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ORIGINAL PAPER

Synthesis and evaluation of anticancer and PDE 5 inhibitory activity of spiro-substituted quinazolin-4-ones

Mohamed A. Ameen[1] · Essam Kh. Ahmed[1] · Mohamed Ramadan[2] · Hisham A. Abd El-Naby[1] · Asmaa A. Abdel-Haseeb[1]

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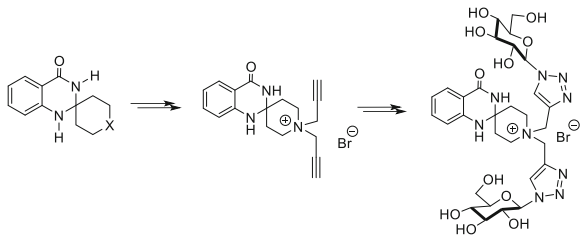
Abstract A series of novel spiro-substituted 2,3-dihydroquinazolin-4(1*H*)-ones was synthesized and structurally confirmed by spectral analysis, screened for their anticancer activity at a concentration of 10 μM against a panel of 56 cell lines derived from nine different types of cancers, including leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate, and breast cancers. The synthesized compounds screened for their PDE 5 inhibitory activity and it showed encouraged activity compared to sildenafil.

Graphical abstract



Keywords Spiro compounds · Cycloadditions · Antitumor agents · Structure elucidation

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✉ Mohamed Ramadan
elbashamohammed@yahoo.com

[1] Chemistry Department, Faculty of Science, Minia University, El Minya, Egypt

[2] Organic Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Assuit, Egypt

Introduction

The rational use of click approach for the discovery of new medicinal agents takes advantages of the structurally unique compounds provided by the rapid increase of molecular complexity [1]. Quinazoline compounds tethered to biomolecules via triazole linker produced by the combination of an unusual quinazolinone scaffold with the possibility of functionalization at N-atoms were not reported. Quinazoline and its derivatives are versatile nitrogen heterocyclic compounds, which known as a promising class of biologically active compounds. Quinazolin-4-one derivatives exhibited a wide range of pharmacological properties such as CNS depressant, sedative, hypnotic, antidepressant, anesthetic, tranquilizer, anticonvulsant, muscle relaxant, antiviral, antibacterial, antifungal, analgesic, anti-inflammatory, antipyretic, antiulcer, antihypertensive, spore germination inhibition in *Drechslera rostrata* and *Fusarium oxysporum*, antihistaminic, hypoglycemic, MAO inhibitor, insecticidal, radioprotective, spasmolytic, contraceptive, and H_2-antagonist [2]. They are also useful as cytotoxic agents [3], PDE 5 inhibitors [4, 5] antitubercular [6], antipsychotic [7], dihydrofolate reductase (DHFR) inhibition [8].

Male erectile dysfunction (MED) is a common medical problem affecting many of men worldwide. Phosphodiesterase 5 (PDE 5) inhibitors are very attractive therapeutic agents for the treatment of MED [9]. Although Sildenafil (Viagra®, $IC_{50} = 3$ nM; Fig. 1) was the lead compound and the most famous PDE 5 inhibitor, but due to its low selectivity against other PDEs, mainly towards PDE 6 which found in the retina, and its inhibition may cause visual disturbances, perception of bluish haze, and increased light sensitivity [10]. In the last few decades, the focus on the design of more selective PDE 5 inhibitors

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expanded. PDE 5 belongs to a superfamily of enzymes that catalyzes the hydrolysis of cyclic nucleotides cAMP and cGMP to the corresponding 5-nucleoside monophosphate.

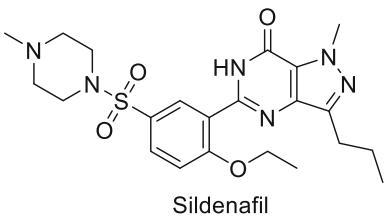
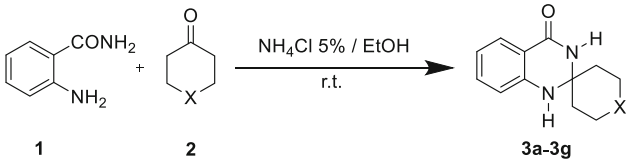


Fig. 1 Sildenafil

Scheme 1



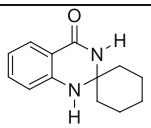
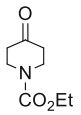
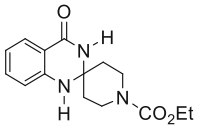
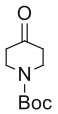
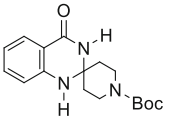
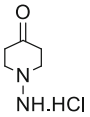
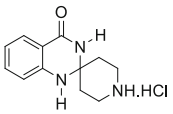
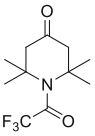
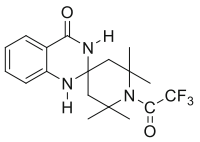
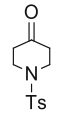
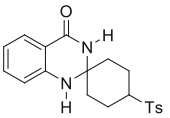
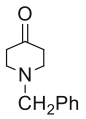
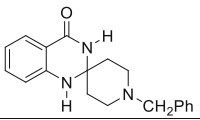
Right now, 11 different isoforms of phosphodiesterases (PDE 1–11) and their subtypes are known, distinguished by substrate specificities and tissue concentration. The phosphodiesterase plays a vital role in the cellular signaling process [11, 12].

Synthetic transformations based on the click approach expand the medicinal potential of different heterocyclic classes. So we illustrated the potential of acetylenic quinazoline derivatives as a structural scaffold in the design of therapeutic agents. The synthesized compounds were screened for their anticancer and PDE 5 inhibitory activity.

