Journal of Steroid Biochemistry & Molecular Biology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Original Research

Synthesis of combretastatin A4 analogues on steroidal framework and their anti-breast cancer activity

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ARTICLE INFO

Article history:
Received 12 November 2012
Received in revised form 28 January 2013
Accepted 19 February 2013

Keywords:
Combretastatin A4
Breast cancer
Tubulin polymerization inhibition
Estrogenicity
Antiestrogenicity
Acute oral toxicity

ABSTRACT

Combretastatin A4 analogues were synthesized on steroidal framework from gallic acid with a possibility of anti-breast cancer agents. Twenty two analogues were synthesized and evaluated for cytotoxicity against human breast cancer cell lines (MCF-7 & MDA-MB 231). The best analogue 22 showed potent antitubulin effect. Docking experiments also supported strong binding affinity of 22 to microtubule polymerase. In cell cycle analysis, 22 induced apoptosis in MCF-7 cells significantly. It was found to be non-toxic up to 300 mg/kg dose in Swiss albino mice in acute oral toxicity.

This article is part of a Special Issue entitled 'Synthesis of Steroids'.

1. Introduction

Cancer, the uncontrolled proliferation of cells, is not just one disease, but a large group of almost 100 diseases with the characteristics of uncontrolled growth of the cells and ability to migrate from the original site and spread to distant sites. If the spread is not controlled, cancer can result in death and as per the present statistics, it accounts for 13% of total deaths globally [1]. Breast cancer is the leading cause of cancer deaths among women worldwide. More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue rising with an estimated over 13.1 million deaths in 2030 [1]. These troubling numbers are a constant reminder that new therapeutic approaches that will improve patient's survival are still desperately needed. So this requires the cutting-edge research that will lead to more effective strategies for breast cancer treatment and prevention. Even though there are continuous efforts globally to eradicate cancer, it is second major cause of deaths after cardiovascular disease.

Plants have immense contribution in the development of drugs and especially in the treatment of cancer [2]. Natural compounds are often used in traditional medicines for their various therapeutic effects. Among these, stilbenes (1,2-diaryl ethylenes) are widely distributed in nature and exhibit a wide range of biological activities [3]. Resveratrol (**1**), combretastatin A4 (**2**) and some of their glycosides like piceid (**3**) and rhapontin (**4**) etc. are notable naturally occurring anticancer compounds (Fig. 1) [4]. Resveratrol was isolated from *Polygonum cuspidatum*, a Japanese knotweed and now several other sources have also been identified [5]. It modulates mRNA transcript genes related to redox metabolism and cell proliferation in cancer cells [6]. Both, resveratrol and combretastatin A4 induce apoptosis and act as antiangiogenic agents [7].

In the present communication, we have synthesized combretastatin A4 (CA4) analogues on steroidal framework. From structure-activity relationship of combretastatin A4, a diaryl system should be separated through a linker group having restricted rotation and one of the aromatic rings should have a 3,4,5-trimethoxyphenyl unit. A cis isomer is more active than the trans isomer. Thus, we designed steroidal stilbenes at 2-position of estradiol unit. In our pharmacophore, one of the phenyl rings is introduced through wittig salt, while ring A of steroidal ring acts as second aryl group to fulfil the requirement of diaryl system. Twenty two steroidal stilbenes have been synthesized and evaluated for anticancer activity against human breast carcinoma cells

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http://dx.doi.org/10.1016/j.jsbmb.2013.02.009

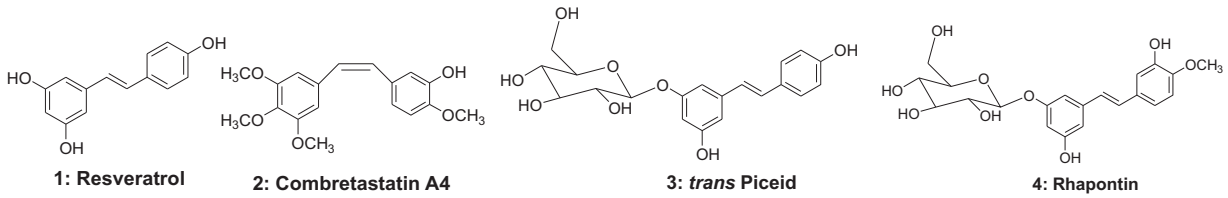

Fig. 1. Structures of plant based stilbenes.

(MCF-7 & MDA-MB-231). Compounds have also been evaluated for in vivo estrogenicity/antiestrogenicity. The analogues with significant cytotoxicity were evaluated for tubulin polymerisation inhibition, induction of lactose dehydrogenase (LDH) and in vivo acute oral toxicity. The anticancer target has also been validated by in-silico docking of lead molecules.

2. Materials and methods

2.1. General procedures

Melting points were determined in open capillaries on E-Z Melt automated melting point apparatus, Stanford Research System, USA and are uncorrected. Merck silica gel thin layer chromatography (TLC, UV_{254nm}) aluminium sheets were used to monitor the reactions and 2% ceric sulphate in 10% aqueous sulphuric acid was used as spraying reagent to visualise the TLC after charring at 80–100 °C. Dry solvents were prepared as per standard methods. Column chromatography was carried out on silica gel (100–200 mesh, Thomas Baker). Solvents were evaporated under reduced pressure in rotavapor. NMR experiments were obtained on Bruker Avance DRX-300 MHz instrument using tetramethylsilane (TMS) as an internal standard and chemical shifts are given in δppm. NMR abbreviations for signal patterns are as; s, singlet; d, doublet; t, triplet, m, multiplet & bs, broad singlet. ^{1}H and ^{13}C spectral data are reported. Electrospray ionization (ESI) mass spectra were recorded on Shimadzu LC–MS after dissolving the compounds in methanol. FT-IR spectra were recorded on Perkin–Elmer SpectrumBX after making KBr pellets. Nomenclature of steroid derivatives has been given as per the recommendations published by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC [8]. Estrone, podophyllotoxin, combretastatin A4, MTT etc. were procured from Sigma-Aldrich USA.

2.2. Chemical synthesis

The detailed initial synthetic steps from estrone to compounds **5**–**7** have already been described earlier [9]. Further steps are described below.

General procedure for the preparation of Wittig salts

Three step preparation. Step-I (Preparation of benzyl alcohols): benzaldehyde derivatives (1 mmol) in methanol (15 mL), sodium borohydride (2 mmol) and stirred at room temperature for 1 h. Usual work-up, quantitative yield.

Step-II (Preparation of benzyl bromides): benzyl alcohols (1 mmol) in dry benzene (15 mL) and phosphorus tribromides (0.5 mL) and stirred at room temperature to get respective benzyl bromides in quantitative yields, usual work-up.

Step-III (Preparation of Wittig salts): Benzyl bromide (1 g) in dry toluene (15 mL). To this tripheylphosphine (1.5 g) was added and the reaction mixture was refluxed for 1–3 h at 120 °C to get the wittig salt as precipitate. It was filtered and washed with dry benzene (yield 62–87%).

General procedure for synthesis of stilbenes **10**, **12**, **13**, **16**, **18**, **19**, **22**, **24**, **25**, **28**–**31** by Wittig reaction

Sodium hydride (150 mg, 6.25 mmol) was first washed with dry petroleum ether (3 × 5 mL) and solvent was decanted. To this dry toluene (6 mL) and benzyltriphenylphosphonium bromide (Wittig salt, 500 mg, 1.16 mmol) were added and stirred at room temperature. After 20 min aldehyde **7** (250 mg, 0.65 mmol) was added to it and the reaction mixture was further stirred at room temperature for 5 h. On completion, it was acidified with dil HCl (5%, 2 mL) and extracted with ethyl acetate (3 × 20 mL). Combined organic layer was washed with water and dried over anhydrous sodium sulphate. The solvent was evaporated in vacuo and residue was purified through silica gel column using hexane–ethyl acetate as eluants. In most of the cases both cis and trans stilbenes were obtained.

2.2.1. Z-2-(2-Phenylvinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**10**)

^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H, 18-CH_3), 2.04 (s, 3H, 17-$OCOCH_3$), 2.19 (s, 3H, 3-$OCOCH_3$), 1.2–1.82 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.83 (bs, 2H, 6-CH_2), 4.64 (t, 1H, 17-CH), 6.40–6.44 (d, 1H, CH-vinyl, *J* = 12 Hz), 6.60–6.64 (d, 1H, CH-vinyl, *J* = 12.3 Hz), 6.75 (s, 1H, 4-CH), 7.12 (s, 1H, 1-CH), 7.18–7.22 (m, 5H, phenyl ring); ^{13}C NMR ($CDCl_3$): δ 12.30, 21.02, 21.31, 23.61, 26.08, 27.36, 27.96, 29.54, 37.23, 38.69, 43.27, 44.21, 50.39, 83.00, 122.41, 125.17, 127.45, 127.54, 127.86, 128.45, 128.45, 129.18, 129.18, 131.81, 137.65, 137.74, 138.05, 146.87, 169.52, 171.21; ESI mass (MeOH): 357 $[M-H]^+$. IR (KBr, cm^{-1}): 2929, 1752, 1733, 1607, 1509, 1245.

2.2.2. Z-2-[2-(3-Methoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**12**)

^{1}H NMR ($CDCl_3$): δ 0.81 (s, 3H, 18-CH_3), 2.02 (s, 3H, 17-$OCOCH_3$), 2.18 (s, 3H, 3-$OCOCH_3$), 0.84–1.85 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.79 (bs, 2H, 6-CH_2), 3.62 (s, 3H, OCH_3), 4.59–4.64 (t, 1H, 17-CH, *J* = 8.3 Hz), 6.38–6.42 (d, 1H, CH-vinyl, *J* = 12 Hz), 6.54–6.58 (d, 1H, CH-vinyl, *J* = 12.3 Hz), 6.69–7.12 (m, 6H, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 12.26, 21.00, 21.28, 23.61, 26.11, 27.37, 27.96, 29.50, 37.24, 38.76, 43.26, 44.20, 50.38, 55.37, 83.00, 113.79, 114.44, 121.84, 122.39, 125.43, 127.62, 127.89, 129.41, 131.76, 137.72, 138.06, 138.87, 146.90, 159.90, 169.47, 171.19; ESI mass (MeOH): 511 $[M+Na]^+$, Negative mode: 487 $[M-H]^-$; IR (KBr, cm^{-1}): 2923, 1761, 1734, 1598, 1246.

