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Regioselective one-pot synthesis and anti-proliferative and apoptotic effects of some novel tetrazolo[1,5-*a*]pyrimidine derivatives

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ABSTRACT

An easy and efficient route for the synthesis of some tetrazolo[1,5-*a*]-pyrimidine derivatives was described through the reaction of sodium salts of formyl cycloalkanones with 5-aminotetrazole monohydrate. The derivative 6,7,8,9-tetrahydrotetrazolo[1,5-*a*]quinazoline **(6b)** has profound anti-tumor cytotoxic effects against Ehrlich ascites carcinoma (EAC) both in vivo and in vitro and against hepatocellular carcinoma (HepG2) cell line in vitro. These anti-tumor effects may be mediated via stimulation of cell cycle arrest and apoptosis through down-regulation of Bcl-2 and up-regulation of p53 transcription factors.

1. Introduction

Tetrazolopyrimidines have been reported to be used in the treatment of obesity, diabetes, atherosclerosis, hypertension, coronary heart disease, hypercholesterolemia, hyperlipidemia, thyroid cancer, hypothyroidism, depression, glaucoma, cardiac arrhythmias, and congestive heart failure.[1–6]

Despite significant advances in medical technology for protection and treatment, cancer is still widely producing threat mortality. Thereby, the search for new anti-cancer chemopreventive and chemotherapeutic agents continues to be an active area of research at many companies and research centers.[7–10]

Apoptosis, programmed cell death, has been recognized as a tightly controlled mechanism invoked to prevent unrestricted growth.[11] The recognition of tumor development involves an imbalance between proteins promoting cell viability like Bcl-2, and those mediate cell death or cell cycle arrest, p53,[12,13] which is the current dogma in tumor biology.[14]

Pyrimidine is found as a core structure in large variety of compounds that exhibit biological activity.[15,16] Some novel substituted pyrimidine derivatives showed anti-tumor cytotoxicity effects against different human carcinoma cell lines.[10,16,17] Also, some substituted tetrazolo compounds had anti-tumor effects against murine cell line B16.[18] Although many investigators are interested in the synthesis of new tetrazolopyrimidine derivatives,[19,20] there are scarce publications concerning the anti-tumor effects of these compounds with tetrazole and pyrimidine nuclei together.

Thus, this study was designed to synthesize new tetrazolo[1,5-*a*]pyrimidine derivatives. One of these derivatives, 6,7,8,9-tetrahydrotetrazolo[1,5-*a*]quinazoline **(6b)**, was tested for its anti-tumor cytotoxicity effects against Ehrlich ascites carcinoma (EAC) and hepatocellular carcinoma (HepG2) cell line. In addition, the effect on the anti-apoptotic protein Bcl-2 and tumor suppressor protein p53 was also investigated.

2. Results and discussion

2.1. Chemistry (synthesis)

Since the direct introduction of some specific substances for construction of tetrazolopyrimidines nucleus is not always easy and the most promising methods for synthesis of these compounds are multi-step reactions, our method has succeeded to prepare some novel tetrazolo[1,5-*a*]pyrimidines through the reaction of readily available starting materials and in a one-step fast reaction. Thus, treatment of 5-amino-1*H*-tetrazole hydrate **(4)** with the formyl salts of cycloalkanones, namely sodium (2-oxocycloalkylidine)methenoates **(2)**[21–24] in the presence of aqueous piperidine acetate and acetic acid, afforded in a good yield (69–73%) of the

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solid reaction products. The reaction products were suggested to be cycloalkane ring-fused tetrazolo[1,5-*a*]pyrimidines **(6)** and not the isomeric tetrazolopyrimidines **(8)** as outlined in Chart 1.

The reaction mode for the formation of the products is suggested to proceed through the initial nucleophilic attack by the exocyclic amino group at the formyl group of compound **(3)**, that formed in situ due to the reaction of the formyl salts **(2)** with water, followed by cyclization through the elimination of two water molecules leading to the formation of the reaction products **(6)**.

