Ph	O
23	4-ClPh	O
24	2-ClPh	O
25	2,4-Cl_2Ph	O
26	2,6-Cl_2Ph	O
27	3-OHPh	O
28	4-OHPh	O
29	3-OH,4-OMePh	O
30	2-Pyridyl	O
31	3-Pyridyl	O
32	4-Pyridyl	O
33	2-Thienyl	O
34	5-Me-2-furyl	O
35	2-Furyl	O
36	4-OMePh	S
37	2,5-$(OMe)_2$Ph	S
38	3,4,5-$(OMe)_3$Ph	S
39	4-ClPh	S
40	2,4-Cl_2Ph	S
41	2,6-Cl_2Ph	S
42	4-OHPh	S
43	2-Pyridyl	S
44	3-Pyridyl	S
45	4-Pyridyl	S
46	2-Thienyl	S
47	5-Me-2-furyl	S



Scheme 1. Synthesis of 3,5-dicyanopyridines **20–47**. Reagents and condition: (i) X–CHO, MeCN, reflux, 30 min.

In this paper, we developed an alternative and simple synthetic pathway to obtain 2-amino-4-aryl-6-dialkylamino-3,5-dicyanopyridines with high yields and purity. These compounds were obtained through an one-pot reaction starting from the key intermediates 3-amino-3-morpholino-propenenitrile **1** and 3-amino-3-thiomorpholino-propenenitrile **2** that were prepared according to a previously described procedure.[14] These were treated with the appropriate aryl or heteroarylaldehyde in 2:1 molar ratio, in MeCN solution. The reaction mixture was heated at reflux and, after a short time, 2-amino-4-aryl-6-dialkylamino-3,5-dicyano-pyridines precipitated. As shown in Scheme 2, we assume that the pathway of formation of 3,5-dicyanopyridine ring was initiated by condensation of two molecules of 3-amino-3-dialkylaminopropenenitriles **1**, **2** with the aldehyde, to give the intermediate **A**. Intramolecular cyclization with loss of one of the dialkylamino moieties and aromatization produced the target dicyanopyridines **20–47**. All the newly synthesized compounds gave corrected analytical data. The IR and NMR spectral data were consistent with the assigned structure.



Scheme 2. Pathway of formation of dicyanopyridines **20–47**.

Table 2. Overview of the results[a] of the anticancer screening for compounds **11** and **20–47**[b]

Compound	No. of the cell lines investigated	Number of the cell lines giving positive $\log GI_{50}$, $\log TGI$, and $\log LC_{50}$[c]					
		$\log GI_{50}$ (M)		$\log TGI$ (M)		$\log LC_{50}$ (M)	
		No.	Range	No.	Range	No.	Range
11	57	56	−5.77 to −4.02	44	−5.24 to −4.06	18	−4.59 to −4.04
20	57	57	−6.08 to −4.30	35	−5.21 to −4.06	5	−4.14 to −4.02
21	57	27	−4.92 to −4.02	2	−4.24 to −4.01		
22	57	18	−5.79 to −4.02	3	−4.40 to −4.10		
23	52	49	<−8.00 to −4.09	13	−8.00 to −4.01	1	−4.20
24	52	50	−7.63 to −4.03	11	−5.55 to −4.05		
25	52	52	−5.49 to −4.61	49	−4.96 to −4.04	16	−4.24 to −4.02
26	57	57	−7.74 to −4.48	50	−5.61 to −4.02	12	−5.04 to −4.02
27	52	26	<−8.00 to −4.03	6	<−8.00 to −4.10		
30	57	11	<−8.00 to −4.13	3	−7.23 to −6.32		
31	57	36	−5.06 to −4.01	12	−4.36 to −4.02		
32	57	32	−5.04 to −4.02	5	−4.29 to −4.01		
34	45	43	−4.81 to −4.06	7	−4.36 to −4.09		
42	57	57	−5.00 to −4.29	39	−4.58 to −4.01	15	−4.25 to −4.07

[a] Data obtained from the NCI's in vitro disease-oriented human tumor cells screen (see Refs. 15–17 for details).
[b] Compounds **10**, **28**, **29**, **33**, **35–41**, and **43–47** were inactive.
[c] The response parameters: $\log GI_{50}$, $\log TGI$ and $\log LC_{50}$ are interpolated values representing the molar concentrations at which percentage growth is +50, 0 and −50, respectively.

