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Original article

Novel synthesized aminosteroidal heterocycles intervention for inhibiting iron-induced oxidative stress

Gamal A. Elmegeed [a,*], Hanaa H. Ahmed [a], Jihan S. Hussein [b]

[a] *Hormones Department, National Research Center, Dokki, Giza, Egypt*
[b] *Medical Biochemistry Department, National Research Center, Dokki, Giza, Egypt*

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Abstract

The objective of this study was to elucidate the potential role of novel synthesized aminosteroidal heterocyclic compounds **2**, **5**, **9b** and **10c** against iron-induced oxidative stress with particular insight on erythrocyte ghosts in male rats. Chronic iron supplementation (3000 mg kg^{-1} diet) for 6 weeks significantly increased plasma iron and ferritin levels. It also produced significant increase in plasma TNF-α and NO levels. Lipid metabolism was also affected by excess iron, so that plasma and erythrocyte membrane total cholesterol, triglycerides, phospholipids and total lipid levels were significantly elevated. In consequence, a significant increase in plasma leptin level was detected. Iron overload clearly induces oxidative stress as indicated by the significant increase in both plasma and erythrocyte membrane lipid peroxidation levels. Noteworthy, excess iron not only decreased the mean value of erythrocyte membrane protein but also caused marked alterations in the membrane protein fractions with concomitant inhibition in erythrocyte membrane ATPases activity. On the other hand, treatment with the aminosteriodal heterocyclic compounds especially compounds **5**, **2**, and **10c** in an oral dose of 5 mg kg^{-1} B.W. per day could ameliorate almost all of the changes in plasma and erythrocyte ghosts components induced by iron overload. The efficacious role of these novel synthesized aminosteriods in preventing iron-induced oxidative stress may be mediated through their iron chelating properties, anti-lipid peroxidation activities and membrane stabilizing actions. The encouraging results obtained in the present study lend credence to substantial investigation to assess the use of these compounds as a potent line of therapy to retard the pathogenesis of iron overload diseases.
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1. Introduction

Iron is an essential metal for normal cellular physiology, but an excess iron can result in cell injury. This is because it plays a catalytic role in the initiation of free radical reactions. The resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates; the result is wide-ranging impairment in cellular function and integrity [1]. The mechanism by which iron can cause this deleterious effect is that iron can react with superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) to produce the hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction [2]. These radicals can also lead to the formation of other reactive oxygen species (ROS) [3]. The overproduction of ROS can directly attack the polyunsaturated fatty acids of the cell membranes and induce lipid peroxidation [4]. The inhibition of ion pump ATPases in plasma membranes can also occur as a result of iron-promoted formation of ROS and subsequent lipid peroxidation [5]. Moreover, the reactive oxygen species can activate the transcription factors as nuclear factor-kB (NF-kB), which up-regulates the transcription of adhesion molecules, cytokines and enzymes, all involved in the inflammatory responses [6].

Series of aminosteroids have been established as antioxidant agents [7] and a growing body of evidence has confirmed the ability of these compounds to limit free radical-mediated cellular injury [8]. The exact mechanisms by which aminosteroids protect against cellular injury are unknown. One hypothesis is that the beneficial effects of aminosteroids are due to their antioxidant and free radical-scavenging properties [7]. The other proposed mechanism is stabilization of

* Corresponding author. Tel.: +20 2 568 2070; fax: +20 2 337 0931.
E-mail address: gamalae@hotmail.com (G.A. Elmegeed).

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biological membranes [9]. These agents could inhibit lipid peroxidation, prevent the release of free arachidonic acid from injured cell membranes [10] and, thus, blunt the damage from the secondary wave of injury which follows any insults to cell membranes [11]. Also, it has been suggested that aminosteroids act as an antioxidant against lipid peroxidation through a physicochemical and not a pure chemical mechanism and the physical interaction with the cell membrane may facilitate the inhibition of lipid peroxidation by these compounds [12].

It has long been believed that small alterations in the structure of certain steroids can greatly affect their receptor binding affinity and biological activity [13]. Several studies have demonstrated that the introduction of various heterocyclic rings to ring A or ring D of various steroids was effective in the production of variety of compounds possessing biological activities [14,15].

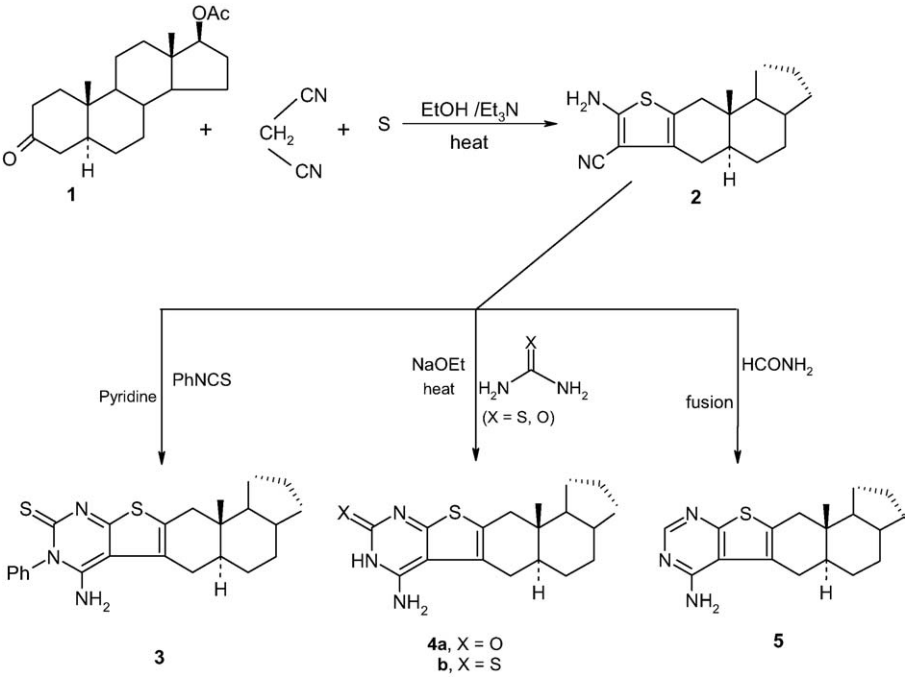
The main purposes of the present study were the synthesis of novel aminosteroidal heterocyclic derivatives using 17β-acetoxy-5α-androstan-3-one **(1)** and 3β-acetoxy-5α-androstan-17-one **(6)** as starting materials. Furthermore, this study was undertaken to shed light on the potential role of some of these novel aminosteroidal heterocycles against oxidative damage, particularly on the erythrocyte ghosts, induced by iron overload in experimental rat models.

