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Original article

Novel modified steroid derivatives of androstanolone as chemotherapeutic anti-cancer agents

Mohamed El-Far [a], Gamal A. Elmegeed [b,*], Emad F. Eskander [b], Hanaa M. Rady [c], Mohamed A. Tantawy [b]

[a] Division of Biochemistry, Faculty of Science, Mansoura University, Mansoura, Egypt
[b] Hormones Department, National Research Center, Dokki, Giza, Egypt
[c] Chemistry of Natural Compounds Department, National Research Center, Dokki, Giza, Egypt

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ABSTRACT

The aim of the present study is to synthesize and evaluate new potential chemotherapeutic anti-tumor agents. Several thiazolo-, pyrido-, pyrano- and lactam steroid derivatives were obtained using 17β-hydroxy-5α-androstan-3-one (androstanolone) **1** as starting steroid. The structure of the novel steroid derivatives was confirmed using the analytical and spectral data. The most pure and structurally promising compounds **7a**, **10a**, **12b**, **18** and **23** were evaluated as anti-tumor agents. The in vitro cytotoxic activity was evaluated against hepatoma cell lines using MTT assay. Also the in vivo anti-tumor activity was evaluated against Ehrlich ascites carcinoma (EAC). The results of the in vitro study showed that at incubation time 72 h, in olive oil, compound **7a** was the most effective cytotoxic compound with IC_{50} of 30 μM, while the effects of compounds **18** and **23** were approximately similar with IC_{50} of 37 μM and 35 μM respectively. While the tested compounds when dissolved in DMSO showed approximately the same IC_{50} at both 48 and 72 h incubation period, compound **23** was the most effective cytotoxic with IC_{50} 42 μM at 48 h and 40 μM at 72 h. The results of the in vivo study showed that all the tested novel compounds at 25 mg/kg were effective against EAC. Our novel steroid derivatives are promising candidates as anti-cancer agents, none of the mice treated with our novel derivatives showed any toxic symptoms, but they also completely inhibited tumor growth and retained the hemoglobin content, body weight, and WBCs near normal values and similar to what obtained for the standard drug 5-flurouracil.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignancies that carries poor prognosis worldwide; it is the fifth most common cancer and the third leading cause of cancer death [1]. The incidence of HCC varies greatly worldwide, reflecting the prevalence of its common etiologic factors such as hepatitis B (HBV) and hepatitis C (HCV) virus infections, heavy alcohol consumption and high dietary exposure to aflatoxins [2]. In Egypt, HCC is the second most common malignancy in males and the fifth in females. The major risk factor is infection by HCV, which accounts for 20% of acute hepatitis, 70% of chronic hepatitis, 40% of cirrhosis and up to 90% of HCC cases [3]. Egypt has the highest prevalence of hepatitis C virus (HCV) in the world with an average of approximately 13.8% in the general population [4]. Because of the mutlifocal nature of liver carcinoma, most cancer patients are considered non-resectable. In these patients, chemotherapy is the only choice of treatment. Unfortunately, development of drug resistance in tumor after treatment is always a major obstacle to the successful management of liver cancer [5]. Thus, developing new therapeutic agents that can overcome drug resistance becomes an urgent need for cancer patients.

Hybrid anti-cancer agents, which combine two active compounds in one, such as steroidal alkylators, contain steroidal moiety as biological vectors for cytotoxic agents in order to diminish toxicity and to enhance specificity was recently demonstrated [6]. These agents attain duplicate effects on cancer cells. A variety of steroids with unusual and interesting structures have been synthesized and evaluated for their anti-tumor activity [7,8]. Also the anti-tumor activity of many compounds containing heterocyclic ring has been reviewed [9,10].

In view of the above facts, we set as a goal to prepare some new steroid derivatives by combining heterocyclic moiety of potent cytotoxic activity. We report here the facile synthesis of new

* Corresponding author. Tel.: +20 235682070; fax: +20 23370931.
E-mail address: gamalae@hotmail.com (G.A. Elmegeed).

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steroidal heterocyclic derivatives. Furthermore, the more structurally promising compounds were examined for their cytotoxicity in vitro against a panel of hepatoma cell lines ($HepG_2$) using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Also, the new compounds as anti-cancer agents were examined in vivo against Ehrlich ascites carcinoma (EAC) in mice.

