Full Paper

Synthesis, *in-vitro* Cytotoxicity, and a Preliminary Structure-Activity Relationship Investigation of Pyrimido[4,5-*c*]quinolin-1(2*H*)-ones

Kamel Metwally[1], Ashraf Khalil[1], Harris Pratsinis[2], and Dimitris Kletsas[2]

[1] Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia
[2] Laboratory of Cell Proliferation and Ageing, Institute of Biology, National Centre of Scientific Research ''Demokritos'', Athens, Greece

As part of our ongoing research effort to develop new antimitotic agents based on the recently reported pyrimido[4,5-*c*]quinoline-1(2*H*)-one ring skeleton, we were interested in identifying structural elements that contribute to the cytotoxicity of this class of compounds. The effect of several quinoline-ring substituents was examined and the new compounds were evaluated *in vitro* for cytotoxicity against three human cancer cell lines namely, lung fibrosarcoma HT-1080, colon adenocarcinoma HT-29, and breast carcinoma MDA-MB-231. Most of the compounds showed cytotoxic activity in the low micromolar and sub-micromolar range. Structure-activity relationship information revealed that a combination of electronic and steric factors may be involved. Flow cytometric cell cycle analysis performed on HT-1080 cells revealed that the most cytotoxic compounds **48**, **50**, **54**, **59**, and **63** inhibit the S-phase and arrest the cells in the G2/M phase of the cell cycle suggesting an antimitotic action of these compounds.

Keywords: Antimitotic agents / Pyrimido[4,5-*c*]quinolin-1(2*H*)-ones / Structure-activity relationship (SAR)

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Introduction

Interfering with microtubule dynamics has been proved to be a successful strategy for enriching the clinical oncology arsenal with new chemotherapeutic drug candidates [1]. As was the case with most major drug discoveries, lead antimitotic agents, also called spindle poisons, were inspired by nature [2]. Antimitotic agents were categorized depending on their ability to polymerize tubulin or depolymerize microtubules into two major classes. Agents which promote tubulin polymerization thereby blocking microtubule disassembly are referred to as ''microtubule-stabilizing agents'' and are typified by the taxoids such as paclitaxel and the epothilones. On the other hand, agents such as colchicine, podophyllotoxin, combretastatin A-4, and the vinca alkaloids such as vinblastine and vincristine, inhibit tubulin polymerization thereby blocking microtubule assembly and are therefore called ''microtubule-destabilizing agents'' [3, 4]. Whether microtubule-stabilizing or -destabilizing, the ultimate biological consequence is the alteration of spindle dynamics and cell-cycle arrest during mitosis [5]. Furthermore, it has been recently established that some of these agents, particularly those binding to the colchicine domain, act as vascular-disrupting agents through selectively targeting microtubules of endothelial cells of the tumor-associated microvasculature system resulting in inhibition of blood supply to the growing tumor [6–10]. Some vascular-disrupting agents are currently in ongoing clinical trials to be used alone or in combination with other cancer chemotherapeutic agents [8, 10]. Noteworthy in this respect is that vascular-disrupting agents are different from anti-angiogenic agents, which inhibit tumor neovascularization rather than affecting already established tumor blood vessels [11–13]. With these unique mechanistic aspects, antimitotic agents comprise a good alternative to DNA-interacting drugs in cancer chemotherapy. Although some of these agents are

Correspondence: Kamel Metwally, Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Kingdom of Saudi Arabia.
E-mail: kametwally@hotmail.com
Fax: +966 3 581-7174

currently used in the treatment of certain human malignancies, their narrow therapeutic window, limited bioavailability, drug resistance, and complex syntheses or isolation procedures significantly detract from their clinical success [14]. Therefore, there is a need for the development of new antimitotic agents with improved pharmacological profile. In this regard, we have previously reported the synthesis and *in-vitro* cytotoxic activity of 2-amino-3,5,9-trisubstituted-pyrimido[4,5-*c*]quinolin-1(2*H*)-ones (**A**) as a novel class of antimitotic agents (Fig. 1). It was generally concluded that the presence of a chloro-substituent at C-9 has a major positive impact on cytotoxic activity and the presence of a 4-chlorophenyl substituent at C-3 further enhances activity. With respect to C-5 substituents, a 4-chlorophenyl was slightly better than a 4-bromophenyl in terms of cytotoxicity. The most potent compounds dose-dependently inhibited tubulin polymerization and arrested the cell cycle in the G2/M phase [15]. In another study, we examined the effect of replacing the 2-amino group by hydrophobic aryl residues. It was found that the 2-aryl analogues (**B**) retain cytotoxicity of their 2-amino counterparts [16]. In continuation of this research effort, we were interested in exploring the influence of quinoline-ring substituents on the cytotoxic activity through formulating a preliminary structure-activity relationship for this new class of antimitotic agents. In the present work, pyrimido[4,5-*c*]quinolin-1(2*H*)-ones having substituents with different electronic and steric characteristics (**C**) were synthesized and evaluated for *in-vitro* cytotoxicity and their mechanism of action was investigated through cell cycle analysis.

