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Novel synthesis of nicotinamide derivatives of cytotoxic properties

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Abstract—A variety of 2-substituted-4,6-diaryl-3-pyridinecarboxamides **5** were synthesized through aromatic nucleophilic substitution reaction of secondary amines with 2-bromo analogues **4**. The latter were obtained via bromination of 2-cyano-3,5-diaryl-5-oxo-N-substituted pentamides **3** in glacial acetic acid. Moreover, pentamide derivatives **3** were prepared through base-catalyzed Michael addition of cyanacetanilides **2** with 1,3-diaryl-2-propen-1-ones **1**. Otherwise, reaction of 2-bromo-3-pyridinecarboxamides **4** with primary aromatic amines in refluxing pyridine afforded the corresponding 2-(arylamino)-3-pyridinecarboxamides **6** besides the unexpected 2-unsubstituted amino analogues **7**. Antitumor properties of the synthesized pyridinecarboxamides utilizing 59 different human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate as well as kidney, were screened. Many of the tested compounds show considerable in vitro antitumor properties especially **5c** and **7a**, which reveal moderate activities against most of the used human tumor cell lines. It has also been achieved that, all the tested nicotinamide derivatives reveal promising antitumor properties against MDA-MB-231/ATCC (breast cancer).

1. Introduction

Nicotinamide (3-pyridinecarboxamide) 'vitamin B_3' has been mainly used to treat pellagra in past clinical practice[1] and is an effective agent in treating some neurodegenerative diseases 'for example, Alzheimer's disease, Parkinson's disease and other cognitive disorders of mammals'.[2] It has been also reported to be a potential therapeutic agent for the treatment of retinal degeneration.[3] Other publications mentioned that, nicotinamide and its derivatives both prevent and reverse neuronal and vascular cell injury.[4–6] Moreover, nicotinamide is helpful in the maintenance of cellular energy balance during ischemia and reperfusion conditions (e.g., cardiac, testicles, and liver injury). Many administrations of nicotinamide suggested the therapeutic interest in preventing the development of stroke by rescuing the still viable but injured cells and partially preventing infarction.[7–9] Others were used for treating acquired immunodeficiency syndrome (AIDS),[10,11] inflammatory, allergic, and respiratory diseases.[12,13]

Many publications stated that, treatment with nicotinamide prevents or delays insulin-deficient diabetes (anti-diabetogenic activity) via improving beta cell function.[14–20] It has also been achieved that, nicotinamide is an oral antimicrobial active agent for co-infection with *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) which is responsible for one-third of all deaths due to acquired immunodeficiency syndrome (AIDS).[21] Numerous pharmacological compositions containing nicotinamide were appeared to be effective as antidandruff, anti-itching, hair growth promoting, gray hair preventing, increasing hair elasticity, and treating acne, fine lines as well as age spots.[22–31]

Moreover, various substituted nicotinamides were used as fungicides,[32,33] pesticides[34–39] or for treatment of benign prostatic hyperplasia.[40] Nikethamide '*N*,*N*-diethyl-3-pyridinecarboxamide' is a well-known drug used as respiratory analeptic.[1]

In continuation of our previous work directed toward construction of heterocycles bearing natural product residue related compounds of anticipated biological and/or pharmacological properties,[41,42] it is intended in the present work to investigate the synthesis of novel 3-pyridinecarboxamide derivatives utilizing easily accessible starting materials and facile synthetic approaches. The interest for the synthesis of these analogues is also

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originated due to activities associated with the structure of 2-(substituted amino)-3-pyridinecarboxamide derivatives as antitumor[43–45] in addition to inhibitory properties against the proliferation of human immunodeficiency virus (HIV) as well as hepatitis B virus (HBV) and hepatitis C virus (HCV).[46] The antitumor properties of the newly synthesized 3-pyridinecarboxamide derivatives against a variety of human tumor cell lines will be also screened.

2. Results and discussion

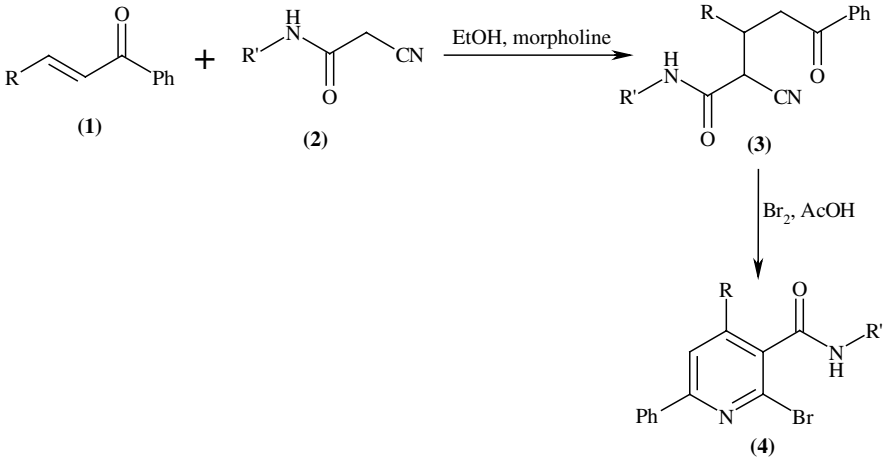
2.1. Chemistry

Reaction of 1,3-diaryl-2-propen-1-ones **1a** and **b** with cyanacetamides **2a** and **b** in refluxing ethanol in the presence of a catalytic amount of morpholine as a basic catalyst afforded the corresponding 2-cyano-3,5-diaryl-5-oxo-N-substituted pentamides **3a–c** in good yields. The structure of the latter was established through spectroscopic (IR and ^{1}H NMR) as well as elemental analyses data.

Addition of bromine to pentamides **3a–c** in glacial acetic acid at 60–70 °C gave directly the 2-bromo-4,6-diaryl-N-substituted-3-pyridinecarboxamides **4a–c** in 82–77% yield. The structure of **4a–c** was deduced through spectroscopic (IR, ^{1}H NMR, and MS) and elemental analyses data (Scheme 1).

Reaction of 2-bromonicotinamides **4a–c** with secondary amines 'piperidine and morpholine' in refluxing tetrahydrofuran afforded smoothly, the 2-substituted nicotinamide derivatives **5a–f** in good yields. On the other hand, reaction of **4a–c** with aromatic amines 'aniline, *p*-toluidine, *p*-anisidine, and *p*-chloroaniline' in refluxing pyridine gave two products which were separated and purified by silica gel 60G F_{254} TLC. The structures of which were established to be 2-(arylamino)nicotinamides **6a–i** and the unexpected 2-(unsubstituted amino)nicotinamide analogues **7a–c** based on spectroscopic (IR, ^{1}H NMR, and MS) and elemental analyses data (Scheme 2).