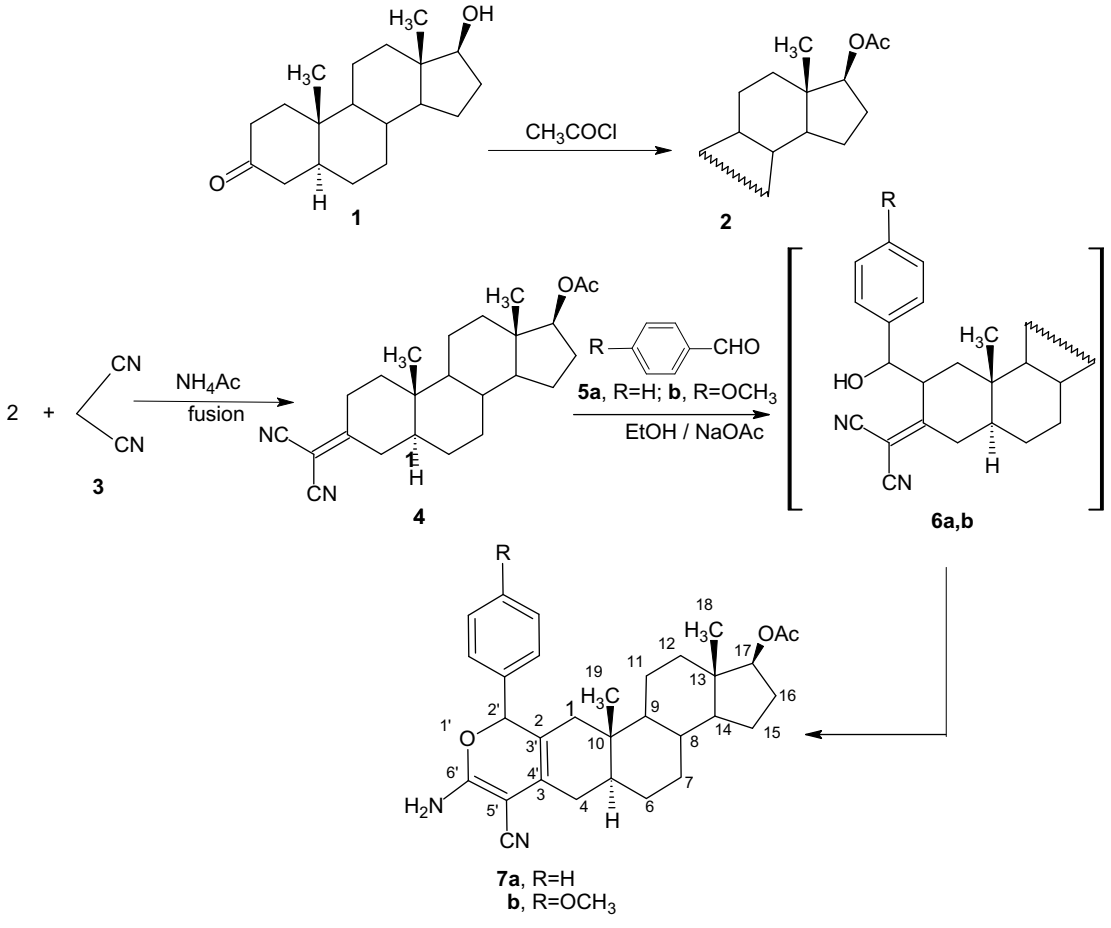
2. Results and discussion

2.1. Chemistry

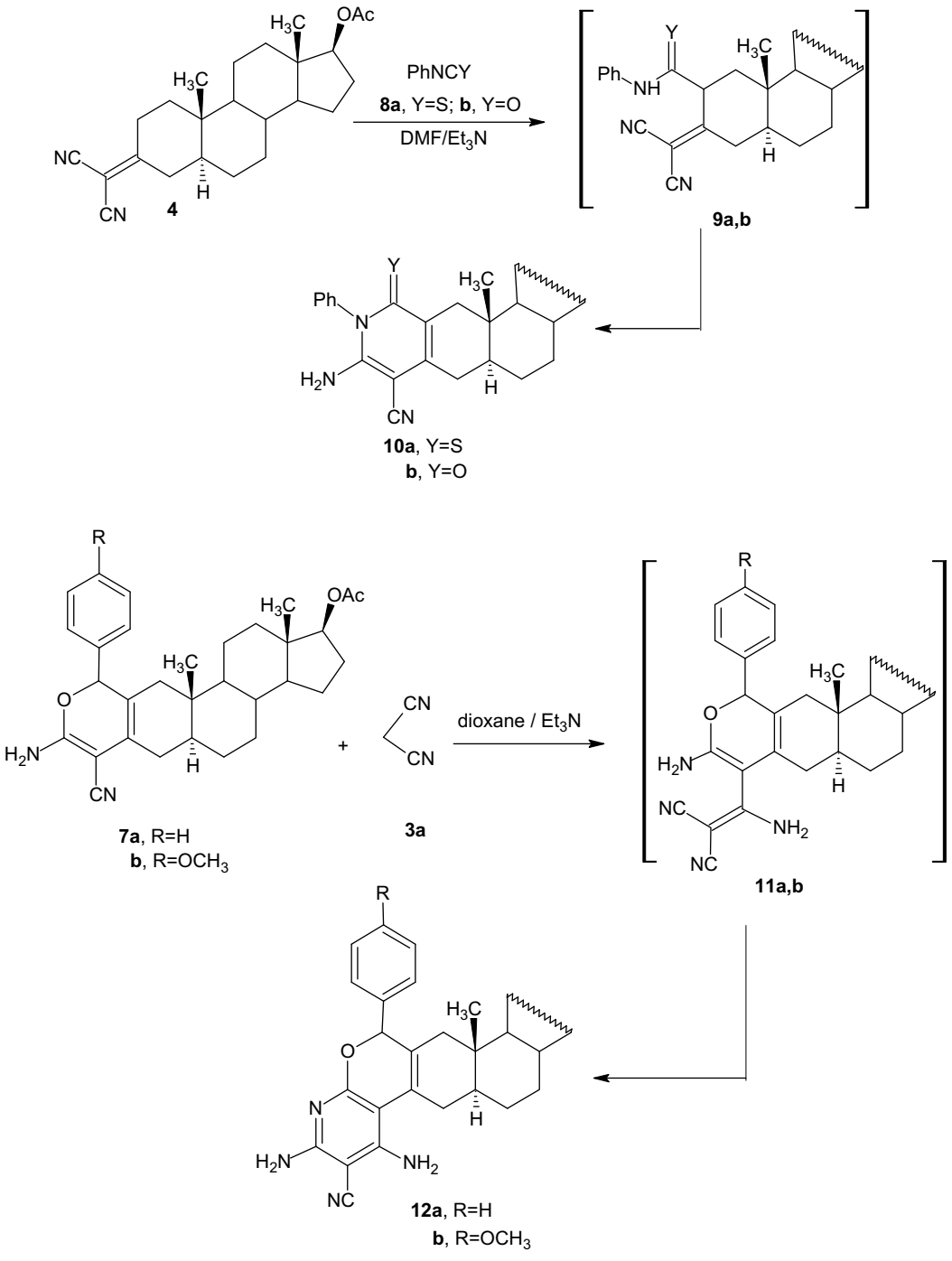
Pyrane, pyridine, thiazole, thiophene and lactam rings represent molecular frameworks that serve as a platform for developing pharmaceutical agents for various applications. Many derivatives of these rings proved as anti-tumor agents [11–13]. Simple acetylation of 17β-hydroxy-5α-androstan-3-one (androstanolone **1**) afforded 17β-acetoxy-5α-androstan-3-one **2**. Compound **2** was fused with malononitrile **3** in the presence of ammonium acetate to form the Knoevenagel condensed product, 3-dicyanomethylene-5α-androstane derivative **4** (Scheme 1) in 75% yield. The mass spectrum of compound **4** (EIMS) revealed a molecular ion peak at $m/z = 380$ (28%) and the IR spectrum showed two cyano groups stretching at ν 2220 and 2225 cm^{-1}.

The presence of the α,β-unsaturated nitrile moiety in compound **4** showed pronounced activity towards several chemical reagents. The reaction of compound **4** with either benzaldehyde **5a** or anisaldehyde **5b** in refluxing ethanol in the presence of sodium acetate did not give the corresponding arylidine but afforded compounds containing amino groups which were identified, according to the analytical and spectral data (cf. Materials and methods), as 6′-aminopyrano[3′,4′:2,3]androstane derivatives **7a**,**b** (Scheme 1). The formation of these compounds was assumed to proceed via the non-isolable aldol adduct intermediates **6a**,**b** followed by cyclization (Scheme 1).

The reaction of compound **4** with either phenyl isothiocyanate **8a** or phenyl isocyanate **8b** in dimethylformamide using triethylamine as a catalyst afforded the corresponding non-isolable intermediates **9a**,**b** which readily underwent intramolecular cyclization to give the 6′-aminopyrido[3′,4′:2,3]androstane derivatives **10a**,**b** respectively (Scheme 2). Elucidation of the proposed structures **7a**,**b** and **10a**,**b** was based on their correct elemental analyses and compatible IR, ^1H NMR, ^{13}C NMR and mass spectral data (cf. Materials and methods). The reactivity of enaminonitrile moiety of compounds **7a** and **7b** towards active methylene reagents was studied in the aim of further confirmation of their structures and to form annulated aminopyridopyrano-steroids with potential biological activities. Thus, the reaction of compound **7a** or **7b** with equimolar amounts of malononitrile **3** in boiling dioxane solution containing a catalytic amount of triethylamine afforded the corresponding diaminopyrido[2″,3″:6′,5′]pyrano[3′,4′:2,3]androstane derivatives **12a**,**b** respectively (Scheme 2). The reaction takes place via an initial addition of the active methylene group of malononitrile to the cyano group of compounds **7a**,**b** to give the non-isolable intermediates **11a**,**b** which undergo intramolecular cyclization to afford the isolable products **12a**,**b** in 78% and 72% yield respectively (Scheme 2). The mass spectrum of compound **12a** revealed



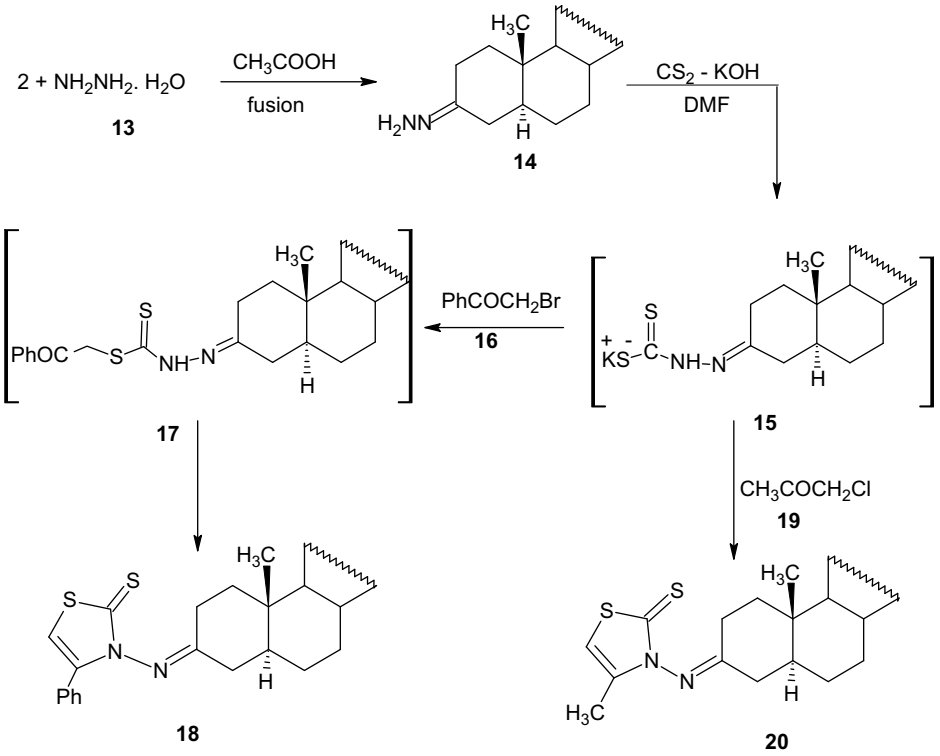
Scheme 1.



Scheme 2.

a molecular ion peak ($M^+ - 1$) at $m/z = 551$ (25%) and its IR spectrum showed absorption peaks at ν 3450–3230 cm^{-1} ($2NH_2$) and ν 2220 cm^{-1} (CN). The ^1H NMR spectrum of compound **12a** revealed the presence of two D_2O-exchangeable singlets (4H) at $\delta = 4.89$ and 5.62 ppm which are characteristic for the NH_2 groups. Also, all the analytical and spectral data of compound **12b** are in agreement with the proposed structure (cf. Materials and methods). Mixture of distereoisomers at position C-2′ in compounds **7a**,**b** and **12a**,**b** was isolated. The isolation and identification of these isomers were beyond the scope of this study.

The reactivity of 17β-acetoxy-5α-androstan-3-one **2** towards hydrazines was studied in the aim to form aminosteroids. Thus, compound **2** reacted with hydrazine hydrate **13** by fusion in the presence of glacial acetic acid drops to form the 3-hydrazono-5α-androstane derivative **14** in 80% yield (Scheme 3). The reaction of amines with carbon disulfide followed by cyclization with α-haloketones to yield the corresponding thiazole with potential biological activities has been reported [14,15]. Thus, the reaction of compound **14** with carbon disulfide in dimethylformamide, containing potassium hydroxide, afforded the non-isolable intermediates, *N*-potassium thiocarbamate salt **15** (Scheme 3). The latter reacted in situ with phenacyl bromide **16** to give the phenyl thiazolyl androstane derivative **18** in 73% yield via the non-isolable intermediate **17**. The mass spectrum of compound **18** revealed



Scheme 3.

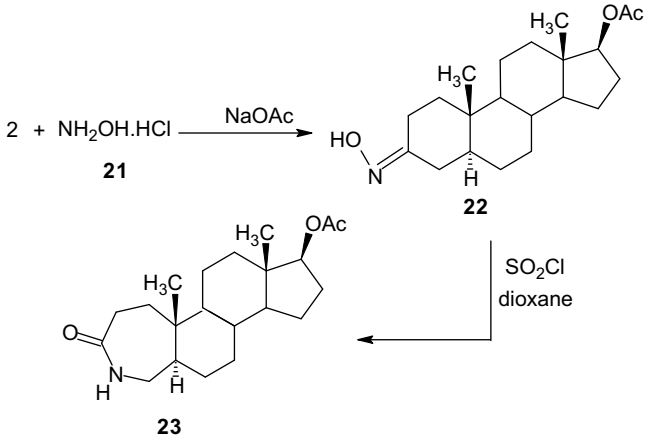
molecular ion peak at $m/z = 522$ (33%) and its IR spectrum showed absorption peak at $\nu = 1195\ cm^{-1}$ for the C=S group. Moreover, the ^1H NMR spectrum revealed the disappearance of NH_2 group singlet and the presence of singlet at δ 6.47 for the thiazole 5-H and a multiplet (5H) at δ 7.46–7.84 for the aromatic protons. Similarly, the reaction of the non-isolable intermediate **15** with chloroacetone **19** under the same experimental conditions provided the methyl thiazolyl androstane derivative **20** in 68% yield (Scheme 3). The structure of compound **20** was confirmed by their elemental analysis and spectral data (cf. Materials and methods).

