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Investigating the anti-proliferative activity of styrylazanaphthalenes and azanaphthalenediones

Anna Mrozek-Wilczkiewicz [a,c], Danuta S. Kalinowski [b], Robert Musiol [a], Jacek Finster [a], Agnieszka Szurko [c,d], Katarzyna Serafin [a], Magdalena Knas [a], Sishir K. Kamalapuram [b], Zaklina Kovacevic [b], Josef Jampilek [e,f], Alicja Ratuszna [c], Joanna Rzeszowska-Wolny [d], Des R. Richardson [b,*], Jaroslaw Polanski [a,*]

[a] Department of Organic Chemistry, Institute of Chemistry, University of Silesia, PL-40006 Katowice, Poland
[b] Department of Pathology and Bosch Institute, University of Sydney, Sydney, New South Wales 2006, Australia
[c] Department of Solid State Physics, Institute of Physics, University of Silesia, PL-40007 Katowice, Poland
[d] Department of Experimental and Clinical Radiobiology, Center of Oncology-M. Sklodowska-Curie Memorial Institute, PL-44101 Gliwice, Poland
[e] Zentiva a.s., CZ-10237 Prague 10, Czech Republic
[f] Department of Chemical Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, CZ-61242 Brno, Czech Republic

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ABSTRACT

A group of styrylazanaphthalenes and azanaphthalenediones were synthesized and tested for their anti-proliferative activity. Most of the compounds were obtained with the use of microwave-assisted synthesis. The lipophilicity of the compounds was measured by RP-HPLC and their anti-proliferative activity was assayed against the human SK-N-MC neuroepithelioma and HCT116 human colon carcinoma cell lines. Active compounds were also tested in clonogenity and comet assays. Several quinazolinone and styrylquinazoline analogues were found to have markedly greater anti-proliferative activity than desferoxamine and cis-platin.

1. Introduction

The quinoline moiety is present in many classes of biologically active compounds. A number of them have been clinically used as anti-fungal, anti-bacterial, anti-protozoic[1–3] and anti-tuberculotic agents.[4,5] Some quinoline-based compounds also have anti-neoplastic, anti-asthmatic and anti-platelet activity,[6–11] as well as other biological effects.[12,13]

Styrylquinolines are another class of interesting quinoline-related compounds.[14–20] The anti-proliferative effects of quinoline-5,8-diones and styarylquinoline-carboxylic acids on tumor cell lines have been observed and recently reported.[21–26] In this respect, compound **I** (2-[2-(4-chlorophenyl)vinylquinoline-8-ol]) (Fig. 1) demonstrated marked anti-proliferative effects (IC_{50} = 0.77 μM).[20] Quinolinedione is the main fragment of lavendamycine and related compounds, which are known for their broad spectrum activity. Further, a series of 7-amino-quinaldine-5,8-diones with anti-proliferative activity has been reported recently.[21–25] In this context, compound **V** (R = Ph–CH_2CH_2) appeared to be very active, having an IC_{50} of 1.44 μM.[21]

An important structure–activity relationship observed was that anti-proliferative activity of quinoline analogues is often potentiated with the introduction of a second nitrogen atom to the azanaphtalene moiety.[27,28] Jiang et al. synthesized a large series of quinazolines (**III**) in the search for new tubulin polimerization inhibitors[29] and some were found to be highly active against L1210 leukemia cells. The 6-methoxy derivative (**III**, R = OMe) showed anti-proliferative activity at micromolar concentrations (IC_{50} = 3.59 μM). The authors optimized the anti-proliferative activity of the most interesting analogues by introducing various substituents to the C-6 position of quinazoline moiety. However, structural modifications to the styryl moiety have not been reported.

A lack of detailed structure–activity relationship studies make new drug discovery of these promising compounds difficult. On this basis, we present the anti-proliferative activity of modified quinoline- and styrylquinoline-related azanaphthalene compounds.

This drug design idea was based on our former results[20,21] and is shown in Figure 1. We have designed new structures by incorporating molecular fragments from known anti-proliferative agents onto the diazanaphthalene moiety. The introduction of the second nitrogen atom into the structures of the previously investigated quinoline compounds[21–26] provided three diazanaphthalene scaffolds **VI–VIII** which were investigated in this publication.

* Corresponding authors. Tel.: +48 32 3591197; fax: +48 32 2599978 (J.P.).
E-mail addresses: d.richardson@med.usyd.edu.au (D.R. Richardson), polanski@us.edu.pl (J. Polanski).