Formation of **7** probably took place through imino-form isomerization originated from the primary aromatic amines under the used basic reaction conditions (refluxing pyridine), which via hydrolysis 'due to unavoidable moisture' liberated ammonia. The latter due to aromatic nucleophilic substitution with the used starting 2-bromo-3-pyridinecarboxamides **4** gave finally, the 2-amino-3-pyridinecarboxamides **7**. It has been noticed that, the yields of **7a–c** (39–30%) were greater in case of using aromatic amine substituted with deactivating moiety '*p*-chloroaniline' compared with the cases, when the amines were substituted with electron-donating or-activating functions (*p*-toluidine 'yields of **7a–c** are 16–15%' and *p*-anisidine 'yields of **7a** and **b** are 11–10%'). This observation supports the role of substituent attached with the used aromatic amine in deriving the reaction mechanistic route toward the unexpected product **7** formation, which coincides with the role of substitution favoring the imino-form process isomerization during the reaction course. Eventually, it could be concluded that, the opportunity of **7** formation under the described basic reaction conditions appeared greater when the used primary aromatic amine substituted with deactivating moieties. Formation of **7** during the reaction course seems similar to what was previously reported about the yielding of 2-amino-3-pyridinecarbonitrile derivatives through the reaction of 2-bromo analogues with α-primary amino acid esters (glycine or alanine esters) in refluxing pyridine.[41] It was assumed in the



(1) (2) (3) (4)

1a; R = 4-ClC_6H_4

1b; R = 4-$H_3CC_6H_4$

2a; R = Ph

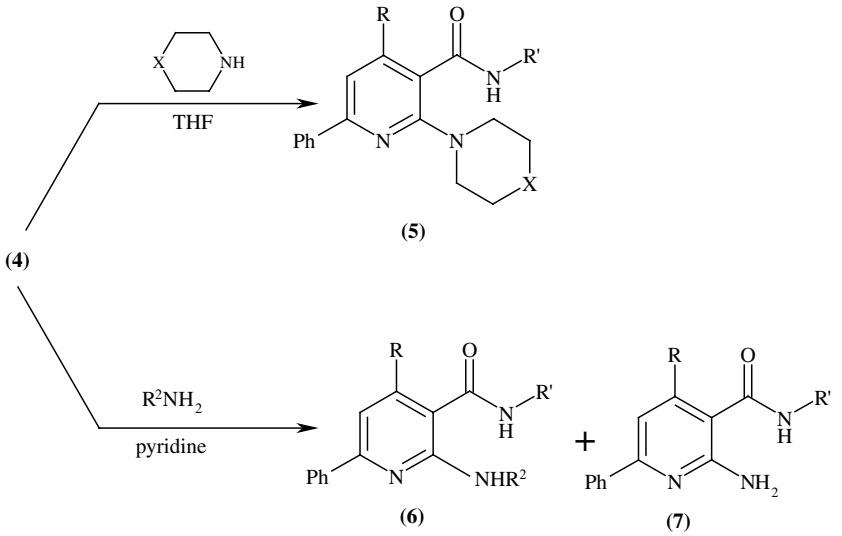
2b; R = 4-$H_3CC_6H_4$

3a, **4a**; R = 4-ClC_6H_4, R' = Ph

3b, **4b**; R = 4-ClC_6H_4, R' = 4-$H_3CC_6H_4$

3c, **4c**; R = 4-$H_3CC_6H_4$, R' = Ph

Scheme 1.



5a; R = 4-ClC_6H_4, R' = Ph, X = CH_2

5b; R = 4-ClC_6H_4, R' = Ph, X = O

5c; R = 4-ClC_6H_4, R' = 4-$H_3CC_6H_4$, X = CH_2

5d; R = 4-ClC_6H_4, R' = 4-$H_3CC_6H_4$, X = O

5e; R = 4-$H_3CC_6H_4$, R' = Ph, X = CH_2

5f; R = 4-$H_3CC_6H_4$, R' = Ph, X = O

7a; R = 4-ClC_6H_4, R' = Ph

7b; R = 4-ClC_6H_4, R' = 4-$H_3CC_6H_4$

7c; R = 4-$H_3CC_6H_4$, R' = Ph

6a; R = 4-ClC_6H_4, R' = R^2 = Ph

6b; R = 4-ClC_6H_4, R' = Ph, R^2 = 4-$H_3CC_6H_4$

6c; R = 4-ClC_6H_4, R' = Ph, R^2 = 4-$H_3COC_6H_4$

6d; R = R^2 = 4-ClC_6H_4, R' = Ph

6e; R = 4-ClC_6H_4, R' = R^2 = 4-$H_3CC_6H_4$

6f; R = 4-ClC_6H_4, R' = 4-$H_3CC_6H_4$, R^2 = 4-$H_3COC_6H_4$

6g; R = R^2 = 4-ClC_6H_4, R' = 4-$H_3CC_6H_4$

6h; R = R^2 = 4-$H_3CC_6H_4$, R' = Ph

6i; R = 4-$H_3CC_6H_4$, R' = Ph, R^2 = 4-ClC_6H_4

Scheme 2.

latter reaction that, the mechanistic pathway proceeded analogously to the famous ninhydrin reaction with α-amino acids,[47] where the amino acids isomerized to the corresponding imino-acid forms under the effect of applied reaction conditions. Then, upon hydrolysis, due to unavoidable moisture, ammonia was liberated which in turn interacted with 2-bromo-3-pyridinecarbonitriles giving the 2-amino derivatives. Another observation was also reported about the formation of 2-aminonicotinate esters through the reaction of 2-bromonicotinates with primary aromatic amines under similar reaction conditions.[42]

2.2. Antitumor activity

Nicotinamides are a well-known class of drugs that contain many analogues having radio- and chemosensitizing properties. Particularly, the N-substituted derivatives which distinguished because they are: (i) susceptible to radiolysis, (ii) induce cytotoxicity by apoptosis but not necrosis, (iii) inhibit cell proliferation, (iv) activate poly adenosine diphosphate ribosyl transferase, and (v) have a much-reduced effect on microregional tumor blood perfusion.[48,49] Multiple mechanistic properties were presumed explaining the well-established activities for example, they are known to affect blood flow, inhibit DNA repair, and enhance DNA damage induction.[50] Other reports assumed that the potent antitumoral nicotinamide properties were attributed to the primary mechanism of action regulated by inhibition at the gene transcription level.[51]

Antitumor activity screening for the synthesized nicotinamides utilizing 59 different human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate as well as kidney, was carried out according to the previously reported standard procedure.[52–54] The obtained results (Table 1) represent concentrations of the used investigated compounds resulting in growth inhibition of 50% (GI_{50}) for the tested human tumor cell lines.

From the in vitro observed data it has been noticed that, 2-(unsubstituted amino)-3-pyridinecarboxamides **7a** and **b** reveal better antitumor properties compared with the corresponding 2-arylamino-3-pyridinecarboxamides **6**. Moreover, 2-(1-piperidinyl)-*N*-(4-methylphenyl)-3-pyridinecarboxamide **5c** exhibits enhanced antitumor properties than the corresponding tested analogues **5a**, **b**, and **d**. It has also been noticed that, all the tested compounds show considerable antitumor activities against MDA-MB-231/ATCC (breast cancer). Generally, compounds **5c** and **7a** exhibit moderate antitumor properties against most of the tested human tumor cell lines

Table 1. Concentrations resulting in growth inhibition of 50% (GI_{50}, mg/l) of in vitro human tumor cell lines

