International Journal of Medicine and Medical Sciences Vol 1(4) pp.126-131, April, 2009
Available online http://www.academicjournals.org/ijmms

Full length Research paper

Aminothienoandrostane: Novel promising anti-tumor agent

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Accepted 26 February, 2009

The sensitivity of liver to chemotherapy provides the basis of novel investigational treatment. An amino thienoandrostane derivative was synthesized and tested *in vitro* for anti-tumor activity against heaptoma cell lines ($HepG_2$) using MTT assay. Also the *in vivo* anti-tumor activity was evaluated against Ehrlich ascites carcinoma (EAC). After 24 h of tumor inoculation, the tested compound was administered intraperitoneal (i.p.) with concentration of 25 mg/kg day after day for 14 days. The effect of aminothienoandrostane on the growth of transplantable tumor and simultaneous alterations in hematological profile was estimated. Aminothienoandrostane in olive oil induced significantly high cytotoxic effect against $HepG_2$ cell line (IC_{50} = 36). Aminothienoandrostane completely inhibited tumor growth and maintained the hemoglobin content, body weight, and WBCs near normal values and similar to what obtained for standard drug 5-flurouracil which is one of the most commonly used drugs to treat cancers.

Key words: Anti-cancer, androstane, hepatoma (Hep-G_2), Ehrlich ascites carcinoma (EAC), cytotoxicity.

INTRODUCTION

The sensitivity of liver to chemotherapy provides the basis of novel investigational treatment. Because of the multifocal nature of liver carcinoma, most cancer patients are considered non-resistible. 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 (Wu et al., 2006). Thus, developing new therapeutic agents that can overcome drug resistance becomes an urgent need for cancer patients. A variety of steroids with unusual and interesting structures have been synthesized and evaluated for their anti-tumor activity (Thibeault et al., 2008; Evgenija et al., 2008). Also the anti-tumor activity of many compounds containing heterocyclic ring have been reviewed (Preobrazhenskaya, 1985; Kidwai et al., 2002). The biological and medical activity of steroidal heterocyclic compounds has stimulated considerable interest in the chemistry of steroids (Jindal et al., 2001).

Based on the above observations, in the present work we evaluate the activity of very promising amino thienoandrostane derivative (Figure 1) against hepato-cellular carcinoma ($HepG_2$ cell lines) *in vitro* and against Ehrlich ascites carcinoma (EAC) *in vivo.*

MATERIALS AND METHODS

Synthesis of 17β-Acetoxy-5'-amino-4'-amidothieno [2',3':2,3]-5α-androstane

Equimolar amounts of 17 β-acetoxy-5α-androstan-3-one (1.66 g, 5 mmol), sulfur (0.16 g, 5 mmol) and cyanoacetamide (0.42 g, 5 mmol) in absolute ethanol (30 ml) containing a catalytic amount of triethylamine (0.5 ml) were heated under reflux for 3 h until all the starting materials had disappeared as indicated by TLC. The reaction mixture was left to cool over night at room temperature. The solid product that formed, in each case, was collected by filtration and crystallized from the proper solvent.

Compound aminothienoandrostane

Yellow crystals from methanol yield 1.76 g (82%), mp 113 - 115°C, IR (KBr, cm^{-1}): 3486, 3340 ($2NH_2$), 2935, 2851 (CH_3, CH_2), 1728,1695 (2C=O), 1575 (C=C). ^1H NMR (DMSO-d_6 , ppm): δ= 0.78 (s, 3H, CH_3-19), 1.03 (s, 3H, CH_3-18), 2.07 (s, 3H, $COCH_3$), 3.45-3.65 (m,1H, C_5-αH), 4.65, 4.87 (2s, 4H, $2NH_2$, D_2O-exchangeable , ^{13}C NMR ($CDCL_3$, ppm). δ = 35.7 (C-1), 140.5, 127.2 (fused