2.2. Pharmacology

The compounds **20–47** were submitted to the US National Cancer Institute (NCI; Bethesda, MD), for in vitro testing against a panel of approximately 60 tumor cell lines, derived from nine different cancer types: leukemia, lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast. The compounds were tested at five concentrations at 10-fold dilution. A 48 h continuous drug exposure protocol was used and sulforhodamine B (SRB) protein assay was used to estimate cell growth. Details of this system and the information which is encoded by the activity pattern over all cell lines have been published.[15–17] The antitumoral activity of tested compounds is given by three parameters for each cell line: $\log GI_{50}$ value (GI_{50} = molar concentration of the compound that inhibits 50% net cell growth), $\log TGI$ value (TGI = molar concentration of the compound leading to total inhibition), and $\log LC_{50}$ value (LC_{50} = molar concentration of the compound leading to 50% net cell death). Furthermore, a mean graph midpoint (MG-MID) is calculated for each of the mentioned parameters, giving an averaged activity parameter over all cell lines. For the calculation of the MG-MID, insensitive cell lines are included with the highest concentration tested. Selectivity of the compound with respect to one or more cell lines of the screen is characterized by a high deviation (Δ) of the particular cell line parameter compared to the MG-MID value. The following is to be noted regarding the tumor cell growth inhibition data with the tested compounds: (a) relatively broad spectrum of tumor cell growth inhibition was found for the compounds **20**, **23**, **24**, **25**, **26**, and **42** (Table 2), while the compounds **22**, **27**, **30**, and **34** demonstrated a moderate to high selectivity toward one or more tumor cell lines ($\Delta \log GI_{50}$ ranged from 1.11 to 3.77; Table 3); (b) the compounds **28**, **29**, **33**, **35–41**, and **43–47** were inactive ($\log GI_{50}$ [M] > −4), whereas the other compounds **21**, **31**, **32** exhibited antiproliferative activity against a few human cancer cell lines (Tables 2 and 3). Dicyanopyridine **26** proved to be the most active member within the series, showing potent and broad spectrum of antitumoral activity (MG-MID value −5.31). Compound **26** showed an in vitro chemosensitive profile toward 48 different cancer cell lines with GI_{50} values lying in the concentration range between submicromolar to micromolar. Compound **26** displayed selectivity on colon cancer HCC 2998 cell line at GI_{50} ($\log GI_{50}$ value −7.74), TGI ($\log TGI$ value −5.61), and LC_{50} levels ($\log LC_{50}$ value −4.70). Although less potent when compared to **26**, compounds **20**, **23**, **24** and **25** showed activity against most of the tested cell lines with MG-MID values −4.77, −4.56, −4.56, and −4.84, respectively. However, compound **24** displayed high antiproliferative activity against NCI-H522 non-small cell lung cancer cell line ($\log GI_{50}$ value −7.63 and $\log TGI$ value −5.55) with high selectivity ($\Delta \log GI_{50}$ 3.07; Table 3). Compounds **23** and **27** selectively exhibited high potency against renal cancer UO31 cell line ($\log GI_{50}$ and TGI values <−8, $\Delta \log GI_{50}$ 3.44 and 3.77, respectively). Compound **20** inhibits the growth of all tested cell lines, showing its best activity against breast cancer T47-D cell line ($\log GI_{50}$ value −6.08).

The displacement of benzylamino groups bound to C-2 and C-6 of the pyridine with a morpholine or thiomorpholine at C-6 and a primary amino group at C-2 led to new derivatives **20–47** endowed with a pattern of antiproliferative activity extremely different with respect to the reference 2,6-dibenzylamino-3,5-dicyanopyridine compounds. From Tables 2 and 3, it is interesting to note that an aryl substituent at 4-position of 3,5-dicyanopyridines apparently plays an important role in the activity of these compounds. Compounds where phenyl ring has been replaced by an heterocyclic ring displayed weak activity. These are the only findings in accordance with previously reported structure–activity relationships.[11] On the contrary, the conversion of 2,6-bis(benzylamino)-4-(3-hydroxyphenyl)pyridine-3,5-dicarbonitrile **11** into the corresponding 2-amino-6-morpholino analog **27** leads

Table 3. The in vitro activity[a] and selectivity toward most sensitive tumor cell lines for compounds **11**, **20**–**27**, **30**–**32**, **34**, and **42**