Taken into account the structural requirements for the cytotoxicity, several new hydroximinosteroid derivatives had been prepared and evaluated for their cytotoxic activity [16,17]. Compound **2** reacted with hydroxylamine hydrochloride **21** in ethanolic sodium acetate solution to give (*E*)-3-hydroxyiminoandrostane derivative **22** in 80% yield (Scheme 4). The structure of compound **22** was determined on the basis of its analytical and spectral data. IR spectra bands at 3267 cm^{-1} for the (*E*)-isomer indicate the presence of a hydroximine group. In the ^1H NMR spectrum of compound **22**, the signal for H_β–C(4) was shifted downfield, appearing at 3.25 ppm as a doublet of doublets due to the deshielding influence of the hydroxy oxygen of the oxime which confirmed the (*E*) configuration. Also, the ^1H NMR revealed the presence of broad singlet (1H) at $\delta = 9.36$ ppm for the NOH group.

In view of the importance of steroid containing alkylating agents, modified steroids, such as aza-homosteroids, characterized by the amide group in the steroidal nucleus as biological carrier that transports the alkylating agent to the tumor site was reported [18]. It is well known that Beckman rearrangement of steroidal keto-oximes in the *E*-configuration usually leads to the formation of enamine-type lactams [19,20]. Thus, treatment of (*E*)-3-hydroximinoandrostane derivative **22** with thionyl chloride in dioxane solution gave, as a single product, an enamide-type lactam, 4-aza-A-homoandrostan-3-one **23**, in 72% yield (Scheme 4) free from the isomer 3-aza-A-homoandrostan-3-one. The IR spectrum of compound **23** revealed cyclic carbonyl group at $\nu = 1682\ cm^{-1}$ in addition to the acetate-carbonyl at $\nu = 1734\ cm^{-1}$ and its ^1H NMR spectrum showed broad singlet at 8.03 ppm for the amide proton. All the analytical and spectral data of compound **23** are in accordance with the proposed structures.

2.2. In vitro evaluation of the cytotoxic activity

In this study, the most structurally promising compounds **7a**, **10a**, **12b**, **18** and **23** were investigated individually as anti-tumor agents against Hepatoma ($HepG_2$) cell lines. The inhibition of proliferation of $HepG_2$ cells was determined using MTT assay. The



Scheme 4.

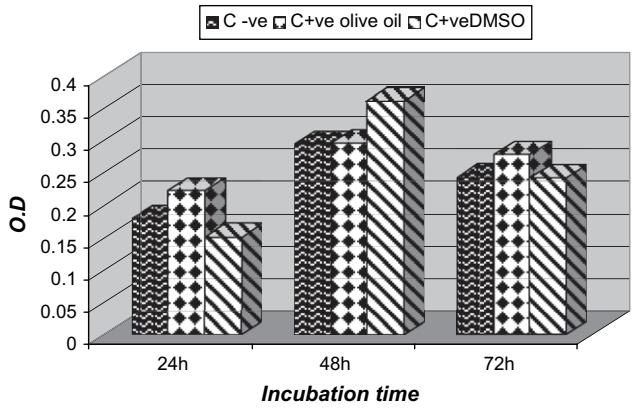


Fig. 1. Effect of DMSO and olive oil (100 μl) on growth of $HepG_2$ cells with 100 μl volume, at different time intervals; C = control.

usage of olive oil or DMSO as a solvent at a volume of 100 μl (the-maximum volume used to dissolve the tested compounds) had non-significant effect on the viability of $HepG_2$ cells when treated for 24 h, 48 h and 72 h (Fig. 1). Data in Tables 1 and 2 are expressed as percentage of cell growth inhibition of the treated cells vs controls ± S.D. calculated on the average of the experiments performed in triplicate. The concentration 10 μM/ml did not show any significant effect on viability of hepatoma cells at 24, 48, and 72 h incubation time for any of the tested compounds in olive oil or in DMSO. The results in Table 1 and Fig. 2 showed the effect of compounds **7a**, **10a**, **12b**, **18** and **23**, dissolved in olive oil, on $HepG_2$ cells at 24, 48 and 72 h incubation time. At 24 h incubation and concentration 50 μM/ml: compound **7a** increased $HepG_2$ cells growth (40%, $P < 0.05$) while compound **10a** showed significant inhibition of $HepG_2$ cells growth (26%, $P < 0.05$). The other tested compounds **12b**, **18** and **23** showed non-significant cell growth inhibition of $HepG_2$ cells. At a concentration of 100 μM/ml, compound **10a** significantly inhibited the proliferation of $HepG_2$ cells (38%, $P > 0.01$), while the other tested compounds showed non-significant effect on $HepG_2$ cells. At 48 h incubation, there was significant cell growth inhibition of hepatoma cells by all the tested compounds with the same effect at both 50 and 100 μM/ml concentration. The cytotoxic effect of all tested compounds at 72 h incubation was highly significant ($P > 0.001$) and more than that obtained at 48 h incubation, the percent of cell growth of compounds **7a**, **10a**, **12b**, **18** and **23** was 66, 74, 65, 64 and 65% respectively.

The results in Table 2 and Fig. 3 showed the effect of compounds **7a**, **10a**, **12b**, **18** and **23** dissolved in DMSO on $HepG_2$ cells at 24, 48 and 72 h incubation time. At 24 h incubation, and concentration 50 μM/ml: compounds **7a**, **18** and **23** non-significantly ($P < 0.05$) inhibited the cell proliferation of $HepG_2$ cells. Also at a concentration of 100 μM/ml, all tested compounds non-significantly inhibited the cell growth of $HepG_2$ cells. At 48 h incubation, at both 50 and 100 μM/ml concentration, all tested compounds significantly ($P > 0.01$) inhibited the cell proliferation of $HepG_2$ cells. At 72 h incubation, and concentration 50 μM/ml: compounds **7a**, **18** and **23** significantly ($P > 0.01$) inhibited the cell proliferation of $HepG_2$ cells, while compounds **10a** and **12b** showed very weak cytotoxic effect on $HepG_2$ cells compared with that obtained at 48 h of incubation time. At a concentration of 100 μM/ml, compounds **7a**, **18** and **23** showed highly significant ($P > 0.01$) cytotoxic effect on $HepG_2$ cells with percent of inhibition 69, 55 and 68% respectively. Also compounds **10a** and **12b** could overcome the cells resistance and significantly ($P > 0.01$) inhibited the cell growth of $HepG_2$ cells with the rates 38 and 41% respectively.

Table 3 revealed the IC_{50} of the tested compounds. Actually, all tested compounds gave the best results when dissolved in olive oil in 50 and 100 μM/ml and incubation time 72 h. Compound **7a** was the most effective one among the other tested compounds ($IC_{50} = 30$ μM), while the effect of compounds **18** and **23** was approximately similar ($IC_{50} = 37$ μM and 35 μM respectively). In the case of using DMSO as solvent, IC_{50} of all tested compounds were elevated as compared to that obtained in the case of olive oil. However, compound **23** showed also good inhibition of $HepG_2$ cells when dissolved in DMSO in 50 or 100 μM/ml at incubation time 72 h ($IC_{50} = 40$ μM). The results revealed that olive oil is the suitable vehicle for anti-tumor drugs and is better than DMSO. Olive oil has not synergic effect with anti-tumor activity [21] as shown in Fig. 1. These results were confirmed also via the indirect effect of the tested compounds on morphology of $HepG_2$ cells. The cells underwent dramatic morphological changes, shrunken and the ratio of cytolysis increased after exposure to tested compounds that were dissolved in olive oil, while the cells that were treated with compounds dissolved in DMSO still have some viable cells and less cytolysis compared to that obtained in the case of olive oil (Photograph 1 for compound **7a**, as example).

2.2.1. Structure–activity relationship

The analysis of the structure–activity relationship indicates that the cytotoxicity of the tested compounds seems to be linked to the presence of heterocyclic moiety fused to the steroid moiety. This is identified clearly in comparison with the published cytotoxic activity of some steroids [22]. In olive oil at 72 h incubation time the aminopyranoandrostane derivative **7a** is the most cytotoxic compound followed by the lactam derivative **23** and thiazolyl androstane **18**. Compound **7a** is more effective than the amino-pyridoandrostane derivative **10a** and this may be attributed to the presence of pyrane ring with its oxygen atom at position 2. Also, the addition of pyridine ring to the pyranoandrostane moiety to form compound **12b** decreased the cytotoxic activity of the pyranoandrostane moiety of compound **7a**. Also, 4-aza-A-homoandrostan-3-one derivative **23** is the most cytotoxic derivative in DMSO at 48 and 72 h incubation time. This compound is characterized by the presence of the amide group in the steroid nucleus, which acts as a biological carrier that transports the alkylating agent to the tumor site. The presence of amide group is important in order to lower

Table 1
Effect of tested compounds using olive oil as solvent on $HepG_2$ cell line proliferation, data expressed as percent of cell growth ± S.D. $^{*}P < 0.05$ was considered as statistically significant. $^{**}P < 0.01$ was considered as highly significant.

