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In vitro antitumor activity evaluation of some 1,2,4-triazine derivatives bearing piperazine amide moiety against breast cancer cells

Leyla Yurttaş [a,*], Şeref Demirayak [b], Sinem Ilgın [c], Özlem Atlı [c]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskisehir, Turkey
[b] Department of Pharmaceutical Chemistry, School of Pharmacy, Medipol University, 34083 Istanbul, Turkey
[c] Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Anadolu University, 26470 Eskisehir, Turkey

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ABSTRACT

A series of 1,2,4-triazine derivatives bearing piperazine amide moiety has been synthesized and investigated for their potential anticancer activities. 1-[4-(5,6-Bis(4-subtituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-substituted phenyl)piperazin-1-yl]ethanone derivative (**1**–**32**) compounds were synthesized by a four step synthetic procedure. The activity studies were evaluated using XTT method, BrdU method and flow cytometric analysis on MCF-7 breast cancer cells and NIH/3T3 (mouse embryonic fibroblast cells) healthy cells. Compounds **5** with 3-chlorophenyl and compound **7** with 4-chlorophenyl substitutions were found to be promising antiproliferative agents comparing with an effective anticancer drug, cisplatin.

1. Introduction

Breast cancer is one of the most common cancer in females all over the world. According to the latest data observed in United Kingdom, it was shown that breast cancer is the most common cancer in women (31%).[1] Additionally, according to the data obtained from United States in 2012, it was suggested that breast cancer occurred in 29% of newly diagnosed cancers in women.[2] Treatments against breast cancer are surgery, drugs (hormonal therapy and chemotherapy), radiation and/or immunotherapy. However, an effective drug for therapy and prognosis after surgery still do not exist.[3] Therefore an effective new drug development process is seemed to be necessary.

One important approach to antineoplastic agents is the design of an antimetabolite drug whose structure is related those of pyrimidines and purines involved in the biosynthesis of DNA. Antimetabolites often similar in structure to the metabolite that they interfere with DNA synthesis and consequently block crucial metabolic pathways essential for cell growth.[4–6] Various nucleosides containing interchanged nitrogen and carbon atoms in their base moieties have shown considerable activity as antimetabolic agents. 6-Aza analogues of the naturally occurring nucleic acids components represent isosteres with the C-6 carbon atom of the pyrimidine nucleoside base being replaced by nitrogen.[7,8] Certain azanucleosides, 6-azauracil and 6-azacytosine), structurally based on 1,2,4-triazine nucleus have displayed an impressive array of biological activities, among which antitumor,[9–19] antiviral,[20] antimicrobial,[21] antiinflammatory,[22] antiplatelet,[23] antimalarial,[24] and antifungal[25] properties. Additionally, tertiary amines in the linkers reported to be positively charged at physiological pH in order to favor electrostatic interactions in the minor groove with the anionic phosphate DNA backbone.[26]

On the other hand, a comprehensive literature study shows that two carbon linker piperazines have potential antiproliferative effect.[27,28] Additionally, structure–activity relationships (SARs) of a farnesyltransferase inhibitor anticancer drug lonafarnib (SCH 66336) have been explored and several studies have established that piperazine is a suitable replacement for the piperidine core, producing compounds with comparable potency and pharmacokinetic (PK) profiles. In fact, compounds including α-alanine piperazine amide structure improves therapeutic level of these compounds.[29]

Based on the literature above, we designed a novel series of polyamines existing a 'Y shape' pharmacophore model that contains three basic center, three hydrophobic domains, and an amide linker and synthesized thirty two 1-[4-(5,6-bis(4-subtituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-substituted phenyl)piperazin-1-yl]ethanone derivatives by adopting the bioisosteric-replacement

* Corresponding author. Tel.: +90 222 3350580/3783; fax: +90 222 3350750.
E-mail address: lyurttas@anadolu.edu.tr (L. Yurttaş).

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principle. Cytotoxic activities of the titled compounds were evaluated against human breast adenocarcinoma cell line, MCF-7 and mouse embryonic fibroblast cell line, NIH3T3. To determine potential anticancer activity, DNA synthesis inhibition abilities and apoptotic properties of the indicated compounds were also specified and reported within the study.

2. Results and discussion

2.1. Chemistry

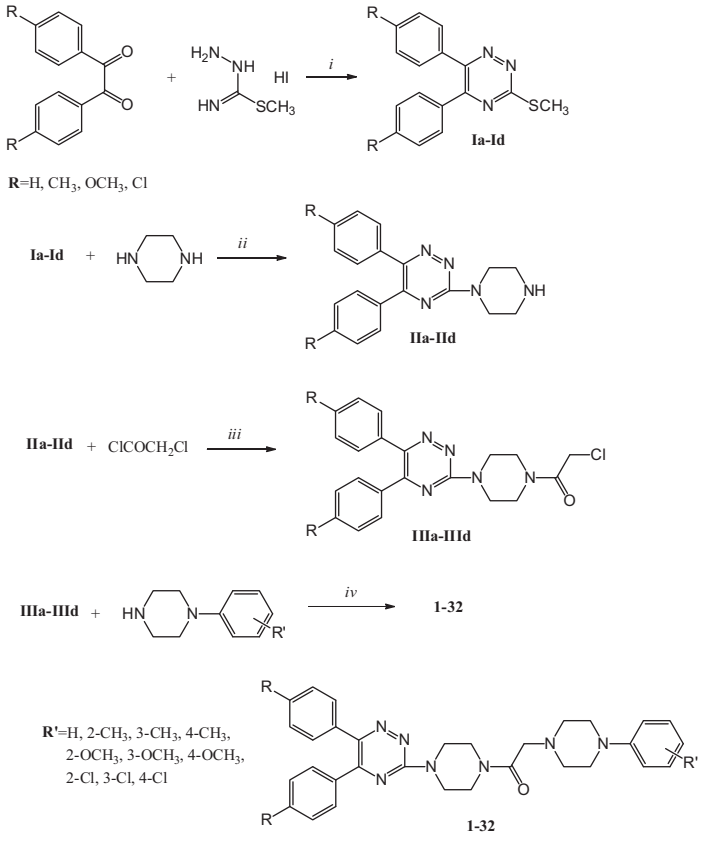
Compounds **Ia–Id** were synthesized by the condensation reaction of benzil derivatives and *S*-methylthiosemicarbazide hydrogen iodide with sodium carbonate in methanol to give 5,6-bis(4-substituted phenyl)-3-methylthio-1,2,4-triazine (Scheme 1). The obtained 3-methylthio derivatives of 1,2,4-triazines were reacted with twofold excess piperazine in pyridine to give 5,6-bis(4-substituted phenyl)-3-(piperazin-1-yl)-l,2,4-triazine (**IIa–IId**) derivatives. The amino group of the piperazine ring was acetylated with chloroacetyl chloride in dimethylformamide and basic medium was provided by triethylamine to gain intermediate products **IIIa–IIId**. Lastly, final compounds **1–32** were obtained by the reaction of 2-chloro-1-[4-(5,6-bis(4-substituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]ethanone derivatives (**IIIa–IIId**) and 1-substituted piperazine derivatives in triethylamine and dimethylformamide by refluxing and yielding 66–83%. All final compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR elemental analyses and mass spectra. In IR spectrum of the compounds, bands which were seen in between 1645–1669 cm^{-1} and 1296–1612 cm^{-1} were confirmed the presence of amide C=O and also C=C, C=N bonds, respectively. In ^{1}H NMR of the intermediate compounds **IIIa–IIId**, signals belonging to $COCH_2$ protons vicinal to chlorine atom were observed at about 3.49–3.50 ppm. In the spectra of the final compounds (**1–32**), same protons were seen much further to low-field and at about 3.28–3.42 ppm. Piperazine CH_2 protons were assigned in between 2.59–4.01 ppm as broad singlets and two or four overlapping protons. Aromatic protons were observed at the range of 6.34–8.08 ppm, changing to substituents on phenyl rings. In the ^{13}C NMR spectra of the final compounds, signals belonging to aliphatic carbon atoms were assigned at about 20.70–60.76 ppm; and signals for aromatic carbon atoms were observed at 101.36–162.27 ppm. The carbonyl carbon of amide function was seen at about 167 ppm. In mass spectra of the compounds, $[M+1]^+$ peaks were observed for all final compounds and also elemental analyses results for C, H, and N elements were satisfactory within calculated values of the compounds.

2.2. Biological evaluation

2.2.1. Cytotoxicity test

According to results, the compounds **2**, **3**, **5**, **7**, **10**, **13**, **14**, **15**, **23**, **26**, **27** and **28** showed antiproliferative activities against MCF-7 cell line. Compounds **2**, **5**, **7**, **10**, **14** and **28** were found to have the highest cytotoxic activity against MCF-7 cell line. The IC_{50} values of the compounds are represented in Table 1.

Furthermore, the cytotoxicity of the compounds **2**, **5**, **7**, **10**, **14** and **28**, which were determined to have the highest selective cytotoxic activity against MCF-7 cell line, were measured against NIH/3T3 cell line. According to the results of the XTT test against NIH/3T3 cell line, the compounds **2**, **5**, **7**, **10**, **14** and **28** showed cytotoxicity against NIH/3T3 cell line in higher doses than their antiproliferative doses against MCF-7 cell line. The IC_{50} values of the compounds against NIH/3T3 cell line are represented in Table 2.



Scheme 1. The synthesis of the compounds. Reactants and conditions: (i) $NaHCO_3$, CH_3OH, reflux 3 h; (ii) pyridine, reflux 6 h; (iii) Et_3N, DMF, rt, 45 min; (iv) Et_3N, DMF, rt, 5 h.

