Steroidal dihydrocarbothioic acid amido pyrazoles: synthesis, characterization, cytotoxicity and genotoxicity studies

Ayaz Mahmood Dar¹ · Manzoor Ahmad Gatoo² · Shamsuzzaman¹

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Abstract A new series of steroidal dihydrocarbothioic acid amido pyrazole analogues were synthesized, and after characterization, evaluation for cytotoxicity, comet assay and western blotting was carried out. The synthesis of these analogues is convenient and involves two steps, i.e. aldol condensation as first step followed by nucleophilic addition of thiosemicarbazide across α , β -unsaturated carbonyl as a later step. Quantitative yields of more than 80 % are obtained in both the steps. After characterization by IR, ¹H NMR, ¹³C NMR, MS and analytical data, all the compounds of both series were tested for cytotoxic activity against a panel of different human cancer cell lines by MTT assay, during which compound **3e**, **3f**, **4e**, **4f** and **4h** are very potent especially against HepG2 and MCF-7 cancer cell lines. Cell cycle analysis depicted the cell death in S-phase while as annexin V-FITC/PI analysis showed that compounds effectively induce apoptosis. Apoptotic degradation of DNA of MCF-7 cells in the presence of different steroidal derivatives was analysed by agarose gel electrophoresis and visualized by ethidium bromide staining (comet assay). In western blotting analysis, the relative expressions of relevant apoptotic markers depicted an apoptosis by steroidal dihydropyrazole in MCF-7 cancer cells.

Keywords Dihydropyrazole · Cytotoxicity · Western blotting · Comet assay

Introduction

Medicinal chemistry over the past 20 years has gained enormous popularity for its role in drug discovery. Many natural products, their semi-synthetic analogues and small molecules have been discovered and evaluated for their role in the treatment and cure of various fatal diseases such as cancer, diabetes, microbial infections and cardiovascular diseases [1, 2]. However, there is always scope for the design and development of new and modified analogues as more efficient drug candidates because of the drug resistance and drug tolerance problems. This has been done through the rational design and synthesis of receptor-based lead molecules which still remains an open area. Natural products have extensively been used as starting tools for the design and synthesis of lead therapeutic scaffolds. The use of natural products for curing human ailments dates back to 1550 B.C.; also, natural products have been used in medicine especially for the treatment of cancer and related diseases. A number of plants have been used as folklore medicinal agents. The modern drug discovery programme has made a great success as a number of lead compounds have been developed from the traditionally used medicinal plants. A number of plant-based anticancer compounds such as vincristine (Oncovin), vinblastine (Velban), etoposide (VP-16), vinorelbine (Navelbine), Taxol (paclitaxel), teniposide (VM-26), and most recently irinotecan (Camptosar), topotecan (Hycamtin), and Taxotere (docetaxel), have been approved for use as anticancer drugs by the US FDA [3].

Steroids as natural products have been extensively studied as their biological and clinical importance is now well validated. Steroidal derivatives have been found to have the potential to be developed as drugs for the treatment of a large number of diseases including cardiovascular, autoimmune diseases, brain tumours, breast and prostate cancer and osteoarthritis [4–6].

✉ Ayaz Mahmood Dar
ayazchem09@gmail.com

¹ Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India

² Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202002, India

The promise of using steroids for the development of lead molecules lies in their regulation of a variety of biological processes and being a fundamental class of signalling molecules [7]. We have been interested in the area of steroid-based medicinal chemistry for the past few years, and we have successfully evaluated various steroid-based congeners as potent pharmacological agents showing activities such as cytotoxic, genotoxic, antimicrobial, DNA binding agents, etc. [8]. This is particularly true of pregnenolone which happens to be a precursor of most other steroids and has been extensively studied for various biological activities. It has been found that the molecule holds a great promise against brain disorders, memory loss, schizophrenia, insulin-related disorders and cancer [9]. Thus, in continuation of our programme, we herein report the convenient synthesis, in vitro cytotoxicity (MTT assay) and genotoxicity (comet assay) of two series of steroidal compounds, i.e. steroidal phenylpropenone and their dihydrocarbothioic acid amido pyrazole derivatives.

Experimental

Material and methods

Melting points were recorded on Buchi Melting Point apparatus D-545; IR spectra (KBr discs) were recorded on Bruker Vector 22 instrument. NMR spectra were recorded on Bruker DPX200 instrument (500 MHz) in CDCl_3 with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values are mentioned in δ (ppm), and coupling constants are given in hertz. Mass spectra were recorded on EIMS (Shimadzu) and ESI-esquire 3000 Bruker Daltonics instrument. The progress of all reactions was monitored by TLC on 2×5 cm pre-coated silica gel 60 F254 plates of thickness of 0.25 mm (Merck). The chromatograms were visualized under UV 254–366 nm and iodine. The human cancer cell lines used for the cytotoxicity experiment were SW480, A549, HT-29, HepG2, HL-60, MCF-7, HeLa and A545 and which were obtained from National Cancer Institute (NCI), biological testing branch, Frederick Research and Development Centre, USA. The treated and control cancer cells were viewed with a FluoView FV1000 (Olympus, Tokyo, Japan) confocal laser scanning microscope (CLSM) equipped with argon and HeNe lasers.

General synthesis of 3 β -hydroxy-cholest-5-en-17 β -yl-3'-phenylprop-2'-en-1'-one derivatives (3a–h)

To a solution of pregnenolone **1** (0.316 g, 1 mmol, 1 eq.) in ethanol (10 ml) was added a conc. aq. solution of KOH (2 eq.). Then, aldehyde **2** (1.2 eq.) was added into the reaction mixture

to get the corresponding steroidal phenylpropenone derivative **3** (Scheme 1). After completion as revealed by thin-layer chromatography (TLC) in an average span of around 1 h, the reaction mixture was precipitated using water because of the limited solubility. The precipitate was filtered, dried and monitored through TLC for the purity. Thin-layer chromatography revealed just a single spot which proved the presence of a single product. It is to be mentioned that when non-aromatic aldehydes were used, the product was formed in a very minor quantity and that too not stable enough at ambient conditions. Thus, the study was restricted to the use of aromatic aldehydes only in which the yields were obtained 80–86 %.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-phenylprop-2'-en-1'-one (3a)

