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Synthesis and Antiproliferative Activity of Some Androstene Oximes and Their *O*-Alkylated Derivatives

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In order to study the structure–activity relationship with respect to the cytotoxicity of steroidal oximes, several 6*E*-hydroximino-4-ene steroids and their *O*-alkylated derivatives were synthesized. The oxime ethers were solidified and purified by preparing their corresponding oxalate salts. The new derivatives as well as some previously synthesized ones were evaluated for *in vitro* antineoplastic activity against a panel of 60 cancer cell lines at 10 μM. The oximes and oxime ethers were found to have moderate to good antiproliferative activity against various leukemia, colon, melanoma, and renal cancer cell lines.

Keywords: Antileukemic activity / Cytotoxic steroids / Oxime ethers / Steroidal oximes

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Introduction

The fact that the available cytotoxic cancer chemotherapeutic agents worsen the quality of life has driven the quest to find a safer and target-oriented anticancer agent [1]. However, most of the FDA-approved drugs for the clinical use are either analogs of the older cytotoxic drugs or reintroduced after the patent expiry, indicating their importance either alone or in combination with newer innovative agents for cancer therapy [1]. Several steroids, which are in clinical use to treat the complications associated with malignancy, include prednisone, cyproterone acetate, and fluoxymesterone [2–4]. Inhibition of specific enzymes like aromatase and 5α-reductase are the frontline treatment approaches for breast and prostate carcinoma [5, 6]. Extensive chemical modifications of the steroid skeleton have been carried out to synthesize many potent antineoplastic agents, which could target hormone-dependent cancers like prostate (e.g., finasteride, dutasteride), breast (e.g., exemestane), and uterine carcinomas [7–9].

Literature reports indicate prominent cytotoxicity of steroidal oximes and their *O*-alkylated derivatives on various cancer cell lines [10, 11]. It has been observed that varying the position of the hydroximino group on the parental androsterone skeleton leads to remarkable changes in the antineoplastic activity [10]. Steroidal oximes of natural origin have also emerged as a new perspective of cytotoxic agents in the recent past. Two steroidal oximes, (6*E*)-hydroximinocholest-4-en-3-one and its 24-ethyl analog (Fig. 1), isolated from marine sponges *Cinachyrella alloclada* and *C. apion* [12], have shown good antiproliferative activity against several types of cancer cells [13]. Several endocrine active antitumor steroidal oximes like aromatase or 5α-reductase inhibitors are also reported in the literature [14, 15].

In continuation with our earlier similar studies [10, 11], wherein synthesis of a variety of oximes and their etheral derivatives have been reported as potent cytotoxic agents, it was envisaged to synthesize ether derivatives of 6*E*-hydroximino steroid. Impact of substituting 2-alkylaminoethyl side chain on antiproliferative activity of the parent 6*E*-hydroximinoandrostene framework was also observed. To study the effect of the location of the oxime on the steroid skeleton and establish the structure–activity relationship of steroidal oximes, the hydroximino androstene derivatives with oxime positioned at C_3 (**13**) and C_7 (**14**) of steroid (Fig. 2) were also evaluated for antineoplastic activity.

Results and discussion

Chemistry

Synthesis of the steroidal oxime **8**, 6*E*-hydroximino-androst-4-ene-3,17-dione, starting from dehydroepiandrosterone acetate was achieved based on the methodology available in the

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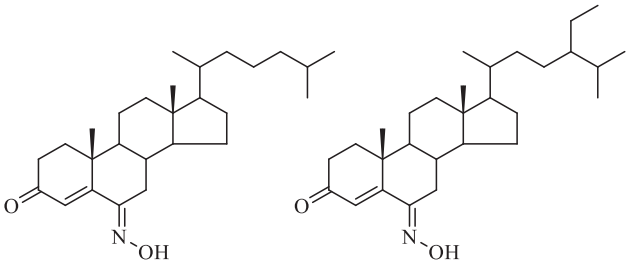


Figure 1. Structures of (6*E*)-hydroximino cholestene derivatives isolated from marine sponges [12, 13].