Compound	Most sensitive tumor cell lines	$\log GI_{50}$ (M)[b]	Selectivity toward tumor cell lines (δ) for $\log GI_{50}$ (M)[c,d]	Mean value for all tested cell lines (MG-MID)[e] for $\log GI_{50}$ (M)
11	Leukemia: CCRF-CEM	−5.47		−5.12
	Leukemia: MOLT-4	−5.48		
	Leukemia: RPMI-8226	−5.51		
	Leukemia: SR	−5.68		
	Non-small cell lung: HOP-92	−5.60		
	Non-small cell lung: NCI-H226	−5.54		
	CNS: SF-295	−5.53		
	Melanoma: LOX IMVI	−5.53		
	Melanoma: SK-MEL-5	−5.77		
	Melanoma: UACC-62	−5.68		
	Ovarian: OVCAR-3	−5.48		
	Renal: A498	−5.70		
	Renal: ACHN	−5.58		
	Renal: CAKI-1	−5.56		
	Renal: SN12C	−5.64		
	Breast: MDA-MB-231/ATCC	−5.65		
	Breast: MDA-MB-435	−5.54		
20	Non-small cell lung: NCI-H522	−5.06		−4.77
	Colon: COLO-205	−5.67		
	Ovarian: IGROV1	−5.51		
	Ovarian: SK-OV-3	−5.30		
	Renal: A498	−5.13		
	Breast: T-47D	−6.08	1.31	
21	CNS: SNB-75	−4.89		−4.14
	Ovarian: SK-OV-3	−4.92		
22	Leukemia: SR	−4.87		−4.14
	Colon: COLO-205	−5.25	1.11	
	Colon: HCC2998	−5.23		
	CNS: SNB-75	−5.79	1.65	
	Breast: T-47D	−4.82		
23	Non-small cell lung: HOP-92	−5.05		−4.56
	CNS: SNB-75	−4.84		
	Ovarian: IGROV1	−4.85		
	Renal: A498	−5.09		
	Renal: RXF393	−6.32	1.76	
	Renal: UO31	−8.00	3.44	
24	Non-small cell lung: NCI-H522	−7.63	3.07	−4.56
	CNS: SNB-75	−6.38	1.82	
	Renal: A-498	−5.64	1.08	
25	Non-small cell lung: HOP-92	−5.48		−4.84
	Non-small cell lung: NCI-H460	−5.03		
	Non-small cell lung: NCI-H522	−5.32		
	CNS: SNB-75	−5.20		
	Ovarian: IGROV1	−5.18		
	Renal: A498	−5.57		
	Renal: RXF393	−5.25		
	Renal: UO31	−5.49		
	Breast: MCF7	−5.06		
	Breast: MDA-MB-435	−5.24		
26	Leukemia: K-562	−5.48		
	Non-small cell lung: HOP-62	−5.45		
	Non-small cell lung: NCI-H522	−5.63		
	Colon: HCC2998	−7.74	2.43	
	Colon: HCT-116	−5.51		
	Colon: HT-29	−5.48		−5.31
	CNS: SNB-75	−5.76		
	CNS: U251	−5.41		

Table 3 (*continued*)

Compound	Most sensitive tumor cell lines	$\log GI_{50}$ (M)[b]	Selectivity toward tumor cell lines (δ) for $\log GI_{50}$ (M)[c,d]	Mean value for all tested cell lines (MG-MID)[e] for $\log GI_{50}$ (M)
	Melanoma: MALME-3M	−5.43		
	Melanoma: M14	−5.61		
	Ovarian: IGROV1	−5.70		
	Ovarian: OVCAR-3	−5.62		
	Ovarian: SK-OV-3	−5.39		
	Renal: A498	−5.43		
	Renal: CAKI-1	−5.65		
	Breast: MCF-7	−5.53		
	Breast: NCI/ADR-RES	−5.63		
	Breast: MDA-MB-231/ATCC	−5.45		
	Breast: MDA-MB-435	−5.81		
	Breast: T-47D	−5.47		
27	CNS: SNB-75	−4.76		−4.23
	Ovarian: IGROV1	−4.73		
	Renal: A498	−4.83		
	Renal: UO31	−8.00	3.77	
30	Non-small cell lung: NCI-H226	−6.29	2.04	−4.25
	Melanoma: UACC-62	−7.47	3.22	
	Renal: SN12C	−7.42	3.17	
	Breast: MDA-MB-231/ATCC	−8.00	3.75	
31	Colon: HCC2998	−5.06		−4.24
32	Colon: HCC2998	−4.87		−4.24
	Melanoma: MALME-3M	−5.04		
	Ovarian: IGROV1	−4.91		
	Renal: A498	−4.73		
	Breast: T-47D	−4.90		
34	CNS: SNB-75	−5.56	1.16	−4.40
42	Leukemia: SR	−4.95		−4.72
	Non-small cell lung: HOP-62	−4.90		
	Non-small cell lung: HOP-92	−5.00		
	CNS: SNB-75	−4.96		

[a] Data obtained from the NCI's in vitro disease-oriented human tumor cells screen (see Refs. 15–17 for details).
[b] The response parameter: $\log GI_{50}$ is interpolated value representing the molar concentration at which percentage growth is +50.
[c] The reported data represent the logarithmic difference between the parametric value referred to the most sensitive cell line and the same mean parameter, δ is considered low if <1, moderate >1 and <3, high if >3.
[d] The value is shown if $\delta > 1$.
[e] MG-MID = mean graph midpoint = arithmetical mean value for all tested cancer cell lines. If the indicated effect was not attainable within the used concentration interval, the highest concentration was used for the calculation.