The suggestion of the formation of the alternative isomeric planer products **(8)** is based on the probability of the initial attack of the indocyclic NH of the tetrazole ring, which is expected to be more nucleophilic at the formyl group in **(3)** followed by cyclization and elimination of water. This suggestion is principally excluded due to the more steric hindrance around the endocyclic nitrogen atom than the exocyclic one that can easily attack the unhindered and the electronically favoured formyl group of compound **(3)**.[23,25]

The characterization of the reaction products was confirmed by using the elemental analysis (C, H, N) and spectral data (IR, MS, ^{1}H NMR). Thus, the IR spectrum of compound **(6b)** showed the absence of the bands related to NH or NH_2 of the start and revealed bands at $\nu = 2948\ cm^{-1}$, (paraffinic CH), $1655\ cm^{-1}$ (C=N), and at $1619\ cm^{-1}$ (C=C). The ^{1}H NMR spectrum revealed the presence of signals at $\delta = 1.96–2.11$ ppm (m, 4H, $2CH_2$); 2.91–3.33 (m, 4H, $2CH_2$), 8.70 (s, 1H, pyrimidine proton). Its mass spectrum showed a molecular ion peak at $m/z = 175$ (32.8%) coincident with the molecular weigh of the compound (175.19) and revealed the base peak at 107 (100%). The fragmentation pattern of compound **(6b)** was illustrated in Scheme 1.

2.2. Biological studies

For determination of lethal dose $(LD)_{50}$ of 6,7,8,9-tetrahydrotetrazolo[1,5-*a*]quinazoline (**6b**), single gradual increasing doses were orally administered to various groups of normal albino mice. After 48 h, the number of dead animals in each group was determined and LD_{50} was calculated. LD_{50} of **6b** was found to be 500 mg/kg b.w. (Table 1). Based on this toxicity study, the therapeutic dose for subsequent in vivo study was chosen to be 10 mg/kg b.w. (1/50 of LD_{50}) which is so far from LD_{50}.

The Ehrlich ascites carcinoma (EAC), in this study, was induced in female mice by transplantation of EAC-cells into peritoneal cav-

