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Full Paper

Synthesis and Cytotoxicity Evaluation of Some Novel 1-(3-Chlorophenyl)piperazin-2-one Derivatives Bearing Imidazole Bioisosteres

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A series of substituted 3-chlorophenylpiperazinone derivatives were synthesised using L-778123 (an imidazole-containing FTase inhibitor) as a model by bioisosteric replacement of the imidazole ring. The final compounds were evaluated against two human cancer cell lines including A549 (lung cancer) and HT-29 (colon cancer) by MTT assay. The results showed that substitution of imidazole ring with 1-amidinourea, semicarbazide, and thiobiuret led to improvement of cytotoxic activity against both cell lines.

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Introduction

Cancer is an important cause of death in developed countries, and the introduction of new anticancer agents is immediately required due to problems with existing drugs, such as toxicities and drug resistance.[1]

Research shows that the mutation of *ras* genes is responsible for 30 % of human cancers.[2] The activation of signal transduction pathways by Ras proteins has a crucial role for cellular growth. They undergo post-translational modifications by several sequential enzymatic steps. Farnesyltransferase is a zinc metalloenzyme, which adds a fanesyl group to the carboxyl terminal of a group of membrane-bound small G-proteins, such as Ras proteins. It has been shown that farnesyltransferase inhibitors (FTIs) can stop Ras protein farnesylation and suppress the growth of Ras-dependent tumour cells.[3,4] Therefore, the search for FTIs for the treatment of cancer has recently generated considerable interest. FTIs consist of three main classes; peptidomimetics, farnesyl pyrophosphate (FPP) analogues, and a class that has features of the other two classes. The potent peptidomimetic FTIs had a free thiol group. Free thiols are associated with several adverse drug effects, therefore the development of FTIs is clearly directed towards non-thiol FTIs. The most frequently used replacements for cysteine are nitrogen-containing heterocycles such as imidazole.[2,5–7] Several potent FTIs such as tipifarnib (Zarnestra®), L-778123, BMS-214662, and lonafarnib have been developed by researchers and were in advanced stages of human clinical trials for the treatment of hematological cancers and solid tumours (Fig. 1).[3,8–10]

Previous studies showed that although the substitution of an imidazole ring with other groups such as pyridine in FTIs can improve cytotoxic activity, farnesyltransferase inhibitor activity decreased. On the other hand, a survey of cancer cell lines has shown that cell lines without mutant Ras proteins were also sensitive to FTIs (more than 70 % of the cell lines).[3,4] These results show that other mechanisms exist in addition to inhibition of the farnesyltransferase (FTase) enzyme.[11–14]

In addition, guanidine, semicarbazide, urea, and thiourea derivatives showed potent anticancer activity with various mechanisms.[15–19]

In the present study, we chose L-778123 as a model and report the synthesis and evaluation of cytotoxic activity of 1-(3-chlorophenyl) piperazin-2-one derivatives. Several classes of bioisosteric replacement of imidazole such as 1-amidinourea, thiobiuret, and semicarbazide were selected, synthesised and evaluated against two human cancer cell lines including A549 (adenocarcinoma human alveolar basal epithelial) and HT-29 (human colonic adenocarcinoma) cell lines.

Results and Discussion

The intermediate 1-(3-chlorophenyl)piperazin-2-one hydrochloride (compound **2**) was prepared according to known methods described previously, as shown in Scheme 1.[20,21]

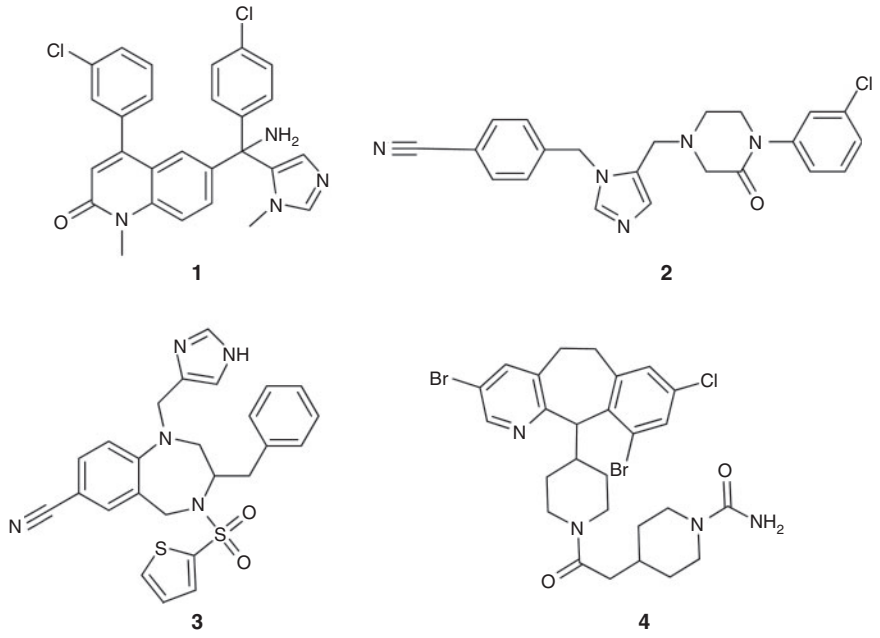
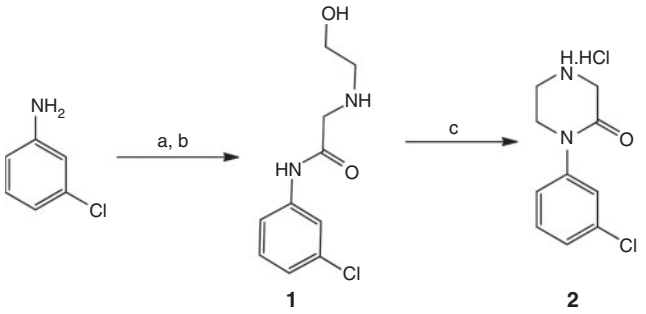


