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ORIGINAL ARTICLE

Synthesis and antitumor testing of certain new fused triazolopyrimidine and triazoloquinazoline derivatives

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Abstract New series of 1,2,4-triazolopyrimidine and 1,2,4-triazoloquinazoline derivatives were designed, synthesized, and evaluated for their antitumor activity. Compounds **6**, **11**, **26**, **29**, **41**, **44**, **48**, **49** and **58** were tested as antitumor agents by the use of DNA-binding assay on TLC-plates, colorimetric assay for the degree of DNA-binding (Methyl green-DNA displacement assay), evaluation of antineoplastic activity against Ehrlich Ascites Carcinoma in mice, and finally modulation of apoptosis. 5-Flurouracil, vitamin C and ethidium bromide were used as positive controls in these techniques. Compound 26 proved to be the most active member of these series as antitumor agent with IC50 value of 47 ± 1. Several characteristic features were observed to be essential for activity such as the morpholine group and the phenylazo group, in addition the electron-withdrawing groups favor the activity than the electron-donating ones.
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1. Introduction

1,2,4-Triazolopyrimidine is one of the important ring systems that has drawn the attention for its different biological activities. Literature survey has revealed the progressive findings about its synthesis and antitumor activity (Hafez and El-Gazzar, 2009; Huang et al., 2012; Takunaka et al., 2005; Zhang et al., 2005). Furthermore, a series of pyrazolo[3,4-*d*]pyrimidine derivatives was found to be potentially useful as cyclin-dependant kinase inhibitors and showed antiproliferative activity on cancer cell line (Maravcova et al., 2003). Moreover, some functional groups such as substituted phenylazo and

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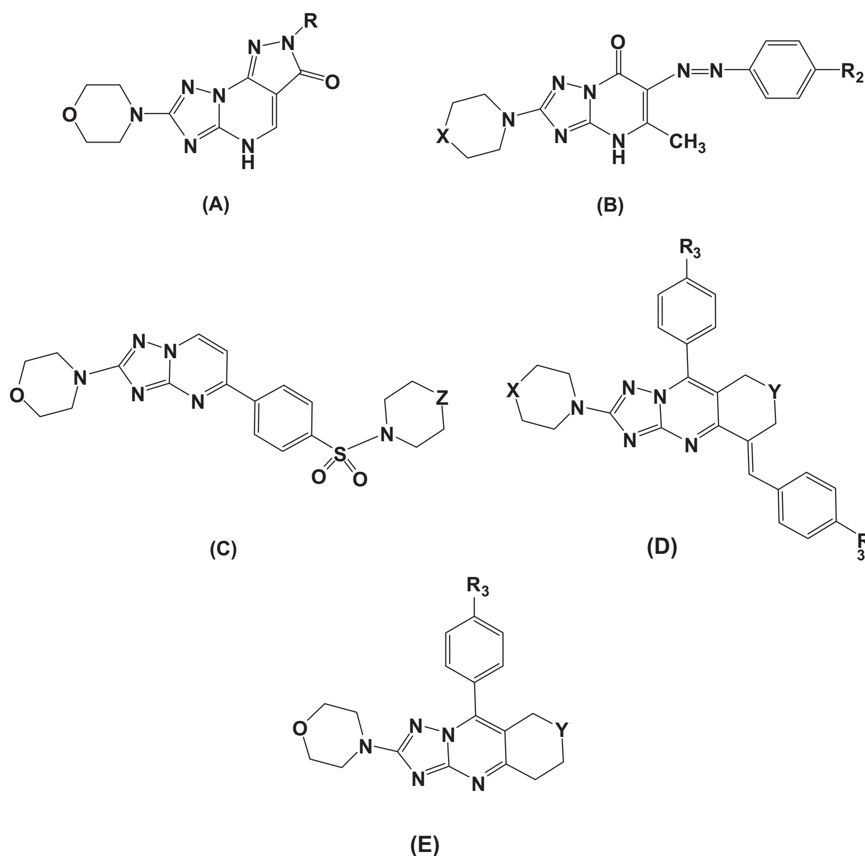


Chart 1 General structures of the proposed compounds.

sulphonylphenyl moieties were reported to enhance the antitumor activity (Saavedra et al., 2006). Furthermore, the significance of the α,β-unsaturated ketone moiety in the cytotoxic activity of certain derivatives (Al-Omar et al., 2005) and their incorporation in many fused ring systems has been reported, in addition, these derivatives proved to be active as antitumor agents (Al-Omary et al., 2012).

On the other hand, alkylating agents and intercalating agents represent two major classes of antitumor drugs that act by direct interaction with DNA. Based on this fact; methods for determination of the interaction of small molecular weight compounds with DNA by DNA-binding assay (Pezzuto et al., 1991) and methyl green-DNA displacement assay (Bronstein and Weber, 2001) have been applied. In addition, apoptosis – a programmed form of cell death by which unwanted cells are removed from the body without causing inflammation-has been tested as most anticancer drugs such as topoisomerase inhibitors, alkylating agents, antimetabolites, and hormone antagonists induce it in sensitive cells. The tendency of a cancer cell to undergo apoptosis may be especially important for the chemotherapy (Sfikakis et al., 1995).

Depending on these entire bases, it was found interesting to synthesize a series of triazolopyrimidine bearing pyrazole ring, phenylazo and sulphonylphenyl moieties to afford compounds of types (**A**–**C**), in addition to design and synthesis of new condensed triazolopyrimidine and triazoloquinazoline analogs of general formula (**D**) and (**E**). The newly synthesized compounds were evaluated for their antitumor activity through screening their ability to interact with DNA which is known as the major mechanism of action of several known antitumor agents.

2. Materials and methods

Melting points were recorded using *Fisher-Johns* melting point apparatus and are uncorrected. Microanalysis was performed in the microanalytical unit, Cairo University. IR was recorded on Mattson 5000 FT-IR spectrophotometer. ^{1}H NMR spectra were performed in (DMSO-d_6) and were obtained on FT-NMR (200 MHz) Gemini Varian spectrometer using TMS as internal standard (chemical shift in ppm, δ units) in the microanalytical unit, Cairo University. MS analyses were performed on JEOL JMS-600H spectrometer in the microanalytical unit, Cairo University. TLC plates (RP-18 F_{254}; 0.25 mm) were purchased from Merck, Germany. DNA, Ethidium bromide, Anisaldehyde, DNA–Methyl green complex, 5-Fluorouracil and ascorbic acid (vitamin C) were purchased from Sigma–Aldrich Co., USA. Methanol, dimethyl sulfoxide (DMSO) and all other chemicals are of high analytical grade, and are obtained from El Nasr Co. for Pharmaceutical Chemicals, Egypt. The cells of Ehrlich ascites tumor were obtained from the National Cancer Institute, Cairo, Egypt. Adult Swiss male albino mice (20–25 gm) of both sexes were used in this experiment. Animals were housed in microlon boxes in a controlled environment (temperature 25 ± 2 °C and 12 h dark/light cycle)

with standard laboratory diet and water regimen. Compounds **3**, **20**–**23**, **36**–**40**, **46**, **47**, **53** were previously prepared (Williams, 1962; Vejdelek et al., 1976; Al-Khamees et al., 1993; El-Subbagh et al., 2000; Dimmock et al., 2003). Yield percentage, melting points and the elemental analysis for all the newly synthesized compounds are recorded in Table 1.