Treatment	24 h			48 h			72 h		
Conc.	10 μM	50 μM	100 μM	10 μM	50 μM	100 μM	10 μM	50 μM	100 μM
7a	99.05 ± 0.27	139.5 ± 0.06**	112.72 ± 0.08	100.04 ± 0.05	46.13 ± 0.04**	45.45 ± 0.09**	97.04 ± 0.01	29.85 ± 0.006**	33.63 ± 0.00**
10a	99.7 ± 0.00	74.20 ± 0.32*	62.98 ± 0.31**	97.5 ± 0.01	47.49 ± 0.27**	43.07 ± 0.55**	96.34 ± 0.02	26.87 ± 0.00**	25.89 ± 0.00**
12b	100.04 ± 0.00	80.66 ± 0.01	95.61 ± 0.01	98.88 ± 0.03	47.91 ± 0.02**	50.86 ± 0.02**	98.56 ± 0.01	35.25 ± 0.06**	35.25 ± 0.00**
18	99.02 ± 0.07	99.5 ± 0.04	112.72 ± 0.04	99.67 ± 0.04	45.71 ± 0.02**	46.72 ± 0.00**	95.37 ± 0.01	32.80 ± 0.07**	34.53 ± 0.00**
23	98.97 ± 0.04	99.09 ± 0.01	115.45 ± 0.03	100.34 ± 0.00	46.27 ± 0.03**	47.01 ± 0.07**	95.88 ± 0.00	35.97 ± 0.04**	35.25 ± 0.02**

Table 2
Effect of tested compounds using DMSO as solvent on $HepG_2$ cell line proliferation, data were expressed as percent of cell growth ± S.D. *$P < 0.05$ was considered as statistically significant. **$P < 0.01$ was considered as highly significant.

Treatment	24 h			48 h			72 h		
Conc.	10 μM	50 μM	100 μM	10μ M	50 μM	100 μM	10 μM	50 μM	100 μM
7a	100.04 ± 0.00	90.00 ± 0.00	91.33 ± 0.01	98.04 ± 0.01	62.98 ± 0.01**	41.98 ± 0.00**	98.05 ± 0.04	67.21 ± 0.02**	31.21 ± 0.00**
10a	98.97 ± 0.04	103.33 ± 0.00	97.33 ± 0.01	99.34 ± 0.03	77.51 ± 0.00**	59.55 ± 0.00**	100.04 ± 0.05	98.34 ± 0.07	62.24 ± 0.028**
12b	99.05 ± 0.27	107.33 ± 0.00	94.00 ± 0.00	98.56 ± 0.07	74.30 ± 0.00**	47.67 ± 0.01**	99.26 ± 0.04	89.21 ± 0.05	58.50 ± 0.01**
18	100.6 ± 0.07	86.66 ± 0.00	84.00 ± 0.00	99.39 ± 0.03	66.66 ± 0.00**	41.79 ± 0.00**	97.53 ± 0.00	71.36 ± 0.01**	45.22 ± 0.02**
23	100.02 ± 0.063	79.33 ± 0.00	72.00 ± 0.00	100.88 ± 0.00	37.56 ± 0.00**	38.12 ± 0.00**	96.74 ± 0.078	34.56 ± 00**	31.95 ± 0.00**

systemic toxicity and improve specificity in cancer treatment [18]. The results verified the importance of the presence of pyrane, thiazole and lactam moieties as pharmacophores for the anti-cancer activity.

2.3. In vivo evaluation of the anti-tumor activity

The anti-tumor activity was evaluated on EAC bearing mice using animal model. All the tested novel steroid derivatives **7a**, **10a**, **12b**, **18** and **23** at dose level of 25 mg/kg completely inhibited the tumor growth in the experimental model and showed zero tumor volume at the end of in vivo experiment, while EAC control showed 4.56 ± 0.37 ml (Table 4, Photograph 2). In cancer chemotherapy one of the major problems is anemia which is mainly due to reduction in RBCs or hemoglobin percentage. Treatment with all novel synthesized compounds retained the hemoglobin content, body

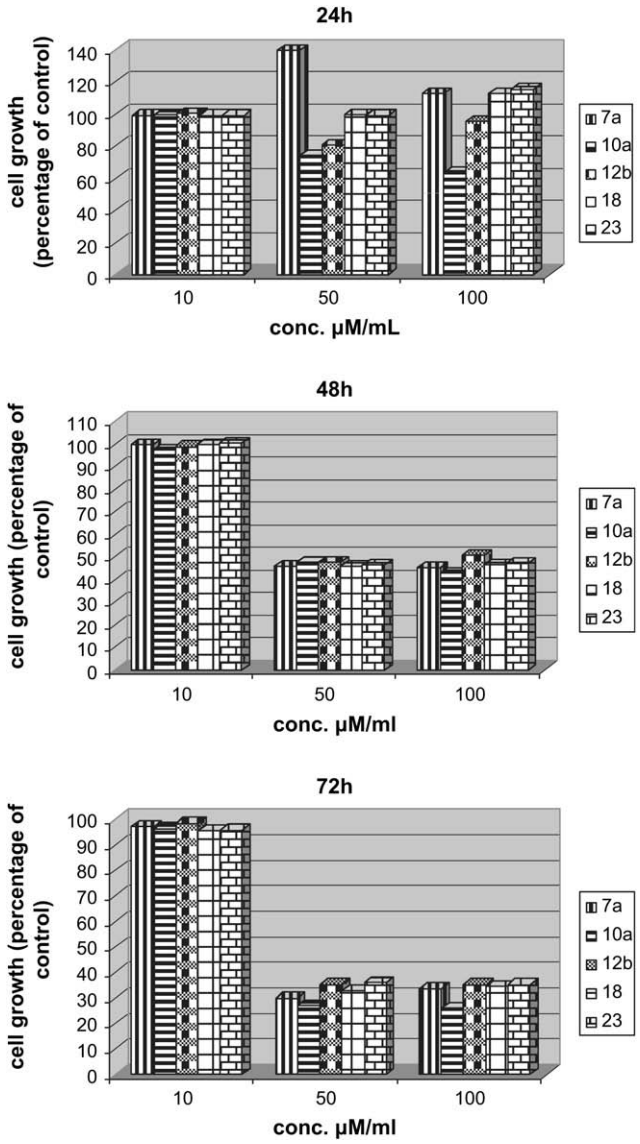


Fig. 2. Effect of tested compounds dissolved in olive oil on $HepG_2$ cells at 24, 48 and 72 h.

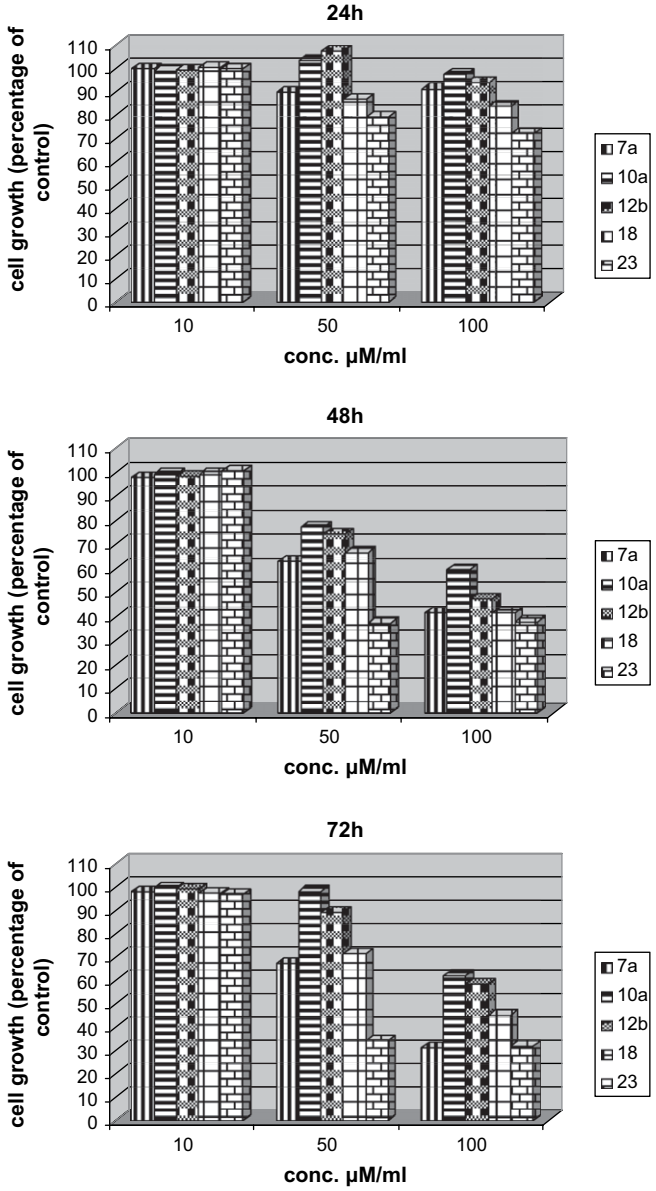


Fig. 3. Effect of tested compounds dissolved in DMSO on $HepG_2$ cells at 24, 48 and 72 h.

Table 3
The in vitro cytotoxic activity of tested compounds on $HepG_2$ cells (IC_{50} in μM).

Compounds	Olive oil		DMSO	
	48 h	72 h	48 h	72 h
7a	46	30	81	73
10a	48	40	>100	>100
12b	48	41	95	>100
18	47	37	85	90
23	47	35	42	40

weight, hematocrit % and WBCs beyond normal values and similar to values observed with the most commonly clinically used drug, 5-flurouracil (Table 4, Fig. 4). Furthermore, none of the treated mice with novel compounds exhibited any abnormal behavioral or any toxicity symptoms of dose used during this study. They were all active as anti-tumor agents with no loss of appetite and showed no dizziness or erection of hairs or hypothermia (Photograph 2). This indicated the safety and high activity of all tested compounds at the specified low dose as chemotherapeutic agents.

2.4. Conclusion

In conclusion, we have described a facile synthesis of novel promising anti-cancer steroid derivatives and investigated also the importance of incorporating heterocyclic moiety to the steroid nucleus to form new effective anti-cancer hybrid molecules. The three compounds namely **7a**, **18**, and **23** dissolved in olive oil showed promising broad spectrum cytotoxic activity. All tested compounds completely inhibited the tumor growth of EAC in experimental model and brought back the hemoglobin content, body weight, hematocrit % and WBCs beyond normal values. Finally, the broad spectrum anti-tumor activity displayed by these compounds may be of interest for further derivatization, further in vivo and clinical studies in the hope of finding more active and selective anti-tumor agents.

