Med Chem Res
DOI 10.1007/s00044-012-0396-0

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antitumor activity of new pyrido[2,3-*d*]pyrimidine derivatives

Magdy M. Gineinah · Magda N. A. Nasr · Sahar M. I. Badr · Walaa M. El-Husseiny

Received: 10 February 2012 / Accepted: 5 December 2012

Abstract New series of pyrido[2,3-*d*]pyrimidines such as; 5-(4-aryl-5-sulfanyl-4*H*-[1,2,4]triazol-3-yl) 1*H*,3*H*,8*H*-pyrido[2,3-*d*]pyrimidine-2,4,7-triones **6**, **7**; *S*-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidin-5-yl)-4-(4-substituted phenyl)-4*H*-[1,2,4]triazol-5-yl]-2-(4-phenylpiperazin-1-yl)ethanethioates **10**, **11**; 2,4,7-trioxo-*N*′-[(4-substituted piperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazides **13–16** and *N*′-arylidene-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazides **17–19** was synthesized through the reaction of the key intermediate 2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazide **3** with different reagents. The structures of the newly synthesized compounds were elucidated through microanalysis, IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. These compounds have been subjected to in vitro antitumor evaluation by bleomycin-dependant DNA damage assay. The most active antitumor compound **6** was selected for further in vivo evaluation of antineoplastic activity against Ehrlich ascites carcinoma in mice. It was observed that our target compound has a potent antitumor activity.

Keywords Pyrido[2,3-*d*]pyrimidines · Piperazines · Triazoles · Synthesis · Antitumor activity

M. M. Gineinah · M. N. A. Nasr · S. M. I. Badr (✉) · W. M. El-Husseiny
Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt
e-mail: saharmbadr@yahoo.com

Introduction

The discovery of new compounds with antitumor activity has become one of the most important goals in medicinal chemistry. Several literatures enlightening the anticancer nature of pyrido[2,3-*d*]pyrimidines such as piritrexim, piritrexim analogs, isopiritrexim, and compounds V–VII (El-Nassan, 2011; Kurumurthy *et al*., 2011; Shi *et al*., 2011; Tu *et al*., 2006; Mohamed *et al*., 2007; Cordeu *et al*., 2007; Zink *et al*., 2004; Gangjee *et al*., 2001; Agrawal *et al*., 2002; Joska and Anderson, 2006; Chan *et al*., 2005; Sanmartin *et al*., 2005) (Fig. 1). In addition, as revealed from the literatures, potential antitumor activity was reported by many hydrazides (De *et al*., 2010; Grande *et al*., 2007), piperazines (Guo *et al*., 2004), and [1,2,4]-triazoles (Al-Soud *et al*., 2004; Formagio *et al*., 2008). From the aforementioned argument, we designed and synthesized the target compounds having pyrido[2,3-*d*] pyrimidine pharmacophore (Fig. 2); this scaffold hybridized with one or more heterocyclic moieties (piperazines or triazoles) and some functionalities that possess antitumor potency; this kind of integration might result in compounds with high efficacy as antitumor agents.

Results and discussion

Chemistry

The target compounds were prepared as outlined in Schemes 1, 2, 3. The key intermediate 2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazide **3** was prepared by refluxing methyl 2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*] pyrimidine-5-carboxylate **2** (Anderson *et al*., 1977) with hydrazine hydrate in ethanol.

Published online: 18 December 2012

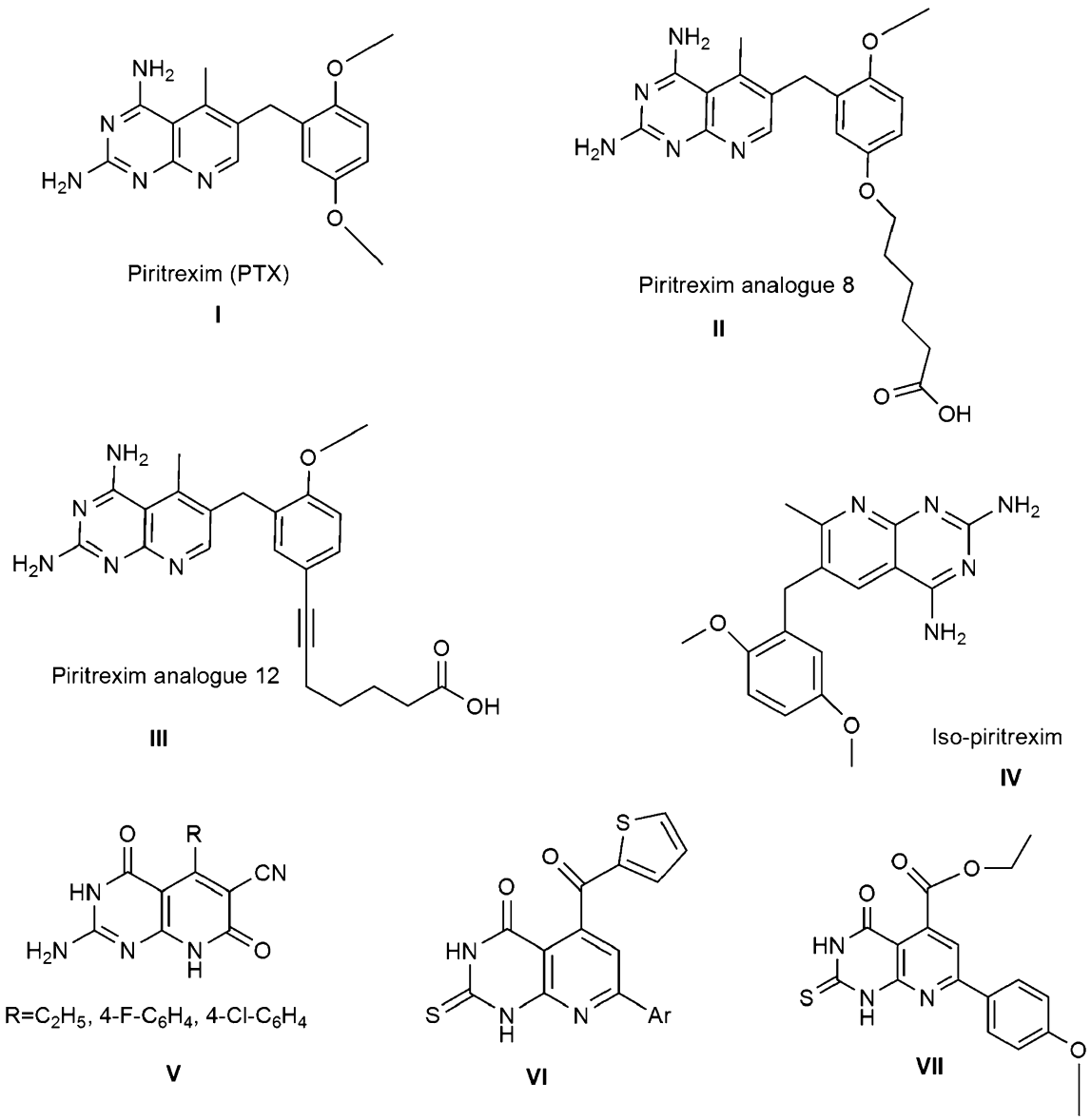


Fig. 1 Structures of active antitumor compounds containing pyrido[2,3-*d*]pyrimidine moiety