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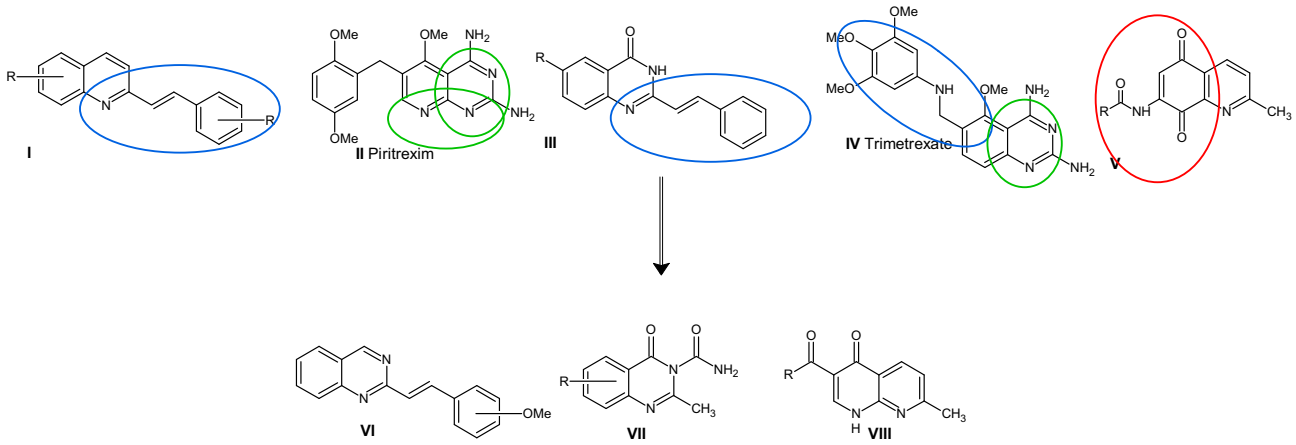
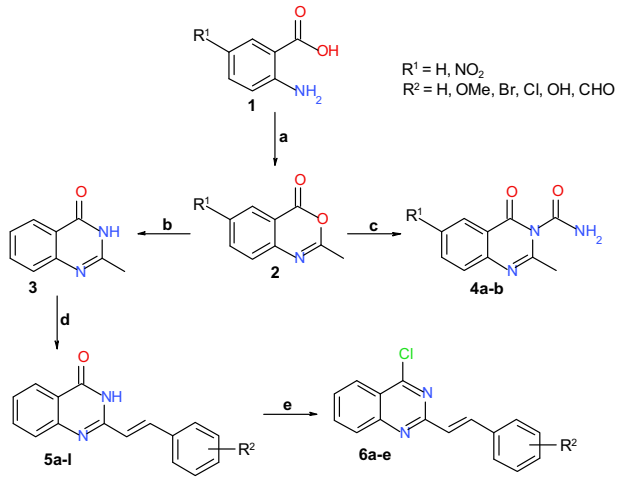
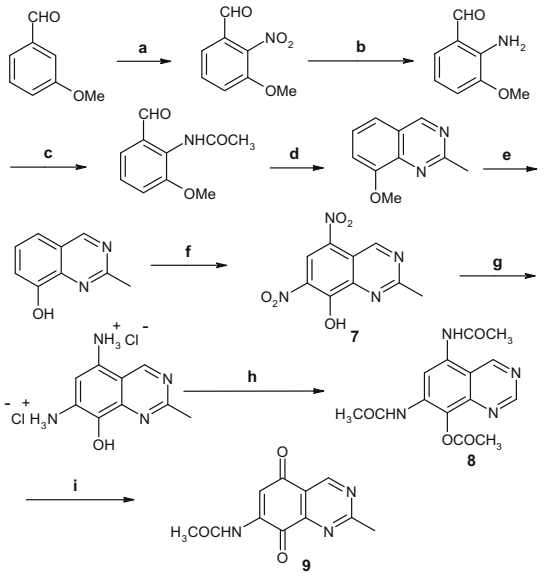


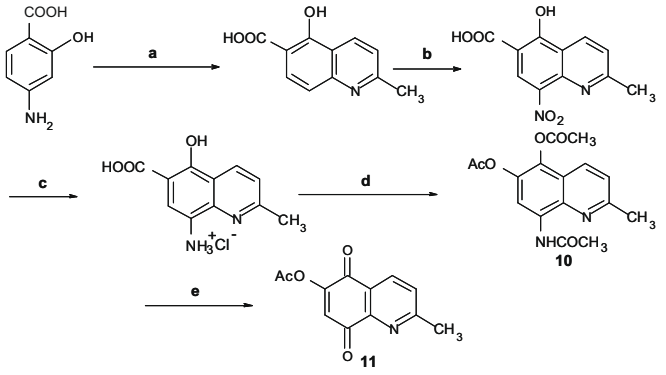
Figure 1. Design of styrylquinazoline and related structures.



Scheme 1. Synthesis of the studied compounds: (a) Ac_2O MW; (b) NH_3aq MW; (c) urea; (d) aldehyde MW; (e) $POCl_3$.



Scheme 2. Synthesis of the compound **9**. Reagents: (a) HNO_3, H_2SO_4; (b) Fe, HCl, EtOH, H_2O; (c) $(CH_3CO)_2O$, CH_2Cl_2; (d) NH_3, EtOH; (e) BBr_3, CH_2Cl_2; (f) HNO_3, H_2SO_4; (g) H_2, Pd/C, HCl, H_2O; (h) $(CH_3CO)_2O$, CH_3COONa, Na_2SO_3; (i) $K_2Cr_2O_7$, CH_3COOH.



Scheme 3. Synthesis of the compound **11**. Reagents: (a) $CH_3CH{=}CHCHO$, CH_3COOH, HCl; (b) HNO_3, H_2SO_4; (c) $Na_2S_2O_4$, HCl, H_2O; (d) $(CH_3CO)_2O$, CH_3COONa, $Na_2S_2O_4$; (e) $K_2Cr_2O_7$, CH_3COOH.

2. Results and discussion

2.1. Chemistry

All studied compounds were prepared according to Schemes 1–3. Microwave-assisted synthesis facilitated the process of obtaining the quinazoline-related structures. Benzoxazin-4-ones (**2**) were synthesized from anthranilic acids and acetic anhydride. Further reaction with ammonia or amines afforded the quinazolin-4-ones (**3**, **4a**, **4b**). Compounds (**5a–5l**) were obtained from appropriate aldehydes using neat microwave-assisted synthesis.[30] Further aromatization with $POCl_3$ yielded the 4-chloro-2-styryl-quinazoline derivatives (**6a–e**). Compounds **9** and **11** were obtained by multistep synthesis according to Scheme 2 or Scheme 3 using 3-methoxybenzaldehyde or 5-hydroxy-6-quinaldic acid, respectively, through subsequent nitration, reduction and oxidation reactions in a similar manner as described earlier.[21,31]

2.2. Lipophilicity

Drugs most frequently cross biological barriers through passive transport, which strongly depends on their lipophilicity.[32] Therefore, log *k* data characterizing the hydrophobicity of the studied compounds are presented, as this parameter is an important factor in membrane permeability. The log *k* values were measured by reverse phase high performance liquid chromatography (RP-HPLC). The procedure was performed under isocratic conditions with methanol as an organic solvent in the mobile phase using an

end-capped non-polar C_{18} stationary RP column. The capacity factors k were determined and subsequent log k values were calculated. These results are shown in Table 1.

2.3. Biological activities

The anti-proliferative activity of the synthesized compounds were assessed by the MTT assay against human SK-N-MC neuroepithelioma cells using standard methods.[20] Some compounds were additionally assessed in the HCT116 (human colon carcinoma) cell line by the MTS assay. Moreover, we performed clonogenic survival tests to measure the long term influence of the active compounds on the cells. Cell survival in response to anti-tumor agents could be an important criterion, as final cell death may occur only after additional cell divisions.[33]

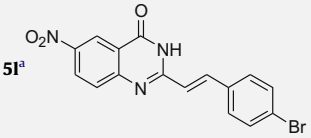
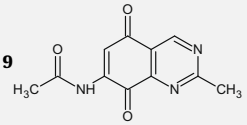
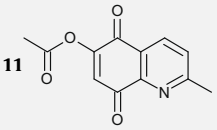
The results from the anti-proliferative activity assays are shown in Tables 1 and 2. All the compounds can be divided into three groups, namely **4**, **5** and **6**, according to their structural scaffolds (Scheme 1). When tested against the SK-N-MC and HCT116 cell lines, compounds **4a** and **4b** had limited anti-proliferative activity.