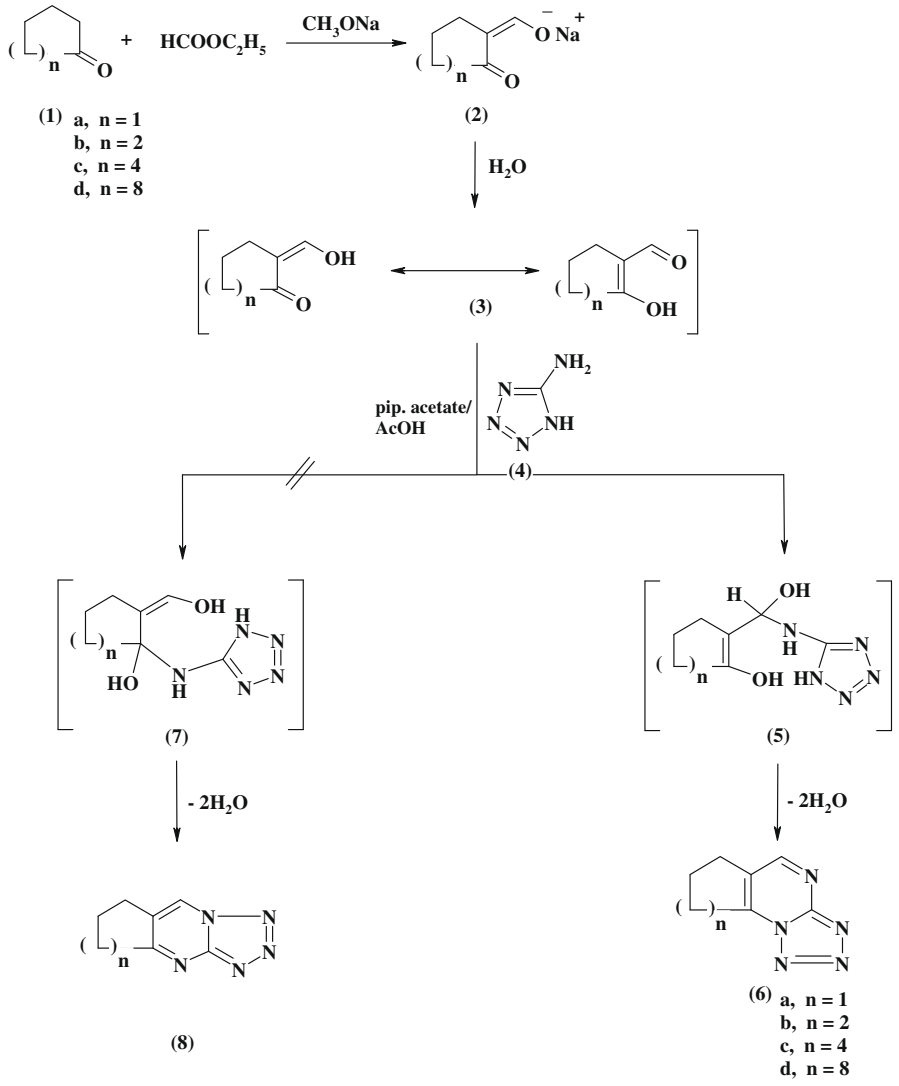
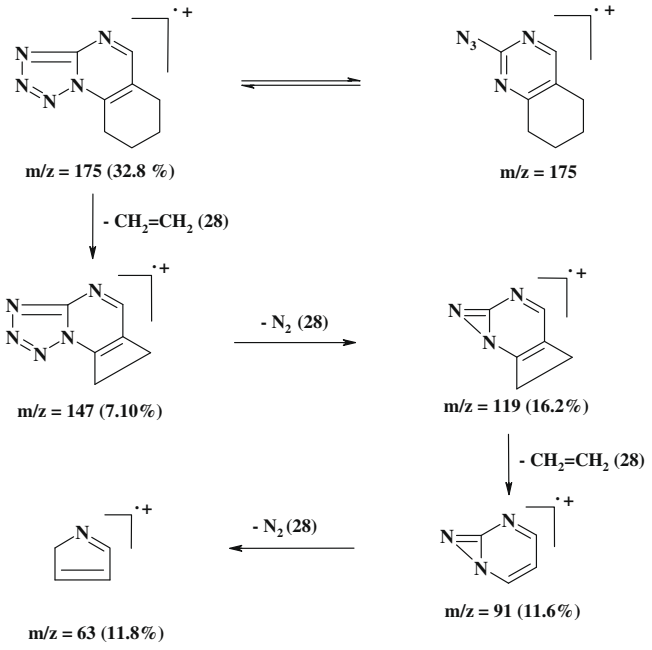


Chart 1.



Scheme 1.

Table 1
Determination of LD_{50} of 6,7,8,9-tetrahydrotetrazolo[1,5-*a*] quinazoline in albino mice

Dose (mg/kg b.w.)	Total number of animals	Number of dead animals	*z*	*d*	∑(*z*·*d*)
0	5	0	—	—	—
200	5	1	0.5	200	100
400	5	2	1.5	200	300
600	5	3	2.5	200	500
800	5	4	3.5	200	700
1000	5	5	4.5	200	900

z: Mean number of dead animals in two successive doses.
d: Constant factor between two successive doses.
LD_{50} = the biggest dose which kill all animals − ∑(*z*·*d*)/*n* = 1000–2500/5 = 500 mg/kg b.w.

ity. The EAC-cell is a spontaneous murine mammory adenocarcinoma adopted to ascites form and carried in outbred mice by serial passage.[26,27]

In the present study, the survival percentage of EAC-bearing mice treated with tetrazolo[1,5-*a*]pyrimidine derivative (**6b**) for 2 weeks was profoundly increased to reach 83.33% that was higher as compared with that of control (66.66%). Thus, this tetrazolo[1,5-*a*]pyrimidine derivative decreased the mortality and improved the survival rate of EAC-bearing mice.