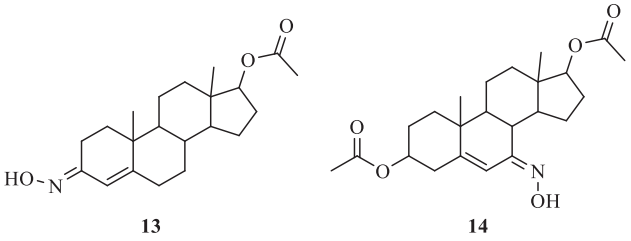


Figure 2. Structures of C_3 and C_7 hydroximino androstene derivatives [10, 11].

literature [13, 16, 17] with some suitable modifications as shown in Scheme 1. The $NaBH_4$ reduction of the C_{17} keto functionality of dehydroepiandrosterone acetate led to the formation of reduced steroid 3-acetoxy-17-hydroxy-androst-5-ene. However, this also resulted in simultaneous hydrolysis of the C_3 acetate group leading to the formation of an 80:20 mixture of 3-acetoxy-17-hydroxy and 3,17-dihydroxy steroid represented by **1** as observed from ^{1}H NMR data. Splitted multiplets were observed for 3α-*H* in the ^{1}H NMR spectrum at δ 4.55–4.64 and 3.49–3.54 ppm, which together (area ratio 80:20) integrated for one proton. The reduced mixture of steroids was subjected to acetylation using acetic anhydride and pyridine [17], which yielded 3β,17β-diacetyl derivative **2**.

The diacetyl product **2** on epoxidation with *m*-chloroperbenzoic acid in ice-cold chloroform afforded a mixture of α/β-isomers of 3β,17β-diacetoxy-5,6-epoxy-androstane (**3**) consistent with literature reports [16, 18]. The isomeric mixture **3** on oxidation with CrO_3 was converted to 3β,17β-diacetoxy-5α-hydroxy-androstan-6-one (**4**), which, on further treatment with $SOCl_2$ and pyridine, dehydrated to 3β,17β-diacetoxy-androst-4-en-6-one (**5**) [16, 19]. Oximation of compound **5** with $NH_2OH{\cdot}HCl$ in the presence of CH_3COONa yielded 3β,17β-diacetoxy-6*E*-hydroximino-androst-4-ene (**6**). The 7β-*H* proton resonated downfield as a double doublet at δ 3.34 ppm ($J_{trans} = 15.16$ Hz, $J_{gem} = 4.64$ Hz), confirming the steroidal oxime **6** being the *E*-isomer consistent with earlier reports [13].

To obtain compounds with higher degree of oxidation in ring A, diacetoxy 6-oxime **6** was hydrolyzed using methanolic KOH to yield 3β,17β-dihydroxy-6*E*-hydroximino-androst-4-ene (**7**), which was further oxidized with CrO_3/pyridine complex to obtain 3,17-dioxo-6*E*-hydroximino-androst-4-ene (**8**) [16].