2. Results and discussion

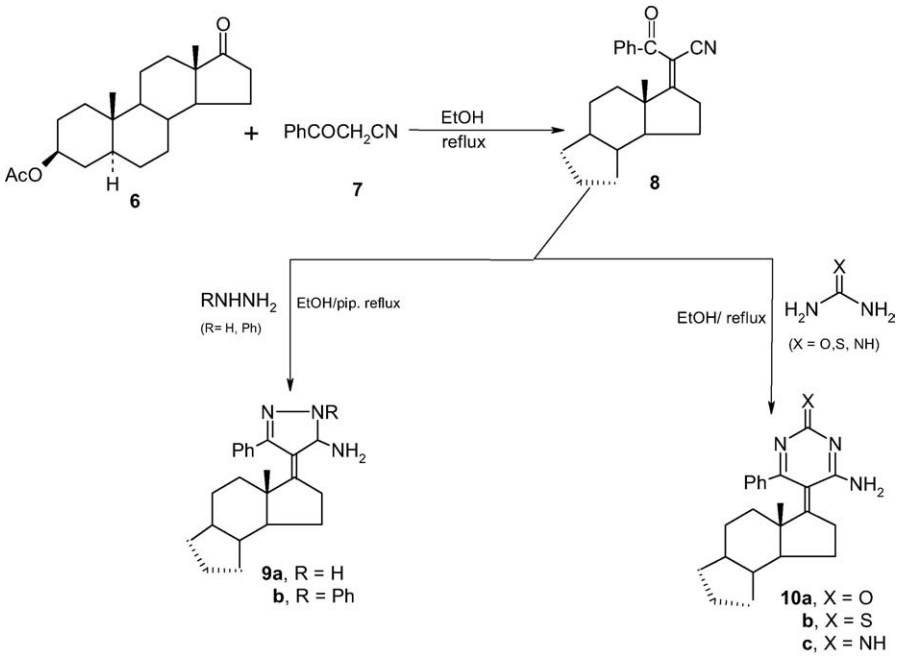
2.1. Chemistry

Our intention was to prepare steroid derivatives with incorporating an active aminoheterocyclic moiety at different structural settings in the steroid molecule. 17β-Acetoxy-5α-androstan-3-one **(1)** reacted with equimolar amounts of malononitrile and sulfur in ethanolic triethylamine solution to afford in 82% yield the corresponding aminothieno[2′,3′:2,3]androstane-4′-carbonitrile **2** (Scheme 1). Identical mass spectra of compound **2** confirmed that the compound obtained is free from the angular isomer aminothieno[3′,2′:3,4]androstane. The mass spectra showed molecular ion peak at *m/z* 412 (58%) besides a base peak at *m/z* 150 with the metastable ion at *m/z* 48.5. Such fragmentation is characteristic mass spectral feature of the steroidal heterocycles in which the heterocyclic ring is fused at the C_2 and C_3 positions of Δ^2 steroids [16]. The IR spectrum of compound **2** revealed the existence of absorption band at ν 2220 cm^{-1} corresponding to the CN group. The ^1H NMR spectrum of compound **2** revealed the presence of D_2O-exchangeable singlet for the NH_2 group at δ 5.76 ppm. On the other hand, the 17-keto steroid, 3β-acetoxy-5α-androstan-17-one **(6)**, was recovered unreacted with malononitrile and sulfur in ethanolic triethylamine solution after refluxing for 7 h.

Steroids condensed with pyrimidine have been proven to be pharmacologically active compounds [17]. The enaminonitrile moiety of compound **2** proved to be highly reactive towards nitrogen nucleophiles to form the corresponding pyrimidines. Thus, compound **2** reacted with phenylisothiocyanate, in refluxing pyridine, to afford the corresponding aminopyrimidino[5″,4″:4′,5′]thieno[2′,3′:2,3]androstane derivative **3** (Scheme 1). Similarly, compound **2** condensed with either urea or thiourea in ethanolic sodium ethoxide solution to give the corresponding aminopyrimidino-[5″,4″:4′,5′]thieno[2′,3′:2,3]androstane derivatives **4a**, **b**, respectively, and condensed also with formamide by fusion in oil bath to afford the aminopyrimidino[5″,4″:4′,5′]-



Scheme 1.



Scheme 2.

thieno[2′,3′:2,3]androstane derivative **5** (Scheme 1). All the obtained analytical and spectral data of compounds **3**, **4a**, **b** and **5** were in accordance with the proposed structures. The 17-keto group of 3*β*-acetoxy-5α-androstan-17-one **(6)** reacted readily with benzoyl acetonitrile **7** in refluxing ethanol to afford the Knoevenagel condensed product 3*β*-acetoxy-17-(2′-benzoylacetonitrile-2′-ylideno)androstane **8**. The *β*-benzoyl unsaturated nitrile moiety of compound **8** showed marked reactivity towards chemical reagents to form heterocyclic derivatives with a wide spectrum of biological activities [18]. Thus, further confirmation for structure **8** was obtained through studying its reactivity towards chemical reagents; compound **8** reacted with either hydrazine hydrate or phenyl hydrazine in refluxing ethanol/piperidine solution to afford the corresponding 17-(5′-aminopyrazol-4′-ylideno)-androstane derivatives **9a**, **b**, respectively. Moreover, compound **8** reacted with either urea or thiourea in sodium ethoxide solution and reacted also with guanidine hydrochloride in ethanolic sodium acetate solution to afford the 17-(6′-aminopyrimidin-5′-ylideno)androstane derivatives **10a**, **b**, **c**, respectively. The structures of all these products were confirmed by their spectral and analytical data (Scheme 2).

2.2. Bioassay

There are several inherited and acquired disorders that can result in chronic iron overload in humans [19]. Iron removal in iron overload subjects can be achieved using chelating therapy. Iron chelating drugs could, in addition to removing iron, offer protection against iron toxicity by inhibiting the catalytic formation of toxic free radicals [20]. Pakala et al. [21] stated that the aminosteroids have both antioxidant and iron chelating properties. These principles encouraged us to study the potential role of the novel aminosteroidal heterocycles **2**, **5**, **9b** and **10c** against oxidative damage-induced by iron overload in experimental animals.

2.2.1. Effect of aminosteroidal heterocycles on plasma iron status

As expected, chronic iron supplementation caused significant elevation ($P < 0.01$) in plasma iron and iron storage protein, ferritin, concentrations as shown in group (Fe^{2+}) compared to the control group receiving a diet with normal amounts of iron (Table 1). This finding is in agreement with the previous reports of Araujo et al. [22] and Silvana et al. [23]. Moreover, Pietrangelo et al. [24] suggested that dietary iron overload in rats can specifically activate target genes in the liver (i.e. L ferritin and procollagen) in the absence of either histological signs of severe liver damage or alterations in differentiated liver functions. Valerio and Petersen [25] supported this suggestion by providing an evidence for the

Table 1
Effect of aminosteroidal heterocycles on plasma iron status in control and iron overloaded rats

Group	Iron (μg dl^{-1})	TIBC (μg dl^{-1})	Ferritin (ng ml^{-1})
Control	69.5 ± 5.5	165.8 ± 3.7	5.8 ± 1.5
Compound **2**	68.9 ± 3.1$^{a\ ns}$	164.4 ± 2.0$^{a\ ns}$	5.6 ± 1.1$^{a\ ns}$
Compound **5**	68.7 ± 3.3$^{a\ ns}$	162.8 ± 2.4$^{a\ ns}$	5.4 ± 1.3$^{a\ ns}$
Compound **10c**	69.2 ± 3.8$^{a\ ns}$	164.8 ± 2.5$^{a\ ns}$	5.7 ± 1.2$^{a\ ns}$
Compound **9b**	69.4 ± 4.7$^{a\ ns}$	165.5 ± 2.9$^{a\ ns}$	5.8 ± 1.1$^{a\ ns}$
Fe^{2+}	99.3 ± 3.7^{a**}	178.3 ± 4.8$^{a\ ns}$	12.6 ± 0.8^{a**}
Fe^{2+} + **2**	85.3 ± 3.9^{b*}	174.2 ± 3.9$^{b\ ns}$	10.5 ± 0.3^{b*}
Fe^{2+} + **5**	80.7 ± 3.5^{b**}	170.8 ± 2.6$^{b\ ns}$	10.1 ± 0.2^{b**}
Fe^{2+} + **10c**	89.9 ± 2.3^{b*}	176.5 ± 2.8$^{b\ ns}$	10.7 ± 0.3^{b*}
Fe^{2+} + **9b**	90.3 ± 3.2$^{b\ ns}$	177.3 ± 2.6$^{b\ ns}$	11.5 ± 0.6$^{b\ ns}$

* $P < 0.05$, ** $P < 0.01$. ns: Nonsignificant.
[a] Comparison related to control group.
[b] Comparison related to iron overloaded group.

increased expression of the heavy-chain isoform of ferritin mRNA in the liver of animals fed with iron-enriched diet compared to the controls.

Results in Table 1 also revealed that treatment with the aminosteroids **5**, **2** and **10c** significantly decreased plasma iron and ferritin levels as shown in groups (Fe^{2+} +**5**, Fe^{2+} +**2** and Fe^{2+} +**10c**) compared to untreated iron overloaded group (Fe^{2+}), whereas, treatment with compound **9b** (Fe^{2+} +**9b**) showed slight decrease in plasma iron and ferritin levels compared to Fe^{2+} group. These findings confirmed the iron chelating properties of these novel aminosteroidal heterocycles as reported by Pakala et al. [21] concerning other aminosteroids.