Fig. 1. Chemical structures of farnesyl transferase inhibitors: Tipifarnib (**1**), L-778123 (**2**), BMS-214662 (**3**) and Lonafarnib (**4**).



Scheme 1. (a) Chloroacetyl chloride, *i*PrAc, 0°C to rt, 1 h. (b) Ethanolamine, *i*PrAc, 55°C, 2 h. (c) Diisopropylazodicarboxylate (DIAD), tributylphosphine, EtOAc, −10°C to rt, 1 h.

The synthetic route to achieve the target compounds **8a**–**g** is shown in Scheme 2. The ethyl *N*-(4-chlorobenzyl)glycine ethyl ester **3** was synthesised in 50 % yield by the reaction of 4-chlorobenzylamine with ethyl chloroacetate. The amine group of intermediate **3** was protected using di-*tert*-butyl dicarbonate ($(Boc)_2O$) to yield the ester **4**.[22,23] The acid **5** was obtained by hydrolysis of ester **4** under basic conditions.[22] The intermediate **6** was prepared from reaction of intermediate **5** and 1-(3-chlorophenyl)piperazin-2-one in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) followed by acidic deprotection to afford intermediate **6**. Carbamoylimidazole **7** was prepared from intermediate **6** in the presence of carbonyldiimidazole (CDI). The carbamoylimidazole salt was then prepared using methyl iodide,[24–26] before addition of the appropriate amine in dichloromethane or acetonitrile to furnish compounds **8a**–**g**.[24]

The results of biological activity of the compounds are summarised as IC_{50} values (in μM) in Table 1. The IC_{50} values of the compounds against both cell lines showed that most compounds had significant cytotoxic activity at concentrations lower than 500 μM. The 1-amidinourea showed the highest cytotoxic activity against both cell lines at concentrations less than 10 μM, which is better than doxorubicin against the A549 cell line. The 2-thiobiuret and semicarbazide derivatives (compounds **8d** and **8f**) were also potent, with IC_{50} values in the range 7–30 μM. Cytotoxic activity of compound **7** didn't change significantly compared with L-778123. The compounds **8e** and **8a** indicated good cytotoxic activity but lower than L-778123 as the standard compound. The compound **8b** showed cytotoxic activity near 500 μM. The intermediate **6** and compound **8c** didn't reveal significant cytotoxic activity on either cell line (>1 mM).