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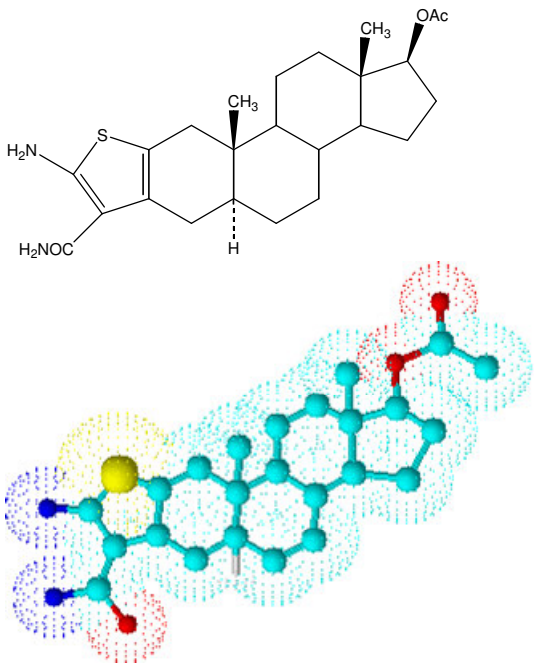


Figure 1. Structure of amino-thienoandrostane.

C-2, C-3), 20.2 (C-4), 43.7 (C-5), 26.7 (C-6), 29.2 (C-7), 36.0 (C-8), 50.7 (C-9), 37.6 (C-10), 22.5 (C-11), 34.9 (C-12), 43.0 (C-13), 51.8 (C-14), 24.2 (C-15), 27.8 (C-16), 82.0 (C-17), 20.7 (C-18), 15.7 (C-19), 170.8 (C=O), 21.0 (CH_3, acetate), 117.5 (C-4′), 163.8 (C-5′), 168.2 (C=O, amide). MS (EI): m/z(%): 430 (M^+, 32), 370 (M^+-CH_3COOH), 262 [M^+-($C_7H_8N_2OS$, retro-Diels Alder fragment), 26], 168 ($C_7H_8N_2OS$, retro-Diels Alder fragment (100). Calc. for $C_{24}H_{34}N_2O_3S$ (430.603): C, 66.94; H, 7.96; N, 6.51; S, 7.45; found: C, 66.71; H, 7.70; N, 6.29; S, 7.27.

In vitro cytotoxic activity

Materials

RPMI-1640, Trypsin, 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). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT was purchased from Duchefa-Biochemie (Haarlem, Amsterdam, Netherlands). Sodium bicarbonate was obtained from Merck Co. Inc (USA).

Cell culture

Human hepatocellular carcinoma cell line ($HepG_2$) was 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.

Growth inhibition assay

The cytotoxic effect of the aminothienoandrostane was investigated using MTT assay (Mosmann, 1989). The human hepatocelluar carcinoma ($HepG_2$ cell lines) at approximately 80% confluence (that is, logarithmically growing cells) were selected for trypsinization, and then counted using trypan blue dye. The percentage of cells that resisted staining ought to be above 97%. Cells were seeded in 96-well microplates, after the cell concentrations were adjusted to 3 X 10^3 cells/well in 100 µl RPMI-1640 culture medium and incubated at 37°C and 5% CO_2 over night. The cells were treated with aminothienoandrostane which was dissolved -individually- in olive oil and in DMSO, in three different concentrations (10, 50 and 100 µg/ml) and re-incubated for 24, 48 and 72 h. Then the cells were washed with sterile phosphate buffer solution (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 aminothienoandrostane on the morphology of treated hepatocellular carcinoma cells was investigated by the light microscopy and then photographed by SONY CYBER-SHORT (Theiszova et al., 2005).

In-vivo anti-tumor activity

Tumor transplantation

A line of Ehrlich Ascites Carcinoma (EAC) was supplied from the National Cancer Institute, Cairo, Egypt. The EAC cells were there after propagated by weekly intraperitoneal (IP) injections of 3 x 10^6 cells, freshly drawn from a donor mouse bearing 7 - 9 day-old ascites tumor suspended in 0.3 ml sterile saline solution (Mady, 2002).

Experimental animals

Twenty five Swiss albino female mice weighting 20 ± 2 g were obtained from the Animal House Colony of the National Research Center, Cairo, Egypt. Five mice in each cage were housed in plastic cages of dimensions of 42L x 26W x 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 5 groups (n = 5) as follows: Group 1 (vehicle control) was left without any treatment for 14 day. Group 2 (negative control) injected intraperioneal (i.p.) with 0.2 ml of EAC, which contains 2 x 10^6 cells for tumor induction and left for 14 day (Gupta et al., 2004). Group 3 (positive control) injected (i.p.) with 0.2 ml of EAC and treated with dose of 20 mg/kg b.wt. i.p. day after day of reference drug 5-flurouracil for 14 days. Group 4 received aminothienoandrostane at dose 25 mg/kg b.wt. (i.p.) dissolved in dimethyl sulfoxide (DMSO) day after day for 14 day which is period of the study of antitumor activity and simultaneous alterations in the hematological profile were estimated as well as tumor volume was measured (Gupta et al., 2004).