Results and discussion

In continuation of our studies on the development of new routes in heterocyclic synthesis [13–16] substituted derivatives of spiro-2,3-dihydroquinazolin-4(1*H*)-one were prepared as outlined in Scheme 1. Since the spirohexyl

Table 1 Reaction time and yield percentage of compounds **3a–3g**

Entry	Ketone	Product **3**	Method A		Method B	
			Yield/%	Time/h	Yield/%	Time/h
A			80	3	45	20
B			77	5	43	24
C			74	6	40	24
D			82	6	51	28
E			69	5	42	30
F			68	6	45	30
G			66	4	44	24

Method A: the reaction performed with catalyst at room temperature

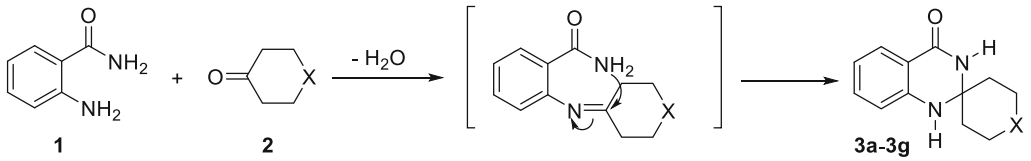
Method B: the reaction performed without catalyst under reflux

type was found to be favorable for high affinity and good pharmacokinetic profiles [17]. It was found that the reaction of equimolar amounts of 2-aminobenzamide and cyclohexanone or N-substituted 4-piperidone derivatives in the presence of 5% ammonium chloride as a catalyst in ethanol at room temperature yielded the target products **3a**–**3g** in good yields as shown in Table 1. When the reactions were performed without a catalyst under reflux, the products were obtained in lower yields and with longer reaction times. So the treatment of aminobenzamide with ketones to afford the spiro systems was best achieved by running the reaction in the presence of catalytic quantities of ammonium chloride at room temperature. The ^{1}H and ^{13}C NMR spectra and mass spectrometry of compounds **3a**–**3g** clearly confirmed the formation of spiro-substituted 2,3-dihydroquinazolin-4(1*H*)-ones.

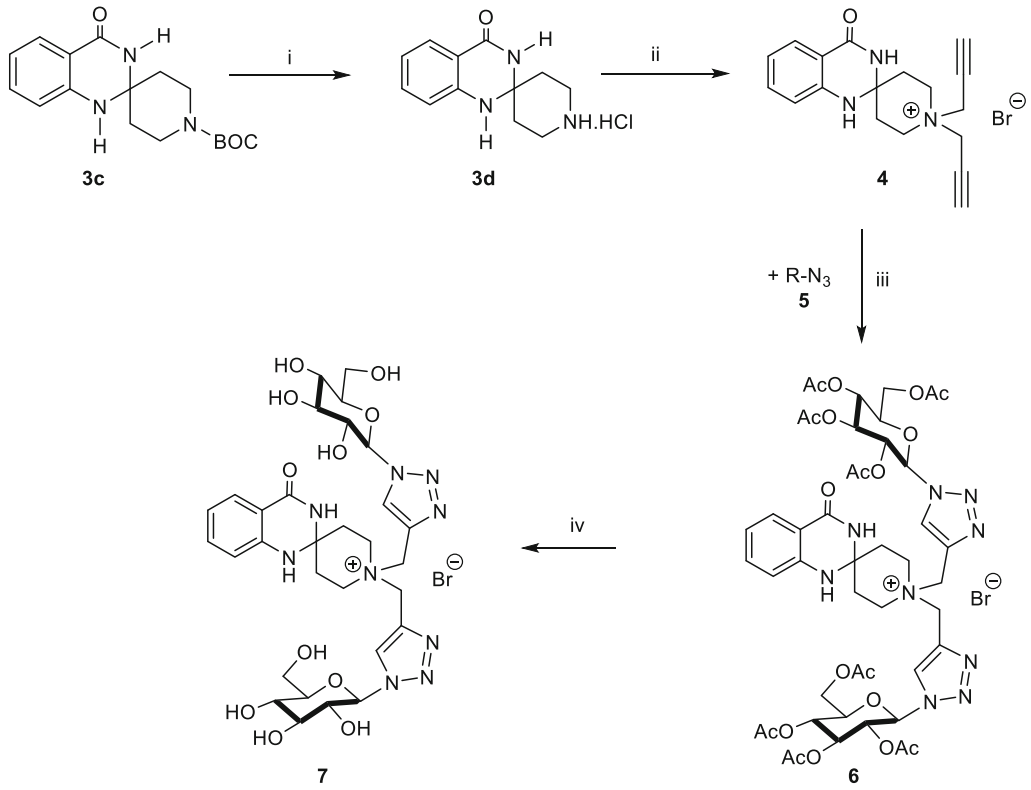
A plausible mechanism for this reaction is suggested in Scheme 2. It is reasonable to assume that the functionalized spiroquinazoline **3a**–**3g** could be obtained from the initial condensation of amino group in 2-aminobenzamide and carbonyl group of the ketone, followed by the attack of the lone pair of N-atom of amide on the positively charged carbon of imine. Finally, N-anion of ketenimine could protonated to form the targeted compounds **3a**–**3g**.

To generate the alkyne core **4**, we examined the reaction of NH-hydrochloride compound **3d** with propargyl

Scheme 2



Scheme 3



(i) HCl, CH_2Cl_2, r.t.; (ii) propargyl bromide, DMF, K_2CO_3, r.t.; (iii) cat. $CuSO_4{\cdot}5H_2O$, Na-ascorbate, H_2O/THF, r.t.; (iv) CH_3ONa, CH_3OH, R = b

bromide in alkaline medium. The mass spectra for the reaction product revealed the formation of 1:2 adduct. Three possible nucleophilic centers are possible cites to be attacked with alkyne electrophile, the spiroquinazoline N1, N3 as well as the piperidine NH. Quinazoline nitrogen were excluded realized in high inactivity [18] as a result of either steric hindrance or tautomerization. Interestingly, the piperidinium salt with attaching two propargyl groups was formed. Accordingly, structure **4** was established for the reaction product. ^{1}H and ^{13}C NMR was in support of the proposed structure.