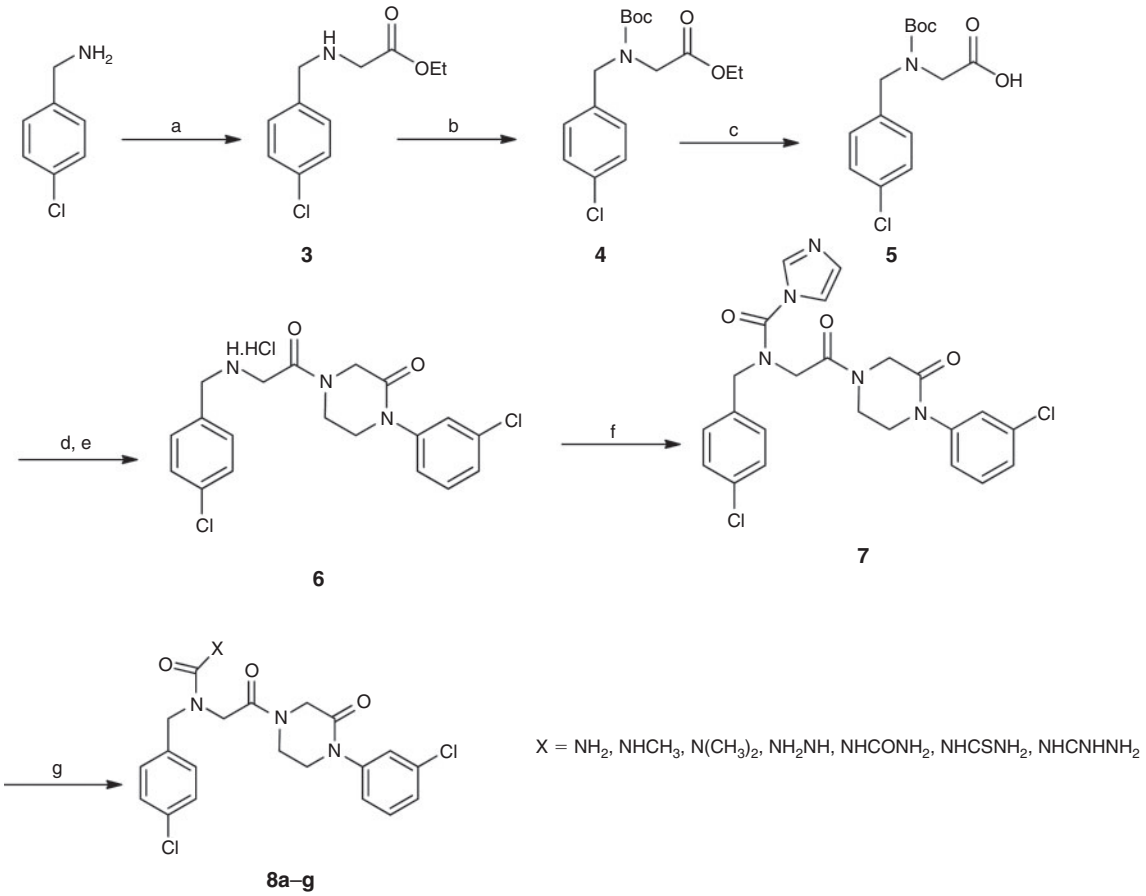
The anticancer mechanism of the 1-(3-chlorophenyl) piperazin-2-one derivatives are not clearly defined. Although it is proposed that they act through inhibition of FTase and consequently inhibit the Ras protein activation, most synthesised compounds which showed poor cytotoxic activity were potent FTIs and vice versa.[11–14,27]

There are various electron withdrawing groups such as chloro- or nitrile- groups at the para position of the benzyl moiety in the 1-(3-chlorophenyl)piperazin-2-one derivatives. There are many farnesyltransferase inhibitors such as tipifarnib which has a 4-Cl instead of a 4-CN group. These inhibitors showed higher potency than L-778123. We guessed that substitution of 4-Cl with 4-CN may improve potency. So we decided to use 4-chlorobenzyl instead of 4-cyanobenzyl in our derivatives.[3,4,28–31]

For cytotoxicity evaluation, lung and colon cancers were chosen because they are the main types of cancer which cause death worldwide, based on reports by the World Health Organization (WHO).[32,33] On the other hand, A549 (lung cancer) and HT-29 (colon cancer) are very common cell lines in cytotoxic activity evaluations. In addition, wild-type K-ras was found in these cell lines.[34,35]

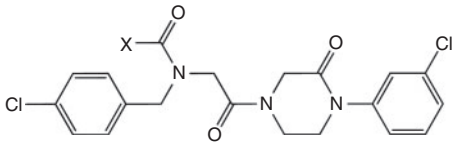
In this study, several novel 1-(3-chloropheny)piperazin-2-one derivatives bearing 1-amidinourea, thiobiuret, semicarbazide, and biuret were synthesised, characterised and evaluated against HT-29 and A549 cell lines to investigate structure-cytotoxic activity relationships.

It seems that terminal nitrogens of substituents have a key role in the cytotoxic activity. The cytotoxic activity of compound **7** showed that all changes (substitution of 4-chlorobenzyl instead of 4-cyanobenzyl) except bioisosteric replacement didn't change the



Scheme 2. (a) Ethyl chloroacetate, sodium carbonate, benzene, reflux overnight. (b) $(Boc)_2O$, CH_2Cl_2, rt. (c) NaOH, $MeOH/H_2O$, rt, overnight. (d) 1-(3-chlorophenyl)piperazin-2-one, DCC, CH_2Cl_2, 0°C to rt. (e) TFA, MeOH, rt, 1 h. (f) CDI, Et_3N, CH_2Cl_2, rt, 48 h. (g) MeI, MeCN, rt, 24 h; then appropriate amine, CH_2Cl_2 or MeCN, Et_3N, rt, 24 h.