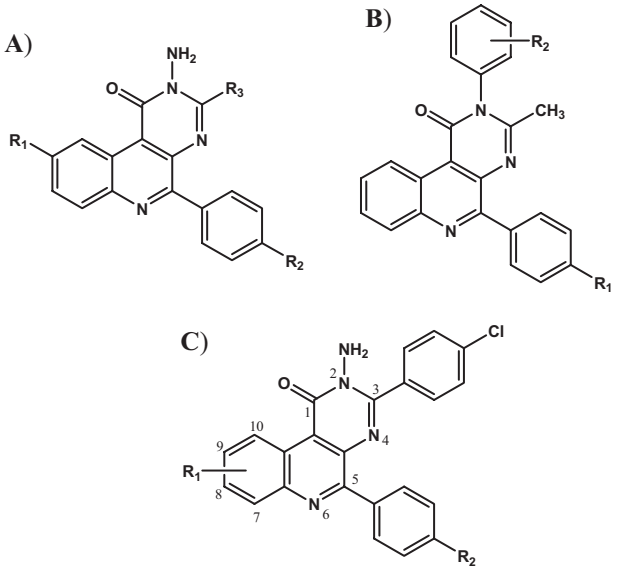


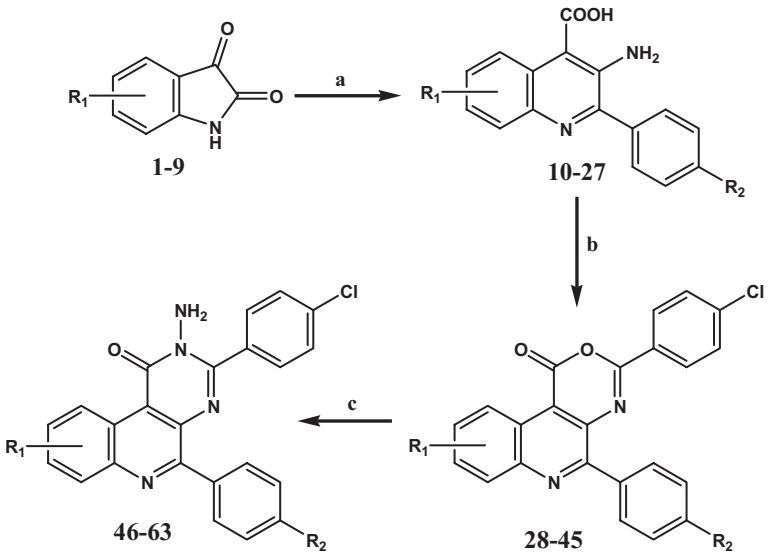
Figure 1. Structures of 2-amino-3,5,9-trisubstituted-pyrimido[4,5-*c*]quinolin-1(2*H*)-ones (**A**), 2-aryl analogues (**B**), and pyrimido[4,5-*c*]quinolin-1(2*H*)-ones having substituents with different electronic and steric characteristics (**C**).

Chemistry

Novel pyrimido[4,5-*c*]quinolin-1(2*H*)-ones **46–63** were synthesized as shown in the general reaction sequence depicted in Scheme 1. The effect of various electron-withdrawing 7- and/or 9-halo substituents was studied through synthesizing analogues **47–52** and **56–61** whereas the synthesis of **53**, **54**, **62**, and **63** allowed us to investigate electron-donating 9-methyl and 9-methoxy substituents. Preliminarily, the 3-aminoquinoline-4-carboxylic acids **10–27** were obtained in good yields following a modified Pfitzinger procedure through reaction of isatins **1–9** with 4-chloro or 4-bromophenacylamine hydrochloride in the presence of sodium hydroxide. Unfortunately, our attempts to prepare 3-amino-2-(4-chlorophenyl)-6-nitroquinoline-4-carboxylic acid and 3-amino-2-(4-bromophenyl)-6-nitroquinoline-4-carboxylic acid starting from 5-nitroisatin under different reaction conditions were unsuccessful. Treatment of the acids **10–27** with 4-chlorobenzoyl chloride in pyridine at room temperature afforded the corresponding lactones **28–45** in 51–63% yields. IR spectra showed the appearance of characteristic absorption bands at 1754–1776 cm^{-1} corresponding to the lactone carbonyl group. Unfortunately, ^{1}H-NMR spectral analysis could not be performed for all the lactones due to lack of adequate solubility in the available deuterated solvents. The desired target compounds **46–63** were synthesized by reacting the lactones **28–45** with hydrazine hydrate in 2-ethoxyethanol under reflux conditions. ^{1}H-NMR spectra showed a characteristic singlet at $\delta = 6.01$–6.09 ppm integrating for two protons corresponding to the amino group. All the synthesized compounds were characterized by spectral data analysis that confirmed the assigned structures. In addition, the new compounds were microanalyzed satisfactorily for C, H, N.

Results and discussion

The antiproliferative activity of the new pyrimido[4,5-*c*]-quinolin-1(2*H*)-ones **47**, **49–54**, **56**, and **58–63** was determined *in vitro* against three human cancer cell lines, namely, lung fibrosarcoma HT-1080, colon adenocarcinoma HT-29, and breast carcinoma MDA-MB-231 using the widely accepted MTT assay. Related compounds from our previous investigation [15], namely **46**, **48**, **55**, and **57** were co-tested with the new compounds under the same assay conditions for comparison purposes. Doxorubicin hydrochloride was included in the experiments as a reference cytotoxic compound. The results were expressed as IC_{50} values which represent the compound concentrations required to produce



46: R_1= H, R_2= Cl
47: R_1= 9-F, R_2= Cl
48: R_1= 9-Cl, R_2= Cl
49: R_1= 9-Br, R_2= Cl
50: R_1= 9-I, R_2= Cl
51: R_1= 7-Cl, R_2= Cl
52: R_1= 7,9-di-Cl, R_2= Cl
53: R_1= 9-Me, R_2= Cl
54: R_1= 9-OMe, R_2= Cl
55: R_1= H, R_2= Br
56: R_1= 9-F, R_2= Br
57: R_1= 9-Cl, R_2= Br
58: R_1= 9-Br, R_2= Br
59: R_1= 9-I, R_2= Br
60: R_1= 7-Cl, R_2= Br
61: R_1= 7,9-di-Cl, R_2= Br
62: R_1= 9-Me, R_2= Br
63: R_1= 9-OMe, R_2= Br

Reagents and conditions:

(**a**) 4-Chloro- or 4-bromophenacylamine hydrochlorides, NaOH, H_2O/EtOH/THF, 85°C, then reflux;

(**b**) 4-chlorobenzoyl chloride, pyridine, room temp.; (**c**) hydrazine hydrate 99%, 2-ethoxyethanol, reflux.