2.1. Chemistry

2.1.1. Ethyl 7-chloro-2-morpholino-[1,2,4]triazolo[1,5-a]pyrimidine-6-carboxylate (**4**)

A mixture of compound **3** (Al-Khamees et al., 1993) (0.29 g, 0.001 mol) and $POCl_3$ (10 ml) was heated under reflux for 6 h. After cooling, the resultant solution was poured into ice water, and neutralized with ammonia solution. The formed precipitate was filtered, washed with water, dried and crystallized from aqueous ethanol to yield 0.11 g (35 %) of **4**, m.p. 195–8 °C. **IR**, 1755(CO). **^{1}H NMR**: δ 1.25 (t, 3H, CH_2CH_3), 2.86–2.91 (m, 4H, morpholine-H), 3.62–3.64 (m, 4H, morpholine-H), 4.31 (q, 2H, CH_2CH_3), 8.45 (s, 1H, pyrimidine-H). MS *m/z* (%); 311 (53.7, M^+), 313 (20.4, M^{+2}).

2.1.2. 7-Morpholino-5H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-a]pyrimidin-3(2H)-one (**5**)

2.1.2.1. Method A. Hydrazine hydrate 98% (2.5 g, 0.05 mol) was added dropwise to a stirred solution of compound **3** (Al-Khamees et al., 1993) (2.93 g, 0.01 mol) in absolute ethanol (15 ml) over a period of 15 min. The reaction mixture was heated under reflux for 14 h. Excess solvent was evaporated under vacuum; the formed precipitate was separated, washed with ethanol and used for the preparation of compounds **10** and **11** without further purification.

2.1.2.2. Method B. Hydrazine hydrate 98% (2.5 g, 0.05 mol) was added dropwise to a stirred solution of compound **4** (3.11 g, 0.01 mol) in absolute ethanol (15 ml) over a period of 15 min. The reaction mixture was heated under reflux for 10 h. Excess solvent was evaporated under vacuum; the formed precipitate was separated, washed with ethanol and used for the preparation of same compounds. **5** ^{1}H NMR; δ 2.54–2.58 (m, 4H, morpholine-H), 3.68–3.84 (m, 4H, morpholine-H), 7.55 (brs, 1H, NH-pyrimidine, D_2O-exchang), 8.48 (brs, 1H, NH-pyrazole, D_2O-exchang.), 10.03 (s, 1H, pyrimidine-H). MS *m/z* (%); 261 (1.25, M^+), 43 (100).

2.1.3. 7-Morpholino-2-substituted phenyl-5H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-a]pyrimidin-3(2H)-ones (**6**–**9**)

Compound **3** (2.93 g, 0.01 mol) was added portionwise to a stirred solution of the appropriate phenyl hydrazine (0.015 mol) in ethanol (50 ml). The reaction mixture was heated under reflux for 16-18 h. On cooling, the separated solid was filtered, washed with ice water, dried and crystallized from ethanol. Yield percentage and melting points are recorded in Table 1. **6** ^{1}H NMR: δ 2.61–2.63 (m, 4H, morpholine-H), 3.75–3.89 (m, 4H, morpholine-H), 6.68–7.12 (m, 5H, Ar–H), 7.61 (brs, 1H, NH-pyrimidine, D_2O-exchang), 9.58 (s, 1H, pyrimidine-H). MS *m/z* (%); 337 (0.54, M^+), 128 (100). **7** ^{1}H NMR: δ 2.51–2.54 (m, 4H, morpholine-H), 3.78–3.85 (m, 4H, morpholine-H), 6.53–6.92 (m, 4H, Ar-H), 7.65 (brs, 1H, NH-pyrimidine, D_2O-exchang.), 9.43 (s, 1H, pyrimidine-H). MS *m/z* (%); 371 (0.54, M^+), 373 (0.21, M^{+2}). **8** ^{1}H NMR: δ 2.49–2.53 (m, 4H, morpholine-H), 3.66–3.76 (m, 4H, morpholine-H), 4.15 (s, 3H, OCH_3), 5.44 (brs, 1H, NH-pyrimidine, D_2O-exchang.), 6.83–6.91 (m, 4H, Ar–H), 8.45 (s, 1H, pyrimidine-H). MS *m/z* (%); 367 (3.41, M^+). **9** ^{1}H NMR: δ 2.64–2.69 (m, 4H, morpholine-H), 3.75–3.83 (m, 4H, morpholine-H), 6.37–6.68 (m, 3H, Ar–H), 7.42 (brs, 1H, NH-pyrimidine, D_2O-exchang.), 8.96 (s, 1H, pyrimidine-H). MS *m/z* (%); 427 (3.41, M^+).

2.1.4. 7-Morpholino-2-((4-substituted) phenyl)sulphonyl-5H-pyrazolo[4,3-e][1,2,4]triazolo [1,5-a]pyrimidin-3(2H)-ones (**10**, **11**)

A mixture of compound **5** (0.261 g, 0.001 mol) and the appropriate benzenesulphonyl chloride (0.0015 mol) was refluxed in pyridine (10 ml) for 6 h. After cooling, the produced solution was poured into ice water. The separated solid was filtered, washed with water and dried. Yield percentage and melting points are recorded in Table 1. **10** ^{1}H NMR: δ 2.76–2.92 (m, 4H, morpholine-H), 3.39–3.63 (m, 4H, morpholine-H), 7.85–8.21 (m, 6H, Ar–H, NH-pyrimidine, D_2O-exchang.), 8.51 (s, 1H, pyrimidine-H). MS *m/z* (%); 401 (1.63, M^+), 114 (100). **11** ^{1}H NMR; δ 2.19 (s, 3H, CH_3), 2.84–2.96 (m, 4H, morpholine-H), 3.35–3.69 (m, 4H, morpholine-H), 8.21 (m, 5H, Ar–H, NH-pyrimidine, D_2O-exchang.), 8.64 (s, 1H, pyrimidine-H). MS *m/z* (%); 415 (2.48, M^+).

