Bioorg. Med. Chem. 42 (2021) 116252

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Design, synthesis and broad spectrum antibreast cancer activity of diarylindoles *via* induction of apoptosis in aggressive breast cancer cells

Yashveer Gautam [a,1,3], Sharmistha Das [b,1], Hamidullah Khan [b,4], Nandini Pathak [a,c], Hina Iqbal [a], Pankaj Yadav [a], Vijay Kumar Sirohi [b], Sana Khan [a,c], Dushyant Singh Raghuvanshi [a], Anila Dwivedi [b,c], Debabrata Chanda [a,c], Karuna Shanker [a,c], Feroz Khan [a,c], Rituraj Konwar [b,c,*,2], Arvind S. Negi [a,c,*]

[a] CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), P.O. CIMAP, Kukrail Picnic Spot Road, Lucknow 226 015, U.P., India
[b] Endocrinology Division, CSIR-Central Drug Research Institute, Jankipuram Extension, Lucknow 226031, U.P., India
[c] Academy of Scientific and Innovative Research (AcSIR), New Delhi 110001, India

ARTICLE INFO

Keywords:
Diarylindoles
Breast cancer
Apoptosis
In-vivo efficacy
Acute oral toxicity

ABSTRACT

Breast cancer is the second leading cause of cancer deaths in women with significant morbidity and mortality. Present study describes design, synthesis and detailed pharmacology of indole derivatives exhibiting remarkable broad spectrum antiproliferative activity against breast cancer cells. Detailed mechanistic evaluations confirmed induction of G0/G1 arrest, apoptosis induction, loss of mitochondrial integrity, enhanced ROS generation, autophagy, estrogen receptor β-transactivation and increased tubulin polymerization. In *in-vivo* efficacy studies in rodent model, these indole derivatives induced significant regression in mice mammary tumour on 21 days daily oral dose. Moreover, compounds **19** and **23** were safe in Swiss albino mice in safety studies. These diarylindoles may further be optimized for better efficacy.

1. Introduction

Breast cancer is a complex and heterogeneous group of disease. Broadly, there are hormone dependent, and hormone independent type breast cancers. Hormone dependent breast cancer (estrogen receptor, progesterone receptor and HER2 receptor positive, triple positive) which comprises about two third of breast cancer cases has several options for treatment, but triple negative breast cancer (TNBC) constitutes about 10–15% of all cases is more aggressive with limited options for specific and effective therapy. Moreover, hormone dependent breast cancer targeting drugs are mostly ineffective against triple negative breast cancer cells.[1] TNBC has been considered a disease with poor response to molecular target therapy due to less validated biomarkers.[2]

Nitrogen heterocycles play a crucial role in drug discovery. Presently, 59% of all unique small-molecule drugs approved by the FDA have some sort of nitrogen heterocycles.[3] Indole is one of the most attractive nucleus exhibiting diverse pharmacological activities. It is considered as one of the most privileged structures by medicinal chemists due to its ability to bind multiple receptors with high affinity and eliciting wide range of pharmacological activities.[4] Indoles represent the most important bioactive core of all structural classes in drug discovery.[5] This core has been reported to exhibit diverse pharmacological activities i.e. anticancer, anti-inflammatory, analgesic, antitubercular, antihypertensive, and antidiabetic etc.[4–6] Many naturally occurring compounds possess indole framework like reserpine from *Rauwolfia serpentina* as antihypertensive drug,[7] vinca alkaloids from *Catharanthus roseus* as antileukemic and anti-Hodgkin's agents,[8] yahimbine from *Pausinystalia yahimbe* as sedation reversal agent,[9] and ajmalicine from *Rauwolfia serpentina* as antiarrythmic agent [10] etc. Further, several clinical drugs have been developed synthetically on indole core particularly, bazedoxifene as anti-osteoporotic agent,[11] indalpine as selective serotonin re-uptake inhibitor,[12] pravadoline as anti-inflammatory drug,[13]