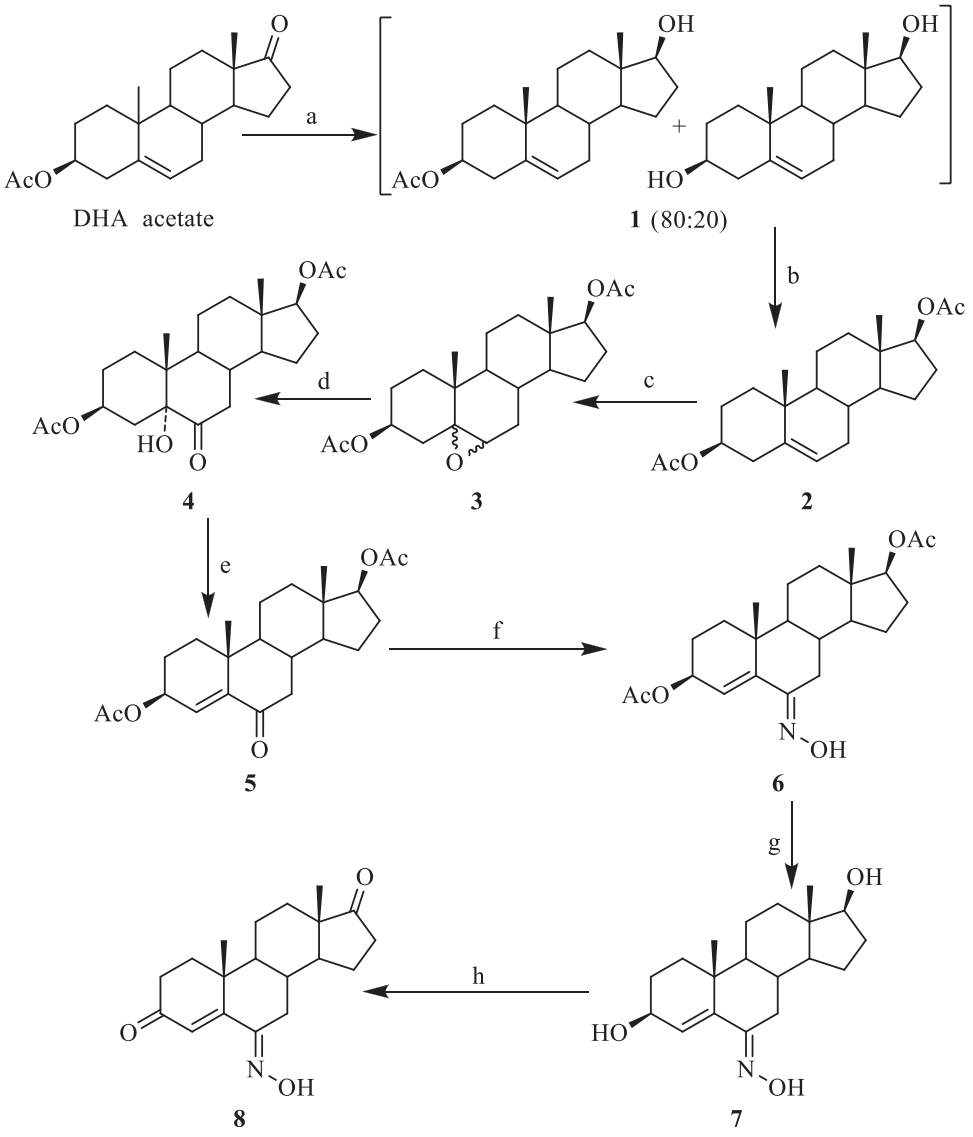
O-Alkylation of 6*E*-hydroximino-androst-4-en-3,17-dione (**8**) with hydrochlorides of 2-chloroethylmorpholine and 2-diethylaminoethyl chloride in the presence of K_2CO_3 and KOH in ethylmethyl ketone afforded a sticky mass of **9** and **11**, respectively (Scheme 2). Attempts to crystallize the dried mass using various solvents remained unsuccessful. Hence, it was decided to prepare the salt of the steroidal oxime ether to obtain solid product. Oxalate salt was prepared by adding a saturated ethereal solution of oxalic acid to a solution of the steroidal oxime ethers in dry solvent ether to obtain the corresponding solid product of 6*E*-(2-morpholinoethoxy-imino)-androsta-4-ene-3,17-dione oxalate dihydrate (**10**) and 6*E*-(2-(diethylamino)ethoxyimino)-androst-4-ene-3,17-dione oxalate dihydrate (**12**).

The broad singlet for $-\overset{\oplus}{N}-(CH_2)_2-$ of morpholine and multiplet of $-CH_2\overset{\oplus}{N}<$ appeared downfield at δ 2.98 and 3.16 ppm, respectively, in the ^{1}H NMR spectrum of **10** due to presence of quaternary nitrogen. Similarly, the multiplets for protons adjacent to the quaternary nitrogen of the oxalate salt **12** appeared downfield at δ 3.08 ($-\overset{\oplus}{N}-(CH_2CH_3)_2$) and 3.31 ppm ($-CH_2-\overset{\oplus}{N}$) in comparison to its tertiary nitrogen steroidal substrate **11** in the proton NMR spectrum. Similarly, the broad singlet for the $-OCH_2-$ of the aminoethyl side chain and singlet for 4-C*H* vinylic proton were observed downfield at $\delta \sim 4.39$ and ~ 5.99 ppm, respectively, for the oxalate dihydrate salts **10** and **12**. The quaternization of the oxime ether nitrogen and formation of the oxalate dihydrate salt was further confirmed by the appearance of the molecular ion [M^+] peaks at 555.4 and 541.3 in the mass spectra of the oxalate salts **10** and **12**.

The C_3 and C_7 hydroximino androstene derivatives **13** and **14** were synthesized according to the literature methods [10, 11].