Panel/cell line	Compound										
	5a	**5b**	**5c**	**5d**	**6a**	**6c**	**6d**	**6f**	**6g**	**7a**	**7b**
Leukemia											
CCRF-CEM	>46.80	>46.99	18.75	>48.40	>47.60	>50.60	16.90	>52.00	>52.44	8.55	3.77
HL-60(TB)	>46.80	1.71	10.79	>48.40	3.22	14.59	10.42	>52.00	>52.44	2.90	0.17
K-562	NT	3.40	6.81	>48.40	12.52	30.49	10.66	NT	>52.44	4.00	1.37
MOLT-4	>46.80	2.41	7.30	>48.40	0.34	1.84	4.65	>52.00	>52.44	3.82	0.67
RPMI-8226	>46.80	4.59	12.11	21.13	>47.60	17.95	0.44	NT	NT	4.59	2.07
SR	1.51	0.65	NT	NT	NT	10.57	10.42	NT	2.88	1.10	NT
Non-small cell lung cancer											
A549/ATCC	>46.80	>46.99	8.00	>48.40	>47.60	>50.60	37.84	>52.00	>52.44	12.64	3.86
EKVX	>46.80	28.32	21.53	>48.40	>47.60	>50.60	39.62	>52.00	>52.44	10.76	2.80
HOP-62	>46.80	>46.99	8.00	44.14	>47.60	>50.60	36.98	>52.00	>52.44	17.86	24.37
HOP-92	>46.80	8.95	9.62	>48.40	>47.60	>50.60	19.86	>52.00	>52.44	6.34	3.86
NCI-H226	>46.80	>46.99	12.39	>48.40	43.41	>50.60	>51.04	>52.00	>52.44	10.52	12.79
NCI-H23	>46.80	21.98	6.65	31.98	>47.60	>50.60	19.40	>52.00	>52.44	8.16	3.44
NCI-H322M	>46.80	>46.99	6.50	15.30	>47.60	>50.60	22.28	NT	NT	11.01	12.50
NCI-H460	>46.80	10.52	7.82	>48.40	>47.60	NT	NT	NT	NT	9.82	3.95
NCI-H522	16.23	3.65	6.81	6.68	20.78	>50.60	18.53	>52.00	>52.44	7.11	4.04
Colon cancer											
COLO 205	>46.80	>46.99	11.30	>48.40	>47.60	9.42	7.21	>52.00	>52.44	7.45	4.86
HCC-2998	>46.80	>46.99	25.88	>48.40	>47.60	>50.60	34.51	>52.00	>52.44	14.19	12.21
HCT-116	19.96	4.09	4.60	13.64	0.85	12.42	5.34	>52.00	21.36	5.91	1.89
HCT-15	>46.80	8.17	9.62	24.82	>47.60	20.61	14.72	>52.00	>52.44	7.62	2.38
HT29	>46.80	2.47	5.93	45.17	12.52	>50.60	5.73	>52.00	>52.44	10.76	2.49
KM12	>46.80	>46.99	15.96	>48.40	>47.60	>50.60	14.72	>52.00	>52.44	11.01	4.24
SW-620	>46.80	>46.99	15.60	>48.40	>47.60	>50.60	27.41	>52.00	2.63	21.47	13.09
CNS cancer											
SF-268	>46.80	>46.99	8.57	>48.40	>47.60	>50.60	8.09	>52.00	>52.44	20.51	20.27
SF-295	>46.80	>46.99	8.57	>48.40	>47.60	>50.60	13.74	>52.00	>52.44	14.52	2.67
SF-539	17.39	20.05	6.21	16.40	>47.60	>50.60	>51.04	>52.00	>52.44	14.52	11.66
SNB-19	>46.80	>46.99	7.64	>48.40	>47.60	>50.60	38.72	>52.00	>52.44	24.09	27.98
SNB-75	>46.80	11.80	8.00	15.30	>47.60	NT	6.00	NT	NT	8.75	20.27
U251	>46.80	27.04	5.66	20.65	>47.60	>50.60	26.79	>52.00	>52.44	8.95	2.86
Melanoma											
LOXIMVI	>46.80	>46.99	5.93	>48.40	>47.60	>50.60	23.87	>52.00	>52.44	10.28	4.75
MALME-3M	>46.80	14.19	6.21	7.50	43.41	>50.60	>51.04	>52.00	>52.44	8.95	5.21
M14	>46.80	>46.99	8.38	43.13	23.85	>50.60	14.39	>52.00	26.28	8.75	4.43
SK-MEL-2	>46.80	>46.99	13.58	>48.40	>47.60	>50.60	>51.04	>52.00	>52.44	18.28	22.23
SK-MEL-28	>46.80	>46.99	29.72	>48.40	>47.60	43.07	12.53	>52.00	9.54	25.23	26.72
SK-MEL-5	>46.80	27.04	>48.20	>48.40	>47.60	>50.60	>51.04	>52.00	0.41	7.80	5.46
UACC-257	>46.80	>46.99	>48.20	>48.40	>47.60	>50.60	40.54	>52.00	>52.44	15.92	14.69
UACC-62	>46.80	8.95	10.79	24.26	>47.60	>50.60	29.37	>52.00	>52.44	5.91	7.36
Ovarian cancer											
IGROV1	>46.80	40.93	9.40	>48.40	>47.60	>50.60	>51.04	>52.00	>52.44	7.98	11.40
OVCAR-3	>46.80	>46.99	7.82	>48.40	>47.60	35.82	27.41	NT	1.95	7.62	3.36
OVCAR-4	>46.80	17.06	8.00	>48.40	>47.60	34.21	10.18	>52.00	NT	11.53	7.36
OVCAR-5	>46.80	>46.99	11.83	>48.40	>47.60	>50.60	>51.04	>52.00	>52.44	35.64	>41.39
OVCAR-8	>46.80	>46.99	6.97	>48.40	>47.60	>50.60	>51.04	>52.00	>52.44	28.97	>41.39
SK-OV-3	>46.80	>46.99	14.22	>48.40	>47.60	44.07	12.53	NT	NT	>39.99	>41.39
Renal cancer											
786-0	14.80	9.82	3.83	11.61	12.23	>50.60	>51.04	>52.00	>52.44	6.95	3.07
A498	>46.80	>46.99	8.77	>48.40	>47.60	>50.60	32.20	>52.00	0.81	39.08	>41.39
ACHN	>46.80	31.05	6.07	>48.40	>47.60	>50.60	17.70	>52.00	>52.44	9.16	2.93
CAKI-1	>46.80	>46.99	15.96	22.12	>47.60	>50.60	28.05	>52.00	4.67	11.53	6.87
RXF-393	>46.80	9.38	8.77	5.95	5.72	>50.60	9.08	NT	NT	6.05	3.00
SN12C	>46.80	>46.99	7.47	>48.40	>47.60	>50.60	36.98	>52.00	>52.44	10.28	3.36
TK-10	>46.80	8.95	7.13	10.35	>47.60	>50.60	11.17	>52.00	14.44	8.75	4.14
UO-31	>46.80	3.10	6.81	5.56	>47.60	>50.60	21.77	>52.00	>52.44	7.11	2.61
Prostate cancer											
PC-3	>46.80	8.75	7.30	>48.40	38.69	33.43	11.17	>52.00	>52.44	5.03	1.89
Du-145	>46.80	>46.99	11.83	>48.40	>47.60	>50.60	>51.04	>52.00	>52.44	19.58	2.38

(*continued on next page*)

Table 1 (*continued*)

Panel/cell line	Compound										
	5a	**5b**	**5c**	**5d**	**6a**	**6c**	**6d**	**6f**	**6g**	**7a**	**7b**
Breast cancer											
MCF7	>46.80	26.43	13.58	>48.40	>47.60	NT	NT	NT	NT	7.80	2.17
NCI/ADR-RES	>46.80	>46.99	6.50	>48.40	>47.60	>50.60	44.45	>52.00	>52.44	9.37	4.64
MDA-MB-231/ATCC	30.92	8.17	6.07	9.01	44.42	49.45	9.95	4.63	17.37	9.59	9.05
HS 578T	>46.80	>46.99	13.90	>48.40	>47.60	>50.60	17.70	>52.00	0.47	39.99	>41.39
MDA-MB-435	>46.80	23.55	15.60	>48.40	>47.60	>50.60	25.58	NT	NT	8.95	2.22
BT-549	30.92	>46.99	12.97	>48.40	>47.60	>50.60	>51.04	>52.00	>52.44	13.86	8.26
T-47D	>46.80	>46.99	11.56	>48.40	>47.60	>50.60	6.73	>52.00	20.40	13.55	3.69

NT, Not tested.