Scheme 1. Synthesis of compounds **10–63**.

a 50% inhibition of cell growth after three days of incubation compared to untreated controls (Table 1).

Structure-activity relationships (SAR)

Since it was concluded from our previous work [15] that the 5-(4-chlorophenyl) and 5-(4-bromophenyl) analogues showed only a slight difference in cytotoxic potency, it seemed relevant to conduct the present SAR study on two separate sets of compounds: a set with a 5-(4-chlorophenyl) residue and another with a 5-(4-bromophenyl) residue for additional confirmation. As might be expected, the two sets of compounds displayed a similar pattern of structure-activity relationship in the three cell lines. Because previously [15], a 9-chloro substituent was found to be a critical determinant of cytotoxic activity, we were interested initially in examining the influence of chloro substitution on other positions of the ring. The 7-chloro analogue **51** was substantially less active than the 9-chloro analogue **48** exhibiting 9- to 17-fold reduction in cytotoxicity against the three cell lines tested. Double chloro substitution gave the 7,9-dichloro analogue **52** which demonstrated an activity intermediate between **48** and **51**, indicating that the 9-position is the best position for chloro substitution. A similar behavior was observed in the 5-(4-bromophenyl) series. Compound **61** was more active than **60** but exhibited lower cytotoxic activity when compared to **57** against HT-1080 and MDA-MB-231 cell lines.

These initial results urged us to focus on the 9-position of the pyrimido[4,5-*c*]quinolin-1(2*H*)-one ring. Investigating other halogens was our next approach in the present study. Introduction of a 9-fluoro substituent resulted in compounds **47** and **56**, which were generally found to be the least active among the 9-halo substituted analogues in both sets of compounds against all cell lines tested. Compared to the unsubstituted parent compounds **46** and **55**, however, 9-fluoro substitution generally appears to contribute positively to cytotoxic activity.

Interestingly, the 9-iodo analogue **59** showed higher cytotoxicity as compared to the 9-chloro analogue **57** against the three cancer cell lines. Similarly, compound **50** was nearly as potent as **48** against colon adenocarcinoma and breast

Table 1. *In-vitro* cytotoxicity of the target pyrimido[4,5-*c*]quinolin-1(2*H*)-ones using the MTT assay.

Compound	R_1	R_2	Cell line		
			HT-1080	HT-29	MDA-MB-231
46	H	Cl	1.82 ± 0.41	23.42 ± 9.69	50.12 ± 5.90
47	9-F	Cl	1.99 ± 0.03	7.61 ± 1.18	3.37 ± 0.74
48	9-Cl	Cl	1.06 ± 0.33	1.96 ± 0.32	1.47 ± 0.42
49	9-Br	Cl	1.11 ± 0.02	3.89 ± 1.58	2.06 ± 0.16
50	9-I	Cl	0.64 ± 0.04	1.86 ± 0.37	1.34 ± 0.30
51	7-Cl	Cl	9.12 ± 0.57	33.00 ± 8.37	17.77 ± 3.64
52	7,9-di-Cl	Cl	1.15 ± 0.08	9.58 ± 2.65	5.51 ± 1.70
53	9-Me	Cl	1.47 ± 0.18	>100	>100
54	9-OMe	Cl	0.32 ± 0.14	10.10 ± 4.18	2.06 ± 0.21
55	H	Br	7.12 ± 0.71	60.41 ± 24.91	37.27 ± 6.52
56	9-F	Br	2.07 ± 0.31	6.26 ± 2.49	4.76 ± 0.98
57	9-Cl	Br	1.84 ± 0.19	10.76 ± 4.12	3.99 ± 0.70
58	9-Br	Br	0.97 ± 0.27	2.91 ± 0.57	3.04 ± 1.07
59	9-I	Br	1.04 ± 6.2	3.92 ± 1.09	2.32 ± 0.37
60	7-Cl	Br	8.21 ± 0.22	24.11 ± 5.58	10.45 ± 0.33
61	7,9-di-Cl	Br	2.43 ± 0.03	2.99 ± 0.86	9.93 ± 1.77
62	9-Me	Br	0.86 ± 0.14	>100	>100
63	9-OMe	Br	0.49 ± 0.22	6.74 ± 3.23	1.69 ± 0.47
Doxo			0.022 ± 0.08	0.35 ± 0.13	0.030 ± 0.02

IC_{50} values in μM.

carcinoma cells and slightly more active in the lung fibrosarcoma cell line. In the 5-(4-chlorophenyl) series, the 9-halo substituents contributed to cytotoxicity in the order of: I > Cl > Br > F whereas, in the 5-(4-bromophenyl) series, the halogens ranked differently in the three cell lines tested. These results imply that a combination of electronic, steric, and hydrophobic factors may be involved.

The effect of electron-donating groups was also studied through synthesizing and testing compounds **53**, **54**, **62**, and **63**. Methylation of the 9-position to give compounds **53** and **62** resulted in complete loss of activity against colon adenocarcinoma and breast carcinoma cell lines. In the lung fibrosarcoma screen, however, compound **53** was almost equipotent to the unsubstituted parent compound **46**, while compound **62** displayed about 8-fold higher cytotoxicity as compared to its unsubstituted counterpart **55**. The 9-methoxy analogues **54** and **63** were the most potent among the compounds tested against the lung fibrosarcoma cell line exhibiting IC_{50} values of 0.32 and 0.49 μM, respectively, but showed only modest cytotoxic activity against other cell lines tested. In terms of selectivity, the tested compounds demonstrated generally higher selectivity to lung fibrosarcoma HT-1080 cell line as compared to other cell lines used in the assay with colon adenocarcinoma cells being the least susceptible.