2.1.5. 5-Methyl-2-morpholino (or 4-methylpiperazino)-6-(4-substituted phenylazo)-4H,7H-[1,2,4]triazolo [1,5-a]pyrimidin-7-ones (**25**–**29**)

A mixture of the appropriate ethyl 2-(4-substituted phenylhydrazono)-3-oxobutanoates (**20**–**23**) (Williams, 1962,Vejdelek et al., 1976) (0.001 mol) and 5-amino-3-substituted-1*H*-1;2,4-triazoles (**1**, **24**) (0.001 mol) was refluxed in glacial acetic acid (10 ml) for 5 h. After cooling, the reaction mixture was concentrated under vacuum. The obtained solid was filtered, dried and crystallized from petroleum ether. Yield percentage and melting points are recorded in Table 1. **25**: ^{1}H NMR; δ 2.04 (s, 3H, CH_3), 2.64–2.68 (m, 4H, morpholine-H), 3.56–3.63 (m, 4H, morpholine-H), 7.83–8.20 (m, 4H, Ar–H), 8.24 (brs, 1H, NH-pyrimidine, D_2O-exchang.). MS *m/z* (%); 373 (2.26, M^+), 374 (0.23, M^{+1}), 108 (100). **26**: ^{1}H NMR; δ 2.30 (s, 3H, CH_3), 2.54–2.59 (m, 4H, morpholine-H), 3.45–3.49 (m, 4H-morpholine-H), 7.57–8.26 (m, 4H, Ar–H), 8.36 (brs, 1H, NH-pyrimidine, D_2O-exchang.). MS *m/z* (%); 384 (31.84, M^+), 279 (100). **27**: ^{1}H NMR; δ 2.22 (s, 3H, CH_3), 2.53–2.55 (m, 4H, morpholine-H), 3.73–3.82 (m, 4H, morpholine-H), 3.93 (s, 3H, OCH_3), 7.07–7.73 (m, 4H, Ar–H), 8.32 (brs, 1H, NH-pyrimidine, D_2O-exchang.). MS *m/z* (%); 369 (18.3, M^+). **28**: ^{1}H NMR; δ 2.20 (s, 3H, CH_3), 2.35 (s, 3H, N–CH_3), 2.56–2.79 (m, 8H, piperazine-H), 7.38–7.51 (m, 4H, Ar–H), 8.29 (brs, 1H, NH-pyrimidine, D_2O-exchang.). MS *m/z* (%); 431 (21.5, M^+), 433 (0.34, M^{+2}), 125 (100). **29**: ^{1}H NMR; δ 2.21 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.56–2.69 (m, 8H, piperazine-H), 4.01 (s, 3H, OCH_3), 7.51–8.05 (m, 4H, Ar–H), 8.33 (brs, 1H, NH-pyrimidine, D_2O-exchang.). MS *m/z* (%); 382 (8.3, M^+).

Table 1 Physical properties, yields, molecular formulae and elemental analysis of compounds (5–59).

No.	X	Y	Z	R	R_1	R_2	R_3	Yield %	m.p. (°C)	Molecular Formulae[a]	Analysis	
											Calc.	Found
5	O	–	–	H	–	–	–	44	Decomp.at 202	$C_{10}H_{11}N_7O_2$	C 45.98 H 4.24 N 37.53	46.02 4.51 37.84
6	O	–	–	C_6H_5	–	–	–	58	208–10	$C_{16}H_{15}N_7O_2$	C 56.97 H 4.48 N 29.07	57.11 4.65 29.25
7	O	–	–	4-Cl–C_6H_4	–	–	–	38	235–7	$C_{16}H_{14}ClN_7O_2$	C 51.69 H 3.80 N 26.37	51.93 4.11 26.72
8	O	–	–	4-$CH_3OC_6H_4$	–	–	–	49	175–9	$C_{17}H_{17}N_7O_3$	C 55.58 H 4.66 N 26.69	55.82 4.99 26.82
9	O	–	–	2,4-$(NO_2)_2C_6H_3$	–	–	–	36	246–9	$C_{16}H_{13}N_9O_6$	C 44.97 H 3.07 N 29.50	45.21 3.24 29.85
10	O	–	–	–	H	–	–	64	>300	$C_{16}H_{15}N_7O_4S$	C 47.88 H 3.77 N 24.43	48.01 3.93 24.74
11	O	–	–	–	CH_3	–	–	55	275–8	$C_{17}H_{17}N_7O_4S$	C 49.15 H 4.12 N 23.60	49.38 4.42 23.89
25	O	–	–	–	–	Cl	–	58	278–80	$C_{16}H_{16}ClN_7O_2$	C 51.41 H 4.31 N 26.23	51.58 4.72 26.30
26	O	–	–	–	–	NO_2	–	78	108–9	$C_{16}H_{16}N_8O_4$	C 50.00 H 4.20 N 29.15	50.37 4.59 29.42
27	O	–	–	–	–	OCH_3	–	53	209–11	$C_{17}H_{19}N_7O_3$	C 55.28 H 5.18 N 26.54	55.53 5.31 26.73
28	NCH_3	–	–	–	–	Br	–	66	249–50	$C_{17}H_{19}BrN_8O$	C 47.34 H 4.44 N 25.98	47.63 4.60 26.13
29	NCH_3	–	–	–	–	OCH_3	–	48	Decomp. at 195	$C_{18}H_{22}N_8O_2$	C 56.53 H 5.80 N 29.30	56.80 6.01 29.69
41	O	CH_2	–	–	–	–	H	56	265–7	$C_{26}H_{25}N_5O$	C 73.74 H 5.95 N 16.54	74.00 6.11 16.87
41	O	CH_2	–	–	–	–	Br	78	Decomp.at 240	$C_{26}H_{23}Br_2N_5O$	C 53.72 H 3.99 N 12.05	53.99 4.00 12.39
43	N–CH_3	CH_2	–	–	–	–	Cl	59	230–2	$C_{27}H_{26}Cl_2N_6$	C 64.16 H 5.18 N 16.63	64.32 5.28 16.84
44	O	N–CH_3	–	–	–	–	OCH_3	52	230–1	$C_{28}H_{30}N_6O_3$	C 67.45 H 6.06 N 16.86	67.61 6.29 17.11
45	N–CH_3	N–CH_3	–	–	–	–	H	40	210–1	$C_{27}H_{29}N_7$	C 71.81 H 6.47 N 21.71	72.09 6.77 21.94
48	O	CH_2	–	–	–	–	Cl	56	251–3	$C_{19}H_{20}ClN_5O$	C 61.70 H 5.45 N 18.94	61.91 5.75 19.14
49	O	CH_2	–	–	–	–	OCH_3	48	199–201	$C_{20}H_{23}N_5O_2$	C 65.73 H 6.34 N 19.16	65.99 6.54 19.37
54	–	–	N–CH_3	–	–	–	–	91	136–9	$C_{13}H_{18}N_2O_3S$	C 55.30 H 6.43 N 9.92	55.64 6.49 10.05
56	–	–	O	–	–	–	–	78	150–3	$C_{15}H_{20}N_2O_4S$	C 55.54 H 6.21 N 8.64	55.72 6.38 8.84

Table 1 (*continued*)

No.	X	Y	Z	R	R_1	R_2	R_3	Yield %	m.p. (°C)	Molecular Formulae[a]	Analysis Calc.	Analysis Found
57			N–CH_3				–	71	170–2	$C_{15}H_{20}N_2O_4S$	C 56.95 H 6.87 N 12.45	57.03 6.95 12.70
58	O	–	O	–	–	–	–	80	221–3	$C_{19}H_{22}N_6O_4S$	C 53.01 H 5.15 N 19.52	53.24 5.40 19.75
59	O	–	NCH_3	–	–	–	–	62	187–9	$C_{20}H_{25}N_7O_3S$	C 54.16 H 5.68 N 22.11	54.46 5.86 22.46

[a] Analyzed for C,H,N; results were within ±0.4 % of the theoretical values for the formulae given.