* Corresponding authors at: Academy of Scientific and Innovative Research (AcSIR), New Delhi 110001, India.
E-mail addresses: rituraj.konwar@neist.res.in (R. Konwar), arvindcimap@rediffmail.com, arvindcimap@gmail.com (A.S. Negi).
[1] Equal authorship.
[2] Present address: CSIR-North East Institute of Science and Technology, Jorhat, Assam-785006, India.
[3] Present address: Department of Chemistry, Pandit Prithi Nath PG College, 96/12, Mahatma Gandhi Marg, Kanpur 208001, Uttar Pradesh, India.
[4] Present address: Department of Dermatology, University of Wisconsin-Madison, Madison, WI 53706, USA.

https://doi.org/10.1016/j.bmc.2021.116252
Received 19 March 2021; Received in revised form 23 May 2021; Accepted 28 May 2021
Available online 5 June 2021

panobinostat, a histone deacetylase inhibitor as anticancer agent,[14,15] and many more as potential bioactive lead compounds.[4] Herein, we report design and synthesis of 1-benzyl-2-arylindoles as possible anti-breast cancer agents. We designed dual action hybrid molecules having anti-estrogenic and anti-tubulin effect as well. For anti-estrogenic property the molecule should have affinity to estrogen receptor and an amino alkyl group should be placed either at C7 or C-11 position parallel to estradiol which induces anti-estrogenic effect after binding to anti-estrogenic binding site. Thus, we planned to have aminoalkyl chain at C11 and also at both C7 and C11 positions which has not been tried so far. Simultaneously, for anti-tubulin effect, we placed a 3,4,5-trimethoxyphenyl fragment at C-2 position of indole pharmacophore to induce anti-tubulin effect (Fig. 1).[16] This fragment is supposed to inhibit tubulin polymerization and induce cell cycle arrest. Thus, these dual acting compounds are designed to have cytotoxicity not only against estrogen receptor positive breast cancers *via* their anti-estrogenic component, but also against ER-negative breast cancer cases through their co-existing anti-tubulin effect. Thus a broad spectrum of breast cancer can be addressed simultaneously.

2. Results and discussion

2.1. Chemistry

As outlined in Scheme 1. 2-arylindole core was synthesized by using modified Fischer indole synthesis. 4-methoxyphenylhydrazine hydrochloride (**1**) and 3,4,5-trimethoxyacetophenone (**2**) underwent condensation reaction in presence of *N,N'*-dimethylurea (DMU) and L (+) tartaric acid to afford 5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indole **(3)** in 35% yield.[17] The indole core was benzylated at nitrogen with benzyloxybenzyl chloride, using NaH in dry DMF in an ice-bath to get 1-(4-(benzyloxy)benzyl)-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indole **(4)** in 41% yield. Interestingly, we got a dibenzylated product also where C-benzylation also took place at C3 position along with *N*-benzylation (1,3-bis-(4-(benzyloxy)benzyl)-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indole) **(5)** in 45% yield. The *O*-benzyl group (**5** & **7**) was deprotected by hydrogenolysis using 10% Pd-C in dry THF and hydrogen gas in a balloon for 4–5 h at RT to afford 4-(5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indol-1-yl)methyl)phenol **(8)** in 40% yield and 1,3-bis(4-(hydroxy)benzyl)-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indole **(9)** in 56% yield. Phenolic compounds **8** and **9** were treated with ethyl bromoacetate in potassium carbonate dry acetone system to afford **11** and **18** in excellent yields. Further, various aminoalkyl chains were hooked-up at phenolic OH of **8** & **9** using dry acetone/K_2CO_3, at reflux for 2–3 h, to achieve the desired product in excellent yield (82–89%). (**12**–**16** & **19**–**23**). The indole core was further benzylated with plain benzyl chloride to afford 1-benzyl-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indole **(4)** in 40% yield and 1,3-dibenzyl-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1*H*-indole **(6)** in 58% yield. All the intermediates and final products were confirmed by spectroscopy [Supplementary information].

2.1.1. Purity profile of potent cytotoxic compounds

The purity of all active compounds **12**, **14**, **16**, **19**, and **20**–**23** was checked by UPLC. All these compounds possessed purity 95.21% to 98.52%.