Interestingly, these compounds were also among the most hydrophilic compounds analyzed. These results suggested that they may have poor membrane permeability which limits their biological activity. Some compounds of the styrylquinazolinone series (**5a**–**l**) showed promising anti-proliferative efficacy. Compounds **5a**, **5b** and **5h** demonstrated the highest anti-proliferative activity (IC_{50}: 1.39–2.88 μM) within this series against both SK-N-MC and HCT116 cells. Generally, substitution at the $C_{(3)}$ position resulted in a decrease of anti-cancer activity, while substitution at $C_{(2)}$ or $C_{(4)}$ appeared to be more advantageous. Within the interesting sub-series formed by the OMe-substituted styrylquinazolinones (**5b**, **5d**, **5g**, **5j**), the 2-OMe substitution (**5b**) appeared to be optimal for anti-cancer activity, while the dimethoxy substitution resulted in the largely inactive compound **5j**.

It is evident that the pattern of anti-cancer activity for styryl-modified quinazolones **5** and **6** differs between the two investigated cell lines (compare **5a**, **5b**, **6b**, and **6e**). Compound **6b** showed the highest anti-proliferative activity (IC_{50} = 0.58 μM) of all the groups of compounds when screened against the SK-N-MC line, but only moderate activity when tested using HCT116 cells (IC_{50} = 9.40 μM).

In Table 1 we have also listed the Hammett constant (σ) values. Accordingly, it can be concluded that anti-proliferative activity is dependent, only to some extent, on the Hammett constant (σ) and lower σ values result in higher anti-proliferative activity within series **5**. The substitution pattern within series **6** is too homogenous to estimate the influence of this parameter on anti-cancer effects.

Table 1
Anti-cancer activity and cytotoxic data of the studied compounds (ND—not determined)

Compd	R^1/R^2	Activity IC_{50} (μM)		Cytotoxicity IC_{50}^* (μM)	log k	σ[a]
		SK-N-MC	HCT116	NIH3T3		
4a	H	>6.25	>600	ND	0.553	0.00
4b	NO_2	>6.25	>92	ND	0.958	0.71
5a	H	1.39 ± 0.04	2.53 ± 0.31	2.4 ± 0.05	1.115	0.00
5b	2-OMe	2.88 ± 0.21	1.58 ± 0.23	1.17 ± 0.07	1.151	–
5c	2-Br	>6.25	>46	ND	1.483	–
5d	3-OMe	5.13 ± 0.08	ND	ND	1.184	0.12
5e	3-Br	>6.25	ND	ND	1.590	0.39
5f	3-Cl	>6.25	ND	ND	1.506	0.37
5g	4-OMe	>6.25	>46	>100[b]	1.135	−0.11
5h	4-Br	2.85 ± 2.33	ND	ND	1.593	0.23
5i	4-CHO	>6.25	ND	ND	1.035	1.03
5j	2,4-OMe	>6.25	ND	ND	1.198	–
5k	2,3,4-OH	ND	>46	ND	0.551	–
5l[a]		>6.25	ND	ND	1.675	0.94
6a	H	4.96 ± 0.03	ND	3.04 ± 0.09	1.109	0.00
6b	2-OMe	0.58 ± 0.05	9.40 ± 0.48	>100[b]	1.365	–
6c	3-OMe	5.28 ± 0.06	ND	>100[b]	1.183	0.12
6d	4-OMe	4.34 ± 0.17	ND	>100[b]	1.134	−0.11
6e	2,4-OMe	1.75 ± 1.67	23	34.09 ± 0.09[b]	1.195	–
9		ND	17	ND	0.017	–
11		ND	10.9 ± 1.15	ND	–	–
I		0.7	ND	ND	1.539	–
DFO		9.89 ± 0.05	–	–	–	–
Doxorubicin		0.12 ± 0.03	5.95 ± 0.5	–	–	–
Cisplatin		3.80 ± 0.36	–	–	–	–

Results are expressed as mean ± SD of 3–5 experiments.
[a] According to[34,35].
[b] Compounds with toxicity index (IC_{50}^*/IC_{50}) higher than 10.

Table 2
Prolonged cytotoxicity as survival fraction (SF) of tumor cells after incubation with the compound

Compd	Concentration (μM)	SF after 24 h incubation	SF after 96 h incubation
6b[a]	0.58	0.43	0.0
	46	0.03	0.0
9	0.58	1.2[b]	0.26[b]
	46	0.0[a]	0.0[a]
11[b]	0.58	1.07	0.21
	46	0.04	0.0

[a,b] Results are expressed as mean of 3 and 2 experiments, respectively

Interestingly, the most potent anti-proliferative compound of series **5** against the SK-N-MC cell line, **5a**, possessed the lowest lipophilicity. Higher log *k* values of compounds in series **5** resulted in a decrease in anti-proliferative activity. However, in contrast, the relatively lipophilic compound, **5h** (log *k* = 1.593), also demonstrated moderate anti-cancer activity, opposing this trend.

Within series **6**, compound **6b** possessed the most potent anti-proliferative efficacy, while also being the most lipophilic. This compound also has the highest lipophilicity of all compounds in the styrylquinazoline series. This suggests that membrane permeability could play an important role in its anti-proliferative effects. Hence, compound **6b** may permeate the cell membrane and gain access to the intracellular environment to exert its anti-cancer effects more efficiently.

In Table 1, we examined the cytotoxic effects of a number of the most potent compounds against the normal NIH3T3 fibroblast cell line to determine their selectivity. The relatively high toxicity of the quinolinedion system is a well known fact in the literature.[23] This was also observed in our study. Thus, compounds **5a** and **5b**, when tested against NIH3T3 fibroblasts, appeared to be almost as cytotoxic as they were against the cancer cell lines. Conversely, the **6** group of compounds all showed lower anti-proliferative activity against fibroblasts, with the exception of the un-substituted **6a** analogue. This demonstrated that the most potent anti-cancer agent, **6b**, has selective anti-proliferative activity with little effect on the growth of normal cells and may be less toxic when administered in vivo.

