Synthesis and Anticancer Evaluation of Some New 4-Amino-3-(*p*-methoxybenzyl)-4,5-dihydro-1,2,4-triazole-5-one Derivatives

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4-Amino-3-*p*-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid ethyl ester (**2**) was prepared from 4-amino-4,5-dihydro-1*H*-1,2,4-triazole-5-one (**1**) and ethyl bromoacetate. Compound **3** was synthesized by the condensation of **2** with hydrazine hydrate. The treatment of compound **3** with various aromatic aldehydes resulted in the formation of arylidene hydrazides as *cis-trans* conformers **4a** – **g**. Thiosemicarbazide derivative **5** was prepared by the reaction of compound **3** with phenylisothiocyanate. Cyclization of **5** with sodium hydroxide resulted in the formation of compound **6**. Treatment of **6** with benzyl bromide gave compound **7**. Four of the newly synthesized compounds were screened for their anticancer activity against a panel of 60 cell lines derived from nine cancer types, namely, non-small cell lung, colon, breast, ovarian, leukemia, renal, melanoma, prostate and CNS cancers at a fixed dose of 10 μM.

Key words: 1,2,4-Triazoles, *E*/*Z* Geometrical Isomers, *cis/trans* Amide Conformers, Benzylidenehydrazides, Anticancer Activity

Introduction

Cancer has always been among the most incurable diseases, and man has been prone to this deadly illness for ages. Even today, we lack the effective medicine or treatment that can be applied to most cancer sufferers. Thus, the quest for certain effective and less harmful cancer drugs is the priority of science [1].

It was reported that compounds having triazole moieties, such as vorozole, letrozole and anastrozole appeared to be very effective aromatase inhibitors, which in turn prevented breast cancer [2 – 4]. Some 1,2,4-triazole compounds are considered interesting heterocycles since they possess important pharmacological activities such as antifungal and antiviral activities. For example, fluconazole [5], itraconazole [6], ravuconazole [7], ICI 153066 [8], posaconazole [9], and voriconazole [10] are antifungal drugs. Furthermore, certain 1,2,4-triazole derivatives have been reported as anticonvulsants [11], antimicrobial [12], antimalarial [13], antihypertensive [14], analgesic [15], antiviral [16], antiinflammatory [17], antioxidant [18, 19], antitumor [20, 21], anti HIV [22], fungicidal [23], insecticidal [24], herbicidal [25], and pesticidal agents [26]. In addition to these, *N*-benzylidene derivatives of acid hydrazides are also associated with diverse biological activities [27, 28]. In an attempt to obtain new compounds with possible anticancer properties, some novel 1,2,4-triazole-5-one derivatives were designed and synthesized, and their anticancer properties were evaluated.

Results and Discussion

Chemistry

The synthetic pathway leading to the title compounds is given in Scheme 1. The starting compound, 3-(4-methoxybenzyl)-4-amino-4,5-dihydro-1,2,4-triazole-5-one (**1**), was prepared according to the literature [29]. Treatment of **1** with ethyl bromoacetate in Na/absolute ethanol gave 1,2,4-triazol-1-yl-acetic acid ethyl ester **2**. The IR spectrum of compound **2** showed absorption bands at 3291 and 3210 cm^{-1} corresponding to the NH_2 and at 1755 and 1709 cm^{-1} corresponding to ester C=O and triazole C=O groups, respectively. 1,2,4-Triazol-1-yl-acetic acid hydrazide **3** was prepared by the reaction of **2** and hydrazine hydrate in butanol.

Compound **3** was condensed with various aromatic aldehydes in ethanol to give the corresponding Schiff

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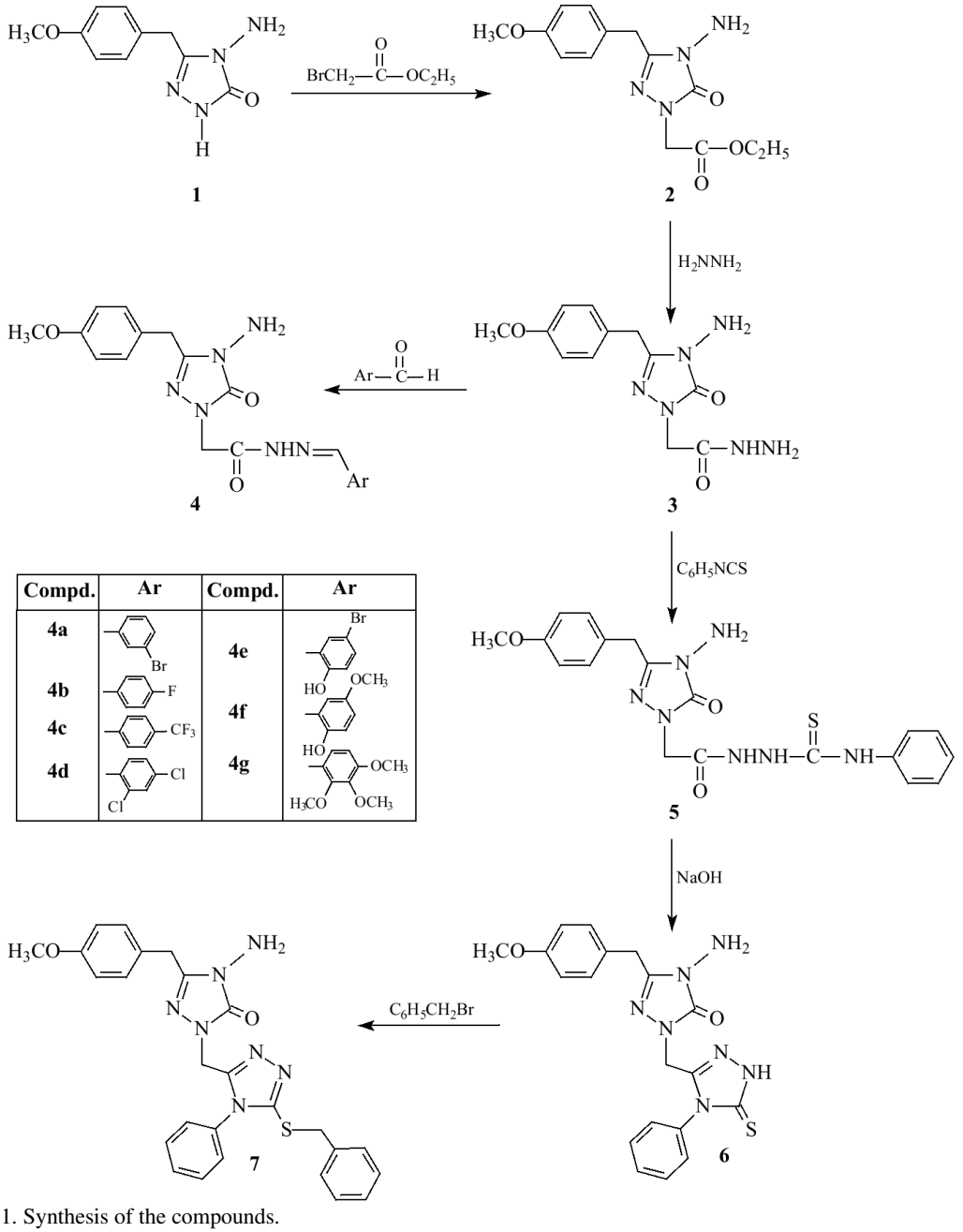