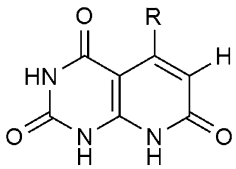
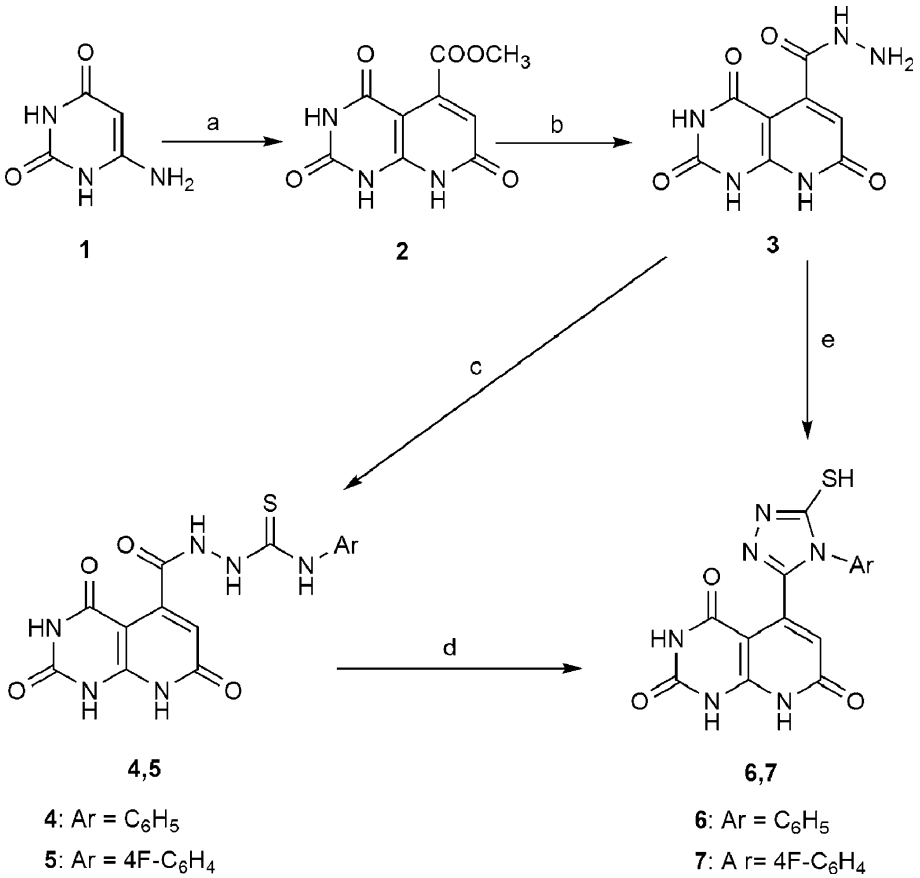


Fig. 2 Structure of the proposed pyrido[2,3-*d*]pyrimidine pharmacophore in the present study

Then 5-(4-aryl-5-sulfanyl-4*H*-[1,2,4]triazol-3-yl) 1*H*,3*H*, 8*H*-pyrido[2,3-*d*]pyrimidine-2,4,7-triones **6**, **7** were prepared through two different routes. In the first one, the acid hydrazide **3** was reacted with aryl isothiocyanates in absolute ethanol to yield *N*-aryl-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine)-5-carbohydrazidocarbo thioamides **4**, **5** which on heating under reflux with 5 % potassium hydroxide solution underwent cyclization to the corresponding title compounds **6**, **7**. While in the second route, the acid hydrazide **3** was heated under reflux with aryl isothiocyanates in alcoholic potassium hydroxide followed by acidification with hydrochloric acid (Scheme 1). Furthermore, 2-chloro-*S*-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidin-5-yl)-4-(4-substituted phenyl)-4*H*-[1,2,4]triazol-4-yl]ethanethioates **8**, **9** were prepared by refluxing compounds **6** or **7** with chloroacetyl chloride in toluene in the presence of triethylamine (TEA) as a base. Their IR spectroscopy showed the peak at 2,600 cm^{-1} characteristic for (SH) group disappeared, while peaks at about 1,658–1,662 and 3,000–3,016 cm^{-1} attributed to (C=O) and (CH_2), respectively, were observed. Then,



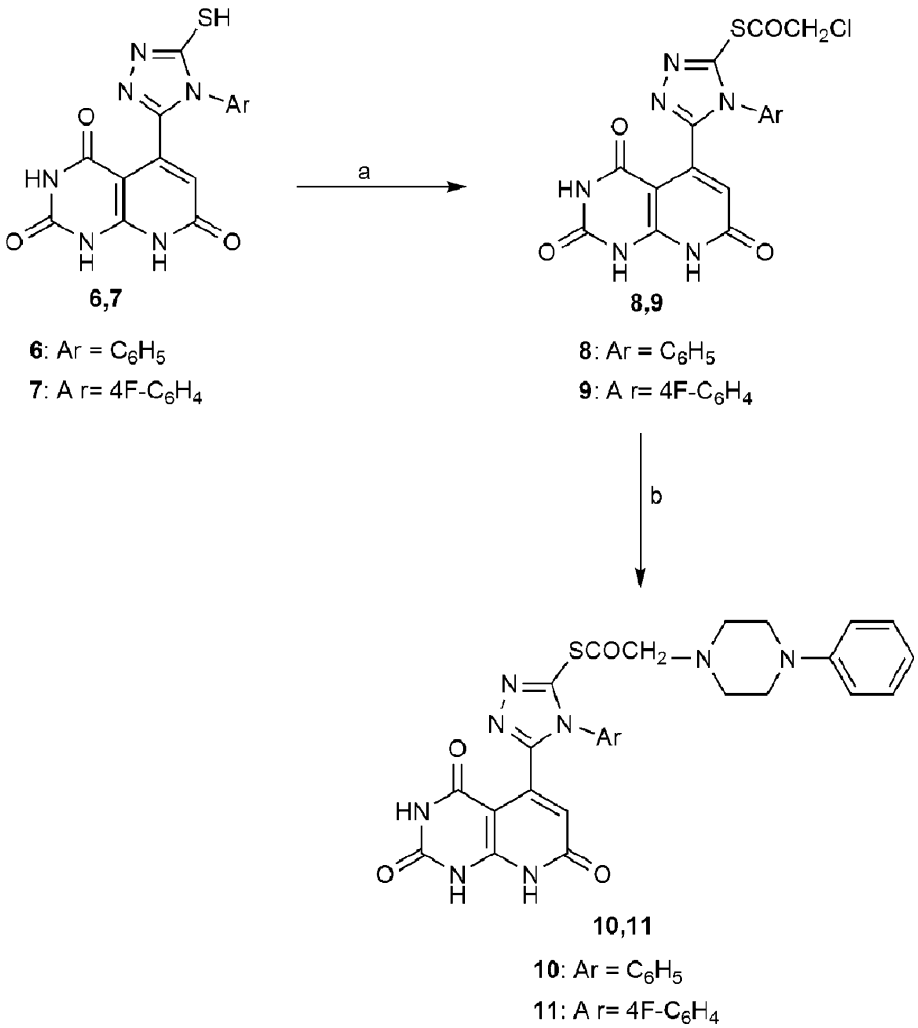
Scheme 1 Synthesis of 5-(4-aryl-5-sulfanyl-4*H*-[1,2,4]triazol-3-yl)1*H*,3*H*,8*H*-pyrido[2,3-*d*]pyrimidine-2,4,7-triones **6**,**7**. Reagent and condition: *a* dimethylacetylenedicarboxylate, *b* hydrazine hydrate, EtOH, reflux, *c* appropriate isothiocyanate, EtOH, reflux, *d* KOH, reflux, *e* KOH, appropriate isothiocyanate, reflux