Table 1. Cytotoxic activity (IC_{50}, μM) of intermediates 6 and 7 and compounds 8a–g against HT-29 (colon cancer) and A549 (lung cancer) cell lines



Compounds	X	A549	HT-29
6	–	>1000	>1000
7	Imidazole	105 ± 2	126 ± 3
8a	$-NH_2$	201 ± 2	198 ± 2
8b	$-NHCH_3$	307 ± 4	501 ± 3
8c	$-N(CH_3)_2$	>1000	>1000
8d	$-NHNH_2$	22 ± 5.22	26 ± 3
8e	$-NHCONH_2$	124 ± 1	171 ± 2
8f	$-NHCSNH_2$	7.3 ± 0.4	11 ± 0.6
8g	$-NHC(NH)NH_2$	3 ± 0.3	7.1 ± 0.2
L-778123	–	101 ± 2	125 ± 2
Doxorubicin	–	4.1 ± 0.1	3 ± 0.1

cytotoxic activity significantly. Replacement of imidazole with 1-amidinourea, 2-thiobiuret, and semicarbazide (compounds **8d**, **8f**, and **8g**) resulted in better cytotoxic activity against HT-29 and A549 cell lines in comparison with the standard compound. The increase in cytotoxic activity may be due to the higher electron density on terminal nitrogens.[36–38] Anticancer activity of potent compounds (**8f** and **8g**) may be attributed only to cytotoxic activity of bioisosteres of imidazole which have cytotoxic activity by various mechanisms such as inosine monophosphate dehydrogenase inhibition by guanidine-based compounds[15–19,27] Other substitutions didn't significantly improve the cytotoxic activity in comparison with the mentioned substituents, perhaps due to lower electron density of the amide groups. Compound **8a** indicated better cytotoxic activity than compounds **8b** and **8c**, which may be a result of steric effects.

In future research, more studies should be done to determine the mechanisms of the action of most potent compounds.

Conclusion

In conclusion, a series of substituted 1-(3-chlorophenyl)piperazin-2-one derivatives bearing bioisosteres of imidazole were synthesised, characterised by IR, ^{1}HNMR, ^{13}CNMR, and liquid chromatography mass spectrometry (LC-MS), and evaluated as cytotoxic agents. The 3-chlorophenylpiperazinone derivatives with 1-amidinourea (**8g**) showed higher potency with IC_{50} equal to 3 μM and 7 μM against A549 and HT-29 cell lines respectively, which is better than doxorubicin against A549. Thus, compound **8g** is a good lead compound for designing new anticancer agents. The thiobiuret and semicarbazide groups can be potent bioisosteres instead of the imidazole ring. Methyl substitution on amine groups decreased cytotoxic activity and the intermediate **6** (without an imidazole group) didn't show

significant cytotoxic activity. It seems that electron density and steric effects of terminal nitrogens of substituents are critical factors in cytotoxic activity.

Experimental

Chemistry

All reagents and solvents were purchased from Merck and Sigma Aldrich. L-778123 was prepared according to known methods.[20,21] Melting points were measured with an Electrothermal-9100 melting point apparatus and are uncorrected. The IR spectra were recorded on a Shimadzu 4300 spectrophotometer (potassium bromide disks). ^{1}HNMR and ^{13}CNMR spectra were recorded on Varian unity 500, 400, and 300 spectrometers and chemical shifts (δ) are reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. The mass spectra were run on an Agilent 6410 LC-MS at 70 eV. Merck silica gel 60 F254 plates were used for analytical TLC. Column chromatography was performed on silica gel 60 (Merck, particle size 0.06–0.20 mm).

N-*(4-Chlorobenzyl) Glycine Ethyl Ester Hydrochloride (**3**)*

A mixture of 4-chlorobenzylamine (10 mL, 82 mmol), ethyl chloroacetate (9.21 mL, 90 mmol), and anhydrous sodium carbonate (13 g, 123 mmol) in 50 mL of benzene was refluxed overnight. After cooling, the salts were removed by filtration. The solvent was evaporated under reduced pressure and diethyl ether was added to the residue and stirred for 1 h at room temperature. The mixture was filtered and hydrochloric acid (4.0 N in isopropanol) was added to the filtrate. The white precipitate was filtered, washed three times with diethyl ether, and then dried.

The title compound (10.35 g, 55 %) was obtained as a white solid. Mp 140–142°C; ν_{max} (KBr)/cm^{-1}: 3325, 1700; δ_H (500 MHz, DMSO-d$_6$)7.49 (t, *J* 1.5, 2H, phenyl), 7.47 (t, *J* 1.5, 2H, phenyl), 4.18 (s, 2H, $NH_2^+CH_2$phenyl), 4.11 (s, 2H, $COCH_2NH_2^+$), 4.07 (q, *J* 7.1, 2H, OCH_2), 1.16 (t, *J* 7.1, 3H, CH_3); δ_C (125 MHz, DMSO-d$_6$)169.06, 136.18, 130.25, 128.76, 126.21, 61.34, 52.73, 50.37, 13.41; *m/z* (ESI) 228.59 [M+H]; Anal. Calcd. for $C_{11}H_{15}Cl_2NO_2$: C 50.02, H 5.72, N 5.30; found: C 50.18, H 5.52, N 5.41 %.