to drastic reduction of activity. Furthermore, the conversion of the inactive 2,6-bis(benzylamino)-4-(2,6-dichlorophenyl) pyridine-3,5-dicarbonitrile **10** into the corresponding 2-amino-6-morpholino analog **26** produces noteworthy enhancement of the inhibitory activity giving the most active compound of the two series. The presence of functionality as chlorine atoms at 2 and 6 positions of phenyl ring has an enhancing effect on anticancer potency. Thus, 2-amino-4-(2,6-dichlorophenyl)-6-morpholino pyridine-3,5-dicarbonitrile **26** is the most active member within the series. The shift of one chlorine atom from the 6-position to the 4-position on phenyl ring leads to **25** that retains antitumoral activity on the same cell lines but at higher concentrations. Further reduction of the antiproliferative activity is induced by the presence of only one chlorine atom on phenyl moiety (compounds **23** and **24**). The shift of chlorine from 2-position (compound **24**) to 4-position (compound **23**) on phenyl ring does not affect activity. As a matter of fact compounds **23** and **24** showed the same MG-MID value (−4.56, Table 3). Significant antiproliferative activity is induced by a 4-methoxy group on phenyl moiety (compound **20**). The introduction on phenyl ring of two or more methoxy substituents is detrimental for the activity as well as the presence of a 3- or 4-hydroxy moiety. Furthermore, we can note that the presence of the morpholino group is very important for the activity. Replacement of morpholino group with a thiomorpholino produces negative effect on activity except for compound **42** that retained a good activity.

A COMPARE[18] analysis was performed with the more active compound **26** to investigate whether it resembles anticancer drugs of the NCI standard agent database and to probably predict its mechanism of action. The COMPARE algorithm was developed to determine the

Table 4. COMPARE correlation coefficients (PCC) using GI_{50} values of compound **26** (NSC736022) as seed, tested in the US NCI 60 Cell lines in vitro screen

Rank	NSC	PCC	No of common cell lines	Compound
1	3051	0.504	57	*N*-Methylformamide
2	148958	0.465	57	Tegafur
3	13875	0.457	57	Altretamine
4	192965	0.376	57	Spirogermanium
5	330500	0.362	57	MacbecinII
6	77037	0.351	57	D-Tetrandine

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.[19] Using GI_{50} values of dicyanopyridine **26** (NSC736022) as seed, COMPARE analysis shown that compounds in the database (Table 4) had a Pearson's correlation coefficient (PCC) <0.55. The weakly correlated compounds, showed in Table 4, are cytotoxic through diverse mechanisms of action, including DNA alkylation (altretamine), induction of apoptosis (*N*-methylformamide), and alteration of cell cycle progression involving the MAPK pathway (tetrandine). All in all the COMPARE analysis for the representative compound **26** against the standard agent database showed poor or no correlation indicating that mechanism of action for the novel dicyanopyridines may differ from that of the standard antitumor drugs. Therefore, antitumoral activity of the novel dicyanopyridines may be caused by a new and unknown mechanism.

In conclusion, we have synthesized a series of 2-amino-4-aryl-6-dialkylamino-3,5-dicyanopyridines. Some of these had excellent growth inhibition activity against most of the cancer cell lines tested. Dicyanopyridine **26** was found to be more potent than dicyanopyridine **11** previously described. These findings have encouraged us to continue the development and testing of novel dicyanopyridine derivatives and to conduct further studies to investigate SAR and their mechanisms of action.

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-morpholino-propenenitrile **1** was obtained with a previously described procedure.[14]

3.1.1. 3-Amino-3-thiomorpholino-propenenitrile (2). According to our previously described procedure,[14] thiomorpholine (1.03 g, 10 mmol) was added to a solution of 3-amino-3-ethoxypropenenitrile (1.10 g, 10 mmol) in anhydrous acetonitrile (10 mL). The resulting solution was kept at room temperature for 24 h. The formed precipitate was filtered off and washed with diethyl ether. Yield 85%. Mp 125 °C. ^{1}H NMR ($CDCl_3$) δ 2,58, 3.53 (m, 8H, thiomorpholinyl), 3.14 (s, 1H, CH), 4.29 (s, 2H, NH_2). IR (Nujol) 3450, 3333, 3239, 2162, 1646, 1551 cm^{-1}. Anal. Calcd for $C_7H_{11}N_3S$: C, 49.68; H, 6.55; N, 24.83. Found: C, 49.72; H, 6.54; N, 24.80.

3.1.2. General procedure for the synthesis of 3,5-dicyanopyridines (20–47). The appropriate aldehyde (2.5 mmol) was added to a solution of 3-amino-3-morpholino-propenenitrile **1** or 3-amino-3-thiomorpholino-propenenitrile **2** (5 mmol) in anhydrous acetonitrile (10 mL). The resulting solution was refluxed for 30 min. After cooling, the formed precipitate was filtered off and washed with diethyl ether to give the target dicyanopyridines.