compounds *S*-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidin-5-yl)-4-(4-substituted phenyl)-4*H*-[1,2,4]triazol-5-yl]-2-(4-phenylpiperazin-1-yl)ethanethioates **10**, **11** were prepared by stirring compounds **8**, **9** with 1-phenylpiperazine in dimethylformamide using potassium carbonate as a base (Scheme 2). Moreover, the reaction of pyrido[2,3-*d*]pyrimidine carbohydrazide **3** with chloroacetyl chloride in ethanol afforded *N*′-chloroacetyl-2,4,7-tri oxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbo hydrazide **12**, which on stirring with the appropriate 1-substituted piperazine in dimethylformamide in the presence of potassium carbonate as a base gave 2,4,7-trioxo-*N*′-[(4-substituted piperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazides **13**–**16**. Finally, the reaction of compound **3** with the appropriate aromatic aldehyde in ethanol yielded *N*′-arylidene-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*] pyrimidine-5-carbohydrazides **17**–**19**. Their IR spectroscopy showed the peak characteristic for (NH_2) of the acid hydrazide **3** disappeared while peak at about 1,600 cm^{-1} characteristic for (C=N) was observed (Scheme 3).

All compounds were spectroscopically characterized and the spectral data agree with the proposed structures.

Biologic screening

The therapeutically active antitumor agents should possess high and potential efficacy with minimal side effects. As a result, interaction of antitumor agents with DNA should produce as little DNA damage as possible. The newly synthesized compounds had been subjected to bleomycin-dependant DNA damage assay (Ayyad *et al*., 2011a, b) to screen their antitumor activity and the degree of DNA damage caused by these compounds. Table 1 shows the effect of the newly synthesized compounds on the degree of DNA damage described in terms of sample absorbance (*A*). As sample absorbance (*A*) increases, DNA damage increases and the sample efficiency as antitumor agent decreases. Compounds **2**–**4**, **6**, **7**, **9**, **11**–**14**, and **18** showed the lowest absorbance, and hence the highest antitumor activity, while compounds **5** and **19** showed moderate activity. We can conclude that compound **6** exhibited the highest

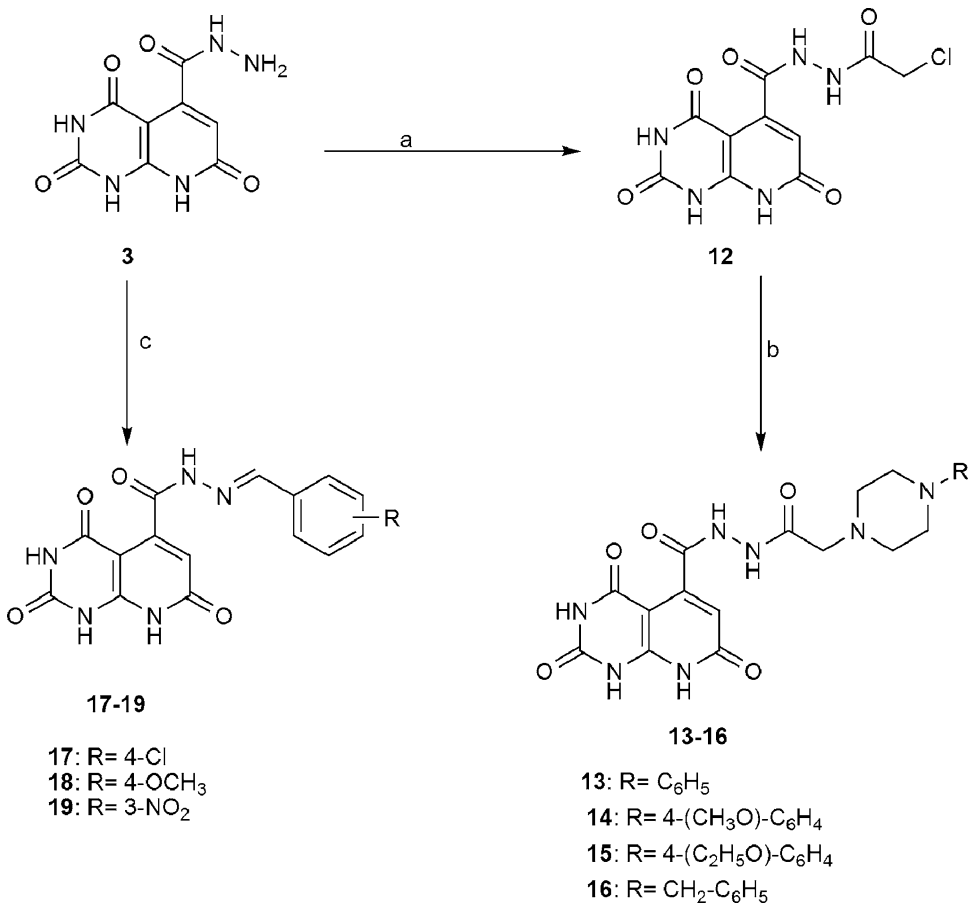


Scheme 2 Synthesis of *S*-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidin-5-yl)-4-(aryl)-4*H*-[1,2,4]triazol-5-yl]-2-(4-phenylpiperazin-1-yl)ethanethioates **10**, **11**. Reagent and condition: *a* chloroacetyl chloride, toluene, TEA, reflux, *b* 1-phenylpiperazine, K_2CO_3, DMF, rt

antitumor activity, and as a result it was subjected for antineoplastic activity against Ehrlich ascites carcinoma (EAC) in mice (Ayyad *et al*., 2011a, b; Qureshi *et al*., 2001; Bala *et al*., 2010). The prolongation of life span of EAC-bearing hosts, the reduction in viable tumor cell count, and the recovery of normal biochemical and hematologic profiles are three important measures that have been used in this in vivo testing for evaluation of the antineoplastic activity. Effect on survival time was the first measure used to detect the antineoplastic activity of the tested compound where an increase in survival time for this group over the control group was detected. 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 and then the percent increase in life span for each group over the control group was calculated as follows:

