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Synthesis and anticancer activity of new 1-[(5 or 6-substituted 2-alkoxyquinoxalin-3-yl)aminocarbonyl]-4-(hetero)arylpiperazine derivatives

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ABSTRACT

A series of novel quinoxalinyl-piperazine compounds, 1-[(5 or 6-substituted alkoxyquinoxalinyl)aminocarbonyl]-4-(hetero)arylpiperazine derivatives were synthesized and evaluated as an anticancer agent. From screening of quinoxalinyl-piperazine compound library, we identified that many compounds inhibited proliferation of various human cancer cells at nanomolar concentrations. Among them, one of the fluoro quinoxalinyl-piperazine derivatives showed its IC_{50} values ranging from 11 to 21 nM in the growth inhibition of cancer cells. This compound also displayed a more potent effect than paclitaxel against paclitaxel resistant HCT-15 colorectal carcinoma cells. The potency of this novel compound was further confirmed with the synergistic cytotoxic effect with several known cancer drugs such as paclitaxel, doxorubicin, cisplatin, gemcitabine or 5-fluorouracil in cancer cells. This strong cell killing effect was derived from the induction of apoptosis. Mechanistic studies have shown that this quinoxalinyl-piperazine compound is a G2/M-specific cell cycle inhibitor and inhibits anti-apoptotic Bcl-2 protein with p21 induction. Thus the results suggest that our compound has potential use in the growth inhibition of drug resistant cancer cells and the combination therapy with other clinically approved anticancer agents as well.

1. Introduction

The importance of G2/M-specific cell cycle inhibitor in the mediation of normal and pathological processes has motivated considerable efforts to identify cell proliferation. Especially compounds to induce G2/M arrest and apoptosis in cancer cells were well known; for example, paclitaxel and related taxens,[1,2] *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a DNA alkylating agent,[3] benzazepine (BBL22),[4] curcumin,[5] etc.

To identify small molecule inhibitors of G2/M-specific cell cycle, we screened 1200 drug-like compounds using a cell-based reporter assay that measures the transcriptional activity of G2/M-specific cell cycle. The G2/M-specific cell cycle transcriptional activity was dependent on cell proliferation and could be used to monitor the activity of the cancer cell proliferation pathway. The 1200 compounds that were screened represent 20,000 structurally diverse and druggable, heterocyclic compound libraries, including benzopyrans,[6] oxazoles,[7] pyrazoles,[8] oxadiazoles,[9] thiadiazoles,[9] various thiazoles,[10,11] and pyrimidinediones.[12] Inhibition of cell proliferation of various cancer cells by the 1200 compounds were screened at a compound concentration of 5 μM and compounds that reproducibly inhibited growth by over 50% were selected. In the first round of screening, quinoxalinyl-piperazine derivatives were shown to reproducibly have IC_{50} below 5 μM. Therefore, in connection with a project for exploring the novel potent G2/M-specific cell cycle inhibitors, we have designed some target compounds for quinoxalinyl-piperazine-based library for optimization of the structural features of the primary screening results through high-throughput screening efforts. Although quinoxalinyl-piperazine compounds which have an unsubstituted quinoxaline ring were presented[13] and one of the unsubstituted quinoxalinyl-piperazine compounds was shown to exhibit microtubule-inhibiting activities as a candidate for anticancer agent with the ability to inhibit the angiogenesis of endothelial cells and to induce the apoptosis of tumor cells,[14] little attention was directed towards substituted quinoxalinyl-piperazine compounds at quinoxaline ring as a new therapeutic molecule for the treatment of disorders such as tumors.

In this Letter, a series of novel 5 or 6-substituted compounds at quinoxaline ring of 1-[(2-alkoxyquinoxalin-3-yl)aminocarbonyl-4-

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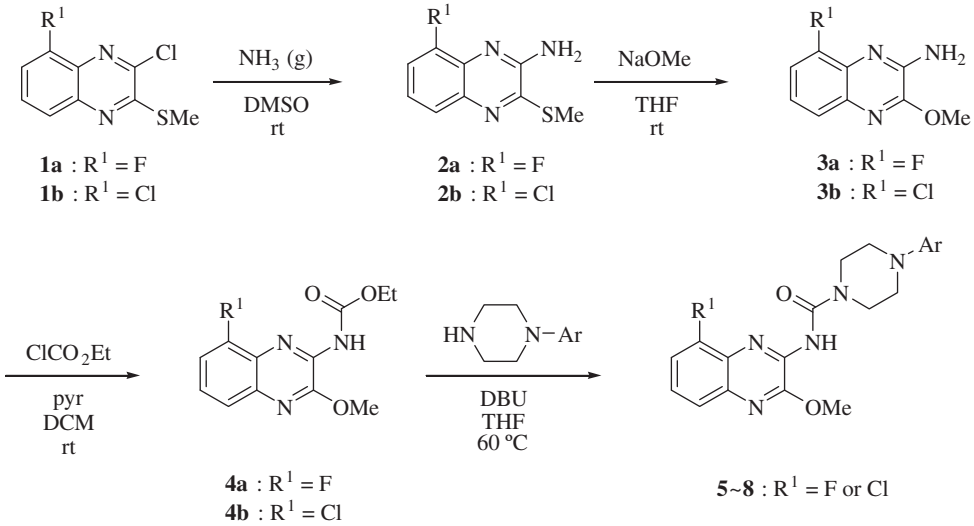
(hetero)arylpiperazine derivatives were made and tested as a prominent anticancer agent in various types of human cancer cell lines. The best compound was further characterized through combination assay with several known cancer drugs, drug resistant cells, and apoptosis assay.