2.2.3. E-2-[2-(3-Methoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**13**)

^{1}H NMR ($CDCl_3$): δ 0.82 (s, 3H, 18-CH_3), 2.02 (s, 3H, 17-$OCOCH_3$), 2.33 (s, 3H, 3-$OCOCH_3$), 0.84–2.24 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.86 (bs, 2H, 6-CH_2), 3.82 (s, 3H, OCH_3), 4.65–4.71 (t, 1H, 17-CH, *J* = 8.4 Hz), 6.76 (s, 1H, 1-CH), 7.54 (s, 1H, 4-CH), 6.78–7.27 (m, 6H, 2xCH-vinyl, 4xCH phenyl ring), 6.54–6.58 (d, 1H, CH-vinyl, *J* = 12.3 Hz), 6.69–7.12 (m, 6H, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 12.40, 21.22, 21.39, 23.65, 26.53, 27.41, 28.00, 29.64, 37.34, 38.70, 43.30, 44.43, 50.43, 55.63, 83.04, 112.57, 113.65, 119.62, 122.89, 123.46, 124.07, 127.36, 129.94, 130.30, 138.17, 138.67, 139.44, 146.63, 160.41, 169.71, 171.36; ESI mass (MeOH): 511 $[M+Na]^+$, Negative mode: 487 $[M-H]^-$; IR (KBr, cm^{-1}): 2932, 1751, 1736, 1598, 1496, 1248.

Please cite this article in press as: S. Parihar, et al., Synthesis of combretastatin A4 analogues on steroidal framework and their anti-breast cancer activity, J. Steroid Biochem. Mol. Biol. (2013), http://dx.doi.org/10.1016/j.jsbmb.2013.02.009

*2.2.4. Z-2-[2-(3,4-Dimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**16**)*

^{1}H NMR ($CDCl_3$): δ 0.74 (s, 3H, 18-CH_3), 0.87–1.95 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.09 (s, 3H, 17-OCOCH_3), 2.28 (s, 3H, 3-OCOCH_3), 2.82 (bs, 2H, 6-CH_2), 3.58 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 4.60–4.66 (t, 1H, 17-CH, J = 8.3 Hz), 6.32–6.36 (d, 1H, CH-vinylic, J = 12 Hz), 6.51–6.55 (d, 1H, CH-vinylic, J = 12 Hz), 6.69–7.15 (m, 5H, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 12.28, 20.97, 21.25, 23.60, 26.24, 27.40, 27.96, 29.49, 37.22, 38.83, 43.24, 44.24, 50.37, 55.93, 56.34, 82.96, 111.79, 112.97, 122.43, 123.99, 127.88, 128.28, 130.47, 131.66, 137.50, 138.16, 146.98, 149.07, 169.47, 171.15; ESI mass (MeOH): 541 [M+Na]$^+$, 557 [M+K]$^+$, Negative mode: 517 [M−H]$^-$; IR (KBr, cm^{-1}): 2929, 1752, 1736, 1655, 1510, 1246.

*2.2.5. Z-2-[2-(3,5-Dimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**18**)*

^{1}H NMR ($CDCl_3$): δ 0.78 (s, 3H, 18-CH_3), 0.84–1.91 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.05 (s, 3H, 17-OCOCH_3), 2.19 (s, 3H, 3-OCOCH_3), 2.83 (bs, 2H, 6-CH_2), 3.63 (s, 6H, 2xOCH_3), 4.61–4.67 (t, 1H, 17-CH, J = 8.4 Hz), 6.41–6.45 (d, 1H, CH-vinylic, J = 12 Hz), 6.53–6.57 (d, 1H, CH-vinylic, J = 12 Hz), 6.28–7.17 (m, 5H, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 12.25, 21.02, 21.30, 23.61, 26.15, 27.38, 27.97, 29.47, 37.23, 38.80, 43.24, 44.19, 50.34, 55.49, 55.49, 82.99, 100.48, 107.40, 107.40, 122.37, 125.64, 127.65, 127.93, 131.87, 137.71, 138.03, 139.30, 146.89, 161.01, 161.01, 169.47, 171.20; ESI mass (MeOH): 519 [M+H]$^+$, 541 [M+Na]$^+$, 557 [M+K]$^+$, Negative mode: 517 [M−H]$^-$; IR (KBr, cm^{-1}): 2931, 1761, 1737, 1591, 1510, 1246, 1207, 1157.

*2.2.6. E-2-[2-(3,5-Dimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**19**)*

^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H, 18-CH_3), 1.21–2.34 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.12 (s, 3H, 17-OCOCH_3), 2.34 (s, 3H, 3-OCOCH_3), 2.81 (bs, 2H, 6-CH_2), 3.82 (s, 6H, 2xOCH_3), 4.60–4.66 (t, 1H, 17-CH, J = 8.4 Hz), 6.86–6.91 (d, 1H, CH-vinylic, J = 16.2 Hz), 6.95–7.00 (d, 1H, CH-vinylic, J = 16.5 Hz), 6.32–7.5 (m, 5H, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 12.41, 21.20, 21.37, 23.66, 26.54, 27.41, 28.06, 29.63, 37.35, 38.70, 43.31, 44.41, 50.44, 55.72, 55.72, 83.03, 100.47, 105.32, 105.32, 122.91, 123.70, 124.12, 127.28, 130.40, 138.21, 138.66, 140.02, 146.68, 161.51, 161.51, 169.64, 171.28; ESI mass (MeOH): 519 [M+H]$^+$, 541 [M+Na]$^+$, 557 [M+K]$^+$, Negative mode: 517 [M−H]$^-$; IR (KBr, cm^{-1}): 2937, 1762, 1722, 1593, 1459, 1250, 1205, 1156.

*2.2.7. Z-2-[2-(3,4,5-Trimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**22**)*

^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H, 18-CH_3), 1.17–2.23 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.82 (bs, 2H, 6-CH_2), 3.64 (s, 6H, 2xOCH_3), 3.82 (s, 3H, OCH_3), 4.60–4.65 (t, 1H, 17-CH, J = 8.3 Hz), 6.39–6.43 (d, 1H, CH-vinylic, J = 13 Hz), 6.44 (s, 2H, Ar—H), 6.51–6.55(d, 1H, CH-vinylic, J = 12 Hz), 6.78 (s, 1H, 1-CH), 7.14 (s, 1H, 4-CH); ^{13}C NMR ($CDCl_3$): δ 12.25, 20.96, 21.23, 23.59, 26.23, 27.39, 27.95, 29.47, 37.18, 38.88, 43.21, 44.26, 50.35, 61.07, 82.91, 107.37, 107.37, 122.44, 125.05, 127.92, 128.07, 131.86, 132.64, 137.60, 137.60, 138.09, 147.02, 153.28, 169.41, 171.09; EI mass (MeOH): 548 [M$^+$], 549 [M+1]$^+$; IR (KBr, cm^{-1}): 3449, 2851, 2370, 1736, 1720, 1597, 1509, 1244, 1127.

*2.2.8. Z-2-[2-(3,4-Methylenedioxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**24**)*

^{1}H NMR ($CDCl_3$): δ 0.79 (s, 3H, 18-CH_3), 1.21–1.88 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.05 (s, 3H, 17-OAc), 2.19 (s, 3H, 3-OAc), 2.84 (bs, 2H, 6-CH_2), 4.61–4.67 (t, 1H, 17-CH), 5.87 & 5.91 (two distinct singlets, 2H, 6-CH_2), 6.29–6.32 (d, 1H, CH, vinylic, J = 12 Hz), 6.48–6.52 (d, 1H, CH, vinylic, J = 12.3 Hz), 6.66–7.17 (m, 5H, Ar—H); ^{13}C NMR ($CDCl_3$): δ 12.30, 21.02, 21.30, 23.61, 26.22, 27.35, 27.98, 29.52, 37.32, 38.72, 43.28, 44.24, 50.40, 83.01, 101.16, 108.39, 109.37, 122.45, 123.38, 124.10, 127.61, 127.75, 131.32, 131.68, 137.73, 138.12, 146.83, 147.15, 147.77, 169.47, 171.21; ESI mass (MeOH): 503 [M+H]$^+$, 525 [M+Na]$^+$, 541 [M+K]$^+$, Negative mode: 501 [M−H]$^-$; IR (KBr, cm^{-1}): 2925, 1753, 1723, 1508, 1490, 1243.

*2.2.9. E-2-[2-(3,4-Methylenedioxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**25**)*

^{1}H NMR ($CDCl_3$): δ 0.84 (s, 3H, 18-CH_3), 1.23–2.29 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.05 (s, 3H, 17-OAc), 2.37 (s, 3H, 3-OAc), 2.85 (t, 2H, 6-CH_2, J = 3.6 Hz), 4.67–4.73 (t, 1H, 17-CH, J = 8.4 Hz), 5.97 (s, 2H, —*O*—CH_2—*O*—), 6.3 (d, 1H, CH, vinylic, J = Hz), 6.60 (d, 1H, CH, vinylic, J = Hz), 6.80–7.53 (m, 5H, Ar—H); ^{13}C NMR ($CDCl_3$):δ 12.37, 21.14, 21.28, 23.64, 26.54, 27.43, 28.01, 29.58, 37.39, 38.76, 43.33, 44.45, 50.51, 83.02, 101.42, 106.08, 108.73, 121.57, 121.76, 122.82, 123.84, 127.55, 130.06, 132.64, 137.79, 138.61, 146.57, 147.82, 148.60, 169.54, 171.20; ESI mass (MeOH): 525 [M+Na]$^+$, 541 [M+K]$^+$; IR (KBr, cm^{-1}): 2928, 1735, 1720, 1507, 1498, 1249.

*2.2.10. E-2-[2-(4-Fluorophenyl)vinyl)-estra 1,3,5(10)-trien-3-hydroxy,17-acetate (**28**)*

^{1}H NMR ($CDCl_3$): δ 0.85 (s, 3H, 18-CH_3), 1.24–2.39 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.07 (s, 3H, 17-OAc), 2.82 (bs, 2H, 6-CH_2), 4.68–4.73 (t, 1H, 17-CH, J = 8.4 Hz), 6.53 (s, 1H, 1-CH), 7.02–7.07 (d, 1H, CH, vinylic, J = 16.2 Hz), 7.04 (s, 1H, 4-CH), 7.21–7.19 (d, 1H, CH, vinylic, J = 15.9 Hz), 7.44–7.51 (m, 4H, Ar—H); ESI mass (MeOH): 459 [M+Na]$^+$.