Antineoplastic activity

The newly synthesized 6*E*-oxime steroidal derivatives **6–8**, **10**, and **12** and previously reported steroidal oximes **13** and **14** were submitted to NCI, Bethesda, USA, for anticancer screening. Out of these, compounds **6–8** and **12–14** were selected for the preliminary *in vitro* anticancer screening. The screening begins with the evaluation of selected compounds at a single dose of 10 μM against the 60 cell lines, which include cells from eight melanomas, six leukemias, eight breast cancers, two prostate, nine lung, seven colon, six ovary, eight kidney, and six central nervous system cancers. A 48-h continuous drug exposure protocol was used, and a sulforhodamine B (SRB) protein assay was used to estimate

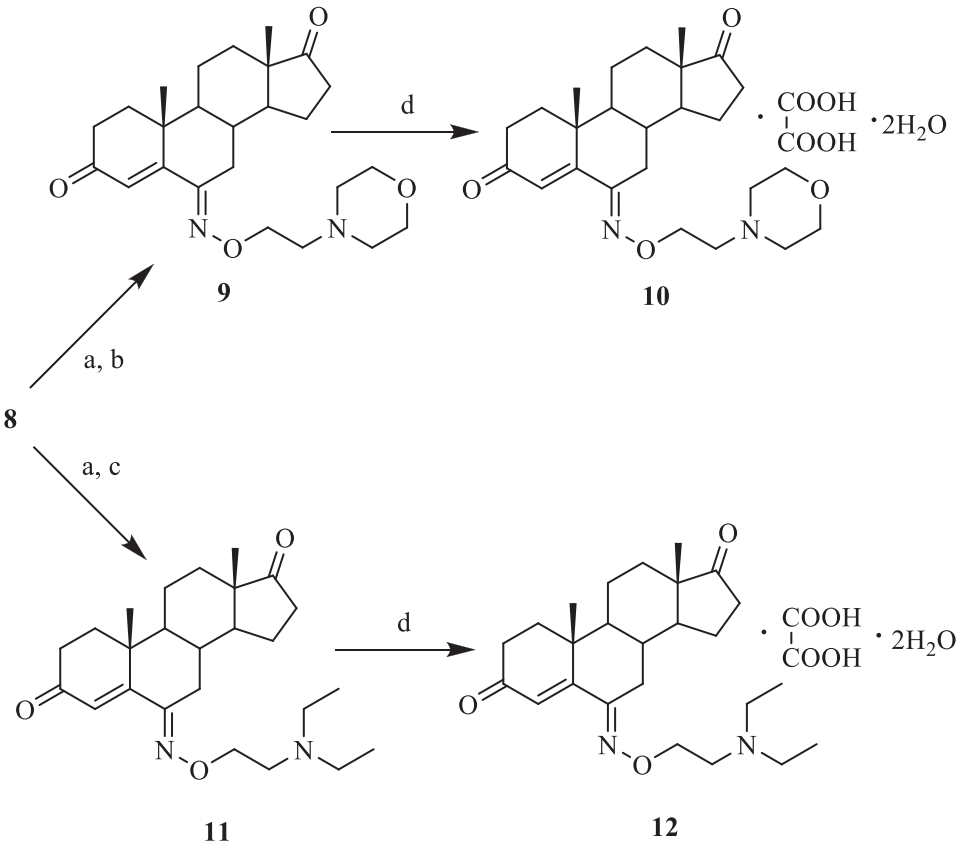


Scheme 1. Reagents and reaction conditions: (a) $NaBH_4$/MeOH, stirring, rt, 4 h, (b) Ac_2O/pyridine, 100°C, 2 h, (c) MCPBA, 0°C, stirring, 12 h, (d) CrO_3/H_2O/ethylmethyl ketone, stirring, 0°C, 1 h, (e) $SOCl_2$/pyridine, stirring, 0°C, 1 h, (f) $NH_2OH \cdot HCl/CH_3COONa$, stirring, rt, overnight, (g) KOH/MeOH, reflux, 1 h, and (h) CrO_3/pyridine, stirring, 1 h.

the cell viability or growth [20]. The percentage growth of some selected cancer cell lines treated with various steroidal derivatives at 10 μM is summarized in Table 1.