Compd.	Ar	Compd.	Ar
4a	4-Br	4e	3-Br, 4-HO, 5-OCH_3
4b	4-F	4f	2-HO, 3-OCH_3
4c	4-CF_3	4g	2-HO, 3-OCH_3, 4-H_3CO, 5-OCH_3
4d	2-Cl, 4-Cl		

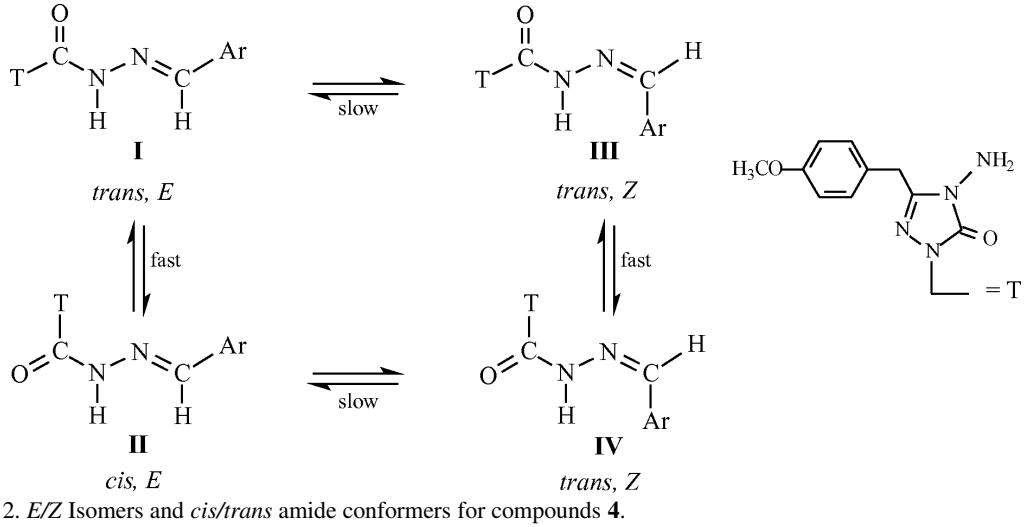
Scheme 1. Synthesis of the compounds.

bases **4a – g** in very good yield. In the ^{1}H NMR spectra of compounds **4**, NCH_2, N=CH, and NH proton signals were recorded as double singlets, and in the ^{13}C NMR spectra of these compounds NCH_2, triazole C-3, triazole C-5, and N=CH carbon signals were also recorded as double peaks. According to the literature, *N*-benzylidene derivatives of acid hydrazides may exist as *E*/*Z* geometrical isomers about C=N double bonds and *cis/trans* amide conformers (Scheme 2) [30 – 33]. It is known that the N=CH double bond restricts rotation and gives rise to the formation of *E* and *Z* isomers with the *E* isomer dominating, and that the equilibration rate is rather low [30 – 33]. It has been reported that when acid

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts and percentage of *cis/trans* conformers[a].

Compound	Conformer	NMR	NCH_2	N=CH	OH	NH	Triazole C-3	Triazole C-5	Percentage of *trans/cis*
4a	*trans*	H	4.84	7.95	–	11.72	–	–	71.4
		C	47.24	143.01	–	–	147.81	154.38	
	cis	H	4.42	8.15	–	11.77	–	–	28.7
		C	47.64	146.19	–	–	148.10	164.07	
4b	*trans*	H	4.82	7.97	–	11.64	–	–	72.7
		C	46.33	142.68	–	–	146.96	153.53	
	cis	H	4.40	8.17	–	11.64	–	–	27.3
		C	46.67	146.00	–	–	147.21	163.00	
4c	*trans*	H	4.88	8.07	–	11.86	–	–	70.9
		C	46.39	142.21	–	–	147.08	153.56	
	cis	H	4.46	8.28	–	11.86	–	–	29.2
		C	46.87	146.77	–	–	147.32	163.38	
4d	*trans*	H	4.85	8.31	–	11.83	–	–	73.2
		C	46.39	138.81	–	–	147.07	153.54	
	cis	H	4.42	8.52	–	11.91	–	–	26.9
		C	46.92	142.05	–	–	147.34	163.29	
4e	*trans*	H	4.84	8.22	10.37	11.63	–	–	61.0
		C	46.45	139.10	–	–	146.94	153.57	
	cis	H	4.44	8.37	11.02	11.93	–	–	39.0
		C	46.85	144.76	–	–	147.36	163.11	
4f	*trans*	H	4.81	8.26	9.56	11.53	–	–	56.8
		C	46.40	140.84	–	–	146.95	153.54	
	cis	H	4.41	8.39	10.38	11.80	–	–	43.2
		C	46.77	146.65	–	–	147.31	162.90	
4g	*trans*	H	4.79	8.16	–	11.50	–	–	69.6
		C	46.36	141.37	–	–	146.98	153.58	
	cis	H	4.37	8.35	–	11.59	–	–	30.4
		C	46.85	142.66	–	–	147.26	162.68	

[a] 200 MHz spectrometer; all spectra in [D_6]DMSO.