Table 3
Effect of 6,7,8,9-tetrahydrotetrazolo[1,5-*a*]quinazoline on percent inhibition of EAC-cell viability in vitro after 2 h of incubation

Compound	% inhibition of cell viability		
	25 μg/ml	50 μg/ml	100 μg/ml
Tetrazolo[1,5-*a*]pyrimidine	10.00 ± 1.29	25.00 ± 2.24**	45.00 ± 6.71**

** p <0.01: Values are highly significantly different as compared with value at lowest concentration.

The inhibitory effect of tetrazolo[1,5-*a*]pyrimidine derivative (**6b**) on EAC-cells in vivo was examined in terms of EAC-aliquot volume and number of total, alive and dead EAC-cell number and dead EAC-cells percent of EAC-bearing mice treated with vehicle or compound. The obtained data (Table 2) revealed that administration of **6b** for 2 weeks induced a profound decrease (p <0.01) of EAC-aliquot volume, total number of EAC-cells and number of alive cells, on one hand, and marked increase of count (p <0.05) and percent (p <0.01) of dead EAC-cells, on the other. Similarly, to in vivo, the tested derivative (**6b**) has potent anti-tumor cytotoxic effects on EAC-cells and HepG2 cell line in vitro. The treatment of EAC-cells in vitro with **6b** at doses 25, 50 and 100 μg/ml produced 10.00 ± 1.29, 25.00 ± 2.24 and 45.00 ± 6.71 percent inhibition of cell viability (Table 3). Furthermore, the incubation of HepG2 cell line with gradual increased concentrations of the tested compound (**6b**) for 48 h produced moderate dose-dependent inhibition of cell growth and anti-tumor activity (Fig. 1).

In trial to explain the cytotoxic and anti-proliferative effects, the effect of tetrazolo[1,5-*a*]pyrimidine derivative **(6b)** on anti-apoptotic mediator Bcl-2 and tumor suppressor protein p53 was checked by immunohistochemical techniques. Bcl-2 has been reported to function primarily by blocking the apoptosis pathway.[28] On the other hand, p53 activates the transcription of downstream genes such as p21 and Bax to induce apoptotic process, inhibiting the growth of cells with damaged DNA or cancer cells.[29,30] It is required for cells to initiate apoptotis.[31,32] Thus, whether a cell becomes committed to apoptosis partly depends upon the balance between proteins that mediate death such as p53 and proteins that promote cell viability, such as Bcl-2.[33–35] The photomicrographs of immunohistochemically stained EAC sections revealed that the concentration of Bcl-2 in the cytoplasm of EAC-cells was profoundly decreased in EAC-bearing mice treated with tetrazolo[1,5-*a*]pyrimidine derivative (**6b**) (Fig. 2b and 2d) as compared with EAC-bearing mice control (Fig. 2a and 2c). On the other hand, the amount of p53 was potentially increased in EAC-bearing mice treated with the tested derivative (**6b**) (Fig. 2f) as compared with EAC-bearing mice control (Fig. 2e). Based on these results, it could be suggested that the decreased number of total and alive EAC-cells and increased number and percent of dead cells, may be attributed, at least in part, to the apoptotic effects of the tested tetrazolo[1,5-*a*]pyrimidine derivative (**6b**). In addition, the stimulat-

Table 2
Effect of 6,7,8,9-tetrahydrotetrazolo[1,5-*a*]quinazoline administration for 2 weeks on EAC-aliquot volume, EAC-cells number and percent of dead cells in EAC-bearing mice

Group	Parameter				
	EAC-aliquot volume (ml)	Total EAC-cells number·10^7	Alive EAC-cells number·10^7	Dead EAC-cells number·10^7	Percent of dead EAC-cells
EAC-bearing mice control (n = 8)	5.77 ± 0.39	125.76 ± 8.35	122.83 ± 8.24	2.64 ± 0.29	2.28 ± 0.22
EAC-bearing mice treated with tetrazolo[1,5-*a*] pyrimidine (n = 10)	2.52 ± 0.25**	71.01 ± 2.99**	66.39 ± 2.74**	4.62 ± 0.61*	8.24 ± 1.28**
LSD at the 5% level	0.94	17.26	16.83	1.56	3.09
LSD at the 1% level	1.29	23.79	23.18	2.16	4.26

Data are expressed as mean ± standard error (SE).
* p <0.05: Difference is significant.
** p <0.01: Difference is highly significant.