As shown in Table 2, the studied compounds prevented clonogenity of cancer cells, with compound **6b** being the most active in this context. However, all tested compounds resulted in a marked decrease in survival fraction after a 96 h incubation (Table 2). Further studies with these promising compounds are warranted to fine-tune their anti-tumor efficacy.

3. Conclusion

A series of **21** azanaphthalene related compounds were designed on the basis of known anti-proliferative agents. A structurally diverse set of styrylquinazolines and related compounds were prepared and characterized. Their lipophilicity and purity was analyzed using RP-HPLC and their anti-proliferative and anti-clonogenic activity was also examined using HCT116 and SK-N-MC cells. Structure–activity relationships were also studied by examining their lipophilicity and Hammett constants. Some compounds were found to be more active than desferrioxamine (DFO) and the clinically used agent, cisplatin.

4. Experimental

4.1. General

All reagents were purchased from Aldrich. Kieselgel 60, 0.040–0.063 mm (Merck, Darmstadt, Germany) was used for column chromatography. TLC experiments were performed on alumina-backed silica gel 40 F254 plates (Merck, Darmstadt, Germany). The plates were illuminated under UV (254 nm) and evaluated in iodine vapor.

The melting points were determined on a Boetius PHMK 05 instrument (VEB Kombinat Nagema, Radebeul, Germany) and are uncorrected. Elemental analyses were carried out on an automatic Perkin–Elmer 240 microanalyser (Boston, USA) for C, H, N and are within 0.4% of theoretical values. The purity of the final compounds was checked by HPLC. A detection wavelength of 210 nm was chosen for detection. The peaks in the chromatogram of the solvent (blank) were deducted from the peaks in the chromatogram of the sample solution. The purity of individual compounds was determined from the area peaks in the chromatogram of the sample solution.

UV spectra (λ, nm) were determined on a Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA) in methanolic solution (ca. 6×10^{-4} mol) and log ε (the logarithm of molar absorption coefficient, ε) was calculated for the absolute maximum λ_{max} of individual target compounds. All ^{1}H NMR spectra were recorded on a Bruker AM-500 (499.95 MHz for ^{1}H), Bruker BioSpin Corp., Germany. Chemical shifts are reported in ppm (δ) against the internal standard, $Si(CH_3)_4$. Easily exchangeable signals were omitted when diffuse.

Syntheses were performed on Plazmatronika RM-800PC microwave reactor with monomode cavity, magnetic stirrer and external IR temperature measurements. Microwave power was automatically adjusted to achieve the desired temperature unless specified otherwise.

4.2. HPLC determination of purity and lipophilicity (capacity factor *k*/calculated log *k*)

The HPLC separation module, Waters Alliance 2695 XE and Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA), were used. The Symmetry® C_{18} 5 μm, 4.6 × 250 mm, Part No. WAT054275, (Waters Corp., Milford, MA, USA) chromatographic column was used. The HPLC separation process was monitored by Millennium32® Chromatography Manager Software, Waters 2004 (Waters Corp., Milford, MA, USA). The mixture of MeOH p.a. (55.0%) and H_2O-HPLC—Milli-Q Grade (45.0%) was used as a mobile phase. The total flow of the column was 0.9 mL/min, using an injection volume of 30 μL, a column temperature of 30 °C and a sample temperature of 10 °C. The detection wavelength of 210 nm was chosen. The KI methanolic solution was used for the dead time (t_D) determination. Retention times (t_R) were measured in minutes.

The capacity factors *k* were calculated using the Millennium32® Chromatography Manager Software according to formula $k = (t_R - t_D)/t_D$, where t_R is the retention time of the solute, whereas t_D denotes the dead time obtained via an unretained analyte. Log *k*, calculated from the capacity factor *k*, was used as the lipophilicity index converted to log *P* scale. The log *k* values of the individual compounds are shown in Table 1.

4.3. Synthesis

4.3.1. General synthesis of compounds 4a–b

A mixture of benzoxazin-4-one (0.01 mol) and urea (0.02 mol) in absolute ethanol (10 mL) was refluxed for 3 h, then cooled and filtered. The separated solid was purified by crystallization from ethanol.

The following compounds were prepared in this manner.

4.3.1.1. 2-Methyl-4-oxo-*4H*-quinazoline-3-carboxylic acid amide (4a). Yield 33% of white solid; mp 150 °C; ^{1}H NMR

($(CD_3)_2CO$) δ 2.41 (s, 3H, CH_3); 7.54 (d, J = 8.1, 1H, Ar-H); 7.58 (t, J = 7.8, 1H, Ar-H); 7.89 (t, J = 7.8, 1H, Ar-H); 8.12 (d, J = 7.7, 1H, Ar-H); 11.30 (s, 2H, NH_2); IR (KBr) [cm^{-1}]: 3323; 3246; 1758; 1682; 1641; 1621; 1518; 1466; 1381; 776; purity: 92.88%; UV: λ_{max} = 314.9; log ε = 3.57.

4.3.1.2. 2-Methyl-6-nitro-4-oxo-*4H*-quinazoline-3-carboxylic acid amide (4b). Yield 65% of yellow solid; mp 159 °C; ^{1}H NMR ($(CD_3)_2CO$) δ 2.27 (s, 3H, CH_3); 8.43 (d, J = 9.4, 1H, Ar-H); 8.83 (s, 1H, Ar-H); 8.90 (d, J = 9.4, 1H, Ar-H); 11.30 (s, 2H, NH_2); IR (KBr) [cm^{-1}]: 3273; 3133; 1716; 1684; 1616; 1587; 1514; 1473; 1372; 872; 857; 749; purity: 95.37%; UV: λ_{max} = 304.9; log ε = 3.51.

4.3.2. General method for synthesis of styryl-compounds 5a–l

A mixture of quinazolin-4-one (0.01 mol) and the appropriate aldehyde (0.02 mol) was mixed thoroughly and irradiated in a monomode cavity of the microwave reactor using pulse sequence (3 × 5 min with 30 s intervals) at 250 W. During irradiation, the temperature was controlled between the range of 150–180 °C. After the reaction, the mixture was cooled and washed with boiling ether. The product was crystallized from acetic acid.

The following compounds were prepared in this manner.