Yield 83 %, m.p: 129–130 °C; IR (KBr) cm^{-1} : 3429, 2943, 1794, 1632, 1412, 1071, 694; ^1H NMR (500 MHz, CDCl_3): 0.63 (s, 3H), 1.00 (s, 3H), 1.61–1.90 (m, 6H), 2.20–2.38 (m, 3H), 2.82 (t, $J=8.80$, 1H); 3.51 (m, 1H); 6.78 (s, $J=16.00$, 1H), 7.39–7.55 (m, 6H, arom.); ^{13}C NMR (CDCl_3): 13.29, 18.97, 21.87, 22.41, 24.91, 32.04, 32.66, 32.86, 38.31, 40.89, 45.77, 48.33, 48.72, 49.0, 49.22, 49.41, 50.3, 57.19, 61.90, 72.12, 122, 125.5, 127.4, 129.3, 131.2, 134.69, 137.19, 140.93, 197.25; ESI-MS: 405 (M+H); Anal. Calcd. for $\text{C}_{28}\text{H}_{36}\text{O}_2$: C, 83.12; H, 8.97; Found C, 83.3; H, 8.83.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*o*-tolyl)-phenylprop-2'-en-1'-one (3b)

Yield 85 %, m.p: 209–210 °C; IR (KBr) cm^{-1} : 3410, 2955, 1781, 1657, 1412, 1069, 748; ^1H NMR (500 MHz, CDCl_3): 0.61 (s, 3H), 1.1 (s, 3H), 1.61–1.9 (m, 6H), 2.2–2.33 (m, 3H), 2.36 (s, 3H), 2.82 (t, $J=8.4$, 1H); 3.51 (m, 1H), 5.41 (s, 1H), 6.68 (d, $J=15.78$, 1H), 7.40 (m, 3H), 7.56 (d, $J=6.79$, 1H), 7.82 (d, $J=15.88$, 1H); ^{13}C NMR (CDCl_3): 13.611, 19.33, 19.91, 21.38, 22.41, 24.48, 30.14, 31.90, 32.09, 36.61, 37.27, 37.42, 39.32, 42.32, 45.00, 50.13, 57.15, 62.2, 71.8, 71.66, 121.88, 121.92, 126.29, 126.41, 127.98, 129.22, 130.74, 130.33, 134.37, 138.27, 139.71, 140.53, 140.9, 200.51; ESI-MS: 441 (M+Na); Anal. Calcd. for $\text{C}_{29}\text{H}_{38}\text{O}_2$: C, 83.21; H, 9.15; Found C, 83.11; H, 8.96.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*m*-tolyl)-phenylprop-2'-en-1'-one (3c)

Yield 84 %, m.p: 211–213 °C; IR (KBr) cm^{-1} : 3420, 2927, 1800, 1776, 1633, 1407, 1067, 761; ^1H NMR (500 MHz, CDCl_3): 0.63 (s, 3H), 1.00 (s, 3H), 1.61–1.9 (m, 6H), 2.20–2.34 (m, 3H), 2.44 (s, 3H), 2.86 (t, $J=8.73$, 1H); 3.54 (m, 1H), 5.36 (s, 1H), 6.75 (d, $J=15.93$, 1H), 7.27 (m, 4H), 7.51 (d, $J=15.93$, 1H); ^{13}C NMR (CDCl_3): 12.30, 19.36, 20.07,

Scheme 1 Synthesis of steroidal phenylpropenone derivatives (**3a–h**) and dihydrocarbothioic acid amido pyrazole derivatives (**4a–h**)

20.52, 21.86, 24.20, 30.71, 30.92, 31.10, 34.94, 36.69, 38.61, 43.33, 45.6, 50.1, 55.31, 62.51, 71.81, 120.44, 124.94, 127.29, 128.65, 131.18, 139.63, 140.54, 198.42; ESI-MS: 441 (M+Na); Anal. Calcd. for $\text{C}_{29}\text{H}_{38}\text{O}_2$: C, 83.21; H, 9.15; Found C, 83.11; H, 9.26.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*p*-tolyl)-phenylprop-2'-en-1'-one (3d)

Yield 81 %, m.p: 243–245 °C; IR (KBr) cm^{-1} : 3386, 2933, 1804, 1778, 1641, 1399, 1222, 1045, 751; ^1H NMR (500 MHz, CDCl_3): 0.63 (s, 3 H), 1.00 (s, 3H), 1.61–1.90 (m, 6H), 2.20–2.38 (m, 3H), 2.45 (s, 3H), 2.85 (t, $J=8.43$, 1H); 3.52 (m, 1H), 5.36 (s, 1H), 6.74 (d, $J=15.98$, 1H), 7.22 (d, $J=7.82$, 2H), 7.48 (d, $J=7.82$, 2H), 7.54 (d, $J=15.98$, 1H); ^{13}C NMR (CDCl_3): 12.41, 18.34, 20.13, 20.48, 21.66, 23.49, 30.70, 30.86, 32.41, 35.22, 36.34, 38.43, 41.81, 44, 50, 56.2, 60.99, 70.73, 120.44, 124.94, 127.29, 128.65, 131.08, 139.73, 140.6, 199.42; ESI-MS: 441 (M+Na); Anal. Calcd. for $\text{C}_{29}\text{H}_{38}\text{O}_2$: C, 83.21; H, 9.15; Found C, 83.15; H, 8.98.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*m*-fluoro)-phenylprop-2'-en-1'-one (3e)