Table 2. Cell-cycle phase distribution (%). [§]

Compound	G_0/G_1	S	G_2/M
48	44.0	10.2	45.8
50	42.3	15.7	42.0
54	48.8	17.2	34.1
59	44.7	16.7	38.7
63	45.4	5.7	49.0
Control	45.0	27.4	27.6

[§] One out of two similar experiments is depicted.

Effect on cell-cycle progression

Compounds that had the highest activity in cytotoxicity assays, namely, **48**, **50**, **54**, **59**, and **63** were selected for cell cycle studies in human lung fibrosarcoma HT-1080 cells through flow cytometry after staining with propidium iodide. As shown in Table 2, all the compounds tested inhibited the S-phase and caused a significant accumulation of cells at the G_2/M phase of the cell cycle relative to the untreated control, which is in agreement with the microtubule-disrupting effect of **48** shown previously [15].

Conclusion

We have formulated a preliminary structure-activity relationship for pyrimido[4,5-*c*]quinoline-1(2*H*)-ones recently identified as cytotoxic antimitotic agents. Several substituents with different electronic and steric properties at different positions of the quinoline ring were investigated for their contribution to cytotoxicity of this class of compounds. Among the substituents examined, chloro, iodo, and methoxy groups at *C*-9 were generally shown

to give the most potent cytotoxic compounds. In the three cell lines tested, substantially lower activity was displayed by 7-chloro analogues as compared to their 9-chloro counterparts. Interestingly, the 9-methyl analogues exhibited remarkable selectivity for HT-1080 cells and were devoid of activity against HT-29 and MDA-MB-231 cells. Cell cycle studies indicated that the cytotoxic effects of these compounds are most probably produced through disruption of mitosis.

Experimental

Chemistry

Melting points were determined on a Barnstead Electrothermal 9100 melting point apparatus (Barnstead, Dubuque IA, USA) and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8400S spectrophotometer (Shimadzu, Tokyo, Japan) as KBr pellets. ^{1}H-NMR spectra were recorded at 300 MHz on a Varian-Mercury 300BB spectrometer (Varian, Palo Alto, CA, USA) in DMSO-d_6. Chemical shifts were reported as parts per million (ppm) downfield from tetramethylsilane (TMS), and coupling constants (*J*) are given in Hertz (Hz). Elemental analyses (C, H, N) were performed at the Microanalytical Unit, Cairo University, Cairo, Egypt. All compounds were routinely checked by thin-layer chromatography (TLC) on aluminum-backed silica gel plates. All solvents were dried by standard methods. Compounds **10–12**, **15**, **16**, **19–21**, **24**, **25**, **28**, **30**, **37**, **39**, **46**, **48**, **55**, and **57** were reported previously [15, 17].

*General procedure for 3-amino-2-(4-halophenyl)-6-substituted-quinoline-4-carboxylic acids **10–27***

To a solution of the appropriate isatin (10 mmol) and sodium hydroxide (80 mmol) in water (10 mL) at 85°C a solution of the appropriate phenacylamine hydrochloride (14 mmol) in a water/ethanol/THF mixture (25 mL:25 mL:10 mL was added) in a dropwise manner over a period of 2 h and the mixture was then heated at reflux for additional 30 min. After cooling, the reaction mixture was concentrated under reduced pressure and filtered through celite. The filtrate was acidified with acetic acid and the solid obtained was filtered, washed with water, and dried. The crude product was purified by recrystallization from ethanol/ethyl acetate.

*3-Amino-6-bromo-2-(4-chlorophenyl)quinoline-4-carboxylic acid **13***

Yield: 76%, m. p.: 230–233°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 7.51–7.55 (dd, J = 2.21, 2.20 Hz, 1H, Ar-H), 7.59–7.63 (m, 2H, Ar-H), 7.68–7.71 (m, 2H, Ar-H), 7.75–7.78 (d, J = 8.76 Hz, 1H, Ar-H), 8.66–8.67 (d, J = 2.14 Hz, 1H, Ar-H). Anal. calcd. for $C_{16}H_{10}BrClN_2O_2$: C, 50.89; H, 2.67; N, 7.42. Found: C, 50.67; H, 2.72; N, 7.32.

*3-Amino-2-(4-chlorophenyl)-6-iodoquinoline-4-carboxylic acid **14***

Yield: 71%, m. p.: 255–258°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 7.58–7.62 (m, 3H, Ar-H), 7.66–7.71 (m, 3H, Ar-H), 8.85–8.86 (d, J = 1.85 Hz, 1H, Ar-H). Anal. calcd. for $C_{16}H_{10}ClIN_2O_2$: C, 45.26; H, 2.37; N, 6.60. Found: C, 45.67; H, 2.51; N, 6.34.

*3-Amino-2-(4-chlorophenyl)-6-methylquinoline-4-carboxylic acid **17***

Yield: 75%, m. p.: 241–243°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 2.46 (s, 3H, CH_3), 7.24–7.27 (dd, J = 1.55, 1.34 Hz, 1H, Ar-H), 7.58–7.61 (m, 2H, Ar-H), 7.68–7.74 (m, 3H, Ar-H), 8.13 (s, 1H, Ar-H). Anal. calcd. for $C_{17}H_{13}ClN_2O_2$: C, 65.29; H, 4.19; N, 8.96. Found: C, 65.04; H, 4.02; N, 8.68.

*3-Amino-2-(4-chlorophenyl)-6-methoxyquinoline-4-carboxylic acid **18***

Yield: 72%, m. p.: 234–235°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 3.86 (s, 3H, OCH_3), 7.06–7.10 (tt, J = 4.67, 4.64 Hz, 1H, Ar-H), 7.57–7.61 (m, 2H, Ar-H), 7.67–7.70 (m, 2H, Ar-H), 7.73–7.77 (dd, J = 1.74, 1.75 Hz, 1H, Ar-H), 8.85–8.86 (t, J = 4.36 Hz, 1H, Ar-H). Anal. calcd. for $C_{17}H_{13}ClN_2O_3$: C, 62.11; H, 3.99; N, 8.52. Found: C, 62.19; H, 3.70; N, 8.16.