*2.2.11. Z-2-[2-(4-Methylphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**29**)*

^{1}H NMR ($CDCl_3$): δ 0.78 (s, 3H, 18-CH_3), 1.08–2.21 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.26 (s, 3H, 4′-CH_3), 2.70 (bs, 2H, 6-CH_2), 3.64–3.69 (t, 1H, 17-CH, J = 8.3 Hz), 6.93–7.31 (m, 8H, Ar—H, 2xCH vinylic); ^{13}C NMR ($CDCl_3$):δ 11.45, 21.49, 23.55, 26.83, 27.62, 29.73, 30.03, 31.01, 37.19, 39.36, 43.70, 44.38, 56.64, 82.37, 116.36, 122.79, 123.21, 124.41, 126.73, 126.73, 129.47, 129.67, 129.67, 133.35, 135.65, 137.49, 137.91, 151.54; ESI mass (MeOH): 427 [M+K]$^+$; IR (KBr, cm^{-1}): 3398, 2926, 1510, 1422, 1281, 1051.

*2.2.12. E-2-[2-(4-Methylphenyl)vinyl)-estra 1,3,5(10)-trien-3-hydroxy,17-acetate (**30**)*

^{1}H NMR ($CDCl_3$): δ 0.84 (s, 3H, 18-CH_3), 1.25–2.35 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.07 (s, 3H, 17-OAc), 2.35 (s, 3H, 4′-CH_3), 2.81 (bs, 2H, 6-CH_2), 4.67–4.72 (t, 1H, 17-CH, J = 8.4 Hz), 6.54 (s, 1H, 1-CH), 7.01–7.06 (d, 1H, CH, vinylic, J = 16.2 Hz), 7.14–7.17 (d, 2H, 3′ & 5′-CH of aromatic ring, J = 8.1 Hz), 7.24–7.29 (d, 1H, CH, vinylic, J = 16.2 Hz), 7.40 (s, 1H, 4-CH), 7.40–7.43 (d, 2H, 2′ & 6′-CH of aromatic ring); ^{13}C NMR ($CDCl_3$): δ 12.43, 21.40, 21.48, 23.66, 26.72, 27.60, 28.01, 29.69, 30.02, 37.38, 39.10, 43.37, 44.23, 50.39, 83.22, 116.37, 122.82, 123.21, 124.49, 126.73, 126.73, 129.67, 129.67, 133.27, 135.64, 137.50, 137.83, 151.55, 171.59; ESI mass (MeOH): 431 [M+H]$^+$; IR (KBr, cm^{-1}): 3448, 2930, 1702, 1510, 1422, 1276, 1249.

*2.2.13. Z-2-[2-(2-Methoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diacetate (**31**)*

^{1}H NMR ($CDCl_3$): δ 0.75 (s, 3H, 18-CH_3), 0.84–2.25 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.04 (s, 3H, 17-OAc), 2.21 (s, 3H, 3-OAc), 2.79 (bs, 2H, 6-CH_2), 3.83 (s, 3H, OCH_3), 4.60–4.66 (t, 1H, 17-CH, J = 8.4 Hz), 6.44–6.48 (d, 1H, CH, vinylic), 6.71 (s, 1H, 1-CH), 7.09 (s, 1H, 4-CH), 6.74–7.19 (m, 5H, 4-Ar—H & CH vinylic); ^{13}C NMR ($CDCl_3$): δ 11.67, 20.52, 20.89, 22.92, 25.28, 26.65, 27.20,

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28.90, 36.47, 37.85, 42.54, 43.44, 49.48, 55.13, 82.38, 110.10, 120.03, 121.63, 124.10, 125.88, 126.75, 126.81, 127.21, 128.27, 128.70, 136.85, 137.21, 145.93, 156.75, 169.36, 170.98; ESI mass (MeOH): 487 $[M-H]^+$, 488 $[M^+]$, 527 $[M+K]^+$; IR (KBr, cm^{-1}): 2930, 1736, 1719, 1578, 1544, 1246, 1209.

General procedure for hydrolysis of acetylated steroidal stilbenes to 3, 17-dihydroxy derivatives (**11**, **14**, **15**, **17**, **20**, **21**, **23**, **26** & **27**)

100 mg of 3,17-diacetylated steroidal stilbene derivative was taken in 5 mL of 1% aqueous methanolic KOH (1:1). The reaction mixture was heated for 45 min at 60 °C. On completion, the reaction mixture was poured in water, acidified with dil. HCl (5%) and extracted with ethyl acetate (3 × 10 mL). The combined organic layer was dried over anhydrous sodium sulphate and dried in vacuo. The residue thus obtained was recrystallised with chloroform:hexane (1:5) to get the authentic hydrolysed product.

*2.2.14. E-2-(2-Phenylvinyl)-estra 1,3,5(10)-trien-3,17-diol (**11**)*

^{1}H NMR ($CDCl_3$):δ 0.79 (s, 3H, 18-CH_3), 0.81–2.39 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.82 (bs, 2H, 6-CH_2), 3.76 (t, 1H, 17-CH, J = 8.4 Hz), 6.55 (s, 1H, 1-CH), 7.1 (d, 1H, CH-vinyl, J = 16.5 Hz), 7.31–7.54 (m, 7H, 4-CH, vinyl-CH & Ar—H of phenyl ring; ^{13}C NMR ($CDCl_3$): δ 11.41, 23.49, 27.59, 29.72, 30.46, 31.06, 37.21, 39.35, 43.70, 44.38, 50.67, 82.31, 116.34, 117.36, 124.19, 124.58, 126.79, 127.65, 127.65, 128.93, 129.61, 129.61, 133.16, 138.18, 138.38, 151.52; ESI mass (MeOH): 373 $[M-H]^+$.IR (KBr, cm^{-1}): 3449, 2927, 1509, 1422, 1249, 1051.

*2.2.15. Z-2-[2-(3-Methoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**14**)*

^{1}H NMR ($CDCl_3$): δ 0.79 (s, 3H, 18-CH_3), 1.16–2.15 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.81 (bs, 2H, 6-CH_2), 3.69 (s, 3H, OCH_3), 3.69–3.74 (t, 1H, 17-CH, J = 8.6 Hz), 6.54–7.18 (m, 8H, 2xCH-vinyl, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 11.35, 23.54, 26.64, 27.61, 29.69, 31.07, 37.16, 39.41, 43.68, 44.29, 50.69, 55.34, 82.29, 114.16, 114.44, 116.04, 121.65, 121.82, 125.59, 126.95, 129.54, 129.68, 132.25, 133.11, 138.33, 150.80, 160.05; ESI mass (MeOH): 831 $[2M+Na]^+$, Negative mode: 403 $[M-H]^-$; IR (KBr, cm^{-1}): 3485, 2928, 1606, 1508, 1263, 1051.

*2.2.16. E-2-[2-(3-Methoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**15**)*

^{1}H NMR ($CDCl_3$): δ 0.80 (s, 3H, 18-CH_3), 0.96–2.42 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.79 (bs, 2H, 6-CH_2), 3.83 (s, 3H, OCH_3), 3.72–3.78 (t, 1H, 17-CH, J = 8.3 Hz), 6.53 (s, 1H, 1-CH), 7.23–7.28 (d, 1H, CH-vinylic, J = 15 Hz), 7.32–7.37 (d, 1H, CH-vinylic, J = 16.5 Hz), 6.78–7.25 (m, 5H, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 11.40, 23.53, 26.82, 27.58, 29.70, 31.02, 37.20, 39.35, 43.70, 44.37, 50.67, 55.64, 82.34, 112.22, 113.61, 114.15, 116.38, 119.61, 122.54, 124.59, 129.46, 129.85, 133.49, 138.23, 139.90, 151.59, 160.45; ESI mass (MeOH): 405 $[M+H]^+$, 427 $[M+Na]^+$, Negative mode: 403 $[M-H]^-$; IR (KBr, cm^{-1}): 3448, 2928, 1654, 1598, 1509, 1283, 1156.

*2.2.17. Z-2-[2-(3,4-Dimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**17**)*

^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H, 18-CH_3), 0.79–2.33 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.79 (bs, 2H, 6-CH_2), 3.57 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.69–3.75 (t, 1H, 17-CH, J = 8.4 Hz), 6.44–6.48 (d, 1H, CH-vinylic, J = 12 Hz), 6.61–6.65 (d, 1H, CH-vinylic, J = 12.3 Hz), 6.54–7.18 (m, 5H, 1-CH, 4-CH & Ar—H); ^{13}C NMR ($CDCl_3$): δ 11.37, 23.53, 26.76, 27.64, 29.70, 31.04, 37.16, 39.47, 43.67, 44.35, 50.67, 55.85, 56.27, 82.27, 111.86, 112.35, 115.92, 122.00, 122.61, 123.48, 126.79, 129.76, 132.49,133.14, 138.16,149.29, 150.78; ESI mass (MeOH): 457 $[M+Na]^+$, Negative mode: 433 $[M-H]^-$; IR (KBr, cm^{-1}): 3448, 2928, 1619, 1510, 1262, 1138.

*2.2.18. Z-2-[2-(3,5-Dimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**20**)*

^{1}H NMR ($CDCl_3$): δ 0.76 (s, 3H, 18-CH_3), 0.86–2.12 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.79 (bs, 2H, 6-CH_2), 3.69 (s, 6H, 2xOCH_3), 3.73 (t, 1H, 17-CH), 6.31–7.30 (m, 7H, 2xCH-vinylic, 5xArH); ^{13}C NMR ($CDCl_3$):δ 11.35, 23.53, 26.65, 27.62, 29.68, 30.99, 37.13, 39.42, 43.65, 44.27, 50.61, 55.49, 55.49, 82.30, 101.02, 105.13, 116.02, 116.02, 121.68, 125.84, 126.98, 132.28, 133.04, 138.31, 138.72, 150.85, 161.13, 161.49; EI mass (MeOH): 434 $[M^+]$, 435 $[M+1]^+$; IR (KBr, cm^{-1}): 3485, 3401, 2929, 1578, 1510, 1251, 1054.