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

By comparing the percentage increase in life span over the control group in the treated group, it was found that compound **6** had shown increase in the life span higher than that of 5-fluorouracil (5-FU, Table 2). Effect on EAC viable cell count, after 5 days of treatment: 100 µl samples were taken from Ehrlich ascites cells from three mice in each of the treated groups and from the control group. 20-fold dilution was made for the taken cells in saline. The cells in the final dilution were stained with Giemsa stain and the number of the viable cells was counted under the microscope. As shown in Table 3, compound **6** showed significant reduction in viable tumor cell count. Effect on biochemical and hematologic parameters: on the day 14, the biochemical and hematologic parameters, as regard to hemoglobin level (Hb/g%), hematocrits (HCT/g%), and



Scheme 3 Synthesis of 2,4,7-trioxo-*N*′-[(4-substituted piperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazides **13–16** and *N*′-arylidene-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carbohydrazides **17–19**. Reagent and condition: *a* chloroacetyl chloride, EtOH, reflux, *b* appropriate substituted piperazine, K_2CO_3, DMF, rt, *c* appropriate aromatic aldehyde, EtOH, reflux

Table 1 Absorbance degree (*A*) of tested compounds as estimated by bleomycin-dependant DNA damage assay

Compound numbers	Sample absorbance (*A*)
Ascorbic acid	0.068
2	0.055
3	0.064
4	0.054
5	0.097
6	0.052
7	0.057
9	0.074
11	0.062
12	0.060
13	0.071
14	0.053
18	0.057
19	0.079

leukocytes counts (WBCs/10^3 mm^3), were compared in the group treated with the standard drug 5-FU and the group treated with the newly synthesized drug **6** with the values obtained from normal and control groups. As shown in Table 4, the biochemical and hematologic parameters in the group treated with the compound **6** has been nearly recovered completely to be within the normal values. The values are better than those obtained from the group treated with 5-FU.

Studying structure–activity relationship of the tested compounds revealed that regardless of changes in the structure of the starting compound **2**, the biologic activity was retained due to the presence of the main moiety pyridopyrimidine. However, the ester transformation into the acid hydrazide, compound **3**, reduced the activity, introduction of *N*-phenylthiosemicarbazide moiety, compound **4**, at position five of the pyridopyrimidine ring system led to better activity in comparing to the carbohydrazide **3** itself, while introducing the electron-withdrawing fluoro group at the four position of the phenyl ring, compound **5**, decreased the activity. Compounds **6** and **7** are more active in general than their non-cyclized counterparts, compounds **4** and **5** indicating that cyclization step of thiosemicarbazide containing compounds into [1,2,4]-triazoles affords higher and more potent antitumor activity. Moreover, compound **9** showed lower activity than compounds **6** and **7;** thus, we can conclude that free sulfanyl group (–SH) manifests more potent activity. On the other hand, compound **11** is highly active as antitumor agent comparing to

Table 2 The percentage increase in life span for each treated group over the control group

Group no.	Treatment (IP)	Percent increase in life span
1	Normal (untreated)	–
2	Control (Ehrlich only)	9.1
3	Compound **6**	18.6
4	5-FU (20 mg/kg)	16.7

Table 3 The number of viable cells for each group

Group no.	Group type	Count cells
2	Control (Ehrlich only)	220
3	Compound **6**	153.6
4	5-FU	123.0

Table 4 The biochemical and hematologic parameters for each group

Group type	Hb/g%	HCT/g%	WBCs/10^3 mm^3
Normal	13.9	53.6	8.4
Control	8.7	35.5	38.6
Compound **6**	11.7	51.6	11.2
5-FU	10.0	42.3	13.8

Hb/g%, hemoglobin level; HCT/g%, hematocrits; WBCs/10^3 mm^3, leukocytes counts

compound **9**; it means that piperazine moiety potentiates antitumor activity. Likewise, compounds **13** and **14** showed high activity due to the presence of piperazine moiety. However, the introduction of methoxy group at position four of the 1-phenylpiperazine reduces the absorbance; thus, compound **14** is more potent than compound **13**. This indicates that better activity could be attained by the presence of a piperazine moiety substituted at position four with phenyl group substituted with electron-donating group, but not with electron-withdrawing or even unsubstituted group. It was shown that methoxy group at position four of the Schiff base phenyl ring compound **18** displays higher activity than nitro group at the same position as in compound **19**. Again, the presence of an electron-donating group on an aromatic nucleus displays, in part, higher antitumor activity.

Conclusion

A new series of pyrido[2,3-*d*]pyrimidines **6**, **7**; **10**, **11**; **13**–**16**, and **17**–**19** has been synthesized and evaluated for their antitumor activity. Our target compound **6** showed potential antitumor activity.

Experimental

Chemistry

Melting points were determined on Fisher-Johns melting point apparatus and are uncorrected. Microanalyses were performed at the microanalytical center, Cairo University, and the values are found within ±0.4 % of theoretic values. IR spectra were recorded on Mattson 5000 FT-IR spectrometer (υ in cm^{-1}) using KBr disk at the Faculty of Science, Mansoura University. ^{1}H NMR and ^{13}C NMR spectra were recorded on FT-NMR spectrometer (200 MHz) using TMS as internal standard (chemical shifts in ppm, δ units) at the microanalytical center, Cairo University. MS analyses were performed on JEOL JMS-600H spectrometer at Cairo University. TLC analysis was carried out on silica gel-protected aluminum sheets (Type 60 F 254, Merck) and the spots were detected under UV-Lamp at λ 254 nm. Methyl 2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-*d*]pyrimidine-5-carboxylate **2** was prepared as reported method (Anderson *et al*., 1977).

*Preparation of 2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide (**3**)*

To a suspension of the ester **2** (2.37 g, 10 mmol) in absolute ethanol (40 ml), hydrazine hydrate 98 % (1 g, 20 mmol) was added. The reaction mixture was heated under reflux for 24 h and then cooled to room temperature. The product was collected by filtration, washed with ethanol, dried, and recrystallized from water.

Yield 84 %, mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 3.7 (s, 2H, NH_2; D_2O exchangeable), 6.3 (s, 1H, C_6–H), 8.6 (s, 1H, CONH; D_2O exchangeable), 10.2 (brs, 1H, NH; D_2O exchangeable), 11.0 (brs, 1H, NH; D_2O exchangeable), 11.3 (brs, 1H, NH; D_2O exchangeable). Anal. calcd. for $C_8H_7N_5O_4$: C 40.51; H 2.97; N 29.53. Found: C 40.48; H 2.95; N 29.58.

*Preparation of N-(aryl) (2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)carbohydrazidocarbothioamides **4**, **5***

To a suspension of **3** (2.37 g, 10 mmol) in absolute ethanol (50 ml), substituted isothiocyanate (10 mmol) was added. The reaction mixture was heated under reflux for 24 h and then cooled to room temperature. The product was collected by filtration, washed with ethanol, dried, and recrystallized from acetic acid.