4.3.2.1. 2-((*E*)-Styryl)-*3H*-quinazolin-4-one (5a). Yield 50% of white solid; mp 253–255 °C (lit. mp 252 °C[28]); ^{1}H NMR DMSO-d_6) δ 7,00 (d, J = 16.23 Hz, 1H, C=C–H); 7.41 (t, J = 7.5, 1H, Ar-H); 7.42–7.49 (m, 3H, Ar-H); 7.65–7.68 (m, 3H, Ar-H); 7,80 (t, J = 7.8, 1H, Ar-H); 7.95 (d, J = 16.16 Hz, 1H, C=C–H); 8.10 (d, J = 7.57, 1H, Ar-H); 12.35 (s, 1H, N–H); purity: 97.95%; UV: λ_{max} = 321.3; log ε = 3.53.

4.3.2.2. 2-((*E*)-2-(2-Methoxy-phenyl)-vinyl)-*3H*-quinazolin-4-one (5b). Yield 76% of white solid; mp 234–236 °C (lit. mp 234–236 °C[28]); ^{1}H NMR (DMSO-d_6) δ 3.90 (s, 3H, OCH_3); 7.02 (t, J = 7.45 1H, Ar-H); 7.07 (d, J = 16.25, 1H, C=C–H); 7.11 (d, J = 7.0, 1H, Ar-H); 7.39 (t, J = 7.4, 1H, Ar-H); 7.45 (t, J = 7.6, 1H, Ar-H); 7.60 (d, J = 7.69, 1H, Ar-H); 7.67 (d, J = 7.5, 1H, Ar-H); 7.79 (t, J = 7.75, 1H, Ar-H); 8.09 (d, J = 7.65, 1H, Ar-H); 8.15 (d, J = 16.2, 1H, C=C–H); 12.36 (s, 1H, N–H); purity: 94.04%; UV: λ_{max} = 343.4; log ε = 3.62.

4.3.2.3. 2-((*E*)-2-(2-Bromo-phenyl)-vinyl)-*3H*-quinazolin-4-one (5c). Yield 71% of white solid; mp 279 °C; ^{1}H NMR ($CDCl_3$) δ 6.93 (d, J = 16.45, 1H, C=C–H); 7.40 (t, J = 7.6, 1H, Ar-H); 7.50 (t, J = 7.6, 1H, Ar-H); 7.67 (d, J = 7.9, 1H, Ar-H); 7.74–7.82 (m, 4H, Ar-H); 8.12 (d, J = 16.45, 1H, C=C–H); 8.36 (d, J = 7.8, 1H, Ar-H); 10.96 (s, 1H, NH); purity: 98.84%; UV: λ_{max} = 326.9; log ε = 3.59.

4.3.2.4. 2-((*E*)-2-(3-Methoxy-phenyl)-vinyl)-*3H*-quinazolin-4-one (5d). Yield 68% of white solid; mp 239–241 °C; ^{1}H NMR (DMSO-d_6) δ 3.81 (s, 3H, OCH_3); 6.98 (d, J = 8.1, 1H, Ar-H); 7.01 (d, J = 16.8, 1H, C=C–H); 7.22 (s, 1H, Ar-H); 7.23 (d, J = 7.6, 1H, Ar-H); 7.37 (t, J = 7.4, 1H, Ar-H); 7.47 (t, J = 7.55, 1H, Ar-H); 7.66 (d, J = 7.5, 1H, Ar-H); 7.80 (t, J = 7.9, 1H, Ar-H); 7.91 (d, J = 16.1, 1H, C=C–H); 8.10 (d, J = 8.2, 1H, Ar-H); 12.31 (s, 1H, NH); purity: 96.82%; UV: λ_{max} = 326.4; log ε = 3.58.

4.3.2.5. 2-((*E*)-2-(3-Bromo-phenyl)-vinyl)-*3H*-quinazolin-4-one (5e). Yield 43% of white solid; mp 277–279 °C; ^{1}H NMR ($CDCl_3$) δ 6.91 (d, J = 16.3, 1H, C=C–H); 7.33 (t, J = 7.5, 1H, Ar-H); 7.50–7.56 (m, 2H, Ar-H); 7.55 (s, 1H, Ar-H); 7.73 (d, J = 16.4, 1H, C=C–H); 7.76–7.82 (m, 3H, Ar-H); 8.33 (d, J = 7.9, 1H, Ar-H); 10.31 (s, 1H, NH); purity: 97.34%; UV: λ_{max} = 326.4; log ε = 3.58.

4.3.2.6. 2-((*E*)-2-(3-Chloro-phenyl)-vinyl)-*3H*-quinazolin-4-one (5f). Yield 93% of white solid; mp 289 °C; ^{1}H NMR ($CDCl_3$) δ 6.93 (d, J = 16.4, 1H, C=C–H); 7.39 (d, J = 6.5, 2H, Ar-H); 7.46–7.53 (m, 2H, Ar-H); 7.64 (s, 1H, Ar-H); 7.79 (d, J = 16, 1H, C=C–H); 7.77–7.83 (m, 2H, Ar-H); 8.33 (d, J = 7.5, 1H, Ar-H); 10.64 (s, 1H, NH); purity: 96.51%; UV: λ_{max} = 326.4; log ε = 3.59.

4.3.2.7. 2-((*E*)-2-(4-Methoxy-phenyl)-vinyl)-*3H*-quinazolin-4-one (5g). Yield 33% of white solid; mp 280–281 °C (lit. mp 284–285 °C[28]); ^{1}H NMR (DMSO-d_6) δ 3.80 (s, 3H, OCH_3); 6.84 (d, J = 16.2, 1H, C=C–H); 7.01 (d, J = 8.4, 2H, Ar-H); 7.45 (t, J = 7.8, 1H, Ar-H); 7.60 (d, J = 8.4, 2H, Ar-H); 7.64 (d, J = 8.1, 1H, Ar-H); 7.78 (t, J = 8.0, 1H, Ar-H); 7.90 (d, J = 16.1, 1H, C=C–H); 8.08 (d, J = 7.9, 1H, Ar-H); 12.25 (s, 1H, N–H); purity: 94.36%; UV: λ_{max} = 322.7; log ε = 3.59.