3. Materials and methods

3.1. Synthetic methods, spectral and analytical data

Starting steroid 17β-hydroxy-5α-androstan-3-one **1** (androstanolone) was purchased from Sigma Company, USA. All solvents were dried by distillation prior to use. All melting points were measured using an Electrothermal apparatus and are uncorrected. The IR spectra were recorded in (KBr discs) on a Shimadzu FT-IR 8201 PC spectrometer and expressed in cm^{-1}. The ^1H NMR and ^{13}C NMR spectra were recorded with Jeol instrument (Japan), at 270 and 125 MHz respectively, in DMSO-d_6 or $CDCl_3$ as solvent and chemical shifts were recorded in ppm relative to TMS. Mass spectra were recorded on a GCMS-QP 1000 Ex spectra mass spectrometer operating at 70 eV. Elemental analyses were carried out by Microanalytical Data Unit at National Research Center, Giza, Egypt. Reactions were monitored on Merck aluminum thin layer chromatography (TLC) plates and visualized by UV light (254 nm). For the nomenclature of steroid derivatives, we used the definitive rules for the nomenclature of steroids published by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC [23,24].

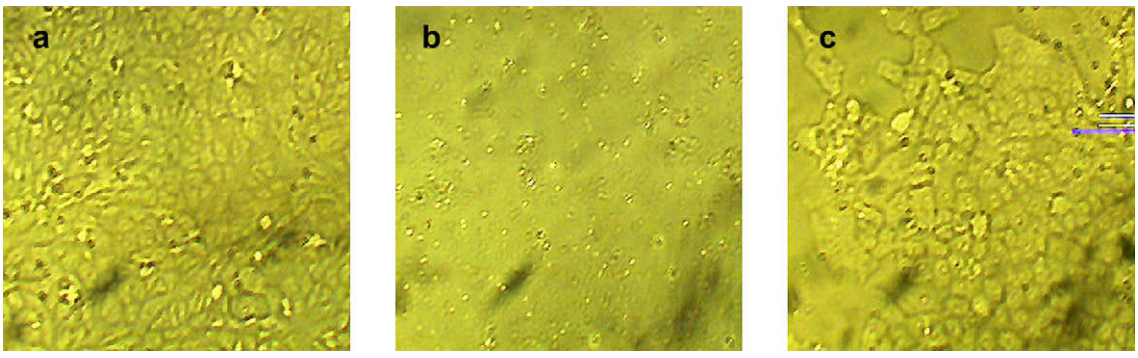
3.1.1. 17β-Acetoxy-3-dicyanomethylen-5α-androstane (*4*)

To a mixture of 17β-acetoxy-5α-androstan-3-one **2** (0.33 g, 1 mmol) and ammonium acetate (0.5 g), equimolar amount of malononitrile **3** (0.06 g, 1 mmol) was added. The reaction mixture was heated in an oil bath at 130 °C for 15 min. The solid product formed upon cooling at room temperature was triturated with ethanol, collected by filtration and crystallized from ethanol to yield dark yellow crystals of compound **4**, yield 0.28 g (75%), mp 178–180 °C, IR (KBr, cm^{-1}): $\nu = 2935$, 2873 (CH_3, CH_2), 2220, 2225 (2CN), 1732 (acetate C=O), 1643 (C=C). ^1H NMR ($CDCl_3$, ppm): $\delta = 0.78$ (s, 3H, CH_3-19), 1.03 (s, 3H, CH_3-18), 2.13 (s, 3H, $COCH_3$), 3.35–3.54 (m, 1H, C_5-αH). MS (EI): *m/z* (%): 380 (M^+, 28), 320 ($M^+ - CH_3COOH$, 33), 273 ($C_{19}H_{29}O$, 100), 257 ($C_{19}H_{29}$, 40). Calc. for $C_{24}H_{32}N_2O_2$ (380.5230): C, 75.75; H, 8.48; N, 7.36; found: C, 75.49; H, 8.32; N, 7.17%.

3.1.2. 17β-Acetoxy-6′-amino-2′-phenyl-2′H-pyrano[3′,4′:2,3]androstan-5′-carbonitrile (*7a*); 17β-acetoxy-6′-amino-2′-(p-methoxyphenyl)-2′H-pyrano[3′,4′:2,3]-androstan-5′-carbonitrile (*7b*): General procedure

To a solution of compound **4** (0.76 g, 2 mmol) in absolute ethanol (30 ml) containing sodium acetate (1.0 g) either benzaldehyde **5a** (0.21 g, 2 mmol) or anisaldehyde **5b** (0.27 g, 2 mmol) was added. The reaction mixture in each case was heated under reflux for 3–5 h until all the reactants had disappeared as indicated by TLC. Sodium acetate was isolated on hot by filtration and the filtrate was concentrated under vacuum. The isolated product upon cooling overnight at room temperature in each case was collected by filtration, dried and crystallized from the appropriate solvent.

*3.1.2.1. Compound **7a**.* Brown crystals from dioxane, yield 0.69 g (72%), mp 138–140 °C, IR (KBr, cm^{-1}): $\nu = 3480$ (NH_2), 3050 (CH-aromatic), 2935, 2850 (CH_3, CH_2), 2225 (CN), 1735 (acetate-C=O), 1615 (C=C). ^1H NMR (DMSO-d_6, ppm): $\delta = 0.75$ (s, 3H, CH_3-19), 0.96 (s, 3H, CH_3-18), 2.03 (s, 3H, $COCH_3$), 3.39–3.55 (m, 1H, C_5-αH), 4.49 (s, 2H, NH_2, D_2O-exchangeable), 7.15–7.45



Photograph 1. The morphology of $HepG_2$ cells after 72 h incubation: (a) control without any treatment, (b) conc. 100 μM of **7a** using olive oil as solvent, (c) conc. 100 μM of **7a** using DMSO as solvent.

Table 4
Effect of the tested compounds on hematological parameters and on body weight of EAC bearing mice. $n = 5$. Mean ± SE. $^{*}P > 0.05$ vs normal group. $^{**}P > 0.01$ vs normal group.

Parameters	Normal	EAC control (2×10^6 cells/mouse)	**7a** (25 mg/kg) + EAC	**10a** (25 mg/kg) + EAC	**12b** (25 mg/kg) + EAC	**18** (25 mg/kg) + EAC	**23** (25 mg/kg) + EAC	Standard 5-flurouracil (20 mg/kg) + EA
Hemoglobin/g %	13.63 ± 0.75	6.86 ± 2.25**	12.50 ± 0.20	12.60 ± 0.14	12.65 ± 0.91	10.70 ± 0.72*	12.96 ± 0.30	10.73 ± 0.15**
Haematocrit %	42.36 ± 1.00	25.9 ± 2.29**	36.6 ± 2.81**	33.8 ± 0.70**	38.3 ± 2.12*	42.7 ± 2.15	38.56 ± 1.15*	40.86 ± 2.17
Total WBC/10^3 mm^{-3}	9.16 ± 0.70	11.16 ± 0.25**	5.9 ± 0.30**	5.75 ± 0.49**	7.35 ± 1.48*	5.66 ± 1.15**	6.46 ± 0.76**	6.40 ± 0.52**
Body weight/g	22.66 ± 0.57	27.33 ± 2.51**	18.66 ± 2.30*	18.00 ± 1.41*	21.5 ± 2.12	20.00 ± 1.50	20.33 ± 0.57	23.0 ± 1.0
Tumor volume/ml	Nil	4.56 ± 0.37	Nil	Nil	Nil	Nil	Nil	Nil

(m, 5H, C_6H_5). ^{13}C NMR (DMSO-d_6, ppm): $\delta = 34.7$ (C-1), 138.9, 137.5 (fused, C-2, C-3), 35.3 (C-4), 49.9 (C-5), 27.2 (C-6), 28.7 (C-7), 36.4 (C-8), 52.7 (C-9), 46.8 (C-10), 21.21 (C-11), 37.0 (C-12), 42.2 (C-13), 52.3 (C-14), 23.1 (C-15), 27.5 (C-16), 82.0 (C-17), 21.1 (C-18), 21.7 (C-19), 170 (C═O), 20.9 (CH_3-acetyl), 74.4 (C-2′), 53.2 (C-5′), 182.0 (C-6′), 117.0 (CN), 127.7, 129.0, 129.2, 141.8 (C-phenyl). MS (EI): *m*/*z* (%): 486 (M^+, 43), 426 ($M^+ - CH_3COOH$, 15), 409 ($M^+ - C_6H_5$, 20), 261 ($C_{19}H_{33}$, 100), 77 (C_6H_5, 38). Calc. for $C_{31}H_{38}N_2O_3$ (486.6450): C, 76.51; H, 7.87; N, 5.76; found: C, 76.33; H, 7.67; N, 5.52.

3.1.2.2. Compound ***7b****.* Yellow crystals from methanol, yield 0.72 g (70%), mp 128–130 °C, IR (KBr, cm^{-1}): $\nu = 3452$ (NH_2), 3030 (CH-aromatic), 2948, 2852 (CH_3, CH_2), 2220 (CN), 1726 (C═O), 1642 (C═C). ^{1}H NMR (DMSO-d_6, ppm): $\delta = 0.78$ (s, 3H, CH_3-19), 1.06 (s, 3H, CH_3-18), 2.18 (s, 3H, $COCH_3$), 3.32–3.50 (m, 1H, C_5-αH), 5.86 (s, 3H, OCH_3), 5.12 (s, 2H, NH_2, D_2O-exchangeable), 7.30 (dd, 2H-aromatic, J_{HH} 9 Hz), 7.50 (dd, 2H-aromatic, J_{HH} 9 Hz). MS (EI): *m*/*z* (%): 516 (M^+, 28), 456 ($M^+ - CH_3COOH$, 61), 409 [$M^+ - (C_6H_4$–$OCH_3)$, 55], 107 (C_6H_4–OCH_3, 100). Calc. for $C_{32}H_{40}N_2O_4$ (516.6713): C, 74.39; H, 7.80; N, 5.42; found: C, 74.50; H, 7.98; N, 5.63.