2. Chemistry

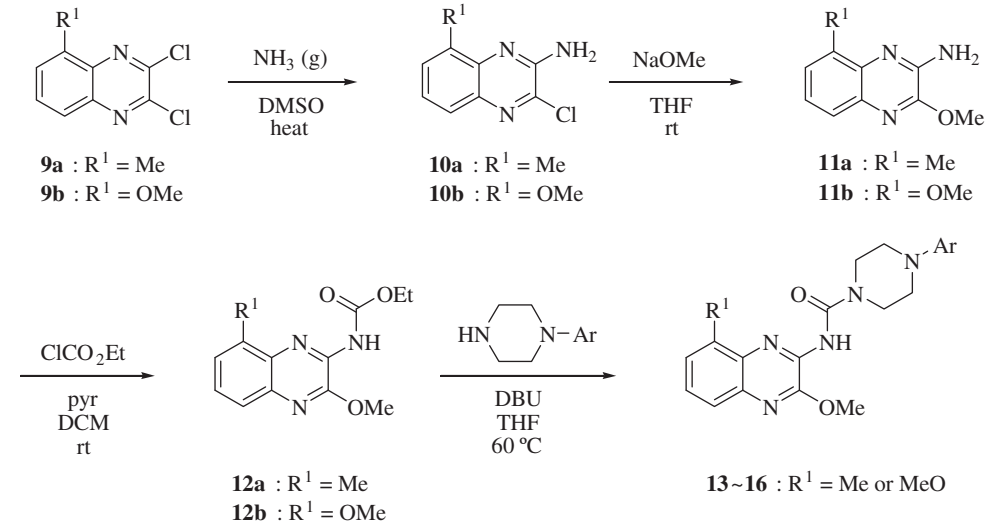
The compounds **5**–**8** with fluorine or chlorine atom at 5 position of quinoxaline ring system were regiospecifically synthesized from the corresponding 3-chloro-2-(methylthio)quinoxalines **1a** and **1b**[15] as described in Scheme 1. Sequential substitution reactions by treatment of the starting materials **1a** and **1b** with ammonia followed by methoxide afforded 3-amino-2-methoxy derivatives **3a** and **3b** through the formation of 3-amino-2-(methylthio)quinoxaline derivatives **2a** and **2b**. The final quinoxalinyl-piperazine compounds **5**–**8** were prepared from the reactions of carbamate derivatives **4a** and **4b** of the intermediates **3a** and **3b** with arylpiperazines. For the preparation of the quinoxalinyl-piperazine derivatives **13**–**16** substituted with 5-methyl or 5-methoxy group, the compounds **11a** and **11b** were synthesized by the application of the same reaction sequence as that for the synthesis of intermediates **3a** and **3b** from compounds **1a** and **1b** to the corresponding 2,3-dichloroquinoxaline starting materials **9a** and **9b**[16] (Scheme 2). The compound **10a** (R_f = 0.20 for *n*-hexane/ethyl acetate (4:1) mixture) was isolated from the corresponding major regioisomer 2-amino-3-chloro-5-methylquinoxaline (R_f = 0.35 for *n*-hexane/ethyl acetate (4:1) mixture) by silica gel column chromatography in the amination step. In the case of **10b**, separation from the major 2-amino-3-chloro regioisomer was difficult and purification by silica gel column chromatography was performed in the next methoxylation step (for *n*-hexane/ethyl acetate (1:1) mixture, **11b**, R_f = 0.13; major 2-amino-3-methoxy regioisomer, R_f = 0.20). The conversion of **11a** and **11b** to the piperazine products **13**–**16** were completed similarly to the examples in Scheme 1. Treatment of 2,3-dichloroquinoxalines **17a**–**d** with ammonia and subsequent recrystallization of the crude products in appropriate solvents (tetrahydrofuran for **18a** and dichloromethane for **18c**–**d**) gave the major regioisomeric products **18a**–**d** in ^{1}H NMR spectroscopically pure form (Scheme 3). The next steps were performed for the preparation of final compounds **21**–**40** in analogous way to the cases in Scheme 2. The regiochemistry of compounds **10** and **18** in Schemes 2 and 3 was determined on the basis of analogy with previously reported related reactions[17,18] and was supported by X-ray crystallographic analysis of compound **19b** (Fig. 1).

3. Result and discussion

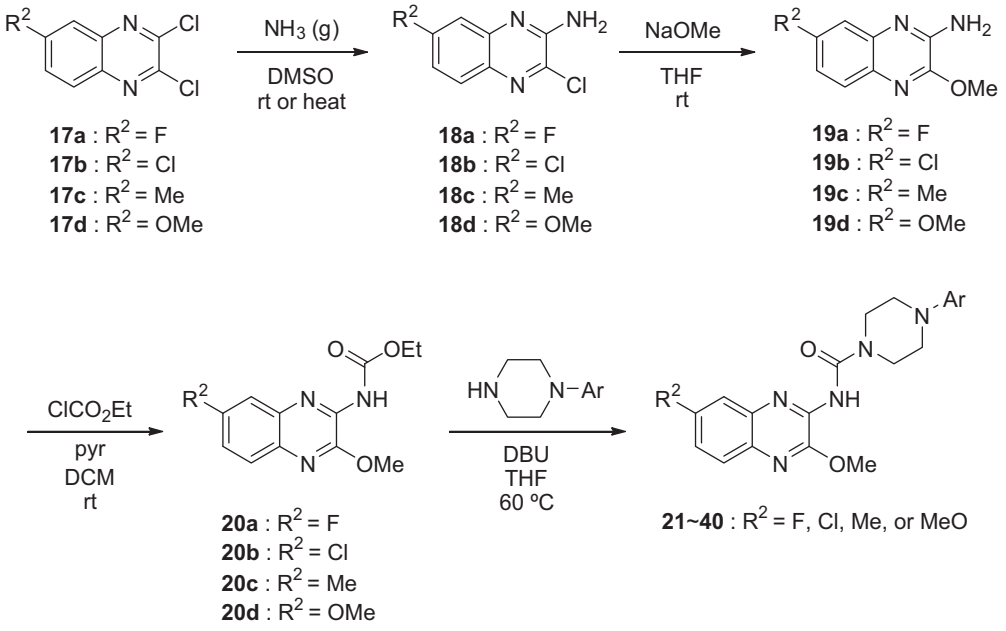
The in vitro cytotoxicity experiments were performed with the synthesized 1-[(5 or 6-substituted alkoxyquinoxalinyl)aminocarbonyl]-4-(hetero)arylpiperazine derivatives (Table 1) against human cancer cell lines using a colorimetric SRB-assay.[19] Initially we tested the compounds against three cancer cell lines such as MDA-MB-231, PANC-1, and UMRC2 at 0.1 and 1 μM. Among the compounds in Table 1, 13 compounds in Table 2 showed more than 50% inhibition at 0.1 μM in at least one of these cell lines and they were further assayed at lower concentrations against more human cancer cell lines to obtain their IC_{50} values (Table 2). Despite their structural similarity to the quinoxalinyl-piperazine core scaffold, the IC_{50} values significantly varied; the activity of the compounds depends on the substitution at quinoxaline ring but also from substituents at the phenyl ring. Although the fluoro (compounds **5** and **6**) or chlorine (compounds **7** and **8**) containing compounds at C-5 position of quinoxaline ring might not possess strong anticancer activity, the introduction of an electron donating group such as methyl or methoxy at C-5 position of quinoxaline ring resulted in a significant increase in cytotoxic potency. In particular, compounds **13** and **15** having 3,5-dimethoxyphenyl-piperazine inhibited the cell growth at IC_{50} values ranged from 29 to 74 nM in the human cancer cells tested, which are more potent than compounds **14** and **16** having 3,5-dimethylphenyl-piperazine that might have more than 0.1 μM of IC_{50} values. Our further efforts using SAR study to improve the cytotoxic activity of compound **13** or **15** resulted in the introduction of fluorine, chlorine, methyl or methoxy at C-6 position of quinoxaline ring. Table 2 showed that among compounds containing F or Cl at C-6 position of quinoxaline ring, 3,5-dimethoxyphenyl-piperazine compounds induced the most cell death. But introduction of methyl or methoxy group at 2 or 4 position in phenyl reduced the cytotoxic activity of compounds by electronic and steric effects. Interestingly, the introduction of methyl (compounds **38** and **39**) or methoxy (compound **40**) at C-6 position of quinoxaline ring resulted in the increase of IC_{50} values which is opposite to C-5 substitution of quinoxaline ring. Compound **30**, which showed 19–31 nM of IC_{50} range and has F-substitution at C-6, was compared to unsubstituted compound **42** (from U.S. Patent No. 6,683,184) with 20–90 nM of IC_{50} range.



Scheme 1.



Scheme 2.



Scheme 3.

This result suggests that introducing fluorine at C-6 in quinoxaline ring imparted the increased cytotoxic activity. Compound **25** among tested compounds strongly inhibited proliferation of human cancer cells of the brain, breast, cervix, colon, kidney, liver, lung, ovary, pancreas, prostate, skin, stomach, and skin (melanoma). The growth inhibition by compound **25** appeared to be dose-dependent and the IC_{50} for cell growth ranged from 11 to 21 nM in the tested 14 human cancer cells (see Table 2), and we further characterized this compound.