Table 1
Cytotoxic activity of the compounds against MCF-7 cell line (μg/mL and μM)

Compound	IC_{50} (μg/mL)	IC_{50} (μM)	Compound	IC_{50} (μg/mL)	IC_{50} (μM)
1	>500	>963.4	**17**	>500	>863.6
2	91.7 ± 3.4	167.0 ± 6.2	**18**	>500	>821.0
3	145.9 ± 2.7	263.3 ± 4.9	**19**	>500	>814.3
4	>500	>910.7	**20**	>500	>821.0
5	31.2 ± 4.5	56.3 ± 8.1	**21**	>500	>814.3
6	>500	>910.7	**22**	>500	>821.0
7	31.2 ± 3.8	56.3 ± 6.9	**23**	372.7 ± 6.8	607.0 ± 11.0
8	>500	>886.5	**24**	>500	>801.2
9	500 ± 6.5	914 ± 11.9	**25**	>500	>850.3
10	34.3 ± 2.2	59.4 ± 4.4	**26**	203.7 ± 3.9	329.6 ± 6.3
11	>500	859.1	**27**	246.4 ± 3.7	395.8 ± 10.1
12	>500	866.6	**28**	91.9 ± 4.2	148.7 ± 6.8
13	463.8 ± 3.5	796.9 ± 6.0	**29**	>500	>803.2
14	30.6 ± 4.0	53.0 ± 6.9	**30**	>500	>809.0
15	384.2 ± 4.1	659.8 ± 7.0	**31**	>500	>803.2
16	>500	844.6	**32**	>500	>789.9
Cisplatin	29.3 ± 2.5	97.7 ± 8.3			

The effects of compounds **2**, **5**, **7**, **10**, **14** and **28** were evaluated on DNA synthesis and apoptosis because they were determined to be the most active derivatives against MCF cell line.

2.2.2. DNA synthesis inhibition assay

Figure 1 shows the DNA synthesis inhibitory activity % of the compounds **2**, **5**, **7**, **10**, **14**, **28** and cisplatin against MCF-7 cells for 24 h. According to the assay, compounds **2** and **14** were determined as the most active compounds. Compound **14** was found to have 56.94, 77.82 and 88.52% DNA synthesis inhibition, respectively compound **2** was found to have 22.44, 76.35 and 81.61% DNA synthesis inhibition at $IC_{50}/2$, IC_{50} and $2 \times IC_{50}$ values after 24 h of incubation, respectively whereas cisplatin was found to have 59.69, 65.96 and 76.53% inhibition at $IC_{50}/2$, IC_{50} and $2 \times IC_{50}$ values, respectively. According to these results, it was observed that compounds **2** and **14** showed more antiproliferative activity against MCF-7 cells than cisplatin at IC_{50} and $2 \times IC_{50}$ values. Compound **5** was found to have 26.06, 31.36 and 41.48% DNA synthesis inhibition at $IC_{50}/2$, IC_{50} and $2 \times IC_{50}$ values, respectively. Additionally, it was determined that compounds **7** and **28** showed less antiproliferative activity than other compounds against the MCF-7 cell line.

The DNA synthesis inhibitory activity % of the compounds **2**, **5**, **7**, **10**, **14**, **28** and cisplatin against MCF-7 cells is shown in Figure 2 for 48 h. DNA inhibition % was increased with 48 h incubation period for all compounds. These increases are obviously significant for the compounds **5**, **7** and cisplatin. It was observed that compounds **2** and **14** showed the highest DNA synthesis inhibitory activities on

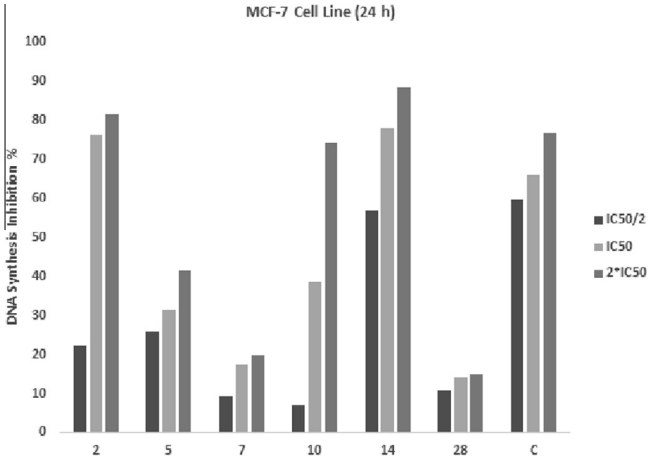


Figure 1. DNA synthesis inhibitory activity % of the compounds **2**, **5**, **7**, **10**, **14**, **28** and cisplatin against MCF-7 cells for 24 h incubation period. Mean percent absorbance of untreated control cells were assumed to be 0% and three different concentrations ($IC_{50}/2$, IC_{50}, $2 \times IC_{50}$) of test compounds and cisplatin were given. Data points represent means for two independent experiments ± SD of four independent wells. p <0.05.

Table 2
Cytotoxic activity of the compounds against NIH/3T3 cell line (μg/mL and μM)

Compound	IC_{50} (μg/mL)	IC_{50} (μM)	Compound	IC_{50} (μg/mL)	IC_{50} (μM)
1	>500	>963.4	**17**	>500	>863.5
2	243.5 ± 5.6	443.5 ± 10.2	**18**	>500	>821.0
3	193.7 ± 2.8	349.6 ± 5.1	**19**	>500	>814.3
4	198.3 ± 4.8	361.2 ± 8.7	**20**	>500	>821.0
5	98.5 ± 4.7	177.8 ± 15.7	**21**	>500	>814.3
6	>500	>910.7	**22**	>500	>821.0
7	62.5 ± 4.9	112.8 ± 8.8	**23**	>500	>814.3
8	>500	>886.5	**24**	>500	>801.3
9	>500	>914.1	**25**	>500	>850.3
10	76.9 ± 6.5	133.3 ± 11.3	**26**	250 ± 4.2	404.5 ± 8.4
11	>500	>859.1	**27**	>500	>803.2
12	>500	>866.5	**28**	125.0 ± 4.8	202.3 ± 7.8
13	500 ± 6.4	859.1 ± 11.0	**29**	>500	>803.2
14	66.8 ± 3.3	115.8 ± 5.7	**30**	>500	>809.1
15	330.1 ± 5.5	567.2 ± 9.5	**31**	>500	>803.2
16	>500	>844.6	**32**	>500	>789.9
Cisplatin	388.2 ± 4.7	1294.0 ± 15.7			

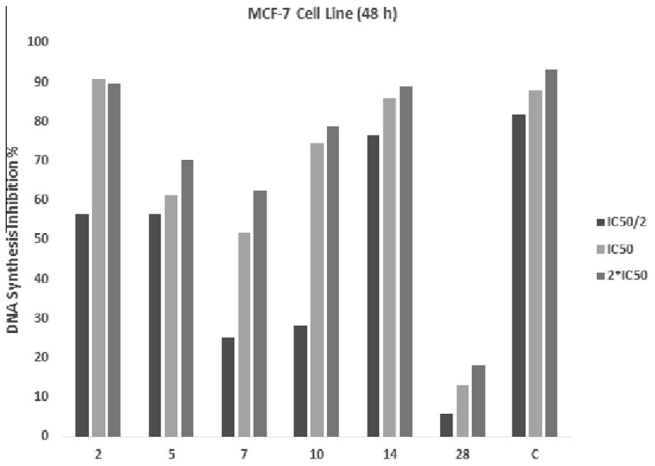


Figure 2. DNA synthesis inhibitory activity % of the compounds **2**, **5**, **7**, **10**, **14**, **28** and cisplatin against MCF-7 cells for 48 h incubation period. Mean percent absorbance of untreated control cells were assumed to be 0% and three different concentrations ($IC_{50}/2$, IC_{50}, $2 \times IC_{50}$) of test compounds and cisplatin were given. Data points represent means for two independent experiments ± SD of four independent wells. p <0.05.

MCF-7 cells. Otherwise, cisplatin showed the most inhibitory activity with 93.31% at a concentration of 62.4 μg/mL. Compound **10** showed 78.50% and 78.71% inhibitory activity after 48 h incubation period, at concentrations of 22.09 and 44.18 μg/mL, respectively whereas compound **14** showed 76.73%, 85.99% and 88.91% inhibitions at concentrations of 15.32, 30.63 and 61.26 μg/mL, respectively. Compound **5** was found to have 56.59%, 61.16% and 70.18% DNA synthesis inhibition at $IC_{50}/2$, IC_{50} and $2 \times IC_{50}$ values, respectively whereas compound **7** was found to have 24.98%, 52.05% and 62.34% DNA synthesis inhibition after 48 h incubation period at $IC_{50}/2$, IC_{50} and $2 \times IC_{50}$ values, respectively. Furthermore, it was determined that compound **28** showed less antiproliferative activity than other compounds against the MCF-7 cell line.