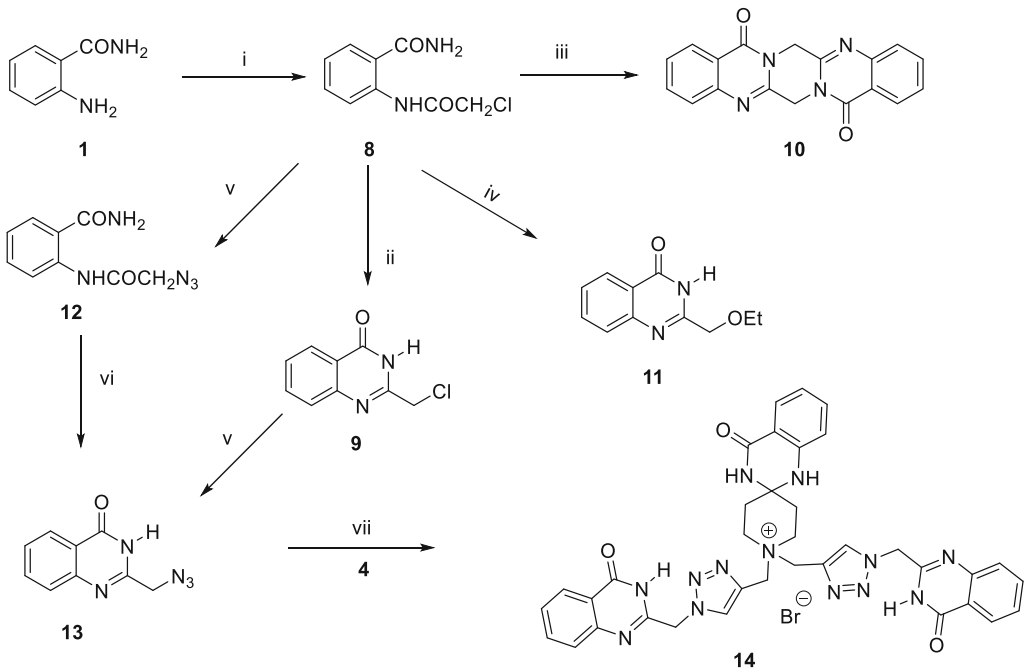
The alkyne product **4** and azide **5** coupling partners were reacted together, under our previously developed CuAAC click conditions [14] to deliver the new library of hybrid triazole-bio ligand **6** in good yield (Scheme 3).

According to what has been previously reported [19] a reasonable mechanism for the formation of clicked products **6** and **14** involves deprotonation of the triple bond forming Cu-acetylide followed by 1,3-dipolar cycloaddition of azide.

Furthermore, we report here the chemical synthesis of azido-alkyne functionalized glucose dimer **7** by hydrolysis of **6** in a diluted sodium methoxide. The structure of **7** was confirmed by spectroscopic data. IR spectrum of compound **7** showed the presence of the broad band at 3407–3365 cm^{-1} corresponding to OH groups. ^{1}H NMR spectrum of compound **7** presents a signal at $\delta = 3.74$ ppm characteristic for 4 OH groups and disappearance of acetyl groups.

We designed the following scheme to optimize the direct formation of compound **14**, proceeding to the desired target through 1,3-dipolar azide-alkyne cycloaddition reaction (Scheme 4). The treatment of aminobenzamide **1** with chloroacetyl chloride to afford the acetylated product **8** was best achieved using an excess amount of chloroacetyl chloride in chloroform [20]. The subsequent cyclization step of compound **8** to afford 2-(chloromethyl)quinazoline **9** was sluggish when conducted in sodium ethoxide affording other products. Refluxing compound **8** in an equivalent sodium ethoxide afforded the pentacyclic ring system **10**. In addition, we investigated the effect of the excess amount of sodium ethoxide at room temperature and then under reflux but the two cases led to the formation of ethoxy methylene product **11**. In contrast when carrying out the reaction in toluene in the presence of catalytic amount of TsOH afforded 2-(chloromethyl)quinazoline **9**. Hence, the azido product **13** was obtained by treating the corresponding compound **9** with sodium azide.

Scheme 4



(i) $ClCH_2COCl$, CH_2Cl_2, r.t.; (ii) TsOH, dry toluene, reflux; (iii) EtONa (1:1), EtOH, reflux; (iv) EtONa (2:1), EtOH, reflux; (v) NaN_3, acetonitrile, reflux; (vi) EtONa (1:4), EtOH, r.t.; (vii) THF:H_2O (1:1), sodium ascorbate, cat.$CuSO_4 \cdot 5H_2O$, r.t.

Table 2 56 cell line in vitro anticancer screening data of compounds **3c**, **3d**, **6**, and **7**

Subpanel tumor cell lines	Growth/%			
	3c NSC:793274	**3d** NSC:793275	**6** NSC:793276	**7** NSC:793277
Leukemia				
CCRF-CEM	97.68	94.87	94.88	93.73
HL-60(TB)	89.47	95.62	93.17	94.27
MOLT-4	88.81	84.74	86.64	88.20
SR	98.83	86.92	85.46	100.27
Non-small cell lung cancer				
A549/ATCC	65.54	69.06	65.56	78.55
EKVX	108.72	109.31	107.12	109.69
HOP-62	90.22	96.57	97.04	97.43
HOP-92	106.41	107.21	108.93	104.48
NCI-H226	98.01	97.67	99.56	97.96
NCI-H23	101.11	100.49	104.48	103.08
NCI-H322 M	108.56	108.77	104.45	101.84
NCI-H460	107.23	110.30	107.37	104.80
NCI-H522	84.49	97.52	92.40	98.06
Colon cancer				
HCC-2998	104.12	103.23	101.62	104.04
HCT-116	103.16	100.40	97.63	96.30
HCT-15	113.68	112.99	116.45	111.49
HT29	93.23	98.18	98.02	100.22
KM12	106.62	105.19	102.81	102.97
SW-620	96.69	102.34	100.39	98.26
CNS cancer				
SF-268	106.94	105.81	101.84	104.10
SF-295	105.34	107.34	105.46	109.91
SF-539	106.41	114.20	106.43	105.44
SNB-19	97.80	96.24	96.83	98.31
SNB-75	91.11	82.77	87.85	88.61
U251	85.25	91.18	91.14	93.28
Melanoma				
LOX IMVI	101.06	101.89	103.24	97.95
MALME-3M	105.62	117.27	102.11	98.84
M14	101.63	100.04	102.47	102.65
MDA-MB-435	106.46	103.74	105.27	107.40
SK-MEL-2	100.08	98.06	108.31	101.82
SK-MEL-28	119.11	116.41	114.48	113.55
SK-MEL-5	107.37	113.99	109.09	104.60
UACC-257	58.33	85.66	62.90	83.07
UACC-62	95.59	96.12	96.23	100.01
Ovarian cancer				
IGROV1	106.42	113.16	109.29	106.74
OVCAR-3	114.43	108.09	108.25	109.43
OVCAR-4	110.84	115.93	113.55	119.53
OVCAR-5	111.66	114.89	112.57	107.17
OVCAR-8	82.31	84.38	76.12	88.65
NCI/ADR-RES	109.36	108.66	107.66	108.21
SK-OV-3	96.11	103.48	79.33	87.49