Scheme 2. *E/Z* Isomers and *cis/trans* amide conformers for compounds **4**.

hydrazide derivatives are dissolved in polar solvents such as [D_6]DMSO, the geometrical *E* isomers of these compounds undergo a rapid *cis/trans* amide equilibrium, in which the *trans* conformer predominates [30 – 33]. The *E* isomers and the *cis/trans* conformer ratios can easily be determined by ^{1}H NMR integration.

The chemical shift values of *cis/trans* conformers belonging to protons of NCH_2, N=CH, OH, and NH in the ^{1}H NMR and to carbons of NCH_2, triazole C-3, tri-

azole C-5, and N=CH in the ^{13}C NMR spectra of compounds **4** and the percentage ratios of *cis/trans* conformers are given in Table 1. These data prove the *E* isomers and *trans* conformer structures (**I**) to be dominant forms among the four possible structures [30–33]. The proton signals of N=CH, NH, and OH of the *trans* conformer are found at higher field than those of the *cis* conformer. In contrast, the N-CH_2 proton signal of the *trans* conformer is found downfield compared to the *cis* conformer, because of steric hindrance [30].

The condensation of the acid hydrazide **3** with phenyl isothiocyanate resulted in the formation of thiosemicarbazide **5**. The IR spectra of **5** exhibited a strong C=S absorption at 1246 cm^{-1}. The ^{13}C NMR signal of this group was observed at δ = 180.71 ppm. The ^{1}H NMR spectrum displayed three singlets due to three different –NH groups at δ = 9.61, 9.73 and 10.29 ppm. Cyclization of **5** with sodium hydroxide resulted in the formation of 1-(5-thioxo-1,2,4-triazol-3-yl)-5-oxo-4,5-dihydro-1,2,4-triazole **6**. The IR spectrum of compound **6** showed a strong C=S absorption at 1358 cm^{-1}, and the ^{13}C NMR signal of this group was observed at δ = 168.14 ppm. The IR spectrum of **6** displayed bands at 3448 and 1358 cm^{-1} associated with the N-H and C=S functionalities. In the ^{1}H NMR spectrum of compound **6**, the NH signal was observed as a singlet at δ = 13.90 ppm. Therefore, the compound was proven to be in the thionic form [34]. Treatment of **6** with benzyl bromide in Na/absolute ethanol gave the S-benzyl derivative **7**. The ^{1}H NMR spectrum of compound **7** showed no signal belonging to an NH group. Instead, the S-CH_2 proton signals appeared at δ = 4.88 ppm as a singlet, and the S-CH_2 carbon signal at δ = 35.98 ppm.

Anticancer activity

The tumor growth inhibition properties of compounds **2**, **3**, **6**, and **7** with the NCI codes NSC 741870, NSC 741871, NSC 741872, and NSC 741873, selected among compounds **1**–**7** by the National Cancer Institute (NCI), USA, were screened on 60 human tumor cell lines, derived from nine cancer types, namely, non-small cell lung, colon, breast, ovarian, leukemia, renal, melanoma, prostate, and CNS cancers [35, 36]. The results are expressed as percentage growth (PG) at the single dose of test compounds of 10 μM. Smaller values represent better growth inhibitory activity. Negative values show the tumoricidal activity. The compounds, in general, showed mild antiproliferative activity on the whole cell panel, although they did not prove cytotoxic or cytostatic at the tested concentration (10 μM). The screening data are presented in Table 2.

Table 2. Sixty cell line *in vitro* anticancer screening (Percent Growth, PG) results for compounds **2**, **3**, **6** and **7**.

Panel/cell line	Percent growth[a]				
	2	**3**	**6**	**7**	Mean
Non-small cell lung Cancer					
A549/ATCC	114.67	106.98	100.43	113.72	109
EKVX	86.90	96.77	110.39	89.65	96
HOP-62	108.56	104.23	114.40	97.75	106
HOP-92	55.63	54.55	–	47.17	52
NCI-H226	103.39	97.73	101.21	94.39	99
NCI-H23	101.48	103.85	102.94	96.20	101
NCI-H322M	110.20	121.03	95.83	83.27	103
NCI-H460	139.00	121.41	119.90	119.78	125
NCI-H522	110.51	98.48	102.39	82.88	99
	103.37	100.56	105.94	91.65	100.2[b]
Colon cancer					
COLO 205	107.29	113.97	121.36	114.60	114
HCC-2998	104.55	107.00	131.84	94.77	110
HCT-116	98.71	108.04	108.19	99.94	104
HCT-15	101.82	105.47	100.23	110.19	104
HT29	109.21	109.69	106.34	111.44	109
KM12	96.37	114.76	99.61	95.44	102
SW-620	101.57	111.59	111.10	111.61	109
Mean:	102.79	110.07	111.24	105.43	107.4[b]
Breast cancer					
BT-549	129.99	107.96	137.86	94.39	118
HS 578T	115.44	116.39	112.05	122.04	116
MCF7	130.23	144.87	120.12	129.31	131
MDA-MB-231/ATCC	99.38	93.23	102.10	95.17	97
MDA-MB-435	119.70	108.70	109.98	94.85	108
NCI/ADR-RES	99.00	106.22	99.72	99.58	101
T-47D	104.46	110.88	103.06	110.94	107
Mean:	114.03	112.61	112.13	106.61	111.3[b]
Ovarian cancer					
IGROV1	120.68	120.40	112.47	30.37	96
OVCAR-3	106.67	112.65	114.79	106.21	110
OVCAR-4	113.36	107.26	122.22	118.54	115
OVCAR-5	91.67	97.17	91.41	96.08	94
OVCAR-8	102.44	104.88	105.41	104.24	104
SK-OV-3	103.79	127.62	106.23	108.51	112
Mean:	106.44	111.66	108.76	93.99	105.2[b]
Leukemia					
CCRF-CEM	66.95	−13.82	−66.63	73.73	15
HL-6O(TB)	114.26	101.95	–	–	108
K-562	93.13	82.05	80.60	–	85
MOLT-4	93.41	109.27	118.54	–	107
RPMI-8226	85.88	70.12	75.16	68.53	75
SR	4.45	92.55	–	–	49
Mean:	76.35	73.69	51.92	71.13	69.5[b]

The performances of the four test compounds were evaluated individually on each cell type as well as all cell lines. The sensitivity orders of the cancer cell types to individual compounds were in the following orders

Table 2 (continued).