*3.1.3. 17β-Acetoxy-6′-amino-1′-phenyl-2′-thioxopyrido[3′,4′:2,3]-5α-androstan-5′-carbonitrile (****10a****); 17β-acetoxy-6′-amino-1′-phenyl-2′-oxopyrido[3′,4′:2,3]-5α-androstan-5′-carbonitrile (****10b****): General procedure*

To a solution of compound **4** (0.76 g, 2 mmol) in dimethylformamide (20 ml) containing a catalytic amount of triethylamine (1 ml) either phenyl isothiocyanate **8a** (0.27 g, 2 mmol) or phenyl isocyanate **8b** (0.23 g, 2 mmol) was added. The reaction mixture, in each case, was heated under reflux for 5–7 h until all the reactants had disappeared as indicated by TLC. The reaction mixture was poured over an ice/water mixture and neutralized with dilute hydrochloric acid. The solid product that formed in each case was filtered off, dried and crystallized from the appropriate solvent.

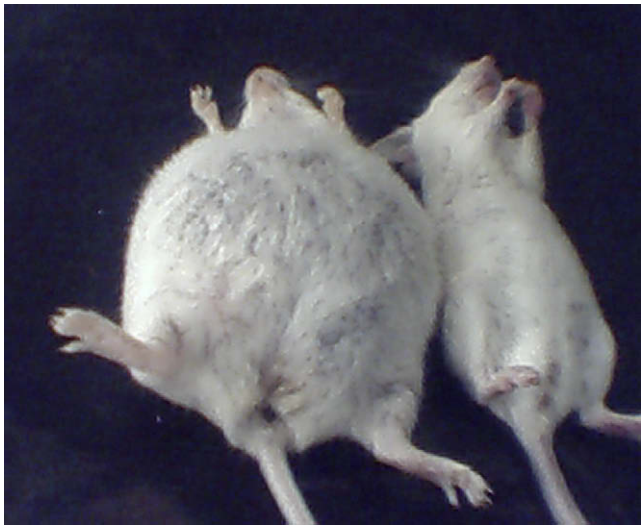
3.1.3.1. Compound ***10a****.* Brown crystals from ethanol, yield 0.78 g (76%), mp 163–165 °C, IR (KBr, cm^{-1}): $\nu = 3376$ (NH_2), 3025 (CH-aromatic), 2930, 2825 (CH_3, CH_2), 2207 (CN), 1730 (C═O), 1597 (C═C), 1195 (C═S). ^{1}H NMR (DMSO-d_6, ppm): $\delta = 0.75$ (s, 3H, CH_3-19), 0.96 (s, 3H, CH_3-18), 2.04 (s, 3H, $COCH_3$), 3.22–3.56 (m, 1H, C_5-αH), 6.12 (s, 2H, NH_2, D_2O-exchangeable), 6.95–7.35 (m, 5H, C_6H_5). ^{13}C NMR (DMSO-d_6, ppm): $\delta = 38.8$ (C-1), 140.7, 148.9 (fused, C-2, C-3), 31.7 (C-4), 50.1 (C-5), 27.4 (C-6), 28.9 (C-7), 36.6 (C-8), 53.4 (C-9), 42.6 (C-10), 21.1 (C-11), 38.5 (C-12), 42.3 (C-13), 52.3 (C-14), 23.3 (C-15), 27.9 (C-16), 82.2 (C-17), 20.2 (C-18), 21.2 (C-19), 170.3 (C═O), 21.0 (CH_3-acetyl), 180.2 (C-2′), 62.8 (C-5′), 157.2 (C-6′), 115.0 (CN), 134.0, 126.5, 129.1, 124.8 (C-phenyl). MS (EI): *m*/*z* (%): 515 (M^+, 43), 455 ($M^+ - CH_3COOH$, 27), 77 (C_6H_5, 100). Calc. for $C_{31}H_{37}N_3O_2S$ (515.7093): C, 72.20; H, 7.23; N, 8.15; S, 6.22; found: C, 72.40; H, 7.45; N, 8.35; S, 6.46.

3.1.3.2. Compound ***10b****.* Yellow crystals from ethanol, yield 0.72 g (73%), mp 158–160 °C, IR (KBr, cm^{-1}): $\nu = 3385$ (NH_2), 3030 (CH-aromatic), 2942, 2835 (CH_3, CH_2), 2220 (CN), 1735, 1710 (2C═O), 1598 (C═C). ^{1}H NMR ($CDCl_3$, ppm): $\delta = 0.78$ (s, 3H, CH_3-19), 0.98 (s, 3H, CH_3-18), 2.18 (s, 3H, $COCH_3$), 3.27–3.63 (m, 1H, C_5-αH), 5.82 (s, 2H, NH_2, D_2O-exchangeable), 7.23–7.63 (m, 5H, C_6H_5). MS (EI): *m*/*z* (%): 498 ($M^+ - 1$, 62), 438 ($M^+ - CH_3COOH$, 50), 77 (C_6H_5, 100). Calc. for $C_{31}H_{37}N_3O_3$ (499.6437): C, 74.52; H, 7.46; N, 8.41; found: C, 74.30; H, 7.23; N, 8.65.

*3.1.4. 17β-Acetoxy-4″,6″-diamino-2′-phenylpyrido[2″,3″:6′,5′]pyrano[3′,4′:2,3]-5α-androstan-5″-carbonitrile (****12a****); 17β-acetoxy-4″,6″-diamino-2′-(p-methoxyphenyl)pyrido[2″,3″,6′,5′]-pyrano[3′,4′:2,3]-5α-androstan-5″-carbonitrile (****12b****): General procedure*

A solution of equimolar amounts of compound **7a** (0.97 g, 2 mmol) or compound **7b** (1.03 g, 2 mmol) and malononitrile **3** (0.13 g, 2 mmol) in dioxane (30 ml) containing triethylamine (0.5 ml) was heated under reflux for 4–5 h until all the reactants had disappeared as indicated by TLC. The reaction mixture was then evaporated under vacuum and the remaining product was triturated with ethanol. The solid product that formed in each case was collected by filtration and crystallized from the appropriate solvent.

3.1.4.1. Compound ***12a****.* Brown crystals from ethanol, yield 0.86 g (78%), mp 183–185 °C, IR (KBr, cm^{-1}): $\nu = 3345$–3230 ($2NH_2$), 3015 (CH-aromatic), 2220 (CN), 1730 (C═O), 1642 (C═N), 1580 (C═C). ^{1}H NMR (DMSO-d_6, ppm): $\delta = 0.74$ (s, 3H, CH_3-18), 0.89 (s, 3H, CH_3-19), 2.04 (s, 3H, OCH_3), 3.32–3.50 (m, 1H, C_5-αH), 4.89, 5.62 (2s, 4H, $2NH_2$, D_2O-exchangeable), 7.35–7.67 (m, 5H, C_6H_5). MS (EI): *m*/*z* (%): 551 ($M^+ - 1$, 25), 492 ($M^+ - CH_3COOH$, 33), 475 ($M^+ - C_6H_5$, 42), 273 ($C_{19}H_{29}O$, 18), 257 ($C_{19}H_{29}$, 100), 77 (C_6H_5, 37). Calc. for $C_{34}H_{40}N_4O_3$ (552.7064): C, 73.88; H, 7.29; N, 10.14; found: C, 73.62; H, 7.04; N, 10.35.



Photograph 2. Effect of **7a** at dose 25 mg/kg b.wt. on mice with EAC (right), compared to an EAC control – non treated-animal (left). Substantial difference observed on treated animals indicating complete response.

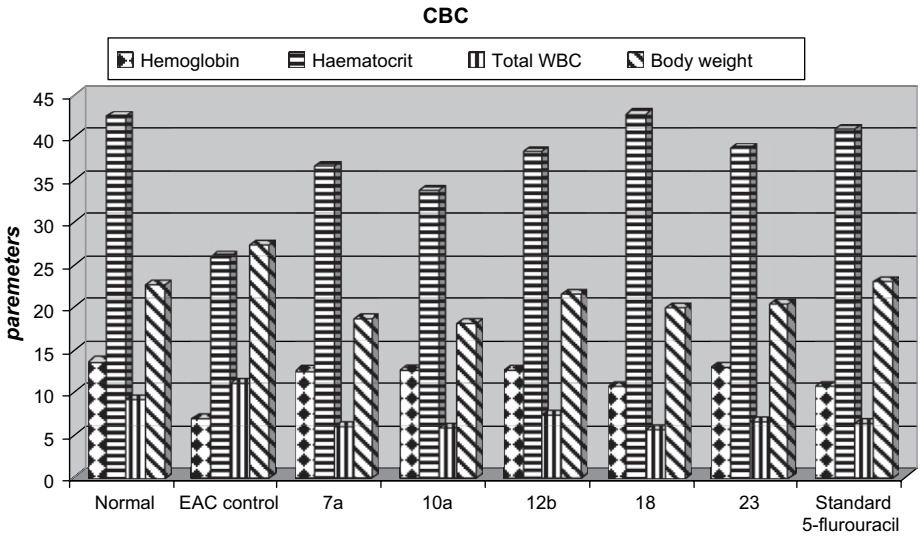


Fig. 4. Effect of the tested compounds on hematological parameters and on body weight of EAC bearing mice.