Table 2 continued

Subpanel tumor cell lines	Growth/%			
	3c NSC:793274	**3d** NSC:793275	**6** NSC:793276	**7** NSC:793277
Renal cancer				
786-0	103.02	107.40	109.68	106.67
A498	98.98	95.47	96.51	106.19
ACHN	113.82	110.38	108.59	108.66
RXF 393	107.50	111.25	119.71	102.48
SN12C	101.86	99.14	97.06	97.57
TK-10	94.65	98.26	94.92	101.84
UO-31	85.71	89.44	90.37	84.14
Prostate cancer				
PC-3	94.78	88.54	92.77	82.27
DU-145	112.69	108.42	112.04	101.90
Breast cancer				
MCF7	96.46	107.36	103.62	97.02
MDA-MB-231/ATCC	108.36	119.57	106.83	104.19
HS 578T	95.49	101.78	102.42	109.19
BT-549	114.28	108.15	113.88	108.48
T-47D	96.61	109.13	102.49	109.11
MDA-MB-468	112.08	98.76	110.27	105.70
Mean	100.32	102.03	100.46	100.85
Delta	41.99	32.97	37.56	22.30
Range	60.78	50.51	56.81	40.98

Range = highest growth percent − lowest growth percent. Delta = mean growth percent − lowest growth percent

An alternative reaction condition was devised using sodium azide to convert the acetylated product **8** into azido product **12** which was then subjected to cyclization in sodium ethoxide to give compound **13**. The copper(I)-catalyzed 1,2,3-triazole-forming reaction between azide **13** and terminal alkyne **4** afforded the interesting two triazolo linkers quinazoline trimer **14** (Scheme 4). The presence of triazolo moieties in **14** was confirmed by the disappearance of the characteristic bands in the IR spectrum for azide group. ^{1}H NMR spectrum also introduced aromatic proton signals for triazole rings at 8.08, 8.12 ppm. ^{13}C NMR showed signals of $2CH_{triazoles}$ and carbonyl groups at 135.0, 161.8, and 163.4 ppm, respectively.

Screening of anticancer activity

Among the synthesized compounds, compound **3c**, **3d**, **6**, and **7** were selected by National Cancer Institute (NCI) based on the protocol of the Drug Evaluation Branch of the National Cancer Institute, Bethesda, USA, for in vitro anticancer screening. The methodology of the NCI anticancer screening has been illustrated in detail elsewhere (http://www.dtp.nci.nih.gov).

The prepared compounds were added at single concentration 10 μM and the culture was incubated for 48 h. End point determinations were made with a protein binding dye, sulforhodamine B (SRB). All the selected compounds evaluated toward the panel of 56 cancer cell lines. The human tumor cell lines were derived from nine different cancer types: leukemia, melanoma, lung, renal, prostate, colon, CNS, ovarian and breast cancers. Results for each compound were reported as the growth percent of the treated cells which are evaluated spectrophotometrically and compared to that of the untreated control cells (Table 2).

The results listed in Table 2 revealed that compounds **3c**, **3d**, **6**, and **7** possess a weak to moderate cytotoxic and/or growth inhibitory effect toward different cell lines.

Screening of PDE 5 inhibition activity

The synthesized compounds were screened for their ability to inhibit PDE 5 compared to sildenafil as a standard reference drug. Most of the synthesized quinazoline-4-one derivatives were found to have PDE 5 inhibitory activity at a concentration of 3 nM (as IC_{50} of sildenafil) (Table 3).

Table 3 PDE 5 inhibition of compounds **3a**–**3d**, **4**, **6**, and **7** at conc. 3 nM

Compound	PDE 5 inhibition/%
Sildenafil	100
3a	100
3b	100
3c	34
3d	47
4	100
6	100
7	95

Conclusion

We designed the first synthesis of quaternary piperidinium salt of quinazoline with two acetylenic groups and their Cu-catalyzed alkyne-azide cycloaddition reaction. The possibility of combining the acetylenic scaffolds with the flexibility of structural "click" modifications could open opportunities for the rational design of spiroquinazoline-based therapeutic agents. The newly synthesized compounds were screened for their anticancer activity and it revealed moderate to weak anticancer activity. Encouragingly, most of the synthesized compounds possess potent PDE 5 inhibitory activity. Despite the fact that this assay is less sensitive compared to radioisotope PDE 5 assays and likely to underestimate the relative inhibitory potencies of the test compounds and further studies needed.

Experimental

All reagents were purchased from Alfa Aesar and Fluka companies and were used without further purification. 2-(2-Chloroacetamido)benzamide (**8**) was synthesized according to the literature [20]. Melting points were measured with a Gallenkamp apparatus and are uncorrected. The reactions and compounds purity were monitored by TLC, on aluminum plates coated with silica gel with fluorescent indicator (Merck, 60 F254). The NMR spectra were performed on a JHA-LAA 400 WB-FT spectrometer (300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR) and a Bruker Avance (400 MHz for ^{1}H, and 100 MHz for ^{13}C) with either deuterated DMSO-d_6 or $CDCl_3$ as a solvent. Chemical shifts are expressed in δ using TMS as a reference. The mass spectra were recorded on a Trace GC 2000/Finnegan Mat SSQ 7000 and a Shimadzu GCMS-QP-1000EX mass spectrometer at 70 eV. Elemental analyses were ascertained with a Euro EA analyzer.

*General procedure for synthesis of **3a–3g***

Ammonium chloride (1 mmol) was added to a mixture of 2-aminobenzamide (1 mmol) and appropriate ketone (1 mmol) in ethanol. Stirring was continued at room temperature till the disappearance of the starting material, followed by TLC. The precipitate was filtered off, washed with ethanol to afford the title compounds **3a**–**3g**.