Yield 86 %, m.p: 229–231 °C; IR (KBr) cm^{-1} : 3398, 2940, 1777, 1681, 1513, 1243, 1052, 762; ^1H NMR (500 MHz, CDCl_3): 0.62 (s, 3H), 1.00 (s, 3H), 1.61–1.90 (m, 6H), 2.20–2.32 (m, 3H), 2.84 (t, $J=8.92$, 1H); 3.52 (m, 1H), 5.36 (s, 1H), 6.70 (d, $J=15.93$, 1H), 7.13 (m, 2H), 7.62 (m, 3H); ^{13}C NMR (CDCl_3): 13.06, 19.28, 20.13, 20.48, 21.78, 25.10, 30.62, 30.86, 31.03, 35.54, 36.27, 38.14, 43.26, 43.96, 49.11, 56.21, 60.99, 71.73, 122.44, 124.94, 126.49, 130.02, 133.8, 139.63, 140.53, 196.92; ESI-MS: 445 (M+Na); Anal. Calcd. For $\text{C}_{28}\text{H}_{35}\text{FO}_2$: C, 79.58; H, 8.35, F, 4.50; Found C, 79.47; H, 8.42, F, 4.30.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*p*-fluoro)-phenylprop-2'-en-1'-one (3f)

Yield 82 %, m.p: 197–198 °C; IR (KBr) cm^{-1} : 3413, 2963, 1776, 1682, 1514, 1228, 1053, 751; ^1H NMR (500 MHz,

CDCl_3): 0.62 (s, 3H), 1.00 (s, 3H), 1.61–1.90 (m, 6H), 2.20–2.33 (m, 3H), 2.83 (t, $J=8.61$, 1 H), 3.63 (m, 1H), 5.37 (s, 1H), 6.75 (d, $J=15.84$, 1H), 7.08 (m, 2H), 7.24–7.32 (m, 2H), 7.50 (d, $J=15.84$, 1H); ^{13}C NMR (CDCl_3): 12.78, 19.52, 20.34, 20.45, 21.87, 23.45, 30.44, 30.69, 31.89, 35.9, 36.51, 38.31, 43.26, 43.96, 49.9, 57.33, 63.41, 71.21, 120.52, 124.94, 127.29, 128.65, 131.18, 139.63, 140.54, 198.33; ESI-MS: 445 (M+Na); Anal. Calcd. For $\text{C}_{28}\text{H}_{35}\text{FO}_2$: C, 79.58; H, 8.35, F, 4.50; Found C, 79.47; H, 8.26, F, 4.39.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*o*-methoxy)-phenylprop-2'-en-1'-one (3g)

Yield 84 %, m.p: 201–203 °C; IR (KBr) cm^{-1} : 3411, 2930, 2851, 1800, 1710, 1639, 1513, 1272, 1045, 752; ^1H NMR (500 MHz, CDCl_3): 0.60 (s, 3H), 1.00 (s, 3H), 1.65–1.90 (m, 6H), 2.20–2.36 (m, 3H), 2.83 (t, $J=8.89$, 1H), 3.53 (m, 1H), 3.87 (s, 3H), 5.36 (s, 1H), 6.65 (d, $J=15.92$, 1H), 6.91 (d, $J=8.72$, 2H), 7.51 (d, $J=8.72$, 2H), 7.53 (d, $J=15.84$, 1H); ^{13}C NMR (CDCl_3): 12.17, 18.29, 21.13, 20.50, 21.78, 24.10, 30.62, 30.81, 32.11, 34.22, 36.27, 38.14, 43.26, 43.96, 49.22, 56.21, 60.99, 71.73, 120.44, 124.94, 127.29, 128.65, 133.16, 139.63, 140.54, 197.54; ESI-MS: 457 (M+Na); Anal. Calcd. for $\text{C}_{29}\text{H}_{38}\text{O}_3$: C, 80.14; H, 8.81; Found C, 80.05; H, 8.73.

3 β -Hydroxy-cholest-5-en-17 β -yl-3'-(*p*-methoxy)-phenylprop-2'-en-1'-one (3h)

Yield 80 %, m.p: 226 °C; IR (KBr) cm^{-1} : 3421, 2944, 1729, 1633, 1600, 1298, 1027, 753; ^1H NMR (500 MHz, CDCl_3): 0.63 (s, 3H), 1.00 (s, 3H), 1.65–1.90 (m, 6H), 2.20–2.32 (m, 3H), 2.89 (t, $J=8.85$, 1H), 3.52 (m, 1H), 3.91 (s, 3H), 5.37 (s, 1H), 6.94 (d, $J=16.16$, 1H), 6.98 (m, 2H), 7.36 (m, 1H), 7.55 (d, $J=6.37$, 1H), 7.88 (d, $J=16.16$, 1H); ^{13}C NMR (CDCl_3): 12.34, 18.34, 20.13, 20.48, 21.78, 25.10, 30.62, 30.86, 31.03, 35.54, 36.27, 38.14, 43.26, 43.96, 49.11, 56.21, 60.99, 71.73, 118.30, 124.94, 127.29, 128.65, 131.18, 139.63, 140.54, 195.23; ESI-MS: 457 (M+Na); Anal. Calcd. for $\text{C}_{29}\text{H}_{38}\text{O}_3$: C, 80.14; H, 8.81; Found C, 80.03; H, 8.77.

General synthesis of 3 β -hydroxy-cholest-5-en-17 β -yl-5'-phenyl-4',5'-dihydro-1'-carbothioic acid amidopyrazole derivatives (4a–h)

To an ethanolic solution of compound **3a–h** (0.418 g, 1 mmol) was added thiosemicarbazide hydrochloride (0.127 g, 1 mmol), and the reaction was refluxed for 2 h at room temperature. A precipitate of the thiosemicarbazone was obtained as a single spot as revealed by the TLC. The reaction mixture was worked up by first evaporating the ethanol and then extracting the solid with EtOAc: H₂O (3×25 ml). Quantitative yields of 90–93 % were obtained for all the compounds **4a–h**.

3 β -Hydroxy-cholest-5-en-17 β -yl-5'-phenyl-4',5'-dihydro-1'-carbothioic acid amido pyrazole (**4a**)

Yield 86 %, m.p: 241 °C; IR (KBr) cm⁻¹: 3415, 3340, 1649, 1620, 1251, 1328, 1065; ¹H NMR (500 MHz, CDCl₃): 8.2 (s, 2H, NH₂, exchangeable with D₂O), 7.39–7.55 (m, 6H, aromatic), 7.9 (s, 1H, OH, exchangeable with D₂O), 5.37 (s, 1H, C₅-H), 4.7 (m, 1H, C₃ α -H, *W* 1/2=15 Hz), 4.1 (q, 1H, C₅'-H), 2.0 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (500 MHz, CDCl₃): 17.6, 20.1, 20.3, 20.6, 21.4, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 53.1, 72.1, 122.5, 125, 127, 129, 130, 132, 149.1, 155.6, 182.2; ESI-MS: 478 (M+H); Anal. Calcd. for C₂₉H₃₉N₃OS: C, 72.91; H, 8.23; N, 8.80; S, 6.71; Found C, 72.83; H, 8.07; N, 8.74; S, 6.68.