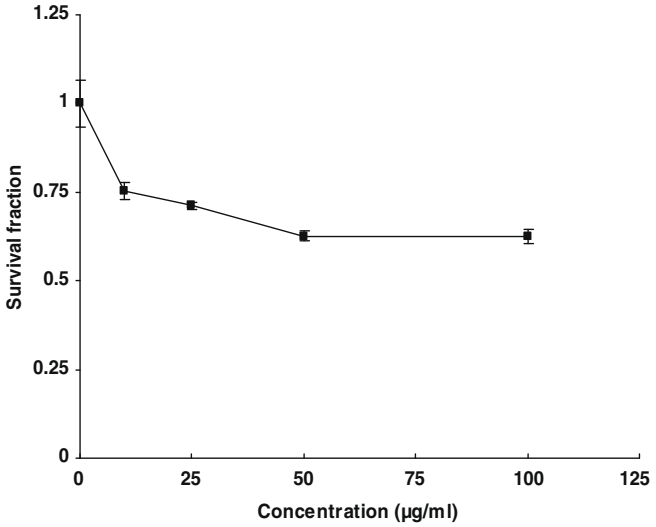


Figure 1. Anti-proliferative effect of 6,7,8,9-tetrahydrotetrazolo[1,5-*a*] quinazoline on HepG2 cell lines in vitro after 48 h of incubation.

ing effect of this tetrazolo[1,5-*a*]pyrimidine derivative on apoptosis is not restricted to one mediator but may be mediated via affecting many apoptotic and cell cycle arrest signal like p53 and anti-apoptotic factors like Bcl-2.

3. Experimental protocols

3.1. Chemistry

All melting points were determined on an electrothermal apparatus and are uncorrected. IR spectra were recorded (KBr discs) on a BRUKER IFS-25 FT-IR spectrophotometer at the region 400–4000 cm^{-1}. ^1H NMR spectra were recorded in $CDCl_3$ and $(CD_3)_2SO$ solutions on a Varian Gemini 300 MHz spectrometer and chemical shifts are expressed in δ units using TMS as an internal reference. Mass spectra were recorded on a GC–MS QP 1000 EX Shimadzu. Elemental analyses were carried out at the Microanalytical Center of the Cairo University, Giza, Egypt. Piperidine acetate was prepared by addition of 5 ml piperidine to a mixture of 4 ml acetic acid and 10 ml water.[21]

3.1.1. Synthesis of tetrazolo[1,5-*a*]pyrimidine derivatives 6a–d

Respective mixtures of 5-aminotetrazole monohydrate **(4)** (0.01 mol) were refluxed in a solution of sodium salts **(2)** (0.012 mol) and piperidine acetate (1.5 ml) for 3–5 min. Acetic acid (1.5 ml) was added to the reaction mixture while boiling, then the mixture was cooled and solid product was collected by filtration and recrystallized from the a suitable solvent (Tables 4 and 5).

3.2. Biology

3.2.1. In vivo studies

3.2.1.1. Acute toxicity study and determination of LD_{50} in normal mice. LD_{50} of the studied compound (**6b**) was determined as described by Afifi et al.[36] In this experiment, six groups of albino mice weighing 20–25 g were used. One group serves as control and other groups of mice were orally administered the tested compound by gastric intubation in gradual increasing doses (200, 400, 600, 800 and 1000 mg/kg b.w.). After 48 h of administration, the number of dead animals in each group, the mean of dead animals in two successive doses (z) and the constant factor between two successive doses (d) were recorded and LD_{50} was calculated as follow:

$$LD_{50} = \text{the biggest dose which kill all animals} - \sum(z \cdot d)/n$$

where n: number of animals in groups = five animals in each group.

3.2.1.2. Induction of EAC-bearing and animal grouping. Normal female albino mice were obtained from animal house, Institute of Ophthalmology, Giza, Egypt. EAC-bearing stock female mice were obtained from Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. To induce EAC in mice for the experimental study, 0.2 ml EAC-aliquot aspirated from stock mice was added to 9.8 ml saline (dilution is 1:50) and 0.2 ml of this diluted EAC was intraperitoneally administered by syringe into each mice. The EAC-injected mice were divided into two groups each of 12 animals. Mice of group 1 (control group) was administered with 10% DMSO as a vehicle in a volume equivalent to that given to treated animals. Group 2 was treated with tetrazolo[1,5-*a*]pyrimidine derivative **(6b)**, dissolved in 10% DMSO, at dose of 10 mg/kg b.w. (1/50 of LD_{50}) The compound and vehicle were orally applied by gastric intubation between 10 and 12 AM daily for 2 successive weeks beginning from the 1st day of EAC-injection.