1′H-Spiro[cyclohexane-1,2′-quinazolin]-4′(3′H)-one (**3a**)

Yield: 0.17 g (80%); white solid; m.p.: 215–216 °C (Ref. [21] 217–219 °C).

Ethyl 4′-oxo-3′,4′-dihydro-1′H-spiro[piperidine-4,2′-quinazoline]-1-carboxylate (**3b**, $C_{15}H_{19}N_3O_3$)

Yield: 0.22 g (77%); white solid; m.p.: 188–190 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.18$ (t, $J = 7.2$ Hz, 3H, $CO_2CH_2CH_3$), 1.71 (m, 4H, $2CH_2$), 3.36–3.57 (m, 4H, $2CH_2$), 4.08 (q, $J = 7.1$ Hz, 2H, $CO_2CH_2CH_3$), 6.62 (t, $J = 7.5$ Hz, 1H, Ar–H), 6.72 (d, $J = 8.1$ Hz, 1H, Ar–H), 7.19 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.21 (s, 1H, NH), 7.56 (d, $J = 8.0$ Hz, 1H, Ar–H), 8.12 (s, 1H, NH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 14.7$ (CH_3), 36.6 (C-3′, C-5′), 39.1 (C-2′, C-6′), 60.9 ($CO_2CH_2CH_3$), 66.6 (C-4′), 114.4 (C-4a), 114.8 (C-8), 117.1 (C-6), 127.3 (C-5), 133.5 (C-7), 146.5 (C-8a), 154.7 (C=O, ester), 164.3 (C=O) ppm; MS: $m/z = 289$ (M^+).

Tert-butyl 4′-oxo-3′,4′-dihydro-1′H-spiro[piperidine-4,2′-quinazoline]-1-carboxylate (**3c**, $C_{17}H_{23}N_3O_3$)

Yield: 0.24 g (74%); white solid; m.p.: 206–208 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.39$ (s, 9H, BOC), 1.71 (m, 4H, $2CH_2$), 3.32–3.52 (m, 4H, $2CH_2$), 6.64 (m, 1H, Ar–H), 6.77 (m, 1H, Ar–H), 7.22 (m, 2H, Ar–H), 7.56 (s, 1H, NH), 8.11 (s, 1H, NH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 28.1$ ($3CH_3$), 36.6 (C-3′, C-5′), 56.8 (C-2′, C-6′), 66.6 (C-4′), 78.9 ($C(CH_3)_3$), 114.4 (C-4a), 114.7 (C-8), 117.0 (C-6), 127.3 (C-5), 133.4 (C-7), 146.5 (C-8a), 154.0 (C=O, ester), 163.2 (C=O) ppm; MS (ESI): m/z calcd. $C_{17}H_{24}N_3O_3$ ($[M + H]^+$) 318.17, found 318.1.

1′-H-Spiro[piperidine-4,2′-quinazolin]-4′(3′H)-one hydrochloride (**3d**, $C_{12}H_{16}ClN_3O$)

Yield: 0.21 g (82%); white solid; m.p.: 269–271 °C (MeOH); IR (KBr): $\bar{\nu} = 3190$ (CH-$_{arom.}$), 3060 (CH-$_{aliph.}$), 1655 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.71$ (m, 4H, $2CH_2$), 3.32 (m, 4H, $2CH_2$), 6.65 (t, $J = 7.5$ Hz, 1H, Ar–H), 6.90 (d, $J = 8.1$ Hz, 1H, Ar–H), 7.22 (bs, 1H, NH.HCl), 7.23 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.57 (d, $J = 8.1$ Hz, 1H, Ar–H), 7.96 (s, 1H, NH), 8.33 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 33.9$ (C-3′, C-5′), 40.1 (C-2′, C-6′), 65.4 (C-4′), 114.8 (C-4a), 115.4 (C-8), 117.7 (C-6), 127.7 (C-5), 133.8 (C-7), 146.7 (C-8a), 163.5 (C=O) ppm; MS: $m/z = 253$ (M^+).

2,2,6,6-Tetramethyl-1-(2,2,2-trifluoroacetyl)-1′H-spiro[piperidine-4,2′-quinazolin]-4′(3′H)-one (**3e**, $C_{18}H_{22}F_3N_3O_2$)

Yield: 0.23 g (69%); white solid; m.p.: 196–198 °C (MeOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.78$ (s, 12H, 4CH_3), 2.19 (m, 4H, 2CH_2), 6.67 (t, $J = 7.5$ Hz, 1H, Ar–H), 6.71 (d, $J = 8.1$ Hz, 1H, Ar–H), 7.28 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.57 (d, $J = 8.1$ Hz, 1H, Ar–H), 7.96 (bs, 1H, NH), 8.63 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 25.1$ (4CH_3), 36.6 (C-3$^\backslash$, C-5$^\backslash$), 50.2 (C-2$^\backslash$, C-6$^\backslash$), 66.6 (C-4$^\backslash$), 114.6 (C-4a), 115.7 (C-8), 118.1 (C-6), 128.1 (C-5), 133.1 (C-7), 145.8 (C-8a), 154.3 (C=O, ester), 162.9 (C=O) ppm; MS: $m/z = 369$ (M^+).

1-Tosyl-1′H-spiro[piperidine-4,2′-quinazolin]-4′(3′H)-one (**3f**, $C_{19}H_{21}N_3O_3S$)

Yield: 0.25 g (68%); white solid; m.p.: 288–290 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 2.43$ (m, 4H, 2CH_2), 3.07 (m, 4H, 2CH_2), 3.32 (s, 3H, CH_3), 6.64–6.73 (m, 3H, Ar–H), 7.19 (s, 1H, Ar–H), 7.45–7.66 (m, 5H, Ar–H, NH), 8.12 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 21.5$ (CH_3), 36.8 (C-3$^\backslash$, C-5$^\backslash$), 42.1 (C-2$^\backslash$, C-6$^\backslash$), 66.1 (C-4$^\backslash$), 114.9 (C-4a), 115.2, 117.4, 127.6, 127.8, 130.0 133.7 (8CH-Ar), 134.2 (CH_3C), 143.8 (SO_2C), 146.8 (C-8a), 163.6 (C=O) ppm; MS: $m/z = 371$ (M^+).