N-(phenyl) (2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido [2,3-d]pyrimidin-5-yl)carbohydrazidocarbothioamide ***(4)*** Yield 80 %, mp > 300 °C. IR (KBr, υ, cm^{-1}): 1548 (C=S), 1660, 1700 (C=O), 3160, 3200 (NH). ^{1}H NMR (DMSO-d_6): δ 6.4 (s, 1H, C_6–H), 6.8 (t, $J = 8.0$ Hz, 1H, Ar–H), 7.3 (t, $J = 8.0$ Hz, 2H, Ar–H), 7.6 (d, $J = 8.0$ Hz, 2H, Ar–H), 8.4 (brs, 1H, NH; D_2O exchangeable), 9.5 (brs, 1H, NH; D_2O exchangeable), 10.4 (brs, 1H, NH; D_2O exchangeable), 10.8 (brs, 1H, NH; D_2O exchangeable), 11.4 (brs, 1H, NH; D_2O exchangeable), 12.0 (brs, 1H, NH; D_2O exchangeable). ^{13}C NMR (DMSO-d_6): δ 99.2, 112.7, 126.5, 128.7, 129.4, 139.0, 144.2, 150.6, 161.3, 162.6, 166.8, 182.4. Mass *m/z* (%): 372 (M^+, 1.80), 77 (100), 206 (46.72), 118 (67.3), 91 (57.01). Anal. calcd. for $C_{15}H_{12}N_6O_4S$: C 48.38; H 3.25; N 22.57. Found: C 48.30; H 3.29; N 22.51.

N-(4-flourophenyl) (2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)carbohydrazidocarbothioamide ***(5)*** Yield 75 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1542 (C=S), 1653, 1720 (C=O), 3170, 3202 (NH)). ^{1}H NMR (DMSO-d_6): δ 6.6 (s, 1H, C_6–H), 7.2 (d, $J = 7.5$ Hz, 2H, Ar–H), 7.7 (d, $J = 7.5$ Hz, 2H, Ar–H), 8.5 (brs, 1H, NH; D_2O exchangeable), 9.7 (brs, 1H, NH; D_2O exchangeable), 10.5 (brs, 1H, NH; D_2O exchangeable), 10.7 (brs, 1H, NH; D_2O exchangeable), 11.2 (brs, 1H, NH; D_2O exchangeable), 12.1 (brs, 1H, NH; D_2O exchangeable). Mass *m/z* (%): 390 (M^+, 7.50), 127 (100), 368 (91.56), 200 (30.00), 168 (54.34). Anal. calcd. for $C_{15}H_{11}FN_6O_4S$: C 46.15; H 2.84; N 21.53. Found: C 46.22; H 2.83; N 21.57.

Preparation of 5-(4-aryl-5-sulfanyl-4H-[1,2,4]triazol-3-yl)1H,3H,8H-pyrido[2,3-d]pyrimidine-2,4,7-triones ***6, 7***

Method 1 A solution of **4** or **5** (10 mmol) in an aqueous potassium hydroxide solution (5 %, 50 ml) was heated under reflux for 2 h and then concentrated. The precipitated solid was dissolved in water and neutralized with concentrated solution of hydrochloric acid. The separated solid was collected by filtration, dried, and recrystallized from water.

Method 2 To a mixture of **3** (2.37 g, 10 mmol) and potassium hydroxide (0.56 g, 10 mmol) in 95 % ethanol (50 ml), substituted isothiocyanate (10 mmol) was added. The reaction mixture was heated under reflux for 12 h and then concentrated. The precipitated solid was dissolved in water and neutralized with concentrated solution of hydrochloric acid. The separated solid was collected by filtration, dried, and recrystallized from water.

5-(4-Phenyl-5-sulfanyl-4H-[1,2,4]triazol-3-yl)1H,3H,8H-pyrido[2,3-d]pyrimidine-2,4,7-trione ***(6)*** Yield 65 % (method 1), 60 % (method 2), mp >300 °C. IR (KBr, υ, cm^{-1}): 1650 (C=O), 3210 (NH), 2600 (SH). ^{1}H NMR (DMSO-d_6): δ 6.3 (s, 1H, C_6–H), 7.1 (t, $J = 8.3$ Hz, 1H, Ar–H), 7.5 (t, $J = 8.3$ Hz, 2H, Ar–H), 7.8 (d, $J = 8.3$ Hz, 2H, Ar–H), 10.2 (brs, 1H, NH; D_2O exchangeable), 10.6 (brs, 1H, NH; D_2O exchangeable), 11.3 (brs, 1H, NH; D_2O exchangeable), 13.2 (s, 1H, SH). ^{13}C NMR (DMSO-d_6): δ 98.8, 122.1, 129.1, 130.2, 132.0, 144.5, 151.1, 156.2, 162.1, 163.5, 170.4. Mass *m/z* (%): 355 (M^++1, 5.28), 236 (100), 268 (39.09), 97 (69.19), 69 (70.33). Anal. calcd. for $C_{15}H_{10}N_6O_3S$: C 50.84; H 2.84; N 23.72. Found: C 50.81; H 2.80; N 23.76.

5-[4-(4-Fluorophenyl)-5-sulfanyl-4H-[1,2,4]triazol-3-yl]1H,3H,8H-pyrido[2,3-d]pyrimidine-2,4,7-trione ***(7)*** Yield 70 % (method 1), 65 % (method 2), mp >300 °C. IR (KBr, υ, cm^{-1}): 1660 (C=O), 3200 (NH), 2605 (SH). ^{1}H NMR (DMSO-d_6): δ 6.7 (s, 1H, C_6–H), 7.3 (d, $J = 7.8$ Hz, 2H, Ar–H), 7.6 (d, $J = 7.8$ Hz, 2H, Ar–H), 10.5 (brs, 1H, NH; D_2O exchangeable), 10.9 (brs, 1H, NH; D_2O exchangeable), 11.4 (brs, 1H, NH; D_2O exchangeable), 13.1 (s, 1H, SH). ^{13}C NMR (DMSO-d_6): δ 99.4, 120.5, 122.2, 126.8, 131.2, 144.1, 151.4, 157.1, 162.3, 163.6, 164.9, 170.6. Mass *m/z* (%): 372 (M^+, 11.02), 227 (100), 200 (34.83), 168 (61.73), 115 (57.32). Anal. calcd. for $C_{15}H_9FN_6O_3S$: C 48.38; H 2.44; N 22.57. Found: C 48.45; H 2.39; N 22.54.

Preparation of 2-chloro-S-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)-4-(aryl)-4H-[1,2,4]triazol-4-yl]ethanethioates ***8, 9***

Chloroacetyl chloride (1.13 g, 10 mmol) was added dropwise to a suspension of compounds **6** or **7** (10 mmol) in toluene (50 ml). TEA (0.5 ml) was added to the reaction mixture and heated under reflux for 6 h and then filtered while hot. The precipitated solid was dried and recrystallized from acetic acid.

