Bioorganic & Medicinal Chemistry 17 (2009) 6158–6165

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and evaluation of anticancer activity of 2-arylamino-6-trifluoromethyl-3-(hydrazonocarbonyl)pyridines

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ARTICLE INFO

Article history:
Received 17 June 2009
Revised 24 July 2009
Accepted 26 July 2009
Available online 30 July 2009

Keywords:
Hydrazones
Anticancer activity
SAR

ABSTRACT

The synthesis and anticancer activity of 2-arylamino-6-trifluoromethyl-3-(hydrazonocarbonyl)pyridines is described. The new trifluoromethylpyridine derivatives were evaluated for their anticancer activity toward human cancer cell lines by the National Cancer Institute (NCI). Most of them had excellent growth inhibition activity, having GI_{50} values in the low micromolar to nanomolar concentration range. The most potent 2,6-dichlorobenzaldehydehydrazone **29** inhibited the growth of all tested cancer cell lines with nanomolar potency, and did not show animal toxicity. Hydrazone **29** has been selected by the Biological Evaluation Committee of NCI for testing in vivo Hollow Fiber Assay.

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1. Introduction

In previous studies, our research group has reported the interesting anticancer activity of a new class of 2-arylamino-6-trifluoromethylnicotinamides. Most compounds of the series were found to have GI_{50} values in the low micromolar or submicromolar concentration range against human cancer cell lines and reaching, in the case of most active derivative **AM-44** (Fig. 1), averaged activity value on all tested cell lines of 2.88×10^{-6} M.[1]

On the other hand several studies have been devoted to the antiproliferative activity of aroylhydrazone derivatives. Thus benzoxazolylhydrazones derived from alpha-(*N*)-acylheteroaromatics are endowed with anticancer activity in vitro against colon, ovarian, and renal cancer cells.[2] While salicylaldehyde hydrazones showed inhibitory effects on the growth of A549 lung cancer cells.[3] A number of hydrazone have been reported to induce apoptosis. For example aroyl hydrazones of 2-phenylindole-3-carbaldehydes were capable to cause cell cycle arrest accompanied by apoptosis in MDA-MB 231 and MCF-7 breast cancer cells.[4] Furthermore the aroylhydrazone PAC-1 directly activates procaspase-3 to caspase-3 in vitro and induces apoptosis in cancerous cells isolated from primary colon tumors in a manner directly proportional to the concentration of procaspase-3 inside these cells.[5] A variety of hydrazones demonstrated their antiproliferative activity through inhibition of kinases. As a result 4-amino-6-arylamino-pyrimidine-5-carbaldehyde hydrazones were identified as potent dual ErbB-2/EGFR kinase inhibitors, resulting in growth inhibition of ErbB-2 over-expressing human cancer cell lines.[6] At the same time as thieno[2,3-*d*]pyrimidin-4-yl hydrazones have been identified as cyclin-dependent kinase 4 (CDK4) inhibitors. In addition, these compounds have antiproliferative activities and act as cytotoxic agents with the ability to prevent cell progression.[7] Furthermore phenylhydrazonopyrazolone sulfonate is as a potent and cell permeable inhibitor of the protein tyrosine phosphatase Shp2 a positive regulator of growth factor signaling. Activating mutations of SHP2 have been identified in about 30% of the most common pediatric leukemia, juvenile myelomonocytic leukemia (JMML), and in myelodysplastic syndrome, acute myeloid leukemia, and some solid tumors.[8] Sulfonylhydrazone substituted imidazo[1,2-*a*]pyridines are potent inhibitors of PI3K p110α enzymatic activity and of tumor cell growth in vitro and in vivo.[9] Hydrazone derivatives, such us EPH136, have been shown to exhibit anticancer activity in vitro and in vivo, through generation of radicals and dissipation of the mitochondrial membrane potential.[10] Related molecular mechanisms responsible of anticancer activity has been reported for guanylhydrazone derivatives. These were found to be inhibitors of

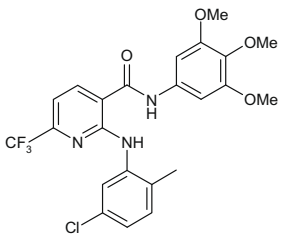

Figure 1. 2-(5-Chloro-2-methylphenylamino)-6-(trifluoromethyl)-*N*-(3,4,5-(trimethoxy)phenyl)nicotinamide (**AM-44**).

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doi:10.1016/j.bmc.2009.07.066

Complex III of the mitochondrial respiratory chain and were able to induce apoptosis in the cell lines HT29 and HL60,[11] in addition they were able to cause oxidative stress and to interfere with cellular energetics.[12] Further guanylhydrazones have been reported to be coactivator binding inhibitors (CBIs), which block estrogen receptor transcription through a different mechanism than traditional antagonists tamoxifen and related drugs. These molecules have been proposed as potential alternative therapy in breast cancer resistant to tamoxifen.[13]

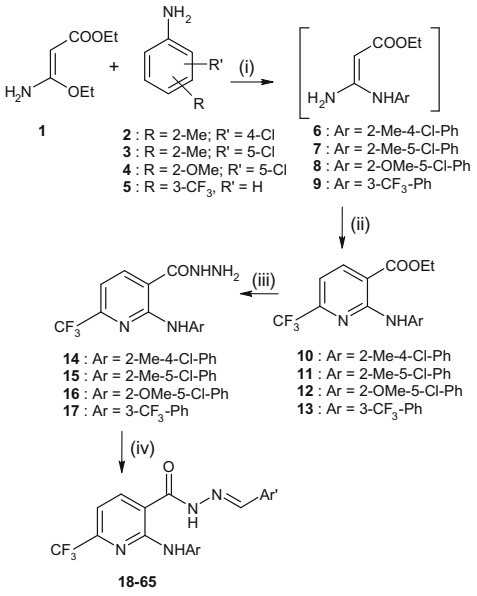
In this context we decided to investigate if the presence of an hydrazono moiety on the 2-arylamino-6-trifluoromethylpyridine skeleton can also give rise to classes of biologically active compounds. Therefore, in the present work we proposed the synthesis and evaluation for in vitro anticancer efficacy against human cancer cell lines of a series of novel 6-trifluomethylpyridines bearing the hydrazone moiety at C-3, expecting that the incorporation of these substituents may improve the anticancer activities of 2-arylamino-6-trifluoromethylpyridines.

2. Results and discussion

2.1. Chemistry

The target nicotinohydrazones **18–65** (Table 1) were synthesized as shown in Scheme 1. We have previously reported an easy and convenient method for the synthesis of alkyl esters of 2-arylamino-6-trifluoromethyl-3-pyridinecarboxylic acids.[1] As reported in Scheme 1, ethyl 3-amino-3-ethoxypropenoate **1** was sequentially treated with the appropriate substituted arylamine **2–5**, then with 1,1,1-trifluoro-4-isobutoxy-3-buten-2-one in MeCN solution to give pyridine derivatives **10–13**. High yields of 3-hydrazinocarbonylpyridines **14–17** were achieved upon refluxing for 3 h an ethanolic solution of esters **10–13** and hydrazine hydrate.

