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Original article

Pyrimido[4,5-*c*]quinolin-1(2*H*)-ones as a novel class of antimitotic agents: Synthesis and in vitro cytotoxic activity

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Abstract

Several 2-amino-pyrimido[4,5-*c*]quinolin-1(2*H*)-ones variously substituted at positions 3, 5, and 9 were prepared from their corresponding lactones. The target compounds were investigated for in vitro cytotoxic activity against a panel of human cancer cell lines, namely, lung fibrosarcoma HT-1080, colon adenocarcinoma HT-29, and breast carcinoma MDA-MB-231. Analysis of data revealed that the presence of chloro at position 9 has a major positive impact on cytotoxic activity. Additional halogen substitution at the *para* position of the 3-phenyl group further enhances activity. Furthermore, compound (**25**) was found to dose-dependently inhibit tubulin polymerization. In accordance, flow cytometric analysis of the most potent compounds (**23**–**26**) indicated that the tested compounds induce cell cycle arrest in the G_2/M phase. The obtained results introduce the rarely described pyrimido[4,5-*c*]quinolin-1(2*H*)-one ring system as a new scaffold for promising antimitotic agents.

Keywords: Cytotoxic agents; Antimitotic; Pyrimido[4,5-*c*]quinolin-1(2*H*)-ones

1. Introduction

Recent revolutionary advances in the field of molecular biology and cancer cell biology have created new targets for antitumor compounds. Microtubules are hollow cylindrical protein polymers composed of α- and β-tubulin heterodimers. The tubulin heterodimers assemble to form the protofilaments which in turn associate longitudinally to form a microtubule as a long tube of 25 nm diameter [1]. During cell division, the microtubules are arranged in a unique manner to form the mitotic spindle which is the key cellular machinery driving mitosis in the metaphase where the replicated chromosomes are congressed to the equator, and the anaphase where chromosomes are segregated towards the spindle poles to generate two new daughter cells [2]. To perform this highly dynamic function, microtubules are required to be highly dynamic. In other words, microtubules should have the ability to lengthen and shorten through a reversible GTP-mediated process of association and dissociation of the α/β-tubulin heterodimers at both ends [3]. Microtubule-targeting drugs alter the dynamic behavior of microtubules through binding to tubulin thereby blocking mitotic cell progression and subsequently leading to apoptotic cell death [4–6]. Evidences indicate that even minor alteration of microtubule dynamics can have major consequences on cell cycle progression at mitosis [7–9]. Noteworthy, it has been postulated that mitosis can be blocked by abnormally rapid microtubule dynamics as well as by inhibited dynamics [10]. Two distinct categories of microtubule-targeting drugs are currently identified: agents that inhibit tubulin polymerization or ''microtubule-destabilizing agents'' such as the vinca alkaloids and colchicines, and agents that promote tubulin polymerization or ''microtubule-stabilizing agents'' such as the taxanes exemplified by paclitaxel and docetaxil [11]. For these reasons, tubulin became an attractive

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target in anticancer drug development. However, development of resistance and neurological, hematological, and other side effects significantly detracts from the therapeutic effectiveness of the currently available agents [12–14]. These findings sparked a worldwide search for new classes of drugs acting by the same mechanism yet enjoying better pharmacological profile.

In the past two decades, there has been a growing interest in 2-phenyl-4-quinolones (A), the aza bioisosteric analogues of flavones (B), as potent cytotoxic antimitotic agents interacting with microtubules [15–21]. Likewise, natural flavonoids have been reported to display potent cytotoxic activity through inhibition of tubulin polymerization [22,23]. Meanwhile, 2-phenyl-4-anilinoquinolines (C) and related condensed heterocyclic compounds were extensively reported to have appreciable cytotoxic activities [24–29]. It was generally concluded that not only the coplanar polycyclic core but also peripheral substituents have considerable impact on cytotoxic activity. In the same context, we report herein the synthesis and the in vitro cytotoxic activity of pyrimidio[4,5-*c*]quinolin-1(2*H*)-ones (D) as a new class of antimitotic agents (Chart 1). The mechanism of action of the new agents was explored through cell cycle analysis performed by a flow cytometric assay and a tubulin polymerization assay.