3 β -Hydroxy-cholest-5-en-17 β -yl-5'-*o*-tolyl-4',5'-dihydro-1'-carbothioic acid amido pyrazole (**4b**)

Yield 80 %, m.p: 247 °C; IR (KBr) cm⁻¹: 3404, 3326, 1646, 1626, 1247, 1323, 1071; ¹H NMR (500 MHz, CDCl₃): 8.2 (s, 2H, NH₂, exchangeable with D₂O), 7.02–6.75 (m, 4H, aromatic), 7.4 (s, 1H, OH, exchangeable with D₂O), 5.37 (s, 1H, C₅-H), 4.7 (m, 1H, C₃ α -H, *W* 1/2=15 Hz), 4.0 (q, 1H, C₅'-H), 2.35 (s, 3H, C₂'-CH₃), 2.1 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.4, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 53.1, 72.1, 122.1, 125.3, 126.4, 127, 129, 136, 139, 149.1, 155.6, 183.1; ESI-MS: 513 (M+Na); Anal. Calcd. for C₃₀H₄₁N₃OS: C, 73.28; H, 8.40; N, 8.55; S, 6.52; Found C, 73.39; H, 8.17; N, 8.34; S, 6.67.

3 β -Hydroxy-cholest-5-en-17 β -yl-5'-*m*-tolyl-4',5'-dihydro-1'-carbothioic acid amido pyrazole (**4c**)

Yield 83 %, m.p: 239 °C; IR (KBr) cm⁻¹: 3400, 3320, 1645, 1621, 1239, 1311, 1069; ¹H NMR (500 MHz, CDCl₃): 8.2 (s,

2H, NH₂, exchangeable with D₂O), 7.09–6.78 (m, 4H, aromatic), 7.6 (s, 1H, OH, exchangeable with D₂O), 5.38 (s, 1H, C₅-H), 4.7 (m, 1H, C₃ α -H, *W* 1/2=15 Hz), 3.9 (q, 1H, C₅'-H), 2.35 (s, 3H, C₃'-CH₃), 1.9 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.3, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 53.1, 72.1, 122.8, 124.1, 127.2, 127.8, 128.7, 137.5, 142.2, 149.0, 155.6, 184.1; ESI-MS: 492 (M+H); Anal. Calcd. for C₃₀H₄₁N₃OS: C, 73.28; H, 8.40; N, 8.55; Found C, 73.17; H, 8.26; N, 8.44.

3 β -Hydroxy-cholest-5-en-17 β -yl-5'-*p*-tolyl-4',5'-dihydro-1'-carbothioic acid amido pyrazole (**4d**)

Yield 83 %, m.p: 256 °C; IR (KBr) cm⁻¹: 3412, 3327, 1648, 1629, 1231, 1321, 1074; ¹H NMR (500 MHz, CDCl₃): 8.1 (s, 2H, NH₂, exchangeable with D₂O), 7.4–7.1 (m, 4H, aromatic), 7.6 (s, 1H, OH, exchangeable with D₂O), 5.38 (s, 1H, C₅-H), 4.7 (m, 1H, C₃ α -H, *W* 1/2=15 Hz), 3.9 (q, 1H, C₅'-H), 2.35 (s, 3H, C₄'-CH₃), 2.1 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.3, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 53.1, 72.1, 122.8, 127.1, 127.3, 129.6, 129.8, 137.6, 139.2, 149.0, 155.6, 184; ESI-MS: 513 (M+Na); Anal. Calcd. for C₃₀H₄₁N₃OS: C, 73.28; H, 8.40; N, 8.55; Found C, 73.13; H, 8.20; N, 8.33.

3 β -Hydroxy-cholest-5-en-17 β -yl-5'-(*m*-fluorophenyl)-4',5'-dihydro-1'-carbothioic acid amido pyrazole (**4e**)

Yield 80 %, m.p: 261 °C; IR (KBr) cm⁻¹: 3414, 3310, 1640, 1646, 1230, 1335, 1272, 1070; ¹H NMR (500 MHz, CDCl₃): 8.0 (s, 2H, NH₂, exchangeable with D₂O), 7.1 (d, 1H), 6.7 (t, 1H), 6.64 (d, 2H), 8.9 (s, 1H, OH, exchangeable with D₂O), 5.38 (s, 1H, C₅-H), 4.7 (m, 1H, C₃ α -H, *W* 1/2=15 Hz), 3.9 (q, 1H, C₅'-H), 2.1 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.3, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 52.5, 72.2, 122.8, 113.2, 114.6, 123.6, 129.9, 144.1, 161.2, 139.2, 149.0, 155.6, 184; ESI-MS: 496 (M+H); Anal. Calcd. for C₂₉H₃₈FN₃OS: C, 70.27; H, 7.73; N, 8.48; F, 3.83. Found C, 70.15; H, 7.56; N, 8.35; F, 3.79.

3 β -Hydroxy-cholest-5-en-17 β -yl-5'-(*p*-fluorophenyl)-4',5'-dihydro-1'-carbothioic acid amido pyrazole (**4f**)

Yield 85 %, m.p: 255 °C; IR (KBr) cm⁻¹: 3413, 3312, 1642, 1635, 1232, 1331, 1274, 1065; ¹H NMR (500 MHz, CDCl₃): 8.0 (s, 2H, NH₂, exchangeable with D₂O), 8.6 (s, 1H, OH,

exchangeable with D₂O), 7.08 (m, 2H), 7.24–7.32 (m, 2H), 5.36 (s, 1H, C₅-H), 4.7 (m, 1H, C₃α-H, $W_{1/2}$ =15 Hz), 3.9 (q, 1H, C₅'-H), 2.2 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.3, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 52.5, 72.2, 122.8, 114.3, 114.7, 128.6, 128.9, 138.5, 161.2, 149.0, 155.6, 184; ESI-MS: 496 (M+H); Anal. Calcd. for C₂₉H₃₈FN₃O₂S: C, 70.27; H, 7.73; N, 8.48; F, 3.83. Found C, 70.19; H, 7.60; N, 8.51; F, 3.69.