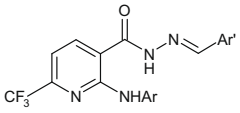
Hydrazones **18–65** were obtained in good to excellent yield by coupling 3-hydrazinocarbonylpyridines **14–17** with the appropriate aldehydes in ethanol. All the newly synthesized compounds gave corrected analytical data. The IR and NMR spectral data are consistent with the assigned structure. According to literature,[14] the presence of single downfield resonating (8.43–8.99 ppm) *CH*=N signal accounts for formation of *E*-isomers exclusively.



Scheme 1. Reagents and conditions: (i) MeCN, rt; (ii) 1,1,1-trifluoro-4-*iso*-butoxy-3-buten-2-one, reflux; (iii) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux; (iv) Ar′CHO, EtOH, reflux.

The antiproliferative properties of this first series of hydrazones **18–65** were then evaluated (see Pharmacological results, Tables 2 and 3). According to our best compounds for a biological point of view, we decided to realize novel modulations on their structure through introduction of seven atom linker between 2,6-dichloro-

Table 1
2-Arylamino-6-trifluomethylpyridin-hydrazone derivatives **18–65**



Compd	Ar	Ar′	Compd	Ar	Ar′
18	2-Me-4-Cl-phenyl	4-NO_2-phenyl	**42**	2-OMe-5-Cl-phenyl	4-NO_2-phenyl
19	2-Me-4-Cl-phenyl	4-$N(Me)_2$-phenyl	**43**	2-OMe-5-Cl-phenyl	4-$N(Me)_2$-phenyl
20	2-Me-4-Cl-phenyl	4-F-phenyl	**44**	2-OMe-5-Cl-phenyl	4-F-phenyl
21	2-Me-4-Cl-phenyl	3-Pyridyl	**45**	2-OMe-5-Cl-phenyl	3-Pyridyl
22	2-Me-4-Cl-phenyl	4-Pyridyl	**46**	2-OMe-5-Cl-phenyl	4-Pyridyl
23	2-Me-4-Cl-phenyl	4-OH-3-OMe-phenyl	**47**	2-OMe-5-Cl-phenyl	4-OH-3-OMe-phenyl
24	2-Me-4-Cl-phenyl	3-OH-phenyl	**48**	2-OMe-5-Cl-phenyl	3-OH-phenyl
25	2-Me-4-Cl-phenyl	3,4,5-$(OMe)_3$-phenyl	**49**	2-OMe-5-Cl-phenyl	3,4,5-$(OMe)_3$-phenyl
26	2-Me-4-Cl-phenyl	2-Cl-phenyl	**50**	2-OMe-5-Cl-phenyl	2-Cl-phenyl
27	2-Me-4-Cl-phenyl	4-Cl-phenyl	**51**	2-OMe-5-Cl-phenyl	4-Cl-phenyl
28	2-Me-4-Cl-phenyl	2,4-Cl_2-phenyl	**52**	2-OMe-5-Cl-phenyl	2,4-Cl_2-phenyl
29	2-Me-4-Cl-phenyl	2,6-Cl_2-phenyl	**53**	2-OMe-5-Cl-phenyl	2,6-Cl_2-phenyl
30	2-Me-5-Cl-phenyl	4-NO_2-phenyl	**54**	3-CF_3-phenyl	4-NO_2-phenyl
31	2-Me-5-Cl-phenyl	4-$N(Me)_2$-phenyl	**55**	3-CF_3-phenyl	4-$N(Me)_2$-phenyl
32	2-Me-5-Cl-phenyl	4-F-phenyl	**56**	3-CF_3-phenyl	4-F-phenyl
33	2-Me-5-Cl-phenyl	3-Pyridyl	**57**	3-CF_3-phenyl	3-Pyridyl
34	2-Me-5-Cl-phenyl	4-Pyridyl	**58**	3-CF_3-phenyl	4-Pyridyl
35	2-Me-5-Cl-phenyl	4-OH-3-OMe-phenyl	**59**	3-CF_3-phenyl	4-OH-3-OMe-phenyl
36	2-Me-5-Cl-phenyl	3-OH-phenyl	**60**	3-CF_3-phenyl	3-OH-phenyl
37	2-Me-5-Cl-phenyl	3,4,5-$(OMe)_3$-phenyl	**61**	3-CF_3-phenyl	3,4,5-$(OMe)_3$-phenyl
38	2-Me-5-Cl-phenyl	2-Cl-phenyl	**62**	3-CF_3-phenyl	2-Cl-phenyl
39	2-Me-5-Cl-phenyl	4-Cl-phenyl	**63**	3-CF_3-phenyl	4-Cl-phenyl
40	2-Me-5-Cl-phenyl	2,4-Cl_2-phenyl	**64**	3-CF_3-phenyl	2,4-Cl_2-phenyl
41	2-Me-5-Cl-phenyl	2,6-Cl_2-phenyl	**65**	3-CF_3-phenyl	2,6-Cl_2-phenyl

Table 2
Overview of the results[a] of the anticancer screening for compounds **18–20, 23, 24, 26, 27, 29 33, 39, 41, 42, 45, 49–51, 53, 56, 58, 63, 65, 75,** Chlorambucil (NSC 3088) and **AM-44**[b]