4.3.2.8. 2-((*E*)-2-(4-Bromo-phenyl)-vinyl)-*3H*-quinazolin-4-one (5h). Yield 66% of white solid; mp 332 °C; ^{1}H NMR (DMSO-d_6) δ 7.03 (d, J = 16.15, 1H, C=C–H); 7.49 (t, J = 8.35, 1H, Ar-H); 7.61 (d, J = 8.2, 1H, Ar-H); 7.64 (d, J = 8.3, 2H, Ar-H); 7.66 (d, J = 8.2, 1H, Ar-H); 7.68 (d, J = 8.2, 1H, Ar-H); 7.81 (t, J = 8.1, 1H, Ar-H); 7.91 (d, J = 16.15, 1H, C=C–H); 8.11 (d, J = 8.35, 1H, Ar-H); 12.36 (s, 1H, N–H); purity: 97.64%; UV: λ_{max} = 326.7; log ε = 3.58.

4.3.2.9. 2-((*E*)-2-(4-Carbaldehyde-phenyl)-vinyl)-*3H*-quinazolin-4-one (5i). Yield 35% of white solid; mp 218 °C; ^{1}H NMR ($CDCl_3$) δ 7.16 (d, J = 16.1, 1H, C=C–H); 7.50 (t, J = 7.3, 1H, Ar-H); 7.69 (d, J = 8.1, 2H, Ar-H); 7.82 (t, J = 8.15, 1H, Ar-H); 7.87 (d, J = 8.15, 1H, Ar-H); 7.97 (d, J = 8.15, 1H, Ar-H); 8.00 (d, J = 16.2, 1H, C=C–H); 8.11 (d, J = 7.9, 2H, Ar-H); 9.95 (s, 1H, CHO); 10.02 (s, 1H, N–H); purity: 96.93%; UV: λ_{max} = 337.9; log ε = 3.69.

4.3.2.10. 2-((*E*)-2-(2,4-Dimethoxy-phenyl)-vinyl)-*3H*-quinazolin-4-one (5j). Yield 57% of white solid; mp 228–230 °C (lit. mp 228–230 °C[28]); ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 3H, OCH_3); 3.90 (s, 3H, OCH_3); 6.63 (d, J = 8.5, 1H, Ar-H); 6.64 (s, 1H, Ar-H); 6.94 (d, J = 16.15, 1H, C=C-H); 7.43 (t, J = 7.7, 1H, Ar-H); 7.53 (d, J=8.4, 1H, Ar-H); 7.64 (d, J = 8.2, 1H, Ar-H); 7.77 (t, J = 8.1, 1H, Ar-H); 8.07 (d, J = 15.2, 1H, C=C–H); 8.08 (d, J = 8.4, 1H, Ar-H); 12.26 (s, 1H, N–H); purity: 96.27%; UV: λ_{max} = 350.1; log ε = 3.67.

4.3.2.11. 2-((*E*)-2-(2,3,4-Trihydroxy-phenyl)-vinyl)-*3H*-quinazolin-4-one (5k). Yield 60% of brown solid; mp 300 °C (decomp.); ^{1}H NMR (DMSO-d_6) δ 6.39 (d, J = 8.5, 1H, Ar-H); 6.83 (d, J = 16.0, 1H, C=C–H); 6.88 (d, J = 8.5, 1H, Ar-H); 7.40 (t, J = 8.1, 1H, Ar-H); 7.63 (d, J = 8.1, 1H, Ar-H); 7.75 (t, J = 8.1, 1H, Ar-H); 8.05 (d, J = 7.9, 1H, Ar-H); 8.10 (d, J = 16.05, 1H, C=C–H); 8.57 (s, 1H, OH); 9.07 (s, 1H, OH); 9.68 (s, 1H, OH); 12.19 (s, 1H, NH); purity: 97.67%; UV: λ_{max} = 365.0; log ε = 3.65.

4.3.2.12. 2-((*E*)-2-(4-Bromo-phenyl)-vinyl)-6-nitro-*3H*-quinazolin-4-one (5l). Yield 32% of brown solid; mp 320 °C (decomp.); ^{1}H NMR (DMSO-d_6) δ 7.07 (d, J = 16.1, 1H, C=C–H); 7.65 (d, J = 8.5, 2H, Ar-H); 7.69 (d, J = 8.5, 2H, Ar-H); 7.84 (d, J = 7.9, 1H, Ar-H); 8.04 (d, J = 16.1, 1H, C=C–H); 8.54 (d, J = 7.0, 1H, Ar-H); 8.80 (s, 1H, Ar-H); 12.81 (s, 1H, N–H); purity: 96.63%; UV: λ_{max} = 339.9; log ε = 3.67.

4.3.3. General method for synthesis of compounds 6a–e

A mixture of compound **5a** (or **5b**, **5d**, **5g**, **5j**) (0.01 mol), *N*,*N*-dimethylaniline (0.02 mol) and phosphorus oxychloride (0.015 mol) in dry benzene (50 mL) was stirred and heated under reflux for 3 h. The reaction mixture was then cooled and filtered. The filtrate was diluted with benzene (30 mL) and the solution washed with water (50 mL), twice with 20% aqueous NaOH (50 mL) and finally twice with water. After drying with $MgSO_4$, the organic solvent was evaporated and the product obtained was crystallized from heptane.

The following compounds were prepared in this manner.

4.3.3.1. 4-Chloro-2-((*E*)-styryl)-quinazoline (6a). Yield 81% of orange solid; mp 104 °C (lit. mp 100–101 °C[27]); ^{1}H NMR (DMSO-d_6) δ 7.34 (d, J = 15.9, 1H, C=C–H); 7.41 (t, J = 8.3, 1H, Ar-H); 7.47 (t, J = 7.9, 2H, Ar-H); 7.77–7.80 (m, 3H, Ar-H); 8.02 (d, J = 8.3, 1H, Ar-H); 8.06 (t, J = 8.15, 1H, Ar-H); 8.12 (d, J = 16.0, 1H, C=C–H); 8.26 (d, J = 8.25, 1H, Ar-H); purity: 99.34%; UV: λ_{max} = 314.9; log ε = 3.67.

4.3.3.2. 4-Chloro-2-((*E*)-2-(2-methoxy-phenyl)-vinyl)-quinazoline (6b). Yield 86% of light yellow solid; mp 153 °C; ^{1}H NMR (DMSO-d_6) δ 3.98 (s, 3H, OCH_3); 7.04 (t, J = 8.0, 1H, Ar-H); 7.12 (d, J = 8.3, 1H, Ar-H); 7.37 (d, J = 16.15, 1H, C=C–H); 7.40 (t, J = 8.1, 1H, Ar-H); 7.78 (t, J = 8.1, 1H, Ar-H); 7.82 (d, J = 8.05, 1H, Ar-H); 8.01 (d, J = 8.1, 1H, Ar-H); 8.06 (t, J = 8.15, 1H, Ar-H); 8.27 (d, J = 8.2, 1H, Ar-H); 8.47 (d, J = 16.15, 1H, C=C–H); purity: 99.95%; UV: λ_{max} = 345.1; log ε = 3.67.