2. Chemistry

Synthetic route to the target pyrimido[4,5-*c*]quinolin-1(2*H*)-ones (**16–26**) is illustrated in Scheme 1. Phenacylamine hydrochlorides (**1** and **2**) were prepared according to a standard method, as reported previously [30]. The starting 3-amino-2-arylquinoline-4-carboxylic acids (**3–6**) were synthesized following a modified Pfitzinger procedure by reacting isatin or 5-chloroisatin with 4-chloro- or 4-bromophenacylamine hydrochlorides (**1** and **2**) under basic conditions [30,31]. Subsequently, the acids were cyclized with acetic anhydride under reflux conditions to afford the key lactones 3-methyl-5-aryl-1*H*-[1,3]oxazino[4,5-*c*]quinolin-1-ones (**7** and **8**) in good yields. Alternatively, reacting the starting acids (**3–6**) with benzoyl chloride or 4-chlorobenzoyl chloride at room temperature followed by cyclization of the resulting intermediate using acetic anhydride gave the lactones (**9–15**) in moderate overall yields. Finally, the requisite pyrimido[4,5-*c*]quinolin-1(2*H*)-ones (**16–26**) were obtained via hydrazinolysis of the intermediate lactones (**7–15**) through heating with hydrazine hydrate in 2-ethoxyethanol as a high boiling solvent.

Structure of the target pyrimido[4,5-*c*]quinolin-1(2*H*)-ones (**16–26**) as well as the intermediate lactones (**7–15**) was confirmed by means of IR, ^{1}H NMR, mass spectral data and microanalysis. IR spectra of (**7–15**) showed a characteristic lactone carbonyl stretching frequency around 1765 cm^{-1}. Mass spectra revealed the appearance of isotopic peaks owing to the presence of halogens. Unfortunately, these intermediate lactones could not be characterized by ^{1}H NMR spectrometry due to lack of solubility in the available ^{1}H NMR solvents such as $CDCl_3$, DMSO-d_6, CD_3OD, CD_3CD_2OD and CD_3COCD_3. In the ^{1}H NMR spectra of the target compounds (**16–26**), a singlet peak in the region δ 6.0–6.3 integrating for two protons was characteristic of the NH_2 group. The methyl protons of compounds (**16**), (**17**) and (**22**) appeared as a sharp singlet at δ 2.7. Aromatic protons appeared at their expected chemical shifts. All the new compounds were microanalyzed satisfactorily for C, H, N.

3. Results and discussion

The widely accepted MTT assay was used for the in vitro cytotoxic evaluation of the target compounds (**16–26**) against

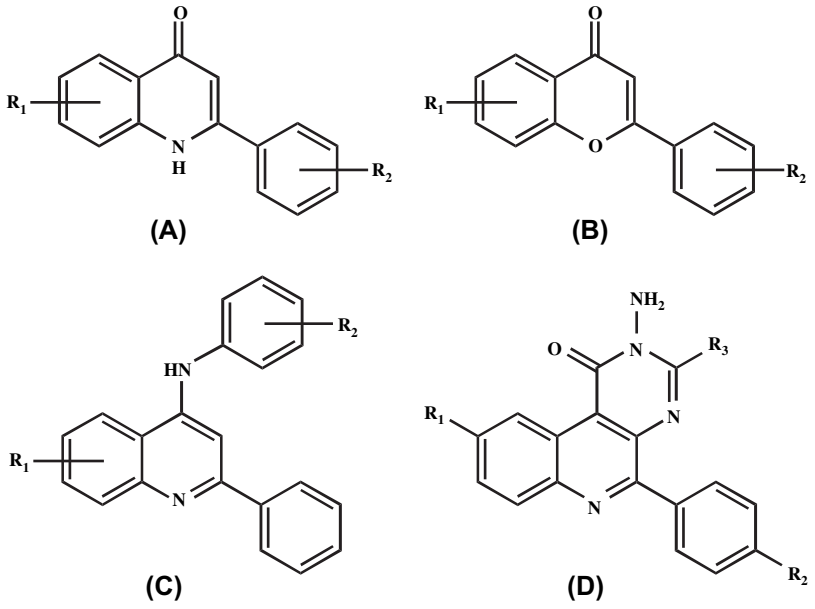
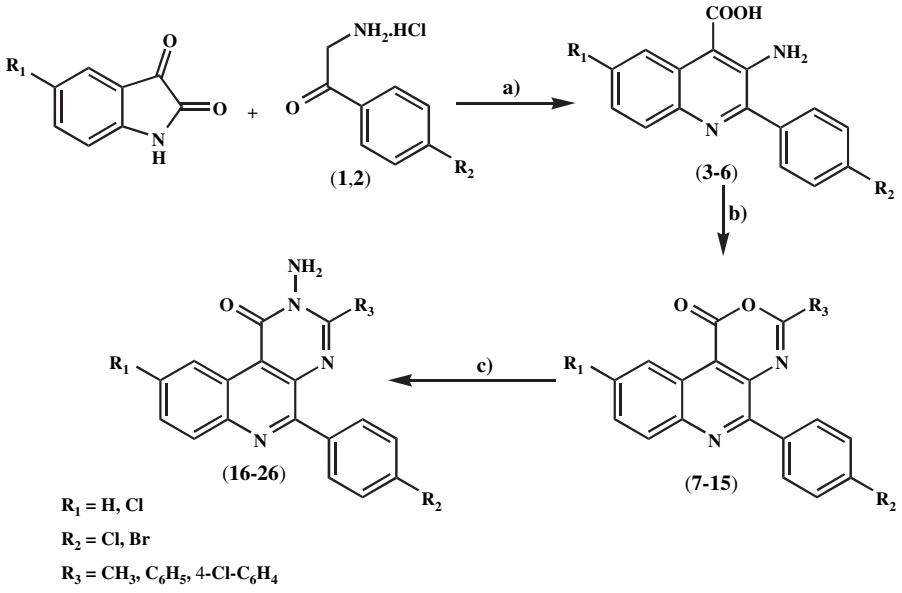