Panel/cell line	Percent growth[a]				
	2	**3**	**6**	**7**	Mean
Renal cancer					
786-0	108.53	103.92	110.48	98.53	105
A498	130.75	115.06	124.05	106.33	119
ACHN	104.97	110.18	104.89	113.93	108
CAKI-I	76.07	99.87	106.26	90.42	93
RXF393	111.03	102.18	103.19	112.18	107
SN12C	94.31	93.22	95.54	103.54	97
TK10	116.48	117.49	103.60	115.12	113
UO-31	81.24	91.20	95.92	78.83	87
Mean:	102.92	104.14	105.49	102.36	103.7[b]
Melanoma					
LOX IMVI	93.86	92.31	93.93	101.73	95
M14	109.69	119.63	125.46	108.83	116
MALME-3M	114.42	142.34	100.12	93.23	113
SK-MEL-2	105.68	103.51	100.53	100.31	103
SK-MEL-28	116.19	115.78	125.39	124.19	120
SK-MEL-5	106.93	104.65	108.61	102.10	106
UACC-257	107.82	103.71	107.31	106.32	106
UACC-62	93.79	93.32	97.14	92.37	94
Mean:	106.05	109.41	107.31	103.64	106.6[b]
Prostate cancer					
DU-145	130.53	110.11	135.38	99.34	119
PC-3	89.38	101.56	119.36	86.31	99
Mean:	109.96	105.84	127.37	92.83	109.0[b]
CNS cancer					
SF-268	165.75	108.21	114.75	110.93	125
SF-295	111.88	100.50	107.52	94.17	104
SF-539	91.16	85.94	103.43	94.88	94
SNB-19	99.85	107.86	103.18	104.04	104
SNB-75	104.27	100.36	107.55	107.78	105
U251	101.03	102.03	96.28	100.52	100
Mean:	112.32	100.82	105.45	102.05	105.2[b]
Overall mean:	103.57	103.40	104.66	99.29	

[a] Percent growth values represent the percentage of the cell growth with respect to the initial cell count at a single compound concentration (10 μM); [b] these values are averages of all the percent growth values for all four test compounds but for a single organ specific cancers, *e. g.* non-small cell lung cancers.

(average PG values are provided in parantheses):

Compound **2**: leukemia (76.35) > colon cancer (102.79) > renal cancer (102.92) > non-small cell lung cancer (103.37) > melanoma (106.05) > ovarian cancer (106.44) > prostate cancer (109.96) > CNS cancer (112.32) > breast cancer (114.03).

Compound **3**: leukemia (73.69) > non-small cell lung cancer (100.56) > CNS cancer (100.82) > renal cancer (104.14) > prostate cancer (105.84) > melanoma (109.41) > colon cancer (110.07) > ovarian cancer (111.66) > breast cancer (112.61).

Compound **6**: leukemia (51.92) > CNS cancer (105.45) > renal cancer (105.49) > non-small cell lung cancer (105.94) > melanoma (107.31) > ovarian cancer (108.76) > colon cancer (111.24) > breast cancer (112.13) > prostate cancer (127.37).

Compound **7**: leukemia (71.13) > non-small cell lung cancer (91.65) > prostate cancer (92.83) > ovarian cancer (93.99) > CNS cancer (102.05) > renal cancer (102.36) > melanoma (103.64) > colon cancer (105.43) > breast cancer (106.61).

The overall sensitivity order of the cell types to all four test compounds is leukemia (69.5) > non-small cell lung cancer (100.2) > renal cancer (103.7) > CNS cancer (105.16) > ovarian cancer (105.21) > melanoma (106.6) > colon cancer (107.4) > prostate cancer (109.0) > breast cancer (111.3).

The order of overall effectiveness of the test compounds against all tested cell lines is **7** (99.26) > **3** (103.40) > **2** (103.57) > **6** (104.66).

The highest antiproliferative activity was observed against the CCRF-CEM leukemia cell line with average PG value of 15. Tumoricidal activity was observed only with compounds **3** and **6** against CCRF-CEM cell line with PG values of −13.82 and −66.63, respectively. Because all four compounds showed promising antitumor potential against the CCRF-CEM leukemia cell line among all cancer cell lines, and compounds **3** and **6** were even tumoricidal, synthesis and anticancer evaluation of structural analogs and various derivatives of these compounds deserve attention.

Conclusion

In conclusion, a series of 4-amino-3-(*p*-methoxybenzyl)-4,5-dihydro-1,2,4-triazole-5-one derivatives was synthesized as potential anticancer agents. Benzylidenehydrazide derivatives **4** appear as both configurational (*E*/*Z*) and conformational (*cis/trans*) isomers. The *E* geometrical isomers and *trans* amide conformers are dominant forms based on ^{1}H and ^{13}C NMR data. Compounds **2**, **3**, **6**, and **7** were selected for a full 60-cell panel anticancer screening at a fixed dose of 10 μM, where they showed mild activity. The highest activity was observed against leukemia cell types, with the lowest PG values. The leukemia subtype CCRF-CEM was the most susceptible to anticancer activity of the test compounds, and compounds **3** and **6** were even tumoricidal against this cell line with negative PG values. The synthesis of new analogs or derivatives of these compounds and testing of their anticancer activity especially against leukemia cancers should provide explanation in terms

of structure – activity relationships and will probably yield better anticancer compounds.