C_3-substituted (**13**) and C_7-substituted (**14**) androstene oximes displayed considerable inhibitory effect on the entire panel of 60-cell lines at 10 μM, being more pronounced on leukemia cell lines. They exhibited significant growth reduction against five leukemia cell lines at 10 μM with mean growth percentage of 53.52 and 57.41, respectively, but C_6-substituted androstene oximes **6**–**8** produced negligible antiproliferative effects in comparison to the standard drug adriyamycin, which has a pronounced cytotoxic effect on these cell lines. This is also in sharp contrast to earlier reports indicating the good antiproliferative activity of (6*E*)-hydroximino cholestane analogs, isolated from marine sponges, against several types of cancer cells [13].

Although steroidal 6*E*-oximes of the current androstene series possess all important structural features such as elevated oxidation in ring A and presence of double bond at $C_{4,5}$ required for the biological activity [13], the compounds are almost inactive, suggesting that cholesterol type steroidal skeleton not only is playing the major role, but might be an essential factor for biological activity of 6*E*-oximes.



Scheme 2. Reagents and reaction conditions: (a) K_2CO_3/KOH/ethylmethyl ketone, (b) 2-chloroethylmorpholine · HCl, reflux, 18 h, (c) 2-diethylaminoethyl chloride · HCl, reflux, 18 h, and (d) dry ether, oxalic acid, stirring.

The oxime ether **12** at 10 μM has shown moderate activity against HT29 (colon cancer) and UO-31 (renal cancer) cell lines with percentage growth inhibition of 57.41% and 64.48%, respectively. The oxime ethers **10** and **12** (as these were not screened by NCI against leukemia cell lines) were tested for cytotoxicity against the K562 leukemia cell line at the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India. The typical SRB assay

Table 1. Percentage growth of some selected cancer cell lines treated with various steroidal derivatives at 10 μM.

	Percentage growth						
Cell lines	**6**	**7**	**8**	**12**	**13**	**14**	**Adriyamycin**
Leukemia							
HL-60 (TB)	99.17	–	93.47	–	23.05	67.11	−21.8
K562	87.33	–	85.26	–	45.90	10.07	2.1
MOLT-4	92.52	–	91.99	–	72.61	50.55	−12.0
RPMI-8226	99.23	–	101.67	–	89.35	75.01	−17.4
SR	92.76	–	80.61	–	36.72	29.88	−5
Colon cancer							
HT29	102.89	98.04	99.80	57.41	76.04	88.60	4.7
Melanoma							
MDA-MB-435	107.86	90.72	102.07	88.48	20.33	46.36	−83.9
Renal cancer							
A498	53.29	–	73.14	–	53.11	72.87	–
UO-31	73.86	66.19	76.30	64.48	65.11	72.59	4.0

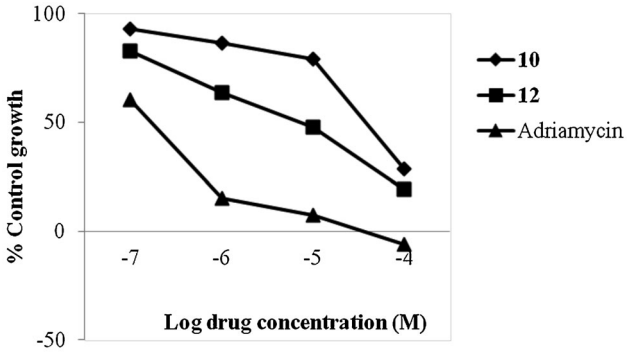


Figure 3. Dose–response curves of compounds **10**, **12**, and adriamycin depicting percentage growth of K562 human leukemia cell line at various concentrations.

protocol was used for the *in vitro* testing of each compound at four dose levels (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M). Each experiment was repeated thrice along with appropriate negative controls and the standard drug adriamycin. Dose–response curve of compounds **10** and **12** depicting percentage growth of K562 human leukemia cell line at various concentrations is shown in Fig. 3. Various dose–response parameters such as GI_{50} (drug concentration resulting in a 50% reduction in the net protein increase), total growth inhibition (drug concentration of TGI), and LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) of compounds **10** and **12** against K562 cell line are summarized in Table 2. The compounds **10** and **12** were found to be moderately active with $GI_{50} = 65.0$ and 35.7 μM, respectively, in comparison to the standard drug adriyamycin ($GI_{50} \leq 1$ μM).