Chart 1.



Scheme 1. Reagents and conditions: (a) NaOH, EtOH, H_2O, THF, reflux, 30 min. (b) For (**7**) and (**8**): $(CH_3CO)_2O$, reflux 5 h; for (**9**–**15**): C_6H_5COCl or 4-Cl–C_6H_4COCl, pyridine, room temperature, 12 h followed by $(CH_3CO)_2O$, reflux 5 h. (c) $NH_2NH_2 \cdot H_2O$, 2-ethoxyethanol, reflux, 24 h.

a panel of three human cancer cell lines, i.e., lung fibrosarcoma HT-1080, colon adenocarcinoma HT-29, and breast carcinoma MDA-MB-231. Close inspection of cytotoxic data revealed that a 9-chloro substituent is the critical determinant of cytotoxic activity in the 3-aryl series (Table 1). Evidently, removal of the 9-chloro of (**23**), (**24**), (**25**), and (**26**) to give (**18**), (**19**), (**20**), and (**21**), respectively, resulted in a remarkable decrease of cytotoxicity against the three cell lines used in the assay. In general, compounds (**22**–**26**) were more potent than their 9-unsubstituted counterparts. Moreover, introduction of a chloro at the *para* position of the 3-phenyl ring enhances cytotoxic activity, particularly in the 9-unsubstituted type of compounds as evidenced by the appreciably higher potency of (**20**) and (**21**) compared to (**18**) and (**19**), respectively. On the other hand, the type of halogen at the 5-phenyl ring appeared to have variable influence on cytotoxic activity. Within this set of compounds, it was found that compounds of the 3-aryl series are generally more potent than their 3-methyl analogues. Compound (**16**) which has neither 9-chloro nor 3-aryl was found to be devoid of cytotoxicity against the three cell lines tested. In general, the tested compounds were more active against fibrosarcoma cells HT-1080 with breast carcinoma MDA-MB-231 cells being the least sensitive.

The mechanistic aspects of the synthesized compounds were investigated through flow cytometry and tubulin polymerization assay. To this end, after incubation of human fibrosarcoma cells for 36 h with the most active compound (**25**) or with control compounds (colcemide and paclitaxel), polymerized tubulin was separated from soluble tubulin and the two fractions were assessed by Western analysis, as described in

Table 1
In vitro cytotoxicity of the target pyrimido[4,5-*c*]quinolin-1(2*H*)-ones using the MTT assay (IC_{50} values in μM)[a]