(Figs. 1 and 2). It has also been noticed that, compound **7b** reveals considerable antitumor properties among all the tested cancer cell lines except OVCAR-5, OVCAR-8, SK-OV-3 (ovarian cancer), A498 (renal cancer), and HS 578T (breast cancer). Similarly, compound **6d** reveals moderate antitumor activities against all the tested cancer cell lines except, NCI-H226 (non-small cell lung cancer), SF-539 (CNS cancer), MALME-3M, SK-MEL-2, SK-MEL-5 (melanoma), IGROV1, OVCAR-5, OVCAR-8 (ovarian cancer), 786-0 (renal cancer), Du-145 (prostate cancer), and BT-549 (breast cancer). It has also been noticed that, compound **7b** seems to be the most active prepared nicotinamide derivative against all the tested leukemia, colon, and breast cancer cell lines considering the observed effective low GI_{50} concentrations. However, compound **5c** reveals the best detected antitumor affinities against most of the tested CNS and ovarian cancer cell lines. In addition, few prepared compounds show remarkable antitumor GI_{50} properties against the tested human tumor cell lines at low concentrations (<1.0 mg/l) particularly, **5b** (GI_{50} = 0.65 mg/l against SR), **6a** (GI_{50} = 0.34, 0.85 mg/l against MOLT-4 and HCT-116, respectively), **6d** (GI_{50} = 0.44 mg/l against RPMI-8226), **6g** (GI_{50} = 0.41, 0.81, and 0.47 mg/l against SK-MEL-5, A498, and HS 578T, respectively), and **7b** (GI_{50} = 0.17, 0.67 mg/l against HL-60(TB) and MOLT-4, respectively). From all the above data, especially those observed through compounds **7a** and **b**, it could be concluded that, 2-amino-*N*-arylnicotinamide analogues may be a hint for determining a highly effective broad spectrum antitumor agent.

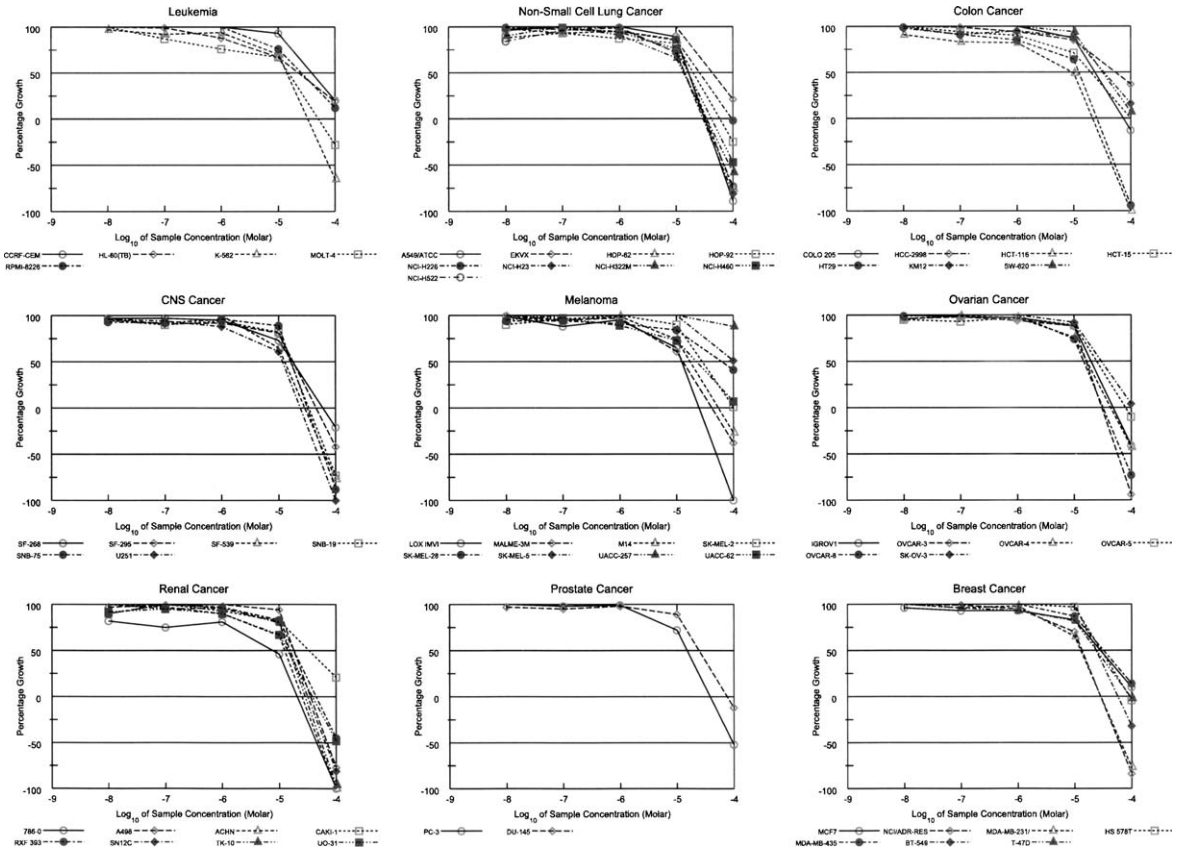


Figure 1. Dose–response curves for the tested human tumor cell lines of compound **5c**.