In order to detect the influence of effect of aminothienoandrostane on the hematological status of EAC-bearing mice, after administration of the last dose followed by 18 h fasting, all mice were then sacrificed; a comparison was made among four groups (n = 5) of mice on the 14th. Blood was drawn from each mouse by the retro orbital plexus method and the white blood cell count (WBC), hemoglobin and haematocrit % were determined (D'Amour et al., 1965; Lowry et al., 1951). Blood collected from mice should be immediately placed in a tube containing anticoagulant, for routine hematologic testing is ethylene diamine tetra-acetic acid (EDTA). The report of results from standard hematological evaluation is called complete is called complete blood count (CBC) which was determined using

Table 1. Effect of aminothienoandrostane using olive oil or DMSO as solvent on hepatoma cell line proliferation.

minothienoandrostane	24h			48h			72h		
	10 µM	50 µM	100 µM	10 µM	50 µM	100 µM	10 µM	50 µM	100 µM
In olive oil	99.78±0.02	121.36±0.08	117.72±0.04	97.02±0.00	46.44±0.00**	48.13±0.00**	96.67±0.00	33.09±0.00**	30.57±0.00**
In DMSO	98.078±0.09	88.66±0.00	81.33±0.01	97.63±0.00	43.64±0.02**	59.11±0.00**	98.27±0.00	48.13±0.03**	71.36±0.00**

Data expressed as percent of cell growth inhibition ± SD.
P<0.05 was considered as statistically significant (*), and P < 0.01 was considered as statistically highly significant (**).

Table 2. The *in vitro* cytotoxic activity of aminothienoandrostane (IC_{50} in µM).

Compound	Olive oil		DMSO	
	48 h	72 h	48 h	72 h
Aminothienoandrostane	47	36	43	50

automated hematology analyzers (cell- Dyn, 3500) (Hedrich et al., 2004).

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 high significant **.

RESULTS AND DISCUSSION

The tested compound aminothienoandrostane (Figure 1) was synthesized via the reaction of 17β-acetoxyandrostan-3-one with cyanoaceta-mide and sulfur in ethanolic triethylamine solution to afford the corresponding aminothieno-(2`,3`:2,3) androstane derivative in 82% yield. Identical mass spectra of the latter product indicate that the compound are free from the angular isomer aminothieno[2`,3`:4,3]androstane derivative. The mass spectra showed the molecular ion peaks at m/z = 430 (32%), besides a base peak at m/z = 168 for the retro-Diels Alder fragment.

In vitro evaluation of the cytotoxic activity

The inhibition of proliferation of $HepG_2$ cells was determined using MTT assay. The usage of olive oil or DMSO as a solvent at a volume of 100 µl (the maximum volume used to dissolve the tested compound) had no significant effect on the viability of $HepG_2$ cells when treated for 24, 48 and 72 h (Figure 2). Data in Table 1 are expressed as percentage of cell growth inhibition of treated cells versus controls ± S.D. calculated on the average of the experiments performed in triplicate. The proliferation (cell growth) of $HepG_2$ cells when treated with aminothienoandrostane was signify-cantly inhibited in a dose and time dependent manner, especially at 48 and 72 h incubation time when olive oil used as solvent and at 24 and 48 h incubation time when DMSO was used as solvent. Significantly, there was no growth inhibition effect observed at 24 h when olive oil or DMSO was used as solvents. After 72 h incubation when DMSO was used as solvent, the growth inhibition was 52% at 50 µM and decreased to 29% at 100 µM (Figure 4). The best results were obtained at 72 h when olive oil was used as solvent at 50 and 100 µM where the growth inhibition rate was 67 and 70%, respectively (Figure 3).