2.2.2. Effect of aminosteroidal heterocycles on plasma TNF-α and NO

Table 2 comprises the effect of treatment with aminosteriodal heterocycles on plasma tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) levels in iron overloaded rats. TNF-α was significantly ($P < 0.01$) increased in iron overloaded group (Fe^{2+}) compared to the control group. Gressner et al. [26] have proposed the presence of activated hepatic stellate cells in the pre-inflammatory period of liver injury. Activated hepatic stellate cells have been demonstrated in rat models of iron overload [27] suggesting that iron plays a role in hepatic stellate cells activation. Hepatic stellate cells are known to be responsive to stimulation by a number of cytokines involving TNF-α in their transformation to active myofibroblastic phenotype [28]. Moreover, the oxidative damage produced by iron overload led to the production of ROS that activate the NF-kB which plays a key role in the inflammatory process [6]. It is well known that NF-kB is activated by a wide range of agents and cytokines including TNF-α secreted from the injured hepatic macrophages [29].

It has been reported that TNF-α could induce NO production from the activated polymorphonuclear leukocytes in response to tissue inflammation [30,31]. As shown in Table 2 the iron overload in group (Fe^{2+}) shows significant ($P < 0.01$) increase in plasma NO level compared to the control group. The presence of nitrogen reactive species explains the iron sequestration pattern that characterizes macrophages under inflammatory conditions. There is a complex relationship between iron and NO so that iron overload resulted predominantly in iron uptake by the liver Kupffer cells that led to an increased NO level in the blood through the induction of nitric oxide synthase (iNOS) [32].

Table 2
Effect of aminosteroidal heterocycles on plasma TNF-α and NO levels in control and iron overloaded rats

Group	TNF-α (pg ml^{-1})	NO (μmol l^{-1})
Control	52.8 ± 1.0	35.2 ± 1.4
Compound **2**	52.2 ± 1.4$^{a\ ns}$	33.7 ± 1.2^{ans}
Compound **5**	52.0 ± 1.1$^{a\ ns}$	32.8 ± 1.0$^{a\ ns}$
Compound **10c**	52.5 ± 1.3$^{a\ ns}$	34.1 ± 2.0$^{a\ ns}$
Compound **9b**	52.7 ± 1.2$^{a\ ns}$	34.7 ± 0.7$^{a\ ns}$
Fe^{2+}	72.9 ± 1.6^{a**}	53.7 ± 4.9^{a**}
Fe^{2+} + **2**	63.3 ± 2.9^{b*}	41.4 ± 1.4^{b*}
Fe^{2+} + **5**	60.5 ± 3.2^{b**}	37.5 ± 1.5^{b**}
Fe^{2+} + **10c**	64.6 ± 3.4^{b*}	42.9 ± 1.1^{b*}
Fe^{2+} + **9b**	68.3 ± 3.2$^{b\ ns}$	46.2 ± 1.5$^{b\ ns}$

* $P < 0.05$, ** $P < 0.01$. ns: Nonsignificant.
[a] Comparison related to control group.
[b] Comparison related to iron overloaded group.

Treatment with the aminosteroidal heterocycles **5**, **2** and **10c** produced significant decrease in each of plasma TNF-α and plasma NO levels as shown in groups (Fe^{2+} + **5**, Fe^{2+} + **2** and Fe^{2+} + **10c**), whereas treatment with compound **9b** (group Fe^{2+} +**9b**) produced insignificant decrease in plasma TNF-α and NO levels compared to untreated iron overloaded group (Fe^{2+}) as presented in Table 2. Sadrzadeh and Naji [33] reported the ability of aminosteriods in reducing the levels of proinflammatory stimuli such as lipid peroxidation, TNF-α and COX-2. Also Lehmann et al. [34] demonstrated that the administration of aminosteroids decreased TNF-α release during experimental endotoxemia. Palma-Vargas et al. [35] stated that aminosteroids could significantly improve liver functions, after total liver ischemia, via blocking neutrophil infiltration which is independent on nitrite/nitrate levels. They could not find statistical difference in plasma nitrite/nitrate levels after treatment with aminosteroids which may be due to the use of single dose of these compounds in short duration.

2.2.3. Effect of aminosteroidal heterocycles on plasma lipid profile and leptin

Table 3 depicts the effect of aminosteroids administration on plasma lipid profile and leptin level among the different studied groups. Iron overload (group Fe^{2+}) could significantly increase plasma total cholesterol, triglycerides ($P < 0.01$), phospholipids and total lipids ($P < 0.05$) levels compared to the control group. These results are consistent with those of Dabbagh et al. [36] and Brunet et al. [37] they suggested their results as that experimental iron overload causes marked perturbations in plasma lipid transport and hepatobiliary sterol metabolism. Additionally, iron-catalyzed lipid peroxidation may well be one of the mechanisms involved in lipid profile alterations.

The results in Table 3 reveal that the aminosteriods **5**, **2** and **10c** administration (groups Fe^{2+} + **5**, Fe^{2+} + **2** and Fe^{2+} + **10c**) results in marked decrease in plasma total cholesterol, triglycerides, phospholipids and total lipids levels compared to untreated iron overloaded group (Fe^{2+}). It has been reported that the aminosteriods are potent inhibitors of free radical production by stimulated monocytes and also they are active scavengers of lipid peroxyl radicals [38]. Therefore, these compounds are able to protect LDL from oxidation leading to a decrease in the chemotactic activity of LDL towards monocytes as well as a reduction of cholesterol accumulation. Thus, aminosteriods could have a beneficial role against the development of atherosclerosis, not only via its antioxidant action towards LDL, but also through reducing the biological alterations of the oxidized LDL [39].

The results in Table 3 show that plasma leptin level is significantly ($P < 0.01$) increased in iron overloaded group (Fe^{2+})

Table 3
Effect of aminosteroidal heterocycles on plasma lipid profile and leptin level in control and iron overloaded rats

Group	Total cholesterol (mg dl^{-1})	Triglycerides (mg dl^{-1})	Phospholipids (mg dl^{-1})	Total lipids (mg dl^{-1})	Leptin (ng ml^{-1})
Control	81.8 ± 1.4	56.0 ± 1.2	120.0 ± 5.7	276.2 ± 7.1	12.7 ± 0.8
Compound **2**	78.0 ± 2.9$^{a\ ns}$	52.8 ± 2.4$^{a\ ns}$	117.3 ± 1.7$^{a\ ns}$	262.2 ± 3.3$^{a\ ns}$	11.8 ± 0.5$^{a\ ns}$
Compound **5**	74.3 ± 5.1$^{a\ ns}$	50.0 ± 3.5$^{a\ ns}$	111.7 ± 1.8$^{a\ ns}$	259.5 ± 5.3$^{a\ ns}$	11.5 ± 0.5$^{a\ ns}$
Compound **10c**	80.3 ± 3.6$^{a\ ns}$	54.3 ± 1.6$^{a\ ns}$	117.8 ± 3.7$^{a\ ns}$	269.3 ± 8.9$^{a\ ns}$	12.4 ± 0.7$^{a\ ns}$
Compound **9b**	81.0 ± 5.0$^{a\ ns}$	54.8 ± 2.9$^{a\ ns}$	118.0 ± 3.5$^{a\ ns}$	272.3 ± 3.3$^{a\ ns}$	11.25 ± 0.2$^{a\ ns}$
Fe^{2+}	94.6 ± 2.1^{a**}	69.9 ± 1.5^{a**}	137.5 ± 4.9^{a*}	305.7 ± 8.8^{a*}	16.8 ± 0.7^{a*}
Fe^{2+} + **2**	85.8 ± 2.2^{b*}	60.8 ± 3.3^{b*}	127.0 ± 5.5$^{b\ ns}$	282.6 ± 5.1^{b*}	13.7 ± 0.9^{b*}
Fe^{2+} + **5**	83.6 ± 3.0^{b**}	59.4 ± 4.3^{b*}	122.3 ± 4.0^{b*}	277.0 ± 3.9^{b**}	12.9 ± 0.6^{b**}
Fe^{2+} + **10c**	87.2 ± 2.5^{b*}	63.0 ± 3.1$^{b\ ns}$	130.1 ± 4.8$^{b\ ns}$	284.4 ± 4.6^{b*}	14.5 ± 0.5^{b*}
Fe^{2+} + **9b**	92.8 ± 3.3$^{b\ ns}$	64.2 ± 4.2$^{b\ ns}$	133.0 ± 6.4$^{b\ ns}$	294.0 ± 3.6$^{b\ ns}$	15.0 ± 0.6$^{b\ ns}$

* $P < 0.05$, ** $P < 0.01$. ns: Nonsignificant.
[a] Comparison related to control group.
[b] Comparison related to iron overloaded group.

compared to control group. The information concerning the changes in serum leptin levels in various hematological diseases are very limited [40]. In vitro study by Mcabee et al. [41] revealed the appearance of rat hepatic leptin-1 (RHL-1) multimers when hepatocyte nonheme iron content has increased by no more than two to fivefold. Additionally, a positive correlation between plasma total cholesterol level, plasma ferritin level and serum leptin level was determined [42].