3.1.3. 2-Amino-4-(4-methoxyphenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (20). Yield 82%. Mp 199–200 °C. ^{1}H NMR (DMSO-d_6) δ 3.65 (m, 8H, morpholinyl), 3.77 (s, 3H, OCH_3), 7.02 (d, J = 8.8 Hz, 2H, aryl), 7.35 (s, 2H, NH_2), 7.40 (d, J = 8.8 Hz, 2H, aryl). IR (Nujol) 3400, 3315, 3205, 2209, 1647, 1609, 1579 cm^{-1}. Anal. Calcd for $C_{18}H_{17}N_5O_2$: C, 64.47; H, 5.11; N, 20.88. Found: C, 64.43; H, 5.13; N, 20.90.

3.1.4. 2-Amino-4-(2,5-dimethoxyphenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (21). Yield 87%. Mp 229-230 °C. ^{1}H NMR (DMSO-d_6) δ 3.62–4.44 (m, 14H, morpholinyl and OCH_3), 6.80, 7.02 (m, 3H, aryl), 7.35 (s, 2H, NH_2). IR (Nujol) 3427, 3332, 3227, 2201, 1640 cm^{-1}. Anal. Calcd for $C_{19}H_{19}N_5O_3$: C, 62.46; H, 5.24; N, 19.17. Found: C, 62.43; H, 5.23; N, 19.20.

3.1.5. 2-Amino-6-morpholin-4-yl-4-(3,4,5-trimethoxyphenyl)-pyridine-3,5-dicarbonitrile (22). Yield 82%. Mp 219–220 °C. ^{1}H NMR (DMSO-d_6) δ 3.56–3.74 (m, 17H, $OCH_3$and morpholinyl), 6.79 (s, 2H, aryl), 7.39 (s, 2H, NH_2). IR (Nujol) 3422, 3334, 3232, 2204, 1644, 1590 cm^{-1}. Anal. Calcd for $C_{20}H_{21}N_5O_4$: C, 60.75; H, 5.35; N, 17.71. Found: C, 60.83; H, 5.33; N, 17.69.

3.1.6. 2-Amino-4-(4-chlorophenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (23). Yield 78%. Mp 210–211 °C. ^{1}H NMR (DMSO-d_6) δ 3.64, 3.69 (m, 8H, morpholinyl), 7.49–7.57 (m, 6H, aryl and NH_2). IR (Nujol) 3397, 3313, 3202, 2210, 1649 cm^{-1}. Anal. Calcd for $C_{17}H_{14}ClN_5O$: C, 60.09; H, 4.15; N, 20.61. Found: C, 60.03; H, 4.16; N, 20.64.

3.1.7. 2-Amino-4-(2-chlorophenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (24). Yield 75%. Mp 174–175 °C.

^{1}H NMR (DMSO-d_6) δ 3.65, 3.71 (m, 8H, morpholinyl), 7.41–7.62 (m, 6H, aryl and NH_2). IR (Nujol) 3416, 3309, 3214, 2207, 1637 cm^{-1}. Anal. Calcd for $C_{17}H_{14}ClN_5O$: C, 60.09; H, 4.15; N, 20.61. Found: C, 60.13; H, 4.14; N, 20.57.

3.1.8. 2-Amino-4-(2,4-dichlorophenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (25). Yield 90%. Mp 199–200 °C. ^{1}H NMR (DMSO-d_6) δ 3.64, 3.73 (m, 8H, morpholinyl), 7.50, 7.59, 7.84 (m, 5H, aryl and NH_2). IR (Nujol) 3388, 3318, 3205, 2211, 1651 cm^{-1}. Anal. Calcd for $C_{17}H_{13}Cl_2N_5O$: C, 54.56; H, 3.52; N, 18.71. Found: C, 54.53; H, 3.54; N, 18.74.

3.1.9. 2-Amino-4-(2,6-dichlorophenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (26). Yield 93 %. Mp 174–175 °C. ^{1}H NMR (DMSO-d_6) δ 3.65–3.77 (m, 8H, morpholinyl), 7.53–7.68 (m, 5H, aryl and NH_2). IR (Nujol) 3335, 3226, 2209, 1624 cm^{-1}. Anal. Calcd for $C_{17}H_{13}Cl_2N_5O$: C, 54.56; H, 3.50; N, 18.71. Found: C, 54.60; H, 3.49; N, 18.68.