2.2.3. Induction of apoptosis

FITC Annexin V staining is a technique to show the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Hence, Annexin V-FITC staining is typically accompanied by a vital dye staining such as PI to identify the formation of apoptotic cells in the early stages (Annexin V-FITC positive, PI negative).Viable cells with intact membranes exclude PI, whereas the membranes of dead

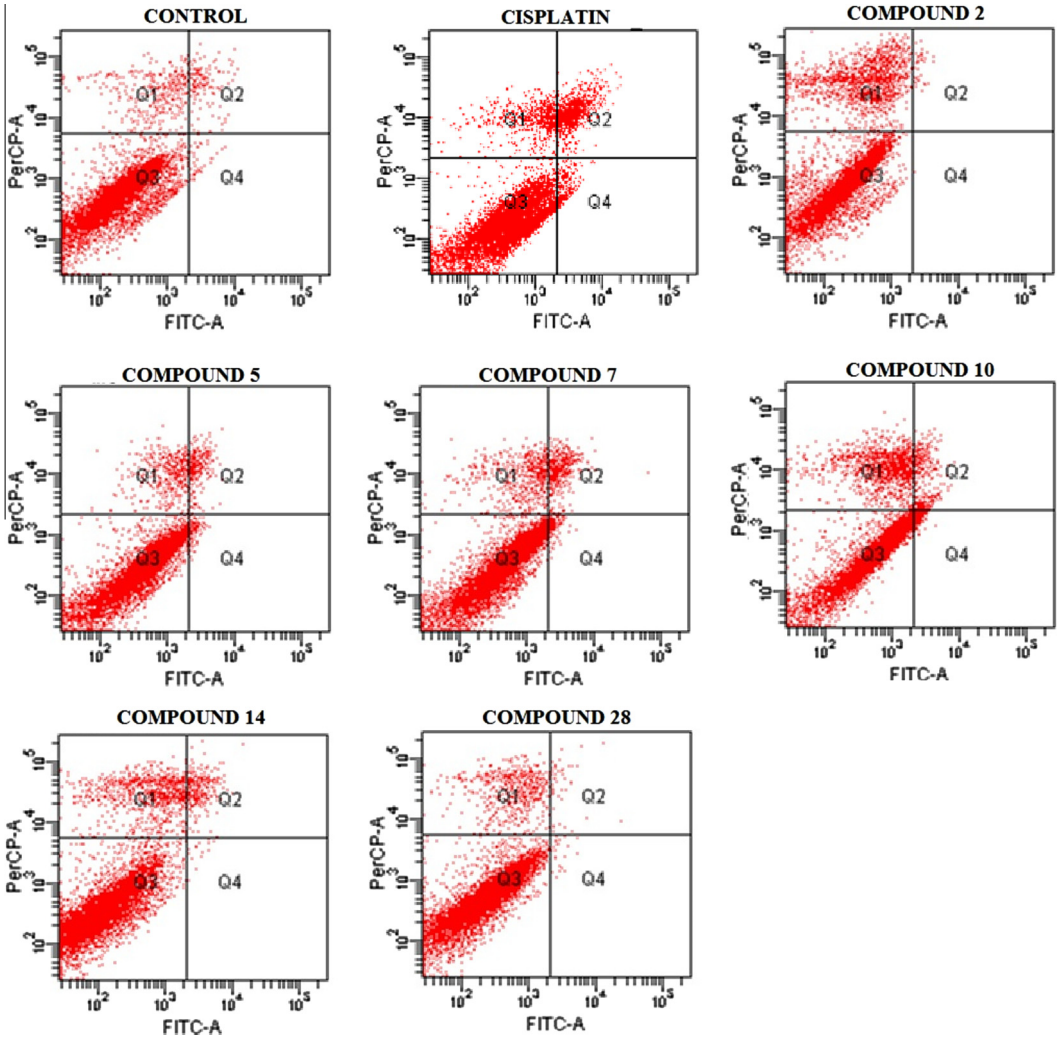


Figure 3. The flow cytometric analysis diagram of compound **2**, **5**, **7**, **10**, **14**, **28** and cisplatin for MCF-7 cell line. Annexin V-PI analysis in MCF-7 cells, following 24 h incubation period of compounds **2**, **5**, **7**, **10**, **14**, **28** and cisplatin by Annexin-V-FITC method at IC_{50} concentrations, which are 91.71, 31.20, 31.20, 34.27, 30.63, 91.85 and 29.31 μg/mL, respectively. Percentages of Q1, Q2, Q3, and Q4 are measured as 3.66, 1.52, 94.36, and 0.46% for control; 17.1, 0.4, 82.5 and 0% for compound **2**; 11.5, 9.4, 76.9 and 2.2% for compound **5**; 11.2, 10.3, 76.4 and 2.1% for compound **7**; 28.2, 5.8, 63.4 and 2.6% for compound **10**; 26.7, 6.2, 66.5 and 0.6% for compound **14**; 24.5, 3.3, 71.3 and 0.9% for compound **28**; 7.0, 9.1, 80.9 and 3.0% for compound cisplatin, respectively.

and damaged cells are permeable to PI. After this procedure, cells can be classified from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death).[30]

In Figure 3, the flow cytometric analysis diagram is showed for MCF-7 cell line. Compound **7** showed the highest population of apoptotic cells (12.3%) whereas cisplatin showed 12.1% apoptotic cells. Compound **5** produced a comparable population of apoptotic cells with a percentage of 11.6% according to cisplatin's percentage. However compounds **2**, **10**, **14** and **28** provoked necrotic induction in MCF-7 cells after 24 h treatment.

3. Conclusion/discussion

One of the important criteria for an anticancer agent, is to show minimum or no side-effects on healthy cells of the patients receiving chemotherapy. We evaluated the antiproliferative potential of newly synthesized compounds on human estrogen-dependent MCF-7 human breast adenocarcinoma cell line. In addition, the cytotoxic activities of these compounds were also evaluated against normal mouse embryonic fibroblast cell line, NIH/3T3, for determining the selectivity of potential anticancer agents. Significant differences in IC_{50} values of the compounds **2**, **5**, **7**, **10**, **14** and **28** were observed between breast carcinoma and normal cell lines.

Structures of the final compounds differ from each other due to substituents on phenyl rings and belong to four main structures including 5,6-diphenyl-1,2,4-triazine (**1**–**8**); 5,6-bis(4-methylphenyl)-1,2,4-triazine (**9**–**15**); 5,6-bis(4-methoxyphenyl)-1,2,4-triazine (**16**–**23**) and 5,6-bis(4-chlorophenyl)-1,2,4-triazine (**24**–**32**). Considering the substituent effect on cytotoxic activity, compounds **5** and **7** bearing 3- and 4-chloro phenyl piperazines has attracted attention with higher activities. Also compounds **10** and **14** containing 2- and 4-methoxy phenyl piperazine moiety had remarkable influence on the cytotoxic activities. Compounds synthesized from 4,4′-dimethoxybenzil (**16**–**23**) and 4,4′-dichlorobenzil (**24**–**32**) did not show prominent activity. Additionally, electron withdrawing substituent nitro on phenyl ring produced inactive compounds (**8**, **16**, **24** and **32**).

According to the DNA synthesis inhibition studies, compounds **2**, **5**, **7**, **10** and **14** inhibited DNA synthesis against MCF-7 cell line and there was a time dependent increase of inhibition ratios, especially in compounds **5** and **7**. Otherwise, the compounds **5** and **7** were determined that they affected breast cancer cell by the apoptotic pathway and compounds **2**, **10** and **14** by the necrotic pathway. Apoptosis, or programmed cell death, plays an essential role in controlling cell death. Our results illustrated that compounds **5** and **7** exhibits significant antiproliferative effect on MCF-7 human breast cells concomitant with the induction of apoptosis.

Our present in vitro studies demonstrate the antiproliferative effects of newly synthesized compounds **5** and **7** against MCF-7 and NIH/3T3 cell lines. Comparing compounds on the basis of biological activity, it was revealed that the molecules with *-meta* and *-para* chloro substituted phenyl ring displayed the highest activity. Further studies are required to determine the anticancer activity of the compounds **5** and **7** in animal models. Besides, it is thought to design new compounds with *-meta* and *-para* chloro aryl substitution, improving synthetic and activity screening methods.

4. Experimental section

4.1. General

All chemicals were purchased from Sigma–Aldrich Chemical Co (Sigma–Aldrich Corp., St. Louis, MO, USA) and Merck Chemicals (Merck KGaA, Darmstadt, Germany). All melting points (mp) were determined by Electrothermal 9100 digital melting point apparatus (Electrothermal, Essex, UK) and are uncorrected. All the reactions were monitored by thin-layer chromatography (TLC) using Silica Gel 60 F254 TLC plates (Merck KGaA, Darmstadt, Germany). Spectroscopic data were recorded with the following instruments: IR, Shimadzu 8400S spectrophotometer (Shimadzu, Tokyo, Japan); ^{1}H NMR, Bruker DPX 500 NMR spectrometer (Bruker Bioscience, Billerica, MA, USA); ^{13}C NMR, VARIAN Mercury 100 FT spectrometer (Varian Inc, Palo Alto, CA, USA) in DMSO-d_6, using TMS as internal standard; M+1 peaks were determined by AB Sciex-3200 Q-TRAP LC/MS/MS system (AB Applied Biosystems Co., MA, USA). Elemental analyses were performed on a Leco TruSpec Micro CHN/CHNS elemental analyzer (Leco, Michigan, USA).

4.2. General procedure for the synthesis of 5,6-bis(4-substituted phenyl)-3-methylthio-l,2,4-triazine (Ia–Id)

Equimolar quantities of 4,4′-substituted benzil (0.3), *S*-methylthiosemicarbazide hydrogen iodide (0.3 mol) and sodium bicarbonate (0.3 mol) were refluxed in methanol (600 mL) for 3 h. After cooling, the mixture was filtrated to obtain raw product. The obtained product was washed with water and ethanol then crystallized from ethanol.[31]

4.3. General procedure for the synthesis of 5,6-bis(4-substituted phenyl)-3-(piperazin-1-yl)-l,2,4-triazine (IIa–IId)

A mixture of 5,6-bis(4-substituted phenyl)-3-methylthio-l,2,4-triazine (**Ia**–**Id**) derivative (0.2 mol) and piperazine (0.4 mol) in pyridine (400 mL) was heated at reflux for 6 h until there was no odor of mercaptan. After cooling, the mixture was treated with water and the separated precipitate was collected by filtration, washed with water, and dried to provide product. Recrystallization from ethanol was afforded for purification.[32]

4.4. General procedure for the synthesis of 2-chloro-1-[4-(5,6-bis(4-substituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl] ethanone (IIIa–IIId)

Chloroacetyl chloride (0.035 mol) in 50 mL dimethylformamide was added drop wise over 45 minutes to a magnetically stirred solution of compound **IIa**–**IId** (0.03 mol) and triethylamine (0.035 mol) in dimethylformamide (300 mL). After completion of reaction, the solvent was evaporated to dryness under reduced pressure. Water was added to wash the resulting solid and the mixture was filtered, dried and recrystallized from ethanol to give compounds **IIIa**–**IIId**.