*3.1.4.2. Compound **12b**.* Dark brown crystals from ethanol, yield 0.84 g (72%), mp 194–196 °C, IR (KBr, cm^{-1}): $\nu = 3382–3320$ ($2NH_2$), 3020 (CH-aromatic), 2215 (CN), 1720 (C═O), 1640 (C═N), 1571 (C═C). ^1H NMR ($CDCl_3$, ppm): $\delta = 0.73$ (s, 3H, CH_3-18), 0.91 (s, 3H, CH_3-19), 2.09 (s, 3H, $COCH_3$), 3.28–3.45 (m, 1H-C_5–αH), 3.80 (s, 3H, OCH_3), 4.67, 5.23 (2s, 4H, $2NH_2$, D_2O-exchangeable), 7.42 (dd, 2H-aromatic, J_{HH} 9 Hz), 7.62 (dd, 2H-aromatic, J_{HH} 9 Hz). ^{13}C NMR (DMSO-d_6, ppm): $\delta = 33.7$ (C-1), 142.8, 130.7 (fused, C-2, C-3), 31.4 (C-4), 48.5 (C-5), 29.4 (C-6), 28.9 (C-7), 37.8 (C-8), 52.7 (C-9), 48.7 (C-10), 21.7 (C-11), 30.5 (C-12), 45.6 (C-13), 50.3 (C-14), 23.5 (C-15), 27.9 (C-16), 82.7 (C-17), 22.1 (C-18), 17.3 (C-19), 170.1 (C═O), 20.7 (CH_3-acetyl), 82.7 (C-2′), 102.3, 162.3 (fused C-5′, C-6′), 162.5 (C-4″), 67.2 (C-5″), 161.1 (C-6″), 117.8 (CN). MS (EI): *m/z* (%): 582 (M^+, 15), 522 ($M^+ - CH_3COOH$, 28), 475 [$M^+ - C_6H_4$–OCH_3, 100], 107 (C_6H_4–OCH_3, 38). Calc. for $C_{35}H_{42}N_4O_4$ (582.7323): C, 72.14; H, 7.26; N, 9.61; found: C, 72.39; H, 7.49; N, 9.80.

*3.1.5. 17β-Acetoxy-3-hydrazono-5α-androstane (**14**)*

To a dry mixture of compound **2** (1.66 g, 5 mmol) and hydrazine hydrate **13** (0.25 g, 5 mmol), few drops of glacial acetic acid were added. The reaction mixture was heated in an oil bath at 120 °C for 30 min and then left to cool at room temperature. The solid product obtained upon triturating the fused result with ethanol followed by pouring onto ice/water mixture was collected by filtration, dried and crystallized from 1,4-dioxane to afford compound **14** as white crystals, yield 1.38 g (80%), mp 239–240 °C, IR (KBr, cm^{-1}): $\nu = 3498$ (NH_2), 2985, 2878 (CH_3, CH_2), 1725 (C═O), 1670 (C═N). ^1H NMR (DMSO-d_6, ppm): $\delta = 0.82$ (s, 3H, CH_3-19), 1.07 (s, 3H, CH_3-18), 2.05 (s, 3H, $COCH_3$), 3.27–3.53 (m, 1H, C_5-αH), 5.57 (s, 2H, NH_2, D_2O-exchangeable). ^{13}C NMR (DMSO-d_6, ppm): $\delta = 37.8$ (C-1), 30.4 (C-2), 161.6 (C-3), 23.3 (C-4), 42.4 (C-5), 28.5 (C-6), 31.1 (C-7), 36.0 (C-8), 53.6 (C-9), 45.7 (C-10), 20.4 (C-11), 34.9 (C-12), 40.4 (C-13), 40.3 (C-14), 53.8 (C-15), 27.3 (C-16), 82.4 (C-17), 22.8 (C-18), 16.5 (C-19), 170.6 (C═O), 21.0 (CH_3-acetate). MS (EI): *m/z* (%): 347 ($M^+ + 1$, 25), 286 ($M^+ - CH_3COOH$, 36), 257 ($C_{19}H_{29}$, 100). Calc. for $C_{21}H_{34}N_2O_2$ (346.5068): C, 72.79; H, 9.89; N, 8.08; found: C, 72.52; H, 10.12; N, 7.87.

*3.1.6. 17β-Acetoxy-3-*N*-(4′-phenyl-2′-thioxo-1′,3′-thiazol-3′-yl)androstane (**18**); 17β-acetoxy-3-*N*-(4′-methyl-2′-thioxo-1,3-thiazol-3′-yl)androstane (**20**): General procedure*

To a solution of compound **14** (0.69 g, 2 mmol) in DMF (30 ml) a solution of KOH (0.11 g, 2 mmol) in 5 ml H_2O was added with stirring followed by carbon disulfide (0.15 g, 2 mmol). The reaction mixture was heated in water bath at 80 °C for 1 h, then left to cool to 20 °C. The α-halogenated compound, phenacyl bromide **16** (0.39 g, 2 mmol) or chloroacetone **19** (0.18 g, 2 mmol), was added dropwise to the cooled reaction mixture with stirring. The reaction mixture, in each case, was stirred at room temperature for 24 h. The solid product formed upon pouring the reaction mixture into ice containing few drops of hydrochloric acid was collected by filtration, dried and crystallized from the proper solvent.

*3.1.6.1. Compound **18**.* Dark yellow crystals from ethanol, yield 0.76 g (73%), mp 99–100 °C, IR (KBr, cm^{-1}): $\nu = 3030$ (CH-aromatic), 2970, 2848 (CH_3, CH_2), 1732 (C═O), 1657 (C═N), 1591 (C═C), 1195 (C═S). ^1H NMR ($CDCl_3$, ppm): $\delta = 0.65$ (s, 3H, CH_3-19), 0.87 (s, 3H, CH_3-18), 2.15 (s, 3H, $COCH_3$), 3.32–3.62 (m, 1H, C_5–αH), 6.47 (s, 1H, thiazole C_5–H), 7.46–7.84 (m, 5H, C_6H_5). ^{13}C NMR (DMSO-d_6, ppm): $\delta = 37.5$ (C-1), 22.9 (C-2), 162.0 (C-3), 28.1 (C-4), 44.0 (C-5), 27.0 (C-6), 30.7 (C-7), 35.2 (C-8), 49.8 (C-9), 45.8 (C-10), 22.3 (C-11), 34.5 (C-12), 42.1 (C-13), 52.8 (C-14), 23.5 (C-15), 27.6 (C-16), 81.8 (C-17), 20.3 (C-18), 16.9 (C-19), 170.2 (C═O), 20.7 (CH_3, acetate), 210.3 (C-2′, C═S), 158.0 (C-4′), 107.6 (C-5′), 134.0, 126.4, 128.7, 127.8 (C-phenyl). MS (EI): *m/z* (%): 522 (M^+, 33), 462 ($M^+ - CH_3COOH$, 21), 316 ($M^+ - C_9H_6N_2S_2$, 86), 77 (C_6H_5, 100). Calc. for $C_{30}H_{38}N_2O_2S_2$ (522.7649): C, 68.93; H, 7.33; N, 5.36; S, 12.27; found: C, 68.74; H, 7.56; N, 5.17; S, 12.54.

*3.1.6.2. Compound **20**.* Yellow crystals from ethanol, yield 0.62 g (68%), mp 108–110 °C, IR (KBr, cm^{-1}): $\nu = 2934$, 2851 (CH_3, CH_2), 1730 (C═O), 1643 (C═N), 1515 (C═C), 1192 (C═S). ^1H NMR ($CDCl_3$, ppm): $\delta = 0.78$ (s, 3H, CH_3-19), 0.91 (s, 3H, CH_3-18), 2.03 (s, 3H, $COCH_3$), 2.85 (s, 3H, CH_3), 3.32–3.54 (m, 1H, C_5–αH), 6.57 (s, 1H, thiazole C_5–H). MS (EI): *m/z* (%): 460 (M^+, 37), 400 ($M^+ - CH_3COOH$, 21), 254 ($M^+ - C_9H_6N_2S_2$, 28), 77 (C_6H_5, 100). Calc. for $C_{25}H_{36}N_2O_2S_2$ (460.6955): C, 65.18; H, 7.88; N, 6.08; S, 13.92; found: C, 65.35; H, 8.05; N, 5.92; S, 13.75.

*3.1.7. 17β-Acetoxy-3-hydroximinoandrostane (**22**)*

To a solution of compound **2** (1.66 g, 5 mmol) in absolute ethanol (30 ml) containing anhydrous sodium acetate (1 g), a solution of hydroxylamine hydrochloride **21** (0.41 g, 5 mmol) in water (5 ml) was added. The reaction mixture was stirred at 25 °C for 30 min then poured into ice/water mixture. The solid product formed was collected by filtration and crystallized from ethanol to afford white crystals of compound **22** in 80% yield (1.38 g), mp 122–124 °C, IR

(KBr, cm^{-1}): $\nu = 3267$ (N–OH), 2931, 2851 (CH_3, CH_2), 1736 (C═O), 1648 (C═N), 1542 (C═C). ^1H NMR (DMSO-d_6, ppm): $\delta = 0.79$ (s, 3H, CH_3-19), 1.06 (s, 3H, CH_3-18), 2.13 (s, 3H, $COCH_3$), 3.25 (dd, $J = 3.6$, 1H, H_β–C4), 3.38–3.52 (m, 1H, C_5–αH), 9.36 (br s, 1H, NOH). MS (EI): m/z (%): 347 (M^+, 28), 287 ($M^+ - CH_3COOH$), 257 ($C_{19}H_{29}$, 100). Calc. for $C_{21}H_{33}NO_3$ (347.4916): C, 72.58; H, 9.57; N, 4.03; found: C, 72.35; H, 9.36; N, 3.87.