Treatment with the aminosteroidal heterocycles **5** or **2** or **10c** (groups Fe^{2+} + **5**, Fe^{2+} + **2** or Fe^{2+} + **10c**) significantly reduces plasma leptin level compared to untreated iron overloaded group (Fe^{2+}). The authors suggest that the cholesterol-lowering effect of the aminosteroids **5**, **2** and **10c** shown in the present study may be responsible for their ability in reducing plasma leptin level. Noteworthy, the treatment with compound **9b** (group Fe^{2+} + **9b**) shows non appreciable change in plasma lipid profile and leptin level (Table 3).

2.2.4. Effect of aminosteroidal heterocycles on plasma and erythrocyte membrane lipid peroxidation

Iron overload in group (Fe^{2+}) reveal significant ($P < 0.01$) increase in plasma as well as in erythrocyte membrane lipid peroxidation levels compared to control group (Table 4). Fischer et al. [43] reported that iron overload increase plasma thiobarbituric acid reactive substances and/or conjugated dienes in rats. Ferrali et al. [44] stated that excess iron induces oxidative damage to erythrocyte membrane as a result of its role in promoting free radical generation and lipid peroxidation.

Table 4
Effect of aminosteroidal heterocycles on plasma and erythrocyte membrane lipid peroxidation (measured by thiobarbituric acid test) in control and iron overloaded rats

Group	Plasma lipid peroxidation (μmol l^{-1})	Lipid peroxidation in cell membrane (μmol l^{-1})
Control	0.85 ± 0.22	1.50 ± 0.12
Compound **2**	0.75 ± 0.20$^{a\ ns}$	1.18 ± 0.18$^{a\ ns}$
Compound **5**	0.65 ± 0.22$^{a\ ns}$	1.15 ± 0.10^{a*}
Compound **10c**	0.78 ± 0.23$^{a\ ns}$	1.23 ± 0.14$^{a\ ns}$
Compound **9b**	0.82 ± 0.31$^{a\ ns}$	1.30 ± 0.13$^{a\ ns}$
Fe^{2+}	1.80 ± 0.15^{a**}	3.5 ± 0.28^{a**}
Fe^{2+} + **2**	1.25 ± 0.17^{b*}	2.5 ± 0.19^{b*}
Fe^{2+} + **5**	1.10 ± 0.15^{b**}	2.1 ± 0.15^{b**}
Fe^{2+} + **10c**	1.30 ± 0.16^{b*}	2.7 ± 0.18^{b*}
Fe^{2+} + **9b**	1.40 ± 0.21$^{b\ ns}$	3.1 ± 0.25$^{b\ ns}$

* $P < 0.05$, ** $P < 0.01$. ns: Nonsignificant.
[a] Comparison related to control group.
[b] Comparison related to iron overloaded group.

Treatment with aminosteroidal heterocycles **5** or **2** or **10c** in groups (Fe^{2+} + **5**, Fe^{2+} + **2** and Fe^{2+} + **10c**) results in significant decrease in each of plasma and erythrocyte membrane lipid peroxidation level while, treatment with compound **9b** (group Fe^{2+} + **9b**) shows insignificant decrease compared to the iron overloaded group (Fe^{+2}) as shown in Table 4. The positive effect of the tested compounds on plasma lipid peroxidation is consistent with that of Fernandes et al. [45] who stated that the aminosteroids family has a potent ability to inhibit iron dependent lipid peroxidation through scavenging lipid radicals in a way quite analogous to alpha tocopherol [46]. Our results are greatly supported by Malmstrom et al. [47] who studied the antioxidant profile of newly synthesized thiophene derivatives and its analogues. They found that these compounds have a potent capacity to inhibit lipid peroxidation in the liver microsomes stimulated by iron and can catalyze the decomposition of hydrogen peroxide. Also, the pyrimidine derivatives bearing an amino moiety in their nucleus may act as agents against lipid peroxidation [48,49]. In addition the in vivo study by Sultatos [50] revealed that pre-treatment of rats with pyrazole derivatives could partially inhibit lipid peroxidation induced by administration of a single dose of ethanol.

Regarding the protective effect of the tested aminosteroids against erythrocyte membrane lipid peroxidation (Table 4), Saniova [51] reported that the aminosteroids are good inhibitors of membrane lipid peroxidation and they appear to function as free radical scavengers. Inhibition of membrane lipid peroxidation may arise from two main actions of these compounds, including their capacity: 1) to compete with α-tocopherol for reactive oxygen species and meanwhile attenuate the consumption of vitamin E that would otherwise occur, and 2) to impede, via a membrane stabilizing action, the mobility of reactive oxygen species in lipid

domains and meanwhile, prevent propagation of lipid peroxdation [52].

2.2.5. Effect of aminosteroidal heterocycles on erythrocyte membrane lipids

The results in Table 5 shows that iron overload significantly increase the mean values of membrane lipids (total cholesterol, triglycerides, phospholipids and total lipids) as shown in group (Fe^{2+}) compared to control group. London and Schwarz [53] reported that the rise in the concentration of erythrocyte cholesterol is concordant to the rise in the plasma cholesterol. Pietrangelo et al. [54] stated that the hepatic iron overload induces a modification of fatty acid profile in cellular structures consistent with the in vivo lipid peroxidation. Smuts et al. [55] demonstrated that iron status influences fatty acid metabolism of specific n-3 fatty acids in the erythrocyte membranes. Thus, in the present study, the increase in membrane lipids resulted from the enhanced iron-induced disturbances in lipid metabolism as reported by Dabbagh et al. [36].

Table 5 also represents the effect of the newly synthesized aminosteroidal heterocycles **5**, **2**, **10c** and **9b** on the erythrocyte membrane lipids. Compounds **5** and **2** could significantly reduce membrane lipids in groups (Fe^{2+} + **5** and Fe^{2+} + **2**) while, compounds **10c** and **9b** could partially decrease membrane lipids in groups (Fe^{2+} + **10c** and Fe^{2+} + **9b**) compared to untreated iron overloaded group (Fe^{2+}). The lipophilic property of aminosteriods has been reported by Noguchi et al. [12]. Furthermore, the physical membrane stabilizing effect of the lipophilic heterocyclic aminosteroids has been demonstrated [56]. These compounds are incorporated into the lipid bilayer of the membrane where they occupy strictly defined positions and orientations. Complexation with fatty acyl chains may enhance membrane antioxidant activity by hindering free radical chain propagation [56].

2.2.6. Effect of aminosteroidal heterocycles on erythrocyte membrane protein and its fractions

The results in Table 6 show that iron overload (group Fe^{2+}) decrease the mean value of total erythrocyte membrane protein as well as causing a disturbance in its fractions compared to the control group. In vitro study of Rohn et al. [57] indicated that inhibition of ion pump ATPase and cross-linking proteins of red blood cell membrane occurred over the course of several hours incubation in the presence of ferrous sulfate and EDTA. They explained these findings as a result of iron-promoted formation of superoxide anion and subsequent lipid peroxidation which can be prevented by free radical scavengers. Also, Sochaski et al. [58] supported this suggestion as they stated that iron overload increases lipid peroxidation with concomitant chemical modification of tissue proteins. Moreover, ultra-structural study by Schwartz et al. [59] confirmed this event since they found the iron particles in membrane-bound structures.

Treatment with aminosteroidal heterocycles **5** or **2** to iron overloaded rats (groups Fe^{2+} + **5** and Fe^{2+} + **2**) results in detectable increase in the mean value of total erythrocyte membrane protein compared to untreated iron overloaded group (Fe^{2+}), while treatment with compounds **10c** or **9b** (groups Fe^{2+} + **10c** and Fe^{2+} + **9b**) shows non appreciable change as shown in Table 6. In vitro study by Monyer et al. [60] demonstrated that the aminosteroids are capable to relieve almost all of the damage-induced by iron exposure through inhibition of free radical-mediated lipid peroxidation.