N-*(*tert*-Butoxycarbonyl)*-N-*(4-chlorobenzyl) Glycine Ethyl Ester (**4**)*

A solution of **3** as free base (5 g, 20 mmol) and $(Boc)_2O$ (4.36 g, 20 mmol) in 40 mL of CH_2Cl_2 was stirred for 4 h at room temperature. Then 100 mL of ethyl acetate was added to the reaction mixture and the solution was transferred to a separating funnel. The resulting solution was washed sequentially with 50 mL of 1 N HCl, 50 mL saturated aqueous sodium bicarbonate, and 30 mL brine, and then dried with anhydrous sodium sulfate, filtered, and the solvent was removed under reduced pressure to give **4**.

The title compound (5.75 g, 80 %) was obtained as a colourless oil. ν_{max} (KBr)/cm^{-1}: 1700, 1660; δ_H (500 MHz, DMSO-d$_6$) 7.49 (d, *J* 8.5, 2H, phenyl), 7.34 (d, *J* 8.5, 2H, phenyl), 4.12 (s, 2H, NCH_2phenyl), 3.80 (s, 2H, $COCH_2N$), 3.73 (br d, *J* 4.5 Hz, 2H, OCH_2), 1.61 (t, *J* 5.1, 3H, -CH_2CH_3), 1.47 (s, 9H, CH_3); δ_C (125 MHz, DMSO-d$_6$) 167.15, 157.21, 134.20, 130.55, 128.70, 127.91, 77.87, 63.13, 51.94, 45.51, 28.15, 14.71; *m/z* (ESI) 329.61 [M+H]; Anal. Calcd. for $C_{16}H_{22}ClNO_4$: C 58.62, H 6.76, N 4.27; found: C 58.80, H 6.62, N 4.12 %.

N-*(4-Chlorobenzyl)*-N-*(*tert*-butoxycarbonyl) Glycine (**5**)*

A solution of **4** (5 g, 15.23 mmol) and NaOH (1.8 g, 45 mmol) in MeOH/H_2O (50 mL, 50 : 50) was stirred at room temperature overnight. The MeOH was removed and the residual aqueous suspension was diluted with H_2O (200 mL) and washed with Et_2O (2 × 100 mL). To the residual solution (aqueous layer) was added 1 N HCl (pH <3) and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give a colourless oil.

The title compound (4.57 g, 76 %) was obtained as a colourless oil. ν_{max} (KBr)/cm^{-1}: 3200, 1760, 1650; δ_H (500 MHz, DMSO-d$_6$) 7.38 (d, *J* 6.4, 2H, phenyl), 7.29 (d, *J* 6.4, 2H, phenyl), 4.39 (d, *J* 6.15, 2H, NCH_2phenyl), 3.83 (s, 2H, $COCH_2N$), 1.36 (s, 9H, CH_3); δ_C (125 MHz, DMSO-d$_6$) 172.89, 154.45, 134.35, 130.51, 128.83, 128.34, 78.12, 50.58, 45.34, 27.76; *m/z* (ESI) 300.66 [M+H]; Anal. Calcd. for $C_{14}H_{18}ClNO_4$: C 56.10, H 6.05, N 4.67; found: C 56.36, H 6.22, N 4.33 %.

*4-[*N*-(4-Chlorobenzyl)glycyl]-1-(3-chlorophenyl)piperazin-2-one (**6**)*

A solution of **5** (5 g, 16.72 mmol) in 60 mL of CH_2Cl_2 was added to a solution of DCC (4.6 g, 22.29 mmol) in 60 mL of CH_2Cl_2. The mixture was stirred at room temperature for 10 min and then a solution of 1-(3-chlorophenyl)piperazin-2-one (2.35 g, 11.14 mmol) in 30 mL of CH_2Cl_2 was added. The mixture was stirred at room temperature overnight. The *N*,*N*′-dicyclohexylurea (DCU) was filtered off and the filter cake was washed with CH_2Cl_2 (2 × 20 mL). The organic filtrate was washed with 15 % aqueous citric acid solution (3 × 20 mL). The aqueous layer was basified with 20 mL concentrated sodium bicarbonate, and then extracted with CH_2Cl_2 three times. The combined organic layers were washed with water (20 mL) and brine (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to obtain the desired amides. To a stirred solution of the resulting product (4.92 g, 10 mmol) in 15 mL MeOH was added trifluoroacetic acid (TFA, 15 mL, 0.19 mol) and the resulting mixture was stirred at room temperature for 1 h. Excess reagent and solvent were evaporated under reduced pressure. To the resulting oil was added 50 mL EtOAc, followed by stirring for 30 min. The white precipitate was filtered, washed with EtOAc, and dried to give **6**.