Experimental Section

Chemistry

All chemicals used in this study were of high purity and purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Melting points were determined on a Büchi oil heated melting point apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra (δ, ppm) were recorded on a Varian-Mercury 200 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) using tetramethylsilane as an internal reference. The IR spectra (λ, cm^{-1}) were run on a Perkin-Elmer 1600 FTIR spectrophotometer (Perkin-Elmer, Beaconfields, England) by using KBr pellets. Microanalyses were performed on a Carlo Erba 1106 elemental analyzer (Carlo Erba, Milan, Italy). Starting compound **1** was synthesized by a published method [29].

*Synthesis of 4-amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid ethyl ester (**2**)*

A mixture of **1** (0.01 mol) and Na (0.01 mol) was refluxed in absolute ethanol for 2 h. Then, ethyl bromoacetate (0.01 mol) was added and refluxed for an additional 6 h. After evaporating, the solid was recyrstallized from ethanol-water (1 : 2). M. p. 97 – 98 °C. Yield 65 %. – IR (KBr): ν = 3291, 3210 (NH_2), 1755 (ester C=O), 1709 (triazole C=O), 1654 (C=N), 1240 (C–O) cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 1.22 (t, 3H, J = 6.0 Hz, CH_3), 3.75 (s, 3H, OCH_3), 3.85 (s, 2H, benzyl CH_2), 4.18 (q, 2H, J = 6.0 Hz, OCH_2), 4.55 (s, 2H, NCH_2), 5.39 (s, 2H, NH_2), Ar-H [6.90 (d, J = 7.4 Hz), 7.22 (d, J = 7.4 Hz)]. – ^{13}C NMR ([D_6]DMSO): δ = 13.90 (CH_3), 29.35 (benzyl CH_2), 46.42 (NCH_2), 54.91 (OCH_3), 60.94 (OCH_2), Ar-C: [113.70 (2C), 127.43, 129.72 (2C), 157.92], 147.53 (triazole C-3), 153.17 (triazole C-5), 167.86 (ester C=O). – Anal. for $C_{14}H_{18}N_4O_4$ (306.32): calcd. C 54.89, H 5.92, N 18.29; found C 54.81, H 5.95, N 18.34.

*Synthesis of 4-amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid hydrazide (**3**)*

A solution of **2** (0.01 mol) and hydrazine hydrate (0.01 mol) was refluxed in butanol for 4 h. The mixture was cooled, and the solid that separated was recrystallized from ethanol. M. p. 200 – 201 °C. Yield 70 %. – IR (KBr): ν = 3306 – 3166 (NH + 2NH_2), 1726 (triazole C=O), 1668 (hydrazide C=O), 1613 (C=N), 1249 (C–O) cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 3.70 (s, 3H, OCH_3), 3.77 (s, 2H, benzyl CH_2), 4.18 (s, 2H, NCH_2), 4.25 (s, 2H, hydrazide NH_2), 5.24 (s, 2H, NH_2), Ar-H: [6.85 (d, 2H, J = 7.8 Hz), 7.16 (d, 2H, J = 7.8 Hz)], 9.16 (s, 1H, NH). – ^{13}C NMR ([D_6]DMSO): δ = 29.41 (benzyl CH_2), 46.27 (NCH_2), 54.93 (OCH_3), Ar-C: [113.70 (2C), 127.56, 129.79 (2C), 157.91], 147.14 (triazole C-3), 153.28 (triazole C-5), 165.92 (hydrazide C=O). – Anal. for $C_{12}H_{16}N_6O_3$ (292.30): calcd. C 49.31, H 5.52, N 28.75; found C 49.29, H 5.52, N 28.74.

*General method for the synthesis of compounds **4***

Compound **3** (0.01 mol) was refluxed with 0.01 mol of the appropriate aldehyde in ethanol for 4 h. The mixture was cooled, and the solid that separated was recrystallized from an appropriate solvent to afford the desired compound.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 3-bromo-benzylidenehydrazide (**4a**)*

M. p. 202 – 203 °C (DMSO-water). Yield 87 %. – IR (KBr): ν = 3339 (NH), 3220, 3186 (NH_2), 1724 (triazole C=O), 1672 (hydrazide C=O), 1635, 1589 (C=N) cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 3.70 (s, 3H, OCH_3), 3.81 (s, 2H, benzyl CH_2), 4.84 and 4.42 (s, 2H, NCH_2, *trans* and *cis* conformers), 5.31 (s, 2H, NH_2), Ar-H: [6.84 (d, 2H, J = 8.4 Hz), 7.18 (d, 2H, J = 8.4 Hz), 7.33 – 7.43 (m, 1H), 7.57 – 7.71 (m, 2H)], 7.95 and 8.15 (s, 1H, N=CH, *trans* and *cis* conformers), 11.72 and 11.77 (s, 1H, NH, *trans* and *cis* conformers). – ^{13}C NMR ([D_6]DMSO): δ = 30.20 (benzyl CH_2), 47.24 and 47.64 (NCH_2, *trans* and *cis* conformers), 55.71 (OCH_3), Ar-C: [114.50 (2C), 122.91, 126.87, 128.38, 129.73, 130.52 (2C), 131.60, 133.20, 137.09 and 137.21 (*trans* and *cis* conformers), 158.70], 143.01 and 146.19 (N=CH, *trans* and *cis* conformers), 147.81 and 148.10 (triazole C-3, *trans* and *cis* conformers), 154.38 and 164.07 (triazole C-5, *trans* and *cis* conformers), 167.92 (hydrazide C=O). – Anal. for $C_{19}H_{19}BrN_6O_3$ (459.30l): calcd. C 49.69, H 4.17, N 18.30; found C 49.69, H 4.15, N 18.32.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 4-fluoro-benzylidenehydrazide (**4b**)*