4.4.1. 2-Chloro-1-[4-(5,6-diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]ethanone (IIIa)

Yield 76–79%, mp 205–207 °C. IR (KBr, cm^{-1}): ν_{maks} 3031 (aromatic C–H), 2887 (aliphatic C–H), 1668 (C=O), 1556–1323 (C=C and C=N), 1235–1012 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.67 (t, 4H, *J*: 5.0 Hz, piperazine-H), 3.95 (br s, 2H, piperazine-H), 4.0 (br s, 2H, piperazine-H), 4.49 (s, 2H, –CO–CH_2), 7.36–7.7.39 (m, 7H, Ar-H), 7.44–7.48 (m, 3H, Ar-H). MS $[M+1]^+$: *m*/*z* 394.

4.4.2. 2-Chloro-1-[4-(5,6-bis(4-methylphenyl)-1,2,4-triazin-3-yl) piperazin-1-yl]ethanone (IIIb)

Yield 75–77%, mp 198–201 °C. IR (KBr, cm^{-1}): ν_{maks} 3025 (aromatic C–H), 2879 (aliphatic C–H), 1669 (C=O), 1569–1333 (C=C and C=N), 1237–1014 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.33 and 2.33 (2s, 6H, CH_3), 3.66 (t, 4H, *J*: 5.0 Hz, piperazine-H), 3.93 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 4.49 (s, 2H, –CO–CH_2), 7.18 (d, 4H, *J*: 8.25 Hz, Ar-H), 7.27 (d,

2H, *J*: 8.0 Hz, Ar-H), 7.38 (d, 2H, 2H, *J*: 8.0 Hz, Ar-H). MS $[M+1]^+$: *m*/*z* 422.

4.4.3. 2-Chloro-1-[4-(5,6-bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]ethanone (IIIc)

Yield 81–83%, mp 166–169 °C. IR (KBr, cm^{-1}): ν_{maks} 3042 (aromatic C–H), 2913 (aliphatic C–H), 1671 (C=O), 1571–1315 (C=C and C=N), 1243–1015 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.66 (br s, 4H, piperazine-H), 3.78 (s, 6H, OCH_3), 3.92 (br s, 2H, piperazine-H), 3.97 (br s, 2H, piperazine-H), 4.50 (s, 2H, –CO–CH_2), 6.93–6.96 (m, 4H, Ar-H), 7.33 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.47 (d, 2H, *J*: 8.5 Hz, Ar-H). MS $[M+1]^+$: *m*/*z* 454.

4.4.4. 2-Chloro-1-[4-(5,6-bis(4-chlorophenyl)-1,2,4-triazin-3-yl) piperazin-1-yl]ethanone (IIId)

Yield 82–84%, mp 176–181 °C. IR (KBr, cm^{-1}): ν_{maks} 3024 (aromatic C–H), 2876 (aliphatic C–H), 1667 (C=O), 1572–1328 (C=C and C=N), 1237–1014 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.67 (t, 4H, *J*: 5.0 Hz, piperazine-H), 3.95 (br s, 2H, piperazine-H), 4.0 (br s, 2H, piperazine-H), 4.49 (s, 2H, –CO–CH_2), 7.40–7.42 (m, 2H, Ar-H), 7.46–7.48 (m, 2H, Ar-H), 7.50 (s, 4H, Ar-H). MS $[M+1]^+$: *m*/*z* 462.

4.5. General procedure for the synthesis of 1-[4-(5,6-bis(4-subtituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-substituted phenyl)piperazin-1-yl]ethanone derivatives (1–32)

2-Chloro-1-[4-(5,6-bis(4-substituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]ethanone derivative (**IIIa–IIId**) (1.5 mmol) and 1-substituted piperazine derivative (1.6 mmol) were stirred in dimethylformamide with the presence of triethylamine (1.6 mmol) for 5 h. After duration of reaction, the solvent was evaporated and the residue was reacted with water. Filtrated product was crystallized, after drying.

4.5.1. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-(4-phenylpiperazin-1-yl)ethanone (1)

Yield 70–76%, mp 153–154 °C. IR (KBr, cm^{-1}): ν_{maks} 3024 (aromatic C–H), 2836 (aliphatic C–H), 1664 (C=O), 1587–1316 (C=C and C=N), 1237–976 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.64 (br s, 4H, piperazine-H), 3.17 (br s, 4H, piperazine-H), 3.42 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.78 (t, 1H, *J*: 8.0 Hz, Ar-H), 6.94 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.21 (t, 2H, *J*: 7.5 Hz, Ar-H), 7.36–7.38 (m, 7H, Ar-H), 7.43–7.47 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.84, 43.05, 43.66, 44.73, 48.17, 52.40, 55.92, 60.55, 115.32, 118.74, 128.16, 128.19, 128.80, 128.81, 129.33, 130.14, 148.42, 150.89, 155.31, 159.18, 162.20, 167.59. For $C_{31}H_{33}N_7O$ calculated: 71.65% C, 6.40% H, 18.87% N; found: 71.74% C, 6.42% H, 18.83% N. MS $[M+1]^+$: *m*/*z* 520.

4.5.2. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-methoxyphenyl)piperazin-1-yl)]ethanone (2)

Yield 68%, mp 187 °C. IR (KBr, cm^{-1}): ν_{maks} 3031 (aromatic C–H), 2838 (aliphatic C–H), 1659 (C=O), 1571–1315 (C=C and C=N), 1243–976 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.15 (br s, 4H, piperazine-H), 3.39 (s, 2H, –CO–CH2), 3.66 (br s, 2H, piperazine-H), 3.72 (s, 3H, OCH3), 3.78 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.41–6.43 (m, 2H, Ar-H), 6.56 (d, 1H, *J*: 8.0 Hz, Ar-H), 7.13 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.38–7.39 (m, 7H, Ar-H), 7.45–7.49 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.88, 43.08, 43.70, 44.75, 50.04, 52.72, 55.28, 60.76, 111.99, 117.93, 120.77, 122.30, 128.15, 128.18, 128.83, 129.32, 130.12, 136.05, 136.15, 141.17, 148.45, 151.95, 155.30, 159.22, 167.70. For $C_{32}H_{35}N_7O_2$ calculated: 69.92% C, 6.42% H, 17.84% N; found: 69.91% C, 6.48% H, 17.80% N. MS $[M+1]^+$: *m*/*z* 550.

4.5.3. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-chlorophenyl)piperazin-1-yl)]ethanone (3)

Yield 69–73%, mp 225–226 °C. IR (KBr, cm^{-1}): ν_{maks} 3016 (aromatic C–H), 2842 (aliphatic C–H), 1662 (C=O), 1587–1319 (C=C and C=N), 1235–976 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.65 (br s, 4H, piperazine-H), 3.02 (br s, 4H, piperazine-H), 3.39 (s, 2H, –CO–CH_2), 3.67 (br s, 2H, piperazine-H), 3.78 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 4.01 (br s, 2H, piperazine-H), 7.16 (t, 1H, *J*: 7.5 Hz, Ar-H), 7.18 (d, 1H, *J*: 8.0 Hz, Ar-H), 7.29 (t, 1H, *J*: 7.5 Hz, Ar-H), 7.37–7.39 (m, 7H, Ar-H), 7.42 (d, 1H, *J*: 8.0 Hz, Ar-H), 7.44–7.49 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.87, 43.08, 43.70, 44.75, 50.82, 52.57, 60.56, 120.81, 123.76, 127.54, 127.94, 128.16, 128.19, 128.83, 129.33, 130.14, 130.21, 136.05, 136.14, 148.45, 148.90, 155.31, 159.22, 167.67. For $C_{31}H_{32}ClN_7O$ calculated: 67.20% C, 5.82% H, 17.70% N; found: 67.25% C, 5.96% H, 17.81% N. MS $[M+1]^+$: *m*/*z* 554.

4.5.4. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-methoxyphenyl)piperazin-1-yl)]ethanone (4)

Yield 66–69%, mp 126–128 °C. IR (KBr, cm^{-1}): ν_{maks} 3024 (aromatic C–H), 2836 (aliphatic C–H), 1661 (C=O), 1581–1321 (C=C and C=N), 1235–976 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 3.16 (br s, 4H, piperazine-H), 3.38 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.71 (s, 3H, OCH_3), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.38 (d, 1H, *J*: 8.0 Hz, Ar-H), 6.45 (br s, 1H, Ar-H), 6.53 (d, 1H, *J*: 8.0 Hz, Ar-H), 7.10 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.36–7.37 (m, 7H, Ar-H), 7.43–7.47 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.86, 43.08, 43.69, 44.73, 48.18, 52.38, 54.78, 60.56, 101.44, 104.14, 108.0, 128.14, 128.18, 128.82, 129.32, 129.47, 130.11, 136.04, 136.14, 148.44, 152.29, 155.29, 159.22, 160.13, 167.62. For $C_{32}H_{35}N_7O_2$ calculated: 69.92% C, 6.42% H, 17.84% N; found: 69.46% C, 6.44% H, 17.85% N. MS $[M+1]^+$: *m*/*z* 550.

4.5.5. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-chlorophenyl)piperazin-1-yl)]ethanone (5)

Yield 66–69%, mp 149–150 °C. IR (KBr, cm^{-1}): ν_{maks} 3026 (aromatic C–H), 2825 (aliphatic C–H), 1659 (C=O), 1593–1325 (C=C and C=N), 1240–977 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.20 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.93 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.78 (d, 1H, *J*: 8.0 Hz, Ar-H), 6.90 (d, 1H, *J*: 8.5 Hz, Ar-H), 6.95 (br s, 1H, Ar-H), 7.21 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.34–7.37 (m, 7H, Ar-H), 7.43–7.47 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.85, 43.08, 43.67, 44.72, 47.66, 52.18, 60.43, 113.57, 114.49, 117.94, 128.15, 128.18, 128.82, 129.31, 130.12, 130.27, 133.72, 136.04, 136.14, 148.44, 152.14, 155.29, 159.22, 167.59. For $C_{31}H_{32}ClN_7O$ calculated: 67.20% C, 5.82% H, 17.70% N; found: 67.23% C, 5.86% H, 17.81% N. MS $[M+1]^+$: *m*/*z* 554.