2.2. Biological evaluation

2.2.1. Indole derivatives induce loss of cell viability in cancer cell lines

Out of twenty compounds screened for anti-cancer activity against human breast cancer cell lines (MCF-7 and MDA-MB-231), and prostate cancer cell line (PC-3) nine compounds showed significant anticancer activity against all the cell lines and rest of the compounds are moderately active (Table 1). Further, compounds, **19**, **20**, **22** and **23** were also investigated against non-malignant human epithelial kidney cell line (HEK-293) to assess the safety aspect. Among the potent compounds, **19**, **20**, **22** and **23** possessed relatively better cytotoxicity in the series against all the cancer cell lines whereas poor cytotoxicities against normal cell line (HEK-293) (Table 1).

Structure activity relationship. In the two series of indole derivatives, Series I possessed mono-*N*-benzylated indoles (**10**–**16**) and series II, 1,3-dibenzylated indoles (**7**, **9**, **17**–**23**). In general in both series (I & II) indole derivatives possessing aminoalkyl chains exhibited significant anticancer activity against all four human cancer cell lines. Only compound **8** with *N*-benzylated phenol derivatives showed moderate cytotoxicity against MDA-MB-231 and PC-3 cell lines.

In series I, only *N*-benzylated indoles with amino alkyl chains were active against all three cancer cell lines. These compounds (**12**–**16**) mainly differed at aminoalkyl chains. Compounds **12** and **14** with *N,N'*-dimethyl chain and pyrrolidine chain were most active against MCF-7 cell line. Compounds **13** and **15** possessing aminoalkyl chains with higher carbons *N,N'*-diethyl or piperidine possessed low cytotoxicities. The corresponding dibenzylated derivatives of compounds **12** (i.e. **19**)