M. p. 267 – 268 °C (DMSO-water). Yield 84 %. – IR (KBr): ν = 3324 (NH), 3266, 3206 (NH_2), 1719 (triazole C=O), 1695 (hydrazide C=O), 1676, 1610 (C=N) cm^{-1}. – ^{1}H NMR ([D_6]DMSO): δ = 3.70 (s, 3H, OCH_3), 3.81 (s, 2H, benzyl CH_2), 4.82 and 4.40 (s, 2H, NCH_2, *trans and cis* conformers), 5.31 (s, 2H, NH_2), Ar-H: [6.84 (d, 2H, J = 8.4 Hz), 7.18 (d, 2H, J = 8.8 Hz), 7.28 (d, 2H, J = 8.8 Hz), 7.57 (d, 2H, J = 8.4 Hz)], 7.97 and 8.17 (s, 1H, N=CH, *trans* and *cis* conformers), 11.64 (s, 1H, NH). – ^{13}C NMR ([D_6]DMSO): δ = 29.36 (benzyl CH_2), 46.33 and 46.67 (NCH_2, *trans* and *cis* conformers), 54.87 (OCH_3), Ar-C: [113.65 (2C), 115.47 (2C), 127.54, 128.90 (2C), 129.67 (2C), 130.46, 157.86, 165.13], 142.68 and 146.00 (N=CH, *trans* and *cis* conformers), 146.96 and 147.21 (triazole C-3, *trans* and *cis* conformers), 153.53 and 163.00 (triazole C-5, *trans* and *cis* conformers), 167.92 (hydrazide C=O). – Anal. for $C_{19}H_{19}FN_6O_3$

(398.40): calcd. C 57.28, H 4.81, N 21.09; found C 57.21, H 4.73, N 21.03.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 4-(trifluoromethyl)-benzylidenehydrazide (**4c**)*

M. p. 248 – 249 °C (DMSO-water). Yield 75 %. – IR (KBr): ν = 3333 (NH), 3270, 3212 (NH_2), 1716 (triazole C=O), 1693 (hydrazide C=O), 1616, 1569 (C=N) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.73 (s, 3H, OCH_3), 3.84 (s, 2H, benzyl CH_2), 4.88 and 4.46 (s, 2H, NCH_2, *trans* and *cis* conformers), 5.35 (s, 2H, NH_2), Ar-H: [6.85 (d, 2H, *J* = 8.4 Hz), 7.20 (d, 2H, *J* = 8.4 Hz), 7.78 (d, 2H, *J* = 8.2 Hz), 7.93 (d, 2H, *J* = 8.2 Hz)], 8.07 and 8.28 (s, 1H, N=CH, *trans* and *cis* conformers), 11.86 (s, 1H, NH). – ^{13}C NMR ([D_6]DMSO): δ = 29.40 (benzyl CH_2), 46.39 and 46.87 (NCH_2, *trans* and *cis* conformers), 54.88 (OCH_3), 125.56 (CF_3), Ar-C: [113.66 (2C), 126.67, 127.44 (2C), 127.55 (2C), 128.08, 129.72 (2C), 137.79, 157.88], 142.21 and 146.77 (N=CH, *trans* and *cis* conformers), 147.08 and 147.32 (triazole C-3, *trans* and *cis* conformers), 153.56 and 163.38 (triazole C-5, *trans* and *cis* conformers), 168.25 (hydrazide C=O). – Anal. for $C_{20}H_{19}F_3N_6O_3$ (448.40): calcd. C 53.57, H 4.27, N 18.74; found C 53.78, H 4.29, N 18.78.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 2,4-dichlorobenzylidenehydrazide (**4d**)*

M. p. 270 – 271 °C (DMSO-water). Yield 92 %. – IR (KBr): ν = 3336 (NH), 3287, 3225 (NH_2), 1711 (triazole C=O), 1695 (hydrazide C=O), 1615, 1589 (C=N) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.71 (s, 3H, OCH_3), 3.81 (s, 2H, benzyl CH_2), 4.85 and 4.42 (s, 2H, NCH_2, *trans* and *cis* conformers), 5.30 (s, 2H, NH_2), Ar-H: [6.84 (d, 2H, *J* = 8.4 Hz), 7.18 (d, 2H, *J* = 8.4 Hz), 7.43 – 7.51 (m, 1H), 7.69 (bs, 1H), 7.90 – 8.04 (m, 1H)], 8.31 and 8.52 (s, 1H, N=CH, *trans* and *cis* conformers), 11.83 and 11.91 (s, 1H, NH, *trans* and *cis* conformers). – ^{13}C NMR ([D_6]DMSO): δ = 29.38 (benzyl CH_2), 46.39 and 46.91 (NCH_2, *trans* and *cis* conformers), 54.88 (OCH_3), Ar-C: [113.65 (2C), 127.82, 128.14, 129.20, 129.71 (2C), 130.15, 133.52, 134.86, 135.85, 157.87], 138.81 and 142.05 (N=CH, *trans* and *cis* conformers), 147.07 and 147.34 (triazole C-3, *trans* and *cis* conformers), 153.54 and 163.29 (triazole C-5, *trans* and *cis* conformers), 168.18 (hydrazide C=O). – Anal. for $C_{19}H_{18}Cl_2N_6O_3$ (449.30): calcd. C 50.79, H 4.04, N 18.70; found C 50.76, H 4.11, N18.70.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 5-bromo-2-hydroxybenzylidenehydrazide (**4e**)*

M. p. 234 – 235 °C (DMSO-water). Yield 78 %. – IR (KBr): ν = 3348 (NH+OH), 3275, 3191 (NH_2), 1719 (triazole C=O), 1670 (hydrazide C=O), 1610, 1588 (C=N) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.70 (s, 3H, OCH_3), 3.81 (s, 2H, benzyl CH_2), 4.84 and 4.44 (s, 2H, NCH_2, *trans* and *cis* conformers), 5.32 (s, 2H, NH_2), Ar-H: [6.83 – 6.87 (m, 3H), 7.18 (d, 2H, *J* = 7.6 Hz), 7.34 – 7.42 (m, 1H), 7.86 and 7.75 (s, 1H, *trans* and *cis* conformers)], 8.22 and 8.37 (s, 1H, N=CH, *trans* and *cis* conformers), 10.37 and 11.02 (s, 1H, OH, *trans* and *cis* conformers), 11.63 and 11.93 (s, 1H, NH, *trans* and *cis* conformers). – ^{13}C NMR ([D_6]DMSO): δ = 29.41 (benzyl CH_2), 46.45 and 46.85 (NCH_2, *trans* and *cis* conformers), 54.88 (OCH_3), Ar-C: [110.77 and 110.35 (*trans* and *cis* conformers), 113.66 (2C), 118.20 and 118.47 (*trans* and *cis* conformers), 122.39 and 121.07 (*trans* and *cis* conformers), 129.71 (2C), 130.02, 133.32, 137.02, 155.40 and 156.14 (*trans* and *cis* conformers), 157.87], 139.10 and 144.75 (N=CH, *trans* and *cis* conformers), 146.94 and 147.36 (triazole C-3, *trans* and *cis* conformers), 153.57 and 163.11 (triazole C-5, *trans* and *cis* conformers), 167.90 (hydrazide C=O). – Anal. for $C_{19}H_{19}BrN_6O_4$ (475.30): calcd. C 48.01, H 4.03, N 17.68; found C 48.05, H 4.03, N 17.67.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 2-hydroxy-5-methoxybenzylidenehydrazide (**4f**)*