*2.1.6. 2-Morpholino (or 4-methylpiperazino)-5-(4-substituted benzylidene)-9-(4-substituted phenyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[5,1-b]quinazolines (**41–43**), and 7-methyl-2-morpholino (or 4-methylpiperazino)-5-(4-substituted benzylidene)-9-(4-substituted phenyl)-5,6,7,8-tetrahydro-pyrido[4,3-d][1,2,4]triazolo[1,5-a]pyrimidines (**44, 45**)*

Sodium metal (0.5 g, 0.02 mol) was added portionwise to absolute ethanol (20 ml) over a period of 15 min. To the resulted solution, the appropriate ketone (El-Subbagh et al., 2000; Dimmock et al., 2003) (**36–40**) (0.01 mol) and the appropriate triazole derivative (**1**, **24**) (0.01 mol) were added. The resulting mixture was heated at reflux for 15 h. The solvent was evaporated under reduced pressure; the solid formed was separated and crystallized from ethanol to yield compounds (**41–45**). Yield percentage and melting points are recorded in Table 1. **41**: ^{1}H NMR; δ 2.38–2.50 (m, 6H, C_6–H, C_7–H, C_8–H), 2.55–2.57 (m, 4H, morpholine-H), 3.68–3.71(m, 4H, morpholine-H), 7.45–7.88 (m, 11H, Ar–H and CH=C). MS m/z (%); 423 (100, M^+). **42**: ^{1}H NMR; δ 1.68–2.40 (m, 6H, C_6–H, C_7–H, C_8–H), 2.57–2.61 (m, 4H, morpholine-H), 3.54–3.62 (m, 4H, morpholine-H), 7.47–7.76 (m, 8H, Ar-H), 8.02 (s, 1H, CH=C). MS m/z (%); 581 (100, M^+), 583 (28.75, M^{+2}). **43**: ^{1}H NMR; δ 2.21 (s, 3H, N–CH_3), 2.45–2.46 (m, 6H, C_6–H, C_7–H, C_8–H), 2.54–3.26 (m, 8H, piperazine-H), 7.46–7.64 (m, 8H, Ar–H), 8.04 (s, 1H, CH=C). MS m/z (%); 504 (22.8, M^+). **44**: ^{1}H NMR; δ 2.29 (s, 3H, N–CH_3), 2.51–3.31 (m, 8H, morpholine-H, C_6–H, C_8–H), 3.62–3.65 (m, 4H, morpholine-H), 3.95 (s, 6H, OCH_3), 7.02–7.63 (m, 8H, Ar–H), 8.05 (s, 1H, CH=C). MS m/z (%); 498 (100, M^+). **45**: ^{1}H NMR; δ 2.23 (s, 3H, N–CH_3), 2.50 (s, 3H, N–CH_3), 2.49–3.30 (m, 12H, C_6–H, C_8–H, piperazine-H), 7.22–7.84 (m, 10H, Ar–H), 8.53 (s, 1H, CH=C). MS m/z (%); 451 (100, M^+).

*2.1.7. 2-Morpholino-9-(4-substituted phenyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolines (**48, 49**)*

Sodium metal (0.5 g, 0.02 mol) was added portionwise to absolute ethanol (20 ml) over a period of 15 min. To the resulting solution, the appropriate ketone (Dimmock et al., 2003) (**46**, **47**) (0.01 mol) and 5-amino-3-morpholino-1*H*-1,2,4-triazole (**1**) (1.69 g, 0.01 mol) were added. The resulting solution was heated under reflux for 17 h. The solvent was then evaporated under vacuum; the solid formed was filtered and crystallized from ethanol to yield compounds (**48**, **49**).Yield percentage and melting points are recorded in Table 1. **48**: ^{1}H NMR; δ 1.58–2.35 (m, 8H, C_5–H, C_6–H, C_7–H, C_8–H), 2.85–2.93 (m, 4H, morpholine-H), 3.53–3.60 (m, 4H, morpholine-H), 7.20–7.53 (m, 4H, Ar–H).MS$m/z$ (%); 369 (100, M^+), 370 (11.39, M^{+1}). **49**: ^{1}H NMR; δ 1.54–2.21 (m, 8H, C_5–H, C_6–H, C_7–H, C_8–H), 2.79–2.82 (m, 4H, morpholine-H), 3.59–3.68 (m, 4H, morpholine-H), 3.93 (s, 3H, OCH_3) 6.88–7.43 (m, 4H, Ar–H). MS m/z (%); 365 (100, M^+).

*2.1.8. 1-(4-(4-methylpiperazin-1-ylsulfonyl)phenyl)ethanone (**54**)*

A mixture of 4-acetylbenzene sulphonylchloride (**50**, 2.18 g, 0.01 mol), 1-methyl piperazine (**52**, 2.53 g, 0.01 mol) and catalytic amount of triethylamine (three drops) in toluene (10 ml) was heated to reflux for 7 h. The solvent was evaporated under vacuum and the remaining solid was crystallized from ethanol to yield the desired compounds. Yield percentage and melting points are recorded in Table 1. **54**: ^{1}H NMR; δ 2.21 (s, 3H, N–CH_3), 2.44 (s, 3H, CH_3), 2.49–2.75 (m, 4H, piperazine-H), 3.25–3.55 (m, 4H, piperazine-H), 7.85–8.22 (m, 4H, Ar–H). MS m/z (%); 282 (3.15, M^+)

*2.1.9. 3-Dimethylamino-1-(4-(substituted sulphonyl)phenyl)prop-2-en-1-ones (**56, 57**)*

A mixture of the appropriate acetophenones (**53**, **54**) (0.01 mol) and DMF-DMA (**55**) (10 ml) was heated under reflux for 7 h. After cooling, the reaction mixture was evaporated under vacuum; the produced solid was separated and crystallized from ethanol to yield the desired compounds. Yield percentage and melting points are recorded in Table 1. **56**: ^{1}H NMR; δ 2.55–2.83 (m, 4H, morpholine-H), 3.35 (s, 6H, CH_3), 3.63–3.71 (m, 4H, morpholine-H), 5.94 (d, 2H, CH=CH), 7.76–8.13 (m, 4H, Ar–H). MS m/z (%); 324 (34.18, M^+). **57**: ^{1}H NMR; δ 2.24 (s, 3H, N-CH_3), 2.61–2.75 (m, 4H, piperazine-H), 3.01 (s, 6H, CH_3), 3.68–3.87 (m, 4H, piperazine-H), 5.37 (d, 2H, CH=CH), 7.41–8.08 (m, 4H, Ar–H). MS m/z (%); 337 (100, M^+).