*2.2.19. E-2-[2-(3,5-Dimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**21**)*

^{1}H NMR ($CDCl_3$): δ 0.78 (s, 3H, 18-CH_3), 0.80–2.30 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.70 (bs, 2H, 6-CH_2), 3.54 (s, 3H, OCH_3), 3.70 (s, 3H, 2xOCH_3), 3.65 (t, 1H, 17-CH, J = 8.7 Hz), 6.95–6.89 (d, 1H, CH-vinylic, J = 16.5 Hz), 7.23–7.29 (d, 1H, CH-vinylic, J = 16.5 Hz), 6.23 (s, 1H, 1-CH), 7.54 (s, 1H, 4-CH), 6.24 (s, 1H, Ar—H), 6.51 (s, 1H, Ar—H), 7.02 (s, 1H, Ar—H); ^{13}C NMR ($CDCl_3$): δ 11.44, 23.53, 26.82, 27.59, 29.73, 30.01, 37.17, 39.33, 43.68, 44.35, 50.62, 55.50, 55.75, 82.33, 105.09, 107.23, 116.02, 116.40, 124.80, 125.86, 126.99, 132.23, 138.27, 140.52, 151.73, 161.09, 161.45; ESI mass (MeOH): 435 $[M+H]^+$, 457 $[M+Na]^+$, 473 $[M+K]^+$, Negative mode: 433 $[M-H]^-$; IR (KBr, cm^{-1}): 3449, 2929, 1594, 1459, 1153, 1058.

*2.2.20. Z-2-[2-(3,4,5-Trimethoxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**23**)*

^{1}H NMR ($CDCl_3$):δ 0.76 (s, 3H, 18-CH_3), 1.20–2.17 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.79 (bs, 2H, 6-CH_2), 3.63 (s, 6H, 2xOCH_3), 3.70 (t, 1H, 17-CH), 3.81 (s, 3H, OCH_3), 6.47 (s, 2H, Ar—H), 6.54–6.61 (m, 2H, vinylic —CHx2), 6.74 (s, 1H, 4-CH); ^{13}C NMR ($CDCl_3$):δ 11.37, 23.51, 26.73, 27.64, 29.70, 29.99, 30.98, 37.10, 39.48, 43.63, 44.33, 50.60, 56.29, 56.29, 61.13, 82.25, 107.06, 107.06, 115.94, 121.84, 124.70, 126.83, 132.11, 132.49, 133.08, 138.24, 138.76, 150.90, 153.38, 153.38; ESI mass (MeOH): 565 $[M+H]^+$, 487 $[M+Na]^+$, Negative mode: 463 $[M-H]^-$; IR (KBr, cm^{-1}): 3448, 3423, 2928, 2371, 1579, 1509, 1499, 1238, 1127.

*2.2.21. Z-2-[2-(3,4-Methylenedioxyphenyl)vinyl]-estra 1,3,5(10)-trien-3,17-diol (**26**)*

^{1}H NMR ($CDCl_3$): δ 0.79 (s, 3H, 18-CH_3), 1.17–2.19 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.82 (t, 2H, 6-CH_2), 3.72 (t, 1H, 17-CH, J = 8.4 Hz), 5.91 (s, 2H, —*O*—CH_2—*O*—), 6.41–6.45 (d, 1H, CH, vinylic, J = 12 Hz), 6.58–6.63 (d, 1H, CH, vinylic, J = 12.3 Hz), 6.58–7.10 (m, 5H, Ar—H), 6.60 (d, 1H, CH, vinylic, J = Hz), 6.80–7.53 (m, 5H, Ar—H); ^{13}C NMR ($CDCl_3$):δ 11.43, 23.55, 26.67, 27.60, 29.75, 31.03, 37.15, 39.29, 43.67, 44.27, 50.58, 82.32, 101.36, 108.65, 108.84, 116.04, 122.57, 123.68, 124.30, 126.87, 130.95, 131.76, 133.09, 138.32, 147.68, 148.02, 150.60; ESI mass (MeOH): 419 $[M+H]^+$, 457 $[M+K]^+$, Negative mode: 417 $[M-H]^-$; IR (KBr, cm^{-1}): 3424, 2924, 1501, 1489, 1249, 1040.

*2.2.22. E-2-[2-(3,4-Methylenedioxyphenyl)vinyl)-estra 1,3,5(10)-trien-3,17-diol (**27**)*

^{1}H NMR ($CDCl_3$): δ 0.80 (s, 3H, 18-CH_3), 1.15–2.08 (m, 13H, rest of the 5xCH_2 and 3xCH of steroidal ring), 2.60 (bs, 2H, 6-CH_2), 3.69 (t, 1H, 17-CH), 5.99 (s, 2H, —*O*—CH_2—*O*—), 7.14–7.19 (d, 1H, CH, vinylic, J = 15.9 Hz), 7.31–7.37 (d, 1H, CH, vinylic, J = 16.5 Hz), 6.40 (s, 1H, 1-CH), 6.59 (d, 1H, 6-CH of Aromatic, methylenedioxy ring), 6.81 (d, 1H, 5-CH of methylenedioxy ring), 6.99 (s, 1H, 4-CH), 7.30 (s, 1H, 2-CH of methylenedioxy ring), 8.10 (s, 1H, exchangeable, phenolic OH); ^{13}C NMR (Acetone-d6): δ 11.12, 23.36, 26.84, 27.62, 28.43, 30.68, 37.37, 39.62, 43.62, 44.48, 50.70, 81.46, 101.41, 105.72, 108.51, 116.20, 121.17, 122.54, 123.23, 123.37, 125.35, 132.40, 133.73, 137.54, 147.35, 148.68, 152.95; ESI mass (MeOH):

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418 [M^+], Negative mode: 417 [M−H]$^-$; IR (KBr, cm^{-1}): 3424, 2926, 1502, 1490, 1249, 1040.

2.3. Pharmacology

2.3.1. Cell culture

Human breast cancer cell lines, MCF-7 and MDA-MB-231 were originally obtained from American type of cell culture collection (ATCC), USA and stock were maintained in laboratory. HEK-293 cells were obtained from institutional cell repository of animal tissue culture facility (CSIR-CDRI). Cells were grown in tissue culture flasks in DMEM (Dulbecco modified Eagle medium, Sigma) supplemented with 10% foetal bovine serum with 1X stabilized antibiotic-antimycotic solution (Sigma) in a CO_2 incubator (Sanyo, Japan) at 37 °C with 5% CO_2 and 90% relative humidity. The cells at subconfluent stage were harvested with 1X porcine pancreatic trypsin (Sigma) and seeded in required density in tissue culture plates for assay.

2.3.2. In vitro cell inhibition assay

The cell inhibiting activity of the compounds of the series were determined using MTT assay as described earlier [10]. In brief, cells were seeded @ 1×10^4 cells/well in each well of 96-well microculture plates in 200 μl DMEM (Sigma), supplemented with 10% FBS and 1× stabilized antibiotic–antimycotic solution (Sigma) and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds were diluted to the desired concentrations in culture medium DMEM without phenol red, supplemented with 0.5% FBS, and were added to the wells with respective vehicle control. After 24 h of incubation, 20 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Sigma) was added to each well and the plates were further incubated for 3 h. Then the supernatant from each well was carefully removed without disturbing the formazan crystals. The formazan crystals were dissolved in 200 μL of dimethyl sulphoxide (DMSO) using plate shaker (Biosan) and absorbance at 570 nm wavelength was recorded in a microplate reader (Microquant; BioTek).

2.3.3. Cell cycle analysis

Cell cycle analysis using PI-staining of cells was carried out as per earlier reported method [11]. MCF-7 cells were plated and treated with 10 μM of compound **22** for 24 h, and harvested. For flowcytometry analysis, collected cells were washed with cold PBS, resuspended at the rate of 2×10^6 cells/mL, fixed in absolute ethanol, treated with RNase A (10 mg/mL), and then stained with propidium iodide (50 μg/mL; Sigma, St. Louis, MO, USA) for 30 min at room temperature. The DNA content of the cells was measured using a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) and CellQuest software.

2.3.4. LDH release assay

Colorimetric assay to measure LDH was performed following already reported method [12]. Briefly, cells were treated with 1 μM, 3 μM and 10 μM of compound **22** with vehicle control wells for 24 h, the media following treatment were collected and LDH release measured using commercially available LDH Cytotoxicity Detection kits (Cat 11 644 793 001; Roche Diagnostics, USA) as per manufacturers' instruction.

2.3.5. Tubulin polymerisation inhibition assay

Tubulin polymerization experiment was done as per reported method using 'assay kit' from Cytoskeleton, USA [13,14]. In brief, tubulin protein (3 mg/mL) in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP and 15% glycerol) was placed in pre-warmed 96-well microtiter plates at 37 °C in the presence of test compounds with variable concentrations. All samples were mixed well and polymerization was monitored kinetically at 340 nm every min for 1 h using Spectramax plate reader. Podophyllotoxin (PDT) was used as standard inhibitor of tubulin polymerase and DMSO as negative control. The IC_{50} value was determined from dose-dependent analysis and is defined as the concentration that inhibits the rate of polymerization by 50%.

2.3.6. In vivo oestrogenicity

Twenty-one days old immature female Sprague–Dawley rats were bilaterally ovariectomized under light ether anaesthesia and after post-operative rest for 7 days were randomized into different treatment groups. Each group comprised of four to five animals. Each rat received the compound once daily for three consecutive days by oral route. A separate group of animals received only the vehicle for similar duration served as control. The rats were sacrificed 24 h after the last treatment and stripped of adipose tissue and luminal tissue was removed by blotting onto absorbent paper then they were weighed. Increase in uterine fresh weight was taken as parameters for evaluation of oestrogen agonistic activity in comparison to rats of vehicle control group. The objective was to evaluate oestrogen agonistic effect of the compounds on the uterus [15].

2.3.7. In vivo anti-oestrogenicity

Twenty-one days old immature female Sprague–Dawley rats were bilaterally ovariectomized under light ether anaesthesia and after post-operative rest for 7 days were randomized into different treatment groups. Each group comprised of four or five animals. Each rat received the compound of the invention and 0.02 mg/kg dose of 17α-ethynylestradiol in 10% ethanol–distilled water once daily for three consecutive days by oral route. A separate group of animals receiving only 17α-ethynylestradiol (0.02 mg/kg) in 10% ethanol–distilled water for similar duration was used for comparison. During autopsy uterus was carefully excised, gently blotted and weighed. Inhibition in ethynylestradiol induced increase in uterine fresh weight was taken as parameters for evaluation of oestrogen antagonistic effect of the compounds [15].