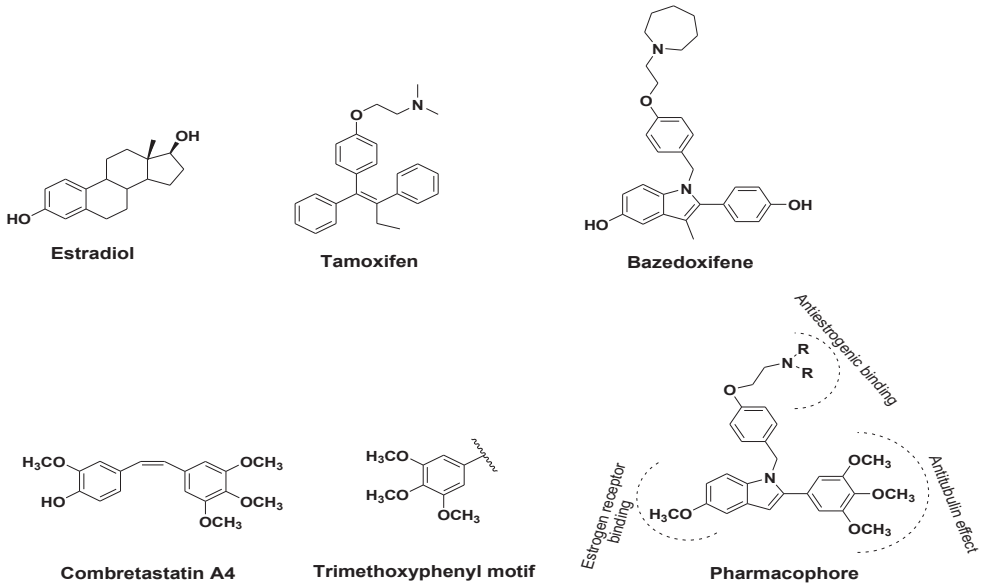
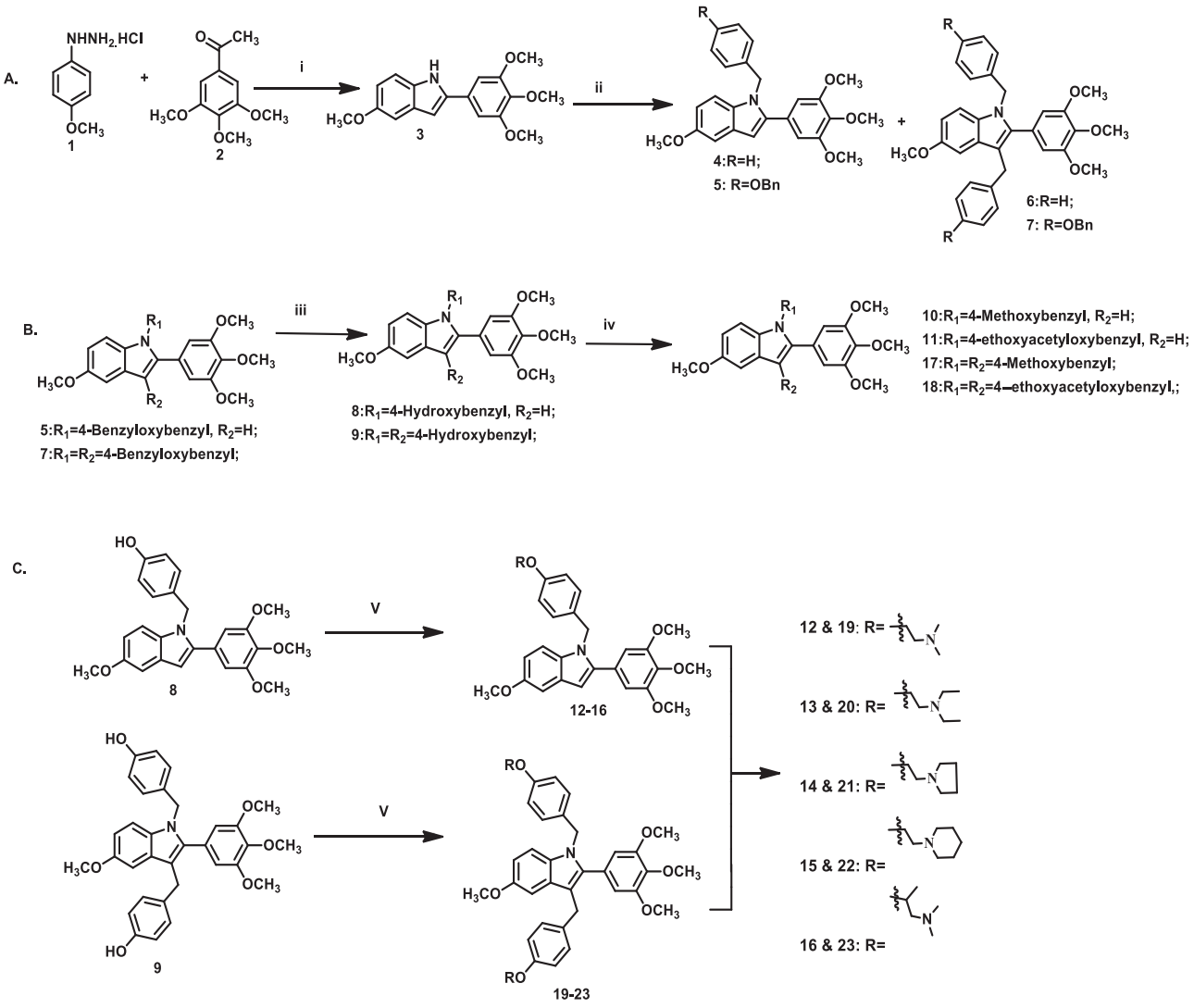


Fig. 1. Structure of estradiol, tamoxifen, bazedoxifene, combretastatin A4, trimethoxyphenyl motif and designed pharmacophore.



Scheme 1. Synthesis of compounds **3**–**23**. Reagents and conditions: i) DMU, L(+) tartaric acid, neat, 120 °C, 3–4 h; 35%; ii) For **4** & **6** benzylbromide/for **5** & **6** benzyloxybenzylchloride, NaH, dry DMF, 1 h, 40–58%; iii) Dry THF, 10% Pd-C, H_2 balloon, 3 h, **8**: 40%, **9**: 56%; iv) For **10** & **17**: Me_2SO_4, K_2CO_3, dry acetone, reflux, 86–91%, for **11** & **18**: ethylbromoacetate, dry acetone, K_2CO_3, reflux, 3–4 h, 92–94%; v) various aminoalkyl chains, dry acetone, K_2CO_3, reflux, 4 h, 82–89%.

and **14** (i.e. **23**) were more cytotoxic in series II against MCF-7 cells. In series-II, compounds **20**, **22** and **23** exhibited potential cytotoxicity against MDA-MB-231 cells, which might be due to antitubulin effect of these compounds.