Compound	R_1	R_2	R_3	Cell line		
				HT-1080	HT-29	MDA-MB-231
16	H	Cl	CH_3	>100	>100	>100
17	H	Br	CH_3	44.8 (±6.2)	77.8 (±11.1)	>100
18	H	Cl	C_6H_5	23.0 (±0.8)	>100	>100
19	H	Br	C_6H_5	47.4 (±3.7)	89.9 (±5.0)	>100
20	H	Cl	4-Cl–C_6H_4	6.84 (±1.60)	18.1 (±7.1)	55.3 (±21.6)
21	H	Br	4-Cl–C_6H_4	10.8 (±0.8)	23.2 (±9.1)	34.2 (±20.1)
22	Cl	Cl	CH_3	34.3 (±7.2)	52.0 (±24.0)	>100
23	Cl	Cl	C_6H_5	2.90 (±0.58)	4.68 (±0.79)	15.8 (±5.1)
24	Cl	Br	C_6H_5	1.48 (±0.06)	2.14 (±0.62)	13.7 (±4.4)
25	Cl	Cl	4-Cl–C_6H_4	1.23 (±0.22)	1.97 (±0.07)	1.82 (±0.53)
26	Cl	Br	4-Cl–C_6H_4	1.84 (±0.40)	2.82 (±0.28)	2.89 (±0.27)
Doxorubicin				0.022 (±0.08)	0.35 (±0.13)	0.030 (±0.020)

[a] The results represent the mean (±standard deviation) of three independent experiments and are expressed as IC_{50}, the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

detail in Section 4. In this assay, colcemide, which was used as a known tubulin polymerization inhibitor, reduced the amount of polymerized tubulin detected (Fig. 1), while paclitaxel, known to stabilize microtubule assembly, increased the fraction of polymerized tubulin, in accordance with the data appearing in the literature [32]. As shown in Fig. 1, compound (**25**) dose-dependently inhibited tubulin polymerization in comparison with the untreated culture, showing a mode of action similar to colcemide. Furthermore, cell cycle analysis using staining with the DNA binding dye propidium iodide, followed by flow cytometry, showed that incubation with compound (**25**) for 36 h caused arrest of HT-1080 cells in the G_2/M phase of the cell cycle and suppression of the S-phase, in agreement with the observed inhibition of tubulin polymerization (Table 2). Moreover, compounds (**23**), (**24**) and (**26**) also block cell proliferation by arresting the G_2/M phase of the cell cycle. Finally, the tested compounds seem to provoke cell apoptosis, as estimated by the appearance of a sub-diploid peak in the DNA-content histograms (percentage of total events collected by the flow cytometer: 2.21% for (**23**), 5.51% for (**24**), 7.62% for (**25**), 9.14% for (**26**) compared to 1.27% for control).

4. Experimental protocols

4.1. Synthesis

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. IR spectra were recorded on a Pye Unicam SP 1000 IR spectrophotometer using KBr pellets. ^{1}H NMR spectra were recorded on a Varian-Mercury 200 MHz spectrometer in DMSO-d_6. Chemical shifts were expressed in parts per million (ppm) with tetramethylsilane (TMS) as an internal standard. MS spectra were measured with an HP 5995 instrument. Elemental analyses (C, H, N) were performed at the Microanalytical Unit, Cairo University and the National Research Centre, 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 (**1**–**6**) were reported previously [30].

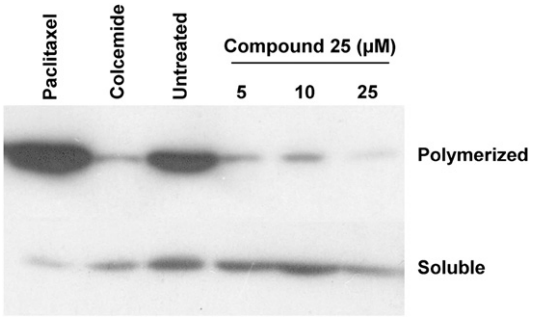


Fig. 1. Inhibition of cellular tubulin polymerization by compound (**25**). Cells were treated with the indicated concentrations of compound (**25**) or control compounds and tubulin polymerization was assessed by Western analysis as described in Section 4.

Table 2
Cell cycle phase distribution (%)[a]

Compound	G_0/G_1	S	G_2/M
23	50.68	26.86	22.46
24	45.78	28.00	26.22
25	57.25	11.26	31.49
26	49.76	22.48	27.75
Control	51.53	29.82	18.65

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

4.1.1. General procedure for 3-methyl-5-aryl-1H-[1,3] oxazino[4,5-c]quinolin-1-ones (*7* and *8*)

A mixture of the appropriate 3-amino-2-aryl-4-quinolinecarboxylic acid (10 mmol) and acetic anhydride (10 mL) was heated under reflux for 5 h. The reaction mixture was cooled and the obtained solid was filtered, washed with cold ethanol, dried, and recrystallized from DMF/EtOH.