To evaluate whether any cooperative effect may exist between cytotoxic drugs and compound **25**, we tested the effect of compound **25** in combination with known anticancer drugs of different classes in the MDA-MB-231 breast cancer cell line. Compound **25** alone at a dose of 20 nM showed an inhibition of MDA-MB-231 cell growth of approximately 14% (Fig. 2). Therefore, we selected this low concentration to study whether any cooperative antiproliferative effect may occur between compound **25** and a series of cytotoxic drugs acting by different mechanisms of action. When MDA-MB-231 cells were treated with compound **25** and a taxane paclitaxel, a platinum derivative cisplatin, a topoisomerase II-selective agent doxorubicin and antimetabolites gemcitabine and fluorouracil, a synergistic growth inhibitory effect was observed (see Fig. 1). Treatment with 20 nM of compound **25**, which inhibits 13.8% of the growth of MDA-MB-231 cells, in combination with 2 or 3 nM paclitaxel, which used alone showed 8.0% and 60.8% growth inhibition, respectively, caused a 77.4% and 90.6% inhibition, respectively. In cells treated with 5 or 10 nM of doxorubicin, which alone cause 4.2% and 59.2% growth inhibition, respectively, addition of 20 nM of compound **25** showed a growth inhibition of 41.1% and 76.2%, respectively. The growth inhibitory effect by gemcitabine at 0.1 μM (1.0%) or 0.2 μM (17.7%) was increased to 35.0% and 56.3%, respectively, with the addition of 20 nM of compound **25** to MDA-MB-231 cells. Further study with 5-fluorouracil or cisplatin occurred also the synergistic inhibition of growth of MDA-MB-231 cells. In fact, in cells treated with 3 or 5 μM 5-fluorouracil, which alone cause 3.3% and 41.6% growth inhibition, respectively, addition of 20 nM of compound **25** caused a growth inhibition of 56.6% and 83.1%, respectively. Cisplatin at 300 μM

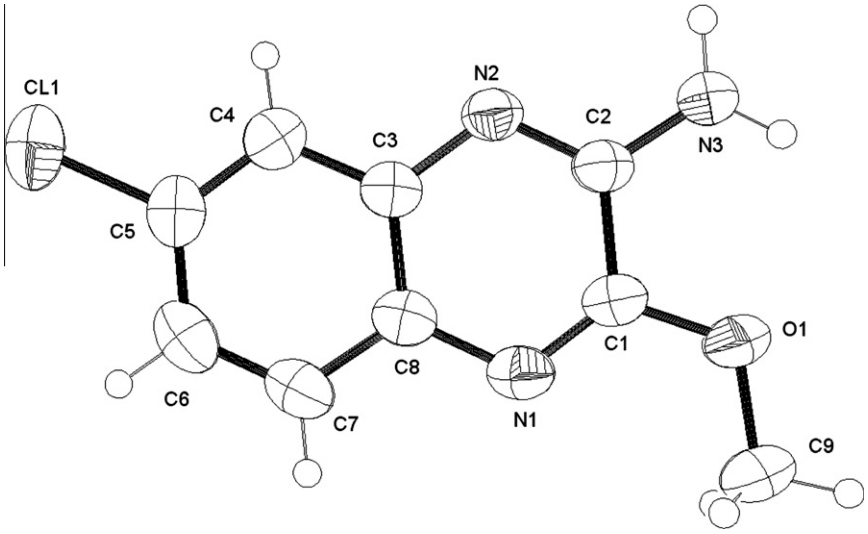
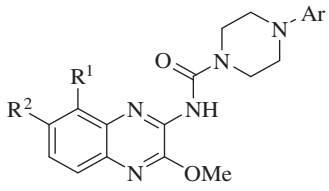


Figure 1. The X-ray structure of compound **19b**.

Table 1
Structure of 1-[(5 or 6-substituted 2-alkoxyquinoxalin-3-yl)aminocarbonyl]-4-(hetero)arylpiperazine derivatives



Compound	R^1	R^2	Ar
5	F	H	3,5-Dimethoxyphenyl
6	F	H	3.5-Dimethylphenyl
7	Cl	H	3,5-Dimethoxyphenyl
8	Cl	H	3.5-Dimethylphenyl
13	Me	H	3,5-Dimethoxyphenyl
14	Me	H	3.5-Dimethylphenyl
15	MeO	H	3,5-Dimethoxyphenyl
16	MeO	H	3.5-Dimethylphenyl
21	H	F	Phenyl
22	H	F	2-Methoxyphenyl
23	H	F	3-Methoxyphenyl
24	H	F	4-Methoxyphenyl
25	H	F	3,5-Dimethoxyphenyl
26	H	F	3,4,5-Trimethoxyphenyl
27	H	F	2-Methylphenyl
28	H	F	3-Methylphenyl
29	H	F	2,6-Dimethylphenyl
30	H	F	3,5-Dimethylphenyl
31	H	F	2-Fluorophenyl
32	H	F	4-Fluorophenyl
33	H	F	2-Chlorophenyl
34	H	F	3-Chlorophenyl
35	H	F	4-Chlorophenyl
36	H	Cl	3,5-Dimethoxyphenyl
37	H	Cl	3,5-Dimethylphenyl
38	H	Me	3,5-Dimethoxyphenyl
39	H	Me	3,5-Dimethylphenyl
40	H	OMe	3,5-Dimethylphenyl
42[*]	H	H	3,5-Dimethylphenyl

[*] Compound **42**: from U.S. Patent No. 6,683,184.

(5.0%) or 500 μM (31.6%) showed also the 78.0% and 90.2% of inhibition with the addition of 20 nM of compound **25**, respectively. The combination result suggest that compound **25** can be combined with known anticancer drugs of low concentration to treat the cancer patients more effectively by reducing the side effects of known anticancer drugs that comes from the usage of high concentration.

The development of multidrug resistance (MDR) in tumor cells by known anticancer drugs has become a serious issue and a major obstacle in clinical oncology. Our further investigation of compound **25** was extended to examine the effect in drug resistant cancer cells. In experiment using HCT116 and paclitaxel resistant HCT-15 cancer cells, compound **25** showed the same potent activity in both cancer cells, even stronger effect in drug resistant HCT-15 cells (see Table 3) with 0.72 of Resistant Index (RI). But anticancer activity of paclitaxel was decreased by 70-fold in paclitaxel resistant HCT-15 cells. This result implies that compound **25** can control and overcome drug resistance that has been a major problem in anticancer therapy.

Many potential chemotherapeutic agents have been shown to kill tumor cells by inducing apoptosis (programmed cell death), without eliciting an inflammatory response in the surrounding normal tissue. For example, gemcitabine used as an anticancer agent in various carcinomas including pancreatic, non-small cell lung, bladder, and breast cancers induces apoptosis.[20–22] We confirmed the occurrence of apoptosis by compound **25** treatment in cancer cells using APOPercentage™ Apoptosis Assay that detects the membrane alteration in apoptotic cells (unpublished result). Compound **25** also induced the apoptotic cell death through DNA fragmentation and PARP fragmentation (unpublished data) and this apoptosis induction by compound **25** was associated with an increase in $p21^{WAF1}$ protein, an inhibition of cell cycle progression at G2/M (Fig. 3) and an inhibition of anti-apoptotic Bcl-2 protein and an increase of pro-apoptotic Bad protein as shown in Figure 4.