2.3.8. In vivo acute oral toxicity

The acute oral toxicity of the best analogue of the series i.e. **22** was carried out in Swiss albino mice. Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). For this experiment, 20 mice (10 male and 10 female) were taken and divided into four groups comprising 2 male and 2 female mice in each group weighing between 17 and 24 g. The animals were maintained at 22 ± 5 °C with humidity control and also on an automatic dark and light cycle of 12 h. The animals were fed with the standard mice feed and drinking water was provided ad libitum. Mice of group 1 were kept as control and animals of groups 2, 3, 4 and 5 were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. The test compound was solubilized in minimum volume of ethanol (few drops) and then suspended in carboxymethyl cellulose (0.7%) and was given at 5, 50, 300 and 1000 mg/kg body weight to animals of groups 2, 3, 4 and 5 respectively. Control animals received only vehicle.

The animals were checked for mortality and any signs of ill health at hourly interval on the day of administration of drug. There after a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection, respiratory patterns etc. Also changes in gait, posture and response to handling were also recorded [16]. In addition to observational study, body weights were recorded and blood and serum samples were collected from all the animals on 7th day of

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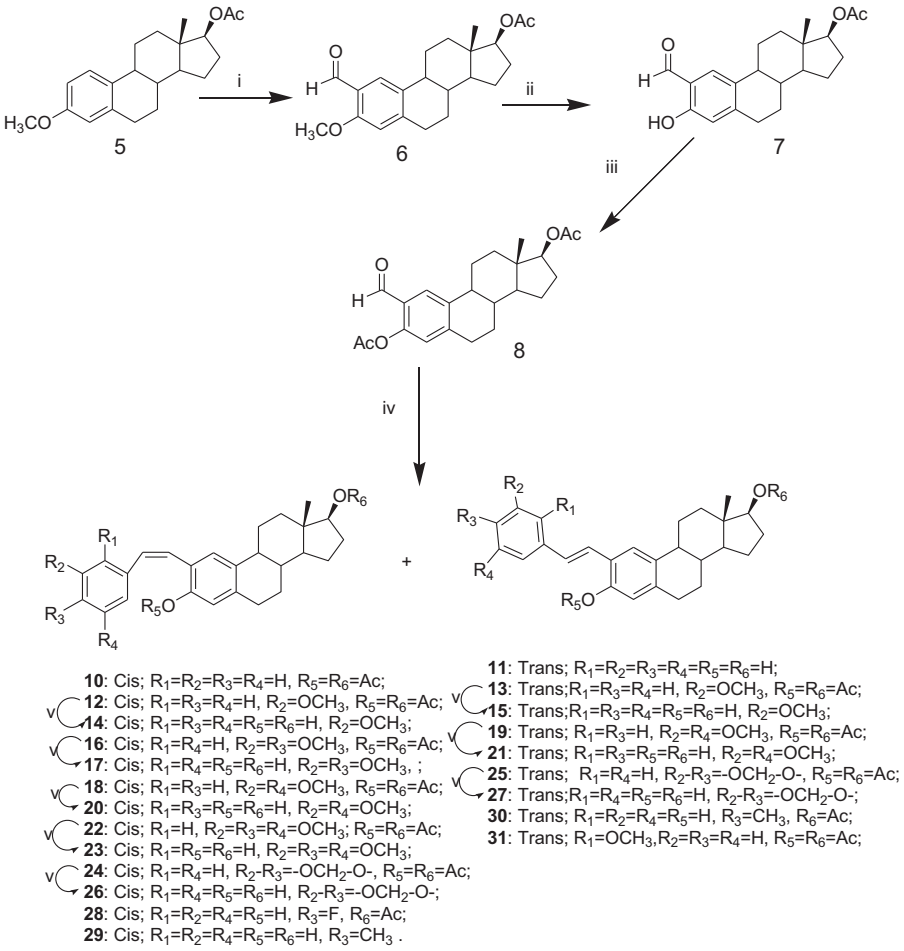

Scheme 1. Reagents and conditions: (i) DMF-$POCl_3$, 0–10 °C, 1 h, then 120 °C for 4 h, 72%; (ii) Anhydrous $AlCl_3$–Dichloromethane, RT, 4 h, 81%; (iii) Ac_2O, dry pyridine, RT, overnight, 93%; (iv) NaH, dry toluene, various Wittig salts, RT, 6–16 h, cis %, trans %; (v) 0.5–1% aqueous methanolic KOH (1:1), 60 °C, 30–45 min.

the experiment in acute oral toxicity. The samples were analysed for total RBC, WBC, differential leucocytes count, haemoglobin percentage and biochemical parameters like ALKP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, serum protein, tissue protein, malonaldehyde and reduced GSH activity. The animals were then sacrificed and were necropsed for any gross pathological changes. Weights of vital organs like liver, heart, kidney were recorded [17].

2.3.9. In-silico studies

The chemical structures of the molecules were constructed using the ChemDraw Ultra. Energy minimization of the compounds with cleaned geometries was achieved through the 'ChemDraw 3D ultra pro'. The augmented molecular mechanics (MM2/MM3) parameter was used to optimize the energy of each molecule up to its lowest stable energy state. This energy minimization process was performed until the energy change was less than 0.001 kcal/mol or the molecules had been updated almost 300 times [18].

The 3D chemical structures of known drugs were retrieved from the PubChem compound database at NCBI (http://www.pubchem.ncbi.nlm.nih.gov). Crystallographic 3D structures of target proteins were retrieved from the Brookhaven Protein Databank (http://www.pdb.org). Hydrogen atoms were added to the protein targets to achieve the correct ionization and tautomeric states of amino acid residues such as His, Asp, Ser, and Glu. Molecular docking of the compounds against selected target was achieved using the 'AutoDock Vina'. To perform the automated docking of ligands into the active sites, we used a Lamarkian genetic algorithm.

2.3.10. Statistical analysis

Data were expressed as means. Statistical analysis was done by ANOVA and Newman Keul's test. A value of p values <0.05 were considered a statistically significant difference.

3. Results

3.1. Chemistry

The synthetic strategy was as depicted in Scheme 1. Estrone was used as the starting compound and gallic acid was used to form wittig salt. The initial synthetic steps up from estrone to compounds **5**–**7** have already been described earlier [9]. Briefly, estradiol 3-methyl ether 17-acetate (**5**) was formylated at 2-position by Vilsmeier reaction using dry dimethylformamide-phosphorus oxychloride (DMF-$POCl_3$) to afford 2-formyl estradiol 3-methyl ether 17-acetate (**6**) which was further demethylated using $AlCl_3$–DCM system to get 2-formyl estradiol 17-acetate (**7**) in good yields. Compound **7** was acetylated with acetic anhydride in dry pyridine to get 2-formyl estradiol 3,17-diacetate (**8**). On the other hand, gallic acid was methylated with dimethylsulphate to get 3,4,5-trimethoxybenzoic acid methyl ester. This ester was reduced to 3,4,5-trimethoxybenzyl alcohol by using lithium borohydride in methanol at room temperature. 3,4,5-trimethoxybenzyl alcohol was treated with phosphorus tribromide in dry benzene to

Table 1
Cell inhibiting and tubulin polymerization inhibition activity of steroidal stilbene series of compounds.

Compound	Yield (%)[a]	M.P. (°C)	Human cancer cell lines IC_{50} (μM)[b]		Human kidney cell lines IC_{50} (μM)	Tubulin polymerization inhibition IC_{50} (μM)[c]
			MCF-7 (breast)	MDA-MB-231 (breast)	HEK-293 (kidney)	
10	41	158–60	39.27	54.15	50	1.34
11	39	193–95	12.29	11.62	n.d.	Inactive
12	44	Oil	93.7	56	>100	Inactive
13	37	75–77	Inactive	Inactive	>100	Inactive
14	83	125–28	Inactive	11.22	n.d.	Inactive
15	87	204–06	10.00	41.47	n.d.	Inactive
16	40	85–87	34.1	Inactive	>100	Inactive
17	84	88–90	Inactive	40	>100	Inactive
18	39	140–42	Inactive	13.83	>100	0.81
19	36	156–58	Inactive	Inactive	~100	Inactive
20	86	98–100	Inactive	Inactive	>100	Inactive
21	79	135–37	Inactive	Inactive	—	Inactive
22	83	176–78	7.5	5.5	~100	0.96
23	89	60–62	38.3	7.3	>100	Inactive
24	34	130–32	97.48	Inactive	n.d.	Inactive
25	37	86–88	Inactive	Inactive	n.d.	Inactive
26	77	138–40	Inactive	58.26	>100	Inactive
27	82	120–22	12.35	12.55	n.d.	Inactive
28	64	Oil	Inactive	Inactive	n.d.	n.d.
29	39	123–25	Inactive	Inactive	>100	Inactive
30	44	168–70	Inactive	44.43	~100	Inactive
31	63	142–45	Inactive	31.2	>100	???
Podophyllotoxin	–	–	64.99	35.7	50	0.74
Paclitaxel	–	–	0.0025	0.0024	n.d.	–
Tamoxifen	–	–	9.00	10	n.d.	–
Combretastatin A4	–	–	0.01	0.11	78.2	1.2[d]

n.d.-not determined.
[a] Isolated yields.
[b] Considered inactive if IC_{50} > 100 μM.
[c] Inactive if IC_{50} > 5.0 μM.
[d] Ref. no. [19].

get 3,4,5-trimethoxybenzyl bromide. 3,4,5-trimethoxybenzyl bromide was treated with triphenyl phosphine in dry toluene to get the Witting salt with overall 58% yield in four steps. Similarly various other Wittig salts were prepared. Finally, the steroidal aldehyde (**8**) was treated with Wittig salts in presence of sodium hydride in dry toluene to get the cis and trans steroidal stilbenes (**10**–**31**) in moderate to good yield. The acetylated cis & trans stilbenes were hydrolysed with 1% aqueous alkaline KOH to get the 3-hydroxy stilbenes. All the compounds were confirmed by spectroscopy.

3.2. Biology

All the steroidal stilbenes (**10–31**) were evaluated for cytotoxicity against human breast cancer cell lines MCF-7 and MDA-MB-231 (Table 1) by MTT assay. Among these stilbene analogues only fourteen compounds possessed significant anti-cancer activity and rest eight analogues were found inactive (IC_{50} > 100 μM). Analogues **11**, **15, 22** and **27** possessed potent anticancer activity ($IC_{50} \leq 12$ μM) against MCF-7 cell lines, while compounds **11**, **14, 18**, **22** and **23** exhibited strong cytotoxicities against MDA-MB-231 cell lines. Compounds **11** and **22** showed high activity against both the cell lines. Among these, compound **22** was the most active in both MCF-7 and MDA-MB-231 cells with IC_{50} of 7.5 μM and 5.5 μM respectively. **11** showed activity against MCF-7 and MDA-MB-231 cells with IC_{50} 22.29 μM and 11.62 μM respectively. The cytotoxicities of **11** and **22** were better than podophyllotoxin and comparable with tamoxifen, but much below the paclitaxel. The significant cellular death induced by **22** as observed under phase contrast microscopy has been given in Fig. 2.