4.1.1.1. 5-(4-Chlorophenyl)-3-methyl-1H-[1,3]oxazino[4,5-c] quinolin-1-one (7). Yield 79%, mp 194–195 °C (DMF/EtOH); IR (KBr, cm^{-1}): 1763 (lactone C=O). MS (*m/z*): 323 (M^+, 13.3%), 251 (base peak). Anal. calcd for $C_{18}H_{11}ClN_2O_2$: C, 66.99; H, 3.44; N, 8.68. Found: C, 67.16; H, 3.67; N, 8.76.

4.1.1.2. 5-(4-Bromophenyl)-3-methyl-1H-[1,3]oxazino[4,5-c] quinolin-1-one (8). Yield 74%, mp 187–190 °C (DMF/EtOH); IR (KBr, cm^{-1}): 1763 (lactone C=O). MS (*m/z*): 367 (M^+, 34.7%), 295 (base peak). Anal. calcd for $C_{18}H_{11}BrN_2O_2$: C, 58.88; H, 3.02; N, 7.63. Found: C, 58.75; H, 2.83; N, 7.53.

4.1.2. General procedure for 3,5-diaryl-1H-[1,3]oxazino [4,5-c]quinolin-1-ones (*9*–*15*)

A mixture of the appropriate 3-amino-2-aryl-4-quinolinecarboxylic acids (**3**–**6**; 10 mmol) and benzoyl chloride or 4-chlorobenzoyl chloride (11 mmol) in dry pyridine (20 mL) was allowed to stir at room temperature for 12 h. The reaction mixture was poured into ice water and the obtained solid was filtered, washed with ethanol, and dried. The solid was suspended in acetic anhydride (10 mL) and the mixture was heated at reflux for 5 h. After cooling, the crude product was filtered, washed with ethanol, dried, and recrystallized from the appropriate solvent.

4.1.2.1. 5-(4-Chlorophenyl)-3-phenyl-1H-[1,3]oxazino[4,5-c] quinolin-1-one (9). Yield 64%, mp 257–258 °C (DMF/EtOH); IR (KBr, cm^{-1}): 1766 (lactone C=O). MS (*m/z*): 429 (M^{+1}, 16.1%), 104 (base peak). Anal. calcd for $C_{23}H_{13}ClN_2O_2$: C, 71.79; H, 3.40; N, 7.28. Found: C, 71.65; H, 3.91; N, 7.36.

4.1.2.2. 5-(4-Bromophenyl)-3-phenyl-1H-[1,3]oxazino[4,5-c] quinolin-1-one (10). Yield 61%, mp 255–257 °C (DMF/EtOH); IR (KBr, cm^{-1}): 1766 (lactone C=O). MS (*m/z*): 429 (M^+, 28.3%), 76 (base peak). Anal. calcd for

$C_{23}H_{13}BrN_2O_2$: C, 64.35; H, 3.05; N, 6.53. Found: C, 64.55; H, 3.03; N, 6.59.

*4.1.2.3. 5-(4-Bromophenyl)-3-(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one (**11**).* Yield 59%, mp 268–270 °C (DMF); IR (KBr, cm^{-1}): 1763 (lactone C=O). MS (*m/z*): 465 (M^{+1}, 19.2%), 139 (base peak). Anal. calcd for $C_{23}H_{12}BrClN_2O_2$: C, 59.57; H, 2.61; N, 6.04. Found: C, 59.80; H, 2.47; N, 6.14.

*4.1.2.4. 3,5-Di(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one (**12**).* Yield 58%, mp 269–272 °C (DMF); IR (KBr, cm^{-1}): 1766 (lactone C=O). MS (*m/z*): 421 (M^{+2}, 13.1%), 138 (base peak). Anal. calcd for $C_{23}H_{12}Cl_2N_2O_2$: C, 65.89; H, 2.88; N, 6.68. Found: C, 65.82; H, 2.73; N, 6.69.