2-Chloro-S-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)-4-(phenyl)-4H-[1,2,4]triazol-4-yl]ethanethioate ***(8)*** Yield 65 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1658 (C=O), 3016 (CH_2), 3181 (NH). Mass *m/z* (%): 430 (M^+, 40.05), 86 (100), 348 (41.8), 277 (41.8), 58 (65.8). Anal. calcd. for $C_{17}H_{11}ClN_6O_4S$: C 47.39; H 2.57; N 19.51. Found: C 47.44; H 2.60; N 19.56.

2-Chloro-S-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)-4-(4-fluorophenyl)-4H-[1,2,4]triazol-4-yl]ethanethioate ***(9)*** Yield 70 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1662 (C=O), 3000 (CH_2), 3176 (NH). ^{1}H NMR (DMSO-d_6): δ 6.8 (s, 1H, C_6–H), 7.2

(d, $J = 7.6$ Hz, 2H, Ar–H), 7.7 (d, $J = 7.6$ Hz, 2H, Ar–H), 10.2 (brs, 1H, NH; D_2O exchangeable), 10.8 (brs, 1H, NH; D_2O exchangeable), 11.4 (brs, 1H, NH; D_2O exchangeable). Mass *m/z* (%): 448 (M^+, 61.8), 109 (100), 312 (79.4), 176 (32.4), 55 (55.9). Anal. calcd. for $C_{17}H_{10}ClFN_6O_4S$: C 45.49; H 2.25; N 18.72. Found: C 45.54; H 2.32; N 18.76.

*Preparation of S-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)-4-(aryl)-4H-[1,2,4]triazol-5-yl]-2-(4-phenylpiperazin-1-yl)ethanethioates **10, 11***

A mixture of compounds **8** or **9** (4 mmol), 1-phenylpiperazine (1.2 g, 8 mmol), and potassium carbonate (0.54 g, 4 mmol) in DMF (10 ml) was stirred at room temperature for 24 h. The reaction mixture was filtered. The residue was washed with water, dried, and recrystallized from acetic acid.

*S-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)-4-(phenyl)-4H-[1,2,4]triazol-5-yl]-2-(4-phenylpiperazin-1-yl)ethanethioate **(10)*** Yield 55 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1554 (C=N), 1631 (C=O), 2839, 2993 (CH_2), 3422 (NH). Mass *m/z* (%): 556 (M^+, 12.5), 178 (100), 100 (23), 89 (18.4), 63 (16.4). Anal. calcd. for $C_{27}H_{24}N_8O_4S$: C 58.26; H 4.35; N 20.13. Found: C 58.32; H 4.42; N 20.19.

*S-[3-(2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidin-5-yl)-4-(4-fluorophenyl)-4H-[1,2,4]triazol-5-yl]-2-(4-phenylpiperazin-1-yl)ethanethioate **(11)*** Yield 62 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1550 (C=N), 1638 (C=O), 2843, 2996 (CH_2), 3425 (NH). ^{1}H NMR (DMSO-d_6): δ 2.8–3.0 (m, 8H, piperazine), 3.6 (s, 2H, CH_2), 6.3 (s, 1H, C_6–H), 6.8 (t, $J = 8.5$ Hz, 1H, Ar–H), 7.1 (t, $J = 8.5$ Hz, 2H, Ar–H), 7.3 (d, $J = 8.0$ Hz, 2H, Ar–H), 7.5 (d, $J = 8.5$ Hz, 2H, Ar–H), 7.7 (d, $J = 8.0$ Hz, 2H, Ar–H), 10.1 (brs, 1H, NH; D_2O exchangeable), 10.6 (brs, 1H, NH; D_2O exchangeable), 11.2 (brs, 1H, NH; D_2O exchangeable). Mass *m/z* (%): 574 (M^+, 48.5), 120 (100), 416 (54.5), 163 (36.4), 77 (57.6). Anal. calcd. for $C_{27}H_{23}FN_8O_4S$: C 56.44; H 4.03; N 19.50. Found: C 56.00; H 4.08; N 19.55.

*Preparation of N′-(chloroacetyl)-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide **(12)*** Chloroacetyl chloride (1.13 g, 10 mmol) was added dropwise to a suspension of compound **3** (2.37 g, 10 mmol) in ethanol (40 ml). The reaction mixture was heated under reflux for 6 h and then filtered while hot. The precipitated solid was dried and recrystallized from DMF.

Yield 38 %, mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 4.3 (s, 2H, CH_2), 6.7 (s, 1H, C_6–H), 8.4 (brs, 1H, NH; D_2O exchangeable), 9.7 (brs, 1H, NH; D_2O exchangeable), 10.2 (brs, 1H, NH; D_2O exchangeable), 10.5 (brs, 1H, NH; D_2O exchangeable), 11.4 (brs, 1H, NH; D_2O exchangeable). Mass *m/z* (%): 313 (M^+, 4.37), 219 (100), 178 (6.57), 135 (14.36), 92 (10.23). Anal. calcd. for $C_{10}H_8ClN_5O_5$: C 38.29; H 2.57; N 22.33. Found: C 38.33; H 2.50; N 22.39.

*Preparation of 2,4,7-trioxo-N′-[(4-substituted piperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazides **13–16***

A mixture of compound **12** (1.22 g, 4 mmol), the appropriate 1-substituted piperazine (8 mmol), and potassium carbonate (0.54 g, 4 mmol) in DMF (10 ml) was stirred at room temperature for 24 h. The product was collected by filtration, washed with water, dried, and recrystallized from acetic acid.

*2,4,7-Trioxo-N′-[(4-phenylpiperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide **(13)*** Yield 85 %, mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 3.0–3.3 (m, 8H, piperazine), 3.7 (s, 1H, CH_2), 6.2 (s, 1H, C_6–H), 6.8–7.4 (m, 5H, Ar–H), 8.1 (brs, 1H, CONH; D_2O exchangeable), 9.2 (brs, 1H, NHCO; D_2O exchangeable), 10.3 (brs, 1H, NH; D_2O exchangeable), 11.2 (brs, 1H, NH; D_2O exchangeable), 11.3 (brs, 1H, NH; D_2O exchangeable). Anal. calcd. for $C_{20}H_{21}N_7O$: C 54.67; H 4.82; N 22.31. Found: C 54.59; H 4.75; N 22.26.

*2,4,7-Trioxo-N′-[4-(4-methoxyphenylpiperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide **(14)*** Yield 75 %, mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 3.3–3.7 (m, 8H, piperazine), 2.5 (s, 2H, CH_2), 3.7 (s, 3H, $-OCH_3$), 6.4 (s, 1H, C_6–H), 6.7 (d, $J = 8.2$ Hz, 2H, Ar–H), 7.5 (d, $J = 8.2$ Hz, 2H, Ar–H), 8.5 (brs, 1H, CONH; D_2O exchangeable), 9.1 (brs, 1H, NHCO; D_2O exchangeable), 10.2 (brs, 1H, NH; D_2O exchangeable), 11.1 (brs, 1H, NH; D_2O exchangeable), 11.4 (brs, 1H, NH; D_2O exchangeable). ^{13}C NMR (DMSO-d_6): δ 50.4, 53.1, 56.7, 59.1, 99.2, 112.9, 116.1, 116.4, 143.5, 147.4, 151.1, 153.7, 161.8, 162.9, 166.4, 172.2. Anal. calcd. for $C_{21}H_{23}N_7O_6$: C 53.73; H 4.94; N 20.89. Found: C 53.66; H 4.90; N 20.95.