1-Benzyl-1′H-spiro[piperidine-4,2′-quinazolin]-4′(3′H)-one (**3g**, $C_{19}H_{21}N_3O$)

Yield: 0.2 g (66%); white solid; m.p.: 282–284 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 2.08$ (s, 4H, 2CH_2), 2.50 (s, 4H, 2CH_2), 4.26 (s, 2H, CH_2Ph), 6.70 (t, $J = 7.5$ Hz, 1H, Ar–H), 6.74 (d, $J = 8.0$ Hz, 1H, Ar–H), 6.99 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.27 (d, $J = 8.1$ Hz, 1H, Ar–H), 7.46 (m, 3H, 2Ar–H, NH), 7.58 (m, 3H, Ar–H), 8.26 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 33.9$ (C-3$^\backslash$, C-5$^\backslash$), 47.1 (C-2$^\backslash$, C-6$^\backslash$), 58.6 (CH_2), 65.5 (C-4$^\backslash$), 115.7 (C-4a), 117.8, 127.6, 128.1, 128.7, 131.8 (9CH-Ar), 133.6 (CH_2C), 149.7 (C-8a), 163.5 (C=O) ppm; MS: $m/z = 307$ (M^+).

4′-Oxo-1,1-di(prop-2-yn-1-yl)-3′,4′-dihydro-1′H-spiro[piperidine-4,2′-quinazolin]-1-ium bromide (**4**, $C_{18}H_{20}BrN_3O$)

Propargyl bromide (0.9 g, 7 mmol) was added portionwise to a well-stirred mixture of 0.5 g of compound **3d** (2 mmol) and anhydrous potassium carbonate (6 mmol) in 10 cm^3 of dry DMF. Stirring was continued overnight at room temperature, the reaction mixture was poured into 50 cm^3 of ice water. The obtained white precipitate was filtered off and dried well. Yield: 0.34 g (58%); m.p.: 236–237 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 2.31$ (m, 4H, 2$CH_{2\text{-spiro}}$), 3.72 (m, 2H, 2CH-$_{\text{alkyne}}$), 4.10 (m, 4H, 2$CH_{2\text{-spiro}}$), 4.54 (s, 2H, $CH_{2\text{-alkyne}}$), 4.68 (s, 2H, $CH_{2\text{-alkyne}}$), 6.74 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.07 (d, 1H, $J = 8.1$ Hz, Ar–H), 7.31 (t, 1H, $J = 7.5$ Hz, Ar–H), 7.53 (s, 1H, NH), 7.63 (d, 1H, $J = 8.1$ Hz, Ar–H), 7.83 (s, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 31.6$ (C-3$^\backslash$, C-5$^\backslash$), 49.3 ($CH_{2\text{-alkyne}}$), 51.2 ($CH_{2\text{-alkyne}}$), 54.6 (C-2$^\backslash$, C-6$^\backslash$), 65.2 (C-4$^\backslash$), 71.4, 71.6 (2CH-$_{\text{alkyne}}$), 84.1, 84.3 (2$C_{q\text{-alkyne}}$), 114.4 (C-4a), 115.5 (C-8), 118.1 (C-6), 127.7 (C-5), 134.0 (C-7), 146.5 (C-8a), 163.3 (C=O) ppm; MS (ESI): *m/z* calcd. $C_{18}H_{20}BrN_3O$ ([M-Br]$^+$) 294.16, found 294.1.

1,1-Bis[(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-1H-1,2,3-triazol-4-yl)methyl]-4′-oxo-3′,4′-dihydro-1′H-spiro-[piperidine-4,2′-quinazolin]-1-ium bromide (**6**, $C_{46}H_{58}BrN_9O_{19}$)

Compound **4** (1 mmol) was added to glucose azide **5** (2 mmol) in THF/H_2O (1:1), then sodium ascorbate (0.4 mmol) and $CuSO_4{\cdot}5H_2O$ (0.2 mmol) were added. The reaction mixture was stirred at room temperature overnight. The reaction progress was monitored by TLC. After completion of the reaction, the reaction mixture was poured into 50 cm^3 of ice water and then extracted with DCM. The solvent was removed under reduced pressure to yield the title compounds. Yield: 0.68 g (65%); m.p.: 190–192 °C (MeOH); IR (KBr): $\bar{\nu} = 1753$ (C=O), 1661 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.80$, 1.84, 1.97, 2.01, 2.02, 2.08 (s, 24H, 8$COCH_3$), 2.37 (m, 4H, 2$CH_{2\text{-spiro}}$), 3.57 (m, 4H, 2$CH_{2\text{-spiro}}$), 4.11–4.19 (m, 6H, 2C_5H-CH_2-OCO, 2C_5H-CH_2-OCO), 4.45 (m, 2H, C_3H), 4.59 (s, 2H, CH_2-C_{triazole}), 4.66 (s, 2H, CH_2-C_{triazole}), 5.26 (m, 2H, 2C_1H-N_{triazole}), 5.71 (m, 4H, 2C_2H, 2C_4H,), 6.40 (t, $J = 7.5$ Hz, 1H, Ar–H), 6.77 (d, $J = 8.0$ Hz, 1H, Ar–H), 6.90 (bs, 1H, NH), 7.35 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.62 (d, $J = 8.0$ Hz, 1H, Ar–H), 8.09 (bs, 1H, NH), 8.86 (s, 1H, CH_{triazole}), 8.89 (s, 1H, CH_{triazole}) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 19.97$, 20.0, 20.2, 20.3, 20.5 (8CH_3), 31.0 (C-3$^\backslash$, C-5$^\backslash$), 36.6 (2NCH_2), 53.7 (C-2$^\backslash$, C-6$^\backslash$), 61.7 (2OCH_2), 66.6 (C-4$^\backslash$), 67.4 (2C_4H), 70.7 (2C_2H), 71.5 (2C_3H), 73.8 (2C_5H), 84.3 (2C_1H-N_{triazole}), 114.1(C-4a), 114.9, 117.9, 127.3, 128.0 (4CH-Ar), 133.8 (2CH-$_{\text{triazole}}$), 135.3 ($C_{\text{-triazole}}$), 145.9 (C-8a), 162.9 (C=O), 169.1, 169.3, 169.4 169.9 (8C=O) ppm.