2.2.7. Effect of aminosteroidal heterocycles on erythrocyte membrane ATPases

The altered fatty acid composition of the erythrocyte membrane results in altered fluidity, which in turn affects several physical and physiological properties of the membrane such as deformability as well as membrane-bound enzyme activity [61]. The results in Table 7 reveal that iron overload (group Fe^{2+}) causes significant decrease in erythrocyte membrane Na–K ATPase level with concomitant inhibition in its activity compared to the control group. These results are in good agreement with Rohn et al. [57]. Similar alterations in erythrocyte membrane Ca–Mg ATPase were detected in iron overloaded group (Fe^{2+}) compared to the control group (Table 7). Leclerc et al. [62] stated that exposure of red blood cells to iron can result in modification of cell components and alter-

Table 5
Effect of aminosteroidal heterocycles on erythrocyte membrane lipids in control and iron overloaded rats

Group	Total cholesterol mg ml^{-1} RBCs	Triglycerides mg ml^{-1} RBCs	Phospholipids mg ml^{-1} RBCs	Total lipids mg ml^{-1} RBCs
Control	1.63 ± 0.04	0.16 ± 0.015	2.90 ± 0.09	4.2 ± 0.41
Compound **2**	$1.47 \pm 0.16^{a\ ns}$	$0.12 \pm 0.016^{a\ ns}$	$2.84 \pm 0.05^{a\ ns}$	$3.4 \pm 0.28^{a\ ns}$
Compound **5**	$1.28 \pm 0.14^{a*}$	$0.11 \pm 0.014^{a*}$	$2.82 \pm 0.06^{a\ ns}$	$3.1 \pm 0.10^{a*}$
Compound **10c**	$1.53 \pm 0.03^{a\ ns}$	$0.14 \pm 0.013^{a\ ns}$	$2.88 \pm 0.05^{a\ ns}$	$3.6 \pm 0.19^{a\ ns}$
Compound **9b**	$1.57 \pm 0.03^{a\ ns}$	$0.15 \pm 0.012^{a\ ns}$	$2.90 \pm 0.04^{a\ ns}$	$3.8 \pm 0.20^{a\ ns}$
Fe^{2+}	$2.15 \pm 0.11^{a**}$	$0.30 \pm 0.017^{a**}$	$3.72 \pm 0.05^{a**}$	$6.3 \pm 0.36^{a**}$
Fe^{2+} + **2**	$1.75 \pm 0.10^{b*}$	$0.21 \pm 0.026^{b*}$	$3.22 \pm 0.20^{b*}$	$5.1 \pm 0.20^{b*}$
Fe^{2+} + **5**	$1.65 \pm 0.12^{b**}$	$0.18 \pm 0.023^{b**}$	$3.00 \pm 0.21^{b**}$	$4.5 \pm 0.23^{b**}$
Fe^{2+} + **10c**	$1.79 \pm 0.11^{b*}$	$0.25 \pm 0.020^{b\ ns}$	$3.35 \pm 0.25^{b\ ns}$	$5.3 \pm 0.21^{b*}$
Fe^{2+} + **9b**	$1.90 \pm 0.07^{b\ ns}$	$0.26 \pm 0.028^{b\ ns}$	$3.55 \pm 0.18^{b\ ns}$	$5.8 \pm 0.23^{b\ ns}$

* $P < 0.05$, ** $P < 0.01$. ns: Nonsignificant.
[a] Comparison related to control group.
[b] Comparison related to iron overloaded group.

Table 6
Effect of aminosteroidal heterocycles on the mean value of erythrocyte membrane total protein and its fractions in control and iron overloaded rats

Group		Total protein (μg ml^{-1} RBCs)	Albumin (μg ml^{-1} RBCs)	Alpha 1 (μg ml^{-1} RBCs)	Alpha 2 (μg ml^{-1} RBCs)	Beta (μg ml^{-1} RBCs)	Gamma (μg ml^{-1} RBCs)
Control	Mean	146	69.8	6.3	32.7	32.1	5.1
	%	100	47.8	4.3	22.4	22.0	3.5
Compound **2**	Mean	148	43.4	59.6	5.8	30.2	9.0
	%	100	29.3	40.3	3.9	20.4	6.1
Compound **5**	Mean	150	0.0	62.7	30.6	23.3	33.5
	%	100	-	41.8	20.4	15.5	22.3
Compound **10c**	Mean	147	103.6	4.41	20.3	14.3	4.4
	%	100	70.5	3.0	13.8	9.7	3.0
Compound **9b**	Mean	147	65.9	26.5	27.0	27.6	0.0
	%	100	44.8	18.0	18.4	18.8	-
Fe^{2+}	Mean	128	90.8	17.7	12.5	5.5	1.5
	%	100	70.9	13.8	9.8	4.3	1.2
Fe^{2+} + **2**	Mean	136	114.9	2.9	4.9	6.0	7.3
	%	100	84.5	2.1	3.6	4.4	5.4
Fe^{2+} + **5**	Mean	140	122.2	4.9	6.4	6.4	0.0
	%	100	87.3	3.5	4.6	4.6	-
Fe^{2+} + **10c**	Mean	133	105.7	16.8	10.5	0.0	0.0
	%	100	79.5	12.6	7.9	-	-
Fe^{2+} + **9b**	Mean	135	88.4	4.8	13.8	17.3	10.7
	%	100	65.5	3.6	10.2	12.8	7.9

ation in cell behavior. It has been reported that the oxidative processes and the hyperproduction of free radicals which are involved in the destabilization of red blood cell membranes may be responsible for the alterations in Ca–Mg ATPase activity [63]. Leclerc et al. [64] supported this suggestion as they stated that the nonheme iron inhibits the erythrocyte Ca–Mg ATPase activity, at least in part, through the preoxidation of phospholipids of the membrane bilayer.

The data in Table 7 show that treatment with compounds **5** or **2** to iron overloaded rats (Fe^{2+} + **5** and Fe^{2+} + **2**) significantly increases erythrocyte Na–K as well as Ca–Mg ATPases activities compared to untreated iron overloaded group (Fe^{2+}). In this concern, it has been reported that since the time course of formation of thiobarbituric acid reactive substance (TBARS), due to iron overload, is closely paralleled with the inhibition of the erythrocyte ATPases and since the treatment with aminosteroids results in the prevention of TBARS formation, they are able to limit the inhibition of the erythrocyte ATPases activities [57,65].

In conclusion, the results of the present work suggest that this novel class of aminosteriodal heterocyclic derivatives may be proved useful as preventive or as therapeutic interventions in situations where plasma or erythrocyte components are subjected to oxidative stress and in situations related to iron-induced oxidative damage. Noteworthy, the insignificant changes recorded for both the lipid profile and lipid peroxidation in the healthy animals treated with the tested compounds as compared to the control group possibly can give a rough indication that these compounds have no marked toxicity. The tested aminosteroidal heterocycles **2**, **5**, **9b** and **10c** show activities with various intensities depending on the structure of each compound. The aminopyrimidinothienosteroid

Table 7
Effect of aminosteroidal heterocycles on erythrocyte membrane ATPases in control and iron overloaded rats