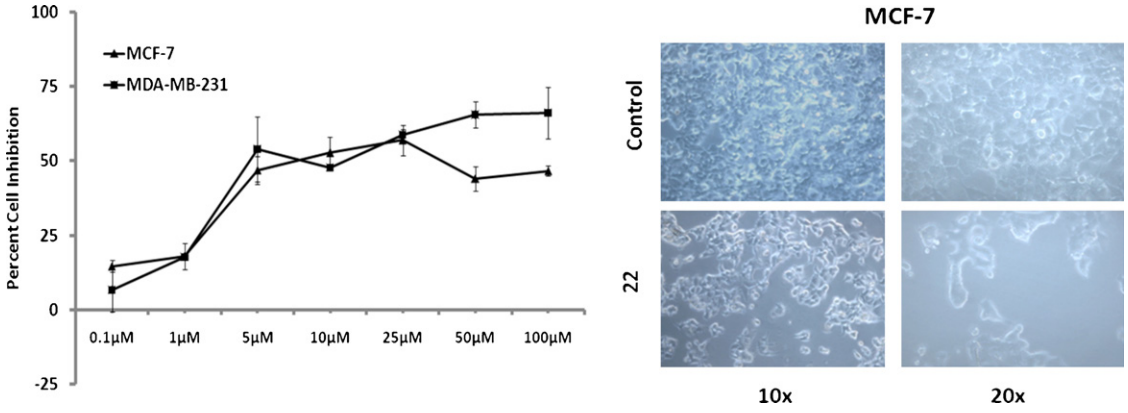

Fig. 2. Cell inhibiting activity of **22**; (a) plotted as percent cancer cell inhibition measured by MTT assay with various concentrations, and (b) observed with phase contrast microscopy. MCF-7 cells were seeded in T25 flask treated with 1 μM of compound **22** and vehicle control for 24 h.

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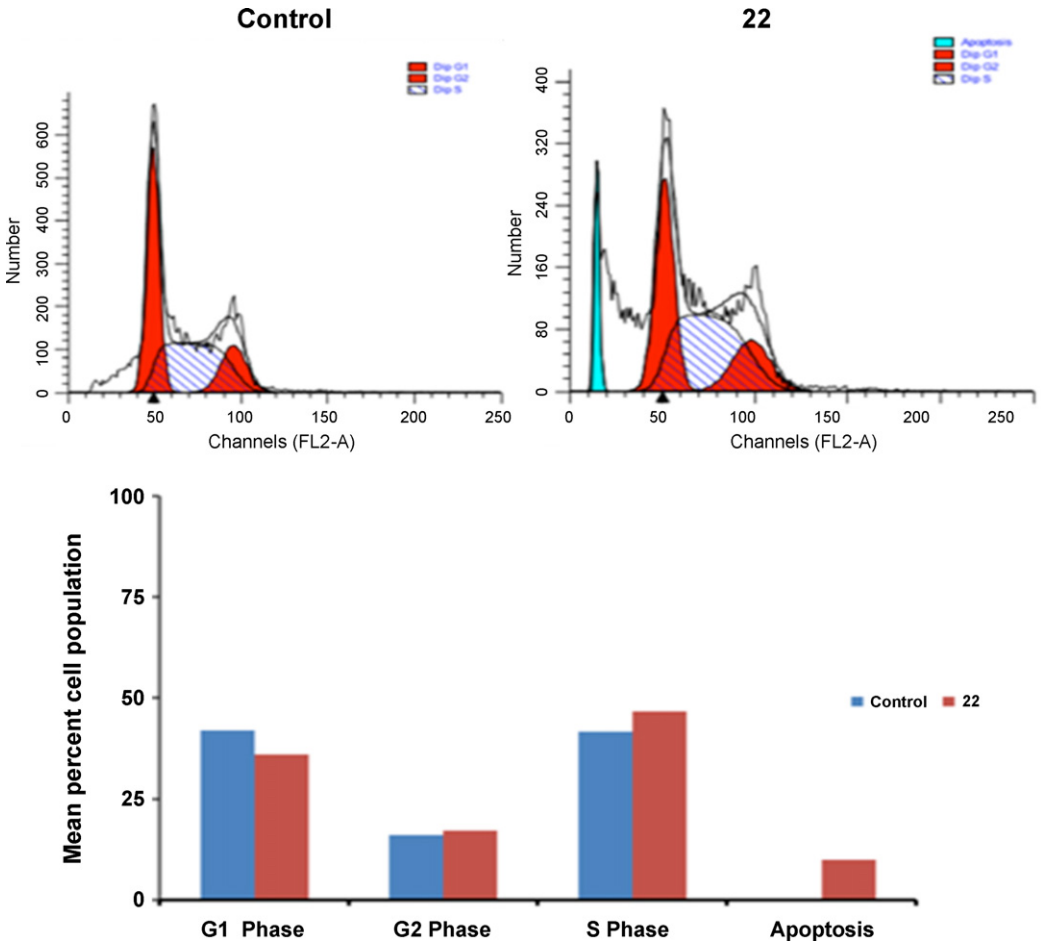

Fig. 3. Effect of **22** in cell cycle phases of MCF-7 cells. Cells were seeded in T25 flask treated with 10 μM of **22** and vehicle control for 24 h, stained with propidium iodide (PI) and recorded with flowcytometer.

In terms of antitubulin activity of these compounds, compound **18** was most efficient with IC_{50} of 0.81 μM followed by **22** (IC_{50} = 0.96 μM) and **10** (IC_{50} = 1.34 μM). But, the antitubulin effect of these compounds was less than the standard compound podophyllotoxin (IC_{50} = 0.74 μM). Rest of the compounds failed to show significant tubulin polymerizing inhibition (IC_{50} > 5.0 μM) (Table 1).

We established structure-activity relationship of these analogues for the cytotoxicity against MCF-7 cell lines. In 3-acetyl estradiol series (**10**, **12**, **13**, **16**, **19**, **22**, **25** and **31**), cis analogues were more active than their trans counterparts. But, it was reversed in case of 3-hydroxy estradiol series (**11**, **14**, **15**, **17**, **20**, **21**, **23**, **26** and **27**–**29**) here trans analogues were more active than their cis isomers. It might be due to better binding of 3-hydroxy derivatives with oestrogen receptor which is essentially required for binding. Also, cis analogues might create hindrance for oestrogen receptor binding due to presence of bulky phenyl group at 2-position as compared to trans analogues which will be away from 3-hydroxy of estradiol unit.

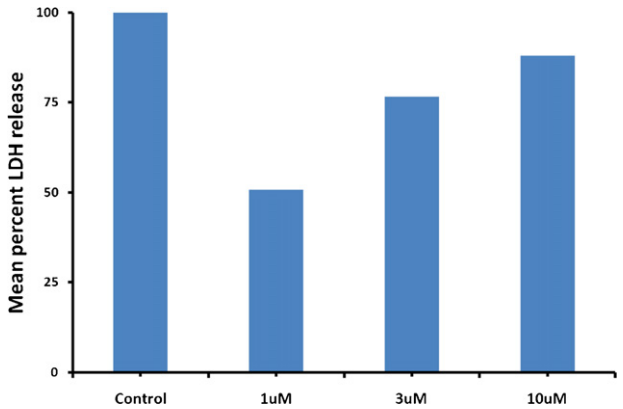

Fig. 4. LDH release induced by **22**. MCF-7 cells were seeded in T25 flask treated with 1 μM, 3 μM and 10 μM of **22** and vehicle control for 24 h, supernatant was estimated for released LDH.

In 3-acetyl series phenyl ring without any substitution exhibited moderate cytotoxicity (**10**, 39.27 μM). On introducing one methoxy group to this phenyl, cytotoxicity was reduced drastically (**12**, 93.7 μM), but a dimethoxylated analogue exhibited improved activity (**16**, 34.1 μM). Cytotoxicity was further improved in case of 3,4,5-trimethoxylated analogue (**22**, 7.5 μM). But, on introducing other groups like 4-methyl (**28**) or 3,4-methylenedioxy (**24**, 97.48 μM) groups at phenyl ring, analogues lost the activity.

22 caused cell cycle arrest at G2 phase in MCF-7 cells at 10 μM concentration (Fig. 3). At 24 h time point, the majority of arrested cells in G2/M possibly due to defective mitotic spindle formation entered into apoptosis. Therefore, the low arrest in G2/M phase due to compound **22** in comparison to vehicle control may corroborate with significantly higher induced sub-diploid population which denotes cells undergone apoptosis. Further, **22** induced release of LDH from MCF-7 cells in a concentration-dependent manner. This indicates higher concentrations induced significant cancer cell membrane damage (Fig. 4).

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Table 2
Estrogenicity, antiestrogenicity of steroidal stilbenes in mice.