The title compound (5.43 g, 50 %), was obtained as a white solid. Mp 220–222°C; ν_{max} (KBr)/cm^{-1}: 3325, 1675, 1580; δ_H (400 MHz, DMSO-d$_6$) 7.60–7.32 (m, 8H, Phenyl), 4.22 (s, 2H, C-3 of piperazinone), 4.14 (s, 2H, $COCH_2NH$), 4.12 (s, 2H, $NHCH_2$phenyl), 3.73 (t, *J* 3.8, 2H, C-5 of piperazinone), 3.47 (dt, *J* 3.8, 2H, C-6 of piperazinone); δ_C (100 MHz, DMSO-d$_6$) 165.35, 165.18, 142.07, 135.63, 135.13, 130.41, 129.19, 129.01, 127.44, 125.67, 118.17, 117.47, 49.17, 45.94, 45.12, 44.70, 42.30; *m/z* (ESI) 393.15 [M+H]; Anal. Calcd. for $C_{19}H_{20}Cl_3N_3O_2$: C 53.23, H 4.70, N 9.80; found: C 53.38, H 4.92, N 9.61 %.

*4-[*N*-(4-Chlorobenzyl)*-N*-(1*H*-imidazol-1-ylcarbonyl) glycyl]-1-(3-chlorophenyl)piperazin-2-one (**7**)*

Intermediate **6** (4.28 g, 10 mmol) and triethylamine (1.4 mL, 10 mmol) was added to a solution of CDI (1.78 g, 11 mmol) in 30 mL CH_2Cl_2. The mixture was stirred for 48 h at room temperature and then washed with water (2 × 30 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under vacuum to yield white solid.

The title compound (2.42 g, 50 %) was obtained as a white solid. Mp 220–222°C; ν_{max} (KBr)/cm^{-1}: 1690, 1580; δ_H (300 MHz, $CDCl_3$) 7.96 (s, 1H, imidazole), 7.77 (s, 1H, imidazole), 7.38–7.13 (m, 8H, phenyl), 7.09 (s, 1H, imidazole), 4.46 (s, 2H, NCH_2phenyl), 4.42 (s, 2H, $COCH_2N$), 4.22 (s, 2H, C-3 of

piperazinone), 3.98 (t, *J* 5.1, 2H, C-6 of piperazinone), 3.86 (t, *J* 5.4, 2H, C-5 of piperazinone); δ_C (75 MHz, $CDCl_3$) 165.05, 164.55, 157.12, 150.42, 141.98, 137, 134.89, 134.38, 130.41, 130.34, 127.8, 125.9, 123.63, 120.89, 117.72, 50.44, 49.03, 48.67, 43.44, 35.86; *m/z* (ESI) 487.35 [M+H]; Anal. Calcd. for $C_{23}H_{21}Cl_2N_5O_3$: C 56.80, H 4.35, N 14.40; found: C 56.48, H 4.62, N 14.13 %.

*General Procedure for the Synthesis of Compounds **8a–g***

Methyl iodide (1.54 mL, 24.7 mmol) was added to a solution of intermediate **7** (3 g, 6.17 mmol) in 30 mL acetonitrile and stirred at 25°C overnight. The solvent was removed under reduced pressure to give the carbamoylimidazolium salt.

To a solution of the above carbamoylimidazolium salt (0.5 g, 0.8 mmol) in 10 mL CH_2Cl_2 or 10 mL acetonitrile was added to the appropriate amine (8 mmol) and triethylamine (0.11 mL, 0.8 mmol). The mixture was stirred at room temperature for 24 h and then washed with water (2 × 5 mL) and brine (5 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, concentrated under reduced pressure, and purified by column chromatography on silica gel eluting with *n*-hexane/ethyl acetate to yield compounds **8a**–**g**.

*1-(4-Chlorobenzyl)-1-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl] Urea (**8a**)*

The title compound (0.26 g, 58 %) was obtained as a white solid. Mp 215–217°C; ν_{max} (KBr)/cm^{-1}: 3300, 3250, 1740, 1680; δ_H (500 MHz, DMSO-d_6) 7.40–7.22 (m, 8H, phenyl), 4.41 (s, 2H, NCH_2phenyl), 4.27 (s, 2H, COCH_2N), 4.22 (s, 2H, C-3 of piperazinone), 4.09 (t, *J* 6.7, 2H, C-6 of piperazinone), 4.05 (t, *J* 6.8, 2H, C-5 of piperazinone); δ_C (125 MHz, DMSO-d_6) 170.55, 169.5, 154.83, 146.20, 137.67, 137.09, 131.67, 130.73, 130.41, 129.52, 128.17, 118.55, 117.50, 53.85, 50.86, 50.26, 48.97, 48.78; *m/z* (ESI) 436.49 [M+H]; Anal. Calcd. for $C_{20}H_{20}Cl_2N_4O_3$: C 55.18, H 4.63, N 12.87; found: C 55.42, H 4.82, N 12.58 %.