3β-Hydroxy-cholest-5-en-17β-yl-5'-(*o*-methoxyphenyl)-4',5'-dihydro-1'-carbothioic acid amido pyrazole (4g)

Yield 86 %, m.p: 250 °C; IR (KBr) cm⁻¹: 3421, 3318, 1641, 1628, 1236, 1339, 1070, 1075; ¹H NMR (500 MHz, CDCl₃): 8.2 (s, 2H, NH₂, exchangeable with D₂O), 8.6 (s, 1H, OH, exchangeable with D₂O), 7.04 (m, 2H), 6.74–6.91 (m, 2H), 5.36 (s, 1H, C₅-H), 4.7 (m, 1H, C₃α-H, $W_{1/2}$ =15 Hz), 4.2 (q, 1H, C₅'-H), 3.81 (s, 3H, C₄'-CH₃), 2.3 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.3, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 52.5, 56.8, 72.2, 122.8, 114.1, 114.6, 128.2, 128.6, 135.2, 160.7, 149.0, 155.6, 184; ESI-MS: 508 (M+H); Anal. Calcd. for C₃₀H₄₁FN₃O₂S: C, 70.97; H, 8.14; N, 8.28; S, 6.32. Found C, 70.81; H, 8.02; N, 8.17; S, 6.29.

3β-Hydroxy-cholest-5-en-17β-yl-5'-(*p*-methoxyphenyl)-4',5'-dihydro-1'-carbothioic acid amido pyrazole (4h)

Yield 80 %, m.p: 243 °C; IR (KBr) cm⁻¹: 3426, 3313, 1654, 1625, 1231, 1345, 1073, 1071; ¹H NMR (500 MHz, CDCl₃): 8.2 (s, 2H, NH₂, exchangeable with D₂O), 8.6 (s, 1H, OH, exchangeable with D₂O), 7.1 (d, 1H), 6.7 (t, 1H), 6.64 (d, 2H), 5.36 (s, 1H, C₅-H), 4.7 (m, 1H, C₃α-H, $W_{1/2}$ =15 Hz), 4.2 (q, 1H, C₅'-H), 3.81 (s, 3H, C₄'-CH₃), 2.3 (d, 2H, C₄'-H₂), 1.18 (s, 3H, C₁₀-CH₃), 1.16 (s, 3H, C₁₃-CH₃), 0.97 & 0.83 (other methyl protons); ¹³C NMR (CDCl₃): 15.3, 17.6, 20.1, 20.3, 20.6, 21.3, 29.3, 30.2, 32.2, 35.3, 36.4, 36.9, 39.5, 42.1, 42.1, 42.8, 43.1, 46.2, 52.5, 56.8, 72.2, 122.8, 112.3, 113.7, 129.3, 119.4, 143.8, 161.3, 149.0, 155.6, 184; ESI-MS: 529 (M+Na); Anal. Calcd. for C₃₀H₄₁FN₃O₂S: C, 70.97; H, 8.14; N, 8.28; S, 6.32. Found C, 70.85; H, 8.05; N, 8.09; S, 6.39.

In vitro anticancer activity

Cell culture and conditions Human cancer cell lines **A545** (lung carcinoma cells)/ATCC (CRL-2579), **SW480** (colon adenocarcinoma cells)/ATCC (CCL-228), **HeLa** (cervical cancer cells)/ATCC (CCL-2), **A549** (lung carcinoma cells)/ATCC (CCL-185), **MCF7** (breast cancer cells)/ATCC (HTB-22),

HepG2 (hepatic carcinoma cells)/ATCC (CRL-8065), **HT-29** (colon cancer cells)/ATCC (HTB-38) and **HL-60** (leukemia cells)/ATCC (CCL-240) were taken for the study. SW480, A549, HT-29, HL-60, A545 and HepG2 cells were grown in RPMI 1640 supplemented with 10 % foetal bovine serum (FBS), 10 U penicillin and 100 µg/ml streptomycin at 37 °C with 5 % CO₂ in a humidified atmosphere. HeLa and MCF7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplanted with FCS and antibiotics as described above for RPMI 1640. Fresh medium was given every second day and on the day before the experiments were done. Cells were passaged at pre-confluent densities, using a solution containing 0.05 % trypsin and 0.5 mM EDTA.

Cell viability assay (MTT) The anticancer activity in vitro was measured using the MTT assay. The assay was carried out according to known protocol [10–12]. Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1 × 10⁴ cells/well. After 24 h incubation at 37 °C under a humidified 5 % CO₂ to allow cell attachment, the cells in the wells were respectively treated with target compounds at various concentrations for 48 h. The concentration of DMSO was always kept below 1.25 %, which was found to be non-toxic to the cells. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/ml in phosphate buffered saline (PBS; 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4). A total of 20 µl of this solution was added to each well. After incubation for 4 h at 37 °C in a humidified incubator with 5 % CO₂, the medium/MTT mixtures were removed, and the formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in 100 µl of DMSO per well. The absorbance of the wells was read with a microplate reader (Bio-Rad Instruments) at 570 nm. Effects of the drug cell viability were calculated using cell treated with DMSO as control.

Data analysis Cell survival was calculated using the formula: survival (%)=[(absorbance of treated cells–absorbance of culture medium)/(absorbance of untreated cells–absorbance of culture medium)] × 100 [13, 14]. The experiment was done in triplicate, and the inhibitory concentration (IC) values were calculated from a dose-response curve. IC₅₀ is the concentration in 'µM' required for 50 % inhibition of cell growth as compared to that of standard drugs, as the values is shown in Table 1. IC₅₀ values were determined from the linear portion of the curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells, by 50 %. Evaluation is based on mean values from three independent experiments, each comprising at least six microcultures per concentration level.