The results imply that the decreased activity of 6*E*-oximes in androstene series may be attributed to missing cholesteryl side chain. The *O*-alkylation of 6*E*-androstene oxime **8** (Scheme 1) marginally improved the antiproliferative potential but was insignificant in comparison to 3-substituted (mean $GI_{50} = \sim 5$ μM) and 17-substituted (mean $GI_{50} = \sim 2.5$ μM) oxime ethers that we reported previously [10]. However, the results obtained in 6*E*-oxime ether series (Scheme 2) are similar to those obtained in 7-substituted steroidal oxime ethers [11]. It seems that *O*-alkylation of various steroidal oximes results in increased antiproliferative activity; however, the location of the oximino moiety on the steroid skeleton is very important. C_3 and C_{17} positions at the far end of the steroidal core are the preferred sites, whereas C_6 and C_7 positions well within the nucleus are less preferred ones for such substitutions.

Table 2. Dose–response parameters (GI_{50}, TGI, and LC_{50}) of compounds **10** and **12** against the K562 cell line.

	Dose–response parameters (μM)		
Compound	**GI_{50}**	**LC_{50}**	**TGI**
10	65.0	>100	>100
12	35.7	>100	>100
Adriamycin	<1	>100	79.1

Conclusions

A new series of hydroximino-4-ene steroids and their *O*-alkylated derivatives were synthesized and studied for antiproliferative potential. It is concluded that steroidal oximes and their ethereal derivatives display potent cytotoxic effects, *O*-alkylation generally results in increased antiproliferative activity. However, the location of the oximino moiety on the steroid skeleton is very significant for these effects.

Experimental

Chemistry

Melting points were determined on a Veego melting point apparatus and are uncorrected. Infrared (wavenumbers in cm^{-1}) spectra were recorded on Perkin-Elmer RX1 FTIR spectrophotometer model using potassium bromide pellets. ^{1}H NMR spectra were obtained on a Bruker Avance II 400 MHz spectrometer using deuterated-chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) as solvents containing tetramethylsilane as internal standard (chemical shifts in δ, ppm). Mass spectra were obtained on an Applied Biosystems API 2000™ mass spectrophotometer. Elemental analyses were carried out on a Perkin Elmer-2400 model CHN analyzer. Plates for thin layer chromatography (TLC) were prepared with silica gel G according to method of Stahl (E. Merck) using ethyl acetate as solvent and activated at 110°C for 30 min. Iodine was used to develop the TLC plates. Anhydrous sodium sulfate was utilized as drying agent. All solvents were distilled prior to use according to standard procedures.

The compounds 3β-acetyloxy-17β-hydroxy-androst-5-ene/3β,17β-dihydroxy-androst-5-ene (**1**), 3β,17β-diacetoxy-androst-5-ene (**2**), 3β,17β-diacetoxy-5α/β,6α/β-epoxy-androstane (**3**), 3β,17β-diacetoxy-5α-hydroxy-androstan-6-one (**4**), and 3β,17β-diacetoxy-androst-4-en-6-one (**5**) were synthesized according to reported methods [16–19].

*3β,17β-Diacetoxy-6E-hydroximino-androst-4-ene (**6**)*

A solution of 3β,17β-diacetoxy-androst-4-en-6-one (**5**; 1.4 g, 3.6 mmol) in methanol (20 mL) was treated with a solution of $NH_2OH \cdot HCl$ (1.1 g, 15.7 mmol) and anhydrous CH_3COONa (1.1 g, 13.09 mmol) in 30% aqueous methanol (20 mL). The reaction mixture was stirred at room temperature overnight and concentrated under vacuum, and cold water was added. The precipitate obtained was filtered, washed, dried, and recrystallized from methanol to give compound **6** (1.2 g, 82.75%), m.p. 115–117°C. FT-IR: 3371, 2938, 1713, 1648, 1453, 1256, 1058, 949. ^{1}H NMR ($CDCl_3$): δ 0.80 (s, 3H, 18-C*H*$_3$), 1.00 (s, 3H, 19-C*H*$_3$), 2.04 (s, 3H, 17β –OCOC*H*$_3$), 2.17 (s, 3H, 3β-OCOC*H*$_3$), 3.34 (dd, 1H, 7β-*H*, $J_{trans} = 15.16$ Hz, $J_{gem} = 4.64$ Hz), 4.62 (t, 1H, 17α-*H*), 5.28 (m, 1H, 3α-*H*), 5.70 (s, 1H, 4-C*H*), 8.2 (br, 1H, =N–O*H*).