*3-Amino-6-bromo-2-(4-bromophenyl)quinoline-4-carboxylic acid **22***

Yield: 73%, m. p.: 241–244°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 7.51–7.52 (tt, J = 3.69, 3.65 Hz, 1H, Ar-H), 7.61–7.64 (m, 2H, Ar-H), 7.74–7.78 (m, 3H, Ar-H), 8.67–8.68 (t, J = 3.36 Hz, 1H, Ar-H). Anal. calcd. for $C_{16}H_{10}Br_2N_2O_2$: C, 45.53; H, 2.39; N, 6.64. Found: C, 45.38; H, 2.55; N, 6.53.

*3-Amino-2-(4-bromophenyl)-6-iodoquinoline-4-carboxylic acid **23***

Yield: 72%, m. p.: 264–266°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 7.57–7.70 (complex m, 4H, Ar-H), 7.73–7.77 (dd, J = 2.10, 2.06 Hz, 2H, Ar-H), 8.85–8.86 (t, J = 3.79 Hz, 1H, Ar-H). Anal. calcd. for $C_{16}H_{10}BrIN_2O_2$: C, 40.97; H, 2.15; N, 5.97. Found: C, 40.78; H, 1.99; N, 6.03.

*3-Amino-2-(4-bromophenyl)-6-methylquinoline-4-carboxylic acid **26***

Yield: 74%, m. p.: 245–247°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 2.45 (s, 3H, CH_3), 7.24–7.27 (dd, J = 1.80, 1.77 Hz, 1H, Ar-H), 7.60–7.65 (m, 2H, Ar-H), 7.71–7.77 (m, 3H, Ar-H), 8.12 (s, 1H, Ar-H). Anal. calcd. for $C_{17}H_{13}BrN_2O_2$: C, 57.16; H, 3.67; N, 7.84. Found: C, 57.30; H, 3.52; N, 7.65.

*3-Amino-2-(4-bromophenyl)-6-methoxyquinoline-4-carboxylic acid **27***

Yield: 70%, m. p.: 240–241°C (EtOH/EtOAc); ^{1}H-NMR (DMSO-d_6) δ: 3.85 (s, 3H, OCH_3), 7.06–7.10 (dd, J = 2.72, 2.70 Hz, 1H, Ar-H), 7.59–7.63 (m, 2H, Ar-H), 7.71–7.76 (m, 3H, Ar-H), 8.85–8.86 (d, J = 2.73 Hz, 1H, Ar-H). Anal. calcd. for $C_{17}H_{13}BrN_2O_3$: C, 54.71; H, 3.51; N, 7.51. Found: C, 54.52; H, 3.33; N, 7.27.

*General procedure for 3-(4-chlorophenyl)-5-(4-chloro- or 4-bromophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-ones **28–45***

To an ice-cooled solution of the appropriate 3-amino-2-aryl-4-quinolinecarboxylic acid (**10–27**; 10 mmol) in dry pyridine (20 mL) 4-chlorobenzoyl chloride (25 mmol) was added in a dropwise manner. After addition was completed, the mixture was allowed to stir at room temperature for 36 h. The reaction mixture was poured into ice water and the obtained solid was filtered, washed with aqueous sodium bicarbonate, water then

ethanol, and dried. The crude product was purified by recrystallization from the appropriate solvent.

*9-Fluoro-3,5-di(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]-quinolin-1-one **29***

Yield: 62%, m. p.: 279–282°C (DMF); IR (KBr, cm^{-1}): 1772 (lactone C=O). Anal. calcd. for $C_{23}H_{11}Cl_2FN_2O_2$: C, 63.18; H, 2.54; N, 6.41. Found: C, 62.90; H, 2.85; N, 6.15.

*9-Bromo-3,5-di(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]-quinolin-1-one **31***

Yield: 53%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1767 (lactone C=O). Anal. calcd. for $C_{23}H_{11}BrCl_2N_2O_2$: C, 55.45; H, 2.23; N, 5.62. Found: C, 55.71; H, 2.50; N, 5.80.

*3,5-Di(4-chlorophenyl)-9-iodo-1H-[1,3]oxazino[4,5-c]quinolin-1-one **32***

Yield: 51%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1770 (lactone C=O). Anal. calcd. for $C_{23}H_{11}Cl_2IN_2O_2$: C, 50.67; H, 2.03; N, 5.14. Found: C, 50.80; H, 2.35; N, 5.29.

*7-Chloro-3,5-di(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one **33***

Yield: 57%, m. p.: 298–300°C (DMF); IR (KBr, cm^{-1}): 1776 (lactone C=O). Anal. calcd. for $C_{23}H_{11}Cl_3N_2O_2$: C, 60.89; H, 2.44; N, 6.17. Found: C, 60.69; H, 2.37; N, 6.34.

*7,9-Dichloro-3,5-di(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]-quinolin-1-one **34***

Yield: 55%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1769 (lactone C=O). Anal. calcd. for $C_{23}H_{10}Cl_4N_2O_2$: C, 56.59; H, 2.06; N, 5.74. Found: C, 56.39; H, 2.00; N, 5.94.

*3,5-Di(4-chlorophenyl)-9-methyl-1H-[1,3]oxazino[4,5-c]-quinolin-1-one **35***

Yield: 51%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1771 (lactone C=O). Anal. calcd. for $C_{24}H_{14}Cl_2N_2O_2$: C, 66.53; H, 3.26; N, 6.47. Found: C, 66.84; H, 2.86; N, 6.67.