*4.1.2.5. 5-(4-Bromophenyl)-9-chloro-3-phenyl-1H-[1,3]oxazino[4,5-c]quinolin-1-one (**13**).* Yield 63%, mp 298–300 °C (DMF/EtOH); IR (KBr, cm^{-1}): 1766 (lactone C=O). MS (*m/z*): 464 (M^{+}, 19.0%), 76 (base peak). Anal. calcd for $C_{23}H_{12}BrClN_2O_2$: C, 59.57; H, 2.61; N, 6.04. Found: C, 59.56; H, 2.19; N, 6.15.

*4.1.2.6. 5-(4-Bromophenyl)-9-chloro-3-(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one (**14**).* Yield 62%, mp > 300 °C (DMF/EtOH); IR (KBr, cm^{-1}): 1766 (lactone C=O). MS (*m/z*): 499 (M^{+}, 22.4%), 138 (base peak). Anal. calcd for $C_{23}H_{11}BrCl_2N_2O_2$: C, 55.45; H, 2.23; N, 5.62. Found: C, 55.83; H, 1.56; N, 5.73.

*4.1.2.7. 9-Chloro-3,5-di(4-chlorophenyl)-1H-[1,3]oxazino[4,5-c]quinolin-1-one (**15**).* Yield 64%, mp > 300 °C (DMF); IR (KBr, cm^{-1}): 1766 (lactone C=O). MS (*m/z*): 456 (M^{+2}, 22.4%), 139 (base peak). Anal. calcd for $C_{23}H_{11}Cl_3N_2O_2$: C, 60.89; H, 2.44; N, 6.17. Found: C, 61.19; H, 2.17; N, 6.30.

*4.1.3. General procedure for 2-amino-5-aryl-pyrimidino[4,5-c]quinolin-1(2H)-ones (**16–26**)*

A mixture of the appropriate 1*H*-[1,3]oxazino[4,5-*c*]quinolin-1-one (**7–15**; 10 mmol) and hydrazine hydrate (85%; 4 mL) in 2-ethoxyethanol (20 mL) was heated at reflux for 24 h. The precipitated solid was filtered, washed with ethanol, dried, and recrystallized from the appropriate solvent.

*4.1.3.1. 2-Amino-5-(4-chlorophenyl)-3-methylpyrimido[4,5-c]quinolin-1(2H)-one (**16**).* Yield 56%, mp 291–293 °C (DMF); ^{1}H NMR (DMSO-d_6) δ: 2.71 (s, 3H, CH_3), 6.27 (s, 2H, NH_2), 7.61–7.65 (m, 2H, Ar-H), 7.85–7.89 (m, 2H, Ar-H), 8.13–8.22 (m, 3H, Ar-H), 9.68–9.73 (m, 1H, Ar-H). Anal. calcd for $C_{18}H_{13}ClN_4O$: C, 64.20; H, 3.89; N, 16.64. Found: C, 63.76; H, 3.89; N, 16.49.

*4.1.3.2. 2-Amino-5-(4-bromophenyl)-3-methylpyrimido[4,5-c]quinolin-1(2H)-one (**17**).* Yield 60%, mp 294–296 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 2.71 (s, 3H, CH_3), 6.27 (s, 2H, NH_2), 7.75–7.79 (m, 2H, Ar-H), 7.84–7.89 (m, 2H, Ar-H), 8.06–8.10 (m, 2H, Ar-H), 8.1–8.2 (m, 1H, Ar-H), 9.67–9.72 (m, 1H, Ar-H). Anal. calcd for $C_{18}H_{13}BrN_4O$: C, 56.71; H, 3.44; N, 14.70. Found: C, 56.26; H, 3.44; N, 14.58.

*4.1.3.3. 2-Amino-5-(4-chlorophenyl)-3-phenylpyrimido[4,5-c]quinolin-1(2H)-one (**18**).* Yield 59%, mp 235–237 °C (DMF); ^{1}H NMR (DMSO-d_6) δ: 6.05 (s, 2H, NH_2), 7.51–7.58 (m, 5H, Ar-H), 7.84–7.86 (m, 2H, Ar-H), 7.93–7.97 (m, 2H, Ar-H), 8.17–8.20 (m, 3H, Ar-H), 9.68–9.72 (m, 1H, Ar-H). Anal. calcd for $C_{23}H_{15}ClN_4O$: C, 69.26; H, 3.79; N, 14.05. Found: C, 69.30; H, 3.20; N, 13.91.