Table 1 Nature of group “R” in the compounds **3a–3h/4a–4h**

Entry	Nature of R	Entry	Nature of R
3a, 4a		3e, 4e
3b, 4b		3f, 4f
3c, 4c		3g, 4g
3d, 4d		3h, 4h

Comet assay

To assess the genotoxic effect of the steroidal derivatives, comet assay [15] was performed in MCF-7 cells. MCF-7 (1×10^6) cells were treated with three different concentrations, 10, 15 and 25 $\mu\text{g/ml}$ of different steroidal derivatives for 24 h. The cells were then washed, and 200 μl of cell suspension in low-melting agarose (LMA) was layered on to the labelled slides pre-coated with agarose (1.5 %). The slides were placed on ice for 10 min and submerged in lysis buffer (2.5 % NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1 % Triton X-100) at pH 10 at 4 °C for more than 1 h. The slides were then equilibrated in alkaline buffer (30 mM NaOH, 1 mM EDTA) at pH 13 at 4 °C, electrophoresed at 0.86 V/cm at 4 °C, neutralized, washed and dried. At the time of image capturing, the slides were stained with ethidium bromide (ETBr, 150 μl 1X), and cover slips were placed over them. For visualization of DNA-damage, ETBr stained slides were observed under 209 objectives of a fluorescent microscope (Olympus BX-51, Japan). The images of 50–100 randomly selected cells were captured per slide using a CCD camera.

Western blot analysis

MCF cells were grown on 100-mm tissue culture discs at a density of 1×10^6 cells per plate and incubated overnight. Then, the cells were exposed to compound **4e** (0, 15 and 25 μM) for 48 h prior to harvest. The cell lysate was prepared by using modified RIPA lysis buffer (50 mM tris,

150 mM NaCl, 0.5 mM deoxycholate, 1 % NP-40, 0.1 % SDS, 1 mM Na_3VO_4 , 5 mM EDTA, 1 mM PMSF, 2 mM DTT, 10 mM β -glycerophosphate, 50 mM NAF, 0.5 % triton X-100, protease inhibitor cocktail). Protein estimation was done by Bradford's method. Proteins were separated in 10 % SDS-PAGE and transferred to PVDF membrane. Membranes were blocked overnight in 10 % skimmed milk in $1 \times \text{TBS-T}$ (Tris-buffered saline containing 0.05 % of Tween-20) at 4 °C and immunoblotted with antibodies anti-Bcl-XL, anti-Bax and anti-PARP. Detection of signals was done using ECL Western blotting reagent, and chemiluminescence was exposed on Kodak X-Omat films. Antibody anti- β -actin was used as loading control. All the antibodies were procured from cell signalling technology, CA, USA.

Measurement of apoptosis by annexin V-FITC/PI analysis

An annexin V-FITC/PI assay was used to evaluate cellular apoptosis. Briefly, cells were harvested after the target compound **4e** treatment with different concentrations (0, 3, 7 and 10 μM) for 24 h and washed with PBS then resuspended in 100 μl **4e** × binding buffer. Then, 5 μl of annexin V-FITC and 5 μl of propidium iodide (PI) were added to the cells (100 μl) and incubated for 1 h at room temperature in the dark. After 400 μl **4e** × binding buffer was added into each tube, the stained cells were analysed by flow cytometry. The rate of cell apoptosis was analysed.

Cell cycle analysis

Flow cytometry was used to evaluate DNA contents and cell cycle analysis. The MCF-7 cells (5×10^5) were incubated with compound **4e** at 0, 3, 7 and 10 μM at 37 °C for 48 h. Cells were then harvested and washed with cold PBS, centrifuged, resuspended in 1 ml of PBS and fixed by the drop wise addition of 9 ml pre-cooled 75 % EtOH overnight at -20 °C. For staining, cells were further washed with cold PBS, digested by 500 μl RNase A (100 $\mu\text{g}/\text{ml}$) at 37 °C for 30 min, and stained with 25 μl PI (50 mg/ml) in the dark at room temperature for 30 min. The distribution of the cell cycle was analysed by using a Becton-Dickinson FACSCalibur.

Results and discussion

Chemistry

Synthesis of highly functional molecules from simple building blocks has always attracted the curiosity of synthetic chemists. We here in report the convenient and efficient synthesis of steroidal phenylpropenone derivatives involving aldol condensation as the first step followed by synthesis of dihydrocarbothioic acid amido pyrazole derivatives of pregnenolones by nucleophilic addition of thiosemicarbazide across α , β -unsaturated carbonyl group as a later step. Also, the importance of different steroidal D-ring heterocycles is now well validated, only few efforts have been reported for their efficient synthesis, to the best our knowledge. This specially refers to the dihydrocarbothioic acid amido pyrazole-based heterocycles at the D-ring of steroids including the very important class of pregnanes. Though there are reports for the synthesis of other such heterocycles, the same is not true for the dihydrocarbothioic acid amido pyrazole derivatives at the D-ring. Natural products of steroidal phenylpropenone are considered as resourceful molecules as far as their pharmacological activities are concerned. Also, many steroidal pyrazoles are known which are found to possess antimicrobial, anti-inflammatory, hypotensive, hypocholesterolemic and diuretic activities. Some of these steroidal heterocycles have been obtained exhibiting potential activity to inhibit cytochrome P₄₅₀ enzyme aromatase and their subsequent clinical application in the treatment of oestrogen-dependent breast cancer. Taking inspiration from the number of reported biological activities associated with structurally related analogues, we, in continuation of our previous work [8], herein report an efficient and simple synthesis of D-ring appended pyrazole derivatives of 20-keto pregnenones and their evaluation as potential anticancer agents. The preparation of the latter involves the following synthetic approach (Scheme 1).

The nature of “R” in compound series **3** and **4** is differently substituted either electron donating or electron withdrawing

groups on aromatic moiety appended at the 5'-position of the dihydropyrazole ring. The nature of “R” in the compound **3a–3h/4a–4h** is given in Table 1.