*2.1.10. 2-Morpholino-5-(4-(substituted sulphonyl)phenyl)-[1,2,4]triazolo[1,5-a] pyrimidines (**58, 59**)*

A mixture of the appropriate compound (**56**, **57**) (0.01 mol), 5-amino-3-morpholino-1*H*-1,2,4-triazole (**1**) (1.69 gm, 0.01 mol) and sodium acetate (0.5 gm) in glacial acetic acid (10 ml) was heated under reflux for 10 h. The solvent was then concentrated under reduced pressure and the solid formed was filtered, washed with water and crystallized from ethanol to

yield the desired products. Yield percentage and melting points are recorded in Table 1. **58**: ^{1}H NMR; δ 2.55–2.83 (m, 8H, morpholine-H), 3.63–3.71 (m, 8H, morpholine-H), 7.47 (d, 1H, C_6–H), 7.95–8.52 (m, 4H, Ar–H), 8.69 (d, 1H, C_7–H). MS m/z (%); 430 (51.0, M^+). **59**: ^{1}H NMR; δ 2.21 (s, 3H, N–CH_3), 2.55–2.83 (m, 8H, morpholine-H, piperazine-H), 3.39–3.66 (m, 8H, morpholine-H, piperazine-H), 7.43 (d, 1H, C_6–H), 7.95–8.52 (m, 4H, Ar–H), 8.64 (d, 1H, C_7–H). MS m/z (%); 443 (1.3, M^+).

2.2. DNA-binding assay on TLC-plates

The TLC plates used in the assay were pre-developed using methanol:water (8:2). Compounds **6**, **11**, **26**, **29**, **41**, **44**, **48**, **49** and **58** were then applied (5 mg/ml in methanol) at the origin, followed by spotting of DNA (1 mg/ml in methanol/water mixture (8:2)) at the same positions at the origin. Ethidium bromide was used as a positive control. After complete spotting, the plates were developed with the same solvent system, and the positions of DNA were visualized by spraying the plates with anisaldehyde, which produces a blue color with DNA. The intensity of the color was proportional to the quantity of DNA added to the plate (Pezzuto et al., 1991).

2.3. Colorimetric assay for the degree of DNA-binding (Methyl green-DNA displacement assay)

DNA–methyl green complex (20 mg) was suspended in 100 ml of 0.05 M Tris–HCl buffer (pH 7.5) containing 7.5 mmole $MgSO_4$ and stirred at 37 °C. The calculated amounts of samples were placed in Eppendorff tubes, and 200 µl of the DNA/methyl green solution was added to each tube. The samples were incubated in the dark at ambient temperature for 24 h, and the final absorbance of each sample was determined at 630 nm. The results were recorded in the form of the IC_{50} of each compound, which is the sample concentration required to produce 50% decrease in the initial absorbance of the DNA–methyl green complex. (Table 2) (Bronstein and Weber, 2001).

2.4. Evaluation of antineoplastic activity against Ehrlich Ascite Carcinoma in mice

The animals were classified into four groups as shown in Table 3. Animals in the treated groups (from 2 to 4) were inoculated with 2×10^6 Ehrlich ascites cells/mouse on the day zero. Treatment was started 24 h after inoculation by intra-protenial (I.P.) injection of the drug. The animals in the group (3) were injected by compound **26** in a dose of 20 mg/kg of body weight, the standard group (group 4) has also received I.P. treatment with 20 mg/kg of body weight of 5-fluorouracil (5-FU). The control group (2) was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given for nine successive days (Table 3) (Hazra et al., 2002).

Table 2 The IC_{50} values of compounds **6**, **11**, **26**, **29**, **41**, **44**, **48**, **49**, **57**.

Compound No.	DNA/methylgreenic$_{50}$, MG/ML
26	47 ± 1
29	59 ± 2
44	63 ± 2
41	63 ± 2
6	66 ± 2
58	67 ± 1
11	69 ± 2
49	71 ± 2
48	73 ± 2
Ethidium Br	1.7 ± 2

Table 3 Grouping of animals in Ehrlich Ascites Carcinoma test.

Group No.	Treatment (I.P.)	No. of mice
1	Normal (untreated)	10
2	Control (Ehrlich only)	10
3	**26** (20 mg/kg)	10
4	**5-FU** (20 mg/kg)	10

2.5. Modulation of apoptosis

Neutrophils (> 98% pure on May-Giemsa stain) were isolated from peripheral blood of normal healthy volunteer donors and from osteoarthritic and rheumatoid arthritic patients by a combination of dextran sedimentation and centrifugation through discontinuous plasma percol gradients. These neutrophils were then re-suspended with 10% autologous platelet rich plasma derived serum (PRPDS) and 100 µg/l of penicillin and streptomycin and divided into five equal volumes in separate culture tubes. Cells were incubated (at 37 °C in a 5% carbon dioxide) as follows: (1) Only cells, (2) Cells + DMSO at 0.01% v/v., (3) Cells + each compound in DMSO at a dose of 50 mmole/ml culture. (The age of neutrophils in culture was calculated while starting the culture at time zero (or base line), 24, 48 and 72 h). At the determined times, cells were removed from culture and counted on a hemocytometer. Cell viability was determined by trypan blue dye exclusion test; one volume of trypan blue (0.4% GiBCo) was added to five volumes of cells at room temperature for 5 min. The neutrophil apoptosis in each culture was assessed: (i) Morphology (Giemsa and Acridine orange stains), (ii) DNA Fragmentation. For assessment of the percentage of cells showing morphology of apoptosis 500 cells/slide were examined for each case at different times (0, 24, 48, and 72 h) in the presence or absence of the drugs used. Neutrophils were considered apoptotic if they exhibited the highly characteristic morphological features of chromatin aggregation, nuclear pyknosis and cytoplasmic vaculation. The apoptotic neutrophil percentage at different times was calculated after addition of tested compounds and the results were then compared statistically using the F-test and student's t-test (Kumagai et al., 1995). Secondly, assessment of chromatin fragmentation in neutrophils was done by the modification of methods previously used for thymocytes (Lahti et al., 1995).

3. Results and discussion

3.1. Chemistry

The strategy for the synthesis of the target compounds is illustrated in schemes 1-5. 5-Amino-3-morpholino-1H-1,2,4-triazole