Compd	No. of the cell lines investigated	Number of the cell lines giving positive log GI_{50}, log TGI and log LC_{50}[b]								
		Log of GI_{50} (M)			Log of TGI (M)			Log of LC_{50} (M)		
		No.	range	MG_MID[c]	No.	range	MG_MID[c]	No.	range	MG_MID[c]
18	58	58	−5.37 to −7.37	−5.99	54	−4.42 to −7.11	−5.33	19	−4.00 to −5.52	−4.32
19	56	56	−5.23 to −6.39	−5.77	8	−4.35 to −5.33	−4.18			
20	59	59	−5.46 to −6.31	−5.80	59	−4.72 to −5.65	−5.15	44	−4.03 to −5.21	−4.34
23	58	58	−5.29 to −6.38	−5.67	58	−4.67 to −5.51	−4.98	51	−4.05 to −5.12	−4.37
24	59	59	−5.20 to −7.77	−5.70	59	−4.74 to −5.81	−4.96	58	−4.31 to −4.98	−4.46
26	60	60	−5.73 to −8.00	−6.13	56	−6.11 to −4.75	−5.29	25	−5.35 to −4.03	−4.34
27	60	60	−5.44 to −6.42	−5.74	59	−4.55 to −5.47	−5.12	45	−4.18 to −5.22	−4.38
29	60	60	−5.87 to −7.90	−6.51	32	−4.82 to −6.53	−4.93	5	−4.86 to −5.17	−4.09
33	58	58	−5.27 to −7.01	−5.63	56	−4.54 to −5.82	−4.90	52	−4.02 to −5.14	−4.37
39	57	57	−5.46 to −6.59	−5.83	57	−4.92 to −5.97	−5.28	52	−4.02 to −5.31	
41	59	59	−7.25 to −5.62	−6.18	50	−6.31 to −4.32	−5.21	15	−5.52 to −4.18	−4.18
42	56	56	−4.51 to −6.01	−5.70	53	−4.20 to −5.49	−5.06	8	−4.06 to −5.11	−4.08
45	59	59	−5.11 to −6.28	−5.53	10	−4.12 to −5.33	−4.10			
49	58	57	−5.29 to − 6.19	−5.70	51	−4.39 to −5.64	−4.98	28	−4.07 to −5.17	−4.30
50	59	59	−5.05 to −7.56	−5.73	47	−4.16 to −6.26	−4.86	9	−4.14 to −5.33	−4.10
51	59	57	−4.13 to −6.97	−5.40	27	−4.16 to −6.03	−4.30	9	−4.02 to −5.17	
53	58	52	−4.41 to −5.98	−5.09	12	−4.14 to − 5.36	−4.13			
56	55	55	−5.58 to −6.49	−5.98	55	−4.92 to −5.82	−5.35	54	−4.19 to −5.29	−4.70
58	56	56	−5.36 to −6.57	−5.69	56	−4.77 to −5.76	−5.06	55	−4.25 to −5.12	−4.49
63	53	53	−5.30 to −7.36	−5.83	52	−4.79 to −6.23	−5.18	49	−4.11 to −5.21	−4.51
65	55	55	−5.48 to −7.35	−6.07	44	−4.03 to −6.38	−4.99	9	−4.11 to −5.14	−4.10
75	59	59	−6.49 to −4.95	−5.56	41	−5.31 to −4.14	−4.40	3	−4.30 to −4.07	−4.01
AM-44	59	59	−4.97 to −8.00	−5.54	59	−4.48 to −7.23	−4.78	52	−4.04 to −5.14	−4.27
NSC 3088	60	60	−4.50 to − 6.10	−5.05	59	−4.10 to −5.10	−4.45	27	−4.01 to − 4.04	−4.02

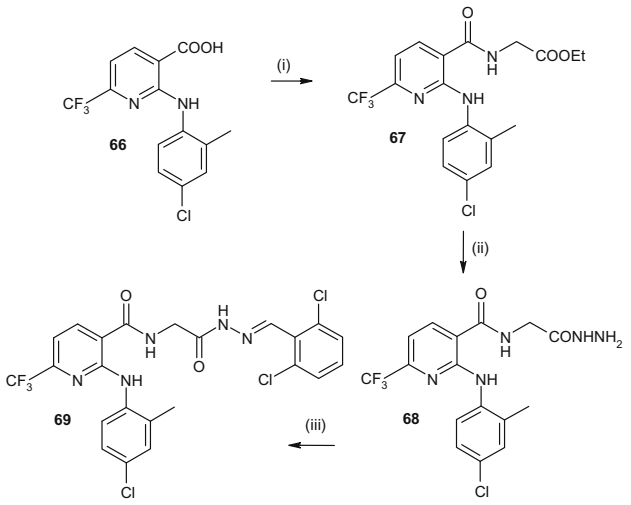
[a] Data obtained from the NCI's in vitro disease-oriented human tumor cells screen (see Refs. 15–17 for details).
[b] The response parameters: log GI_{50}, log TGI and log LC_{50} are interpolated values representing the molar concentrations at which percentage growth is +50, 0 and −50, respectively.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

Table 3
The in vitro activity[a] and selectivity toward most sensitive tumor cell lines for compounds **18, 24, 26, 29, 33, 41, 50, 51, 63,** and **65**

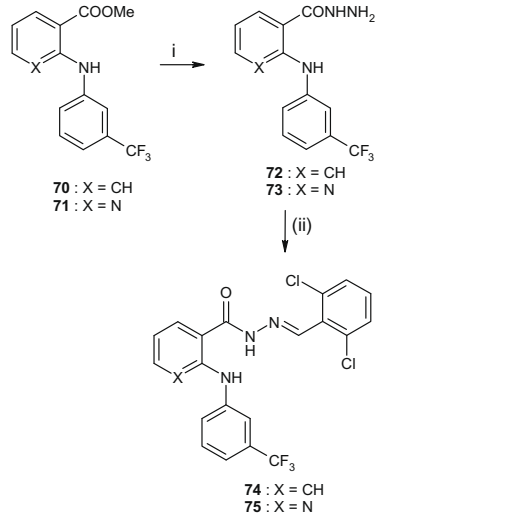
Compd	Most sensitive tumor cell lines	Log GI_{50} (M)	Log TGI (M)	Log LC_{50} (M)	Selectivity toward tumor cell lines (δ) for log GI_{50}/TGI/LC_{50} (M). The value is shown if δ >1[b,c]	GI_{50} MG_MID	TGI MG_MID	LC_{50} MG_MID
18	Leukemia: CCRF-CEM	−7.37	−7.11		GI_{50} 1.38/ TGI 1.12	−5.99	−5.33	
24	Renal: CAKI-1	−7.77	−5.48	−4.62	GI_{50} 2.07	−5.70		
	Renal: RXF 393	−6.71	−4.95		GI_{50} 1.01			
	Breast: T-47D	−7.45	−5.67	−4.67	GI_{50} 1.75			
26	Ovarian: OVCAR-3	−6.72	−6.29	−5.08	TGI 1.0	−6.13	−5.29	
	Renal: A498	<8.00	−5.57	−4.75	GI_{50} 1.87			
29	Non small cell lung: EKVX	−6.71	−6.15		TGI 1.22	−6.51	−4.93	
	Non small cell lung: HOP-92	−6.72	−6.27		TGI 1.34			
	Non small cell lung: NCI-H226	−6.87	−6.28		TGI 1.35			
	CNS: SF-295	−7.62	−6.53		GI_{50} 1.11/ TGI 1.60			
	Renal: CAKI-1	−7.90	−6.49	−4.36	GI_{50} 1.39/ TGI 1.56			
	Renal: TK-10	−6.65	−6.10		TGI 1.17			
	Prostate: PC-3	−6.64	−6.06		TGI 1.13			
	Breast: MCF7	−6.63	−6.13		TGI 1.20			
	Breast: MDA-MB-231/ATCC	−6.81	−6.07		TGI 1.14			
	Breast: BT-549	−6.71	−6.17		TGI 1.24			
	Breast: T-47D	−6.65	−6.11		TGI 1.18			
	Breast: MDA-MB-468	−6.56	−6.07		TGI 1.14			
33	Leukemia: SR	−7.01			GI_{50} 1.38	−5.63		
41	Leukemia: SR	−7.25	−5.57	−4.38	GI_{50} 1.07	−6.18	−5.21	−4.18
	Non small cell lung: HOP-62	−6.60	−6.11	−5.24	LC_{50} 1.06			
	Colon: HCT-116	−6.67	−6.31	−5.48	TGI 1.10 / LC_{50} 1.30			
	Renal: 786-0	−6.60	−6.22	−5.52	TGI 1.01 / LC_{50} 1.34			
50	Leukemia: SR	−7.56	−6.26	−5.33	GI_{50} 1.83	−5.73		
51	Breast: BT-549	−6.97	−6.03		GI_{50} 1.57/ TGI 1.73	−5.40		
63	Non small cell lung: HOP-92	−7.36	−6.23	−4.68	GI_{50} 1.53/ TGI 1.05	−5.83		
65	Non small cell lung: HOP-92	−7.35	−6.38		GI_{50} 1.28/ TGI 1.39	−6.07	−4.99	
	Ovarian: OVCAR-3	−6.72	−6.19	−4.11	TGI 1.20			
	Renal: A498	−6.72	−6.19	−5.03	TGI 1.20			

[a] Data obtained from the NCI's in vitro disease-oriented human tumor cells screen (see Refs. 15–17 for details).
[b] 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.
[c] The value is shown if δ >1.