1,1-Bis[(β-D-glucopyranosyl-1H-1,2,3-triazol-4-yl)methyl]-4′-oxo-3′,4′-dihydro-1′H-spiro[piperidine-4,2′-quinazolin]-1-ium bromide (**7**, $C_{30}H_{42}BrN_9O_{11}$)

Dry freshly prepared solution of NaOMe-MeOH (0.1 equiv) was added to a stirred solution of the glycoside tetraacetate **6** (1.0 equiv) in 20 cm^3 of MeOH at r.t. Stirring was continued for 30 min (monitored by TLC). Neutralize by addition of DOWEX 50 × 8 ion-exchange resin (pH 6), followed by filtration. The filtrate was evaporated to dryness to get the pure unprotected product as colorless crystals in 0.45 g yield (57%). M.p.: 202–204 °C (EtOH); IR (KBr): $\bar{\nu} = 3407$ (OH), 2925 (CH), 1648 (C=O) cm^{-1};

^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 2.39$ (m, 4H, $2CH_{2\text{-spiro}}$), 3.74 (s, 4H, 4OH), 3.79 (m, 4H, $2CH_{2\text{-spiro}}$), 4.05–4.19 (m, 6H, $2C_5H$-CH_2-OCO, $2C_5H$-CH_2-OCO), 4.61 (s, 2H, NCH_2), 4.68 (s, 2H, NCH_2), 5.17 (m, 2H, C_3H), 5.31 (m, 2H, C_2H), 5.42 (m, 2H, C_4H), 5.68 (m, 2H, C_1H-$N_{triazole}$), 6.74 (t, $J = 7.5$ Hz, 1H, Ar–H), 6.92 (d, 1H, $J = 8.2$ Hz, Ar–H), 7.08 (s, 1H, NH), 7.32 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.61 (d, 1H, $J = 8.1$ Hz, Ar–H), 8.14 (s, 1H, NH), 8.82 (s, 2H, $CH_{ar\text{-}triazole}$) ppm.

2-(Chloromethyl)quinazolin-4(3H)-one (**9**)

2-(Chloroacetamido)benzamide (**8**, 0.65 g, 3 mmol) was suspended in 20 cm^3 of toluene with 165 mg of toluenesulfonic acid monohydrate (30 mol %). The suspension was heated under reflux using a Dean-Stark trap equipped with a condenser for 16 h. The mixture was allowed to cool then diluted with 40 cm^3 of ethyl acetate, washed with saturated aqueous sodium hydrogen carbonate (3×20 cm^3), 50 cm^3 of brine, dried over $MgSO_4$, then evaporated to dryness to give compound **9** in 65% yield. M.p.: 246–247 °C (Ref. [20] 247 °C).

Pyrazino[2,1-b:5,4-b′]diquinazoline-8,16(6H,14H)-dione (**10**, $C_{18}H_{12}N_4O_2$)

Sodium ethoxide solution (0.023 g, 1 mmol) in 5 cm^3 of abs. ethanol was added to 0.21 g of compound **8** (1 mmol). The reaction mixture was refluxed for 8 h. The formed solid product was collected by filtration to give compound **10**. Yield: 0.17 g (52%); m.p.: 240–242 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 4.55$ (s, 4H, $2CH_2$), 7.52–7.58 (m, 2H, Ar–H), 7.66–7.69 (m, 2H, Ar–H), 7.81–7.86 (m, 2H, Ar–H), 8.11–8.14 (m, 2H, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 43.7$ ($2CH_2$), 121.7 (C-8a, C-16a), 126.3, 127.7, 135.1 (8CH-Ar), 148.6 (C-4a, C-12a), 152.8 (C-5a, C-13a), 161.9 (C=O) ppm; MS: $m/z = 316$ (M^+).

2-(Ethoxymethyl)quinazolin-4(3H)-one (**11**, $C_{11}H_{12}N_2O_2$)

Sodium ethoxide solution (0.05 g, 2 mmol) in 10 cm^3 of abs. ethanol was added to 2-(2-chloroacetamido)benzamide (**8**, 1 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was evaporated; the residue was dissolved in water. HCl (1 M) was added to reach pH 4. The solid product was filtered off, washed with water and recrystallized from ethanol to give compound **11**. Yield: 0.1 g (48%); m.p.: 140–142 °C (MeOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 1.14$ (t, $J = 7.2$ Hz, 3H, CH_2-CH_3), 3.60 (q, $J = 6.9$ Hz, 2H, CH_2CH_3), 4.36 (s, 2H, CH_2O), 7.48 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.63 (d, 1H, $J = 8.0$ Hz, Ar–H), 7.78 (t, $J = 7.5$ Hz, 1H, Ar–H), 8.09 (d, 1H, $J = 8.1$ Hz, Ar–H), 12.14 (bs, 1H, NH) ppm; MS: $m/z = 204$ (M^+).

2-(2-Azidoacetamido)benzamide (**12**, $C_9H_9N_5O_2$)

To a solution of 2-(2-chloroacetamido)benzamide (**8**, 1 mmol) in acetonitrile, sodium azide (1.5 mmol) was added. The reaction mixture was refluxed for 15 h then cooled to room temperature. The reaction mixture was poured into 20 cm^3 of ice water and then extracted with DCM. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under vacuum. Yield: 0.204 g (93%); m.p.: 120–122 °C (EtOH); IR (KBr): $\bar{\nu} = 3361$ (NH_2), 3186 (CH-$_{aromatic}$), 2114 (N_3), 1676 (C=O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 4.20$ (s, 2H, CH_2N_3), 7.13 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.49 (d, 1H, $J = 7.7$ Hz, Ar–H), 7.65 (bs, 2H, NH_2), 8.09 (t, $J = 7.5$ Hz, 1H, Ar–H), 8.29 (d, 1H, $J = 8.1$ Hz, Ar–H), 12.07 (s, 1H, NH) ppm; MS: $m/z = 219$ (M^+).