4. Conclusion

In conclusion, we screened 1200 heterocyclic small molecules, and identified a novel hit core skeleton of quinoxalinyl-piperazine, selected as a potential G2/M-specific cell cycle inhibitor and demonstrated its effects in inhibiting various cancer cell line proliferation, leading to inhibition of G2/M arrest responses in vitro and pharmacophore analysis. Further characterization of compound **25** showed that this novel piperazine compound specifically induces the apoptosis by the downregulation of Bcl-2 protein level and the G2/M arrest and inhibits the growth of various types of

Table 2
Inhibition of cell growth (IC_{50}, μM) by quinoxalinyl-piperazine compounds against human cancer cell lines

	13	15	22	23	25	28	30	34	36	37	38	39	40	42*
MDA-MB-231	0.060	0.056	0.064	0.063	0.012	0.036	0.023	0.040	0.050	0.037	0.032	0.050	0.057	0.050
Caki-1	0.032	0.033	0.11	0.047	0.011	0.024	0.019	0.064	0.053	0.056	0.029	0.071	0.066	0.035
UMRC2	0.030	0.029	0.10	0.050	0.013	0.032	0.022	0.077	0.065	0.06	0.039	0.050	0.10	0.027
PANC-1	0.054	0.042	0.35	0.062	0.021	0.039	0.024	0.28	0.098	0.24	0.070	0.28	0.33	0.038
A549	0.056	0.064	0.20	0.15	0.021	0.081	0.023	0.10	0.073	0.076	0.055	0.097	0.11	0.021
MKN-45	0.057	0.052	0.093	0.05	0.020	0.023	0.027	0.076	0.064	0.069	0.040	0.080	0.081	0.021
HepG2	0.063	0.057	0.12	0.12	0.019	0.080	0.021	0.077	0.063	0.068	0.045	0.081	0.081	0.060
HCT116	0.050	0.060	0.16	0.064	0.020	0.043	0.025	0.097	0.068	0.076	0.047	0.10	0.11	
HT29	0.055	0.074	0.15	0.090	0.021	0.050	0.031	0.11	0.079	0.11	0.048	0.14	0.12	0.090
PC-3	0.057	0.057	0.22	0.070	0.021	0.060	0.022	0.13	0.076	0.079	0.063	0.11	0.14	0.020
U251	0.054	0.058	0.14	0.070	0.015	0.050	0.019	0.080	0.056	0.069	0.031	0.085	0.075	0.030
HeLa	0.062	0.063	0.15	0.046	0.021	0.056	0.024	0.086	0.073	0.081	0.051	0.10	0.11	0.020
SK-MEL-28	0.064	0.068	0.26	0.068	0.020	0.11	0.022	0.19	0.11	0.11	0.047	0.13	0.14	0.026
OVCAR-3	0.039	0.044	0.076	0.036	0.012	0.024	0.025	0.055	0.042	0.056	0.023	0.065	0.059	0.023

* Compound **42**: from U.S. Patent No. 6,683,184.

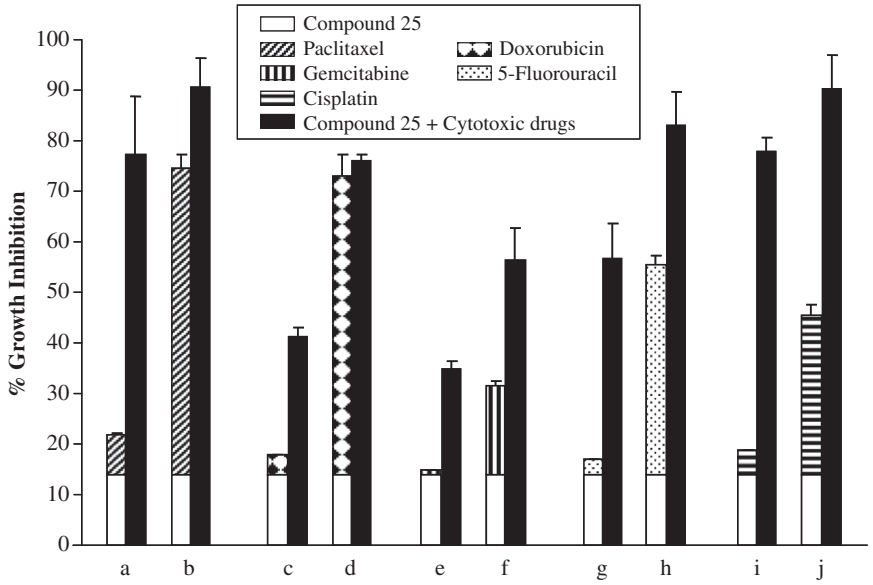


Figure 2. Combination effect of compound **25** with different cytotoxic drugs on the growth of MDA-MB-231 cancer cells. The drugs were used at the following doses: (a–b): 2 and 3 nM paclitaxel; (c–d): 5 and 10 nM doxorubicin; (e–f): 100 and 200 nM gemcitabine; (g–h) 3 and 5 μM fluorouracil; (i–j): 300 and 500 μM cisplatin. Data are expressed as percentage growth inhibition in reference to the growth of untreated control cells. 0.5% DMSO in media was used as a control for drugs. The open portion of the bars represents the percentage growth inhibition values for compound **25**. The striped or squared portion of the bars represents the percentage growth inhibition values for the cytotoxic drugs as indicated in the respective legends. The height of the bars on the left represents the sum of the individual agents' effects and the expected percentage growth inhibition if drugs are additive when used in combination. The total height of the solid bar indicates the actual observed growth inhibition when drugs were used in combination. The data represent means and standard errors of triplicate determination of at least two experiments.

Table 3
Comparison of IC_{50} values (nM)[a] of compound **25** and paclitaxel in HCT116 and HCT-15 cells

Compound	HCT116	HCT-15	Resistant indices[b]
Compound **25**	29 ± 1.4	21 ± 0.98	0.72
Paclitaxel	2 ± 0.08	140 ± 11	70

[a] IC_{50} values were determined after incubation of compound **25** and paclitaxel at various concentrations with the HCT116 cell line (paclitaxel sensitive) and the HCT-15 cell line (paclitaxel resistant) for 96 h. Data are presented as the mean ± SD.
[b] Resistant indices are defined by IC_{50} (HCT-15)/IC_{50} (HCT116).

human cancer cell lines. Compound **25** has also significantly inhibited growth of drug resistant cancer cells, and has potential use in combination therapy with known cancer drugs that could improve its therapeutic index and decrease toxicity to cancer patients although extensive efforts to further characterize this molecule should be followed.

5. Experimental

5.1. Chemistry

5.1.1. General

All chemicals were reagent grade and used as purchased. Reactions were monitored by TLC analysis using Merck silica gel 60 F-254 thin layer plates. Flash column chromatography was carried out on Merck silica gel 60 (230–400 mesh). The crude products were purified by parallel chromatography using Quad3™. ^1H NMR spectra were recorded in δ units relative to deuterated solvent as an internal reference using a Bruker 500 MHz NMR instrument. LC–MS analysis was performed on an ESI mass spectrometer with PDA detection. LC–MS area % purities of all products were determined by LC peak area analysis (XTerraMS C_{18} column, 4.6 mm × 100 mm; PDA detector at 200–400 nm; gradient, 5–95% CH_3CN/H_2O).