3.1.10. 2-Amino-4-(3-hydroxyphenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (27). Yield 88%. Mp 200–201 °C. ^{1}H NMR (DMSO-d_6) δ 3.66 (m, 8H, morpholinyl), 6.83, 7.27 (m, 4H, aryl), 7.43 (s, 2H, NH_2), 9.50 (s, 1H, OH). IR (Nujol) 3440, 3338, 3232, 2203, 1633 cm^{-1}. Anal. Calcd for $C_{17}H_{15}N_5O_2$: C, 63.54; H, 4.71; N, 21.79. Found: C, 63.49; H, 4.70; N, 21.83.

3.1.11. 2-Amino-4-(4-hydroxyphenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (28). Yield 78%. Mp 249–250 °C. ^{1}H NMR (DMSO-d_6) δ 3.65 (m, 8H, morpholinyl), 6.84 (d, J = 8.5 Hz, 2H, aryl), 7.30 (d, J = 8.5 Hz, 2H, aryl), 7.39 (s, 2H, NH_2), 9.94 (s, 1H, OH). IR (Nujol) 3481, 3355, 2204, 1615, 1595 cm^{-1}. Anal. Calcd for $C_{17}H_{15}N_5O_2$: C, 63.54; H, 4.71; N, 21.79. Found: C, 63.59; H, 4.69; N, 21.76.

3.1.12. 2-Amino-4-(4-hydroxy-3-methoxyphenyl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (29). Yield 87%. Mp 269–270 °C. ^{1}H NMR (DMSO-d_6) δ 3.66 (m, 8H, morpholinyl), 3.75 (s, 3H, OCH_3), 6.87 (m, 2H, aryl), 7.05 (s, 1H, aryl), 7.41 (s, 2H, NH_2), 9.52 (s, 1H, OH). IR (Nujol) 3509, 3454, 3300, 3190, 2205, 1639, 1599 cm^{-1}. Anal. Calcd for $C_{18}H_{17}N_5O_3$: C, 61.53; H, 4.88; N, 19.93. Found: C, 61.59; H, 4.89; N, 19.96.

3.1.13. 2-Amino-6-morpholin-4-yl-4-(2-pyridinyl)pyridine-3,5-dicarbonitrile (30). Yield 80%. Mp 160–161 °C. ^{1}H NMR (DMSO-d_6) δ 3.47, 3.53 (s, 8H, morpholinyl), 7.35, 7.47, 7.78, 8.52 (m, 6H, pyridyl and NH_2). IR (Nujol) 3319, 3214, 2210, 1634, 1578 cm^{-1}. Anal. Calcd for $C_{16}H_{14}N_6O$: C, 62.74; H, 4.61; N, 27.44. Found: C, 62.79; H, 4.59; N, 27.41.

3.1.14. 2-Amino-6-morpholin-4-yl-4-(3-pyridinyl)pyridine-3,5-dicarbonitrile (31). Yield 86%. Mp 239–240 °C. ^{1}H NMR (DMSO-d_6) δ 3.63, 3.70 (m, 8H, morpholinyl), 7.47, 8.71 (m, 4H, pyridyl), 7.52 (s, 2H, NH_2). IR (Nujol) 3402, 3305, 3192, 2205, 1643, 1579 cm^{-1}. Anal. Calcd for $C_{16}H_{14}N_6O$: C, 62.74; H, 4.61; N, 27.44. Found: C, 62.69; H, 4.60; N, 27.47.

3.1.15. 2-Amino-6-morpholin-4-yl-4-(4-pyridinyl)pyridine-3,5-dicarbonitrile (32). Yield 83%. Mp 279–280 °C. ^{1}H NMR (DMSO-d_6) δ 3.68 (m, 8H, morpholinyl), 7.52, 7.93, 8.38, 8.66 (m, 6H, pyridyl and NH_2). IR (Nujol) 3418, 3305, 2206, 1651, 1602 cm^{-1}. Anal. Calcd for $C_{16}H_{14}N_6O$: C, 62.74; H, 4.61; N, 27.44. Found: C, 62.70; H, 4.60; N, 27.48.

3.1.16. 2-Amino-6-morpholin-4-yl-4-thiophen-2-yl-pyridine-3,5-dicarbonitrile (33). Yield 84%. Mp 204–205 °C. ^{1}H NMR (DMSO-d_6) δ 3.62, 3.67 (m, 8H, morpholinyl), 7.19, 7.44, 7.82 (m, 5H, thienyl and NH_2). IR (Nujol) 3493, 3364, 2204, 1608 cm^{-1}. Anal. Calcd For $C_{15}H_{13}N_5OS$: C, 57.86; H, 4.21; N, 22.49. Found: C, 57.80; H, 4.20; N, 22.48.