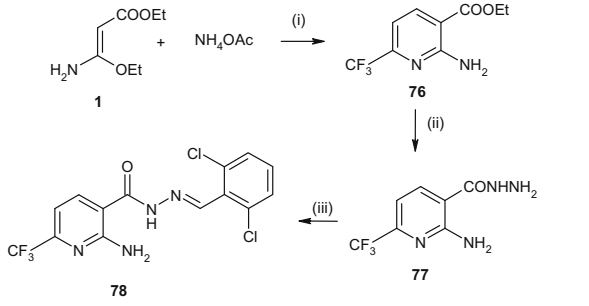
phenyl and trifluoromethylpyridine rings (Scheme 2), by removal of the 6-trifluoromethyl group or replacement of pyridine ring with a phenyl (Scheme 3), or by replacement of 2-arylamino group with a primary amino group (Scheme 4).



Scheme 2. Reagents and conditions: (i) $EtOOCCH_2NH_2{\cdot}HCl$, EDCI, HOBt, TEA, MeCN, rt; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux; (iii) 2,6-dichlorobenzaldehyde, EtOH, reflux.



Scheme 3. Reagents and conditions: (i) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux; (ii) 2,6-dichlorobenzaldehyde, EtOH, reflux.



Scheme 4. Reagents and conditions: (i) 1,1,1-trifluoro-4-*iso*-butoxy-3-buten-2-one, EtOH, reflux; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux; (iii) 2,6-dichlorobenzaldehyde, EtOH, reflux.

Hydrazone **69** was prepared as reported in Scheme 2. Treatment of acid **66** with ethyl glycinate hydrochloride in the presence of 1-(3-dimethylaminopropyl)-3-ethylcardodiimide hydrochloride (EDCI) and hydroxybenzotriazole (HOBt) in MeCN solution gave ester **67**. Following the reaction sequence described for hydrazones 18–**65**, high yields of **69** were obtained.

As shown in Scheme 3, the preparation of hydrazones **74** and **75** was accomplished starting from methyl esters of flufenamic and niflumic acids, using a procedure identical to that described for the preceding hydrazones.

The synthesis of hydrazone **78** was accomplished by refluxing compound **1** with 1,1,1-trifluoro-4-isobutoxy-3-buten-2-one in presence of ammonium acetate, in ethanol, followed by reaction of ester **76** with hydrazine hydrate, in ethanol, under reflux (Scheme 4).

Reaction of **77** with 2,6-dichlorobenzaldehyde produces the target hydrazone **78**.

2.2. Pharmacology

The synthesized hydrazones 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. First the compounds have been evaluated in primary anticancer assay at 10^{-5} M concentration. The compounds showing antiproliferative activity in the primary assay (Table S1, Supplementary data), were then 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 anticancer 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.

From the analysis of the data reported in Tables 2 and 3 we can evince that chlorine atoms on particular positions of benzylidene moiety appears to favorably modulate antiproliferative activity. Thus 2,6-dichlorobenzaldehydehydrazones **29**, **41**, and **65** inhibited the growth of all tested cell lines with micro and submicromolar GI_{50} values.

Hydrazone **29** showed the best activity, as a matter of fact it inhibited the growth of all tested cell lines at nanomolar to micromolar concentrations with MG_MID value −6.51. Compound **29** displayed cytostatic activity at nanomolar concentrations against SF-295 and CAKI-1 as well as high selectivity on the same cell lines (log GI_{50} −7.62 and −7.90). In addition micromolar concentration of **29** totally inhibited the growth of the above cell lines as well as of further 7 cell lines of renal, prostate and breast panels (Δ >1). Although less potent when compared to **29**, hydrazones **41** and **65** (GI_{50} MG_MID −6.18 and −6.07) displayed selectivity on particular cell lines. Compound **41** showed a log GI_{50} value −7.25 against SR leukemia cell line. The same compound exhibited good selectivity at TGI and LC_{50} levels against HCT-116 and 786-0 cell lines. Hydrazone **65** showed selectivity against HOP-92 at GI_{50} and TGI levels (log GI_{50} −7.35, TGI −6.19; Δ 1.28 and 1.39, respectively) and against OVCAR-3 and A498 at TGI level (log TGI −6.19;

Δ 1.20). The shift of one chlorine atom from the 6-position to the 4-position on phenyl ring leads to the inactive compounds **28**, **40**, **52**, and **64**. Substitution of the 2,6-dichlorobenzylidene moiety by a 2-chlorobenzylidene resulted in reduction (compound **26**, GI_{50} MG_MID value −6.13) or loss of activity (compound **62**). However a 2-chlorobenzylidene (compound **50**, GI_{50} MG_MID −5.73) caused an increase in activity respect to the 2,6-dichloro analog **53** (GI_{50} MG_MID −5.09). Compound **50** showed high selectivity against leukemia SR cell line (log GI_{50} −7.56, Δ 1.83).