*4.1.3.4. 2-Amino-5-(4-bromophenyl)-3-phenylpyrimido[4,5-c]quinolin-1(2H)-one (**19**).* Yield 52%, mp 220–221 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 6.11 (s, 2H, NH_2), 7.55–7.59 (m, 3H, Ar-H), 7.74 (d, 2H, $J = 8.79$ Hz, Ar-H), 7.88–7.93 (m, 2H, Ar-H), 7.97–8.02 (m, 2H, Ar-H), 8.11 (d, 2H, Ar-H), 8.21–8.26 (m, 1H, Ar-H), 9.73–9.77 (m, 1H, Ar-H). Anal. calcd for $C_{23}H_{15}BrN_4O$: C, 62.32; H, 3.41; N, 12.64. Found: C, 62.01; H, 3.58; N, 12.41.

*4.1.3.5. 2-Amino-3,5-di(4-chlorophenyl)pyrimido[4,5-c]quinolin-1(2H)-one (**20**).* Yield 57%, mp 280–282 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 6.03 (s, 2H, NH_2), 7.54–7.60 (m, 4H, Ar-H), 7.82–7.88 (m, 2H, Ar-H), 7.95–7.98 (m, 2H, Ar-H), 8.11–8.19 (m, 3H, Ar-H), 9.66–9.69 (m, 1H, Ar-H). Anal. calcd for $C_{23}H_{14}Cl_2N_4O$: C, 63.79; H, 3.26; N, 12.93. Found: C, 63.83; H, 3.51; N, 12.66.

*4.1.3.6. 2-Amino-5-(4-bromophenyl)-3-(4-chlorophenyl)pyrimido[4,5-c]quinolin-1(2H)-one (**21**).* Yield 58%, mp 293–294 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 6.04 (s, 2H, NH_2), 7.58 (d, 2H, $J = 4.59$ Hz, Ar-H), 7.69 (d, 2H, $J = 5.39$ Hz, Ar-H), 7.84–7.88 (m, 2H, Ar-H), 7.96 (d, 2H, $J = 5.39$ Hz, Ar-H), 8.06 (d, 2H, $J = 5.39$ Hz, Ar-H), 8.17–8.20 (m, 1H, Ar-H), 9.68–9.71 (m, 1H, Ar-H). Anal. calcd for $C_{23}H_{14}BrClN_4O$: C, 57.82; H, 2.95; N, 11.73. Found: C, 58.03; H, 2.45; N, 11.83.

*4.1.3.7. 2-Amino-9-chloro-5-(4-chlorophenyl)-3-methylpyrimido[4,5-c]quinolin-1(2H)-one (**22**).* Yield 54%, mp 264–265 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 2.66 (s, 3H, CH_3), 6.23 (s, 2H, NH_2), 7.55–7.59 (m, 2H, Ar-H), 7.78–8.08 (m, 1H, Ar-H), 8.08–8.11 (m, 3H, Ar-H), 9.60 (d, 1H, $J = 1.59$ Hz, Ar-H). Anal. calcd for $C_{18}H_{12}Cl_2N_4O$: C, 58.24; H, 3.26; N, 15.09. Found: C, 57.92; H, 3.39; N, 14.88.

*4.1.3.8. 2-Amino-9-chloro-5-(4-chlorophenyl)-3-phenylpyrimido[4,5-c]quinolin-1(2H)-one (**23**).* Yield 61%, mp 281–282 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 6.14 (s, 2H, NH_2), 7.56–7.64 (m, 5H, Ar-H), 7.93–8.02 (m, 3H, Ar-H), 8.16–8.24 (m, 3H, Ar-H), 9.73 (d, 1H, $J = 1.99$ Hz, Ar-H). Anal. calcd for $C_{23}H_{14}Cl_2N_4O$: C, 63.76; H, 3.26; N, 12.93. Found: C, 63.46; H, 3.54; N, 12.63.