*2,4,7-Trioxo-N′-[4-(4-ethoxyphenylpiperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide **(15)*** Yield 72 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1590, 1640 (C=O), 2820, 2962 (CH_2), 3260, 3295 (NH). Mass *m/z* (%): 483 (M^+, 15.86), 294 (13.0), 179 (11.36), 128 (8.69), 89 (5.68). Anal. calcd. for

$C_{22}H_{25}N_7O_6$: C 54.65; H 5.21; N 20.28. Found: C 54.68; H 5.28; N 20.21.

2,4,7-Trioxo-N′-[(4-benzylpiperazin-1-yl)acetyl]-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide ***(16)*** Yield 84 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1580, 1620 (C=O), 2811, 2941 (CH_2), 3264, 3296 (NH). Mass *m/z* (%): 453 (M^+, 11.76), 55 (100), 275 (13.24), 178 (11.76), 78 (11.76). Anal. calcd. for $C_{21}H_{23}N_7O_5$: C 55.62; H 5.11; N 21.62. Found: C 55.67; H 5.14; N 21.66.

Preparation of N′-arylidene-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazides ***17–19***

To a suspension of compound **3** (2.37 g, 10 mmol) in absolute ethanol (30 ml), the appropriate aromatic aldehyde (20 mmol) was added. The reaction mixture was refluxed for 6 h. On cooling, the precipitated solid was collected by filtration, washed with ethanol, dried, and recrystallized from DMF.

N′-(4-chlorobenzylidene)-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide ***(17)*** Yield 70 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1587 (C=N), 1677 (C=O, ring), 1735 (C=O), 3174, 3243 (NH). Mass *m/z* (%): 359 (M^+, 5.2), 206 (100), 248 (19.6), 178 (15.3), 111 (11.3). Anal. calcd. for $C_{15}H_{10}ClN_5O_4$: C 50.08; H 2.80; N 19.47. Found: C 50.12; H 2.75; N 19.51.

N′-(4-methoxybenzylidene)-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide ***(18)*** Yield 73 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1602 (C=N), 1670 (C=O, ring), 1716 (C=O), 3160, 3243 (NH). ^{1}H NMR (DMSO-d_6): δ 4.1 (s, 3H, CH_3), 6.7 (s, 1H, C_6–H), 7.4 (d, J = 8.2 Hz, 2H, Ar–H), 7.7 (d, J = 8.2 Hz, 2H, Ar–H), 8.1 (s, 1H, CH=N), 8.6 (brs, 1H, NH; D_2O exchangeable), 9.7 (brs, 1H, NH; D_2O exchangeable), 10.5 (brs, 1H, NH; D_2O exchangeable), 11.3 (brs, 1H, NH; D_2O exchangeable). ^{13}C NMR (DMSO-d_6): δ 56.1, 99.0, 113.4, 115.1, 126.4, 131.4, 143.8, 147.4, 150.9, 161.9, 162.7, 163.1, 169.2. Mass *m/z* (%): 355 (M^+, 1.95), 161 (100), 257 (5.41), 178 (5.21), 98 (12.98). Anal. calcd. for $C_{16}H_{13}N_5O_5$: C 54.09; H 3.69; N 19.71. Found: C 54.11; H 3.65; N 19.75.

N′-(3-nitrobenzylidene)-2,4,7-trioxo-1,2,3,4,7,8-hexahydropyrido[2,3-d]pyrimidine-5-carbohydrazide ***(19)*** Yield 68 %, mp >300 °C. IR (KBr, υ, cm^{-1}): 1590 (C=N), 1671 (C=O, ring), 1714 (C=O), 3162, 3241 (NH). ^{1}H NMR (DMSO-d_6): δ 6.9 (s, 1H, C_6–H), 7.5 (t, J = 8.5 Hz, 1H, Ar–H), 7.7 (d, J = 8.5 Hz, 1H, Ar–H), 7.9 (d, J = 8.5 Hz, 1H, Ar–H), 8.1 (s, 1H, Ar–H), 8.4 (s, 1H, CH=N), 8.8 (brs, 1H, NH; D_2O exchangeable), 9.8 (brs, 1H, NH; D_2O exchangeable), 10.4 (brs, 1H, NH; D_2O exchangeable), 11.2 (brs, 1H, NH; D_2O exchangeable). Mass *m/z* (%): 370 (M^+, 1.80), 176 (100), 178 (12.91), 153 (3.02), 122 (4.15). Anal. calcd. for $C_{15}H_{10}N_6O_6$: C 48.66; H 2.72; N 22.70. Found: C 48.62; H 2.76; N 22.77.

Pharmacology

Bleomycin-dependant DNA damage assay (Ayyad et al., 2011a, b)

Materials

(1) DNA, L-ascorbic acid, ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid (TBA), and bleomycin sulfate were purchased all from Aldrich Co., USA.
(2) Magnesium chloride, ferric chloride, and hydrochloric acid are of high analytical grade, and are obtained all from El-Nasr Co. for pharmaceutical chemicals, Egypt.

Method The reaction mixture contained DNA (0.5 mg/ml), bleomycin sulfate (0.05 mg/ml), magnesium chloride (5 mM), ferric chloride (50 μM), and samples to be tested in a concentration of 0.1 mg/ml are dissolved in dimethylsulfoxide. L-Ascorbic acid (0.1 mg/ml) was used as a positive control. The mixture was incubated at 37 °C for 1 h and the reaction was terminated by the addition of 0.05 ml EDTA (0.1 ml). The color was developed by adding 0.5 ml TBA (1 % w/v) and 0.5 ml hydrochloric acid (25 % v/v) followed by heating at 80 °C for 10 min. After centrifugation, the extent of DNA damage was measured by the increase in absorbance at 532 nm (Table 1).

Evaluation of antineoplastic activity against EAC in mice (Ayyad et al., 2011a, b; Qureshi et al., 2001; Bala et al., 2010)

Materials

(1) *Ehrlich ascites cells* The cells of Ehrlich ascites tumor were obtained from the National Cancer Institute (which is a certified institute by National Medical Research Ethics Committee), Cairo, Egypt. After harvesting and preparation of the cells, their total number and viability were determined by counting using Trypan blue. The desired concentration of tumor cells (2×10^6 cells per 0.2 ml) was

obtained by dilution with saline (0.9 % sodium chloride solution). The viability of tumor cells obtained and used in this experiment was always higher than 90 %. Below this percentage, the cells were discarded and the entire procedure was repeated.

(2) 5-FU was obtained from Aldrich Co., USA.