4.3.3.3. 4-Chloro-2-((*E*)-2-(3-methoxy-phenyl)-vinyl)-quinazoline (6c). Yield 81% of light yellow solid; mp 137 °C; ^{1}H NMR (DMSO-d_6) δ 3.90 (s, 3H, OCH_3); 6.98 (d, J = 8.4 Hz, 1H, Ar-H); 7.35 (d, J = 15.75, 1H, C=C–H); 7.35–7.39 (m, 2H, Ar-H); 7.36 (s, 1H, Ar-H); 7.80 (t, J = 8.4, 1H, Ar-H); 8.02 (d, J = 8.4, 1H, Ar-H); 8.06 (d, J = 8.05z, 1H, Ar-H); 8.10 (d, J = 15.8, 1H, C=C–H); 8.28 (d, J = 8.3, 1H, Ar-H); purity: 98.62%; UV: λ_{max} = 342.9; log ε = 3.64.

4.3.3.4. 4-Chloro-2-((*E*)-2-(4-methoxy-phenyl)-vinyl)-quinazoline (6d). Yield 51% of yellow solid; mp 130–131 °C (lit. mp 130–131 °C[27]); ^{1}H NMR (DMSO-d_6) δ 3.87 (s, 3H, OCH_3); 7.03 (d, J = 8.8, 2H, Ar-H); 7.20 (d, J = 15.9, 1H, C=C–H); 7.75 (d, J = 8.6, 2H, Ar-H); 7.76 (t, J = 8.2, 1H, Ar-H); 7.99 (d, J = 8.0, 1H, Ar-H); 8.04 (t, J = 8.45, 1H, Ar-H); 8.08 (d, J = 15.9, 1H, C=C–H); 8.25 (d, J = 8.5, 1H, Ar-H); purity: 97.43%; UV: λ_{max} = 339.9; log ε = 3.64.

4.3.3.5. 4-Chloro-2-((*E*)-2-(2,4-dimethoxy-phenyl)-vinyl)-quinazoline (6e). Yield 48% of yellow solid; mp 172 °C; ^{1}H NMR ($(CD_3)_2CO$) δ 3.88 (s, 3H, OCH_3); 3.98 (s, 3H, OCH_3); 6.64 (d, J = 7.72, 1H, Ar-H); 6.66 (s, 1H, Ar-H); 7.27 (d, J = 16.1, 1H, C=C–H); 7.75 (d, J = 8.0, 1H, Ar-H); 7.60 (t, J = 8.0, 1H, Ar-H); 7.98 (d, J = 7.85, 1H, Ar-H); 8.02 (t, J = 8.0, 1H, Ar-H); 8.25 (d, J = 8.25, 1H, Ar-H); 8.40 (d, J = 16.1, 1H, C=C–H); IR (KBr) [cm^{-1}]: 2980; 2938; 1607; 1556; 1504; 1477; 1450; 1384; 1329; 959; 768; 756; purity: 98.57%; UV: λ_{max} = 355.0; log ε = 3.67.

4.3.4. 8-Hydroxy-2-methyl-5,7-dinitro-quinazoline (7)

8-Hydroxy-2-methyl-quinazoline (3.75 mmol) was added portion wise to a stirred solution of HNO_3 (3.5 mL) and H_2SO_4 (1.5 mL) cooled in an ice-bath. The mixture was allowed to stir in the ice-bath for 2 h and then poured into a beaker containing ice. The mixture was vigorously stirred and the precipitate was filtered, washed with ice-water, diethyl ether and air-dried. Yield 85% of yellow solid; mp 250 °C; ^{1}H NMR (DMSO-d_6) δ 2.78 (s, 3H, CH_3); 9.10 (s, 1H, Ar-H); 10.22 (s, 1H, Ar-H); 11.23 (s, 1H, OH); purity: 95.87%; UV: λ_{max} = 420.6; log ε = 3.65.

4.3.5. 5,7-Diacetamido-8-acetoxy-2-methyl-quinazoline (8)

A suspension of the powdered dinitro compound (1.6 mmol) and 10% Pd/C (0.13 g) in hydrochloric acid solution (0.66 mL of concd HCl in 6 mL H_2O) was hydrogenated until the color changed from yellow to deep red. The mixture was filtered and the residue was washed with a small amount of water. However, the free diamine was found to be very unstable. Hence, it was immediately converted to the more stable triacetic acid derivative. The ammonium salt solution was treated with sodium acetate (1.33 g) and sodium sulfite (0.66 g). To this gently stirred solution, acetic anhydride (4.5 mL) was added dropwise over 10 min. The mixture was allowed to stir for 1 h at room temperature and then for an additional 30 min in an ice-bath. The precipitate was filtered, washed with cold water and air-dried. Yield 50% of white solid; mp 274–276 °C (decomp.); ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 3H, CH_3); 2.34 (s, 3H, CH_3); 2.42 (s, 3H, CH_3); 2.62 (s, 3H, CH_3); 7.88 (s, 1H, Ar-H); 9.47 (s, 1H, Ar-H); 9.57 (s, 1H, N–H); 11.40 (s, 1H, N–H).

4.3.6. 7-Acetamido-2-methyl-quinazoline-5,8-dione (9)

A solution of potassium dichromate (0.2 g) in water (5.5 mL) was added dropwise to a stirred suspension of 5,7-diacetamido-8-acetoxy-2-methyl-quinazoline (0.47 mmol) in glacial acetic acid (5.8 mL). The mixture was stirred and heated in a water bath for 1.5 h. The resulting dark mixture was then extracted with dichloromethane (12 × 3 mL). The combined organic extracts were washed with 3% sodium bicarbonate solution, dried with magnesium sulfate and then evaporated to dryness. Yield 80% of yellow solid; mp 210 °C (decomp.); ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3H, CH_3); 2.98 (s, 3H, CH_3); 7.98 (s, 1H, Ar-H); 8.31 (s, 1H, Ar-H); 9.43 (s, 1H, N–H); purity: 95.98%; UV: λ_{max} = 279.6; log ε = 3.74.