Compound code	Dose (mg/Kg)	E uterine weight (in mg)* Mean ± S.E.	AE uterine weight (in mg)* Mean ± S.E.	%Gain**	%Inhibition***
10	10	34.04 ± 2.67[g]	77.5 ± 1.04[a,b]	13.6	56.43
Control	–	29.96 ± 0.29	–		
EE	0.02	–	139.2 ± 1.2		
11	10	25.82 ± 1.04[g]	72.6 ± 0.76[a,b]	3.3	31.2
Control		26.7 ± 0.73	–		
EE	0.02	–	93.4 ± 1.9		
12	10	32.26 ± 1.64[g]	100.5 ± 2.39[a,h]	12.95	4.63
Control	–	28.56 ± 0.5791	–		
EE	0.02	–	104 ± 2.0		
13	10	29.2 ± 2.12[g]	100 ± 2.54[a,h]	2.24	5.30
Control	–	28.56 ± 0.57	–		
EE	0.02	–	104 ± 2.0		
14	10	49.5 ± 3.68[a]	100.1 ± 4.17[a,b]	65.22	35.79
Control	–	29.96 ± 0.29	–		
EE	0.02	–	139.2 ± 1.2		
15	10	36.15 ± 1.70[g]	93.47 ± 4.39[a,b]	20.6	58.13
Control	–	29.96 ± 0.29	–		
EE	0.02	–	139.2 ± 1.2		
16	10	41.6 ± 2.48[a]	68.5 ± 1.55[a,b]	59.38	60.37
Control	–	26.1 ± 0.84	–		
EE	0.02	–	133.5 ± 1.50		
17	10	30.62 ± 0.55[g]	73.75 ± 3.83[a,b]	2.20	60
Control	–	29.96 ± 0.29	–		
EE	0.02	–	139.2 ± 1.2		
18	10	22.4 ± 2.04[g]	60.1 ± 1.65[a,b]	11.1	66.0
Control	–	25.2 ± 0.48	–		
EE	0.02	–	104.45 ± 0.55		
19	10	38.62 ± 0.99[a]	120.77 ± 4.05[a,b]	53.1	20.6
Control	–	25.2 ± 0.48	–		
EE	0.02	–	104.45 ± 0.55		
20	10	24.77 ± 0.67[g]	98.75 ± 0.58[a,h]	5.3	0.7
Control	–	26.35 ± 0.56	–		
EE	0.02	–	99.2 ± 0.99		
21	10	53.87 ± 1.48[a]	101.62 ± 1.44[a,h]	113.4	5.1
Control	–	25.2 ± 0.48	–		
EE	0.02	–	104.45 ± 0.55		
22	10	31 ± 3.24[g]	109.5 ± 4.40[a,b]	18.77	22.34
Control	–	26.1 ± 0.84	–		
EE	0.02	–	133.5 ± 1.50		
23	10	38.75 ± 2.39[c]	63.27 ± 1.90[a,b]	48.56	65.26
Control	–	26.1 ± 0.84	–		
EE	0.02	–	133.5 ± 1.50		
24	10	19.8 ± 0.90[e]	92.05 ± 2.1[a,h]	25.8	2.1
Control	–	26.7 ± 0.73	–		
EE	0.02	–	93.4 ± 1.9		
25	10	19.95 ± 1.11[e]	93.8 ± 0.49[a,h]	15.4	90.0
Control	–	26.7 ± 0.73	–		
EE	0.02	–	93.4 ± 1.9		
26	10	57.15 ± 0.99[a]	101.65 ± 2.37[a,h]	116.8	3.3
Control	–	26.35 ± 0.56	–		
EE	0.02	–	99.2 ± 0.99, 0.99		
27	10	26.57 ± 0.91[g]	102.12 ± 1.96[a,h]	0.8	3.9
Control	–	26.35 ± 0.56	–		
EE	0.02	–	99.2 ± 0.99		
29	10	39.27 ± 2.14[a]	99.33 ± 0.74[a,h]	49.3	0.1
Control	–	26.35 ± 0.56	–		
EE	0.02	–	99.2 ± 0.99		
30	10	24.6 ± 0.95[g]	125.55 ± 1.71[a,b]	2.4	26.6
Control	–	25.2 ± 0.48	–		
EE	0.02	–	104.45 ± 0.55		
Centchroman	10	36.0 ± 1.5[c]	46.0 ± 2.9[a,b]	106	65.9
Control	–	17.4 ± 0.56	17.4 ± 0.56		
EE	0.02		101.23 ± 2.1		

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Table 2 (*Continued*)

Compound code	Dose (mg/Kg)	E uterine weight (in mg)* Mean ± S.E.	AE uterine weight (in mg)* Mean ± S.E.	%Gain**	%Inhibition***
Tamoxifen	3	52.3 ± 5.3[a]	46.32 ± 2.92[a,d]	248.66	27.36
Control	–	15.0 ± 0.57	15.0 ± 0.57		
EE	0.02		66.35 ± 0.44		

EE = 17α-ethynylestradiol.
Keul's test. p values >0.05 were considered insignificant; p values a <0.001, c <0.01, e <0.05, and g >0.05 vs. control; p values b <0.001, d <0.01, f <0.05 and h >0.05 vs. E_2. %Gain = oestrogenicity and %Inhibition = anti-oestrogenicity.
* Values are mean ± S.E. (n = 4–5).
** Percent of vehicle control group.
*** Percent of 17α-ethynylestradiol per se treated group. Statistical analysis was done by ANOVA and Newman.

Table 3
Effect of **22** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on body weight, haemogram and serum biochemical parameters in Swiss albino mice (mean ± SE; n = 6; a, p < 0.001).

Parameters	Dose of **22** at mg/kg body weight as a single oral dose				
	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight (g)	25.93 ± 0.89	24.79 ± 1.26	25.70 ± 0.85	24.85 ± 0.57	21.96 ± 1.08
RBC (millions/mm^3)	6.40 ± 0.42	5.17 ± 0.82	4.98 ± 0.69	4.62 ± 0.55	3.77 ± 0.36*
WBC (thousands/mm^3)	7.40 ± 0.21	8.24 ± 1.54	6.61 ± 0.92	5.31 ± 1.06	6.32 ± 0.99
SGPT (U/L)	10.36 ± 1.31	13.23 ± 1.22	11.84 ± 0.88	14.10 ± 1.41	22.14 ± 3.67*
SGOT (U/L)	24.58 ± 1.20	30.34 ± 0.53	31.94 ± 3.78	34.30 ± 3.25	34.48 ± 2.00
ALKP (U/L)	212.36 ± 13.49	205.51 ± 5.08	228.39 ± 13.43	218.13 ± 23.83	96.43 ± 6.35*
Cholesterol (mg/dL)	108.59 ± 9.37	129.13 ± 4.54	119.54 ± 12.49	118.88 ± 16.12	94.13 ± 16.64
Triglycerides (mg/dL)	96.14 ± 3.56	83.22 ± 8.19	90.87 ± 4.84	124.00 ± 3.89	97.39 ± 6.69
Creatinine (mg/dL)	1.23 ± 0.05	1.11 ± 0.07	1.14 ± 0.03	1.13 ± 0.03	1.22 ± 0.11
Albumin (mg/dL)	3.93 ± 0.40	4.13 ± 0.14	4.24 ± 0.47	3.27 ± 0.37	3.54 ± 0.42
Serum protein (mg/mL)	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± .0.01
Tissue protein (mg/mL)	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
MDA (nM/mg protein)	3.19 ± 0.29	3.03 ± 0.39	3.24 ± 0.015	3.21 ± 0.37	4.08 ± 0.26
GSH (nM/mg protein)	0.23 ± 0.01	0.24 ± 0.03	0.19 ± 0.04	0.20 ± 0.03	0.18 ± 002

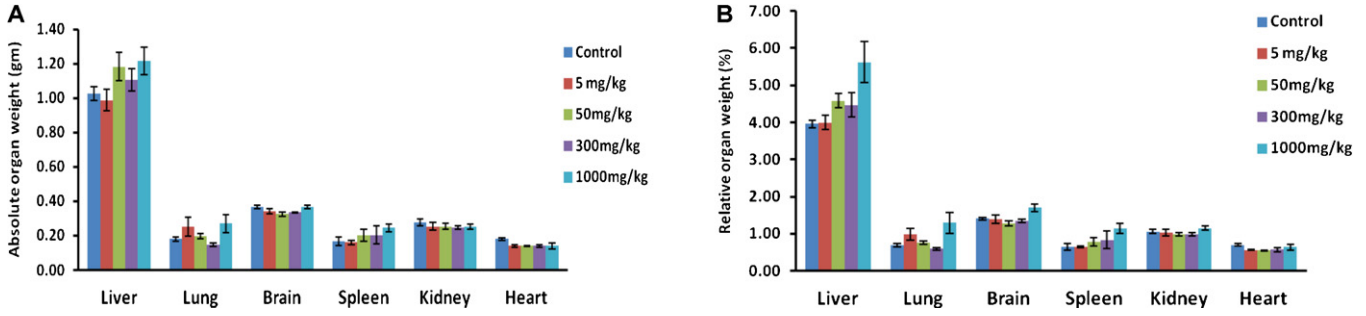

Fig. 5. Effect of **22** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on (A) absolute and (B) relative organ weight in Swiss albino mice (n = 6, non significant changes were found compared to control).

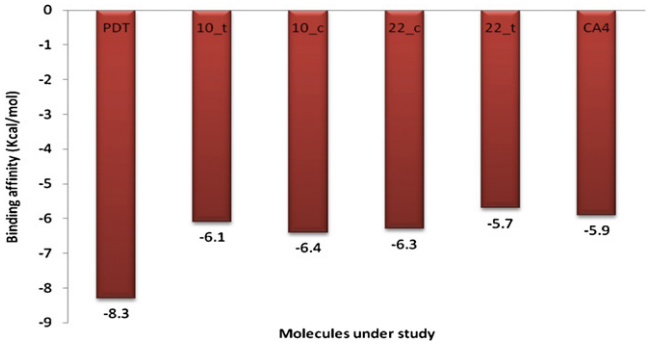

Fig. 6. Binding affinity of PDT, CA4 and derivatives **10** (cis & trans) & **22** (cis & trans) against tubulin polymerase. Negative docking scores (kcal/mol) indicate high binding affinity (t = trans & c = cis isomer).

Since the compounds were designed with steroidal component, we also tested if the compounds could have any untoward estrogenic effect in uterus using in vivo estrogenicity assay (Table 2). Out of the all, compound **26** showed to have significant estrogenic activity with 116.8% gain in total uterine weight, followed by **21** (113.4%), **14** (65.22%), **16** (59.38%) and **19** (53.1%). However, the estrogenic side-effect of these compounds was significantly low as compared to tamoxifen which showed 248.66% gain in uterine weight. In terms of anti-estrogenicity, compound **25** has showed highest loss of total uterine weight of 90% followed by **18** (66%), **23** (65.26%), **16** (60.37%) and **17** (60%).

In acute oral toxicity of **22**, no observational changes, morbidity and mortality were observed throughout the experimental period upto the dose level of 1000 mg/kg body weight. No morbidity or any other gross observation changes could be noticed in the group of animals treated with the test drug at 1000 mg/kg. Blood and serum samples upon analysis showed non-significant changes in all the parameters studied like total haemoglobin level, RBC count, WBC count, differential leucocytes count, SGPT, ALKP, creatinine, triglycerides, Albumin, serum protein, tissue protein, MDA and reduced

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Table 4
Binding pocket's binding residues.