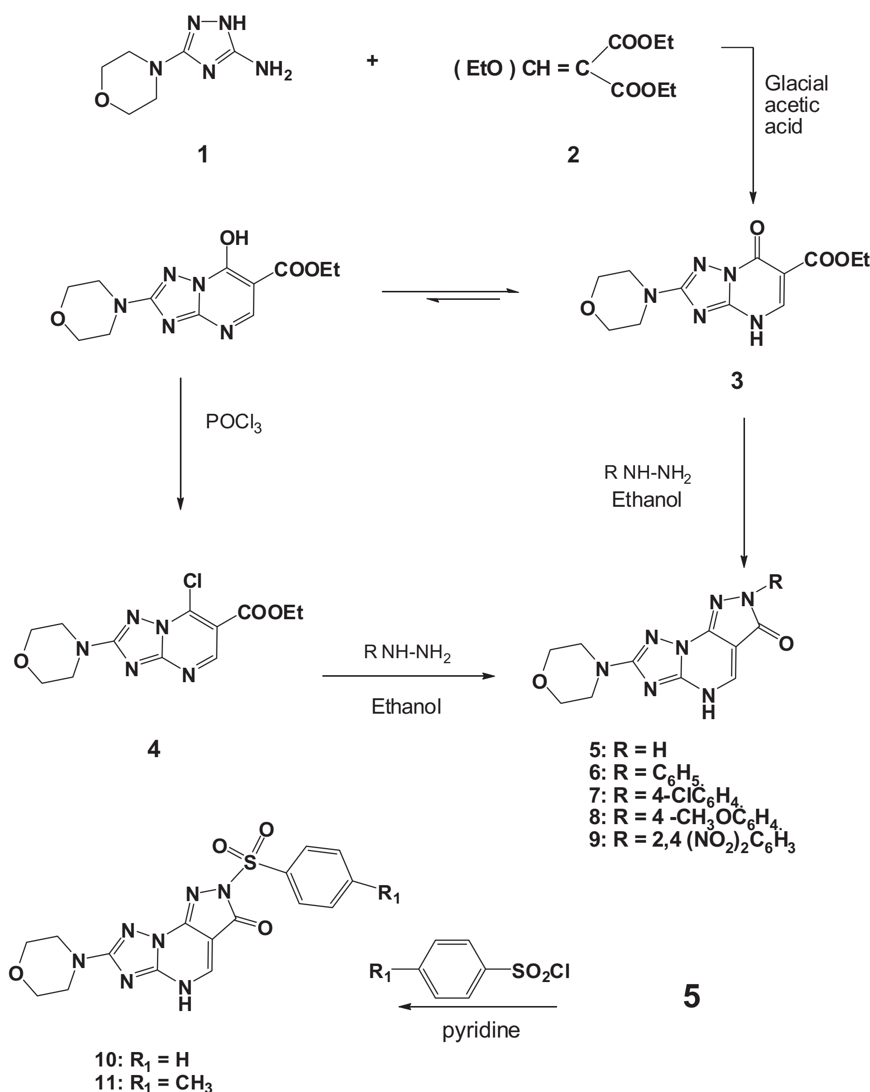
(**1**) was allowed to react with diethyl ethoxymethylenemalonate (**2**) in glacial acetic acid to afford the target compound ethyl 2-morpholino-7-oxo-4*H*,7*H*-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxylate (**3**) in 79% yield. The hydroxyl group of compound (**3**) was replaced with chlorine by refluxing in phosphorus oxychloride to produce ethyl 7-chloro-2-morpholino-[1,2,4]triazolo[1,5-*a*] pyrimidine-6-carboxylate (**4**). 7-morpholino-2-(un)substituted-5*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*a*] pyrimidin-3(2*H*)-ones (**5**–**9**) were prepared *via* either the reaction of compound **3** with either hydrazine hydrate or (substituted) phenyl hydrazine in ethanol, or by the reaction of compound **4** with the same reagents in ethanol. 7-Morpholino-5*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*a*] pyrimidin-3(2*H*)-one (**5**) was allowed to react with the appropriate benzenesulphonyl chloride in pyridine to obtain 7-morpholino-2-(substituted phenyl)sulphonyl-5*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*a*]pyrimidin-3(2*H*)-ones (**10**, **11**) in yields of 64 and 55%, respectively (Scheme 1). (see Chart 1)

5-Methyl-2-substituted-6-(4-substituted phenylazo)-4*H*,7*H*-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-ones (**25**–**29**) were prepared by the condensation of the substituted aminotriazoles (**1**, **24**) with ethyl 2-(4-substituted phenylhydrazono)-3-oxobutanoates (**20**–**23**) in glacial acetic acid to give the required compounds in 48–78% yields (Scheme 2).

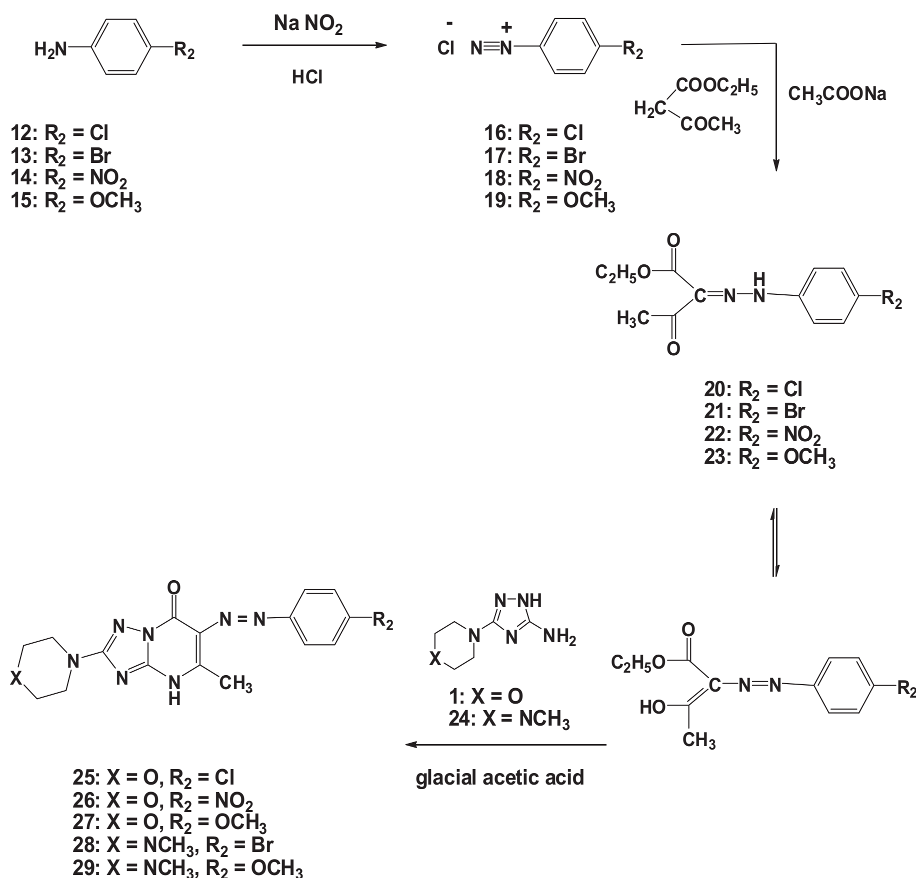
Compounds **41**–**45** were prepared *via* the condensation of either 2,6-*bis*(4-substituted benzylidene)cyclohexanones (**36**–**38**) or 3,5-*bis*(4-substituted benzylidene)-1-methylpiperidin-4-ones (**39**, **40**) with aminotriazoles (**1**, **24**) in freshly prepared sodium ethoxide (Scheme 3).

For the preparation of 2-(substituted benzylidene)cyclohexanones, different molar ratios of the starting materials were used. 2-(4-Substituted benzylidene)cyclohexanones (**46**, **47**) were obtained through reacting cyclohexanone (**30**) and the appropriate aldehyde (**33**, **35**) in a molar ratio of 7:1 for 24 h. Compounds **48** and **49** were prepared by following the same procedure for the preparation of compounds **41**–**45** (Scheme 4).

4-(Substituted sulphonyl)acetophenones (**53**, **54**) were prepared according to the procedure mentioned by Press et al., 1981. A mixture of 4-acetyl benzenesulphonylchloride (**50**) was refluxed with either morpholine (**51**) or *N*-methylpiperazine (**52**) in toluene in the presence of the catalytic amount



Scheme 1 Synthesis of the target compounds **5**–**11**.