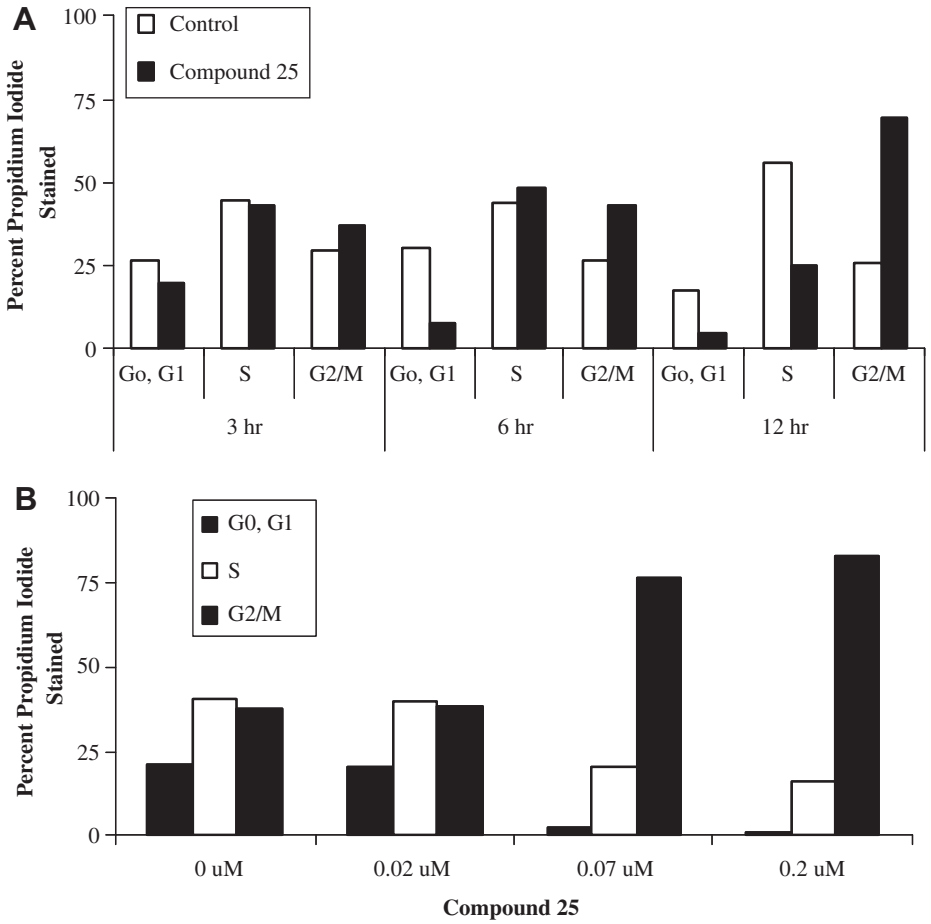


Figure 3. Cell cycle distribution of MDA-MB-231 cells after treatments with compound **25**. The MDA-MB-231 cells were seeded in 10 cm dish and then incubated overnight. Cells were treated with 70 nM of compound **25** for 3, 6, and 12 h (A) or 20 nM, 70 nM and 200 nM of compound **25** for 12 h (B). Compound **25** treated cells were harvested and subject to cell cycle analyses as described in experimental section. PI stained cells were analyzed by Guava PCA-AFP instrument using its cell cycle software program (Guava Technologies, Hayward, CA) and expressed as percentage of cells in G0/G1, S, and G2/M phases of the cell cycle.

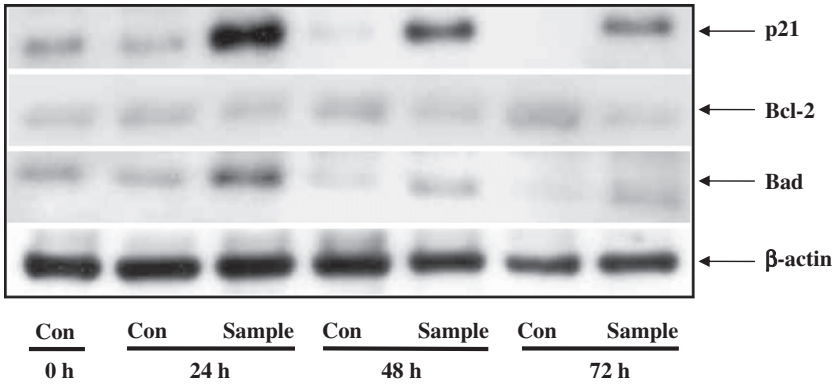


Figure 4. Effect of compound **25** on the expression of $p21^{WAF1}$, Bcl-2, and Bad protein in MDA-MB-231 cancer cells. The cells were treated without (Con) or with sample (compound **25**) for 24, 48, and 72 h and $p21^{WAF1}$, Bcl-2, and Bad protein expression were determined by western blot analysis. β-Actin was determined as a loading control. Representative data are shown from two independent cell culture experiments.

5.1.2. Compounds synthesis

5.1.2.1. Synthesis of 3-amino-5-fluoro-2-methylthioquinoxaline (2a). *Method A*: To a stirred solution of 3-chloro-5-fluoro-2-methylthioquinoxaline (**1a**) (93 mg, 0.41 mmol) in dimethylsulfoxide (20 ml) was injected anhydrous ammonia gas at room temperature. Stirring was continued at room temperature for 48 h and then water was added to the reaction mixture. The mixture was extracted with ethyl acetate and the organic layer was washed with water and dried over $MgSO_4$. After removal of solvent in vacuo, the residue was purified by SiO_2 column chromatography (*n*-hexane/ethyl acetate = 3:1) to yield the desired compound **2a** (50 mg, 58%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.75 (s, 3H), 5.84 (br s, 2H), 7.20 (m, 1H), 7.29 (m, 1H), 7.61 (m, 1H); MS (ESI) *m*/*z* 210 ($[M+H]^+$).

5.1.2.2. Synthesis of 3-amino-5-fluoro-2-methoxyquinoxaline (3a). *Method B*: To a stirred solution of 3-amino-5-fluoro-2-methylthioquinoxaline (**2a**) (47 mg, 0.22 mmol) in tetrahydrofuran (2 ml) was added 25 wt % sodium methoxide in methanol (475 mg, 2.20 mmol) at room temperature. Stirring was continued at room

temperature for 48 h. The resulting mixture was concentrated in vacuo to remove the solvent and then water was added thereto. The mixture was extracted with dichloromethane and the organic layer was washed with water and dried over $MgSO_4$. After removal of solvent in vacuo, the residue was purified by SiO_2 column chromatography (*n*-hexane/ethyl acetate = 3:1) to yield the desired compound **3a** (30 mg, 71%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.16 (s, 3H), 6.28 (br s, 2H), 7.17 (m, 1H), 7.27 (m, 1H), 7.50 (m, 1H); MS (ESI) *m*/*z* 194 ([M+H]$^+$).

5.1.2.3. Synthesis of ethyl *N*-(5-fluoro-2-methoxyquinoxalin-3-yl)carbamate (4a). *Method C*: To a stirred solution of 3-amino-5-fluoro-2-methoxyquinoxaline (**3a**) (26 mg, 0.13 mmol) and ethyl chloroformate (22 mg, 0.20 mmol) in dichloromethane (6 ml) at room temperature was added pyridine (16 mg, 0.20 mmol). The resulting mixture was stirred at room temperature for 40 h, concentrated in vacuo to remove the solvent and purified by SiO_2 column chromatography (*n*-hexane/ethyl acetate = 5:1) to yield the desired compound **4a** (26 mg, 75%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (t, *J* = 7.1 Hz, 3H), 4.17 (s, 3H), 4.34 (q, *J* = 7.1 Hz, 2H), 7.25 (m, 1H), 7.45 (m, 1H), 7.55 (m, 1H), 7.81 (br s, 1H); MS (ESI) *m*/*z* 266 ([M+H]$^+$).