The result expressed as IC_{50} values in µM was reported in Table 2. The IC_{50} of aminothienoandrostane decreased with the increasing of the incubation time when olive oil was used as solvent. On the other hand, the IC_{50} increased with the increasing of the incubation time when DMSO was used as solvent. In comparison with data published about the cytotoxic activity of some ste-roids (Yoshida et al., 2003) and from the structure activity relationship viewpoint, we postulate that the addition of diaminothiophene ring to the steroid moiety increased the cytotoxic activity es-pecially at a concentration of 50 µM and at 72 h incubation period. In general the results at 72 h when olive oil was used as solvent is better than that of DMSO and these results revealed that olive oil is suitable vehicle for anti-tumor drugs and is better than DMSO. These promising results were confirmed by the morphological study and its photographic data of light microscope at photograph 1.

In vivo evaluation of the anti-tumor activity

The anti-tumor activity was evaluated on EAC bearing mice using animal model. Our novel steroid derivative aminothienoandrostane, 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. Our

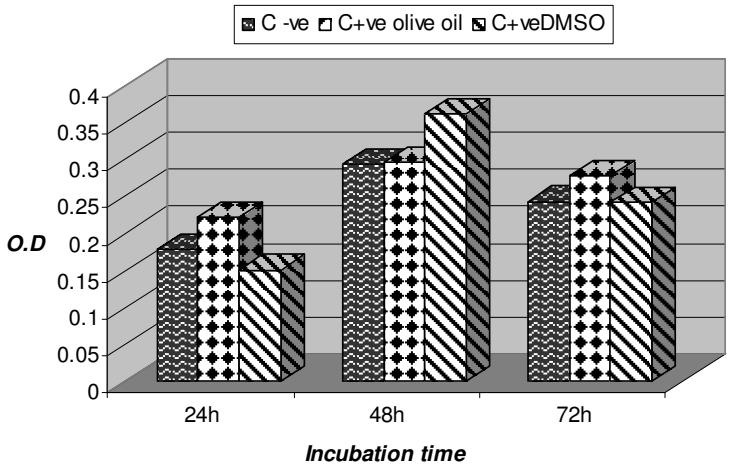


Figure 2. Effect of DMSO and olive oil (100 µl) on growth of ($HepG_2$) cells, at different time intervals. C= control.

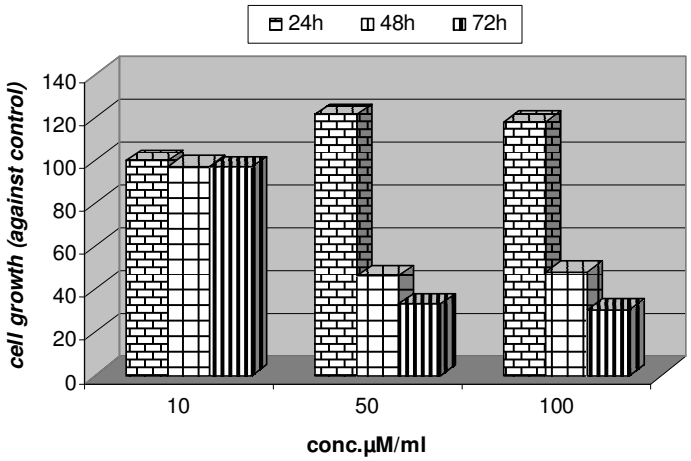


Figure 3. Effect of aminothienoandrostane dissolved in olive oil at *24, 48 and 72 h on the proliferation of $HepG_2$ cells in vitro.*

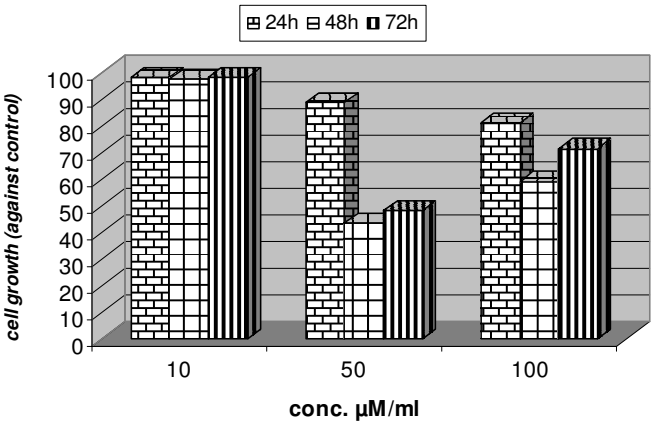
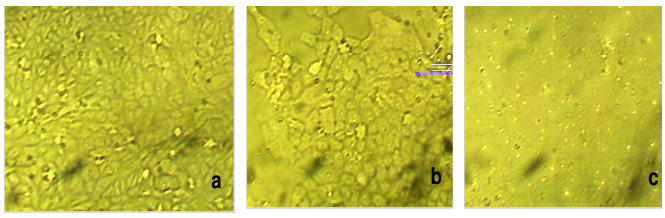