2-(Azidomethyl)quinazolin-4(3H)-one (**13**, $C_9H_7N_5O$)

To a freshly prepared solution of sodium ethoxide (4 mmol in 10 cm^3 of dry ethanol), compound **12** (1 mmol) was added. The reaction mixture was stirred at room temperature overnight. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was poured into 50 cm^3 of ice water and then extracted with DCM. The organic layer was dried using anhydrous Na_2SO_4. The solvent was evaporated under vacuum. Yield: 0.08 g (40%); m.p.: 220–222 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 4.38$ (s, 2H, CH_2N_3), 7.48 (t, $J = 7.5$ Hz, 1H, Ar–H), 7.73 (d, 1H, $J = 8.1$ Hz, Ar–H), 8.05 (t, $J = 7.5$ Hz, 1H, Ar–H), 8.16 (d, 1H, $J = 8.1$ Hz, Ar–H), 12.35 (bs, 1H, NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 51.5$ (CH_2), 121.6 (C-4a), 126.3, 127.3, 134.9 (4CH-Ar), 148.7 (C-8a), 152.7 (C-2), 161.8 (C=O) ppm; MS: $m/z = 201$ (M^+).

4′-Oxo-1,1-bis[[1-[(4-oxo-3,4-dihydroquinazolin-2-yl)methyl]-1H-1,2,3-triazol-4-yl]methyl]-3′,4′-dihydro-1′H-spiro[piperidine-4,2′-quinazolin]-1-ium bromide (**14**, $C_{36}H_{34}BrN_{13}O_3$)

Compound **13** (2 mmol) was added to 1 mmol of **4** in THF/H_2O (1:1), then sodium ascorbate (0.4 mmol) and $CuSO_4$-$5H_2O$ (0.2 mmol) were added. The reaction mixture was stirred for 18 h at room temperature then poured into 50 cm^3 of ice water. The precipitate that formed was filtered and dried well. Yield: 0.43 g (55%); m.p.: 248–250 °C (EtOH); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 2.41$ (m, 4H, $2CH_{2\text{-spiro}}$), 3.21 (m, 4H, $CH_{2\text{-spiro}}$), 4.68 (s, 2H, $CH_2N_{\text{-pip}}$), 4.76 (s, 2H, $CH_2N_{\text{-pip}}$), 5.75 (s, 4H, $2CH_2N_{\text{-triaz}}$), 6.74–6.79 (m, 3H, Ar–H), 7.32 (bs, 1H, NH), 7.44–7.50 (m, 3H, Ar–H), 7.62 (m, 3H, Ar–H), 7.74 (m, 3H, Ar-H), 8.02 (s, 1H, CH-$_{triazole}$), 8.12 (s, 1H, CH-$_{triazole}$), 8.82 (bs, 1H, NH), 12.66 (bs, 2H, 2 NH) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 31.7$ (C-3$^\backslash$, C-5$^\backslash$), 51.9 ($2CH_2N_{\text{-pip}}$), 54.2 (C-2$^\backslash$, C-6$^\backslash$), 59.7 ($2CH_2N_{\text{-triazole}}$), 65.2 (C-4$^\backslash$), 114.1(C-4a), 114.7, 115.5, 118.2, 126.4, 127.5, 127.6, 127.8, 131.2, 134.2 (12CH-Ar), 135.0 ($2CH$-$_{triazole}$), 139.6 ($2C_{\text{-triazole}}$), 148.4 (C-8a), 151.4 (C-2), 161.8 (2C=O), 163.4 (C=O) ppm.

Anticancer screening

The synthesized compounds were screened for their anticancer activity according to NCI protocol. Applying the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of the tested compound at the five concentration levels (Ti)], the percentage growth is calculated at each of the tested compound concentrations levels. Percentage growth inhibition is calculated as: $[(Ti - Tz)/(C - Tz)] \times 100$ for concentrations for which $Ti \geq Tz$ $[(Ti - Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$. Each compound is exposed to 56 human tumor cell lines, including lung, ovarian, breast, colon, melanoma, prostate, and kidney cancers at five different doses for 48 h. Compounds tested initially at a single high dose 10 μM in the full NCI 56 cell panel. The data expressed as a mean graph of the percent growth inhibition of treated cells [22–24]. Mean graph midpoint (MG-MID) (differential activity patterns, bar scale) were created for each cell line to facilitate visual scanning of data for potential NCI patterns of selectivity, with bars depicting the deviation of the individual tumor cell lines from the overall mean value for all the cells tested. In the mean graph, the center point is the mean of all growth inhibition (GI) percentages over all cell lines.

Bars pointed to the right are (positive values) which represent resistance where the inhibition is greater than the average, while bars pointed to the left (negative values), represent sensitivity to the selected cell line where the inhibition is less than the average. The negative numbers indicate cancer cell death.

PDE assay

Inhibition of human PDE5 A1 was determined by the reported protocol [25]. Human cGMP and phosphodiesterase (PDE 5A1) were purchased from Sigma-Aldrich. All other reagents were of analytical grade from Merck.

The enzymatic reaction was carried out in 0.1 M Tris–HCl buffer, pH 8.3, 10 mM $MgCl_2$, 0.1 M KCl at 37 °C. Alfa casein (2 mg) used as a carrier for protein precipitation when protein concentration is low. The final volume ranged between 500 and 100 cm^3. The reaction was started by the addition of cGMP in a final concentration of 0.5×10^{-4} M. Enzyme and time of incubation may be altered and if are not indicated in the specific experimental procedure it will be 60 min. The reaction was finished by transferring the reaction mixture tubes in boiling water bath for 3 min. The sample was then centrifuged and filtered through a nylon-66 filter, 0.2 mm (Rainin corporation). The clear filtrate obtained may be used directly for HPLC assay or stored at −20 °C. A blank with protein, denaturated by boiling water bath for 3 min, with and without substrate was performed. Both incubation time and enzyme concentration were adjusted so that no more than 25% of the substrate was hydrolyzed under the assay conditions. All assays were done in duplicate.

The HPLC system was Agilent Technologies 1200 Series, G1315D DAD. The column used was Zorbax Eclipse AAA rapid resolution 4.6 × 150 mm 3.5 μM particle size. The mobile phase employed for the separation (isocratic elution) consisted in 200 mM ammonium acetate pH 6.0 with 2% acetonitrile (v/v). The flow rate was 1.5 cm^3/min; the detector DAD at 254 nm. The injection volume was 30 mm^3. Peak identities were confirmed by co-elution with standards. Quantitative measurements were carried out by comparison, using standard solutions of known concentrations.

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