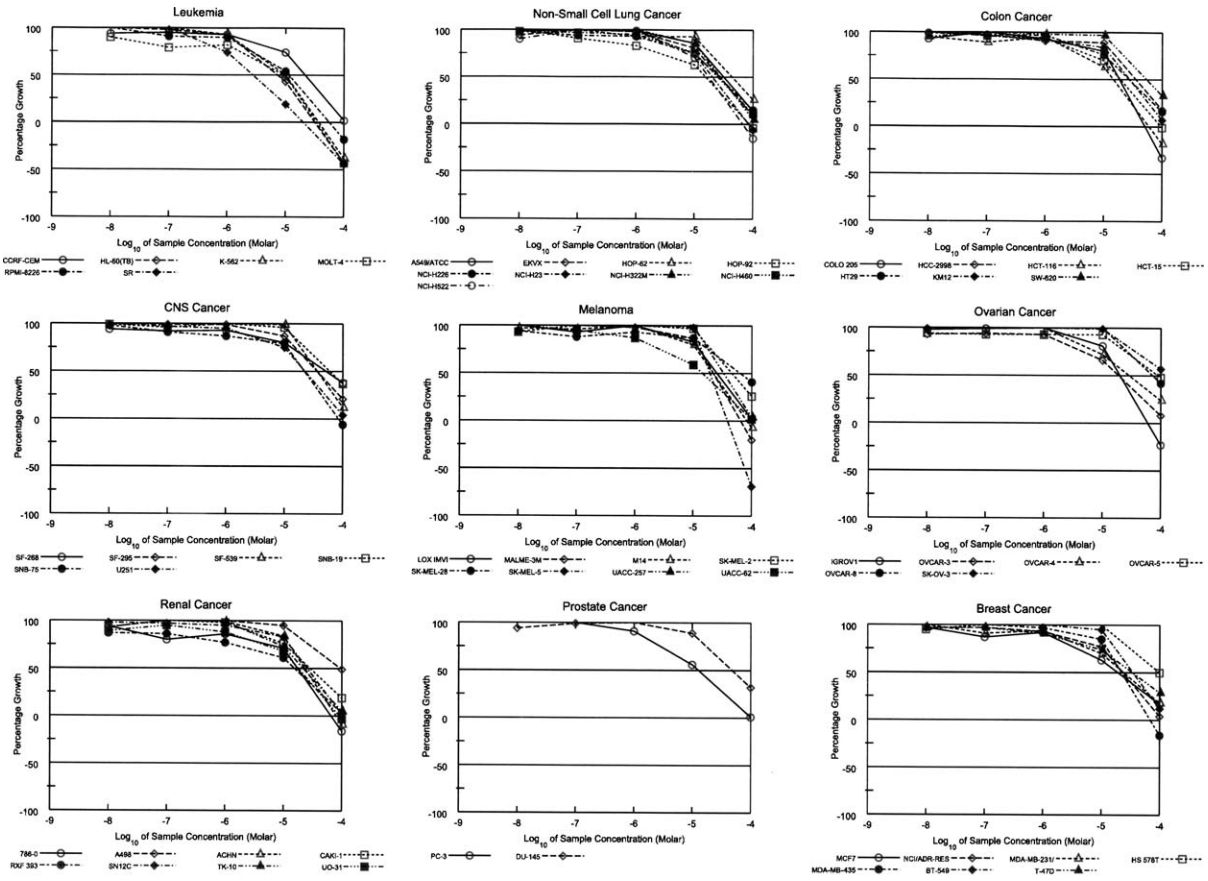


Figure 2. Dose–response curves for the tested human tumor cell lines of compound **7a**.

3. Experimental

Melting points are uncorrected and recorded on an Electrothermal 9100 digital melting point apparatus. IR spectra were recorded (KBr) on a Bruker Vector 22 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian MERCURY 300 (300 MHz). Mass spectra were recorded on a Finnigan SSQ 7000 spectrometer (EI 70 eV). The starting compounds **1a** and **b**,[55] **2a** and **b**[56] were prepared according to the previously reported procedures.

3.1. Synthesis of 2-cyano-3,5-diaryl-5-oxo-N-substituted pentamides (3a–c)

A mixture of equimolar amounts of **1a** and **b** (10 mmol) and the corresponding **2a** and **b** in absolute ethanol (20 ml) containing morpholine (3–5 drops) was boiled under reflux for the appropriate time. The solid separated, while refluxing, was collected and crystallized from a suitable solvent affording the corresponding **3b** and **c**. In case of **3a**, the reaction mixture was evaporated untill dryness under reduced pressure and the remaining residue was triturated with methanol (5 ml). So, the separated solid was collected and crystallized from a suitable solvent giving **3a**.

3.1.1. 3-(4-Chlorophenyl)-2-cyano-5-oxo-5-phenyl-*N*-phenylpentamide (3a). Reaction time 18 h, colorless crystals from ethyl acetate–*n*-hexane mixture as 1:4, v/v, mp 165–167 °C, yield 80%. IR: ν_{max}/cm^{-1} 3299 (NH), 2251 (C≡N), 1687, 1658 (C=O), 1600, 1548 (C=C). ^{1}H NMR ($CDCl_3$): δ 3.57 (dd, 1H, upfield H of CH_2CH, J = 5.1, 17.7 Hz), 3.69 (dd, 1H, downfield H of CH_2CH, J = 8.1, 17.7 Hz), 4.23–4.29 (m, 2H, CH_2CH + CH_2CHCH), 7.10–7.97 (m, 15H, 14 arom. H + NH). Anal. Calcd for $C_{24}H_{19}ClN_2O_2$ (402.863): C, 71.55; H, 4.75; N, 6.96. Found: C, 71.68; H, 4.89; N, 7.22.

3.1.2. 3-(4-Chlorophenyl)-2-cyano-*N*-(4-methylphenyl)-5-oxo-5-phenylpentamide (3b). Reaction time 15 h, colorless crystals from *n*-butanol, mp 201–203 °C, yield 74%. IR: ν_{max}/cm^{-1} 3319 (NH), 2250 (C≡N), 1684 (C=O), 1606, 1542 (C=C). ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 3.45 (dd, 1H, upfield H of CH_2CH, J = 3.9, 17.4 Hz), 3.82 (dd, 1H, downfield H of CH_2CH, J = 9.9, 17.7 Hz), 4.05–4.15 (m, 1H, CH_2CH), 4.36 (d, 1H, CH_2CHCH, J = 8.4 Hz), 7.09–7.95 (m, 13H, arom. H), 10.38 (s, 1H, NH). Anal. Calcd for $C_{25}H_{21}ClN_2O_2$ (416.893): C, 72.02; H, 5.08; N, 6.72. Found: C, 72.22; H, 5.27; N, 6.98.

3.1.3. 2-Cyano-3-(4-methylphenyl)-5-oxo-5-phenyl-*N*-phenylpentamide (3c). Reaction time 20 h, colorless crystals from *n*-butanol, mp 194–196 °C, yield 68%. IR: ν_{max}/cm^{-1} 3322 (NH), 2254 (C≡N), 1691 (C=O), 1606, 1551 (C=C). ^{1}H NMR (DMSO-d_6): δ 2.24 (s, 3H, CH_3), 3.39 (dd, 1H, upfield H of CH_2CH, J = 3.9, 17.1 Hz), 3.77 (dd, 1H, downfield H of CH_2CH, J = 9.3, 17.4 Hz), 3.99–4.07 (m, 1H, CH_2CH), 4.34 (d,

1H, CH_2CHCH, J = 8.7 Hz), 7.09–7.89 (m, 14H, arom. H), 10.46 (s, 1H, NH). Anal. Calcd for $C_{25}H_{22}N_2O_2$ (382.45): C, 78.51; H, 5.80; N, 7.33. Found: C, 78.58; H, 5.84; N, 7.15.

3.2. Synthesis of 2-bromo-4, 6-diaryl-N-substituted-3-pyridinecarboxamides (4a–c)

To a solution of the appropriate **3a**–**c** (5 mmol) in glacial acetic acid (15 ml), heated at 60–70 °C, a solution of bromine (5.5 mmol) in glacial acetic acid (5 ml) was added dropwise while stirring, at such a rate maintaining the same temperature within 1/4 h period. After complete addition, stirring was continued for 3 h at the same temperature. The separated solid was collected and purified on silica gel (60G F_{254}) TLC using chloroform for elution giving **4a**–**c** as colorless crystals.