The number of animals survived in each group was detected at the end of the experiment and the survival percent in each group was calculated as follow:

$$\text{Survival percent} = (\text{number of survived animals}/\textit{total number of animals}) \times 100$$

3.2.1.3. Sampling and detection of EAC-volume and cell number. At the end of the experimental period, animals were sacrificed under diethyl ether anesthesia and 0.2 ml saline was intraperitoneally injected. One minute later, EAC-aliquot was aspirated by a sterile syringe into test tube. The volume of EAC-aliquot for each mouse was measured. The number of alive and dead EAC-cells was determined using trypan blue exclusion assay. Alive and dead EAC-cells were counted by Neubauer haemocytometer. Briefly, 40 µl of EAC-aliquot was added to 4 ml 2% trypan blue (dissolved in 0.9% saline) and the mixture was left for 5 min. One drop from mixture was taken on Neubauer haemocytometer and the number of stained cells (dead cells) and non-stained cells (viable or alive cells) were counted. Total number of EAC-cells and percent of dead EAC-cells were calculated for each EAC-bearing mouse.

Part of EAC-aliquot from each tumor-bearing mouse was centrifuged at 3000 r.p.m. for 15 min and the precipitated EAC-cells were fixed in neutral buffered formalin for immunohistochemical studies.

3.2.1.4. Immunohistochemical investigations. The fixed samples were transferred to the Department of Pathology, National Cancer Institute for processing, blocking, sectioning and mounting onto positive-charged slides (Fisher Scientific, Pittsburgh, PA) to detect the Bcl-2 and p53 reactivity or apoptotic cells using the TUNEL assay.[37] Bcl-2 and p53 reactivity were determined following Hua and Ya-wei[38] method. Briefly, before the incubation with antibodies, endogenous peroxidase activity was quenched, slides washed and then incubated in a blocking solution of hydrogen peroxide 1% in methanol, in darkness for 15 min. Antigen retrieval occurred with citrate buffer 10 mM, pH 6. After cooling, sections were rinsed in tap water and then phosphate buffer saline 1 M. Primary antibodies for either Bcl-2 or p53, diluted 1:150 and 1:100, respectively in PBS, were applied for 1 h at 37 °C. Secondary biotinylated antibody diluted 1:100 and 1:200 in PBS was applied for a period of 30 min at 37 °C. Streptavidin–biotin or avidin–biotin peroxidase (ABC/ HRP) was applied for 10 min at room temperature. Bound antibody com-

plex was visualized by the reaction of 3,3′-diaminobenzidine substrate and counter stained with haematoxylin.

3.2.2. In vitro studies

3.2.2.1. Evaluation of anti-tumor cytotoxicity against EAC-cells. The viability of cells as a result of 3 different concentrations (25, 50 and 100 μg/ml) of tetrazolo[1,5-*a*]pyrimidine derivative (**6b**) was tested by trypan blue exclusion assay according to the method Mclimans et al.[39] Briefly, EAC-cells at concentration of 2.5×10^5 cells/ml suspended in phosphate buffer saline were incubated at 37 °C and 5% CO_2 for 2 h in the presence of three different concentrations of the compound. At the end of incubation period, equal volume of trypan blue solution was added to sample cells, then the stained cells (dead cells) and unstained cells (alive cells) were counted using Neubauer haemocytometer. The percent of dead cells for each test was calculated.