*3,5-Di(4-chlorophenyl)-9-methoxy-1H-[1,3]oxazino[4,5-c]-quinolin-1-one **36***

Yield: 54%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1755 (lactone C=O). Anal. calcd. for $C_{24}H_{14}Cl_2N_2O_3$: C, 64.16; H, 3.14; N, 6.24. Found: C, 63.88; H, 3.36; N, 6.43.

*5-(4-Bromophenyl)-3-(4-chlorophenyl)-9-fluoro-1H-[1,3]oxazino[4,5-c]quinolin-1-one **38***

Yield: 63%, m. p.: 268–269°C (DMF/EtOH); IR (KBr, cm^{-1}): 1771 (lactone C=O). Anal. calcd. for $C_{23}H_{11}BrClFN_2O_2$: C, 57.35; H, 2.30; N, 5.82. Found: C, 56.91; H, 2.39; N, 5.90.

*9-Bromo-5-(4-bromophenyl)-3-(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one **40***

Yield: 61%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1772 (lactone C=O). Anal. calcd. for $C_{23}H_{11}Br_2ClN_2O_2$: C, 50.91; H, 2.04; N, 5.16. Found: C, 50.88; H, 1.87; N, 5.31.

*9-Iodo-3-(4-chlorophenyl)-5-(4-bromophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one **41***

Yield: 59%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1767 (lactone C=O). Anal. calcd. for $C_{23}H_{11}BrClIN_2O_2$: C, 46.85; H, 1.88; N, 4.75. Found: C, 47.00; H, 1.97; N, 5.00.

*5-(4-Bromophenyl)-7-chloro-3-(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one **42***

Yield: 61%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1772 (lactone C=O). Anal. calcd. for $C_{23}H_{11}BrCl_2N_2O_2$: C, 55.45; H, 2.23; N, 5.62. Found: C, 55.52; H, 2.15; N, 5.79.

*5-(4-Bromophenyl)-3-(4-chlorophenyl)-7,9-dichloro-1H-[1,3]oxazino[4,5-c]quinolin-1-one **43***

Yield: 58%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1771 (lactone C=O). Anal. calcd. for $C_{23}H_{10}BrCl_3N_2O_2$: C, 51.87; H, 1.89; N, 5.26. Found: C, 51.99; H, 2.10; N, 5.46.

*5-(4-Bromophenyl)-3-(4-chlorophenyl)-9-methyl-1H-[1,3]oxazino[4,5-c]quinolin-1-one **44***

Yield: 55%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1754 (lactone C=O). Anal. calcd. for $C_{24}H_{14}BrClN_2O_2$: C, 60.34; H, 2.95; N, 5.86. Found: C, 60.62; H, 2.89; N, 6.12.

*5-(4-Bromophenyl)-9-methoxy-3-(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one **45***

Yield: 56%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 1759 (lactone C=O). Anal. calcd. for $C_{24}H_{14}BrClN_2O_3$: C, 58.38; H, 2.86; N, 5.67. Found: C, 58.01; H, 2.68; N, 5.83.

*General procedure for 2-amino-3-(4-chlorophenyl)-5-(4-chloro- or 4-bromophenyl)pyrimido[4,5-c]quinolin-1(2H)-ones **46–63***

Hydrazine hydrate (99%, 1 mL) was added to a suspension of the appropriate lactone (**28–45**; 10 mmol) in 2-ethoxyethanol (10 mL) and the mixture was heated at reflux for 24 h. The precipitated solid was filtered, washed with ethanol, dried, and recrystallized from the appropriate solvent.

*2-Amino-3,5-di(4-chlorophenyl)-9-fluoropyrimido[4,5-c]quinolin-1(2H)-one **47***

Yield: 52%, m. p.: 274–277°C (DMF); IR (KBr, cm^{-1}): 3315, 3218 (NH_2), 1674 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.06 (s, 2H, NH_2), 7.56–7.61 (m, 4H, Ar-H), 7.75–7.82 (ddd, J = 3.03, 2.84, 2.99 Hz, 1H, Ar-H), 7.96–7.99 (d, J = 8.52 Hz, 2H, Ar-H), 8.12–8.15 (d, J = 8.51 Hz, 2H, Ar-H), 8.24–8.29 (m, 1H, Ar-H), 9.35–9.40 (dd, J = 2.90, 2.89 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}Cl_2FN_4O$: C, 61.21; H, 2.90; N, 12.42. Found: C, 61.30; H, 2.81; N, 12.46.

*2-Amino-9-bromo-3,5-di(4-chlorophenyl)pyrimido[4,5-c]quinolin-1(2H)-one **49***

Yield: 47%, m. p.: 295–298°C (DMF); IR (KBr, cm^{-1}): 3298, 3213 (NH_2), 1671 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.56–7.62 (m, 4H, Ar-H), 7.96–8.03 (m, 3H, Ar-H), 8.11–8.16 (m, 3H, Ar-H), 9.88–9.89 (m, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}BrCl_2N_4O$: C, 53.93; H, 2.56; N, 10.94. Found: C, 53.70; H, 2.45; N, 11.13.

2-Amino-3,5-di(4-chlorophenyl)-9-iodopyrimido[4,5-c]quinolin-1(2H)-one ***50***

Yield: 44%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 3288, 3199 (NH_2), 1666 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.57–7.62 (m, 4H, Ar-H), 7.95–8.00 (m, 3H, Ar-H), 8.14–8.18 (m, 3H, Ar-H), 10.12–10.13 (m, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}Cl_2IN_4O$: C, 49.40; H, 2.34; N, 10.02. Found: C, 49.45; H, 2.11; N, 10.03.

2-Amino-7-chloro-3,5-di(4-chlorophenyl)pyrimido[4,5-c]-quinolin-1(2H)-one ***51***

Yield: 48%, m. p.: 258–261°C (DMF/EtOH); IR (KBr, cm^{-1}): 3321, 3221 (NH_2), 1671 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.06 (s, 2H, NH_2), 7.59–7.62 (d, J = 8.57 Hz, 4H, Ar-H), 7.78–7.84 (m, 1H, Ar-H), 7.98–8.05 (m, 3H, Ar-H), 8.22–8.24 (d, J = 8.52 Hz, 2H, Ar-H), 9.67–9.70 (m, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}Cl_3N_4O$: C, 59.06; H, 2.80; N, 11.98. Found: C, 59.30; H, 2.71; N, 11.81.