*1-(4-Chlorobenzyl)-1-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl]-3-methyl Urea (**8b**)*

The title compound (0.3 g) was obtained in 65 % yield. Mp 185–187°C; ν_{max} (KBr)/cm^{-1}: 3350, 1700, 1660; δ_H (400 MHz, $CDCl_3$) 7.39–7.09 (m, 8H, phenyl), 4.44 (s, 2H, NCH_2phenyl), 4.28 (s, 2H, COCH_2N), 4.16 (s, 2H, C-3 of piperazinone), 4.01 (t, *J* 4.9, 2H, C-6 of piperazinone), 3.88 (t, *J* 5.6, 2H, C-5 of piperazinone), 2.75 (s, 3H, CH_3); δ_C (100 MHz, $CDCl_3$) 165.41, 165, 154.52, 143.29, 138.41, 137.52, 132.83, 130.37, 126.37, 125.85, 124.28, 118.4, 117.50, 52.65, 48.55, 47.60, 43.89, 41.10, 40.73; *m/z* (ESI) 450.29 [M+H]; Anal. Calcd. for $C_{21}H_{22}Cl_2N_4O_3$: C 56.13, H 4.94, N 12.47; found: C 56.42, H 4.71, N 12.81 %.

*1-(4-Chlorobenzyl)-1-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl]-3,3-dimethyl Urea (**8c**)*

The title compound (0.28 g) was obtained in 60 % yield. Mp 218–220°C; ν_{max} (KBr)/cm^{-1}: 1660, 1640; δ_H (300 MHz, $CDCl_3$) 7.37–7.19 (m, 8H, phenyl), 4.50 (s, 2H, NCH_2phenyl), 4.38 (s, 2H, COCH_2N), 4.09 (s, 2H, C-3 of piperazinone), 3.82 (t, *J* 12.6, 2H, C-6 of piperazinone), 3.62 (t, *J* 11.7, 2H, C-5 of piperazinone), 2.89 (s, 6H, CH_3, dimethyl); δ_C (75 MHz, $CDCl_3$) 165.71, 163.38, 157.22, 143.77, 136.72, 130.20, 128.35, 128.05, 122.81, 122.11, 121.91, 118.91, 117.01, 51.93, 46.9, 43.28, 42.98, 39.99, 34.29; *m/z* (ESI) 464.39 [M+H]; Anal. Calcd. for $C_{22}H_{24}Cl_2N_4O_3$: C57.03, H 5.22, N 12.09; Found: C 57.41, H 5.46, N 12.30 %.

*4-(4-Chlorobenzyl)-4-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl] Semicarbazide (**8d**)*

The title compound (0.2 g) was obtained in 45 % yield. Mp 201–203°C; ν_{max} (KBr)/cm^{-1}: 3310, 3250, 1650, 1625, 1560; δ_H (400 MHz, $CDCl_3$) 7.37–7.18 (m, 8H, phenyl), 4.17 (s, 2H, NCH_2phenyl), 4.08 (s, 2H, COCH_2N), 4.06 (s, 2H, C-3 of piperazinone), 3.83 (t, *J* 5.6, 2H, C-6 of piperazinone), 3.77 (t, *J* 5.4, 2H, C-5 of piperazinone); δ_C (100 MHz, $CDCl_3$) 164.16, 163.97, 153.29, 145.49, 133.82, 131.71, 130.29, 128.55, 128.02, 126.07, 123.2, 120.76, 118.83, 53.17, 48.01, 45.17, 43.90, 42.01; *m/z* (ESI): 451.29 [M+H]; Anal. Calcd. for $C_{20}H_{21}Cl_2N_5O_3$: C53.34, H 4.70, N 15.55; found: C 53.52, H 4.91, N 15.28 %.