Group	Total ATPase (nmol ml^{-1} RBCs)	Total ATPase specific activity (μmol Pi/mg protein/h)	Na-K ATPase (nmol m^{-1} RBCs)	Na-K ATPase specific activity (μmol Pi/mg protein/h)	Ca- Mg ATPase (nmol ml^{-1} RBCs)	Ca- Mg ATPase specific activity (μmol Pi/mg protein/h)
Control	130 ± 2.9	9.7 ± 0.30	25.4 ± 2.9	1.9 ± 0.20	104.6 ± 1.70	7.8 ± 0.18
Compound **2**	137 ± 3.9 $^{a\ ns}$	10.3 ± 0.42$^{a\ ns}$	31.7 ± 3.1 $^{a\ ns}$	2.3 ± 0.19 $^{a\ ns}$	111.0 ± 3.4$^{a\ ns}$	7.9 ± 0.21 $^{a\ ns}$
Compound **5**	142 ± 3.3 ^{a*}	11.0 ± 0.43 ^{a*}	36.5 ± 3.4 ^{a*}	2.8 ± 0.22 ^{a**}	115.3 ± 2.4^{a**}	8.8 ± 0.27 ^{a**}
Compound **9b**	140 ± 4.5 $^{a\ ns}$	10.0 ± 0.62$^{a\ ns}$	30.1 ± 3.9 $^{a\ ns}$	2.1 ± 0.15 $^{a\ ns}$	109.3 ± 4.6$^{a\ ns}$	7.8 ± 0.41 $^{a\ ns}$
Compound **10c**	133 ± 3.5 $^{a\ ns}$	9.7 ± 0.51a ^{ns}	26.9 ± 2.3 $^{a\ ns}$	1.9 ± 0.25$^{a\ ns}$	105.2 ± 3.9$^{a\ ns}$	7.8 ± 0.44$^{a\ ns}$
Fe^{2+}	116.2 ± 3.9^{a*}	8.4 ± 0.23^{a**}	18.5 ± 1.2^{a*}	1.2 ± 0.10^{a**}	82.1 ± 2.4^{a**}	5.8 ± 0.32^{a**}
Fe^{2+} + **2**	122.3 ± 4.6$^{b\ ns}$	8.9 ± 0.39$^{b\ ns}$	21.9 ± 1.3$^{b\ ns}$	1.6 ± 0.12^{b*}	94.5 ± 3.8^{b*}	6.8 ± 0.25^{b*}
Fe^{2+} + **5**	127.5 ± 2.8^{b*}	9.3 ± 0.32^{b*}	22.5 ± 1.1^{b*}	1.8 ± 0.12^{b**}	97.0 ± 4.5^{b**}	7.3 ± 0.32 ^{b**}
Fe^{2+} + **9b**	119.2 ± 2.5$^{b\ ns}$	8.5 ± 0.51$^{b\ ns}$	20.8 ± 1.9$^{b\ ns}$	1.5 ± 0.11$^{b\ ns}$	88.5 ± 3.8$^{b\ ns}$	6.3 ± 0.38$^{b\ ns}$
Fe^{2+} + **10c**	117.5 ± 4.2$^{b\ ns}$	8.4 ± 0.56$^{b\ ns}$	20.6 ± 1.7$^{b\ ns}$	1.5 ± 0.14$^{b\ ns}$	85.3 ± 3.9$^{b\ ns}$	6.0 ± 0.22$^{b\ ns}$

* $P < 0.05$, ** $P < 0.01$. ns: Nonsignificant.
[a] Comparison related to control group.
[b] Comparison related to iron overloaded group.

5 and the aminothienosteroid **2** have the strongest activity followed by the aminopyrimidinylsteroid **10c** then the aminopyrazolylsteroid **9b**. These novel active aminosteroidal heterocycles seem to have optimal iron chelating properties, activities against lipid peroxidation and membrane stabilizing actions.

3. Experimental

3.1. Synthesis

The starting steroids were purchased from Sigma Company. The appropriate precautions in handling moisture sensitive compounds were undertaken. The melting points were uncorrected, the IR spectra expressed in cm^{-1} and recorded in KBr pellets on a Pa-9721 IR spectrometer. ^1H NMR and ^{13}C NMR spectra were obtained on a Varian EM-390 90 MHz spectrometer in DMSO-d_6 as solvent and TMS as internal reference. Chemical shifts (δ) are expressed in ppm. Mass spectra were recorded on Kratos (75 eV) MS equipment. Elemental analyses were carried out by Microanalytical Data Unit at The National Research Center, Giza, Egypt. The results of elemental analyses were in a range of ± 0.35%. All the described compounds showed the characteristic spectral data of cyclopentanoperhydrophenanthrene moiety of the androstane series similar to those reported in the literature [66]. 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 [67].

*3.1.1. 17β-Acetoxy-5′-aminothieno[2′,3′:2,3]-5α-androstan-4′-carbonitrile (**2**)*

⪾⪾To equimolar amounts of 17*β*-acetoxy-5α-androstan-3-one **(1)** (0.66 g, 0.002 mol), elemental sulfur (0.064 g, 0.002 mol) and malononitrile (0.132 g, 0.002 mol) in absolute ethanol (30 ml), a catalytic amount of triethylamine (0.3 ml) was added. The reaction mixture was heated under reflux for 3 h, then left to cool at room temperature. The formed solid product was collected by filtration and crystallized from ethanol to yield 0.67 g (82%), yellow crystals, m.p. 212–214 °C; $C_{24}H_{32}N_2O_2S$ (412.620). IR (*υ*/cm^{-1}): 3355 (NH_2), 2965, 2850 (CH_3, CH_2), 2225(CN), 1735 (acetate C=O); ^1H NMR (δ ppm): 0.73 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 2.15 (s, 3H, CH_3COO), 3.45–3.65 (m, 1H, C_5-αH), 3.72 (t, 1H, C_{17}-αH), 5.76 (s, 2H, NH_2, D_2O-exchangeable); MS (EI): *m*/*z* (%): 412 (M^+, 58%), 352 (M^+ –CH_3COOH, 25%), 150 (100%), 48.5 (36%).

*3.1.2. 17β-Acetoxy-6″-amino-1″-phenyl-2″-thioxo-pyrimidino[5″,4″:4′,5′]thieno[2′,3′:2,3]-5α-androstane (**3**)*

A mixture of compound **2** (0.82 g, 0.002 mol) and phenylisothiocyanate (0.27 g, 0.002 mol) in pyridine (20 ml) was heated under reflux for 2 h. The reaction mixture was left to cool at room temperature, then poured onto ice/water mixture and neutralized with dilute HCl. The solid product was collected by filtration and crystallized from ethanol to yield 0.84 g (77%), brown crystals, m.p. 233–234 °C; $C_{31}H_{37}N_3O_2S_2$ (547.793); IR (*υ*/cm^{-1}): 3347 (NH_2), 3050 (CH-aromatic), 2970, 2845 (CH_3, CH_2), 1730 (acetate C=O); ^1H NMR (δ ppm): 0.77 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 2.18 (s, 3H, CH_3COO), 3.65 (t, 1H, C_{17}-αH), 5.87 (s, 2H, NH_2, D_2O-exchangeable), 7.37–7.78 (m, 5H, C_6H_5); MS (EI): *m*/*z* (%): 547 (M^+, 45%), 487 (M^+ –CH_3COOH, 35%), 77(C_6H_5, 62%).

*3.1.3. 17β-Acetoxy-6″-amino-1″H-2″-oxopyrimidino-[5″,4″:4′,5′]thieno[2′,3′:2,3]-5α-androstane (**4a**), 17β-acetoxy-6″-amino-1″H-2″-thioxopyrimidino-[5″,4″:4′,5′]thieno[2′,3′:2,3]-5α-androstane (**4b**)*

General procedure. To a suspension of compound **2** (0.82 g, 0.002 mol) in sodium ethoxide (0.01 mol) [prepared by dissolving sodium metal (0.23 g, 0.01 mol) in absolute ethanol (40 ml)], either urea (0.12 g, 0.002 mol) or thiourea (0.15 g, 0.002 mol) was added. The reaction mixture, in each case, was heated in a boiling water bath for 5 h, then poured into ice/water mixture and neutralized with dilute hydrochloric acid. The solid product, in each case, collected by filtration, dried and crystallized from the appropriate solvent.

Compound 4a: Pale brown crystals from methanol, yield 0.59 g (65%), m.p. 193–194 °C; $C_{25}H_{33}N_3O_3S$ (455.623); IR (*υ*/cm^{-1}): 3454–3350 (NH, NH_2), 2967, 2845 (CH_3, CH_2), 1732 (acetate C=O), 1695 (C=O); ^1H NMR (δ ppm): 0.74 (s, 3H, 18-CH_3), 1.05 (s, 3H, 19-CH_3), 2.13 (s, 3H, CH_3COO), 3.82 (t, 1H, C_{17}-αH), 6.90 (s, 2H, NH_2, D_2O-exchangeable), 10.24 (br s, 1H, NH, D_2O-exchangeable); MS (EI): *m*/*z* (%): 455 (M^+, 30%), 395 (M^+ –CH_3COOH, 45%).

Compound 4b: Brown powder from ethanol, yield 0.67 g (72%), m.p. 211–213 °C; $C_{25}H_{33}N_3O_2S_2$ (471.693); IR (*υ*/cm^{-1}): 3504–3367 (NH, NH_2), 2975, 2848 (CH_3, CH_2), 1730 (acetate C=O), 1385 (C=S); ^1H NMR (δ ppm): 0.78 (s, 3H, 18-CH_3), 1.03 (s, 3H, 19-CH_3), 2.21 (s, 3H, CH_3COO), 3.70 (t, 1H, C_{17}-αH), 6.24 (s, 2H, NH_2, D_2O-exchangeable), 10.18 (br s, 1H, NH, D_2O-exchangeable); MS (*m*/*z*,%): 471 (M^+, 55%), 411 (M^+ –CH_3COOH, 24%).