Overall, in case of MCF-7 cell line, in both the series (I & II) an aminoalkyl chain is essentially required for potential cytotoxicity. Among the various type of aminoalkyl chains hooked up, *N,N*-dimethyl (**12** and **19**) and pyrrolidine (**14** and **21**) were relatively more effective than the rest of the chains.

2.2.2. *Compounds **19**, **20**, **22** and **23** inhibit cell cycle progression*

Cell cycle check-points play an important role in the regulation of cell cycle by sensing defects that occur during essential processes and inducing cell cycle arrest in response until the defects are repaired.[18] Induction of cell cycle arrest in cancer cells account for one of the most widespread strategies to stop or halt cancer spreading.[19] Majority of anticancer drugs exert their inhibitory effect on tumor cell growth by interfering with cell cycle progression by arresting cells at different phases to stop or limit cancer spreading.[20] Therefore, to investigate effect of compounds **19**, **20**, **22** and **23** on the cell cycle progression, cell cycle phase distribution was analysed following treatment with compounds for 24 h in MDA-MB-231 cell line (Fig. 2). Compounds **19**, **20**, **22** and **23** retarded the progression of MDA-MB-231 cells at G0/G1 phase. Compounds **19**, **20**, and **23** caused G0/G1 phase of cell cycle arrest and subsequent reduction of G2/M phase. Whereas compound **22** also induced G0/G1 phase of cell cycle arrest but without subsequent decrease in cell population in G2/M phase of cell cycle. However, compound **22** induced stronger reduction in S phase of cell cycle.

2.2.3. *Compounds **19**, **20**, **22** and **23** induce mitochondria-mediated apoptosis*

The major therapeutic approaches of clinical oncology have been the development of therapies promoting effective elimination of cancer cells by apoptosis because dead tumour cells can contribute to clinical response but not to tumour relapse. In order to determine whether cytotoxic response is due to apoptosis or necrosis, we undertook Annexin V-FITC experiment following treatment with indicated concentration of compounds in MDA-MB-231 cells using flow cytometry. As shown in Fig. 3, compounds **19**, **20**, **22** and **23** induced early as well late apoptosis in MDA-MB-231 cells. The apoptotic effects were more pronounced with increasing concentration of test compounds. Whereas, there was no significant difference in PI positive cell population, thus indicating necrosis. There was an increased green fluorescence (JC-1 monomers, indication of depolarised mitochondria) and subsequent

Table 1
Cytotoxicity of the compounds against cancerous and non-cancerous cells in terms of $IC_{50} \pm SE$ in μM.

S. No.	Compound	MCF-7	MDA-MB-231	PC3	HEK-293
1	**3**	>30	>30	>30	—
2	**4**	>30	>30	>30	—
3	**5**	>30	>30	>30	—
4	**6**	>30	>30	>30	—
5	**7**	>30	>30	>30	—
6	**8**	>30	17.3 ± 0.01	>30	—
7	**9**	>30	>30	>30	—
8	**10**	>30	>30	>30	—
9	**11**	>30	>30	>30	—
10	**12**	6.67 ± 0.01	7.84 ± 0.01	6.87 ± 0.02	—
11	**13**	29.8 ± 0.05	13.2 ± 0.01	15.0 ± 0.01	—
12	**14**	5.08 ± 0.03	12.4 ± 0.02	6.03 ± 0.01	—
13	**15**	21.6 ± 0.01	>30	21.8 ± 0.01	—
14	**16**	14.7 ± 0.02	10.6 ± 0.02	13.3 ± 0.03	—
15	**17**	>30	>30	>30	—
16	**18**	>30	>30	17.6 ± 0.06	—
17	**19**	**4.48 ± 0.06**	**3.78 ± 0.01**	5.82 ± 0.03	24.5 ± 0.01
18	**20**	6.36 ± 0.02	**2.34 ± 0.01**	8.31 ± 0.01	25.4 ± 0.01
19	**21**	4.25 ± 0.01	9.45 ± 0.05	7.48 ± 0.03	N.D
20	**22**	6.48 ± 0.03	**2.42 ± 0.01**	7.07 ± 0.01	30.5 ± 0.01
21	**23**	7.06 ± 0.01	**3.88 ± 0.03**	6.72 ± 0.02	21.8 ± 0.01
22	Tamoxifen	8.42 ± 0.02	10.11 ± 0.01	15.8 ± 0.03	22.4 ± 0.02
23	Paclitaxel	0.48 ± 0.01	1.6 ± 0.03	2.6 ± 0.01	5.8 ± 0.02