3.2.1. 2-Bromo-4-(4-chlorophenyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (4a). Mp 237–239 °C, yield 82%. IR: ν_{max}/cm^{-1} 3273, 3246 (NH), 1650 (C=O), 1600, 1547 (C=N, C=C). ^1H NMR (DMSO-d_6): δ 7.10–7.68 (m, 12H, arom. H), 8.08 (s, 1H, pyr. *H*-5), 8.16-8.19 (m, 2H, arom. H), 10.62 (s, 1H, NH). MS: *m*/*z* (%) 466 [(M+4), 2], 464 [(M+2), 8], 462 (M, 6), 371 (13), 370 (62). Anal. Calcd for $C_{24}H_{16}BrClN_2O$ (463.752): C, 62.15; H, 3.48; N, 6.04. Found: C, 62.27; H, 3.57; N, 6.08.

3.2.2. 2-Bromo-4-(4-chlorophenyl)-*N*-(4-methylphenyl)-6-phenyl-3-pyridinecarboxamide (4b). Mp 263–265 °C, yield 80%. IR: ν_{max}/cm^{-1} 3280 (NH), 1653 (C=O), 1602, 1529 (C=N, C=C). ^1H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 7.10–7.67 (m, 11H, arom. H), 8.09 (s, 1H, pyr. *H*-5), 8.16-8.19 (m, 2H, arom. H), 10.56 (s, 1H, NH). MS: *m*/*z* (%) 480 [(M+4), 8], 478 [(M+2), 26], 476 (M, 20), 372 (100), 371 (16), 370 (75). Anal. Calcd for $C_{25}H_{18}BrClN_2O$ (477.772): C, 62.84; H, 3.80; N, 5.86. Found: C, 62.65; H, 3.69; N, 5.71.

3.2.3. 2-Bromo-4-(4-methylphenyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (4c). Mp 258–260 °C, yield 77%. IR: ν_{max}/cm^{-1} 3270, 3242 (NH), 1651 (C=O), 1602, 1550 (C=N, C=C). ^1H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3), 7.07–7.58 (m, 12H, arom. H), 8.04 (s, 1H, pyr. *H*-5), 8.15–8.18 (m, 2H, arom. H), 10.67 (s, 1H, NH). MS: *m*/*z* (%) 444 [(M+2), 14], 442 (M, 14), 351 (22), 350 (100). Anal. Calcd for $C_{25}H_{19}BrN_2O$ (443.329): C, 67.73; H, 4.32; N, 6.32. Found: C, 67.47; H, 4.19; N, 6.25.

3.3. Reaction of 4 with secondary amines

A mixture of **4a**–**c** (5 mmol) and the corresponding secondary amine (10 mmol) in tetrahydrofuran (25 ml) was boiled under reflux for the appropriate time. The reaction mixture was filtered while hot, to remove the secondary amine hydrobromide salt. Then, the mixture was evaporated untill dryness under reduced pressure. The formed solid upon triturating the residual material with methanol (5 ml) was collected and purified on silica gel (60G F_{254}) TLC giving **5a**–**f**.

3.3.1. 4-(4-Chlorophenyl)-6-phenyl-*N*-phenyl-2-(1-piperidinyl)-3-pyridinecarboxamide (5a). Reaction time 40 h, colorless crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:1, v/v, for elution, mp 194–196 °C, yield 69%. IR: ν_{max}/cm^{-1} 3232 (NH), 1643 (C=O), 1597, 1540 (C=N, C=C). ^1H NMR ($CDCl_3$): δ 1.60–1.72 (br m, 6H, pip. 3 CH_2), 3.47 (br s, 4H, pip. 2 NCH_2), 7.11–8.09 (m, 15H, 14 arom. H+pyr. *H*-5), 8.65 (br s, 1H, NH). MS: *m*/*z* (%) 469 [(M+2), 3], 467 (M, 7), 376 (60), 375 (100), 347 (9). Anal. Calcd for $C_{29}H_{26}ClN_3O$ (467.973): C, 74.43; H, 5.60; N, 8.98. Found: C, 74.34; H, 5.57; N, 8.87.

3.3.2. 4-(4-Chlorophenyl)-2-(4-morpholinyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (5b). Reaction time 35 h, colorless crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 5:1, v/v, for elution, mp 223–225 °C, yield 77%. IR: ν_{max}/cm^{-1} 3378 (NH), 1666 (C=O), 1594, 1538 (C=N, C=C). ^1H NMR ($CDCl_3$): δ 3.42 (t, 4H, mor. $2NCH_2$, J = 4.2 Hz), 3.72 (t, 4H, mor. $2OCH_2$, J = 4.2 Hz), 7.06–8.00 (m, 16H, 14 arom. H+pyr. *H*-5+NH). MS: *m*/*z* (%) 471 [(M+2), 4], 469 (M, 9), 378 (42), 377 (100), 349 (11). Anal. Calcd for $C_{28}H_{24}ClN_3O_2$ (469.943): C, 71.56; H, 5.15; N, 8.94. Found: C, 71.35; H, 4.88; N, 9.02.

3.3.3. 4-(4-Chlorophenyl)-*N*-(4-methylphenyl)-6-phenyl-2-(1-piperidinyl)-3-pyridinecarboxamide (5c). Reaction time 50 h, colorless crystals purified by silica gel TLC using chloroform for elution, mp 222–224 °C, yield 67%. IR: ν_{max}/cm^{-1} 3224 (NH), 1640 (C=O), 1596, 1540 (C=N, C=C). ^1H NMR ($CDCl_3$): δ 1.52–1.62 (br m, 6H, pip. 3 CH_2), 2.25 (s, 3H, CH_3), 3.37 (br s, 4H, pip. 2 NCH_2), 7.04–8.01 (m, 14H, 13 arom. H+pyr. *H*-5), 8.35 (br s, 1H, NH). MS: *m*/*z* (%) 483 [(M+2), 2], 481 (M, 2), 376 (33), 375 (100), 347 (5). Anal. Calcd for $C_{30}H_{28}ClN_3O$ (481.993): C, 74.75; H, 5.85; N, 8.72. Found: C, 74.79; H, 5.88; N, 8.82.

3.3.4. 4-(4-Chlorophenyl)-*N*-(4-methylphenyl)-2-(4-morpholinyl)-6-phenyl-3-pyridinecarboxamide (5d). Reaction time 50 h, colorless crystals purified by silica gel TLC using chloroform for elution, mp 231–233 °C, yield 75%. IR: ν_{max}/cm^{-1} 3219 (NH), 1641 (C=O), 1596, 1538 (C=N, C=C). ^1H NMR ($CDCl_3$): δ 2.25 (s, 3H, CH_3), 3.43 (t, 4H, mor. $2NCH_2$, J = 4.2 Hz), 3.72 (t, 4H, mor. $2OCH_2$, J = 4.2 Hz), 7.04–7.99 (m, 15H, 13 arom. H+pyr. *H*-5+NH). MS: *m*/*z* (%) 485 [(M+2), 2], 483 (M, 5), 378 (16), 377 (47), 349 (7). Anal. Calcd for $C_{29}H_{26}ClN_3O_2$ (483.973): C, 71.96; H, 5.42; N, 8.68. Found: C, 72.10; H, 5.50; N, 8.77.