Compound name	Binding pocket residues (4 Å)	No. of H-bond	Amino acid residue involved in docking interaction	Length of H- bond (Å)
PDT	GLY-10, GLN-11, CYS-12, GLN-15, ASN-99, SER-138, GLY-140, GLY-141, GLY-142, THR-143, GLY-144, ASN-204	6	GLN-11	2.8
			GLN-15	3.3
			ASN-99	3.4
			SER-138	3.1
				3.2
				2.7
10	GLY-10, GLN-11, CYS-12, ASP-67, GLU-69, SER-96, ALA-97, ASN-99, SER-138, GLY-140, GLY-141, THR-143, GLY-144, PRO-171, SER-172, LYS-174, GLU-181, ASN-204	4	SER-96	2.9
			THR-143 ASN-204	3.2
				3.0
				3.0
22	GLY-10, GLN-11, CYS-12, ASP-67, GLU-69, GLY-71, THR-72, SER-96, ASN-99, SER-138, GLY-141, GLY-142, THR-143, GLY-144, SER-145, ALA-147, VAL-169, PRO-171, SER-172, THR-178, GLU-181, ASN-204	6	GLY-142	3.5
			THR-143	3.4
			GLY-144 TYR-222	3.9
			ASN-204	3.2
				2.8
				2.8

GSH activity (Table 3 and Fig. 5). However, groups of animals treated with **22** at 1000 mg/kg body weight showed non-significant reduction in body weight as well as significant changes in RBC count, SGPT and ALKP level. The significant reduction in RBC count coupled with reduced ALKP activity showed probability of toxicity in haemopoietic system.

The binding affinity obtained in the docking experiment allowed the activity of steroidal stilbenes to be compared to that of the standard anticancer antitubulins podophyllotoxin [Fig. 6] and combretastatin A4. Both cis and trans isomers of **10** & **22** were docked. All the compounds **10** (cis & trans), **22** (cis & trans) showed high binding affinity (high negative docking energy) against well known human anticancer target microtubule polymerase (PDB ID: 4FFB_beta2). The docking energies of cis isomers of **10** and **22** were −6.4 kcal/mol, −6.3 kcal/mol and for trans isomers −6.1 kcal/mol and −5.7 kcal/mol respectively. The binding energies of these derivatives were comparable to PDT (−8.3 kcal/mol) and CA4 (−5.9 kcal/mol). When we compared the binding site pocket amino acid residues interacted with the derivatives, we found that the consensus having 'GLN-11, ASN-204, SER-138, GLY-141, GLY-142, THR-143, GLY-140, ASN-99′ amino acid residues in reference to PDT and CA4 [Table 4]. The docked view of **10** (cis & trans), **22** (cis & trans), PDT and CA4 with microtubule polymerase have been depicted in Fig. 7.

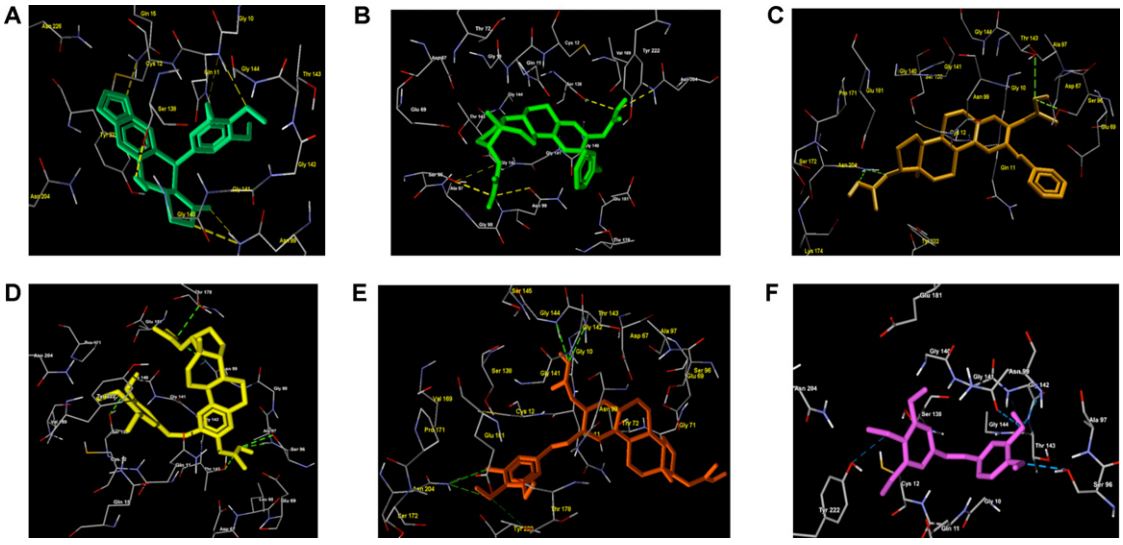

Fig. 7. Docked views of various control (PDT & CA4) and derivatives **10** (cis & trans) & **22** (cis & trans) onto anticancer target microtubule polymerase (A) podophyllotoxin (PDT) was docked with a docking energy of '−8.3' kcal/mol, '6′ H-bonds were observed against binding site residues GLN-11, GLN-15, ASN-99 and SER-138; (B) **10**-cis was docked with a docking energy of −6.4′ kcal/mol. '7′ H-bonds were observed against binding site residues ASN-99, GLY-98, SER-96, SER-138, ASN-204; (C) **10**-trans docked with a docking energy of '−6.1′ kcal/mol, '4′ H-bonds were observed against binding site residues SER-96, THR-143 and ASN-204; (D) **22**-cis was docked with a docking energy of '−6.3′ kcal/mol, '5′ H-bonds were observed against binding site residues TYR-222, GLY-142, ASN-99, SER-96; (E) **22**-trans was docked with a docking energy of '−5.7′ kcal/mol, '6′ H-bonds were observed against binding site residues GLY-142, THR-143, GLY-144, ASN-204 and TYR-222; (F) CA4 docked with a docking energy of '−5.9′ kcal/mol, '5′ H-bonds were observed against binding site residues TYR-222, ASN-99, GLY-142, SER-99.

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4. Discussion

Combretastatin A4 is an investigational drug that binds to β-tubulin at colchicine binding site [20]. It is a tubulin polymerisation inhibitor, arresting cell cycle in metaphase and triggering apoptosis [21]. It acts as a potent antiangiogenic agent by disrupting tumour vascular system [22]. Being a potent anticancer agent its several prodrugs are under various phases of clinical trials [23]. We designed our prototype to have an 3,4,5-trimethoxyphenyl unit similar to these natural tubulin polymerisation inhibitors. This unit is essentially required to induce the cytotoxicity in the molecule [24]. In our steroidal stilbene series **22** exhibited potent anticancer activity. It was a better inhibitor of tubulin polymerase than the model molecule combretastatin A4. In cell cycle analysis **22** induced mild G2/M phase arrest with significant apoptosis in MCF-7 cells.

In tubulin polymerisation inhibition assay, **18** exhibited the highest antitubulin effect, but its cytotoxicity against MCF-7 (ER positive cancer cells) was very poor ($IC_{50} > 100\ \mu M$). It might be due to its high estrogenic response i.e. six times higher than antiestrogenic response (Table 2). This high estrogenicity in turn might exert proliferation of MCF-7 cells. It seems to be supported by its cytotoxicity against MDA-MB-231 (ER negative cancer cell line), where its IC_{50} (13.8 μM) was quite impressive.

Recently, Valdameri et al. have reported several methoxystilbenes of resveratrol and related compounds as potent, specific, untransported and non-cytotoxic inhibitors of breast cancer resistance protein [25]. These stilbene derivatives were shown to act through inhibition of ATP-binding cassate (ABC). Over-expression of multidrug ABC transporters within plasma membranes were found to lower the efficiency of chemotherapeutics in the most frequent MDR mechanism.

Release of lactate dehydrogenase (LDH) is an indicator of membrane integrity and hence cell injury. Tumours show an elevated LDH activity due to high rate of turn over with destroyed cells. So, it is considered as a surrogate marker for tumour burden and response to treatment [26]. LDH activity of MCF-7 cells increased (∼50%) by compound **22** even at 1 μM concentration and increased in a dose-dependent manner. This possibly indicates that **22** causes significant cell membrane damage in cancer cells.

Most of these compounds possessed high to moderate antiestrogenic and low estrogenic activity. Only two of the analogues **21** & **26** exhibited high estrogenic response. Ideally, these compounds are desired to have high anti-estrogenicity with low estrogenicity in breast tissues. Estrogenic effect in uterus may induce possible side-effects like uterine hyperplasia, reported to be observed with Tamoxifen, the drug of choice at present. However, this parameter measures tissue specific action in uterus and may differ from that of breast cells.

In in-silico docking experiments, both **10** (cis & trans) & **22** (cis & trans) showed high binding affinity with microtubule polymerase and were comparable to PDT and CA4. The docking energies of cis isomers of **10** and **22** were higher than the corresponding trans isomers. Thus, cis isomers were more active than the trans isomers in both the case. There were about eight amino acid residues interacting with **10**, **22** with consensus. It indicated that all these compounds occupy the same binding pocket of the tubulin polymerase. Overall, in silico results also support strong affinity of both **10** & **22** with microtubule polymerase.

In acute oral toxicity experiment **22** was well tolerated by the Swiss albino mice up to the dose level of 300 mg/kg bodyweight as a single acute oral dose. However, 1000 mg/kg body weight dose of **22** was found to reduce RBC count and ALK-P level to some extent. Hence, it can be considered safe up 300 mg/kg which is much higher than its IC_{50} (7.5 μM and 5.5 μM) against both the breast cancer cell lines. Hence, it is suggested for a detailed sub-acute study of **22** with special emphasis on haemopoietic organ toxicity. However, lower doses may be considered for revalidation of efficacy, further toxicity and pharmacokinetic studies [27].

5. Conclusion

In conclusion, steroidal analogues of combretastatin A4 have exhibited significant anticancer activity against both ER positive and ER negative breast cancer cells. The most active analogue showed antitubulin activity and induction of apoptosis. This compound was non-toxic up to 300 mg/kg dose in Swiss albino mice. Such analogues can further be optimized for better activity and reduced toxicity. Hence, steroidal stilbenes may be developed as potent anti-breast cancer agents in future.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

The authors are thankful to Directors CSIR-CIMAP and CSIR-CDRI for constant help and support. The financial support from Science and Engineering Research Board, Department of Science & Technology, Govt. of India is duly acknowledged.

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