*1-(4-Chlorobenzyl)-1-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl]-biuret (**8e**)*

The title compound (0.31 g) was obtained in 64 % yield. Mp 246–248°C; ν_{max} (KBr)/cm^{-1}: 3300, 3200, 1740, 1680; δ_H (300 MHz, $CDCl_3$) 7.32–7.19 (m, 8H, phenyl), 4.25 (s, 2H, NCH_2phenyl), 4.16 (s, 2H, COCH_2N), 4.06 (s, 2H, C-3 of piperazinone), 3.79 (br d, *J* 19.8, 2H, C-6 of piperazinone), 3.71 (br d, *J* 19, 2H, C-5 of piperazinone); δ_C (75 MHz, $CDCl_3$) 164.86, 161.47, 152.72, 150, 142.49, 134.82, 130.29, 127.55, 126.02, 123.76, 123.63, 120.89, 118.53, 117.72, 53.17, 51.96, 47.07, 42.93, 37.07; *m/z* (ESI) 479.29 [M+H]; Anal. Calcd. for $C_{21}H_{21}Cl_2N_5O_4$: C 52.73, H 4.43, N 14.64; found: C 52.41, H 4.71, N 14.38 %.

*5-(4-Chlorobenzyl)-5-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl]-2-thiobiuret (**8f**)*

The title compound (0.32 g) was obtained in 63 % yield. Mp. 265–267°C; ν_{max} (KBr)/cm^{-1}: 3425, 3325, 1740, 1640; δ_H (300 MHz, $CDCl_3$) 7.42–7.08 (m, 8H, phenyl), 4.49 (s, 2H, NCH_2phenyl), 4.26 (s, 2H, COCH_2N), 4.17 (s, 2H, C-3 of piperazinone), 3.91 (t, *J* 19.8, 2H, C-6 of piperazinone), 3.71 (t, *J* 19, 2H, C-5 of piperazinone); δ_C (75 MHz, $CDCl_3$) 187.91, 165.8, 164.47, 150, 142.49, 138.48, 135.84, 134.88, 133.84, 131.26, 130.02, 128.76, 118.8, 117.72, 50.47, 49.05, 48.08, 47.30, 36.09; *m/z* (ESI) 495.39 [M+H]; Anal. Calcd. for $C_{21}H_{21}Cl_2N_5O_3S$: C 51.02, H 4.28, N 14.17; found C 51.42, H 4.52, N 14.51 %.

*1-Amidino-3-(4-chlorobenzyl)-3-[2-[1-(3-chlorophenyl)-2-oxopiperazin-4-yl]-2-oxoethyl] Urea (**8g**)*

The title compound (0.15 g) was obtained in 30 % yield. Mp 288–290°C; ν_{max} (KBr)/cm^{-1}: 3400, 3320, 1700, 1650; δ_H (400 MHz, $CDCl_3$) 7.31–7.13 (m, 8H, phenyl), 4.10 (s, 2H, NCH_2phenyl), 4.04 (s, 2H, COCH_2N), 4.02 (s, 2H, C-3 of piperazinone), 3.74 (t, *J* 5.6, 2H, C-6 of piperazinone), 3.65 (t, *J* 5.4, 2H, C-5 of piperazinone); δ_C (100 MHz, $CDCl_3$) 166.67, 164.31, 159.14, 154.88, 139.66, 137.64, 136.51, 132.79, 130.23, 128.87, 128.70, 120.52, 118.50, 117.98, 52.80, 48.35, 47.54, 43.44, 41.28; *m/z* (ESI): 478.29 [M+H]; Anal. Calcd. for $C_{21}H_{22}Cl_2N_6O_3$: C52.84, H 4.65, N 14.85; found: C 53.12, H 4.93, N 14.52 %.

Growth Inhibition Assay

Cytotoxicity of compounds **6**, **7**, **8a**–**g**, L-778123, and doxorubicin was examined by MTT assay at 1–1000 μM concentrations against two human cancer cell lines A549 (adenocarcinoma human alveolar basal epithelial cells) and HT-29 (human colonic adenocarcinoma cells). Cell suspensions were seeded in

96-well plates with concentrations of 8000–10000 A549 cells and 15000–20000 HT-29 cells per well and incubated for 24 h to allow cell attachment. The cells were treated for 72 h with various concentrations of **6**, **7**, **8a**–**g**, L-778123, and doxorubicin. Culture medium was replaced with 150 µL fresh media plus 50 µL MTT (3-(4, 5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide) reagent (2 mg mL^{-1} in PBS). After an additional 4 h of incubation at 37°C the medium was discarded. 200 µL Dimethyl sulfoxide plus 25 µL Sorenson buffer (0.1 M NaCl, 0.1 M glycine, pH: 10.5) was added to each well and the solution was shaken for 15 min at 37°C to dissolve the purple tetrazolium crystals. The absorbance of each well was measured by the plate reader (SUNRISE TECAN, Austria) at a wavelength of 570 nm. The amount of produced purple formazan is proportional to the number of viable cells. Experiments were performed two times in triplicate for determination of sensitivity to each compound. The IC_{50} was calculated by linear regression analysis, expressed in mean ± s.d.[1]

Supplementary Material

Mass spectra for compounds **8a**–**g** are available on the Journal's website.

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