*3.1.4. 17β-Acetoxy-6″-aminopyrimidino[5″,4″:4′,5′]-thieno[2′,3′:2,3]-5α-androstane (**5**)*

A mixture of compound **2** (0.82 g, 0.002 mol) and formamide (0.09 g, 0.002 mol) was heated for 5 h under reflux on an oil bath at 150 °C, after cooling at room temperature the reaction mixture was poured on ice. The solid product thus formed was collected by filtration, washed with distillated water several times and crystallized from DMF/EtOH mixture (1:2) to yield 0.68 g (78%), yellow crystals, m.p. 256–257 °C; $C_{25}H_{33}N_3O_2S$ (439.623); IR (*υ*/cm^{-1}): 3362 (NH_2), 2973, 2845 (CH_3, CH_2), 1728 (acetate C=O); ^1H NMR (δ ppm): 0.76 (s, 3H, 18-CH_3), 0.98 (s, 3H, 19-CH_3), 2.23 (s, 3H, CH_3COO), 3.85 (t, 1H, C_{17}-αH), 6.27 (s, 2H, NH_2, D_2O-exchangeable), 8.47 (s, 1H, pyrimidine H-2); ^{13}C NMR: δ = 14.6 (Me-18), 19.7 (Me-19), 20.8 (C-11), 22.3 (*C*H_3CO),

30.5 (C-12), 30.9 (C-8), 31.8 (C-7), 32.9 (C-15), 33.2 (C-15), 36.7 (C-10), 49.4 (C-13), 50.3 (C-14), 50.6 (C-9), 73.7 (C-17), 83.5 (C-5), 121.8 (C-6), 127.9 (C-3), 129.7 (C-5″), 135.4 (C-4), 136.2 (C-1), 157.1 (C-2″), 165.4 (C-4″), 167.6 (C-2), 169.3 (C-6″), 171.8 (CH_3CO); MS (EI): *m/z* (%): 440 (MH^+, 52%), 379 (M^+ –CH_3COOH, 39%).

*3.1.5. 3β-Acetoxy-17-(2′-benzoylacetonitrile-2′-ylideno)-5α-androstane (**8**)*

3*β*-Acetoxy-5α-androstan-17-one **6** (0.66 g, 0.002 mol) was dissolved in absolute ethanol (20 ml) and benzoyl acetonitrile **7** (0.29 g, 0.002 mol) was added. The reaction mixture was boiled under reflux for 3 h until all starting materials had disappeared as indicated by TLC. Then the reaction mixture was concentrated under *vacuum*, whereby the resulted oily product was triturated with ethyl acetate. The formed solid product was filtered off, dried and crystallized from dioxane to yield 0.69 g (76%), yellowish white crystals, m.p. 198–199 °C; $C_{30}H_{37}NO_3$ (459.633); IR (υ/cm^{-1}): 2978, 2843 (CH_3, CH_2), 2225 (CN), 1738 (acetate C=O), 1705 (C=O); ^{1}H NMR (δ ppm): 0.83 (s, 3H, 18-CH_3), 1.02 (s, 3H, 19-CH_3), 2.25 (s, 3H, CH_3COO), 4.72–4.80 (m, 1H, C_3-αH), 7.25–7.48 (m, 5H, C_6H_5); MS (EI): *m/z* (%): 459 (M^+, 34%), 399 (M^+ –CH_3COOH, 44%), 354 (M^+ –C_6H_5CO, 54%), 105 (C_6H_5CO, 72%).

*3.1.6. 3β-Acetoxy-17-(5′-amino-3′-phenylpyrazol-4′-ylideno)-5α-androstane (**9a**), 3β-acetoxy-17-(5′-amino-1′,3′-diphenylpyrazol-4′-ylideno)-5α-androstane (**9b**)*

General procedure. To a solution of compound **8** (2.29 g, 0.005 mol) in ethanol (40 ml) containing few drops of piperidine, equivalent amount of either hydrazine hydrate (0.25 g, 0.005 mol) or phenylhydrazine (0.51 g, 0.005 mol) was added. The reaction mixture in each case was refluxed for 4 h, concentrated under *vacuum*, triturated with ice/water mixture and then neutralized with few drops of dilute HCl. The solid product thus formed in each case was filtered off, dried and crystallized from the appropriate solvent.

Compound 9a: Yellow crystals from ethanol, yield 1.84 g (78%), m.p. 253–255 °C; $C_{30}H_{40}N_3O_2$ (474.673); IR (υ/cm^{-1}): 3450–3340 (NH, NH_2), 3030 (CH-aromatic), 2975, 2855 (CH_3, CH_2), 1730 (acetate C=O); ^{1}H NMR (δ ppm): 0.84 (s, 3H, 18-CH_3), 1.07 (s, 3H, 19-CH_3), 2.13 (s, 3H, CH_3COO), 4.62–4.75 (m, 1H, C_3-αH), 6.48 (s, 2H, NH_2, D_2O-exchangeable), 7.25–7.53 (m, 5H, C_6H_5), 9.74 (s, 1H, NH, D_2O-exchangeable); MS (EI): *m/z* (%): 474 (M^+, 43%), 414 (M^+ –CH_3COOH, 25%), 397 (M^+ –C_6H_5, 45%), 77 (C_6H_5, 74%).

Compound 9b: Pale brown crystals from ethanol, yield 2.06 g (75%), m.p. 221–222 °C; $C_{36}H_{44}N_3O_2$ (550.764); IR (υ/cm^{-1}): 3347 (NH_2), 3030 (CH-aromatic), 2968, 2755 (CH_3, CH_2), 1727 (acetate C=O), 1605 (C=C); ^{1}H NMR (δ ppm): 0.82 (s, 3H, 18-CH_3), 0.97 (s, 3H, 19-CH_3), 2.26 (s, 3H, CH_3COO), 4.70–4.83 (m, 1H, C_3-αH), 5.94 (s, 2H, NH_2, D_2O-exchangeable), 7.22–7.65 (m, 10H, $2C_6H_5$); MS (EI): *m/z* (%): 551 (M^+ +1, 35%), 490 (M^+ –CH_3COOH, 24%), 77 (C_6H_5, 65%).

*3.1.7. 3β-Acetoxy-17-(4′-amino-2′-oxo-6′-phenylpyrimidin-5′-ylideno)-5α-androstane (**10a**), 3β-acetoxy-17-(4′-amino-2′-thioxo-6′-phenylpyrimidin-5′-ylideno)-5α-androstane (**10b**)*

General procedure. Either urea (0.30 g, 0.005 mol) or thiourea (0.38 g, 0.005 mol) was added to a suspension of compound **8** (2.29 g, 0.005 mol) in sodium ethoxide (0.01 mol) [prepared by dissolving sodium metal (0.23 g, 0.01 mol) in absolute ethanol (40 ml)]. The reaction mixture, in each case, was heated in a boiling water bath for 3 h, then poured into ice/water mixture and neutralized with dilute HCl. The formed solid product, in each case, was collected by filtration, dried and crystallized from the appropriate solvent.

Compound 10a: Pale yellow crystals from dioxane, yield 1.72 g (69%), m.p. 266–267 °C; $C_{31}H_{39}N_3O_3$ (501.673); IR (υ/cm^{-1}): 3350 (NH_2), 3025 (CH-aromatic), 2968, 2857 (CH_3, CH_2), 1725 (acetate C=O), 1695 (C=O); ^{1}H NMR (δ ppm): 0.79 (s, 3H, 18-CH_3), 1.02 (s, 3H, 19-CH_3), 2.23 (s, 3H, CH_3COO), 4.68–4.84 (m, 1H, C_3-αH), 6.27 (s, 2H, NH_2, D_2O-exchangeable), 7.32–7.70 (m, 5H, C_6H_5); MS (EI): *m/z* (%): 501 (M^+, 24%), 424 (M^+ –C_6H_5, 55%), 77 (C_6H_5, 72%).