M. p. 197 – 198 °C (DMSO-water). Yield 95 %. – IR (KBr): ν = 3340 (NH), 3334 (OH), 3275, 3191 (NH_2), 1717 (triazole C=O), 1676 (hydrazide C=O), 1610, 1580 (C=N) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.69 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.81 (s, 2H, benzyl CH_2), 4.81 and 4.42 (s, 2H, NCH_2, *trans* and *cis* conformers), 5.31 (s, 2H, NH_2), Ar-H: [6.82 – 6.86 (m, 4H), 7.19 (d, 2H, *J* = 8.4 Hz), 7.25 (bs, 1H)], 8.26 and 8.39 (s, 1H, N=CH, *trans* and *cis* conformers), 9.56 and 10.38 (s, 1H, OH, *trans* and *cis* conformers), 11.53 and 11.80 (s, 1H, NH, *trans* and *cis* conformers). – ^{13}C NMR ([D_6]DMSO): δ = 29.38 (benzyl CH_2), 46.40 and 46.77 (NCH_2, *trans* and *cis* conformers), 54.88 (OCH_3), 55.34 (OCH_3), Ar-C: [111.71, 113.67 (2C), 117.10, 118.06 and 118.27 (*trans* and *cis* conformers), 120.17 and 118.72 (*trans* and *cis* conformers), 127.56, 129.72 (2C), 150.42 and 151.18 (*trans* and *cis* conformers), 151.99 and 152.13 (*trans* and *cis* conformers), 157.87], 140.84 and 146.65 (N=CH, *trans* and *cis* conformers), 146.95 and 147.31 (triazole C-3, *trans* and *cis* conformers), 153.54 and 162.90 (triazole C-5, *trans* and *cis* conformers), 167.67 (hydrazide C=O). – Anal. for $C_{22}H_{26}N_6O_5$ (470.49): calcd. C 56.33, H 5.20, N 19.71; found C 56.34, H 5.35, N 19.58.

*4-Amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl-acetic acid 2,3,4-trimethoxybenzylidenehydrazide (**4g**)*

M. p. 179 – 180 °C (DMSO-water). Yield 78 %. – IR (KBr): ν = 3334 (NH), 3287, 3173 (NH_2), 1708 (triazole

C=O), 1687 (hydrazide C=O), 1615, 1593 (C=N) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.36 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.82 (s, 2H, benzyl CH_2), 4.79 and 4.37 (s, 2H, NCH_2, *trans* and *cis* conformers), 5.31 (s, 2H, NH_2), Ar-H: [6.82 – 6.92 (m, 3H), 7.18 (d, 2H, J = 8.4 Hz), 7.51 – 7.60 (m, 1H)], 8.16 and 8.35 (s, 1H, N=CH, *trans* and *cis* conformers), 11.50 and 11.59 (s, 1H, NH, *trans* and *cis* conformers). – ^{13}C NMR ([D_6]DMSO): δ = 29.41 (benzyl CH_2), 46.36 and 46.85 (NCH_2, *trans* and *cis* conformers), 54.90 (OCH_3), 55.88 (OCH_3), 60.37 (OCH_3), 61.63 (OCH_3), Ar-C: [108.56, 113.68 (2C), 119.96, 120.52, 127.60, 129.74 (2C), 139.71, 152.36 and 152.47 (*trans* and *cis* conformers), 154.94 and 155.13 (*trans* and *cis* conformers), 157.90], 141.37 and 142.66 (N=CH, *trans* and *cis* conformers), 146.98 and 147.26 (triazole C-3, *trans* and *cis* conformers), 153.58 and 162.68 (triazole C-5, *trans* and *cis* conformers), 167.63 (hydrazide C=O). – Anal. for $C_{22}H_{26}N_6O_5$ (470.49): calcd. C 56.16, H 5.57, N 17.86; found C 56.16, H 5.59, N 17.63.

*Synthesis of 1-(4-amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazol-1-yl)-acetyl-4-phenyl thiosemicarbazide (**5**)*

A mixture of **3** (0.01 mol) and phenyl isothiocyanate (0.01 mol) was refluxed in ethanol for 2 h. The mixture was cooled, and the solid that separated was recrystallized from ethanol. M. p. 179 – 180 °C. Yield 78 %. – IR (KBr): ν = 3338 – 3202 (3NH + NH_2), 1717 (triazole C=O), 1681 (exocyclic C=O), 1611 (C=N), 1246 (C=S) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.70 (s, 3H, OCH_3), 3.79 (s, 2H, benzyl CH_2), 4.46 (s, 2H, NCH_2), 5.31 (s, 2H, NH_2), Ar-H: [6.84 (d, 2H, J = 7.8 Hz), 7.18 (d, 2H, J = 7.8 Hz), 7.29 – 7.43 (m, 5H)], 9.64 (s, 1H, NH), 9.73 (s, 1H, NH), 10.29 (s, 1H, NH). – ^{13}C NMR ([D_6]DMSO): δ = 29.59 (benzyl CH_2), 46.68 (NCH_2), 55.10 (OCH_3), Ar-C: [113.85 (2C), 125.34, 126.02, 127.59 (2C), 128.19 (2C), 129.97 (2C), 139.02, 158.08], 147.49 (triazole C-3), 153.58 (triazole C-5), 166.64 (exocyclic C=O), 180.71 (C=S). – Anal. for $C_{19}H_{21}N_7O_3S$ (427.48): calcd. C 53.38, H 4.95, N 22.94; found C 53.39, H 4.95, N 22.73.