3.1.17. 2-Amino-4-(5-methyl-furan-2-yl)-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (34). Yield 70%. Mp 199–200 °C. ^{1}H NMR (DMSO-d_6) δ 2.31 (s, 3H, CH_3), 3.62 (s, 8H, morpholinyl), 6.36 (d, J = 3.1 Hz, 1H, furyl), 7.13 (d, J = 3.1 Hz, 1H, furyl), 7.36 (s, 2H, NH_2). IR (Nujol) 3454, 3313, 3211, 2208, 1627 cm^{-1}. Anal. Calcd for $C_{16}H_{15}N_5O_2$: C, 62.13; H, 4.89; N, 22.64. Found: C, 62.08; H, 4.90; N, 22.68.

3.1.18. 2-Amino-4-furan-2-yl-6-morpholin-4-yl-pyridine-3,5-dicarbonitrile (35). Yield 83%. Mp 205–206 °C. ^{1}H NMR (DMSO-d_6) δ 3.65 (s, 8H, morpholinyl), 6.74, 7.22, 7.99 (m, 3H, furyl) 7.52 (s, 2H, NH_2). IR (Nujol) 3436, 3316, 3208, 2212, 1634, 1588 cm^{-1}. Anal. Calcd for $C_{15}H_{13}N_5O_2$: C, 61.01; H, 4.44; N, 23.72. Found: C, 61.08; H, 4.43; N, 23.68.

3.1.19. 2-Amino-4-(4-methoxyphenyl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (36). Yield 77%. Mp 214–215 °C. ^{1}H NMR (DMSO-d_6) δ 2.51, 3.76 (m, 8H, thiomorpholinyl), 3.61 (s, 3H, OCH_3), 6.86 (d, J = 6.4 Hz, 2H, aryl), 7.25 (d, J = 6.4 Hz, 2H, aryl), 7.29 (s, 2H, NH_2). IR (Nujol) 3513, 3399, 2199, 1604, 1580 cm^{-1}. Anal. Calcd for $C_{18}H_{17}N_5OS$: C, 61.52; H, 4.88; N, 19.93. Found: C, 61.58; H, 4.90; N, 19.89.

3.1.20. 2-Amino-4-(2,5-dimethoxyphenyl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (37). Yield 78%. Mp 220 °C (dec). ^{1}H NMR (DMSO-d_6) δ 2.68, 3.93 (m, 8H, thiomorpholinyl), 3.69 (s, 6H, OCH_3), 6.83 (s, 1H, aryl), 7.05 (m, 2H, aryl), 7.38 (s, 2H, NH_2). IR (Nujol) 3430, 3338, 3235, 2205, 1640 cm^{-1}. Anal. Calcd for $C_{19}H_{19}N_5O_2S$: C, 59.82; H, 5.02; N, 18.36. Found: C, 59.77; H, 5.00; N, 18.40.

3.1.21. 2-Amino-6-thiomorpholin-4-yl-4-(3,4,5-trimethoxyphenyl)-pyridine-3,5-dicarbonitrile (38). Yield 80%. Mp 220 °C. ^{1}H NMR (DMSO-d_6) δ 2.70, 3.94 (m, 4H, thiomorpholinyl), 3.71, 3.77 (s, 9H, OCH_3), 6.81 (s, 2H, aryl), 7.43 (s, 2H, NH_2). IR (Nujol) 3455, 3340, 3232, 2205, 1639 cm^{-1}. Anal. Calcd for $C_{20}H_{21}N_5O_3S$: C, 58.38; H, 5.14; N, 17.02. Found: C, 58.44; H, 5.15; N, 16.99.

3.1.22. 2-Amino-4-(4-chlorophenyl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (39). Yield 78%. Mp 240 °C. ^{1}H NMR (DMSO-d_6) δ 2.68, 3.95 (m, 8H, thiomorphol-

inyl), 7.48–7.59 (m, 6H, aryl and NH_2). IR (Nujol) 3486, 3442, 3344, 3223, 2204, 1628 cm^{-1}. Anal. Calcd for $C_{17}H_{14}ClN_5S$: C, 57.38; H, 3.97; N, 19.68. Found: C, 57.44; H, 3.99; N, 19.71.

3.1.23. 2-Amino-4-(2,4-dichlorophenyl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (40). Yield 77%. Mp 230 °C. ^1H NMR (DMSO-d_6) δ 2.73, 4.02 (s, 8H, thiomorpholinyl), 7.48, 7.60, 7.85 (m, 5H, aryl and NH_2). IR (Nujol) 3462, 3430, 2170, 1582 cm^{-1}. Anal. Calcd for $C_{17}H_{13}Cl_2N_5S$: C, 52.32; H, 3.36; N, 17.94. Found: C, 52.27; H, 3.35; N, 17.91.