Compound 10b: Pale brown powder from ethanol, yield 1.73 g (67%), m.p. 232–233 °C; $C_{31}H_{39}N_3O_2S$ (517.743); IR (υ/cm^{-1}): 3347 (NH_2), 3028 (CH-aromatic), 2968, 2757 (CH_3, CH_2), 1720 (acetate C=O), 1380 (C=S); ^{1}H NMR (δ ppm): 0.80 (s, 3H, 18-CH_3), 0.98 (s, 3H, 19-CH_3), 2.18 (s, 3H, CH_3COO), 4.72–4.81 (m, 1H, C_3-αH), 6.08 (s, 2H, NH_2, D_2O-exchangeable), 7.29–7.63 (m, 5H, C_6H_5); MS (EI): *m/z* (%): 517 (M^+, 28%), 457 (M^+ –CH_3COOH, 24%), 77 (C_6H_5, 56%).

*3.1.8. 3β-Acetoxy-17-(4′-amino-2′-imino-6′-phenylpyrimidin-5′-ylideno)-5α-androstane (**10c**)*

A mixture of compound **8** (0.92 g, 0.002 mol), guanidine hydrochloride (0.2 g, 0.002 mol) and sodium acetate (0.16 g, 0.002 mol) in ethanol (30 ml) was refluxed for 7 h. The reaction mixture was concentrated under *vacuum* and the residue poured onto ice/water mixture. The solid product was filtered off, washed with water dried and crystallized from methanol to yield 0.82 g (82%) of compound **10c** as colorless crystals, m.p. 179–180 °C; $C_{31}H_{40}N_4O_2$ (500.683); IR (υ/cm^{-1}): 3570–3435 (NH, NH_2), 3035 (CH-aromatic), 2985, 2864 (CH_3, CH_2), 1735 (acetate C=O), 1645 (C=N), 1595 (C=C); ^{1}H NMR (δ ppm): 0.85 (s, 3H, 18-CH_3), 1.12 (s, 3H, 19-CH_3), 2.15 (s, 3H, CH_3COO), 5.56 (s, 2H, NH_2, D_2O-exchangeable), 4.78–4.95 (m, 1H, C_3-αH), 9.32 (br s, 1H, NH, D_2O-exchangeable); ^{13}C NMR: δ = 14.3 (Me-18), 19.3 (Me-19), 20.3 (C-11), 21.4 (CH_3CO), 27.7 (C-2), 30.8 (C-12), 30.9 (C-8), 31.8 (C-7), 32.9 (C-15), 36.7 (C-10), 36.8 (C-1), 38.0 (C-4), 49.7 (C-13), 50.1 (C-14), 50.1 (C-9), 73.7 (C-3), 82.5 (C-5), 121.8 (C-6), 128.4, 128.7, 130.0, 136.6 (Ph), 137.4 (C-16), 140.0 (C-5), 149.9 (C-5′), 165.5 (C-6′), 169.8 (C-4′), 168.1 (C-2′), 170.5 (CH_3CO), 205.0 (C-17); MS (EI): *m/z* (%): 500 (M^+, 28%), 540 (M^+ –CH_3COOH, 54%), 77 (C_6H_5, 76%).

3.2. Bioassay

3.2.1. Experimental design

Eighty male Sprague Dawley rats weighing 120–150 g obtained from the Animal House at National Research Center were used. The animals were housed under standard conditions (12 h light/dark cycles with room temperature of 21–25 °C). Animals were fed with standard diet consisting of casein 10%, salts mixture 4%, vitamins mixture 1%, corn oil 10% and cellulose 5% completed to 100 g with corn starch [68].

After 7 d of acclimation, the animals were divided into 10 groups (eight rats per group). Control group was fed with the standard diet. The second, the third, the fourth and the fifth groups were fed with the standard diet and given one of the compounds **2**, **5**, **10c** and **9b**, respectively, as an oral dose of 5 mg kg^{-1} B.W. per day [69] to confirm the safety of these novel aminosteroidal heterocycles. The sixth group was fed with the same diet with an additional 3000 mg iron (as ferrous sulfate, purchased from Sigma, St. Louis, MO) per kg diet [70]. The seventh, the eighth, the ninth and the 10th groups were fed with the iron diet and received one of compounds **2**, **5**, **10c** and **9b**, as an oral dose of 5 mg kg^{-1} B.W. per day, respectively. The tested compounds were dissolved in 3% Tween. Animals have free access to these diets and tap water for 6 weeks.

3.2.2. Blood sampling

At the end of the experimental period, all animals were fasted for 12 h, and blood was withdrawn from the retro-orbital plexus [71] under diethyl ether anesthesia. The blood samples were collected into heparin-containing and trace element-free vacutainers placed on ice, centrifuged at 3000 × *g* for 10 min at 4 °C. Plasma was separated from all the blood samples and stored at –70 °C for future analysis. The buffy coat was carefully isolated from each blood sample using fine Pasteur pipette. Then the packed erythrocytes from each sample were divided into two equal portions, the first portion (1 ml) was used for the extraction of membrane lipids and the determination of lipid peroxidation, while the second portion (1 ml) was used for the preparation and isolation of ghost cells for protein and ATPases determination.

3.2.3. Methods

3.2.3.1. Extraction of erythrocyte membrane lipids. Total lipids in the red cell membrane were extracted with chloroform: methanol method [72], modified from the method of Bligh and Dyer [73]. The obtained dry lipid extract was dissolved in 2 ml chloroform and stored at –20 °C until used.

3.2.3.2. Preparation and isolation of ghost cells. The method used for erythrocyte ghost preparation is based on the hemolysis of RBCs in hypotonic phosphate buffer pH 7.4 for removal of hemoglobin according to the method described by Dodge et al. [74]. Ghost preparation was performed at 4 °C and the isolated ghost cells were stored at –20 °C until analysis could be completed.

3.2.3.3. Biochemical analysis. Iron concentration and TIBC were determined in plasma by spectrophotometric analysis [75] using commercially available kits. Plasma ferritin level was estimated using a microplate solid phase enzyme immunoassay kit from Biochem ImmunoSystems Co. (Italy) according to the method of Engvall [76]. Quantitative determination of tumor necrosis factor alpha (TNF-α) was done by enzyme linked immunosorbent assay (ELISA) according to the method of Corti et al. [77] using a kit produced by Diaclone Research, France. Plasma nitrate concentration as a stable end product of nitric oxide was estimated by the Griess reaction after quantitative conversion of nitrate to nitrite by nitrate reductase according to the method of Moshage et al. [78] using R&D system GmbH (Germany). Plasma and erythrocyte membrane lipid profiles were determined by assessing total cholesterol [79], phospholipids [80] and total lipids [81] using commercial available kits. Plasma and erythrocyte membrane triglyceride levels were estimated by enzymatic colorimetric procedure [82] using Sentinel CH kits (Italy). Measurement of plasma leptin level was carried out by the method described by Friedman and Halaas [83] using kit produced by BioSource Europe S.A. Plasma and erythrocyte membrane lipid peroxidation (measured by thiobarbituric acid test) was estimated spectrophotometrically according to the method described by Sharma and Wadhwa [84]. Erythrocyte membrane total protein was determined by using the Folin phenol reagent according to Lowry et al. [85] method. Protein fractions of the erythrocyte membrane were separated according to their respective electrical charges at pH 8.8 on a cellulose acetate plate using electrophoresis technique described by Alper [86]. The relative percent of each band is calculated by the following formula:

$$\frac{\text{Number } \textit{of integration units of the band}}{\text{Total } \textit{integartion units}} \times 100 = \text{Relative percent of the band}$$

$$\frac{\text{Relative } \textit{percent of the band}}{\text{Value } \textit{of protein in each band}} \times \text{Total protein} = \text{Absolute}$$

Erythrocyte membrane ATPases were determined according to the method of Post et al. [87]. Enzyme activity is measured as the amount of inorganic phosphate (Pi) released from ATP in the presence of (Ca–Mg ATPase) or absence of (Na–K ATPase) ouabain in lyzed erythrocyte. ATPase activity was expressed as micromole Pi mg^{-1} protein per hour.

3.2.4. Statistical analysis

The statistical analysis was used to evaluate all the above determinations including the calculation of the mean value, standard deviation and standard error. All values were

expressed as mean ± S.E. and the statistical differences were assessed by using Student's two tailed-*t*-tests [88]. A probability value $P \leq 0.05$ was considered to be statistically significant.

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