4.5.6. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-methoxyphenyl)piperazin-1-yl)]ethanone (6)

Yield 69–75%, mp 100–103 °C. IR (KBr, cm^{-1}): ν_{maks} 3041 (aromatic C–H), 2883 (aliphatic C–H), 1665 (C=O), 1603–1315 (C=C and C=N), 1253–1008 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.65 (br s, 4H, piperazine-H), 3.06 (br s, 4H, piperazine-H), 3.33 (s, 2H, –CO–CH_2), 3.67 (br s, 2H, piperazine-H), 3.69 (s, 3H, OCH_3), 3.77 (br s, 2H, piperazine-H), 3.93 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.82 (d, 2H, *J*: 8.0 Hz, Ar-H), 6.91 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.36–7.39 (m, 6H, Ar-H), 7.43–7.47 (m, 4H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm)

δ 40.84, 43.06, 43.65, 44.73, 49.57, 52.50, 55.06, 60.61, 114.12, 117.28, 128.16, 128.19, 128.81, 129.32, 130.14, 135.99, 136.10, 145.27, 148.41, 152.79, 155.31, 159.18, 167.62. For $C_{32}H_{35}N_7O_2$ calculated: 69.92% C, 6.42% H, 17.84% N; found: 69.93% C, 6.46% H, 17.79% N. MS $[M+1]^+$: m/z 550.

4.5.7. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-chlorophenyl)piperazin-1-yl)]ethanone (7)

Yield 75–78%, mp 169–171 °C. IR (KBr, cm^{-1}): ν_{maks} 3033 (aromatic C–H), 2868 (aliphatic C–H), 1663 (C=O), 1588–1303 (C=C and C=N), 1252–1012 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 3.16 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.76 (br s, 2H, piperazine-H), 3.93 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.95 (d, 2H, J: 8.0 Hz, Ar-H), 7.22 (d, 2H, J: 7.5 Hz, Ar-H), 7.34–7.37 (m, 7H, Ar-H), 7.43–7.47 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.88, 43.10, 43.68, 43.67, 44.75, 48.04, 52.23, 60.49, 109.46, 116.78, 122.25, 128.17, 128.21, 128.50, 128.84, 129.34, 130.15, 136.05, 136.14, 148.45, 149.73, 155.32, 159.22, 167.61. For $C_{31}H_{32}ClN_7O$ calculated: 67.20% C, 5.82% H, 17.70% N; found: 67.26% C, 5.88% H, 17.73% N. MS $[M+1]^+$: m/z 554.

4.5.8. 1-[4-(5,6-Diphenyl-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-nitrophenyl)piperazin-1-yl)]ethanone (8)

Yield 68–70%, mp 188–190 °C. IR (KBr, cm^{-1}): ν_{maks} 3018 (aromatic C–H), 2875 (aliphatic C–H), 1662 (C=O), 1613–1302 (C=C and C=N), 1241–1012 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 3.32 (s, 2H, –CO–CH_2), 3.49 (br s, 4H, piperazine-H), 3.65 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 4.0 (br s, 2H, piperazine-H), 7.04 (d, 2H, J: 9.5 Hz, Ar-H), 7.35–7.47 (m, 7H, Ar-H), 7.38 (d, 3H, J: 8.0 Hz, Ar-H), 8.06 (d, 2H, J: 9.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.84, 43.08, 43.66, 44.71, 46.33, 51.89, 60.09, 112.58, 125.59, 128.16, 128.19, 128.82, 129.32, 130.15, 136.04, 136.12, 136.84, 148.45, 154.64, 155.31, 159.22, 167.52. For $C_{31}H_{32}N_8O_3$ calculated: 65.94% C, 5.71% H, 19.85% N; found: 65.87% C, 5.83% H, 19.80% N. MS $[M+1]^+$: m/z 565.

4.5.9. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-(4-phenylpiperazin-1-yl)ethanone (9)

Yield 73–75%, mp 203–205 °C. IR (KBr, cm^{-1}): ν_{maks} 3021 (aromatic C–H), 2890 (aliphatic C–H), 1664 (C=O), 1589–1302 (C=C and C=N), 1231–1003 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.61 (br s, 4H, piperazine-H), 3.20 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.76 (br s, 2H, piperazine-H), 3.91 (br s, 2H, piperazine-H), 3.97 (br s, 2H, piperazine-H), 6.77 (t, 1H, J: 7.5 Hz, Ar-H), 6.94 (d, 1H, J: 8.0 Hz, Ar-H), 7.16–7.22 (m, 6H, Ar-H), 7.27 (d, 2H, J: 8.0 Hz, Ar-H), 7.38 (d, 2H, J: 8.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.70, 20.80, 40.85, 43.05, 43.66, 44.72, 48.20, 52.40, 60.55, 115.32, 118.72, 128.63, 128.77, 128.79, 128.83, 129.28, 133.22, 133.42, 137.52, 140.07, 148.35, 150.91, 155.0, 159.12, 167.61. For $C_{33}H_{37}N_7O$ calculated: 72.37% C, 6.81% H, 17.90% N; found: 72.47% C, 6.88% H, 17.82% N. MS $[M+1]^+$: m/z 548.

4.5.10. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-methoxyphenyl)piperazin-1-yl]ethanone (10)

Yield 71–77%, mp 134–137 °C. IR (KBr, cm^{-1}): ν_{maks} 3028 (aromatic C–H), 2882 (aliphatic C–H), 1661 (C=O), 1581–1313 (C=C and C=N), 1252–927 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.61 (br s, 4H, piperazine-H), 2.98 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.77 (br s, 5H, piperazine-H and OCH_3), 3.91 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 6.84–6.97 (m, 4H, Ar-H), 7.18 (d, 4H, J: 8.0 Hz, Ar-H), 7.27 (d, 2H, J: 8.0 Hz, Ar-H), 7.38 (d, 2H, J: 8.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.70, 20.80, 40.85, 43.06, 43.66, 44.75, 50.03, 52.70, 55.25, 60.75, 111.91, 117.91, 120.74, 122.30, 128.64, 128.76, 128.83, 129.29, 133.22, 133.43, 137.53, 140.08, 141.13, 148.35, 151.92, 155.0, 159.12, 167.68. For $C_{34}H_{39}N_7O_2$ calculated: 70.69% C, 6.80% H, 16.84% N; found: 70.67% C, 6.85% H, 16.87% N. MS $[M+1]^+$: m/z 578.

4.5.11. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-chlorophenyl)piperazin-1-yl]ethanone (11)

Yield 75–76%, mp 152–154 °C. IR (KBr, cm^{-1}): ν_{maks} 3022 (aromatic C–H), 2873 (aliphatic C–H), 1659 (C=O), 1591–1309 (C=C and C=N), 1244–1010 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.64 (br s, 4H, piperazine-H), 3.0 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.91 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 7.04 (t, 1H, J: 8.0 Hz, Ar-H), 7.16–7.18 (m, 5H, Ar-H), 7.26–7.30 (m, 3H, Ar-H), 7.38 (d, 2H, J: 8.0 Hz, Ar-H), 7.41 (d, 1H, J: 8.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.72, 20.82, 40.84, 43.05, 43.66, 44.75, 50.80, 52.56, 60.56, 120.80, 123.77, 127.51, 127.94, 128.64, 128.77, 128.84, 129.31, 130.21, 133.21, 133.41, 137.54, 140.10, 148.33, 148.87, 155.0, 159.10, 167.62. For $C_{33}H_{36}ClN_7O$ calculated: 68.09% C, 6.23% H, 16.84% N; found: 68.12% C, 6.27% H, 16.80% N. MS $[M+1]^+$: m/z 582.

4.5.12. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-methoxyphenyl)piperazin-1-yl]ethanone (12)

Yield 68–71%, mp 111–112 °C. IR (KBr, cm^{-1}): ν_{maks} 3025 (aromatic C–H), 2871 (aliphatic C–H), 1664 (C=O), 1582–1305 (C=C and C=N), 1256–1009 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.59 (br s, 4H, piperazine-H), 3.15 (br s, 4H, piperazine-H), 3.28 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.71 (s, 3H, OCH_3), 3.77 (br s, 2H, piperazine-H), 3.91 (br s, 2H, piperazine-H), 3.97 (br s, 2H, piperazine-H), 6.36 (d, 1H, J: 8.0 Hz, Ar-H), 6.45 (s, 1H, Ar-H), 6.52 (d, 1H, J: 7.5 Hz, Ar-H), 7.09 (t, 1H, J: 8.0 Hz, Ar-H), 7.16 (d, 4H, J: 7.5 Hz, Ar-H), 7.29 (d, 2H, J: 8 Hz, Ar-H), 7.38 (d, 2H, J: 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.71, 20.72, 40.13, 40.90, 43.11, 43.70, 44.80, 48.21, 52.43, 54.79, 60.64, 101.46, 104.13, 108.04, 128.69, 128.81, 128.88, 129.36, 129.52, 133.26, 133.47, 137.57, 140.14, 148.37, 152.32, 155.02, 159.14, 160.14, 167.63. For $C_{34}H_{39}N_7O_2$ calculated: 70.69% C, 6.80% H, 16.97% N; found: 70.72% C, 6.86% H, 16.91% N. MS $[M+1]^+$: m/z 578.

4.5.13. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-chlorophenyl)piperazin-1-yl]ethanone (13)

Yield 71–76%, mp 173–175 °C. IR (KBr, cm^{-1}): ν_{maks} 3013 (aromatic C–H), 2873 (aliphatic C–H), 1663 (C=O), 1612–1319 (C=C and C=N), 1252–1005 (C–N). ^1H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.60 (br s, 4H, piperazine-H), 3.20 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.75 (br s, 2H, piperazine-H), 3.91 (br s, 2H, piperazine-H), 3.97 (br s, 2H, piperazine-H), 6.78 (d, 1H, J: 7.5 Hz, Ar-H), 6.90 (d, 1H, J: 8.5 Hz, Ar-H), 6.94 (s, 1H, Ar-H), 7.16–7.22 (m, 5H, Ar-H), 7.26 (d, 2H, J: 8.0 Hz, Ar-H), 7.37 (d, 2H, J: 8.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.71, 20.81, 40.84, 43.05, 43.63, 44.73, 47.62, 52.18, 60.42, 113.56, 114.46, 117.93, 128.63, 128.76, 128.83, 129.30, 130.27, 133.19, 133.40, 133.69, 137.53, 140.09, 148.33, 152.12, 155.0, 159.09, 167.55. For $C_{33}H_{36}ClN_7O$ calculated: 68.09% C, 6.23% H, 16.84% N; found: 68.15% C, 6.29% H, 16.78% N. MS $[M+1]^+$: m/z 582.