*4.1.3.9. 2-Amino-5-(4-bromophenyl)-9-chloro-3-phenylpyrimido[4,5-c]quinolin-1(2H)-one (**24**).* Yield 55%, mp 269–270 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 6.12 (s, 2H,

NH_2), 7.54–7.57 (m, 2H, Ar-H), 7.72–7.76 (m, 2H, Ar-H), 7.86–7.92 (dd, 1H, $J = 2.39$, 2.39 Hz, Ar-H), 7.95–7.99 (m, 2H, Ar-H), 8.06–8.12 (dd, 2H, $J = 1.39$, 1.79 Hz, Ar-H), 8.17 (d, 2H, $J = 8.79$ Hz, Ar-H), 9.71 (d, 1H, $J = 2.39$ Hz, Ar-H). Anal. calcd for $C_{23}H_{14}BrClN_4O$: C, 57.82; H, 2.95; N, 11.73. Found: C, 57.30; H, 2.95; N, 11.66.

*4.1.3.10. 2-Amino-9-chloro-3,5-di(4-chlorophenyl)pyrimido [4,5-c]quinolin-1(2H)-one (**25**).* Yield 50%, mp 285–288 °C (DMF); ^{1}H NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.54–7.60 (m, 4H, Ar-H), 7.84 (d, 1H, $J = 1.59$ Hz, Ar-H), 7.9–8.0 (d, 2H, $J = 5.79$ Hz, Ar-H), 8.09–8.15 (m, 3H, Ar-H), 9.63 (d, 1H, $J = 1.59$ Hz, Ar-H). Anal. calcd for $C_{23}H_{13}Cl_3N_4O$: C, 59.06; H, 2.80; N, 11.98. Found: C, 58.84; H, 2.96; N, 11.66.

*4.1.3.11. 2-Amino-5-(4-bromophenyl)-9-chloro-3-(4-chlorophenyl)pyrimido[4,5-c]quinolin-1(2H)-one (**26**).* Yield 49%, mp 273–276 °C (DMF/EtOH); ^{1}H NMR (DMSO-d_6) δ: 6.07 (s, 2H, NH_2), 7.58 (d, 2H, $J = 5.59$ Hz, Ar-H), 7.69 (d, 2H, $J = 3.79$ Hz, Ar-H), 7.78 (d, 1H, Ar-H), 7.96 (d, 2H, $J = 5.79$ Hz, Ar-H), 8.04 (d, 2H, $J = 5.59$ Hz, Ar-H), 8.15 (d, 1H, $J = 5.79$ Hz, Ar-H), 9.67 (d, 1H, $J = 1.39$ Hz, Ar-H). Anal. calcd for $C_{23}H_{13}BrCl_2N_4O$: C, 53.94; H, 2.56; N, 10.94. Found: C, 53.67; H, 2.74; N, 10.67.

4.2. Biology

4.2.1. In vitro 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, Rockville, MD), 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; Gibco BRL, Paisley, U.K.) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (media and antibiotics from Biochrom KG, Berlin, Germany) 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 [33,34]. 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}, i.e., the concentration that reduced by 50% the optical density of treated cells with respect to untreated controls.

4.2.2. Cell cycle analysis

Cell cycle analysis was performed following incubation of exponentially growing HT-1080 cells with the test substances (5 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 [34]. DNA content was analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer using the ModFit software (Verity Software House, Topsham, ME, USA).

4.2.3. In vitro inhibition of cellular tubulin polymerization

Exponentially growing HT-1080 cells (approximately 2×10^6 cells/dish) were treated with 5, 10, or 25 μM compound (**25**) for 36 h. Negative control cultures were treated with the corresponding concentrations of the vehicle (DMSO) while positive controls with either 0.5 μM colcemide (Biochrom) or 0.1 μM paclitaxel (Genexol®, Samyang Genex Corporation, Daejeon, Korea – kindly provided by Dr. M. Sagnou) for 18 h. Cultures were then trypsinized, washed in PBS, and polymerized cellular tubulin was separated from soluble tubulin dimers as described by Blagosklonny et al. [35]. Briefly, cells were lysed in a buffer containing 20 mM Tris–HCl (pH 6.8), 1 mM $MgCl_2$, 2 mM EGTA, 0.5% Nonidet P-40 and protease- and phosphatase-inhibitor cocktails (Sigma). Supernatants were collected after centrifugation at 13,000 × g for 10 min. The pellets were dissolved by heating for 5 min at 95 °C in a SDS-polyacrylamide gel electrophoresis (PAGE) sampling buffer and subjected to electrophoresis on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane, and subjected to Western analysis using an anti-α-tubulin monoclonal antibody and peroxidase-conjugated anti-mouse antibody (Sigma). Detection of immunoreactive bands was performed by chemiluminescence (ECL kit, Amersham Biosciences).

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