2-Amino-7,9-dichloro-3,5-di(4-chlorophenyl)pyrimido[4,5-c]-quinolin-1(2H)-one ***52***

Yield: 43%, m. p.: 293–294°C (DMF); IR (KBr, cm^{-1}): 3306, 3213 (NH_2), 1664 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.09 (s, 2H, NH_2), 7.55–7.61 (m, 4H, Ar-H), 7.96–7.99 (d, J = 8.56 Hz, 2H, Ar-H), 8.07–8.08 (d, J = 2.31 Hz, 1H, Ar-H), 8.17–8.19 (d, J = 8.55 Hz, 2H, Ar-H), 9.60–9.61 (d, J = 2.29 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{12}Cl_4N_4O$: C, 55.01; H, 2.41; N, 11.16. Found: C, 55.15; H, 2.20; N, 11.37.

2-Amino-3,5-di(4-chlorophenyl)-9-methylpyrimido[4,5-c]-quinolin-1(2H)-one ***53***

Yield: 43%, m. p.: 281–284°C (DMF); IR (KBr, cm^{-1}): 3271, 3182 (NH_2), 1666 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.60 (s, 3H, CH_3), 6.03 (s, 2H, NH_2), 7.54–7.61 (m, 4H, Ar-H), 7.68–7.72 (dd, J = 1.94, 1.93 Hz, 1H, Ar-H), 7.96–8.00 (m, 2H, Ar-H), 8.06–8.09 (d, J = 8.42 Hz, 1H, Ar-H), 8.10–8.15 (m, 2H, Ar-H), 9.49 (s, 1H, Ar-H). Anal. calcd. for $C_{24}H_{16}Cl_2N_4O$: C, 64.44; H, 3.61; N, 12.53. Found: C, 64.36; H, 3.28; N, 12.62.

2-Amino-3,5-di(4-chlorophenyl)-9-methoxypyrimido[4,5-c]-quinolin-1(2H)-one ***54***

Yield: 46%, m. p.: 271–273°C (DMF); ^{1}H-NMR (DMSO-d_6) δ: 3.97 (s, 3H, OCH_3), 6.01 (s, 2H, NH_2), 7.48–7.60 (m, 5H, Ar-H), 7.96–8.00 (d, J = 8.53 Hz, 2H, Ar-H), 8.08–8.13 (m, 3H, Ar-H), 9.17–9.18 (d, J = 2.83 Hz, 1H, Ar-H). Anal. calcd. for $C_{24}H_{16}Cl_2N_4O_2$: C, 62.22; H, 3.48; N, 12.09. Found: C, 62.33; H, 3.37; N, 12.31.

2-Amino-5-(4-bromophenyl)-3-(4-chlorophenyl)-9-fluoropyrimido[4,5-c]quinolin-1(2H)-one ***56***

Yield: 54%, m. p.: 281–284°C (DMF/EtOH); IR (KBr, cm^{-1}): 3283, 3198 (NH_2), 1665 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.59–7.60 (d, J = 8.52 Hz, 2H, Ar-H), 7.71–7.74 (d, J = 8.48 Hz, 2H, Ar-H), 7.76–7.83 (ddd, J = 3.00, 2.98, 2.96 Hz, 1H, Ar-H), 7.97–7.99 (d, J = 8.49 Hz, 2H, Ar-H), 8.06–8.09 (d, J = 8.47 Hz, 2H, Ar-H), 8.25–8.30 (m, J = 14.96 Hz, 1H, Ar-H), 9.36–9.41 (dd, J = 2.97, 2.95 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}BrClFN_4O$: C, 55.73; H, 2.64; N, 11.30. Found: C, 56.00; H, 2.76; N, 11.26.

2-Amino-9-bromo-5-(4-bromophenyl)-3-(4-chlorophenyl)pyrimido[4,5-c]quinolin-1(2H)-one ***58***

Yield: 50%, m. p.: 287–290°C (DMF/EtOH); IR (KBr, cm^{-1}): 3299, 3215 (NH_2), 1672 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.58–7.61 (d, J = 8.53 Hz, 2H, Ar-H), 7.70–7.73 (d, J = 8.51 Hz, 2H, Ar-H), 7.96–8.02 (m, 3H, Ar-H), 8.05–8.13 (m, 3H, Ar-H), 9.86–9.87(d, J = 2.22 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}Br_2ClN_4O$: C, 49.63; H, 2.35; N, 10.07. Found: C, 49.70; H, 2.27; N, 10.10.

2-Amino-5-(4-bromophenyl)-3-(4-chlorophenyl)-9-iodopyrimido[4,5-c]quinolin-1(2H)-one ***59***

Yield: 43%, m. p.: >300°C (DMF); IR (KBr, cm^{-1}): 3299, 3215 (NH_2), 1666 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.59–7.61 (d, J = 8.47 Hz, 2H, Ar-H), 7.67–7.73 (d, J = 8.48 Hz, 2H, Ar-H), 7.93–7.97 (m, 3H, Ar-H), 8.06–8.09 (d, J = 8.46 Hz, 2H, Ar-H), 8.13–8.16 (dd, J = 1.82, 1.75 Hz, 1H, Ar-H), 10.10–10.11 (d, J = 1.80 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}BrClIN_4O$: C, 45.76; H, 2.17; N, 9.28. Found: C, 45.83; H, 2.37; N, 9.30.