IC_{50} > 30 μM considered as inactive; incubation period = 24 h; — not determined.

decreased red fluorescence (JC-1 aggregates, indication of polarized mitochondria). Collectively, increased mitochondrial membrane depolarization clearly indicates that apoptosis in MDA-MB-231 cells was possibly due to activation of intrinsic apoptotic pathway (Fig. 4). However, the interaction of apoptosis pathways with other signalling mechanisms can also affect cell death. Targeting mechanisms of apoptosis remain a promising strategy in oncology that will continue to evolve in future clinical practice.[21]

2.2.4. Compounds **19, 20, 22** and **23** induce ROS-dependent cell death

Reactive oxygen species is a known mediator of apoptosis and play important role in chemotherapeutics mediated cancer cell death.[22] Therefore, to decipher the ROS generation MDA-MB-231 cells were treated with different concentrations of test compounds for 24 h and ROS generation was detected using ROS sensitive fluorescent probe DCFH-DA using flow cytometer (Fig. 5). We found that all the compounds increased the level of ROS in MDA-MB-231 cells and level of ROS was positively correlated with concentrations of test compounds. To further confirm the contribution of ROS generation on cell viability, ROS level was scavenged with *N*-acetylcysteine (NAC), a widely used ROS inhibitor, along with different compounds and cell viability was evaluated with MTT assay (Fig. 6). Results showed that all the compounds induced loss of cell viability in MDA-MB-231 cells whereas when ROS level was scavenged using NAC compounds-mediated loss of cell viability was abrogated. Collectively, these data support that ROS generation induced by compounds may be the plausible reason of cell death in cancer cells. Nevertheless, many cancer cells become adapted to such stress under persistent intrinsic oxidative stress and develop an augmented, endogenous antioxidant capacity.[23] However, role of ROS in the regulation of apoptosis is well established and accepted.[23]

2.2.5. Compounds **19, 20, 22** and **23** induce autophagy

Macro-autophagy also termed as autophagy is a catabolic and evolutionarily conserved process required for maintenance of cellular biosynthesis during nutrient deprivation or metabolic stress and cancer chemotherapy. Therefore, targeting autophagy is considered as promising therapeutic strategies during development of new cancer therapy.[24] To investigate autophagy induction, cells were stained with acidotropic dye, MDC following treatment with different concentrations of compounds. Autophagy is characterized by formation of acidic autolysosome under fluorescence microscopy when cells were stained with MDC. Results showed that all the compounds increased considerable MDC staining in comparison to untreated vehicle control (Fig. 7). In order to further delineate the cross regulation of autophagy on cell viability, autophagy was inhibited with widely used autophagy inhibitor, chloroquine and cell viability was evaluated by MTT assay. Results showed that inhibition of autophagy led to inhibition of compounds **19** and **20** mediated loss of cell viability (Fig. 8). The effect was more pronounced in case of **19** as compared to **20**. Whereas inhibition of autophagy led no significant difference in case of compounds **22** and **23**. Collectively, these results suggest that compounds **22** and **23** mediated autophagy induction drive cancer cells toward cell death rather than cell survival.