A comparison of substituent effects revealed that the introduction of 4-chlorobenzylidene led to compounds **27**, **39** and **63** endowed with reduced antiproliferative effect (GI_{50} MG_MD −5.74, −5.83 and −5.83, respectively) as compared with 2,6-dichlorobenzylidene analogs **29**, **41** and **65**. Hydrazone **63** displayed good selectivity against HOP-92 non small cell lung line (log GI_{50} value −7.36, log TGI value −6.23, Δ 1.53 and 1.05, respectively). As general trend, hydrazone derived from 4-nitro-, 4-dimethylamino- 3-hydroxy-, 3,4,5-trimethoxy- and 4-hydroxy-3-methoxybenzaldehydes showed reduced antiproliferative activity respect to that derived from 2,6-dichlorobenzaldehyde. As example, hydrazones of the 2-(4-chloro-2-methylphenylamino) series show antiproliferative activity decreasing approximately in the order: **29** (2,6-dichloro) ≫ **18** (4-nitro) > **19** (4-dimethylamino) > **24** (3-hydroxy) > **23** (4-hydroxy-3-methoxy) ≫ **25** (3,4,5-trimethoxy). Although hydrazone **24** showed −5.70 GI_{50} MG_MID value it was high selective against renal CAKI-1 cell line (log GI_{50} −7.77; Δ 2.07). Whereas the presence of 4-fluorobenzylidene versus 2,6-dichlorobenzylidene produces variable effects: in compound **20** led to clear reduction in inhibitory activity (GI_{50} MG_MID −5.80 against −6.51 of **29**), while in compound **56** resulted in marginal reduction of activity (GI_{50} MG_MID −5.98 against −6.07 of **65**). Taking in account the 2-(5-chloro-2-methoxyphenylamino) series, influence of benzylidene substituents on antiproliferative activity is extremely different with respect to hydrazones of the other three series. As matter of fact the antiproliferative activity decreased approximately in the order: **50** (2-chloro, GI_{50} MG_MID −5.73) > **42** and **41** (4-nitro and 3,4,5-trimethoxy, GI_{50} MG_MID −5.70) > **51** (4-chloro, GI_{50} MG_MID −5.40) > **53** (2,6-dichloro, GI_{50} MG_MID −5.09).

Next, the influence on antiproliferative activity of connecting patterns between 2,6-dichlorophenyl and trifluoromethylpyridine rings was evaluated. Introduction of an extra two atom linker between these rings led to drop in activity, as illustrated by the homologous pair **29**/**69**. As matter of fact compound **69** did not show antiproliferative activity in the primary anticancer assay.

The importance of the 6-trifluoromethyl group on pyridine ring was explored by comparing the antiproliferative activity of hydrazones **65** and **75**. The presence of the 6-trifluoromethyl substituent served to enhance activity by a factor of about 3 (compound **75** GI_{50} MG_MID −5.56 vs −6.07 of **65**).

The replacement of pyridine ring of **75** with a phenyl to afford hydrazone **74** caused a further reduction of activity (compound **74**, G% 90.62). The importance of the 2-arylamino group on the trifluoromethylpyridine ring is underlined by the dramatic decrease in activity upon replacement of this group by a primary amino group (compound **78**, G% 101.6).

Animal toxicity experiments were performed on the most active hydrazone **29**. To determine maximum tolerated dose (MTD) this compound was intraperitoneal (ip) administrated to athymic nude mice at the doses of 100, 200 and 400 mg/kg. The highest dose resulted in no body weight loss or lethality, indicating that **29** was well tolerated.

A COMPARE[18] analysis was performed with the most active compound **29** 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 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.[18] Using GI_{50} values of hydrazone **29** (NSC744613) 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, inhibition of cytidine triphoshate synthase (cyclopentenylcytosine) and dihydrofolate reductase (trimetrexate). All in all the COMPARE analysis for the representative compound **29** against the standard agent database showed poor or no correlation indicating that mechanism of action for the novel hydrazones may differ from that of the standard antitumor drugs. Therefore, anticancer activity of the novel hydrazones may be caused by a new and unknown mechanism. Compound **29** that showed reproducible activity in the in vitro anticancer screening as well as no in vivo toxicity, and because it lies outside the category of adequately studied classes of antitumor agents was one of the small percentage which has been selected by the Biological Evaluation Committee of NCI for testing in vivo Hollow Fiber Assay.[19,20]

Table 4
COMPARE correlation coefficients (PCC) using inhibitory values of compound **29** as seeds, tested in the US NCI 60 Cell lines in vitro screen

Standard agent	Endpoint level	PCC	No. of common cell lines
Spiromustine	GI_{50}	0.415	53
Cyanomorpholino-ADR	GI_{50}	0.402	43
Piperazinedione	TGI	0.431	48
Cyclopentenylcytosine	TGI	0.373	51
Penclomedine	LC_{50}	0,507	53
Timetrexate	LC_{50}	0.498	51
O^6-Methylguanine	LC_{50}	0.484	51

In conclusion, we have synthesized a series of *N*′-(arylidene)-2-(arylamino)-6-(trifluoromethyl) nicotinohydrazides. Several of these had excellent growth inhibition activity against the cancer cell lines tested. Furthermore the displacement of 3-amide moiety of 2-arylamino-6-trifluoromethylnicotinamides with an hydrazono group led to new derivatives **18**–**65**, endowed with about 10-fold enhanced antiproliferative activity with respect to the reference nicotinamides. The most potent compound, **29**, inhibited the growth of all tested cancer cell lines with nanomolar potency, and did not show animal toxicity. The potent anticancer activity presented for the synthesized compounds, especially **26**, **29**, **41**, and **65**, together with their easiness of synthesis makes these compounds useful as a template for the design of promising anticancer hydrazone agents.

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 shift are reported in part 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 compounds **1**,[21] **10–11**,[1] **66**,[22] **70**,[23] **71**,[24] and 1,1,1-trifluoro-4-*iso*-butoxy-3-buten-2-one[25] were obtained with previously described procedures.

3.1.1. Ethyl 2-((5-chloro-2-methoxyphenyl)amino)-6-trifluoromethylpyridine-3-carboxylate (12)

5-Chloro-2-methoxyaniline (1.58 g, 10 mmol) was added to a solution of ethyl 3-amino-3-ethoxypropenoate **1** (1.60 g, 10 mmol) in anhydrous acetonitrile (20 mL). The resulting solution was kept at room temperature for 6 days and then 1,1,1-trifluoro-4-*iso*-butoxy-3-buten-2-one (1.96 g, 10 mmol) was added. The resulting mixture was stirred at room temperature for 0.5 h and then refluxed for 3 h. Then solvent was removed to dryness and the resulting residue was treated with isopropyl ether, separated by filtration and crystallized from *n*-hexane. Yield 80%. Mp 149–150 °C. ^{1}H NMR (DMSO-d_6): δ 1.47 (t, J = 6.9 Hz, 3H, CH_3), 4.04 (s, 3H, OCH_3), 4.52 (q, J = 6.9 Hz, 2H, CH_2), 7.20 (m, 2H, aryl), 7.50 (d, 1H, J = 8.1 Hz, pyridyl), 8.64 (d, 1H, J = 8.1 Hz, pyridyl), 8.84 (s, 1H, aryl), 10.92 (s, 1H, NH). IR (Nujol) 3262, 1699, 1612 cm^{-1}. Anal. Calcd for $C_{16}H_{14}ClF_3N_2O_3$: C, 51.28; H, 3.77; N, 7.48. Found: C, 51.32; H, 3.76; N, 7.50.