5.1.2.4. Synthesis of 1-(3,5-dimethoxyphenyl)-4-[(5-fluoro-2-methoxyquinoxalin-3-yl)amino-carbonyl]piperazine (5). *Method D*: To a stirred solution of ethyl *N*-(5-fluoro-2 -methoxyquinoxalin-3-yl)carbamate (**4a**) (10 mg, 0.037 mmol) and 1-(3,5-dimethoxyphenyl) piperazine (12 mg, 0.054 mmol) in tetrahydrofuran (2 ml) at room temperature was added DBU (8 mg, 0.05 mmol). The resulting mixture was stirred at 60 °C for 27 h, concentrated in vacuo to remove the solvent and purified by SiO_2 column chromatography (*n*-hexane/ethyl acetate = 2:1) to yield the desired compound **5** (9 mg, 55%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.25 and 3.35 (2m, 4H), 3.78, 3.85, and 4.14 (2m, 4H), 3.82 (s, 6H), 4.17 and 4.20 (2s, 3H), 6.09 (m, 1H), 6.15 (s, 2H), 7.10–7.20, 7.20–7.27, 7.32, 7.40–7.48, and 7.55–7.60 (5m, 4H); MS (ESI) *m*/*z* 442 ([M+H]$^+$).

Compounds **6**–**8** were synthesized by the same way with the preparation of the compound **5** using methods A, B, C, and D.

5.1.2.5. 1-(3,5-Dimethylphenyl)-4-[(5-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (6). In 66% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.30 and 2.33 (2s, 6H), 3.23 and 3.33 (2m, 4H), 3.79, 3.86, and 4.14 (3m, 4H), 4.17 and 4.20 (2s, 3H), 6.55–6.65 (m, 3H), 7.15–7.20, 7.20–7.26, 7.31, 7.40–7.48, 7.48–7.52, and 7.52–7.60 (6m, 4H); MS (ESI) *m*/*z* 410 ([M+H]$^+$).

5.1.2.6. 1-(3,5-Dimethoxyphenyl)-4-[(5-chloro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (7). In 61% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.25 and 3.33 (2m, 4H), 3.81 (s, 6H), 3.87 and 4.15 (2m, 4H), 4.17 and 4.19 (2s, 3H), 6.08 (s, 1H), 6.10–6.20 (m, 2H), 7.15–7.30, 7.40–7.48, 7.55–7.60, 7.60–7.65, and 7.70–7.75 (5m, 3H); MS (ESI) *m*/*z* 458 ([M+H]$^+$).

5.1.2.7. 1-(3,5-Dimethylphenyl)-4-[(5-chloro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (8). In 72% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.32 (s, 6H), 3.24 and 3.31 (2m, 4H), 3.84, 3.88, and 4.15 (3m, 4H), 4.18 and 4.20 (2s, 3H), 7.55–7.65 (m, 3H), 7.25–7.30, 7.40–7.49, 7.55–7.60, 7.60–7.65, and 7.70–7.75 (5m, 3H); (ESI) *m*/*z* 426 ([M+H]$^+$).

Compounds **13**–**16** in Scheme 2 were prepared using methods A, B, C, and D.

5.1.2.8. Synthesis of 3-amino-2-chloro-5-methylquinoxaline (10a). Prepared from compound **9a** (3.00 g, 14.1 mmol) using method A. The compound **10a** (R_f = 0.20 for *n*-hexane/ethyl acetate (4:1) mixture, 1.07 g, 39%) was purified from the major regioisomeric 2-amino-3-chloro-5-methylquinoxaline (R_f = 0.35 for *n*-hexane/ethyl acetate (4:1) mixture, 1.37 g, 50%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.79 (s, 3H), 5.38 (br s, 2H), 7.34 (t, *J* = 7.9 Hz, 1H), 7.61 (d, *J* = 7.9 Hz, 1H), 7.78 (d, *J* = 7.9 Hz, 1H); MS (ESI) *m*/*z* 194 ([M+H]$^+$).

5.1.2.9. Synthesis of 3-amino-2-methoxy-5-methylquinoxaline (11a). Prepared from compound **10a** (1.04 g, 5.37 mmol) in 99% yield (1.01 g) using method B: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.63 (s, 3H), 4.13 (s, 3H), 5.19 (br s, 2H), 7.23 (d, *J* = 7.7 Hz, 1H), 7.32 (t, *J* = 7.7 Hz, 1H), 7.45 (d, *J* = 7.7 Hz, 1H); MS (ESI) *m*/*z* 190 ([M+H]$^+$).

5.1.2.10. Synthesis of ethyl *N*-(2-methoxy-5-methylquinoxalin-3-yl)carbamate (12a). By method C from compound **11a** (1.01 g, 5.34 mmol) in 73% yield (1.02 g): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (t, *J* = 7.1 Hz, 3H), 2.65 (s, 3H), 4.16 (s, 3H), 4.33 (q, *J* = 7.1 Hz, 2H), 7.43–7.38 (m, 2H), 7.76 (br s, 1H), 7.82 (d, *J* = 8.0 Hz, 1H); MS (ESI) *m*/*z* 262 ([M+H]$^+$).

5.1.2.11. 1-(3,5-Dimethoxyphenyl)-4-[(2-methoxy-5-methylquinoxalin-3-yl)aminocarbonyl] piperazine (13). By method D from compound **12a** (24 mg, 0.092 mmol) in 68% yield (27 mg): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.62 (s, 3H), 3.25–3.27 (m, 4H), 3.79 (s, 6H), 3.85–3.90 (m, 4H), 4.15 (s, 3H), 6.07 (d, *J* = 1.7 Hz, 1H), 6.13 (s, 2H), 7.25–7.27 (m, 2H), 7.30–7.33 (m, 2H); MS (ESI) *m*/*z* 438 ([M+H]$^+$).

5.1.2.12. 1-(3,5-Dimethylphenyl)-4-[(2-methoxy-5-methylquinoxalin-3-yl)aminocarbonyl] piperazine (14). In 86% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.29 (s, 6H), 2.63 (s, 3H), 3.24 (m, 4H), 3.78 (m, 3H), 4.15–4.16 (m, 4H), 6.57–6.59 (m, 3H), 7.27 (br s, 1H), 7.34–7.35 (m, 2H), 7.48–7.50 (m, 1H); MS (ESI) *m*/*z* 406 ([M+H]$^+$).

5.1.2.13. 1-(3,5-Dimethoxyphenyl)-4-[(2,5-dimethoxyquinoxalin-3-yl)aminocarbonyl]piperazine (15). In 60% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.21–3.23 (m, 4H), 3.78–3.83 (m, 7H), 4.00 (s, 3H), 4.13 (s, 6H), 6.05 (br s, 1H), 6.12 (d, *J* = 2.1 Hz, 2H), 6.86–6.89 (m, 1H), 7.22–7.23 (m, 2H), 7.30 (br s, 1H); MS (ESI) *m*/*z* 454 ([M+H]$^+$).

5.1.2.14. 1-[(2,5-Dimethoxyquinoxalin-3-yl)aminocarbonyl]-4-(3,5-dimethylphenyl)piperazine (16). In 50% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.29 (s, 6H), 3.20–3.29 (m, 4H), 3.74–3.83 (m, 3H), 4.00 (s, 3H), 4.13 (m, 4H), 6.56–6.59 (m, 3H), 6.85–6.88 (m, 1H), 7.18–7.26 (m, 2H), 7.40 (br s, 1H); MS (ESI) *m*/*z* 422 ([M+H]$^+$).

Compounds **21**–**40** were synthesized also by the same way with the preparation of the compound **13** using methods A, B, C, and D.

5.1.2.15. Synthesis of 3-amino-2-chloro-6-fluoroquinoxaline (18a). From compound **17a** (21.0 g, 96.8 mmol) in 63% yield (12.1 g) by method A: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.25–7.33 (m, 2H), 7.44 (br, 2H), 7.80 (dd, *J* = 8.7 and 6.2 Hz, 1H); MS (ESI) *m*/*z* 198 ([M+H]$^+$).