2.2.6. Compounds **19, 20, 22** and **23** induce cell death is caspase-dependent cell death

Caspases are considered as primary drivers of apoptotic cell death.[25] Therefore, to investigate the contribution of caspase in anticancer action of these compounds, we blocked caspase using pan caspase inhibitor, Z-VAD-FMK and evaluated the cell viability by MTT assay. Results showed that inhibition of caspase activation leads to abrogation of compounds **20** and **22** induced loss of cell viability (Fig. 9). However, loss of cell viability induced by compounds **19** and **23** in MDA-MB-231 cells was not significantly perturbed by inactivation of caspase. Thus, compounds **20** and **22** induced caspase-dependent apoptosis in breast cancer, whereas compounds **19** and **23** induced cell death was independent of caspase action.

2.2.7. Compounds **19, 20** and **23** enhance ERβ trans-activation without any effect on ERα trans-activation

None of the four compounds significantly altered trans-activation of ERα unlike estradiol or tamoxifen. However, compounds **19**, **20**, and **23** significantly enhanced ERβ trans-activation in MDA-MB-231 cells (Fig. 10). Over-expression of ERα is considered responsible for development of breast cancer[26] while ERβ counteracts hyperproliferative effect of ERα.[27] Hence, activation of ERβ is considered antiproliferative in breast tumour cells.[28] Compound **19** showed highest trans-activation of ERβ and exhibited strong antiproliferative effect (IC_{50} = 4.48 μM) against MCF-7 cells which is highest among the four evaluated compounds. Compounds **22** neither influenced trans-activation of ERα nor that of ERβ, possibly suggesting its action to be independent of ER.

2.2.8. Compounds **19, 20, 22** and **23** enhance tubulin polymerization

Targeting microtubule is an established anticancer target. Microtubule targeting drugs disrupt microtubule dynamics either as a stabilizer or as a destabilizer. Stabilizers promote microtubule assembly by ultimately microtubule aggregation while destabilizers inhibit tubulin polymerization and microtubule assembly.[16] In our tubulin polymerization kinetics study, compounds **19**, **20**, **22** and **23** showed significant enhancement of tubulin polymerisation as compared to vehicle (Fig. 11). Compounds showed higher microtubule formation than positive control paclitaxel. Our results suggest that compounds **19**, **20**, **22** and **23** target MDA-MB-231 cells as stabilizers in tubulin assembly. Modulation of tubulin-microtubules dynamics is one of the most