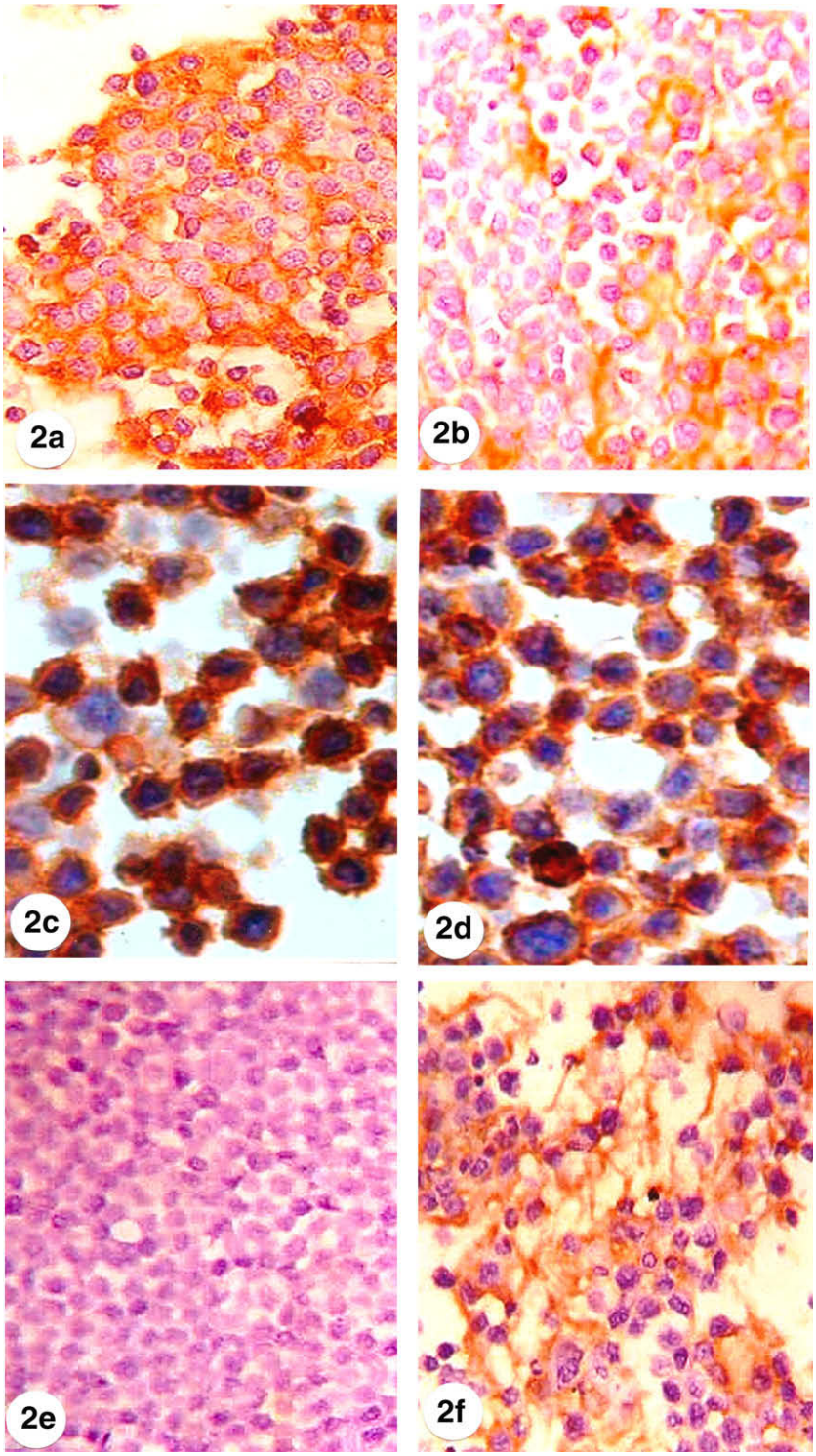


Figure 2. Photomicrographs of EAC-immunohistochemically-stained sections showing the effect of 6,7,8,9-tetrahydrotetrazolo[1,5-*a*]quinazoline on anti-apoptotic marker Bcl-2 (2b; ×100 and 2d; ×400) and apoptotic marker p53 (2f; ×100) expression as compared with the corresponding control (2a; ×100 and 2c; ×400 for Bcl-2 and 2e; ×100 for p53).