Figure 4. Effect of aminothienoandrostane dissolved in DMSO at 24, 48 and 72 h on the proliferation of $HepG_2$ cells *in vitro*



Photograph 1. The morphology of HepG2 cells after 72 h incubation: a) control without any treatment, b) conc. 100 µM of aminothienoandrostane using DMSO as solvent, c) conc. 100 µM of aminothienoandrostane using olive oil as solvent.

novel compound aminothienoandrostane was as good as the most commonly used drug; 5-flurouracil (20 mg/kg) (Table 3, Figure 5). It is well known that 5-flurouracil is

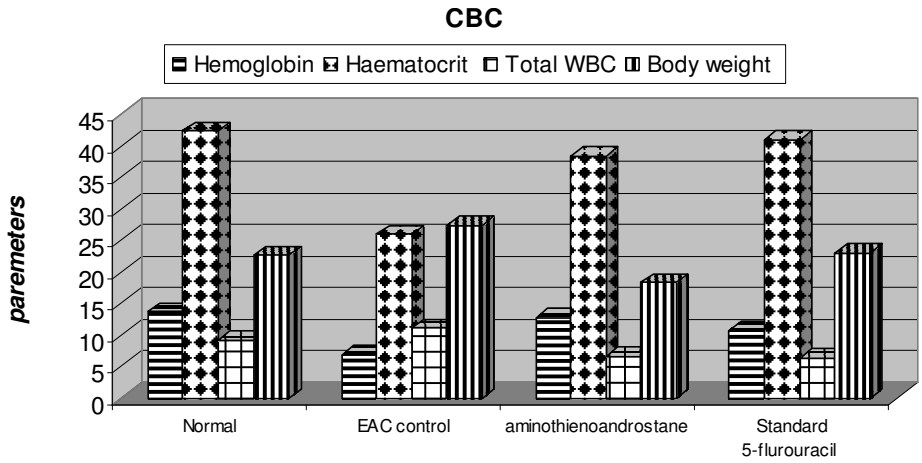


Figure 5. Effect of aminothienoandrostane on hematological parameters and on body weight of EAC bearing mice.

Table 3. Effect of aminothienoandrostane on hematological parameters and on body weight of EAC bearing mice. n = 5. mean ± SD. $^{*}P < 0.05$ vs normal group, $^{**}P < 0.01$ vs normal group.

Parameter	Normal	EAC control (2 x 10^6cells/ mice)	Aminothienoandrostane (25 mg/kg) + EAC	Standard (5-flurouracil (20 mg/kg) + EAC)
Hemoglobin (g%)	13.63±0.75	6.86±2.25**	12.77±0.035	10.73±0.15**
Haematocrit (%)	42.36±1.00	25.9±2.29**	38.32±0.17*	40.86±2.17
Total WBC/10^3 (mm^{-3})	9.16±0.70	11.16±0.25**	6.58±0.1**	6.40±0.52**
Body weight (g)	22.66±0.57	27.33±2.51**	18.27±0.38*	23.0±1.0
Tumor volume (mL)	Nil	4.56±0.37	Nil	Nil

Photograph 2.Shows effects of aminothienoandrostane 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

one of drugs to treat cancer clinically. In cancer chemotherapy one of the major problems is anemia which is mainly due to reduction in RBCs or hemoglobin percentage. Treatment with our novel compound aminothienoandrostane maintains the hemoglobin content, body weight, hematocrit % and WBCs count near to normal values and similar to values observed with 5-flurouracil (Table 3, Figure 5). Furthermore, none of the treated mice with aminothie-noandrostane exhibited any abnormal behavioral or any toxicity symptoms of dose used during this study. Aminothienoandrostane was active with no loss of appetite and showed no dizziness or erection of hair or hypothermia (photograph 2). This indi-cated the safety of using it at specified low dose we used. Finally, the broad spectrum anti-tumor activity dis-played by this compound will be of interest for further de-rivatization, further *in vitro, in vivo* and clinical studies in the hope of finding more active and selective anti-tumor agents

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