3.1.2. Ethyl 2-(-3-(trifluoromethyl)phenylamino)-6-trifluoromethylpyridine-3-carboxylate (13)

3-Trifluoromethylaniline (1.61 g, 10 mmol) was added to a solution of ethyl 3-amino-3-ethoxypropenoate **1** (1.60 g, 10 mmol) in anhydrous acetonitrile (20 mL). The resulting solution was kept at room temperature for 6 days and then 1,1,1-trifluoro-4-*iso*-butoxy-3-buten-2-one (1.96 g, 10 mmol) was added. The resulting mixture was stirred at room temperature for 0.5 h and then refluxed for 3 h. Then solvent was removed to dryness and the resulting residue was treated with isopropyl ether, separated by filtration and crystallized from 2-PrOH. Yield 90%. Mp 196–197 °C. ^{1}H NMR ($CDCl_3$): δ 0.71 (t, J = 7.1 Hz, 3H, CH_3), 3.64 (q, J = 7.1 Hz, 2H, CH_2), 6.66 (m, 1H, aryl), 6.85 (m, 3H, Aryl and pyridyl), 7.08 (d, 1H, J = 8.4 Hz, pyridyl), 7.10 (m, 1H, aryl), 8.98 (s, 1H, NH). IR (Nujol) 3349, 1734, 1720, 1646 cm^{-1}. Anal. Calcd for $C_{16}H_{12}F_6N_2O_2$: C, 50.80; H, 3.20; N, 7.41. Found: C, 50.86; H, 3.19; N, 7.38.

3.1.3. General procedure for the synthesis of hydrazides (14–17, 68, 72, 73, 77)

A mixture of esters **10–13, 67, 70, 71, 76** (2 mmol), and hydrazine monohydrate (1 mL, 20.5 mmol) in EtOH (10 mL) was refluxed for 1.5 h. Then solvent was removed to dryness and the resulting residue tritured with diethyl ether. The resulting precipitate was filtered off an used without further purification.

3.1.3.1. 2-(4-Chloro-2-methylphenylamino)-6-(trifluoromethyl)-nicotinohydrazide (14). Yield 88%. Mp 144–145 °C. ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 7.18–7.26 (m, 6H, Ar, Py and NH_2), 8.18 (d, J = 9.2 Hz, 1H Py), 8.21 (s, 1H, NH), 10.80 (s, 1H, NH). IR (Nujol) 3332, 3280, 3127, 1657, 1612 cm^{-1}. Anal. Calcd for $C_{14}H_{12}ClF_3N_4O$: C, 48.78; H, 3.51; N, 16.25. Found: C, 48.84; H, 3.50; N, 16.23.

3.1.3.2. 2-(5-Chloro-2-methylphenylamino)-6-(trifluoromethyl) nicotinohydrazide (15). Yield 86%. Mp 149–150 °C. ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 7.19–8.21 (m, 8H, Ar, Py, NH), 10.80 (s, 1H, NH). IR (Nujol) 3311, 1616 cm^{-1}. Anal. Calcd for $C_{14}H_{12}ClF_3N_4O$: C, 48.78; H, 3.51; N, 16.25. Found: C, 48.74; H, 3.49; N, 16.28.

3.1.3.3. 2-(5-Chloro-2-methoxyphenylamino)-6-(trifluoromethyl) nicotinohydrazide (16). Yield 92%. Mp 250 °C (dec). ^{1}H NMR (DMSO-d_6): δ 3.85 (s, 3H, OCH_3), 4.65 (s, 2H, NH_2), 6.96 (dd, J = 8.8, 2.3 Hz, 1H, Ar), 7.02 (d, J = 8.8, 1H, Ar), 7.3 (d, J = 7.7, 1H, Py), 8.21 (d, J = 7.7, 1H, Py), 8.65 (d, J = 2.3, 1H, Ar), 10.21 (s, 1H, NH), 11.21 (s, 1H, NH). IR (Nujol) 3287, 1630 cm^{-1}. Anal. Calcd for $C_{15}H_{15}ClF_3N_4O_2$: C, 52.94; H, 4.44; N, 16.46. Found: C, 53.00; H, 4.45; N, 16.41.

3.1.3.4. 6-(Trifluoromethyl)-2-(3-(trifluoromethyl) phenylamino)nicotinohydrazide (17). Yield 81%. Mp 113–115 °C. ^{1}H NMR (DMSO-d_6): δ 4.69 (s, 2H, NH_2), 7.28–7.53 (m, 3H, Ar) 7.66 (d, J = 8.1, 1H, Py), 8.2 (d, J = 7.7, 1H, Py), 8.40 (s, 1H, Ar), 10.28 (s, 1H, NH), 10.95 (s, 1H, NH). IR (Nujol) 3321, 1643, 1598 cm^{-1}. Anal. Calcd for $C_{14}H_{10}F_6N_4O$: C, 46.16; H, 2.77; N, 15.38. Found: C, 46.10; H, 2.78; N, 15.41.

3.1.4. General procedure for the synthesis of hydrazones (18–65, 69, 74, 75, 78)

A mixture of hydrazides **14–17, 68, 72, 73, 77** (1 mmol) and the appropriate aldehyde (1 mmol) in EtOH (10 mL) was refluxed for 3 h. After cooling the formed precipitate was filtered off and purified by crystallization from the adequate solvent to give the hydrazone derivatives. The hydrazones **29**, **41**, **50** and **65** are described as examples. Physical, spectral and analytical data of hydrazones **18–40**, **42–49**, and **51–64** are available in the Supplementary data.

3.1.4.1. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-2-(4-chloro-2-methylphenylamino)-6-(trifluoromethyl) nicotinohydrazide (29). Yield 74%. Mp 201–203 °C (EtOH). ^{1}H NMR (DMSO-d_6): δ 2.20 (s, 3H, CH_3), 7.05 (m, 2H, Ar), 7.25 (m, 2H, Ar), 7.49 (m, 1H, Ar), 7.71 (m, 1H, Ar), 8.02 (d, J = 8.0, 1H, Py), 8.37 (d, J = 8.0, 1H, Py), 8.73 (s 1H, CH), 10.28 (s, 1H, NH), 12.47 (s, 1H, NH). IR (Nujol) 3206, 3266, 1644, 1614 cm^{-1}. Anal. Calcd for $C_{21}H_{14}Cl_3F_3N_4O$: C, 50.27; H, 2.81; N, 11.17. Found: C, 50.34; H, 2.82; N, 11.13.

3.1.4.2. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-2-(5-chloro-2-methylphenylamino)-6-(trifluoromethyl) nicotinohydrazide (41). Yield 62%. Mp 191–192 °C (EtOH). ^{1}H NMR (DMSO-d_6): δ 2.21 (s, 3H, CH_3), 7.24 (m, 3H, Ar), 7.42 (m, 1H, Ar), 7.49 (m, 2H, Ar), 8.03 (d, J = 7.8 1H, Py), 8.35 (d, J = 7.8 1H, Py), 8.56 (s, 1H, CH), 10.27 (s, 1H, NH), 12.47 (s, 1H, NH). IR (Nujol) 3221, 3056, 1644, 1614, 1597 cm^{-1}. Anal. Calcd for $C_{21}H_{14}Cl_3F_3N_4O$: C, 50.27; H, 2.81; N, 11.17. Found: C, 50.22; H, 2.80; N, 11.22.