3.1.8. 17β-Acetoxy-4-aza-A-homoandrostan-3-one (**23**)

To a solution of compound **22** (0.69 g, 2 mmol) in dioxane (20 ml), thionyl chloride (0.15 ml) was added dropwise with stirring. The reaction mixture was stirred for further 30 min at room temperature. Water was added to this solution and the reaction mixture was extracted with diethyl ether (3 × 20 ml). The extract was washed with water, 5% $NaHCO_3$ and water, dried over $CuSO_4$. Removal of the solvent gave a crude compound **23**, which was crystallized from methanol to afford white crystals, yield 0.50 g (72%), mp 273–275 °C, IR (KBr, cm^{-1}): $\nu = 3432$ (NH), 2928, 2852 (CH_3, CH_2), 1734, 1682 (2C═O), 1532 (C═C). ^1H NMR ($CDCl_3$, ppm): $\delta = 0.72$ (s, 3H, CH_3-19), 0.98 (s, 3H, CH_3-18), 2.13 (s, 3H, $COCH_3$), 3.38–3.57 (m, 1H, C_5–αH), 8.03 (br s, 1H, NH, D_2O-exchangable). ^{13}C NMR (DMSO-d_6, ppm): $\delta = 39.2$ (C-1), 33.5 (C-2), 179.2 (C-3), 43.3 (C-4), 49.2 (C-5), 26.3 (C-6), 27.7 (C-7), 35.7 (C-8), 50.9 (C-9), 44.7 (C-10), 22.7 (C-11), 36.3 (C-12), 43.6 (C-13), 50.6 (C-14), 23.5 (C-15), 27.8 (C-16), 82.3 (C-17), 20.0 (C-18), 16.2 (C-19), 171.3 (C═O, acetate), 20.5 (CH_3, acetate), 168.2 (C═O, amide). MS (EI): m/z (%): 348 ($M^+ + 1$, 100), 287 ($M^+ - CH_3COOH$, 18). Calc. for $C_{21}H_{33}NO_3$ (347.4916): C, 72.58; H, 9.57; N, 4.03; found: C, 72.38; H, 9.39; N, 3.94.

3.2. In vitro cytotoxic activity

3.2.1. Materials

Fetal calf serum (FCS), L-glutamine and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin G sodium and streptomycin sulfate were obtained from Bio-Waste Co. (Wexford, Ireland). MTT was purchased from Duchefa-Biochemie (Haarlem, Amsterdam, Netherlands). Sodium bicarbonate was obtained from Merck Co. Inc. (USA).

3.2.2. Cell culture

Human hepatoma cell lines ($HepG_2$) were supplied by Naval American Research Unit, Egypt (NAMRU). Cells were propagated and maintained in RPMI-1640 medium with L-glutamine and supplemented with 10% fetal calf serum for growth and 2% of the maintenance medium [1% of 4% sodium bicarbonate and 1% antibiotic mixture (1,000,000 units of penicillin G sodium and 1,000,000 μg streptomycin sulfate in 100 ml deionized water)] in 75 cm^2 tissue culture flasks.

3.2.3. Growth inhibition assay

The cytotoxic effect of compounds **7a**, **10a**, **12b**, **18** and **23** was done using MTT assay [25]. The human hepatocarcinoma ($HepG_2$ cells) at approximately 80% confluence (i.e. logarithmically growing cells) were selected for trypsinization, and stained with trypan blue and their numbers were recorded. The percentage of cells that resisted staining ought to be above 97%. The cells were seeded in 96-well microplates, after the cell concentrations were adjusted to 3×10^3 cells/well in 100 μl RPMI-1640 culture medium and incubated at 37 °C and 5% CO_2 overnight. The cells were treated with either one of compounds **7a**, **10a**, **12b**, **18** and **23** dissolved in olive oil or DMSO in three concentrations (10, 50 and 100 μg/ml) and re-incubated for 24 h, 48 h and 72 h. Then the cells were washed with sterile phosphate buffer saline (PBS) and 100 μl of the tetrazolium dye (MTT) (0.5 mg/ml) solution was added to each well, and the cells were incubated for an additional 4 h. The medium was discarded; 100 μl of DMSO was added to dissolve the purple formazan crystals formed. The optical density (OD) of solubilized formazan was measured at 570 nm (reference filter 690 nm) using an automatic microplate reader (Wako, Japan). The results are expressed as percent of cell growth inhibition compared with the control. The effect of compounds **7a**, **10a**, **12b**, **18** and **23** on the morphology of treated hepatocellular carcinoma cells was investigated by the light microscope and then photographed by SONY CYBER-SHORT [26].

3.3. In vivo anti-tumor activity

3.3.1. Tumor transplantation

A line of Ehrlich ascites carcinoma (EAC) was supplied from the National Cancer Institute, Cairo, Egypt. Each mouse was injected intraperitoneally (i.p.) with 0.2 ml of EAC, which contained 2×10^6 cells.

3.3.2. Experimental animals

Forty Swiss albino female mice weighing 20 ± 2 g were obtained from the Animal House Colony of the National Research Center, Cairo, Egypt. Five mice/cage were housed in plastic cages of dimensions 42L × 26W × 22H centimeters. The animals were maintained under controlled conditions of humidity, temperature, and diurnal environment of light and dark. The mean ambient temperature in the housing facility was 28 °C. The animals were randomly assigned to 8 groups ($n = 5$) as follows, Group 1 (vehicle control) was left without any treatment for 14 days. Group 2 (negative control) injected intraperitoneally (i.p.) with 0.2 ml of EAC, which contained 2×10^6 cells for tumor induction and left for 14 days. Group 3 (positive control) injected (i.p.) with 0.2 ml of EAC and treated with 20 mg/kg b.wt. of reference drug 5-flurouracil for 14 days. Groups 4, 5, 6, 7 and 8 received either one of the tested compounds **7a**, **10a**, **12b**, **18**, or **23** at doses 25 mg/kg b.wt. dissolved in DMSO (i.p.) day after day for 14 days. After administration of the last dose followed by 18 h fasting, the mice were then sacrificed for observation of anti-tumor activity. The effect of the tested compounds on the growth of transplantable murine tumor, and simultaneous alterations in the hematological profile were estimated.

3.4. Statistics

Data were assessed by the method of analysis of ANOVA followed by t-test, $P < 0.05$ was considered as statistically significant (*), $P < 0.01$ was considered as statistically highly significant (**).

References

[1] P. Pisani, D.M. Parkin, F. Bray, J. Ferlay, Int. J. Cancer 83 (1999) 18–29.
[2] G.D. Kirk, O.A. Lesi, M. Mendy, A.O. Akano, O. Sam, J.J. Goedert, P. Hainaut, A.J. Hall, H. Whittle, R. Montesano, Hepatology 39 (2004) 211–219.
[3] G. Hosny, N. Farahat, H. Tayel, P. Hainaut, Cancer Lett. 264 (2008) 201–208.
[4] S. Deuffic-Burban, M.K. Mohamed, B. Larouze, F. Carrat, A. Valleron, J. Hepatol. 44 (2006) 455–461.
[5] T.H. Wu, R.L. Yang, L.P. Xie, H.Z. Wang, L. Chen, S. Zhang, Y. Zhao, R.Q. Zhang, Cancer Lett. 232 (2006) 199–205.
[6] D.T.P. Trafalis, G.D. Geromichalos, C. Koukoulitsa, A. Papageorgiou, P. Karamanakos, C. Camoutsis, Breast Cancer Res. Treat. 97 (2006) 17–31.
[7] D. Thibeault, J. Roy, P.D. Roy, D. Poirier, Bioorg. Med. Chem. 16 (2008) 5062–5077.
[8] Evgenija A. Djurendíc, Marija N. Sakač, M.P. Zaviš, A.R. Gakovíc, J.J. Canadia, S.A. Andric, O.R. Klisuric, V.V. Kojic, G.M. Bogdanovíc, K.M. Penovgăsi, Steroids 73 (2008) 681–688.
[9] M.N. Preobrazhenskaya, Chem. Heterocycl. Comp. 21 (1985) 13–24.
[10] M. Kidwai, R. Venkataramanan, R. Mohan, P. Sapra, Curr. Med. Chem. 9 (2002) 1209–1228.
[11] M.T. Cocco, C. Congiu, V. Lilliu, V. Onnis, Bioorg. Med. Chem. 15 (2007) 1859–1867.
[12] A.E. Amr, A.M. Mohamed, S.F. Mohamed, N.A. Abdel-Hafez, A.G. Hammam, Bioorg. Med. Chem. 14 (2006) 5481–5488.

[13] L. Brault, E. Migianu, A. Néguesque, E. Battaglia, D. Bagrel, G. KirschMaria, Eur. J. Med. Chem. 40 (2007) 757–763.
[14] G.A. Elmegeed, W.W. Wardakhan, A.R. Baiuomy, Pharmazie 60 (2005) 328–333.
[15] R.M. Mohareb, S.I. Aziza, N.I. Abdel-Sayed, A.H. El-banna, J. Chem. Res. (1999) 101–128.
[16] J. Poza, M. Rega, V. Paz, B. Alonso, J. Rodríguez, N. Salvador, A. Fernández, C. Jimènez, Bioorg. Med. Chem. 15 (2007) 4722–4740.
[17] J. Cui, L. Huang, L. Fan, A. Zhou, Steroids 73 (2008) 252–256.
[18] C. Camoutsis, D. Trafalis, G. Pairas, A. Papageorgiou, Farmaco 60 (2005) 826–829.
[19] N. Krstic, M. Bjelakovic, M.M. Dabovic, L.B. Lorenc, V.D. Pavlovic, J. Serb. Chem. Soc. 69 (2004) 413–420.
[20] C. Camoutsis, P. Catsoulacos, J. Heterocycl. Chem. 25 (1988) 1617–1619.
[21] S. Kunihiro, Eur. Patent, EP0209078, 1987, http://www.freepatentsonline.com/EP0209078A2.html.
[22] S. Yoshida, A. Honda, Y. Matsuzaki, S. Fukushima, N. Tanak, A. Takagiwa, Y. Fujimoto, H. Migazaki, G. Salen, Steroids 68 (2003) 73–83.
[23] IUPAC, Joint Commission on Biochemical Nomenclature (JCBN), Pure Appl. Chem. 61 (1989) 1783–1822.
[24] IUPAC, Joint Commission on Biochemical Nomenclature (JCBN), Eur. J. Biochem. 186 (1989) 429–458.
[25] T. Mosmann, J. Immunol. Methods 65 (1983) 55–63.
[26] M. Theiszova, S. Jantova, J. Dragňova, P. Grznarova, M. Palouc, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc. Czech. Repub. 149 (2005) 393–396.