4.5.14. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-methoxyphenyl)piperazin-1-yl]ethanone (14)

Yield 69–74%, mp 126–129 °C. IR (KBr, cm^{-1}): ν_{maks} 3017 (aromatic C–H), 2839 (aliphatic C–H), 1663 (C=O), 1587–1305 (C=C and C=N), 1261–1002 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.59 (br s, 4H, piperazine-H), 3.15 (br s, 4H, piperazine-H), 3.28 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.72 (s, 3H, OCH_3), 3.78 (br s, 2H, piperazine-H), 3.90 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 6.36 (d, 1H, *J*: 8.0 Hz, Ar-H), 6.85 (d, 2H, *J*: 8.5 Hz, Ar-H), 6.94 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.35–7.38 (m, 3H, Ar-H), 7.40–7.43 (m, 4H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.72, 20.82, 40.84, 43.05, 43.65, 44.75, 49.59, 52.52, 55.06, 60.63, 114.12, 117.29, 128.64, 128.77, 128.83, 129.30, 133.20, 133.41, 137.53, 140.10, 145.28, 148.33, 152.81, 154.99, 159.10, 167.63. For $C_{34}H_{39}N_7O_2$ calculated: 70.69% C, 6.80% H, 16.97% N; found: 70.75% C, 6.89% H, 16.90% N. MS $[M+1]^+$: *m*/*z* 578.

4.5.15. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-chlorophenyl)piperazin-1-yl]ethanone (15)

Yield 72–75%, mp 117–118 °C. IR (KBr, cm^{-1}): ν_{maks} 3015 (aromatic C–H), 2872 (aliphatic C–H), 1669 (C=O), 1602–1303 (C=C and C=N), 1251–925 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.61 (br s, 4H, piperazine-H), 3.16 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.75 (br s, 2H, piperazine-H), 3.91 (br s, 2H, piperazine-H), 3.97 (br s, 2H, piperazine-H), 6.95 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.17 (d, 4H, *J*: 7.5 Hz, Ar-H), 7.22 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.26 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.37 (d, 2H, *J*: 8.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.79, 20.88, 30.74, 35.75, 40.91, 43.11, 43.71, 44.79, 48.05, 52.28, 60.52, 116.81, 122.29, 128.55, 128.70, 128.84, 128.91, 129.36, 133.27, 133.47, 137.60, 140.16, 148.40, 149.76, 155.06, 159.17, 162.27, 167.62. For $C_{33}H_{36}ClN_7O$ calculated: 68.09% C, 6.23% H, 16.84% N; found: 68.06% C, 6.28% H, 16.83% N. MS $[M+1]^+$: *m*/*z* 582.

4.5.16. 1-[4-(5,6-Bis(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-nitrophenyl)piperazin-1-yl]ethanone (16)

Yield 70–72%, mp 254–255 °C. IR (KBr, cm^{-1}): ν_{maks} 3034 (aromatic C–H), 2884 (aliphatic C–H), 1667 (C=O), 1584–1303 (C=C and C=N), 1256–1005 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.31 and 2.32 (2s, 6H, CH_3), 2.61 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.49 (br s, 4H, piperazine-H), 3.65 (br s, 2H, piperazine-H), 3.76 (br s, 2H, piperazine-H), 3.92 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 7.04 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.18 (d, 4H, *J*: 8.0 Hz, Ar-H), 7.27 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.38 (d, 2H, *J*: 8.0 Hz, Ar-H), 8.06 (d, 2H, *J*: 9.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 20.71, 20.73, 40.85, 43.06, 43.66, 44.70, 45.09, 46.35, 51.90, 60.08, 112.59, 125.58, 128.64, 128.77, 128.83, 129.29, 133.24, 133.44, 136.87, 137.54, 140.09, 148.36, 154.64, 155.0, 159.14, 167.51. For $C_{33}H_{36}N_8O_3$ calculated: 66.87% C, 6.12% H, 18.91% N; found: 66.78% C, 6.18% H, 18.82% N. MS $[M+1]^+$: *m*/*z* 593.

4.5.17. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-(4-phenylpiperazin-1-yl]ethanone (17)

Yield 70–73%, mp 174–176 °C. IR (KBr, cm^{-1}): ν_{maks} 3016 (aromatic C–H), 2846 (aliphatic C–H), 1663 (C=O), 1581–1307 (C=C and C=N), 1254–976 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.62 (br s, 4H, piperazine-H), 3.16 (br s, 4H, piperazine-H), 3.32 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.73–3.78 (m, 8H, piperazine-H and OCH_3), 3.90 (br s, 2H, piperazine-H), 3.96 (br s, 2H, piperazine-H), 6.78 (t, 1H, *J*: 7.5 Hz, Ar-H), 6.91–6.96 (m, 6H, Ar-H), 7.21 (t, 2H, *J*: 8.0 Hz, Ar-H), 7.32 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.46 (d, 2H, *J*: 9.0 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.87, 43.08, 43.69, 44.75, 48.22, 52.50, 55.05, 55.22, 60.57, 113.68, 113.78, 115.32, 118.72, 128.13, 128.75, 128.79, 129.99, 131.05, 148.04, 150.92, 154.34, 159.05, 159.20, 160.91, 167.61. For $C_{33}H_{37}N_7O_3$ calculated: 68.37% C, 6.43% H, 16.91% N; found: 68.38% C, 6.48% H, 16.88% N. MS $[M+1]^+$: *m*/*z* 580.

4.5.18. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-methoxyphenyl)piperazin-1-yl]ethanone (18)

Yield 65–67%, mp 114–118 °C. IR (KBr, cm^{-1}): ν_{maks} 3034 (aromatic C–H), 2839 (aliphatic C–H), 1661 (C=O), 1603–1302 (C=C and C=N), 1258–1006 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 2.99 (br s, 4H, piperazine-H), 3.29 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.77–3.79 (m, 11H, piperazine-H and OCH_3), 3.92 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 6.85–6.97 (m, 8H, Ar-H), 7.33 (d, 2H, *J*: 7.0 Hz, Ar-H), 7.47 (d, 2H, *J*: 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.86, 43.05, 43.67, 44.77, 50.01, 52.69, 55.03, 55.22, 55.92, 60.76, 111.81, 113.66, 113.77, 117.88, 120.71, 122.31, 128.08, 128.70, 129.99, 131.07, 141.07, 148.02, 151.88, 154.35, 159.08, 159.16, 160.88, 167.64. For $C_{34}H_{39}N_7O_4$ calculated: 66.98% C, 6.45% H, 16.08% N; found: 66.88% C, 6.50% H, 16.82% N. MS $[M+1]^+$: *m*/*z* 610.

4.5.19. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-chlorophenyl)piperazin-1-yl]ethanone (19)

Yield 69–72%, mp 109–110 °C. IR (KBr, cm^{-1}): ν_{maks} 3018 (aromatic C–H), 2837 (aliphatic C–H), 1656 (C=O), 1601–1305 (C=C and C=N), 1251–974 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.65 (br s, 4H, piperazine-H), 3.01 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.75–3.79 (m, 8H, piperazine-H and OCH_3), 3.91 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 6.92–6.96 (m, 4H, Ar-H), 7.04 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.17 (d, 1H, *J*: 7.5 Hz, Ar-H), 7.29 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.33 (d, 2H, *J*: 7.5 Hz, Ar-H), 7.41 (d, 1H, *J*: 8.0 Hz, Ar-H), 7.47 (d, 2H, *J*: 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.85, 43.05, 43.66, 44.77, 50.80, 52.55, 55.03, 55.21, 60.55, 113.67, 113.77, 120.80, 123.77, 127.49, 127.94, 128.08, 128.69, 129.98, 130.21, 131.07, 148.02, 148.86, 154.35, 159.01, 159.16, 160.88, 167.61. For $C_{33}H_{36}ClN_7O_3$ calculated: 64.54% C, 5.91% H, 15.96% N; found: 64.58% C, 6.0% H, 16.02% N. MS $[M+1]^+$: *m*/*z* 614.

4.5.20. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-methoxyphenyl)piperazin-1-yl]ethanone (20)

Yield 68–71%, mp 91–92 °C. IR (KBr, cm^{-1}): ν_{maks} 3028 (aromatic C–H), 2833 (aliphatic C–H), 1645 (C=O), 1604–1300 (C=C and C=N), 1244–974 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.15 (br s, 4H, piperazine-H), 3.29 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.71 (s, 3H, OCH_3), 3.75–3.78 (m, 8H, piperazine-H and OCH_3), 3.91 (br s, 2H, piperazine-H), 3.96 (br s, 2H, piperazine-H), 6.34 (d, 1H, *J*: 8.0 Hz, Ar-H), 6.45 (s, 1H, Ar-H), 6.53 (d, 1H, *J*: 8.0 Hz, Ar-H), 6.92–6.97 (m, 4H, Ar-H), 7.10 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.33 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.46 (d, 2H, *J*: 7.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.85, 43.05, 43.65, 44.75, 48.13, 52.36, 54.74, 55.03, 55.20, 60.55, 101.36, 104.10, 107.96, 113.66, 113.76, 128.07, 128.70, 129.47, 129.98, 131.06, 148.02, 152.27, 154.35, 159.01, 159.16, 160.07, 160.87, 167.58. For $C_{34}H_{39}N_7O_4$ calculated: 66.98% C, 6.45% H, 16.08% N; found: 66.88% C, 6.48% H, 16.04% N. MS $[M+1]^+$: *m*/*z* 610.