3.1.4.3. (*E*)-*N*′-(2-Chlorobenzylidene)-2-(5-chloro-2-methoxyphenylamino)-6-(trifluoromethyl) nicotinohydrazide (50). Yield 89%. Mp 250 °C (dec). ^{1}H NMR (DMSO-d_6): δ 4.05 (s, 3H, CH_3), 7.18 (m, 4H, Ar), 7.59 (d, J = 6.9, 2H, Ar), 8.20 (m, 1H, Py), 8.61 (d, J = 7.7, 1H, Py), 8.83 (s, 1H, Ar), 8.98 (s, 1H, CH), 11.13 (s, 1H, NH), 12.55 (s, 1H, NH). IR (Nujol) 3236, 1645, 1601 cm^{-1}. Anal. Calcd for $C_{21}H_{15}Cl_2F_3N_4O_2$: C, 52.19; H, 3.13; N, 11.59. Found: C, 52.15; H, 3.12; N, 11.63.

3.1.4.4. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-6-(trifluoromethyl)-2-(3-(trifluoromethyl)phenylamino) nicotinohydrazide (65). Yield 68%. Mp 250 °C dec. (EtOH). ^{1}H NMR (DMSO-d_6): δ 7.04 (m, 1H, Ar), 7.31 (m, 2H, Ar), 7.65 (m, 4H, Ar), 7.96 (d, J = 8.0, 1H, Py), 8.36 (d, J = 8.0, 1H, Py), 8.57 (s, 1H, CH), 10.46 (s, 1H, NH), 12.09 (s, 1H, NH). IR (Nujol) 3359, 3192, 1645, 1606 cm^{-1}. Anal. Calcd for $C_{21}H_{12}Cl_2F_6N_4O$: C, 48.39; H, 2.32; N, 10.75. Found: C, 48.33; H, 2.30; N, 10.72.

3.1.5. Ethyl 2-(2-(4-chloro-2-methylphenylamino)-6-(trifluoromethyl)nicotinamido)acetate (67)

A mixture of acid **66** (0.33 g, 1 mmol), EDCI (1.92 g, 1.1 mmol) and HOBt (0.13 g, 1 mmol) in dry MeCN (10 mL) was stirred at room temperature for 30 min and then treated with the ethyl glycinate hydrochloride (0.28 g, 2 mmol) and TEA (0.2 mL, 2 mmol). The mixture was stirred at room temperature for an additional

2 h. Then the solution evaporated to dryness in vacuo. The residue was dissolved in ethyl acetate (20 mL) and washed with brine (2 × 5 mL), 10% aqueous citric acid (2 × 5 mL), satd aqueous sodium hydrogencarbonate (2 × 5 mL) and water (2 × 5 mL). The organic layer was dried over anhydrous magnesium sulfate. Concentration of the dried extract yielded a residue which was tritured with isopropyl ether. The formed precipitate was filtered off and utilized in the step without further purification. Yield 87%. Mp 128–130 °C. ^{1}H NMR (DMSO-d_6): δ 1.34 (t, J = 6.9, 3H, CH_3), 2.38 (s, 3H, CH_3), 4.12 (m, 2H, CH_2), 4.18 (q, J = 6.9, 2H, CH_2), 7.37 (d, J = 8.7 1H, Ar), 7.44 (s, 1H, Ar), 7.48 (d, J = 8.1 1H, Py), 8.29 (d, J = 8.7 1H, Ar), 8.47 (d, J = 8.1 1H, Py), 9.62 (m, 1H, NH), 10.76 (s, 1H, NH). IR (Nujol) 3431, 1723, 1656 cm^{-1}. Anal. Calcd for $C_{18}H_{17}ClF_3N_3O_3$: C, 52.00; H, 4.12; N, 10.11. Found: C, 52.06; H, 4.14; N, 10.07.

3.1.6. 2-(2-(4-Chloro-2-methylphenylamino)-6-(trifluoromethyl)-nicotinamido)acetohydrazide (68)

Following the general procedure for the synthesis of hydrazides the title compound was obtained in 76% yield. Mp 218–220 °C dec. (EtOH). ^{1}H NMR (DMSO-d_6): δ 2.40 (s, 3H, CH_3), 4.01 (d, J = 5.7, 2H, CH_2), 6.92 (s, 2H, NH_2), 7.37 (d, J = 8.7 1H, Ar), 7.44 (s, 1H, Ar), 7.46 (d, J = 7.8 1H, Py), 8.30 (d, J = 8.7 1H, Ar), 8.48 (d, J = 7.8 1H, Py), 9.37 (m, 1H, NH), 10.85 (s, 1H, NH), 11.28 (s, 1H, NH). IR (Nujol) 3331, 1673, 1616 cm^{-1}. Anal. Calcd for $C_{16}H_{15}ClF_3N_5O_2$: C, 47.83; H, 3.76; N, 17.43. Found: C, 47.89; H, 3.75; N, 17.40.

3.1.7. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-2-(2-(4-chloro-2-methylphenylamino)-6-(trifluoromethyl)nicotinamido)acetohydrazide (69)

Following the general procedure for the synthesis of hydrazones the title compound was obtained in 77% yield. Mp 230–232 °C (EtOH). ^{1}H NMR (DMSO-d_6): δ 2.39 (s, 3H, CH_3), 4.53 (d, J = 5.4, 2H, CH_2), 7.45 (m, 5H, Ar), 7.70 (d, J = 8.1, 1H, Py), 8.31 (d, J = 8.1, 1H, Py), 8.39 (s, 1H, CH), 8.51 (d, J = 7.3, 1H, Ar), 9.43 (m, 1H, NH), 10.84 (s, 1H, NH), 11.99 (s, 1H, NH). IR (Nujol) 3194, 3100, 1689, 1632, 1612 cm^{-1}. Anal. Calcd for $C_{23}H_{17}Cl_3F_3N_5O_2$: C, 49.44; H, 3.07; N, 12.53. Found: C, 49.50; H, 3.06; N, 12.50.

3.1.8. 2-(3-(Trifluoromethyl)phenylamino) benzohydrazide (72)

Following the general procedure for the synthesis of hydrazides the title compound was obtained in 96% yield. Mp 134–136 °C dec. (2-PrOH). Lit.[26] 135–137 °C (EtOH). ^{1}H NMR (DMSO-d_6): δ 7.36 (m, 1H, Ar), 7.33 (d, J = 7.3 Hz, 1H, Ar), 7.49 (m, 6H, Ar and NH_2), 7.58 (d, J = 7.7 Hz, 1H, Ar), 7.70 (d, J = 7.7 Hz, 1H, Ar), 9.97 (s, 1H, NH), 12.16 (s, 1H, NH). IR (Nujol) 3324, 1636, 1584 cm^{-1}. Anal. Calcd for $C_{14}H_{12}F_3N_3O$: C, 56.95; H, 4.10; N, 14.23. Found: C, 56.89; H, 4.12; N, 14.27.