5.1.2.16. Synthesis of 3-amino-6-fluoro-2-methoxyquinoxaline (19a). From compound **18a** (550 mg, 2.78 mmol) in 91% yield (491 mg) by method B: ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.02 (s, 3H), 7.09 (br s, 2H), 7.04–7.21 (m, 2H), 7.60 (dd, *J* = 8.8 and 6.1 Hz, 1H); MS (ESI) *m*/*z* 194 ([M+H]$^+$).

5.1.2.17. Synthesis of ethyl *N*-(6-fluoro-2-methoxyquinoxalin-3-yl)carbamate (20a). From compound **19a** (580 mg,

3.00 mmol) in 93% yield (740 mg) by method C: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36 (t, 3H, J = 7.2 Hz) 4.15 (s, 3H) 4.33 (q, 2H, J = 7.2 Hz) 7.28 (td, 1H, J = 8.7 and 2.7 Hz) 7.40 (dd, 1H, J = 9.3 and 2.7 Hz) 7.74 (s, 1H) 7.93 (m, 1H); MS (ESI) m/z 266 ([M+H]$^+$).

5.1.2.18. Synthesis of 1-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-phenylpiperazine (21). From compound **20a** (27 mg, 0.10 mmol) in 88% yield (34 mg) by Method D: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.29 (s, 4H), 3.77 (s, 3H), 4.14 (s, 4H), 6.89–6.97 (m, 4H), 7.24–7.56 (m, 5H), 7.62–7.71 (m, 1H); MS (ESI) m/z 382 ([M+H]$^+$).

5.1.2.19. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(2-methoxyphenyl)piperazine (22). In 84% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.15 (s, 4H), 3.79–3.87 (m, 6H), 4.11 (s, 4H), 6.86–7.02 (m, 4H), 7.18–7.22 (m, 1H), 7.39–7.50 (m, 1H), 7.65–7.72 (m, 1H); MS (ESI) m/z 412 ([M+H]$^+$).

5.1.2.20. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(3-methoxyphenyl)piperazine (23). In 87% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.28 (s, 4H), 3.80 (s, 6H), 4.13 (s, 4H), 6.45–6.58 (m, 3H), 7.01 (s, 1H), 7.17–7.23 (m, 2H), 7.37–7.70 (m, 2H); MS (ESI) m/z 412 ([M+H]$^+$).

5.1.2.21. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(4-methoxyphenyl)piperazine (24). In 80% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.18 (s, 4H), 3.79 (s, 6H), 4.08–4.15 (m, 4H), 6.85–6.98 (m, 4H), 7.22–7.76 (m, 4H); MS (ESI) m/z 412 ([M+H]$^+$).

5.1.2.22. 1-(3,5-Dimethoxyphenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (25). In 72% yield; mp 149 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.18–3.35 (m, 4H), 3.76 (s, 3H), 3.79 (s, 6H), 4.08–4.15 (m, 4H), 6.07 (s, 1H), 6.11 (s, 2H), 6.83–7.73 (8m, total 4H); MS (ESI) m/z 442 ([M+H]$^+$); MS/MS (amu) 442.152; ^{13}H NMR (125 MHz, $CDCl_3$) δ 45.22 (2 CH_2), 49.42 (2 CH_2), 54.55 (CH_3), 55.43 (2 CH_3), 92.37 (aromatic carbon between 2 OCH_3), 95.75 (two aromatic carbon of *o*-position OCH_3), 111.69 (aromatic carbon of *o*-position F), 111.88 (aromatic carbon of *o*-position F), 116.29 (aromatic carbon of *m*-position F), 128.02 (aromatic carbon of quinoxaline), 128.10 (aromatic carbon of quinoxaline), 153.01 (N-attached aromatic carbon), 153.19 (N attached carbon of quinoxaline), 160.44 (F attached aromatic carbon), 161.71 (O attached two aromatic carbons), 162.39 (O attached quinoxaline carbon).

5.1.2.23. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(3,4,5-trimethoxyphenyl) piperazine (26). In 76% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.22 (s, 4H), 3.79–3.85 (m, 12H), 4.13 (s, 4H), 6.19 (s, 2H), 7.20–7.34 (m, 1H), 7.35–7.36 (m, 1H), 7.44 (s, 1H), 7.67–7.70 (m, 1H); MS (ESI) m/z 472 ([M+H]$^+$).

5.1.2.24. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(2-methylphenyl)piperazine (27). In 73% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.35 (s, 3H), 2.98–3.03 (m, 4H), 3.73–3.78 (m, 3H), 4.10–4.14 (m, 4H), 7.02–7.17 (m, 2H), 7.19–7.29 (m, 2H), 7.36 (s, 1H), 7.48–7.60 (m, 1H), 7.67–7.74 (m, 1H); MS (ESI) m/z 396 ([M+H]$^+$).

5.1.2.25. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(3-methylphenyl)piperazine (28). In 90% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.33 (s, 3H), 3.26–3.30 (m, 4H), 3.74–3.78 (m, 3H), 4.13 (s, 4H), 6.75–6.78 (m, 3H), 7.14–7.28 (m, 2H), 7.36 (s, 1H), 7.44–7.51 (dd, J = 9.8 and 2.4 Hz, 1H), 7.67–7.74 (m, 1H); MS (ESI) m/z 396 ([M+H]$^+$).

5.1.2.26. 1-(2,6-Dimethylphenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (29). In 65% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.26 (s, 3H), 3.20 (s, 4H), 3.71 (s, 3H), 4.12–4.18 (m, 4H), 6.99–7.01 (m, 3H), 7.26–7.32 (m, 2H), 7.53–7.81 (m, 2H); MS (ESI) m/z 410 ([M+H]$^+$).

5.1.2.27. 1-(3,5-Dimethylphenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl] piperazine (30). In 79% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (s, 6H), 3.27 (s, 4H), 3.88 (s, 3H), 4.14 (s, 4H), 6.59 (s, 3H), 7.01–7.10 (s, 1H), 7.24–7.36 (m, 2H), 7.47–7.71 (m, 2H); MS (ESI) m/z 410 ([M+H]$^+$).

5.1.2.28. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(2-fluorophenyl)piperazine (31). In 91% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.18 (s, 4H), 3.78 (s, 3H), 4.13 (s, 4H), 6.93–7.10 (m, 5H), 7.20–7.34 (m, 1H), 7.46–7.60 (m, 1H), 7.67–7.74 (m, 1H); MS (ESI) m/z 400 ([M+H]$^+$).

5.1.2.29. 1-[(6-Fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(4-fluorophenyl)piperazine (32). In 85% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.19 (s, 4H), 3.77 (s, 3H), 4.13 (s, 4H), 6.88–7.02 (m, 4H), 7.23–7.27 (m, 1H), 7.45–7.71 (m, 3H); MS (ESI) m/z 400 ([M+H]$^+$).

5.1.2.30. 1-(2-Chlorophenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]piperazine (33). In 87% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.14 (s, 4H), 3.79 (s, 3H), 4.13 (s, 4H), 6.97–7.05 (m, 2H), 7.22–7.28 (m, 2H), 7.33–7.40 (m, 2H), 7.46–7.51 (d, J = 10.2 Hz, 1H), 7.66–7.73 (m, 1H); MS (ESI) m/z 416 ([M+H]$^+$).

5.1.2.31. 1-(3-Chlorophenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]piperazine (34). In 70% yield: ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.30 (s, 4H), 3.76 (s, 3H), 4.13 (s, 4H), 6.77–6.91 (m, 3H), 7.15–7.33 (m, 3H), 7.44–7.58 (d, J = 10.0 Hz, 1H), 7.58–7.75 (m, 1H); MS (ESI) m/z 416 ([M+H]$^+$).