4.5.21. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-chlorophenyl)piperazin-1-yl]ethanone (21)

Yield 66–70%, mp 186–188 °C. IR (KBr, cm^{-1}): ν_{maks} 3020 (aromatic C–H), 2892 (aliphatic C–H), 1664 (C=O), 1581–1304 (C=C and C=N), 1264–1010 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.20 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.74–3.78 (m, 8H, piperazine-H and OCH_3), 3.91 (br s, 2H, piperazine-H), 3.96 (br s, 2H, piperazine-H), 6.83 (d, 1H, *J*: 8.0 Hz,

Ar-H), 6.89–6.97 (m, 6H, Ar-H), 7.21 (t, 1H, *J*: 8.5 Hz, Ar-H), 7.33 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.47 (d, 2H, *J*: 7.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.85, 43.07, 43.65, 44.74, 47.62, 52.17, 55.03, 55.21, 60.43, 113.56, 113.66, 113.76, 114.46, 117.93, 128.07, 128.70, 129.99, 130.28, 131.06, 133.69, 148.02, 152.12, 154.34, 159.02, 159.16, 160.88, 167.54. For $C_{33}H_{36}ClN_7O_3$ calculated: 64.54% C, 5.91% H, 15.96% N; found: 64.59% C, 6.01% H, 16.03% N. MS $[M+1]^+$: *m*/*z* 614.

4.5.22. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-methoxyphenyl)piperazin-1-yl]ethanone (22)

Yield 66–69%, mp 113–115 °C. IR (KBr, cm^{-1}): ν_{maks} 3034 (aromatic C–H), 2886 (aliphatic C–H), 1668 (C=O), 1574–1307 (C=C and C=N), 1243–1011 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.65 (br s, 4H, piperazine-H), 3.06 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.65 (br s, 5H, piperazine-H and OCH_3), 3.75–3.79 (m, 8H, piperazine-H and OCH_3), 3.91 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 6.83 (d, 2H, *J*: 8.0 Hz, Ar-H), 6.91–6.96 (m, 6H, Ar-H), 7.33 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.47 (d, 2H, *J*: 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.93, 43.13, 43.72, 44.82, 48.07, 52.29, 55.09, 55.27, 56.0, 60.55, 113.74, 113.84, 116.82, 122.30, 128.15, 128.56, 128.78, 130.06, 131.14, 149.77, 154.42, 159.09, 159.24, 160.96, 167.63. For $C_{34}H_{39}N_7O_4$ calculated: 66.98% C, 6.45% H, 16.08% N; found: 66.87% C, 6.34% H, 16.03% N. MS $[M+1]^+$: *m*/*z* 610.

4.5.23. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-chlorophenyl)piperazin-1-yl]ethanone (23)

Yield 69–73%, mp 110–113 °C. IR (KBr, cm^{-1}): ν_{maks} 3033 (aromatic C–H), 2877 (aliphatic C–H), 1663 (C=O), 1588–1305 (C=C and C=N), 1252–1018 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.16 (br s, 4H, piperazine-H), 3.29 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.73–3.78 (m, 8H, piperazine-H and OCH_3), 3.90 (br s, 2H, piperazine-H), 3.96 (br s, 2H, piperazine-H), 6.91–6.96 (m, 6H, Ar-H), 7.23 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.33 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.47 (d, 2H, *J*: 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.89, 42.97, 43.48, 44.51, 52.39, 55.03, 55.08, 55.22, 113.68, 113.78, 114.17, 117.41, 128.06, 128.68, 129.99, 131.07, 131.54, 132.09, 149.12, 151.32, 154.65, 159.22, 159.69, 160.49, 167.31. For $C_{33}H_{36}ClN_7O_3$ calculated: 64.54% C, 5.91% H, 15.96% N; found: 64.59% C, 6.05% H, 16.04% N. MS $[M+1]^+$: *m*/*z* 614.

4.5.24. 1-[4-(5,6-Bis(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-nitrophenyl)piperazin-1-yl]ethanone (24)

Yield 66–72%, mp 210–213 °C. IR (KBr, cm^{-1}): ν_{maks} 3048 (aromatic C–H), 2895 (aliphatic C–H), 1669 (C=O), 1572–1296 (C=C and C=N), 1241–1013 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.49 (br s, 4H, piperazine-H), 3.65 (br s, 2H, piperazine-H), 3.74–3.78 (m, 8H, piperazine-H and OCH_3), 3.91 (br s, 2H, piperazine-H), 3.97 (br s, 2H, piperazine-H), 6.92–6.96 (m, 4H, Ar-H), 7.04 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.32 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.47 (d, 2H, *J*: 8.5 Hz, Ar-H), 8.07 (d, 2H, *J*: 7.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.83, 43.06, 43.64, 44.71, 46.29, 51.89, 55.03, 55.20, 60.10, 112.54, 113.66, 113.76, 125.59, 128.07, 128.70, 129.99, 131.06, 136.78, 148.02, 154.33, 154.62, 159.01, 159.16, 160.89, 167.47. For $C_{33}H_{36}N_8O_5$ calculated: 63.45% C, 5.81% H, 17.94% N; found: 63.49% C, 5.87% H, 17.84% N. MS $[M+1]^+$: *m*/*z* 625.

4.5.25. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-(4-phenylpiperazin-1-yl]ethanone (25)

Yield 69–74%, mp 193–194 °C. IR (KBr, cm^{-1}): ν_{maks} 3018 (aromatic C–H), 2869 (aliphatic C–H), 1664 (C=O), 1582–1319 (C=C and C=N), 1212–975 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 3.17 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.96 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.78 (t, 1H, *J*: 7.5 Hz, Ar-H), 6.94 (d, 2H, *J*: 8.0 Hz, Ar-H), 7.21 (t, 2H, *J*: 8.5 Hz, Ar-H), 7.41 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.45–7.48 (m, 5H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.81, 43.05, 43.66, 44.71, 48.17, 52.39, 60.56, 115.29, 118.73, 128.41, 128.79, 130.59, 131.24, 133.15, 134.65, 134.75, 135.22, 143.75, 135.22, 147.15, 150.88, 154.25, 159.10, 167.60. For $C_{31}H_{31}Cl_2N_7O$ calculated: 63.26% C, 5.31% H, 16.66% N; found: 63.29% C, 5.37% H, 16.64% N. MS $[M+1]^+$: *m*/*z* 588.

4.5.26. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-methoxyphenyl)piperazin-1-yl]ethanone (26)

Yield 65–70%, mp 197–200 °C. IR (KBr, cm^{-1}): ν_{maks} 3034 (aromatic C–H), 2896 (aliphatic C–H), 1669 (C=O), 1582–1301 (C=C and C=N), 1268–1002 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 2.99 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.78 (br s, 2H, piperazine-H and OCH_3), 3.94 (br s, 2H, piperazine-H), 4.0 (br s, 2H, piperazine-H), 6.85–6.97 (m, 4H, Ar-H), 7.41 (d, 2H, *J*: 7.5 Hz, Ar-H), 7.46–7.50 (m, 6H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.82, 43.05, 43.67, 44.73, 50.01, 52.69, 55.22, 60.76, 111.81, 117.85, 120.70, 122.31, 128.41, 130.59, 131.24, 133.16, 134.65, 134.75, 135.23, 141.06, 147.15, 151.87, 154.25, 159.09, 167.68. For $C_{32}H_{33}Cl_2N_7O_2$ calculated: 62.14% C, 5.38% H, 15.85% N; found: 62.19% C, 5.33% H, 15.74% N. MS $[M+1]^+$: *m*/*z* 618.

4.5.27. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(2-chlorophenyl)piperazin-1-yl]ethanone (27)

Yield 68–71%, mp 199–200 °C. IR (KBr, cm^{-1}): ν_{maks} 3036 (aromatic C–H), 2868 (aliphatic C–H), 1658 (C=O), 1563–1325 (C=C and C=N), 1252–976 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.65 (br s, 4H, piperazine-H), 3.12 (br s, 4H, piperazine-H), 3.30 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 4.01 (br s, 2H, piperazine-H), 7.04 (t, 1H, *J*: 7.5 Hz, Ar-H), 7.17 (d, 1H, *J*: 8.0 Hz, Ar-H), 7.29 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.41 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.47–7.50 (m, 7H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.81, 43.05, 43.67, 44.71, 50.79, 52.55, 60.54, 120.78, 123.77, 127.50, 127.94, 128.43, 130.21, 130.59, 131.25, 133.16, 134.66, 134.75, 135.24, 147.17, 148.85, 154.27, 159.11, 167.64. For $C_{31}H_{30}Cl_3N_7O$ calculated: 59.77% C, 4.85% H, 15.74% N; found: 59.79% C, 4.87% H, 15.64% N. MS $[M+1]^+$: *m*/*z* 622.

4.5.28. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-methoxyphenyl)piperazin-1-yl]ethanone (28)

Yield 71–72%, mp 182–186 °C. IR (KBr, cm^{-1}): ν_{maks} 3019 (aromatic C–H), 2873 (aliphatic C–H), 1668 (C=O), 1575–1298 (C=C and C=N), 1232–1021 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.59 (br s, 4H, piperazine-H), 3.15 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.71 (s, 3H, OCH_3), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.98 (br s, 2H, piperazine-H), 6.36 (d, 1H, *J*: 8.0 Hz, Ar-H), 6.45 (br s, 1H, *J*: 8.0 Hz, Ar-H), 6.54 (d, 1H, *J*: 8.5 Hz, Ar-H), 7.11 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.40–7.47 (m, 8H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.82, 43.05, 43.66, 44.70, 48.13, 52.36, 54.74, 60.55, 101.38, 104.08, 107.96, 128.42, 129.47, 130.59, 131.23, 133.15, 134.65, 134.75, 135.22, 147.15, 152.26, 154.26, 159.11, 160.07, 167.60. For $C_{32}H_{33}Cl_2N_7O_2$ calculated: 62.14% C, 5.38% H, 15.85% N; found: 61.19% C, 5.34% H, 15.84% N. MS $[M+1]^+$: *m*/*z* 619.