Table 4
The melting point, color, yield, molecular formula and elemental analysis of the newly synthesized compounds

Compd No.	MP °C solvent	Color yield%	Mol. formula (M.Wt.)	Elemental analysis calcd/found		
				C	H	N
6a	182–183	Pale brown	$C_7H_7N_5$ (161)	52.17	4.35	43.47
	EtOH	69.1		52.22	4.19	43.66
6b	119–121	Orange	$C_8H_9N_5$ (175)	54.85	5.14	40.00
	EtOH	73.3		54.88	5.00	39.79
6c	103–105 EtOH	White 64	$C_{10}H_{13}N_5$ (203)	59.11	6.40	34.48
				58.89	6.65	34.45
6d	110–112 EtOH	White 73	$C_{14}H_{21}N_5$ (259)	64.86	8.11	27.03
				64.49	8.21	27.00

Table 5
The spectral data of the newly synthesized compounds

Compd No.	Spectra
6a	IR ν (cm^{-1}): 2920–2823 (CH paraffinic); 1665 (C=N) and 1594 (C=C) ^1H NMR δ_H (ppm): 1.90–2.22 (m, 2H, CH_2); 2.87–3.16 (m, 4H, $2CH_2$) and 8.72 (s, 1H, pyrimidine H) Mass (*m/z*): 161 ($M^{\cdot+}$, 10.0%); 151 (65.5%); 122 (73.0%); 95 (81.5%) and 67 (100.0%)
6b	IR ν (cm^{-1}): 2948 (paraffinic CH), 1655 (C=N), and 1619 (C=C) ^1H NMR δ_H (ppm): 1.96–2.11 ppm (m, 4H, $2CH_2$); 2.91–3.33 (m, 4H, $2CH_2$) and 8.70 (s, 1H, pyrimidine H) ^{13}C NMR δ_C (ppm): 17.9 ($2CH_2$); 34.6 ($2CH_2$); 140.1 (C=C–C=N); 156.2 (C=C–N); 160.6 (–C=N–C=N); 170.6 (tetrazole C) Mass (*m/z*): 175 ($M^{\cdot+}$, 32.8%); 146 (10.4%); 107 ($M^{\cdot+}$, 100%) and 79 (39.6%)
6c	IR ν (cm^{-1}): 2943–2845 (CH paraffinic); 1658 (C=N) and 1589 (C=C) ^1H NMR δ_H (ppm): 1.36–2.00 (m, 4H, $2CH_2$); 2.67–2.95 (m, 4H, $2CH_2$); 3.48–3.53 (m, 4H, $2CH_2$) and 8.723 (s, 1H, pyrimidine H) Mass (*m/z*): 204 (M^{+}+1, 31.6%); 203 (48.7%); 174 (13.50%); 146 (31.3%); 107 (48.0%) and 81 (48.7%)
6d	IR ν (cm^{-1}): 2920–2853 (CH paraffinic); 1659 (C=N) and 1606 (C=C). Mass (*m/z*): 259 ($M^{+\cdot}$, 7.3%); 233 (56.1%); 192 (18.3%); 176 (36.0%); 148 (48.1%) and 123 (100.0%)

3.2.2.2. Anti-proliferation assay using HepG2 cell line. Hepatoma (HepG2) cell lines were obtained from the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. Cells were maintained in RPMI 1640 medium supplemented with 0.3 g/L glutamine, 2 g/L sodium bicarbonate, 10% foetal calf serum, 100 unit/ml penicillin and 100 μg/ml streptomycin at 37 °C in atmosphere of 5% CO_2.[40]

Cytotoxicity and anti-proliferative activity against HepG2 cell line was detected by sulfo-rhodamine-B (SRB) assay according to the method of Skehan et al.[41] Briefly, Monolayer HepG2 cells plated in 96-multiwell plate were incubated with different concentrations (0, 10, 25, 50 and 100 μg/ml) of each compound for 48 hours at 37 °C in atmosphere of 5% CO_2. At the end of the incubation period, cells were fixed, washed and stained with SRB. Excess stain was washed with acetic acid and attached stain was recovered with tris EDTA buffer and colour intensity was measured by ELISA reader. The relation between survival fraction and compound concentration was plotted.

3.3. Statistical analysis

Data were expressed as mean ± standard error (SE) and were analyzed by one-way ANOVA followed by LSD test using PC-STAT program.[42]

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