3.1.9. 2-(3-(Trifluoromethyl)phenylamino) nicotinohydrazide (73)

Following the general procedure for the synthesis of hydrazides the title compound was obtained in 73% yield. Mp 139–140 °C dec. (2-PrOH). ^{1}H NMR (DMSO-d_6): δ 7.07 (m, 1H, Ar), 6.24 (s, 2H, NH_2), 7.39 (d, J = 8.1 Hz, 1H, Py), 7.63 (m, 1H, Ar), 7.93 (d, J = 8.1 Hz, 1H, Py), 8.23 (m, 1H, Ar), 8.39 (d, J = 8.1 Hz, 1H, Py), 8.49 (m, 1H, Ar), 10.60 (s, 1H, NH), 10.86 (s, 1H, NH). IR (Nujol) 3316, 3204, 3029, 1630, 1602 cm^{-1}. Anal. Calcd for $C_{13}H_{11}F_3N_4O$: C, 52.71; H, 3.74; N, 18.91. Found: C, 52.76; H, 3.73; N, 18.96.

3.1.10. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-2-(3-(trifluoromethyl) phenylamino)benzohydrazide (74)

Following the general procedure for the synthesis of hydrazones the title compound was obtained in quantitative yield. Mp 237–239 °C. (EtOH). ^{1}H NMR (DMSO-d_6): δ 7.00 (m, 2H, Ar), 7.14 (m, 1H, Ar), 7.33 (m, 7H, Ar), 7.68 (m, 1H, Ar), 8.52 (s, 1H, CH), 9.10 (s, 1H, NH), 12.10 (s, 1H, NH). IR (Nujol) 3292, 3174, 3042, 1643, 1604 cm^{-1}. Anal. Calcd for $C_{21}H_{14}Cl_2F_3N_3O$: C, 55.77; H, 3.12; N, 9.29. Found: C, 55.71; H, 3.13; N, 9.25.

3.1.11. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-2-(3-(trifluoromethyl) phenylamino)nicotinohydrazide (75)

Following the general procedure for the synthesis of hydrazones the title compound was obtained in quantitative yield. Mp 250 °C dec. (1-PrOH). ^{1}H NMR (DMSO-d_6): δ 7.07 (m, 1H, Ar), 7.43 (m, 2H, Ar), 7.64 (m, 4H, Ar), 7.98 (d, J = 8.4 Hz, 1H, Py), 8.39 (m, 1H, Py), 8.54 (s, 1H, CH), 8.79 (m, 1H, Py), 10.75 (s, 1H, NH), 12.44 (s, 1H, NH). IR (Nujol) 3322, 3197, 3042, 1641, 1607 cm^{-1}. Anal. Calcd for $C_{20}H_{13}Cl_2F_3N_4O$: C, 53.00; H, 2.89; N, 12.63. Found: C, 52.96; H, 2.90; N, 12.61.

3.1.12. Ethyl 2-amino-6-(trifluoromethyl)nicotinate (76)

Ammonium acetate (2 g, 26 mmol) was added to a solution of ethyl 3-amino-3-ethoxypropenoate **1** (0.80 g, 5 mmol) and 1,1,1-trifluoro-4-*iso*-butoxy-3-buten-2-one (0.98 g, 5 mmol) in anhydrous EtOH (10 mL). The resulting mixture was refluxed for 3 h. Then solvent was removed to dryness. The residue was dissolved in diethyl ether (20 mL) and washed with water (2 × 5 mL). The organic layer was dried over anhydrous magnesium sulfate. Concentration of the dried extract yielded a residue which was tritured with isopropyl ether, separated by filtration to give quantitative yield of the title compound. Mp 66–68 °C (*n*-hexane). ^{1}H NMR (DMSO-d_6): δ 1.47 (t, J = 7.0 Hz, 3H, CH_3), 3.80 (q, J = 7.0 Hz, 2H, CH_2), 7.15 (d, J = 8.1, 1H, Py), 7.70 (s, 2H, NH_2), 8.41 (d, J = 8.1 1H, Py). IR (Nujol) 3440, 3299, 3233, 3185, 1707 cm^{-1}. Anal. Calcd for $C_9H_9F_3N_2O_2$: C, 46.16; H, 3.87; N, 11.96. Found: C, 46.20; H, 3.88; N, 11.92.

3.1.13. 2-Amino-6-(trifluoromethyl)nicotinohydrazide (77)

Following the general procedure for the synthesis of hydrazides the title compound was obtained in 87% yield. Mp 159–160 °C. (2-PrOH). ^{1}H NMR (DMSO-d_6): δ 7.14 (d, J = 7.7 Hz, 1H, Py), 7.40 (s, 4H, NH_2), 8.15 (d, J = 7.7 Hz, 1H, Py), 10.70 (s, 1H, NH). IR (Nujol) 3439, 3338, 3220, 3036, 1658, 1623 cm^{-1}. Anal. Calcd for $C_7H_7F_3N_4O$: C, 38.19; H, 3.20; N, 25.45. Found: C, 38.21; H, 3.22; N, 25.40.

3.1.14. (*E*)-*N*′-(2,6-Dichlorobenzylidene)-2-amino-6-(trifluoromethyl)nicotinohydrazide (78)

Following the general procedure for the synthesis of hydrazones the title compound was obtained in 60% yield. Mp 250 °C dec. (1-PrOH). ^{1}H NMR (DMSO-d_6): δ 6.87 (br s, 2H, NH_2), 7.21 (m, 1H, Ar), 7.58 (m, 2H, Ar and Py), 7.69 (m, 1H, Ar), 8.34 (m, 1H, Py), 8.72 (s, 1H, CH), 12.34 (s, 1H, NH). IR (Nujol) 3479, 3356, 3180, 3038, 1660, 1618 cm^{-1}. Anal. Calcd for $C_{14}H_9Cl_2F_3N_4O$: C, 44.58; H, 2.41; N, 14.86. Found: C, 52.96; H, 2.90; N, 12.61.

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.[27,28] Density of inoculum depends on the type of tumor cell and from its growth characteristics.[17] 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.[29]

3.3. Acute toxicity determination

The determination of maximum tolerated dose (MTD) is performed in a way that conserves compound and minimizes the number of animals sacrificed. Thus, a single mouse is given a single injection (IP) of 400 mg/kg; a second mouse receives a dose of 200 mg/kg and a third mouse receives a single dose of 100 mg/kg. The mice are observed for a period of 2 weeks. They are sacrificed if they lose more than 20% of their body weight or if there are other signs of significant toxicity. If all 3 mice must be sacrificed, the next 3 dose levels (50, 35 and 12.5 mg/kg) are tested in a similar manner. This process is repeated until a tolerated dose is found. This dose is then designated the MTD and is used to calculate the amount of material administered to mice during antitumor testing. The mice are allowed *ad libitum* feed and water. Injections are administered IP. The compounds are solubilized in DMSO and dose volumes were 5 μL, 2.5 μL and 1.25 μL/g of body weight.

Acknowledgement

We thank the Antitumor Evaluation Branch of the National Cancer Institute for performing biological evaluations.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.07.066.

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