4.5.29. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(3-chlorophenyl)piperazin-1-yl]ethanone (29)

Yield 70–74%, mp 160–262 °C. IR (KBr, cm^{-1}): ν_{maks} 3032 (aromatic C–H), 2876 (aliphatic C–H), 1666 (C=O), 1574–1300 (C=C and C=N), 1254–1005 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6,

ppm): δ 2.59 (br s, 4H, piperazine-H), 3.20 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.65 (br s, 2H, piperazine-H), 3.76 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.78 (d, 1H, *J*: 7.5 Hz, Ar-H), 6.91 (d, 1H, *J*: 8.5 Hz, Ar-H), 6.95 (br s, 1H, Ar-H), 7.21 (t, 1H, *J*: 8.0 Hz, Ar-H), 7.41 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.45–7.49 (m, 6H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.82, 43.05, 43.66, 44.70, 47.61, 52.17, 60.41, 113.56, 114.46, 117.94, 128.42, 130.27, 130.59, 131.23, 133.16, 133.69, 134.65, 134.75, 135.23, 147.15, 152.11, 154.26, 159.10, 167.58. For $C_{31}H_{30}Cl_3N_7O$ calculated: 59.77% C, 4.85% H, 15.74% N; found: 59.75% C, 4.87% H, 15.64% N. MS $[M+1]^+$: *m*/*z* 623.

4.5.30. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-methoxyphenyl)piperazin-1-yl]ethanone (30)

Yield 71–76%, mp 187–190 °C. IR (KBr, cm^{-1}): ν_{maks} 3027 (aromatic C–H), 2900 and 2825 (aliphatic C–H), 1646 (C=O), 1525–1379 (C=C and C=N), 1252–974 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 3.04 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.68 (s, 3H, OCH_3), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.81 (d, 2H, *J*: 9.0 Hz, Ar-H), 6.89 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.40 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.45–7.49 (m, 6H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.83, 43.05, 43.66, 44.71, 49.57, 52.50, 55.06, 60.61, 114.11, 117.27, 128.41, 130.59, 131.24, 133.15, 134.65, 134.75, 135.22, 145.26, 147.15, 152.79, 154.26, 159.10, 167.63. For $C_{32}H_{33}Cl_2N_7O_2$ calculated: 62.14% C, 5.38% H, 15.85% N; found: 62.20% C, 5.37% H, 15.74% N. MS $[M+1]^+$: *m*/*z* 619.

4.5.31. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-chlorophenyl)piperazin-1-yl]ethanone (31)

Yield 72–75%, mp 199–203 °C. IR (KBr, cm^{-1}): ν_{maks} 3025 (aromatic C–H), 2877 (aliphatic C–H), 1662 (C=O), 1581–1310 (C=C and C=N), 1259–1003 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.60 (br s, 4H, piperazine-H), 3.16 (br s, 4H, piperazine-H), 3.31 (s, 2H, –CO–CH_2), 3.66 (br s, 2H, piperazine-H), 3.76 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 3.99 (br s, 2H, piperazine-H), 6.95 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.23 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.41 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.46–7.50 (m, 6H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.81, 43.05, 43.66, 44.70, 47.98, 52.21, 60.47, 116.74, 122.22, 128.42, 128.43, 128.48, 130.59, 131.24, 133.16, 134.65, 134.75, 135.24, 147.16, 149.68, 154.26, 159.11, 167.58. For $C_{31}H_{30}Cl_3N_7O$ calculated: 59.77% C, 4.85% H, 15.74% N; found: 59.79% C, 4.87% H, 15.64% N. MS $[M+1]^+$: *m*/*z* 623.

4.5.32. 1-[4-(5,6-Bis(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[4-(4-nitrophenyl)piperazin-1-yl]ethanone (32)

Yield 74–78%, mp 210–214 °C. IR (KBr, cm^{-1}): ν_{maks} 3024 (aromatic C–H), 2856 (aliphatic C–H), 1659 (C=O), 1591–1309 (C=C and C=N), 1244–1010 (C–N). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.61 (br s, 4H, piperazine-H), 3.32 (s, 2H, –CO–CH_2), 3.49 (br s, 4H, piperazine-H), 3.66 (br s, 2H, piperazine-H), 3.77 (br s, 2H, piperazine-H), 3.94 (br s, 2H, piperazine-H), 4.01 (br s, 2H, piperazine-H), 7.04 (d, 2H, *J*: 9.0 Hz, Ar-H), 7.41 (d, 2H, *J*: 8.5 Hz, Ar-H), 7.45–7.49 (m, 6H, Ar-H), 8.08 (d, 2H, *J*: 9.5 Hz, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 40.81, 43.04, 43.64, 44.66, 45.04, 46.26, 51.88, 60.05, 112.56, 125.60, 128.43, 130.59, 131.23, 133.17, 134.65, 134.74, 135.24, 136.80, 147.16, 154.26, 154.61, 159.11, 167.48. For $C_{31}H_{30}Cl_2N_8O_3$ calculated: 58.77% C, 4.77% H, 17.69% N; found: 58.81% C, 4.67% H, 17.64% N. MS $[M+1]^+$: *m*/*z* 634.

4.6. Experimental procedure for antiproliferative activity

4.6.1. Cytotoxicity test

The tetrazolium salt XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) can be used to measure the metabolic activity of viable cells. Tetrazolium salts are reduced to formazan by mitochondrial succinate dehydrogenase, an enzyme which is only active in cells with an intact metabolism and respiratory chain. The formazan is quantified photometrically and correlates with the number of viable cells.[33] NIH/3T3 and MCF-7 cells line were used for cytotoxicity tests. NIH/3T3 and MCF-7 cells were incubated in RPMI medium (Hyclone, Thermo Scientific, USA) supplemented with fetal calf serum (Hyclone, Thermo Scientific, USA), 100 IU/mL penicillin and 100 mg/mL streptomycin (Hyclone, Thermo Scientific, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. NIH/3T3 and MCF-7 cells were seeded at 2×10^4 cells into each well of 96-well plates. After 24 h of incubating period, the culture mediums were removed and compound was added to culture medium at 3.9–500 μg/ml concentrations. After 24 h of incubation, cytotoxicity test was performed using the In Cytotox-XTT 1 Parameter Cytotoxicity Kit (Xenometrix AG, Gewerbertrasse, Switzerland), which measures mitochondrial activity in NIH/3T3 and MCF-7 cells. Firstly, the cells were washed phosphate buffer saline (PBS) and were added 200 μL/well of fresh culture medium. XTTI and XTTII solution were mixed at 1:100 ratio. Then, 50 μL of this mixture was added to all wells. The plate was incubated for 3 h at 37 °C, 5% CO_2. After 3 h, the content of the well was mixed by pipetting up and down. Then, OD of the plate was read at 480 nm with a reference wave length at 680 nm. Inhibition % was calculated each concentration of compound. IC_{50} value was estimated by non-linear regression analysis. Cisplatin was used as positive control. Stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium. The final DMSO concentration was under 0.1%. All experiments were performed in duplicate.

4.6.2. DNA synthesis inhibition assay

The 5-bromo-2′-deoxy-uridine (BrdU) cell proliferation assay was used to detect the proliferation of MCF-7 cells. Sensitive and reproducible colorimetric alternative to quantitate cell proliferation based on the measurement of BrdU incorporation during DNA synthesis in proliferating cells. This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells.[34] This study was performed on six compounds **2**, **5**, **7**, **10**, **14**, and **28**, which were determined to have the highest selective cytotoxic activity by the XTT test. Cisplatin was used as positive control. MCF-7 cells were incubated with the related compounds in three different concentrations ($IC_{50}/2$, IC_{50}, and $IC_{50} \times 2$) for 24 h and 48 h. MCF-7 cells were seeded into the each well of the 96-well plates at a density of 2×10^4 cells. After 24 h of incubation, cytotoxicity test was performed using the Cell Proliferation ELISA, BrdU (Colorimetric) Kit (Roche Diagnostics GmbH, Mannheim, Germany). Firstly, BrdU solution was added and cells were reincubated 2 h at 37 °C. After reincubation, anti-BrdU-POD was added and incubated for 90 min. Then, plates were washed with PBS for three times and the cells were incubated with substrate solution until the color development was adequate. Optical density of the samples were measured at 492 nm. Finally, the percentage of the cell growth was measured versus medium controls. All experiments were performed in duplicate.

4.6.3. Flow cytometric analysis of apoptosis and necrosis using Annexin V/PI dual staining

Apoptosis is a normal physiologic process occurs during embryonic development and in maintenance of tissue homeostasis. The apoptotic process is characterized by loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA.[35,36] Apoptosis was detected by using the FITC-Annexin V Apoptosis Detection

Kit (BD, Pharmingen™, United States) according to the manufacturer's instruction. Apoptosis was induced by adding cisplatin and the related compounds **2**, **5**, **7**, **10**, **14** and **28**, which were determined to have the highest selective cytotoxic activity by the XTT test, in their IC_{50}. After 24 h incubation, cells were harvested by centrifugation at 1200*g* for 5 min at room temperature. Then, cells were rinsed with cold water twice and resuspended at a concentration of 1×10^6 cells/mL in Annexin V-FITC binding buffer. Annexin V-FITC (5 μL) and propidium iodide (PI) (5 μL) were added for staining the cells and the fluorescence was measured using a flow cytometer. The results were analyzed by using FCSExpress software to represent the percentage of normal and apoptotic cells at various stages. The areas in the diagrams represent; necrotic cells in the upper left quadrant, (Q1; Annexin V-negative/PI-positive); the late apoptotic cells in the upper right quadrant (Q2; Annexin V-positive/PI-positive); the viable cell population in the lower left quadrant (Q3; Annexin V-negative/PI-negative) and the early apoptotic cells in the lower right quadrant (Q4; Annexin V-positive/PI-negative), respectively. The experiment was performed in duplicate.

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