(3) *Mice* Adult Swiss male albino mice (20–25 g) of both sexes were used in this experiment. They were housed in microlon boxes in a controlled environment (temperature 25 ± 2 °C and 12 h dark/light cycle) with standard laboratory diet and after regimen.

Method The animals were divided into four groups. All the animals in the treated groups (from groups 2 to 4) were inoculated with 2×10^6 Ehrlich ascites cells/mouse on the day 0. Treatment started 24 h after inoculation by intraperitoneal (IP) injection of the drug. The animals in group 3 were injected by the tested compound in a dose of 100 μg/mouse, which is nearly equivalent to 5 mg/kg of body weight, while the standard group (group 4) had received IP treatment with 5 mg/kg of body weight of 5-FU. The control group (group 2) was treated with the same volume of 0.9 % sodium chloride solution. Treatment was given for nine successive days (Tables 2, 3, 4).

Acknowledgments We would like to thank Prof. Farid A. Badria, Professor of Pharmacognosy, Faculty of Pharmacy, Mansoura University, for his cooperation in performing the biologic screening.

References

Agrawal VK, Sohgaura R, Khadikar PV (2002) QSAR studies on biological activity of piritrexim analogues against *Pc* DHFR. Bioorg Med Chem 10:2919–2926

Al-Soud YA, Al-Dweri MN, Al-Masoudi NA (2004) Synthesis, antitumor and antiviral properties of some 1,2,4-triazole derivatives. IL Farm 59:775–783

Anderson GL, Shim JL, Broom AD (1977) Pyridopyrimidines. 6-Nucleophilic substitutions in the pyrido[2,3-*d*]pyrimidine series. J Org Chem 42:993–996

Ayyad SEN, Makki MS, Al-kayal NS, Basaif SA, El-Foty KO, Asiri AM, Alarif WM, Badria FA (2011a) Cytotoxic and protective DNA damage of three new diterpenoids from the brown alga *Dictyota dichotoma*. Eur J Med Chem 46:175–182

Ayyad SEN, Ezmirly ST, Basaif SA, Alarif WM, Badria AF, Badria FA (2011b) Antioxidant, cytotoxic, antitumor and protective DNA damage metabolites from the red sea brown alga *Sargassum* sp. Pharmacogn Res 3:160–165

Bala A, Kar B, Haldar PK, Mazumder UK, Bera S (2010) Evaluation of anticancer activity of *Cleome gynandra* on Ehrlich's ascites carcinoma treated mice. J Ethnopharmacol 129:131–134

Chan DCM, Fu H, Forsch RA, Queener SF, Rosowsky A (2005) Design, synthesis and antifolate activity of new analogues of piritrexim and other diaminopyrimidine dihydrofolate reductase inhibitors with ω-carboxyalkoxy or ω-carboxy-1-alkynyl substitution in the side chain. J Med Chem 48:4420–4431

Cordeu L, Cubedo E, Bandres E, Rebollo A, Saenz X, Chozas H, Dominguez MV, Echeverria M, Mendivil B, Sanmartin C, Palop JA, Font M, Foncillas JG (2007) Biological profile of new apoptotic agents based on 2,4-pyrido[2,3-*d*]pyrimidine derivatives. Bioorg Med Chem 15:1659–1669

De P, Baltas M, Theys DL, Bruyere C, Kiss R, Belval FB, Saffon N (2010) Synthesis and anticancer activity evaluation of 2(4-alkoxyphenyl)cyclopropyl hydrazides and triazolo phthalazines. Bioorg Med Chem 18:2537–2548

El-Nassan HB (2011) Synthesis and antitumor activity of novel pyrido[2,3-*d*][1,2,4]triazolo[4,3-a]pyrimidin-5-one derivatives. Eur J Med Chem 46:2031–2036

Formagio ASN, Tonin LTD, Foglio MA, Madjarof C, Carvalho JE, Costa WF, Cardoso FP, Sarragiotto MH (2008) Synthesis and antitumoral activity of novel 3-(2-substituted-1,3,4-oxadiazol-5-yl) and 3-(5-substituted-1,2,4-triazol-3-yl) β-carboline derivatives. Bioorg Med Chem 16:9660–9667

Gangjee A, Adair O, Queener SF (2001) Synthesis of 2,4-diamino-6-(thioarylmethyl)pyrido[2,3-*d*]pyrimidines as dihydrofolate reductase inhibitors. Bioorg Med Chem 9:2929–2935

Grande F, Aiello F, Grazia OD, Brizzi A, Garofalo A, Neamati N (2007) Synthesis and antitumor activities of a series of novel quinoxaline hydrazides. Bioorg Med Chem 15:288–294

Guo CC, Tong RB, Li KL (2004) Chloroalkyl piperazine and nitrogen mustard porphyrins: synthesis and anticancer activity. Bioorg Med Chem 12:2469–2475

Joska TM, Anderson AC (2006) Structure activity relationships of *Bacillus cereus* and *Bacillus anthracis* dihydrofolate reductase: toward the identification of new potent drug leads. Antimicrob Agents Chemother 50:3435–3443

Kurumurthy C, Rao PS, Swamy BV, Kumar GS, Rao PS, Narsaiah B, Velatooru LR, Pamanji R, Rao JV (2011) Synthesis of novel alkyltriazole tagged pyrido[2,3-*d*]pyrimidine derivatives and their anticancer activity. Eur J Med Chem 46:3462–3468

Mohamed NR, El-Saidi MMT, Ali YM, Elnagdi MH (2007) Utility of 6-amino-2-thiouracil as a precursor for the synthesis of bioactive pyrimidine derivatives. Bioorg Med Chem 15:6227–6235

Qureshi S, Al-Shabana O, Al-Harbi M, Al-Bekairi A, Raza M (2001) Boric acid enhances in vivo Ehrlich ascites carcinoma cell proliferation in Swiss albino mice. Toxicology 165:1–11

Sanmartin C, Echeverria M, Mendivil B, Cordeu L, Cubedo E, Foncillas JG, Font M, Palop JA (2005) Synthesis and biological evaluation of new symmetrical derivatives as cytotoxic agent and apoptosis inducers. Bioorg Med Chem 13:2031–2044

Shi F, Ding J, Zhang S, Hao WJ, Cheng C, Tu S (2011) Substrate controlled chemoselective synthesis and potent cytotoxic activity of novel 5,6,7-triarylpyrido[2,3-*d*]pyrimidin-4-one derivatives. Bioorg Med Chem Lett 21:1554–1558

Tu S, Zhang J, Zhu X, Xu J, Zhang Y, Wang Q, Jia R, Jiang B, Zhang J (2006) New potential inhibitors of cyclin dependent kinase 4: design and synthesis of pyrido[2,3-*d*]pyrimidine derivatives under microwave irradiation. Bioorg Med Chem Lett 16: 3578–3581

Zink M, Lanig H, Troschutz R (2004) Structural variations of piritrexim, a lipophilic inhibitor of human dihydrofolate reductase: synthesis, antitumor activity and molecular modeling investigations. Eur J Med Chem 39:1079–1088