*3β,17β-Dihydroxy-6E-hydroximino-androst-4-ene (**7**)*

A solution of 3β,17β-diacetoxy-6*E*-hydroximino-androst-4-ene (**6**; 0.5 g, 1.2 mmol) and KOH (0.2 g, 3.5 mmol) in methanol (30 mL) was heated under reflux for 1 h. The reaction mixture was concentrated under vacuum, cold water added, and then neutralized with glacial CH_3COOH. The precipitate obtained was filtered, washed, dried, and recrystallized with aqueous ethanol to yield the compound **7** (0.4 g, 50.63%), m.p. 225–227°C (lit [16] 227–229°C).

*6E-Hydroximino-androst-4-ene-3,17-dione (**8**)*

A solution of compound **7** (0.12 g, 0.37 mmol) in pyridine (2 mL) was added to CrO_3–pyridine complex (0.33 g CrO_3 in 3.5 mL pyridine). The reaction mixture was stirred at room temperature for 1 h. Ethyl acetate was added and the precipitate obtained was filtered. Filtrate was washed successively with 10% HCl and 10% $NaHCO_3$, dried, evaporated, and residue recrystallized from methanol to yield compound **8** (0.08 g, 67.79%), m.p. 135–137°C (lit [16] m.p. 138–140°C).

*6E-(2-Morpholinoethoxyimino)-androst-4-ene-3,17-dione (**9**)*

A mixture of 6*E*-hydroximino-androst-4-ene-3,17-dione (**8**; 0.2 g, 0.63 mmol) and 2-chloroethylmorpholine hydrochloride (0.3 g, 2.2 mmol) in ethylmethyl ketone (50 mL) in presence of K_2CO_3 (2 g) and KOH (0.25 g) was heated under reflux for 18 h. The reaction mixture was filtered hot, the residue was washed with fresh ethylmethyl ketone and washings combined with the filtrate. The combined filtrate was vacuum evaporated to get an oily mass, which was washed several times with cold water to remove unreacted amine and dried in a desiccator over activated silica for 2 days to obtain a sticky mass of **9**. ^{1}H NMR ($CDCl_3$): δ 0.82 (s, 3H, 18-C*H*$_3$), 1.17 (s, 3H, 19-C*H*$_3$), 2.53 (s (br), 4H, −N−(C*H*$_2$)$_2$−, morpholine), 2.73 (m, 2H, −C*H*$_2$N), 3.73 (s (br), 4H, −O−(C*H*$_2$)$_2$−, morpholine), 4.30 (t, 2H, −OC*H*$_2$−), 6.17 (s, 1H, 4-C*H*).

*6E-(2-Morpholinoethoxyimino)-androst-4-ene-3,17-dione oxalate dihydrate (**10**)*

A saturated solution of oxalic acid (0.2 g) in dry ether (10 mL) was added to the solution of compound **9** in dry ether (5 mL). The oxalate salt formed immediately and was kept aside overnight. The yellowish solid obtained was filtered and washed well with dry ether to yield oxalate salt **10** (0.18 g, 52%), m.p. 152–156°C. FT-IR ν_{max} (KBr): 3408, 1732, 1632, 1455, 1379, 1223, 1133, 1036, 914, 701. ^{1}H NMR (DMSO-d_6): δ 0.83 (s, 3H, 18-C*H*$_3$), 1.10 (s, 3H, 19-C*H*$_3$), 2.98 (s (br), 4H, −N−(C*H*$_2$)$_2$−, morpholine), 3.16 (m, 2H, −C*H*$_2$−N), 3.72 (s (br), 4H, −O−(C*H*$_2$)$_2$−, morpholine), 4.38 (s (br), 2H, −OC*H*$_2$−), 5.99 (s, 1H, 4-C*H*). ESI-MS *m/z*: 555.4 [M$^+$]. Anal. calcd. for $C_{25}H_{36}N_2O_4 \cdot C_2H_2O_4 \cdot 2H_2O$: C, 58.47; H, 7.63; N, 5.05. Found: C, 58.39; H, 7.70; N, 4.49.