In vitro cytotoxicity (MTT Assay)

Some studies in recent past have showed that synthetic steroids with α , β -unsaturated ketone core gave the potency against human cancer cell lines [16–18]. Thus, with this institution, an attempt of synthesizing some derivatives from compounds with α , β -unsaturated ketone core was made, and subsequently, in vitro anticancer activity of the synthesized compounds was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [13–15]. The conversion of the soluble yellowish MTT to the insoluble purple formazan by active mitochondrial lactate dehydrogenase of living cells has been used to develop an assay system for the measurement of cell proliferation. Cell viability was measured with the purple formazan that was metabolized from MTT mitochondrial lactate dehydrogenase, which is active only in live cells. The screening of compounds against different cancer cell lines is shown in Table 2. The cytotoxic studies of various steroidal phenylpropenone derivatives and their dihydropyrazole derivatives depicted that these derivatives are cell specific as these were found to be active mostly against the HepG2 and MCF-7 cells.

Further, the dihydropyrazole derivatives of steroids were more potent than the corresponding steroidal phenylpropenone derivatives. This may be attributed to the more hydrophilicity and thus more bioavailability of the dihydropyrazole derivatives. Since the compounds **3e**, **3f**, **4e**, **4f** and **4h** were found to be more active than other analogues, it can be assumed that electron withdrawing groups (fluoro and methoxy) have an effect over the activity. Figure 1 represents the standard error in the IC₅₀ values of the most potent compounds against HepG2 and MCF cell lines. It is evident from the IC₅₀ data that even the position of substituent on the aromatic ring influences the relative cytotoxicity which can be attributed to their differences in either the bioavailability or the protein-binding properties. Overall, it can be concluded that the cytotoxicity of these compounds is cancer cell specific as the IC₅₀ values make it evident.

Microscopic examination of gross morphology of cancer cells and comparison with steroid-treated cancer cells is shown in Fig. 2. The breast cancer cells (MCF), when treated with **3e** and **4e**, the growth was inhibited within 24 h, and the cells were almost completely dead. The lung carcinoma (A545) when treated with **3e** and **4e** also showed the same behaviour, but with **4e**, the cancer cells were almost dead. The hepatic carcinoma cells (HepG2) when treated with **3f** and **4f** also showed the same behaviour, the growth was inhibited within 24 h, but with **4f**, the cancer cells were almost dead.

Table 2 IC₅₀ values (μM) of steroidal phenylpropenone and their pyrazole derivatives against a panel of human cancer cell lines

Entry	SW480	A549	HT29	HL60	A545	HepG2	HeLa	MCF-7	R
3a	6.25	8.12	5.43	2.46	10.29	2.44	5.18	2.46
3b	8.39	3.19	2.51	5.20	3.42	1.89	3.62	1.63
3c	5.21	2.12	2.33	4.88	1.69	3.03	4.17	1.96
3d	6.38	9.73	6.25	11.43	8.20	2.12	6.88	4.91
3e	4.52	2.39	3.50	6.30	1.74	0.97	2.81	0.93
3f	3.81	50.7	48.02	3.19	47.6	1.21	3.61	0.81
3g	9.16	16.15	41.81	6.13	50.7	2.18	9.10	4.7
3h	4.12	3.35	2.49	5.91	2.23	0.92	4.11	1.95
4a	4.21	3.71	2.81	1.77	6.41	1.21	4.15	1.80
4b	6.81	2.84	1.92	3.26	2.71	1.11	2.26	1.24
4c	4.88	1.91	2.01	3.59	1.24	2.26	3.39	1.10
4d	5.11	6.42	4.53	7.82	5.49	1.68	4.51	2.63
4e	2.19	1.08	1.12	3.62	0.96	0.73	0.60	0.31
4f	1.61	0.59	42.13	1.07	36.87	0.91	2.13	0.46
4g	3.15	5.30	8.62	2.82	4.19	1.59	3.18	2.41
4h	1.69	1.24	0.91	0.36	1.12	0.72	2.49	0.39

Fig. 1 Standard error values (% error) of the most active compounds against HepG2 and MCF-7

Genotoxicity (comet assay)

In the comet assay, the images of MCF-7 cells treated with compounds showed the formation of comets. No comet pattern was observed in the control cells. There was dose-dependent increase in tail length when treated with steroidal derivatives. Compound **3f** presented maximum apoptotic

DNA damage among the steroidal dihydropyrazoles studied, which was in accordance with its maximum cytotoxicity as seen in MTT assay. The quantified increase in DNA damage suggested that all steroid derivatives induced dose-dependent fragmentation of chromosomal DNA leading to apoptosis. The images of comet assay for cells treated with 25 μ g/ml of **3e**, **3f**, **4e**, **4f** and **4h** are shown in Fig. 3. Slides were analysed for parameter-like tail length (TL), using image analyser CASP software version 1.2.2. The results of the assay for tail length are shown in Fig. 4.

Western blot analysis

To confirm the apoptotic effect of compound **4e** in MCF-7 cells, a study of protein levels of some apoptotic markers such as caspase-3, Bax, Bcl-XL and PARP cleavage was performed by western blot analysis (Fig. 5). The expression of Bcl-XL was reduced when cells were exposed to compound **4e** of increasing concentrations (0, 15 and 25 μ M). In contrast to Bcl-XL, the expression of Bax and caspase-3

Fig. 2 Microscopic examination of the interaction of cancer cells with steroidal phenylpropenone and corresponding dihydropyrazole derivatives

Fig. 3 Detection of DNA damage in MCF-7 cells. Treated cells (24 h) were layered over agarose gel, lysed, electrophoresed in alkaline buffer and stained with propidium iodide. The DNA fragmentation resulting in a comet-like appearance in MCF-7 cells treated with dihydropyrazole derivatives

was increased. The level of cleaved PARP product (104 KDa) was higher in the treated cells than in untreated cells and increased with increasing concentrations of the compound **4e**. As expected, the expression of β -actin, which served as a loading control, remained unaltered. These relative expressions of relevant apoptotic markers including the increasing Bax/Bcl-XL ratio, caspase-3 and PARP cleavage clearly indicate that compound **4e** causes apoptosis in MCF-7 cancer cells.