4.3.7. 8-Acetamido-5-acetoxy-2-methyl-quinoline-6-carboxylic acid (10)

Powdered 5-hydroxy-8-nitro-quinoline-6-carboxylic acid (4 mmol) was added portion wise to a stirred solution of sodium dithionite (5.33 g) in water (180 mL). The mixture was stirred at room temperature until the color changed from yellow to deep red. The mixture was then filtered and the residue was washed with a small amount of water. Hydrochloric acid solution (0.8 mL of concd HCl in 7 mL H_2O) was added to the filtrate and the mixture was allowed to stir for 10 min. The ammonium salt solution was treated with sodium acetate (0.8 g) and sodium dithionite (0.013 g). Acetic anhydride (14 mL) was added dropwise to this gently stirred solution over 30 min. The mixture was allowed to stir for 1 h at room temperature and then for an additional 45 min in an ice-bath. The precipitate was filtered, washed with cold water and air-dried. Yield 88% of white solid; mp 280 °C (decomp.); ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 3H, CH_3); 2.26 (s, 3H, CH_3); 2.72 (s, 3H, CH_3); 7.51 (d, J = 8.11, 1H, Ar-H); 8.11 (d, J = 8.18, 1H, Ar-H); 8.94 (s, 1H, Ar-H); 9.57 (s, 1H, N–H); 9.70 (s, 1H, COOH).

4.3.8. 2-Methyl-5,8-dioxo-5,8-dihydro-quinoline-6-carboxylic acid (11)

A solution of potassium dichromate (1.2 g) in water (20 mL) was added dropwise to a stirred suspension of 8-acetamido-5-acetoxy-2-methyl-quinoline-6-carboxylic acid (1.3 mmol) in glacial acetic acid (20 mL). The mixture was stirred and heated in a water bath for 3 h. The resulting dark mixture was then extracted with dichloromethane (6 × 40 mL). The combined organic extracts were washed with water, dried with magnesium sulfate and then evaporated to dryness. Yield 65% of yellow solid; mp 232–234 °C; ^{1}H NMR (DMSO-d_6) δ 2.63 (s, 3H, CH_3); 7.67 (d, J = 8.56, 1H, Ar-H); 7.72 (s, 1H, Ar-H); 8.28 (d, J = 8.49, 1H, Ar-H); 9.96 (s, 1H, COOH).

4.4. Biological activity measurements

The human SK-N-MC neuroepithelioma cell line was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). The SK-N-MC cell line was cultured in minimum essential medium (MEM; Gibco, Melbourne Australia) containing 10% (v/v) FBS, 1.0 mM sodium pyruvate (Gibco), 1% (v/v) non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), streptomycin (Gibco) and 0.28 μg/mL fungizone (Squibb Pharmaceuticals, Montreal, Canada).

The human colon adenocarcinoma cells (HCT116) and NIH3T3 fibroblast cells were also obtained from the ATCC. Cells were grown as monolayer cultures in 75 cm^2 flasks (Nunc) in Dulbecco's

modified Eagle's medium supplemented with 12% fetal bovine serum (Gibco-BRL), 100 μg/mL of gentamycin, 100 μg/mL of streptomycin and 100 IU/mL of crystalline penicillin (Polfa). Cells were cultured under standard conditions at 37 °C, in a humidified atmosphere at 5% CO_2.

Twenty four hours before addition of the tested compounds, the cells were seeded in 96-well plates. The assay was performed following a 72 h incubation with varying concentrations of the tested agents. The results were calculated as IC_{50} values. Each individual compound was tested in triplicate in a single experiment, with each experiment being repeated 3–7 times. After a 72 h incubation with tested compounds, 10 μL of MTT solution (MTT: stock solution: 5 mg/mL) was added to each well and incubated for 2 h at 37 °C. After this incubation, 100 μL of the lysis mixture was added to each well. The optical densities of the samples were analyzed at 570 nm.

4.5. Cytotoxicity and anti-proliferative activity

For estimation of anti-proliferative activity of quinazoline derivatives, exponentially growing cells were harvested by trypsinisation of sub-confluent cultures. Cells were then seeded at concentrations of 3.5×10^3, 7×10^3 and 9×10^3 cells per well into 96-well cell culture microtiter plates (Nunc) and cultured for 18 h. After this time, the growth medium was exchanged for medium containing 0.58 μmol/L and 46 μmol/L final concentrations of the compounds. After incubation with the investigated compounds for 24, 48 and 96 h at 37 °C under standard cell culture conditions, medium was replaced with 100 μL of DMEM without phenol red. Metabolic activity of viable cells was determined by adding 20 μL of The CellTiter 96® AQ_{ueous} One Solution—MTS (Promega) to each well followed by incubating for 1 h. The MTS assay is a colorimetric method for determining the number of viable cells. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. A standard solution containing 100 μL of DMEM without phenol red and 20 μL of MTS solution was used to determine 'blank' absorbance. The absorbance was measured at 490 nm using an El_x800 plate reader (BioTek Instruments, USA).

4.6. Clonogenic assay

Clonogenic survival is an important criterion of cell survival in response to anti-tumor agents as final cell death may occur only after additional divisions. The clonogenic assay (also known as a colony formation assay) is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo 'unlimited' division and can be used to determine the effectiveness of cytotoxic agents. Clonogenic potential of cells subjected to studied compounds was assessed as described by Carmichael.[31] For these analyses, 8.5×10^4 or 2.1×10^5 of HCT116 cells were seeded in 35-mm dishes (Nunc). Cells were kept in an incubator for 18 h, and then exposed to various concentrations of compounds (0.58 μM and 46 μM) prepared in culture medium. After 24 h and 96 h, cells were collected by trypsinisation, washed and re-seeded in 60-mm dishes (in the absence of any agents). Cells were then incubated for 9 days at 37 °C in a humidified atmosphere, after which they were fixed in 96% ethanol, washed with distilled water and stained with 0.2% crystal violet/water solution. Thereafter, colonies were counted by microscopic inspection and plating efficiency with clonogenic survival fractions were also calculated. The surviving fraction was calculated as the relative plating efficiency of treated versus non-treated samples. Only colonies with at least 50 cells were counted. Each experiment was repeated on three separate days and on each day triplicates of each dose and treatment combination was performed.

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