Scheme 2 Synthesis of the target compounds **25–29**.

of triethylamine to obtain the target compounds in yield of 88 and 91% respectively (Vejdelek et al., 1976). Refluxing 4-(substituted sulphonyl)acetophenones (**53** and **54**) in an excess of DMFDMA (**55**) yielded compounds **56** and **57** in yield 78 and 71% respectively. The condensation between 5-amino-3-morpholino-1*H*-1,2,4-triazole (**1**) and 3-dimethyl-amino-1-(4-(substituted sulphonyl)phenyl)prop-2-en-1-ones (**56** and **57**) in refluxing glacial acetic acid in the presence of sodium acetate yielded the desired compounds **58** and **59** (Scheme 5).

3.2. Biological screening

Twenty compounds of the newly synthesized ones have been subjected to DNA-binding assay and methyl green-DNA displacement assay in order to study the differences in biological response that might result upon incorporating the triazole moiety into different cyclic structures.

3.2.1. DNA-binding assay on TLC-plates

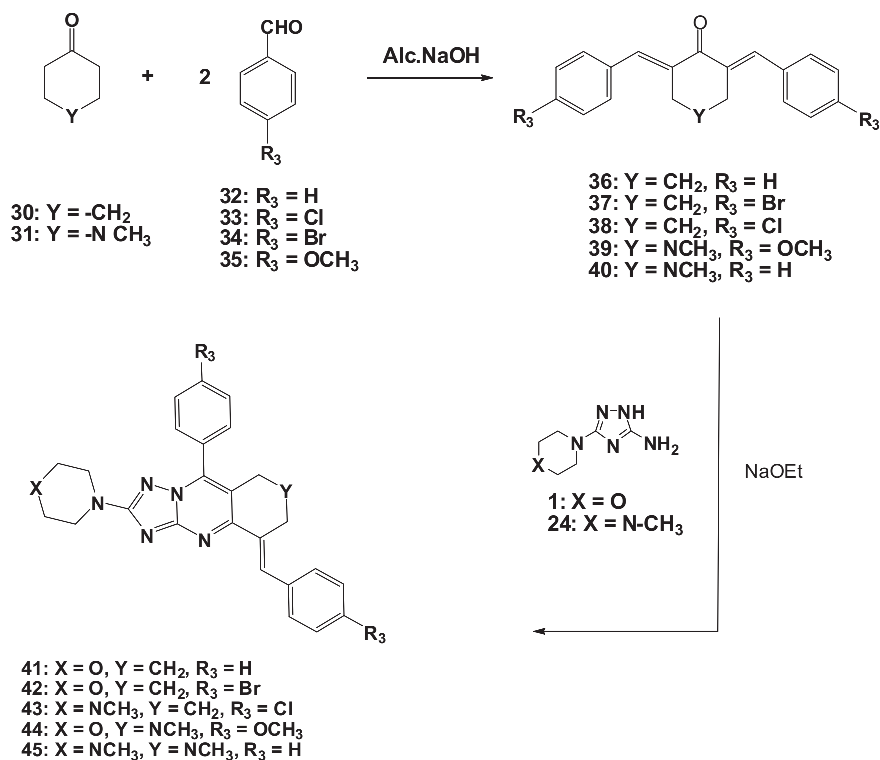
This technique depends on comparing the difference in DNA retaining to the origin between DNA complex with the tested compounds and that with ethidium bromide, a compound known to be good intercalator with DNA. This migration was observed when methanol: water (8:2) was used as an elution solvent. Among all the tested compounds; compound **26** showed the best affinity to DNA between the tested compounds which was demonstrated by retaining the complex at the origin or by its migration for a very short distance. Compounds **6**, **11**, **29**, **41**, **44** and **58** showed moderate affinity. The rest of the tested compounds showed the lowest affinity toward DNA.

3.2.2. Colorimetric assay for the degree of DNA-binding (Methyl green-DNA displacement assay)

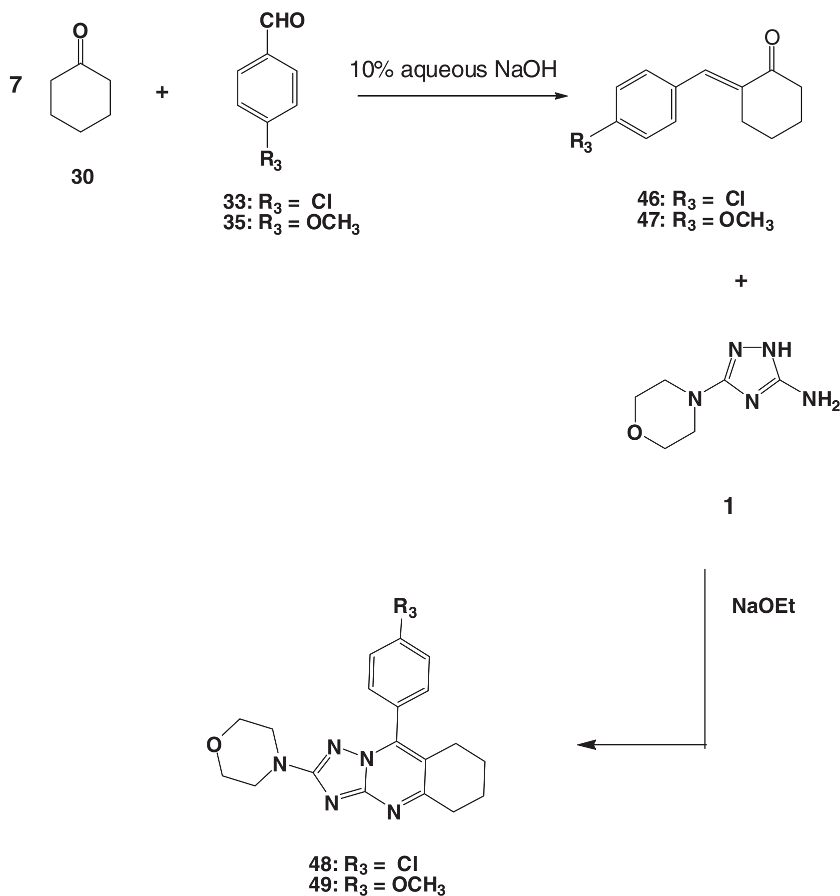
This colorimetric assay was used to measure the degree of displacement of methyl green from DNA by the tested compounds. The degree of displacement was determined spectrophotometrically by a decrease in DNA–methyl green absorbance. The results were highly consistent with those obtained from the DNA-binding assay on TLC-plates. Table 2 showed the most active screened compounds arranged in an increasing order for their IC_{50} values (calculated as μg/ml). The obtained data revealed that compound **26** is the most active member with IC_{50} value (the concentration required to produce 50% decrease in the initial absorbance of the DNA–methyl green complex) equals 47. The recorded values represent the mean ± SD, where $n = 3$–5 separate determinations.