3.3.5. 4-(4-Methylphenyl)-6-phenyl-*N*-phenyl-2-(1-piperidinyl)-3-pyridinecarboxamide (5e). Reaction time 60 h, colorless crystals purified by silica gel TLC using chloroform for elution, mp 221–223 °C, yield 71%. IR: ν_{max}/cm^{-1} 3379 (NH), 1679 (C=O), 1594, 1522 (C=N, C=C). ^1H NMR ($CDCl_3$): δ 1.64–1.74 (br m, 6H, pip. 3 CH_2), 2.40 (s, 3H, CH_3), 3.49 (br s, 4H, pip. 2 NCH_2), 7.11–8.11 (m, 15H, 14 arom. H+pyr. *H*-5), 8.50 (br s, 1H, NH). MS: *m*/*z* (%) 447 (M, 3), 356

(23), 355 (100), 327 (5). Anal. Calcd for $C_{30}H_{29}N_3O$ (447.55): C, 80.50; H, 6.53; N, 9.39. Found: C, 80.74; H, 6.72; N, 9.61.

3.3.6. 4-(4-Methylphenyl)-2-(4-morpholinyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (5f). Reaction time 55 h, colorless crystals purified by silica gel TLC using chloroform for elution, mp 220–222 °C, yield 76%. IR: ν_{max}/cm^{-1} 3377 (NH), 1666 (C=O), 1573, 1538 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.40 (s, 3H, CH_3), 3.55 (br s, 4H, mor. 2NCH_2), 3.84 (br s, 4H, mor. 2OCH_2), 7.12–8.09 (m, 16H, 14 arom. H+pyr. *H*-5+NH). MS: *m*/*z* (%) 449 (M, 8), 358 (27), 357 (100), 329 (4). Anal. Calcd for $C_{29}H_{27}N_3O_2$ (449.53): C, 77.48; H, 6.06; N, 9.35. Found: C, 77.45; H, 6.01; N, 9.51.

3.4. Reaction of 4a–c with aromatic amines

A solution of **4a**–**c** (5 mmol) and the corresponding primary aromatic amine (10 mmol) in pyridine (20 ml) was boiled under reflux for the appropriate time. The solid separated upon pouring the reaction mixture into water (250 ml) and acidification with dil HCl (5%), was collected and washed with water. Then, it was purified on silica gel (60G F_{254}) TLC affording **6a**–**i** and **7a**–**c**.

3.4.1. 4-(4-Chlorophenyl)-6-phenyl-*N*-phenyl-2-(phenylamino)-3-pyridinecarboxamide (6a). Reaction time 75 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:1, v/v, for elution, mp 200–202 °C, yield 29%. IR: ν_{max}/cm^{-1} 3418 (NH), 1663 (C=O), 1621, 1529 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 7.02-8.11 (m, 21H, 19 arom. H+pyr. *H*-5+NH), 9.42 (s, 1H, NH). MS: *m*/*z* (%) 477 [(M+2), 19], 475 (M, 50), 384 (38), 383 (100), 355 (17), 354 (17), 353 (39). Anal. Calcd for $C_{30}H_{22}ClN_3O$ (475.953): C, 75.70; H, 4.66; N, 8.83. Found: C, 75.47; H, 4.48; N, 8.94.

3.4.2. 4-(4-Chlorophenyl)-2-[(4-methylphenyl)amino]-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (6b). Reaction time 65 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:1, v/v, for elution, mp 217–219 °C, yield 37%. IR: ν_{max}/cm^{-1} 3409, 3324 (NH), 1661 (C=O), 1614, 1519 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.36 (s, 3H, CH_3), 6.92 (br s, 1H, NH), 7.08–8.10 (m, 19 H, 18 arom. H+pyr. *H*-5), 9.28 (br s, 1H, NH). MS: *m*/*z* (%) 491 [(M+2), 23], 489 (M, 54), 398 (50), 397 (100), 369 (18), 368 (17), 367 (34). Anal. Calcd for $C_{31}H_{24}ClN_3O$ (489.973): C, 75.99; H, 4.94; N, 8.58. Found: C, 75.93; H, 4.92; N, 8.53.

3.4.3. 4-(4-Chlorophenyl)-2-[(4-methoxyphenyl)amino]-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (6c). Reaction time 60 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 2:1, v/v, for elution, mp 193–195 °C, yield 63%. IR: ν_{max}/cm^{-1} 3369, 3250 (NH), 1635 (C=O), 1600, 1508 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 3.73 (s, 3H, OCH_3), 6.80–7.98 (m, 20 H, 18 arom. H+pyr. *H*-5+NH), 9.15 (br s, 1H, NH). MS: *m*/*z* (%) 507 [(M+2), 23], 505 (M, 59), 414 (42), 413 (65), 412 (100), 385 (5), 384 (7), 383 (12). Anal. Calcd for $C_{31}H_{24}ClN_3O_2$ (505.973): C, 73.58; H, 4.78; N, 8.30. Found: C, 73.73; H, 4.95; N, 8.37.

3.4.4. 4-(4-Chlorophenyl)-2-[(4-chlorophenyl)amino]-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (6d). Reaction time 75 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:1, v/v, for elution, mp 209–211 °C, yield 18%. IR: ν_{max}/cm^{-1} 3417 (NH), 1660 (C=O), 1614, 1532 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 6.90 (br s, 1H, NH), 7.07–8.08 (m, 19 H, 18 arom. H+pyr. *H*-5), 9.44 (br s, 1H, NH). MS: *m*/*z* (%) 513 [(M+4), 7], 511 [(M+2), 34], 509 (M, 45), 418 (69), 417 (100), 389 (10), 388 (8), 387 (11). Anal. Calcd for $C_{30}H_{21}Cl_2N_3O$ (510.40): C, 70.59; H, 4.15; N, 8.23. Found: C, 70.81; H, 4.24; N, 8.37.

3.4.5. 4-(4-Chlorophenyl)-*N*-(4-methylphenyl)-2-[(4-methylphenyl)amino]-6-phenyl-3-pyridinecarboxamide (6e). Reaction time 50 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:1, v/v, for elution, mp 203–205 °C, yield 40%. IR: ν_{max}/cm^{-1} 3414, 3323 (NH), 1659 (C=O), 1612, 1514 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 6.76 (br s, 1H, NH), 6.85–8.00 (m, 18 H, 17 arom. H+pyr. *H*-5), 9.17 (br s, 1H, NH). MS: *m*/*z* (%) 505 [(M+2), 18], 503 (M, 54), 398 (39), 397 (100), 369 (7), 368 (8), 367 (19). Anal. Calcd for $C_{32}H_{26}ClN_3O$ (504.003): C, 76.25; H, 5.20; N, 8.34. Found: C, 76.33; H, 5.31; N, 8.18.

3.4.6. 4-(4-Chlorophenyl)-2-[(4-methoxyphenyl)amino]-*N*-(4-methylphenyl)-6-phenyl-3-pyridinecarboxamide (6f). Reaction time 45 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 20:1, v/v, for elution, mp 179–181 °C, yield 71%. IR: ν_{max}/cm^{-1} 3371, 3275 (NH), 1635 (C=O), 1600, 1507 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3), 3.74 (s, 3H, OCH_3), 6.76 (br s, 1H, NH), 6.81–7.98 (m, 18H, 17 arom. H+pyr. *H*-5), 9.08 (br s, 1H, NH). MS: *m*/*z* (%) 521 [(M+2), 18], 519 (M, 70), 414 (31), 413 (48), 412 (100), 385 (3), 384 (4), 383 (6). Anal. Calcd for $C_{32}H_{26}ClN_3O_2$ (520.003): C, 73.91; H, 5.04; N, 8.08. Found: C, 73.74; H, 4.96; N, 8.19.