Measurement of apoptosis by annexin V-FITC/PI analysis

Apoptotic cells were detected by annexin V-FITC/PI staining. The staining single-positive for annexin V-FITC mostly reflected early apoptotic cells and the staining single-positive for PI mostly reflected necrotic cells, so cells that were stained double-positive could be either necrotic or apoptotic cells. As shown in Fig. 6, annexin V-FITC/PI staining data indicated that percentage of apoptosis was 2.3, 4.2, 10.4 and 16.8 %, respectively, for 24 h. The results showed that compound **4e** could effectively induce apoptosis in MCF-7 cells in a dose-dependent manner.

Fig. 4 Diagram comparing the effect of different concentrations of steroid derivatives on the tail length in comet assay

Cell cycle analysis

The effect of compound **4e** on cell cycle distribution of MCF-7 cells carried out by flow-cytometric analysis further confirmed tumour cell apoptosis. The cell cycle is the series of events that take place in a cell leading to its division and duplication (replication). As shown in Fig. 7, cytometric profiles of the PI-stained DNA show an accumulation of cells in the S phase of the MCF-7 cell cycle treated with **1** for 48 h at 0, 3, 7 and 10 μ M. With the increased concentrations of compound **4e**, S phase populations increased from 41.58 % to 56.27 %, while G1 and G2 phase decreased profoundly, which indicated that compound **4e** could delay or inhibit cell cycle progression through S phase and the percentage of apoptotic cells presented a dose-dependent increase from 3 to 10 μ M (Fig. 7).

Conclusion

In summary, the successfully developed, convenient and operationally simple strategy for a better synthesis of dihydrocarbothioic acid amido pyrazole derivatives of

Fig. 5 Western blot analysis of whole cell extracts. The lower panel (β -actin) represents equal loading of each lane

Fig. 6 Annexin V/propidium iodide assay of MCF-7 cells treated with compound **4e** measured by flow cytometry at concentrations of **4e** (0, 3, 7 and 10 μ M) for 24 h

steroidal phenylpropenone involve the reaction of steroidal phenylpropenone with thiosemicarbazide in ethanol. The reaction completes in 2 h, and on completion, promising yields are obtained. This strategy offered a very straightforward and efficient method for access to steroidal pyrimidines. From the

anticancer screening data, it was found that all the compounds are having promising anticancer activity, and the compound **4e** and **4h** were found to be the most active in this study while as after studying the behaviour of apoptotic markers in the western blotting analysis, it was revealed that apoptosis occurs in

Fig. 7 Diagram depicting the effects of compound **4e** on the MCF-7 cell cycle progression. MCF-7 cells were treated with PBS at various concentrations of **4e** (0, 3, 7 and 10 μ M) for 48 h

MCF-7 cells in the presence of compound **4e**. During cell cycle analysis, delay in cell growth was clear in S phase while as annexin V-FITC/PI analysis depicted that compounds effectively induce apoptosis.

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References

- Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 70:461–477
- Cutler SJ, Cutler HG (2000) Biologically active natural products: pharmaceuticals. CRC, pp 5–23.
- Cragg GM, Kingston DGI, Newman DJ (2005) Anticancer agents from natural products. CRC, Boca Raton
- Dubey RK, Oparil S, Imthurn B, Jackson EK (2002) Sex hormones and hypertension. *Cardiovasc Res* 53:688–708
- Latham KA, Zamora A, Drought H, Subramanian S, Matejuk A, Offner H, Rosloniec EF (2003) Estradiol treatment redirects the isotype of the autoantibody response and prevents the development of autoimmune arthritis. *J Immunol* 171:5820–5827
- Sheridan PJ, Blum K, Trachtenberg M (1988) Steroid receptors and disease. Marcel Dekker, New York, pp 289–564
- Parker MG (1993) Steroid hormone action. IRL, Oxford
- Shamsuzzaman DAM, Khan Y, Sohail A (2013) Synthesis and biological studies of steroidal pyran based derivatives. *J Photochem Photobiol B: Biol* 129:36–47
- Choudhary MI, Alam MS, Atta-ur-Rahman YS, Wud YC, Lind AS, Shaheen F (2011) Pregnenolone derivatives as potential anticancer agents. *Steroids* 76:1554–1559
- Berenyi A, Minorics R, Ivanyi Z, Ocsosvski I, Duczaa E, Thole H, Messinger J, Wolfling J, Motyan G, Mernyak E, Frank E, Schneider G, Zupko I (2013) Synthesis and investigation of the anticancer effects of estrone-16-oxime ethers in vitro. *Steroids* 78:69–78
- Wang XB, Liu W, Yang L, Guo QL, Kong LY (2012) Investigation on the substitution effects of the flavonoids as potent anticancer agents: a structure-activity relationships study. *Med Chem Res* 21: 1833–1849
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Saxena HO, Faridi U, Kumar JK, Luqman S, Darokar MP, Shanker K, Chanotiya CS, Gupta MM, Negi AS (2007) Synthesis of chalcone derivatives on steroidal framework and their anticancer activities. *Steroids* 72:892–900
- Woerdenbag HJ, Moskal TA, Pras N, Malingre TM, El-Feraly FS, Kampinga HH, Konings AW (1993) Cytotoxicity of artemisinin-related endoperoxides to ehrlich ascites tumor cells. *J Nat Prod* 56: 849–856
- Singh NP (2000) Microgels for estimation of DNA-strand breaks, DNA protein crosslinks and apoptosis. *Mutat Res* 455:111–127
- Li C, Qiu W, Yang Z, Luo J, Yang F, Liu M, Xie J, Tang J (2010) Stereoselective synthesis of some methyl-substituted steroid hormones and their in vitro cytotoxic activity against human gastric cancer cell line MGC-803. *Steroids* 75:859–869
- Wang C, Liu L, Xu H, Zhang Z, Wang X, Liu H (2011) Novel and efficient synthesis of 22-alkynyl-13, 24(23)-cyclo-18, 21-dinorchol-22-en-20(23)-one analogues. *Steroids* 76:491–496
- Cui JG, Fan L, Huang L, Liu H, Zhou A (2009) Synthesis and evaluation of some steroidal oximes as cytotoxic agents: structure/activity studies (I). *Steroids* 74:62–72