3.2.3. Evaluation of antineoplastic activity against Ehrlich Ascite Carcinoma in mice

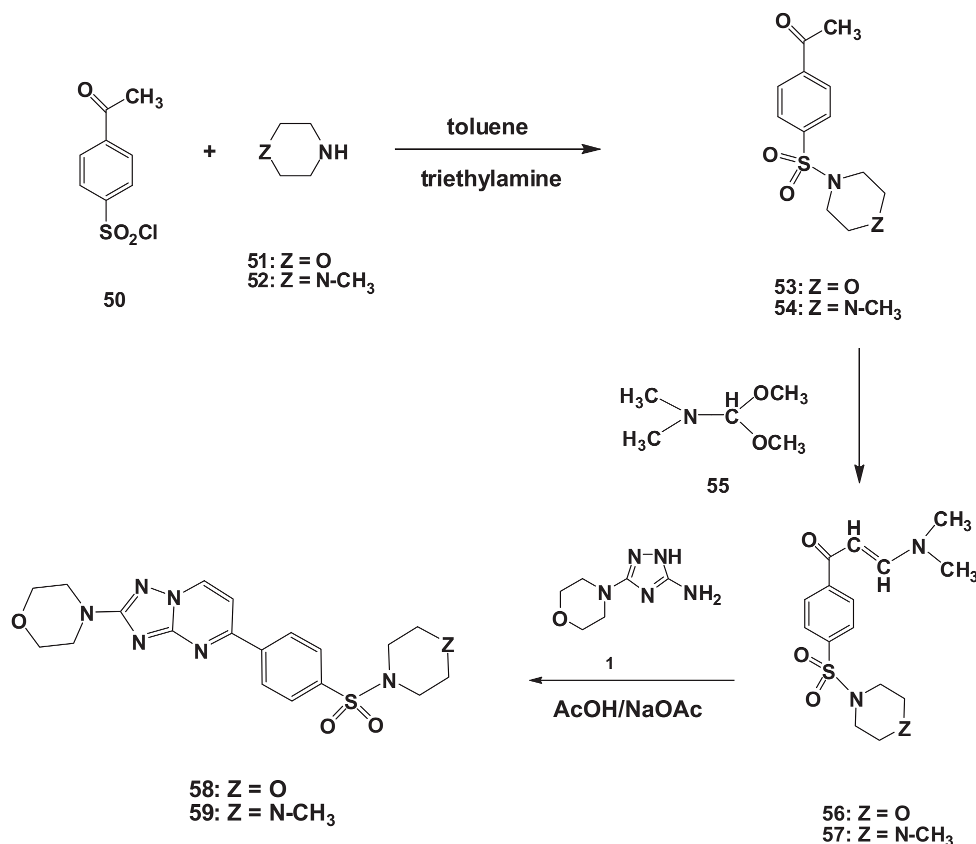
The prolongations of the life span of Ehrlich Ascite Carcinoma (EAC) bearing hosts and the recovery of normal biochemical and hematological profiles are two important measures that



Scheme 3 Synthesis of the target compounds **41–45**.



Scheme 4 Synthesis of the target compounds **48, 49**.



Scheme 5 Synthesis of the target compounds **58, 59**.

Table 4 The % increase in lifespan for treated group over the control group.

Group No.	Treatment (I.P.)	% increase in lifespan
1	Normal (untreated)	71.43
2	Control (Ehrlich only)	Zero
3	**26**	41
4	5-FU	42.86

have been used in this *in vivo* testing for the evaluation of the antineoplastic activity for compound **26**, the most active compound among the newly synthesized derivatives, selected on the basis of the results obtained from the previous evaluation methods, where it showed the highest DNA-affinity. The first measure that can be used to compare between the antineoplastic activities for the tested compounds is the increase in survival time for each treated group over the control group (Gupta et al., 2000). The mean survival time (MST) for each group was calculated by dividing the total survival times for all the mice in that group by the number of mice in the same group, then the percent increase in lifespan for each group over the control group was calculated as follows:

$$\%\text{increase in lifespan over control} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100 - 100$$

By comparing the % increase in lifespan over the control group in each treated group, it was found that compound **26** has shown almost the same increase in life span that produced the standard drug **5-FU** (Table 4).

Table 5 Modulation of apoptosis results.

Comp. No.	% Apoptic Neutrophil (Mean ± SD)			
	0 h	24 h	48 h	72 h
58	0.86 ± 0.01	25 ± 0.02	33 ± 0.02	53 ± 0.05
59	0.79 ± 0.02	20 ± 0.04	33 ± 0.04	52 ± 0.01
44	0.66 ± 0.01	16 ± 0.04	30 ± 0.05	39.1 ± 0.04
41	0.57 ± 0.03	13 ± 0.03	29.22 ± 0.02	29.5 ± 0.04
26	0.28 ± 0.06	8 ± 0.05	27.5 ± 0.05	18.2 ± 0.04
Untreated	0.28 ± 0.4	4.4 ± 0.04	14.40 ± 0.03	13.3 ± 0.03
Vit. C	0.42 ± 0.01	18 ± 0.02	33.2 ± 0.04	58 ± 0.02

3.2.4. Modulation of apoptosis

The present study was designed to investigate the possible involvement of apoptosis of blood neutrophils by the tested compounds. Blood neutrophils were prepared, cultured, and incubated for 24, 48 and 72 h in media with and without compounds. Both morphology and DNA fragmentation methods assessed the percentage of neutrophil apoptosis in each culture. The data obtained indicated that human neutrophils derived from the peripheral blood of normal subjects undergo morphological and chromatin fragmentation changes of programmed cell death (apoptosis). Upon measuring % apoptosis of human neutrophils, the obtained results indicated that the triazolopyrimidines **58** and **59** are the most active members (Table 5). In addition, compounds **41** and **44** showed promising activity. In case of compounds **41**–**45**, the introduction of an electron donating group such as the OCH_3 group at the *p*-position of the phenyl ring favored the activity rather than the electronegative moiety (Table 5). Moreover, compounds **25**–**29** showed the lowest activity, this indicated that these compounds exerted their activity through a mechanism other than apoptosis; they possess higher affinity toward DNA and act through DNA binding.

4. Conclusion

New series of triazolopyrimidine and triazoloquinazoline analogs of general formula (**A**–**E**) have been synthesized (Schemes 1–5). The newly synthesized compounds were evaluated for their antitumor activity through screening their ability to interact with the DNA which is known as the major mechanism of action of several known antitumor agents. By observation of the results of the *in vitro* DNA-binding test and the *in vivo* antitumor testing against Ehrlich ascite carcinoma in mice for the test compounds, the following items were observed: The morpholine ring is essential for activity as most of the test compounds containing this moiety proved to be active more than those containing the N-methyl piperazine moiety. The phenyl azo group proved to enhance *in vitro* activity especially when attached to an electron-withdrawing group at the para position. The diarylidene derivatives proved to be more active than the mono derivatives and this appeared in the difference in activity between compounds **44** and **49**. The order of activity has been changed in both *in vitro* and *in vivo* testing. This inversion in activity order might be owed to pharmacokinetic and pharmacodynamic considerations (such as; metabolism, absorption, distribution, plasma-proteins binding, receptor site interaction). This variation in results between the two testing techniques might be considered also as an indication for the inaccuracy of the DNA-binding as the only testing tool for the evaluation of this class of antitumor compounds. For this reason, the DNA-binding test was used in this investigation only as a preliminary tool for the selection of the compounds that might show high activity in the *in vivo* testing. In addition, the obtained data revealed that the triazolopyrimidinones exhibited their antitumor activity through DNA binding.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2013.04.002.

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