*6E-(2-(Diethylamino)ethoxyimino)-androst-4-ene-3,17-dione (**11**)*

A mixture of 6*E*-hydroximino-androst-4-ene-3,17-dione (**10**; 0.2 g, 0.63 mmol) and 2-diethylaminoethyl chloride hydrochloride (0.3 g, 1.7 mmol) in ethylmethyl ketone (50 mL) in presence of K_2CO_3 (2 g) and KOH (0.25 g) was heated under reflux for 18 h. The reaction mixture was filtered hot, the residue was washed with fresh ethylmethyl ketone, and washings combined with the filtrate. The combined filtrate was vacuum evaporated to get an oily mass, which was washed several times with cold water to remove unreacted amine and dried in a desiccator over activated silica for 2 days to obtain a sticky mass of **11**. ^{1}H NMR ($CDCl_3$): δ 0.82 (s, 3H, 18-C*H*$_3$), 1.11 (s, 3H, 19-C*H*$_3$), 1.18 (t, 6H, −N−(CH$_2$C*H*$_3$)$_2$), 2.65 (m, 4H, −N−(C*H*$_2$CH$_3$)$_2$), 2.98 (m, 2H, −C*H*$_2$−N−), 4.48 (s, 2H, −OC*H*$_2$−), 5.83 (s, 1H, 4-C*H*).

*6E-(2-(Diethylamino)ethoxyimino)-androst-4-ene-3,17-dione oxalate dihydrate (**12**)*

A saturated solution of oxalic acid (0.2 g) in dry ether (10 mL) was added to the solution of compound **11** in dry ether (5 mL). The oxalate salt formed immediately and was kept aside overnight. The yellowish solid obtained was filtered and washed well with dry ether to yield oxalate salt **12** (0.19 g, 55%), m.p. 148–150°C. FT-IR ν_{max} (KBr): 3429, 2927, 1736, 1663, 1459, 1227, 1023, 951. ^{1}H NMR (DMSO-d_6): δ 0.82 (s, 3H, 18-C*H*$_3$), 1.09 (s, 3H, 19-C*H*$_3$), 1.15 (t, 6H, −N−(CH$_2$C*H*$_3$)$_2$), 3.08 (m, 4H, −N−(C*H*$_2$CH$_3$)$_2$), 3.31 (m, 2H, −C*H*$_2$−N−), 4.40 (s, 2H, −OC*H*$_2$−), 5.98 (s, 1H, 4-C*H*). ESI-MS *m/z*: 541.3 [M$^+$]. Anal. calcd. for $C_{25}H_{38}N_2O_3 \cdot C_2H_2O_4 \cdot 2H_2O$: C, 59.98; H, 8.20; N, 5.18. Found: C, 60.01; H, 8.18; N, 5.09.

Antineoplastic activity

The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96-well microtiter plates in 100 μL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs.

After 24 h, two plates of each cell line were fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/mL gentamicin. Additional four, 10-fold or ½ log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations. Following drug addition, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assays were terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μL of cold 50% w/v TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. SRB solution (100 μL) at 0.4% w/v in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM Trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration,

16% TCA). Using the seven absorbance measurements [time zero (Tz), control growth (*C*), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as:

$$\left[\frac{(\mathrm{Ti}-\mathrm{Tz})}{(C-\mathrm{Tz})}\right]\times 100 \text{ for concentrations for which } \mathrm{Ti}\geq \mathrm{Tz}$$

$$\left[\frac{(\mathrm{Ti}-\mathrm{Tz})}{\mathrm{Tz}}\right]\times 100 \text{ for concentrations for which } \mathrm{Ti}< \mathrm{Tz}$$

Three dose–response parameters were calculated for each experimental agent. Growth inhibition of 50% (GI_{50}) was calculated from $[(\mathrm{Ti}-\mathrm{Tz})/(\mathrm{C}-\mathrm{Tz})]\times 100=50$, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in TGI is calculated from $\mathrm{Ti}=\mathrm{Tz}$. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from $[(\mathrm{Ti}-\mathrm{Tz})/\mathrm{Tz}]\times 100=-50$. Values were calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested. Two standard drugs, meaning that their activities against the cell lines are well documented, were tested against each cell line: NSC 19893 (5-fluorouracil) and NSC 123127 (adriamycin).

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