3.1.24. 2-Amino-4-(2,6-dichlorophenyl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (41). Yield 73%. Mp 280 °C. ^1H NMR (DMSO-d_6) δ 2.73, 4.02 (s, 8H, thiomorpholinyl), 7.52–7.68 (m, 5H, aryl and NH_2). IR (Nujol) 3081, 2212, 1556 cm^{-1}. Anal. Calcd for $C_{17}H_{13}Cl_2N_5S$: C, 52.32; H, 3.36; N, 17.94. Found: C, 52.37; H, 3.37; N, 17.97.

3.1.25. 2-Amino-4-(4-hydroxyphenyl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (42). Yield 81%. Mp 225 °C. ^1H NMR (DMSO-d_6) δ 2.68, 3.92 (m, 8H, thiomorpholinyl), 6.83 (d, J = 8.5 Hz, 2H, aryl), 7.30 (d, J = 8.5 Hz, 2H, aryl), 7.39 (s, 2H, NH_2), 9.94 (s, 1H, OH). IR (Nujol) 3475, 3334, 2200, 1624, 1570 cm^{-1}. Anal. Calcd for $C_{17}H_{15}N_5OS$: C, 60.52; H, 4.48; N, 20.76. Found: C, 60.57; H, 4.47; N, 20.74.

3.1.26. 2-Amino-6-thiomorpholin-4-yl-4-(2-pyridinyl)pyridine-3,5-dicarbonitrile (43). Yield 78%. Mp 220 °C. ^1H NMR (DMSO-d_6) δ 2.69, 4.09 (m, 4H, thiomorpholinyl), 7.51, 7.65, 7.96, 8.70 (m, 6H, pyridyl and NH_2). IR (Nujol) 3331, 2206, 1628 cm^{-1}. Anal. Calcd for $C_{16}H_{14}N_6S$: C, 59.61; H, 4.38; N, 26.07. Found: C, 59.57; H, 4.37; N, 26.10.

3.1.27. 2-Amino-6-thiomorpholin-4-yl-4-(3-pyridinyl)pyridine-3,5-dicarbonitrile (44). Yield 77%. Mp 245 °C. ^1H NMR (DMSO-d_6) δ 2.69, 3.97 (m, 8H, thiomorpholinyl), 7.55, 7.94, 8.67 (m, 6H, pyridyl and NH_2). IR (Nujol) 3397, 3193, 2200, 1641, 1579 cm^{-1}. Anal. Calcd for $C_{16}H_{14}N_6S$: C, 59.61; H, 4.38; N, 26.07. Found: C, 59.66; H, 4.39; N, 26.04.

3.1.28. 2-Amino-6-thiomorpholin-4-yl-4-(4-pyridinyl)pyridine-3,5-dicarbonitrile (45). Yield 77%. Mp 290 °C. ^1H NMR (DMSO-d_6) δ 2.69, 3.97 (m, 8H, thiomorpholinyl), 7.48 (d, J = 6.1 Hz, 2H, pyridyl), 7.57 (s, 2H, NH_2), 8.72 (d, J = 6.1 Hz, 2H, pyridyl). IR (Nujol) 3390, 2204, 1663 cm^{-1}. Anal. Calcd for $C_{16}H_{14}N_6S$: C, 59.61; H, 4.38; N, 26.07. Found: C, 59.65; H, 4.37; N, 26.03.

3.1.29. 2-Amino-6-thiomorpholin-4-yl-4-thiophen-2-yl-pyridine-3,5-dicarbonitrile (46). Yield 71%. Mp 210 °C. ^1H NMR (DMSO-d_6) δ 2.70, 3.93 (m, 8H, thiomorpholinyl), 7.20, 7.46, 7.84 (m, 5H, thienyl and NH_2). IR (Nujol) 3433, 3327, 3218, 2205, 1629 cm^{-1}. Anal. Calcd for $C_{15}H_{13}N_5S_2$: C, 55.02; H, 4.00; N, 21.39. Found: C, 55.07; H, 3.99; N, 21.43.

3.1.30. 2-Amino-4-(5-methylfuran-2-yl)-6-thiomorpholin-4-yl-pyridine-3,5-dicarbonitrile (47). Yield 70%. Mp 180 °C. ^1H NMR (DMSO-d_6) δ 2.29 (s, 3H, CH_3), 2.70, 4.01 (m, 8H, thiomorpholinyl), 6.35 (d, J = 3.1 Hz, 1H, furyl), 7.12 (d, J = 3.1 Hz, 1H, furyl), 7.38 (s, 2H, NH_2). IR (Nujol) 3448, 3332, 3220, 2205, 1633 cm^{-1}. Anal. Calcd for $C_{16}H_{15}N_5OS$: C, 59.06; H, 4.65; N, 21.52. Found: C, 59.01; H, 4.66; N, 21.53.

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% 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.[20,21] Density of inoculum depends on the type of tumor cell and on its growth characteristics.[17] These cells are then preincubated on the microtiter 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 were evaluated and the assay results and dose–response parameters were calculated as previously described.[22]

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