*Synthesis of 1-(4-phenyl-5-thioxo-1,2,4-triazol-3-yl)methyl-4-amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazole (**6**)*

Solid thiosemicarbazide **5** (0.01 mol) was added portionwise to 20 mL of a 2 N NaOH solution. The reaction mixture was refluxed for 2 h. The solution was cooled to r. t. and acidified to pH 3 – 4 with 37 % HCl. The precipitated solid was filtered, washed thoroughly with water, dried, and recrystallized from ethanol-water (1 : 2). M. p. 219 – 220 °C. Yield 83 %. – IR (KBr): ν = 3448 (NH), 3275, 3132 (NH_2), 1731 (triazole C=O), 1613, 1516 (C=N), 1358 (C=S) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.70 (s, 3H, OCH_3), 3.72 (s, 2H, benzyl CH_2), 4.63 (s, 2H, NCH_2), 5.04 (s, 2H, NH_2), Ar-H: [6.87 (d, 2H, J = 8.4 Hz), 7.05 – 7.10 (m, 2H), 7.14 (d, 2H, J = 8.4 Hz), 7.27 – 7.28 (m, 3H)], 13.90 (s, 1H, NH). – ^{13}C NMR ([D_6]DMSO): δ = 29.35 (benzyl CH_2), 40.60 (NCH_2), 54.98 (OCH_3), Ar-C: [113.71 (2C), 127.25, 127.61, 127.85 (2C), 128.13 (2C), 129.80 (2C), 136.29, 157.93], 145.78 (triazole C-3), 146.83 (triazole C-3, thioxo ring), 152.34 (triazole C-5), 168.14 (triazole C-5, thioxo ring). – Anal. for $C_{19}H_{19}N_7O_2S$ (409.47): calcd. C 55.73, H 4.68, N 23.95; found C 55.73, H 4.66, N 23.93.

*Synthesis of 1-(4-phenyl-5-benzylthio-1,2,4-triazol-3-yl) methyl-4-amino-3-p-methoxybenzyl-5-oxo-4,5-dihydro-1,2,4-triazole (**7**)*

Compound **6** (0.01 mol) was refluxed with an equivalent amount of sodium in absolute ethanol for 1 h. Then, benzyl bromide (0.01 mol) was added and the mixture refluxed for an additional 5 h. After evaporating, the solid was recyrstallized from ethanol-water (1 : 1). M. p. 82 – 83 °C. Yield 67 %. – IR (KBr): ν = 3321, 3210 (NH_2), 1707 (triazole C=O), 1610, 1584 (C=N) cm^{-1}. – ^1H NMR ([D_6]DMSO): δ = 3.70 (s, 2H, benzyl CH_2), 3.72 (s, 3H, OCH_3), 4.32 (s, 2H, NCH_2), 4.88 (s, 2H, SCH_2), 5.06 (s, 2H, NH_2), Ar-H: [6.86 (d, 2H, J = 6.4 Hz), 7.10 – 7.14 (m, 4H), 7.25 – 7.29 (m, 4H), 7.39 – 7.45 (m, 4H)]. – ^{13}C NMR ([D_6]DMSO): δ = 29.30 (benzyl CH_2), 35.98 (SCH_2), 46.25 (NCH_2), 54.97 (OCH_3), Ar-C: [113.71 (2C), 12.72 (2C), 127.42, 128.39 (2C), 128.86 (2C), 128.97, 129.43 (2C), 129.83 (2C), 129.91, 131.99, 136.87, 157.74], 147.52 (triazole C-3), 148.47 (triazole C-3, benzylthio ring), 152.21 (triazole C-5), 155.92 (triazole C-5, benzylthio ring). – Anal. for $C_{26}H_{25}N_7O_2S$ (499.59): calcd. C 62.51, H 5.04, N 19.63; found C 62.51, H 5.00, N 19.76.

Pharmacology

Sixty human cancer cell line anticancer screening

The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5 % fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96 well microtiter plates in 100 μL samples at plating densities ranging from 5000 to 40000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of the test compounds.

After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of compound addition (Tz). The compounds were solubilized in DMSO and diluted to 20 μM, the complete medium containing 50 μg/mL gentamicin. Aliquots of 100 μL of the compound solution were

added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final compound concentration (10 μM).

Following compound addition, the plates were incubated for an additional 48 h at 37 °C, 5 % CO_2, 95 % air, and 100 % relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were 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 was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL), 0.4 % (w/v) in 1 % acetic acid, was added to each well, and plates were incubated for 10 min at r. t. After staining, unbound dye was removed by washing five times with 1 % acetic acid, and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm.

The measured effect of the compound on a cell line was calculated according to one or the other of the following two expressions and expressed as percent growth (PG):

If $(\text{Mean OD}_{\text{test}} - \text{Mean OD}_{\text{tzero}}) \geq 0$, then

$$\text{PG} = 100 \times (\text{Mean OD}_{\text{test}} - \text{Mean OD}_{\text{tzero}}) / (\text{Mean OD}_{\text{ctrl}} - \text{Mean OD}_{\text{tzero}})$$

If $(\text{Mean OD}_{\text{test}} - \text{Mean OD}_{\text{tzero}}) < 0$, then

$$\text{PG} = 100 \times (\text{Mean OD}_{\text{test}} - \text{Mean OD}_{\text{tzero}}) / \text{Mean OD}_{\text{tzero}}$$

Where:

$\text{Mean OD}_{\text{tzero}}$ = The average of optical density measurements SRB-derived color just before exposure of cells to the test compound.

$\text{Mean OD}_{\text{test}}$ = The average of optical density measurement of SRB-derived color after 48 h exposure of cells to the test compound.

$\text{Mean OD}_{\text{ctrl}}$ = The average of optical density measurements of SRB-derived color after 48 h with no exposure of cells to the test compound.

Smaller positive PG values are indicative of better antiproliferative activity, and negative values show that the compound is tumoricidal, killing already existing cells.

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