2-Amino-5-(4-bromophenyl)-7-chloro-3-(4-chlorophenyl)pyrimido[4,5-c]quinolin-1(2H)-one ***60***

Yield: 49%, m. p.: 263–265°C (DMF/EtOH); IR (KBr, cm^{-1}): 3319, 3209 (NH_2), 1663 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.06 (s, 2H, NH_2), 7.59–7.62 (d, J = 8.54 Hz, 2H, Ar-H), 7.73–7.75 (d, J = 8.53 Hz, 2H, Ar-H), 7.78–7.83 (m, 1H, Ar-H), 7.98–8.00 (d, J = 8.53 Hz, 2H, Ar-H), 8.02–8.05 (m, 1H, Ar-H), 8.14–8.17 (d, J = 8.51 Hz, 2H, Ar-H), 9.66–9.69 (d, J = 8.50 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{13}BrCl_2N_4O$: C, 53.93; H, 2.56; N, 10.94. Found: C, 53.58; H, 2.69; N, 10.77.

2-Amino-5-(4-bromophenyl)-3-(4-chlorophenyl)-7,9-dichloro-pyrimido[4,5-c]quinolin-1(2H)-one ***61***

Yield: 41%, m. p.: 297–299°C (DMF); IR (KBr, cm^{-1}): 3304, 3231 (NH_2), 1664 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 6.09 (s, 2H, NH_2), 7.59–7.62 (d, J = 8.67 Hz, 2H, Ar-H), 7.72–7.75 (d, J = 8.63 Hz, 2H, Ar-H), 7.97–8.00 (d, J = 8.62 Hz, 2H, Ar-H), 8.12–8.15 (m, 3H, Ar-H), 9.67–9.68 (d, J = 2.33 Hz, 1H, Ar-H). Anal. calcd. for $C_{23}H_{12}BrCl_3N_4O$: C, 50.54; H, 2.21; N, 10.25. Found: C, 50.63; H, 2.37; N, 10.03.

2-Amino-5-(4-bromophenyl)-3-(4-chlorophenyl)-9-methylpyrimido[4,5-c]quinolin-1(2H)-one ***62***

Yield: 48%, m. p.: 289–291°C (DMF/EtOH); IR (KBr, cm^{-1}): 3275, 3188 (NH_2), 1666 (C=O); ^{1}H-NMR (DMSO-d_6) δ: 2.59 (s, 3H, CH_3), 6.02 (s, 2H, NH_2), 7.57–7.60 (d, J = 8.49 Hz, 2H, Ar-H), 7.68–7.71 (d, J = 8.42 Hz, 3H, Ar-H), 7.96–7.99 (d, J = 8.47 Hz, 2H, Ar-H), 8.03–8.08 (m, 3H, Ar-H), 9.48 (s, 1H, Ar-H). Anal. calcd. for $C_{24}H_{16}BrClN_4O$: C, 58.62; H, 3.28; N, 11.39. Found: C, 58.33; H, 3.60; N, 11.09.

2-Amino-5-(4-bromophenyl)-3-(4-chlorophenyl)-9-methoxy-pyrimido[4,5-c]quinolin-1(2H)-one ***63***

Yield: 50%, m. p.: 280–282°C (DMF/EtOH); ^{1}H-NMR (DMSO-d_6) δ: 3.99 (s, 3H, OCH_3), 6.02 (s, 2H, NH_2), 7.50–7.54 (dd, J = 2.90, 2.90 Hz, 1H, Ar-H), 7.58–7.61 (d, J = 8.58 Hz, 2H, Ar-H), 7.68–7.71 (d, J = 8.54 Hz, 2H, Ar-H), 7.96–7.99 (d, J = 8.53 Hz, 2H, Ar-H), 8.04–8.13 (m, 3H, Ar-H), 9.19–9.20 (d, J = 2.83 Hz, 1H, Ar-H). Anal. calcd. for $C_{24}H_{16}BrClN_4O_2$: C, 56.77; H, 3.18; N, 11.03. Found: C, 56.87; H, 3.39; N, 11.05.

Biological screening

Cell culture and assessment of cytotoxicity

The new compounds were tested for their cytotoxic activity on the following human solid tumor cell lines: lung fibrosarcoma

HT-1080 (American Type Culture Collection (ATCC), Rockville, MD, USA), mammary adenocarcinoma MDA-MB-231 (ATCC) and colorectal adenocarcinoma HT-29 (European Collection of Cell Cultures, Salisbury, U.K.). All cells were routinely cultured in Dulbecco's minimal essential medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (media and antibiotics were from Biochrom KG, Berlin, Germany, while the serum was purchased from Invitrogen Co., Carlsbad, CA, USA) in an environment of 5% CO_2, 85% humidity, and 37°C, and they were subcultured using a trypsin 0.25%-EDTA 0.02% solution. The cytotoxicity assay was performed by a modification of the MTT method [15]. Briefly, the cells were plated at a density of approximately 5000 cells/well in 96-well, flat-bottomed microplates, and after 24 h, the test compounds were added, appropriately diluted with DMSO. After a 72-h incubation, the medium was replaced with MTT (Sigma) dissolved at a final concentration of 1 mg/mL in serum-free, phenol-red-free DMEM for a further 4 h incubation. Then, the MTT formazan was solubilized in 2-propanol, and the optical density was measured with a microplate reader at a wavelength of 550 nm (reference wavelength 690 nm). Doxorubicin hydrochloride was included in the experiments as positive control. The results represent the mean of three independent experiments and are expressed as IC_{50}, which is the concentration that reduced the optical density of the treated cells by 50% with respect to untreated controls.

Cell cycle analysis

Cell-cycle analysis was performed following incubation of exponentially growing HT-1080 cells with the test substances (2 μM) for 36 h. Treated cultures were then trypsinized, washed in PBS, fixed in 50% ethanol, and stained with an RNAse-containing propidium iodide solution [15]. DNA content was analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the ModFit software (Verity Software House, Topsham, ME, USA).

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