5.1.2.32. 1-(4-Chlorophenyl)-4-[(6-fluoro-2-methoxyquinoxalin-3-yl)aminocarbonyl]piperazine (35). In 95% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.25 (s, 4H), 3.78 (s, 3H), 4.13 (s, 4H), 6.86 (d, J = 8.4 Hz, 2H), 7.22–7.26 (m, 3H), 7.44 (m, 1H), 7.70 (m, 1H); MS (ESI) m/z 416 ([M+H]$^+$).

5.1.2.33. 1-[(6-Chloro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(3,5-dimethoxyphenyl) piperazine (36). In 81% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.21 and 3.29 (2m, 4H), 3.75 (s, 3H), 3.79 (s, 6H), 4.11 and 4.14 (2m, 4H), 6.07 (s, 1H), 6.11 (s, 2H), 7.20–7.97 (8m, 4H); MS (ESI) m/z 458 ([M+H]$^+$).

5.1.2.34. 1-[(6-Chloro-2-methoxyquinoxalin-3-yl)aminocarbonyl]-4-(3,5-dimethylphenyl) piperazine (37). In 79% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (s, 6H), 3.19–3.29 (m, 4H), 3.73–3.89 (m, 3H), 4.11–4.14 (m, 4H), 6.58 (s, 3H), 7.19–7.25 (m, 1H), 7.36–7.64 (m, 2H), 7.67–7.81 (m, 1H); MS (ESI) m/z 426 ([M+H]$^+$).

5.1.2.35. 1-(3,5-Dimethoxyphenyl)-4-[(2-methoxy-6-methylquinoxalin-3-yl)aminocarbonyl] piperazine (38). In 94% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.43 and 2.05 (2s, 3H), 3.21 and 3.30 (2m, 4H), 3.78 (s, 3H), 3.79 (s, 6H), 4.12 (m, 4H), 6.06 (s, 1H), 6.12 (s, 2H), 6.84–7.77 (8m, 4H); MS (ESI) m/z 438 ([M+H]$^+$).

5.1.2.36. 1-(3,5-Dimethylphenyl)-4-[(2-methoxy-6-methylquinoxalin-3-yl)aminocarbonyl] piperazine (39). In 87% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.29 (s, 6H), 2.42–2.48 (m, 3H), 3.20–3.28 (m, 4H), 3.75–3.80 (m, 3H), 4.10–4.13 (m, 4H), 6.59 (s, 3H), 7.00–7.12 (m, 1H), 7.31 (d, J = 8.4 Hz, 1H), 7.48–7.65 (m, 2H); MS (ESI) m/z 406 ([M+H]$^+$).

5.1.2.37. 1-[(2,6-Dimethoxyquinoxalin-3-yl)aminocarbonyl]-4-(3,5-dimethylphenyl)piperazine (40). In 65% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.28 (s, 6H), 3.26 (s, 4H), 3.76–3.87 (m, 6H), 4.11 (s, 4H), 6.59 (s, 2H), 6.90 (m, 1H), 7.14 (d, J = 8.7 Hz, 1H), 7.32 (s, 1H), 7.51 (d, J = 7.5 Hz, 1H), 7.64 (d, J = 8.7 Hz, 1H); MS (ESI) m/z 422 ([M+H]$^+$).

5.2. Biology

5.2.1. Growth of cancer cells

Human cancer cell lines were obtained from the following sources: Caki-1, OVCAR-3, MDA-MB-231, HeLa, PC3, HepG2, A549, PANC-1, SK-MEL-28, HT29, HCT-15, and HCT116 from the American Type Culture Collection (Manassas, VA); U251 from Riken Institutes, Marunouchi, Tokyo, Japan; MKN-45 from DSMZ (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany; UMRC2 (kidney) from the U.S. National Cancer Institute (Bethesda, MD). All cell lines except MDA-MB-231, HCT116, Caki-1, UMRC2, and PANC-1 were grown in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM HEPES, and 100 units/ml penicillin–streptomycin (P/S). MDA-MB-231, HCT116, Caki-1, UMRC2, and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 10 mM HEPES, 100 units/ml P/S, and 2 mM L-glutamine. All cells were incubated at 37 °C under 5% CO_2 and humidified air.

5.2.2. Cell growth inhibition assay

The growth inhibition by the synthesized quinoxalinyl-piperazine compounds against human cancer cells was assessed by the sulforhodamine B (SRB) assay,[15] along with DMSO as a control. Different human cancer cell lines were treated with compounds at various concentrations. After 96 h incubation, surviving cells were fixed with trichloroacetic acid, washed and stained with sulforhodamine B. For the study of combination effect of compound **25** with cytotoxic drugs, MDA-MB-231 cells were seeded in 96 multiwell plate and treated with compound **25** in the presence of different concentrations of the indicated cytotoxic drug for 96 h. Absorbance was measured at 530 nm using Benchmark Plus Microplate reader (Bio-Rad Laboratories, Hercules, CA). The drug concentration which inhibited the cell growth by 50% (IC_{50}) was calculated using Kaledia Graph software program (Synergy software, Reading, PA).

5.2.3. Growth inhibition of drug resistant cancer cells by compound 25

The growth inhibition by compound **25** and paclitaxel (Taxol®) against HCT116 and paclitaxel resistant HCT-15 colon cancer cells was assessed as described in 'cell growth inhibition assay'. Resistance index (RI) was calculated by dividing the IC_{50} value of the resistant cell line by the IC_{50} value of the nonresistant cell line and represents the drug's efficacy against drug resistant cell lines versus the corresponding cancer cell lines (nonresistant).

5.2.4. Cell cycle arrest

MDA-MB-231 cells were plated at 50–70% saturation in a 10 cm dish in DMEM medium with 10% FBS and then incubated overnight in a humidified 37 °C incubator with 5% CO_2. The cells were exposed to compound **25** at various concentrations or for various time intervals. No compound control treatment was also included (0.25% DMSO). Cells were then trypsinized and harvested by centrifugation at 7000 rpm for 5 min. Cell pellets were resuspended in 0.2 ml of PBS containing 0.1% glucose and 2% FBS. Subsequently, 5 ml ice-cold 70% ethanol was added dropwise with shaking and the treated cells were stored at −20 °C at least 30 min. Cells were centrifuged at 2000 rpm for 5 min and washed once with 1 ml PBS with 0.1% glucose and 2% FBS. After removal of the supernatant, cells were resuspended in 0.5 ml of 70 μM propidium iodide (PI) solution containing 0.1% Triton X-100, 40 mM sodium citrate, pH 7.4. RNase was added at 50 μg/ml final concentration and cells were incubated at 37 °C for 30 min. PI stained cells were analyzed by Guava PCA-AFP instrument using its cell cycle software program (Guava Technologies, Hayward, CA) and expressed as percentage of cells in G0/G1, S, and G2/M phases of the cell cycle.

5.2.5. Western blotting analysis

MDA-MB-231 cells were incubated with compound **25** at 70 nM for 24, 48 or 72 h. After the incubation, cells were washed once with PBS and resuspended with the lysis buffer containing 25 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100 and protease inhibitors. Total protein concentration was determined by BCA protein assay according to manufacturer's manual (Pierce Biotechnology, Rockford, IL). Protein was separated on SDS–PAGE gel and transferred to nitrocellulose membranes. The blots were probed against anti-$p21^{WAF1}$ (Santa Cruz, CA), anti-Bcl-2 (Santa Cruz, CA), and anti-Bad antibody (Santa Cruz, CA). Anti-β-actin antibody (Santa Cruz, CA) was used as an internal control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.028.

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