3.4.7. 4-(4-Chlorophenyl)-2-[(4-chlorophenyl)amino]-*N*-(4-methylphenyl)-6-phenyl-3-pyridinecarboxamide (6g). Reaction time 70 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:1, v/v, for elution, mp 209–211 °C, yield 14%. IR: ν_{max}/cm^{-1} 3421 (NH), 1658 (C=O), 1612, 1517 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3), 6.74 (br s, 1H, NH), 6.85–7.98 (m, 18 H, 17 arom. H+pyr. *H*-5), 9.32 (br s, 1H, NH). MS: *m*/*z* (%) 527 [(M+4), 5], 525 [(M+2), 28], 523 (M, 54), 418 (50), 417 (100), 389 (6), 388 (4), 387 (8). Anal. Calcd for $C_{31}H_{23}Cl_2N_3O$ (524.42): C, 70.99; H, 4.42; N, 8.01. Found: C, 70.73; H, 4.24; N, 7.97.

3.4.8. 4-(4-Methylphenyl)-2-[(4-methylphenyl)amino]-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (6h). Reaction time 50 h, yellow crystals purified by silica gel TLC

using chloroform–light petroleum (60–80 °C) mixture as 1:1, v/v, for elution, mp 174–176 °C, yield 43%. IR: ν_{max}/cm^{-1} 3407, 3321 (NH), 1659 (C=O), 1614, 1520 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 6.88 (br s, 1H, NH), 7.02–8.13 (m, 19 H, 18 arom. H+pyr. *H*-5), 9.46 (br s, 1H, NH). MS: *m*/*z* (%) 469 (M, 85), 378 (28), 377 (100), 349 (6), 348 (17), 347 (48). Anal. Calcd for $C_{32}H_{27}N_3O$ (469.56): C, 81.85; H, 5.80; N, 8.95. Found: C, 81.79; H, 5.78; N, 8.82.

3.4.9. 2-[(4-Chlorophenyl)amino]-4-(4-methylphenyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (6i). Reaction time 60 h, pale yellow crystals purified by silica gel TLC using chloroform–light petroleum (60–80 °C) mixture as 3:2, v/v, for elution, mp 185–187 °C, yield 27%. IR: ν_{max}/cm^{-1} 3411 (NH), 1658 (C=O), 1613, 1529 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.43 (s, 3H, CH_3), 6.90 (s, 1H, NH), 7.02–8.10 (m, 19 H, 18 arom. H+pyr. *H*-5), 9.62 (s, 1H, NH). MS: *m*/*z* (%) 491 [(M+2), 20], 489 (M, 36), 398 (42), 397 (78), 396 (100), 369 (6), 368 (10), 367 (13). Anal. Calcd for $C_{31}H_{24}ClN_3O$ (489.973): C, 75.99; H, 4.94; N, 8.58. Found: C, 76.18; H, 4.98; N, 8.66.

3.4.10. 2-Amino-4-(4-chlorophenyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (7a). Colorless crystals, mp 198–200 °C, yield 20%, 15%, 11%, and 30% (during preparation of **6a**, **b**, **c**, and **d**, respectively). IR: ν_{max}/cm^{-1} 3490, 3391, 3268 (NH_2, NH), 1646 (C=O), 1599, 1544 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 6.15 (br s, 2H, NH_2), 6.97–7.91 (m, 16H, 14 arom. H+pyr. *H*-5+NH). MS: *m*/*z* (%) 401 [(M+2), 10], 399 (M, 24), 308 (21), 307 (100). Anal. Calcd for $C_{24}H_{18}ClN_3O$ (399.853): C, 72.09; H, 4.54; N, 10.51. Found: C, 72.34; H, 4.65; N, 10.70.

3.4.11. 2-Amino-4-(4-chlorophenyl)-*N*-(4-methylphenyl)-6-phenyl-3-pyridinecarboxamide (7b). Colorless crystals, mp 177–179 °C, yield 15%, 10%, and 39% (during preparation of **6e**,**f**, and **g**, respectively). IR: ν_{max}/cm^{-1} 3482, 3382, 3316 (NH_2, NH), 1639 (C=O), 1594, 1515 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3), 6.08 (br s, 2H, NH_2), 6.90–7.90 (m, 15H, 13 arom. H+pyr. *H*-5+NH). MS: *m*/*z* (%) 415 [(M+2), 12], 413 (M, 40), 308 (20), 307 (100). Anal. Calcd for $C_{25}H_{20}ClN_3O$ (413.883): C, 72.54; H, 4.87; N, 10.15. Found: C, 72.45; H, 4.86; N, 10.28.

3.4.12. 2-Amino-4-(4-methylphenyl)-6-phenyl-*N*-phenyl-3-pyridinecarboxamide (7c). Colorless crystals, mp 201–203 °C, yield 16% and 36% (during preparation of **6h** and **i**, respectively). IR: ν_{max}/cm^{-1} 3491, 3386, 3267 (NH_2, NH), 1643 (C=O), 1599, 1533 (C=N, C=C). ^{1}H NMR ($CDCl_3$): δ 2.41 (s, 3H, CH_3), 6.13 (s, 2H, NH_2), 6.96 (s, 1H, NH), 7.04–8.02 (m, 15H, 14 arom. H+pyr. *H*-5). MS: *m*/*z* (%) 379 (M, 27), 288 (23), 287 (100). Anal. Calcd for $C_{25}H_{21}N_3O$ (379.44): C, 79.13; H, 5.58; N, 11.07. Found: C, 78.96; H, 5.45; N, 11.01.

3.5. Antitumor activity

Antitumor activity screening for the synthesized nicotinamides utilizing 59 different human tumor cell lines, representing leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate as well as kidney, was carried out according to the previously reported standard procedure.[52–54] The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96-well-microtiter plates in 100 μl at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental test compounds. After 24 h, two plates of each cell lines are fixed in situ with trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of tested compound addition (time zero, T_z). Experimental tested compounds are solubilized in dimethylsulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of the tested compound addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Additional four, 10-fold or 1/2 log serial dilutions are made to provide a total of five tested compound concentrations (10^{-4} to 10^{-8} M concentrations) plus control. Aliquots of 100 μl of these different tested compound dilutions are added to the appropriate microtiter wells already containing 100 μl of medium, resulting in the required final concentrations.

Following the tested compound addition, the plates are incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and the plates are washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air-dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate readed at a wavelength of 515 nm. For cells suspension the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 μl of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero (T_z), control growth (C) and test growth in the presence of the tested compound at the five concentration levels (T_i)], the percentage growth is calculated at each of the tested compound concentration levels.

Percentage growth inhibition is calculated as $[(T_i - T_z)/(C - T_z)] \times 100$ for concentrations for which $T_i \geqslant T_z$. $[(T_i - T_z)/T_z] \times 100$ for concentrations for which $T_i < T_z$.

Growth inhibition of 50% (GI_{50}) is calculated from $[(T_i - T_z)/(C - T_z)] \times 100 = 50$, which is the tested com-

pound concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation (Table 1).

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