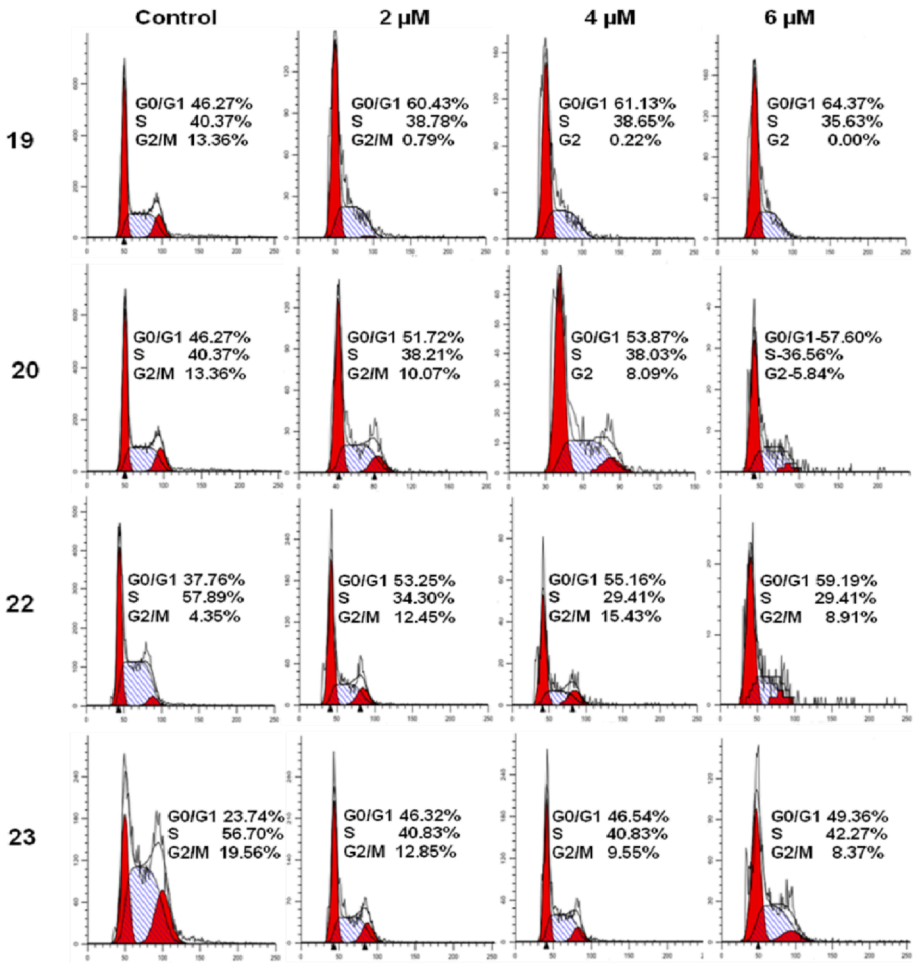


Fig. 2. Effects of compounds **19**, **20**, **22** and **23** on cell cycle in MDA-MB-231 cells. [As indicated above, treatments of compounds **19**, **20**, **22**, and **23** were given in MDA-MB-231 cells and after 24 h cells were harvested and stained with PI and data acquired by flowcytometry]

effective targets for cancer chemotherapeutics.[29]

2.2.9. Molecular docking studies

a) Interaction of indanone derivatives **19**, **20**, **22** and **23** with tubulin at paclitaxel binding site

Docking studies were performed to confirm the interaction of indole derivatives with target protein microtubule. Crystal structure of tubulin dimer was used from a 13-protofilament, paclitaxel stabilized microtubule [PDB: 6WVR]. Among the four indole derivatives, compounds **19** and **22** exhibited very good affinity with target protein which were comparable to paclitaxel (Table 2). There were twelve amino acid residues common to all four indole derivatives and paclitaxel (VAL23, LEU217, HIS229, ALA233, PHE272, LEU275, THR276, ARG278, GLN281, PRO360, ARG369, & LEU371) which clearly indicates that all these occupied similar binding pocket (Supplementary information).

b) Interaction of indoles with α/β-estrogen receptors

All the four indole derivatives **19**, **20**, **22** and **23** were docked with both the estrogen receptor subtypes alpha and beta. Estradiol was used as standard ligand. Human estrogen receptor alpha was taken from ligand binding domain in complex with estradiol [PDB: 2OCF] and estrogen receptor-beta bound to estradiol [PDB: 5TOA]. Estradiol exhibited good affinity with both the estrogen receptor sub-types. All the four Indole derivatives did not show good affinity with both ER-α and ER-β sub-types except compound **22** which showed moderate affinity with ER-β sub-type (Table 3). There were only one or two residual amino acids common to these which indicated that indole derivatives did not occupy the same binding pocket (Supplementary information).

*2.2.10. Compounds **19, 20, 22** and **23** induce tumor regression in 4T1-induced syngeneic mouse mammary tumor model*

In order to confirm the anti-tumor activity and efficacy of compounds **19**, **20**, **22** and **23**, we utilized orthotopically transplanted 4T1 cell line induced mouse mammary tumor model. Daily oral administration of 10 mg/kg and 20 mg/kg bodyweight of compounds **19**, **20**, **22** and **23** led to significant suppression of tumor volume with all the compounds at all tested concentrations as compared to untreated vehicle control (Fig. 12). However, tumor volume regression was highly significant in case of 10 mg/kg bodyweight dose of compound **19** and 20 mg/kg bodyweight dose of compound **20**. Antitumor efficacies of all the four compounds are either comparable or significantly better than paclitaxel. Interestingly, both the compounds lack of any apparent toxicity as no significant difference in body weights and other biochemical parameters was observed. Furthermore, all the compounds showed better survivability than untreated and paclitaxel treated groups. Collectively, these data support that all the compounds show significant anti-cancer effect in both *in vitro* as well as in *in vivo* tumor model.

*2.2.11. Safety studies of compounds **19** and **23** through acute oral toxicity*

Acute oral toxicity describes the adverse effects of potentially

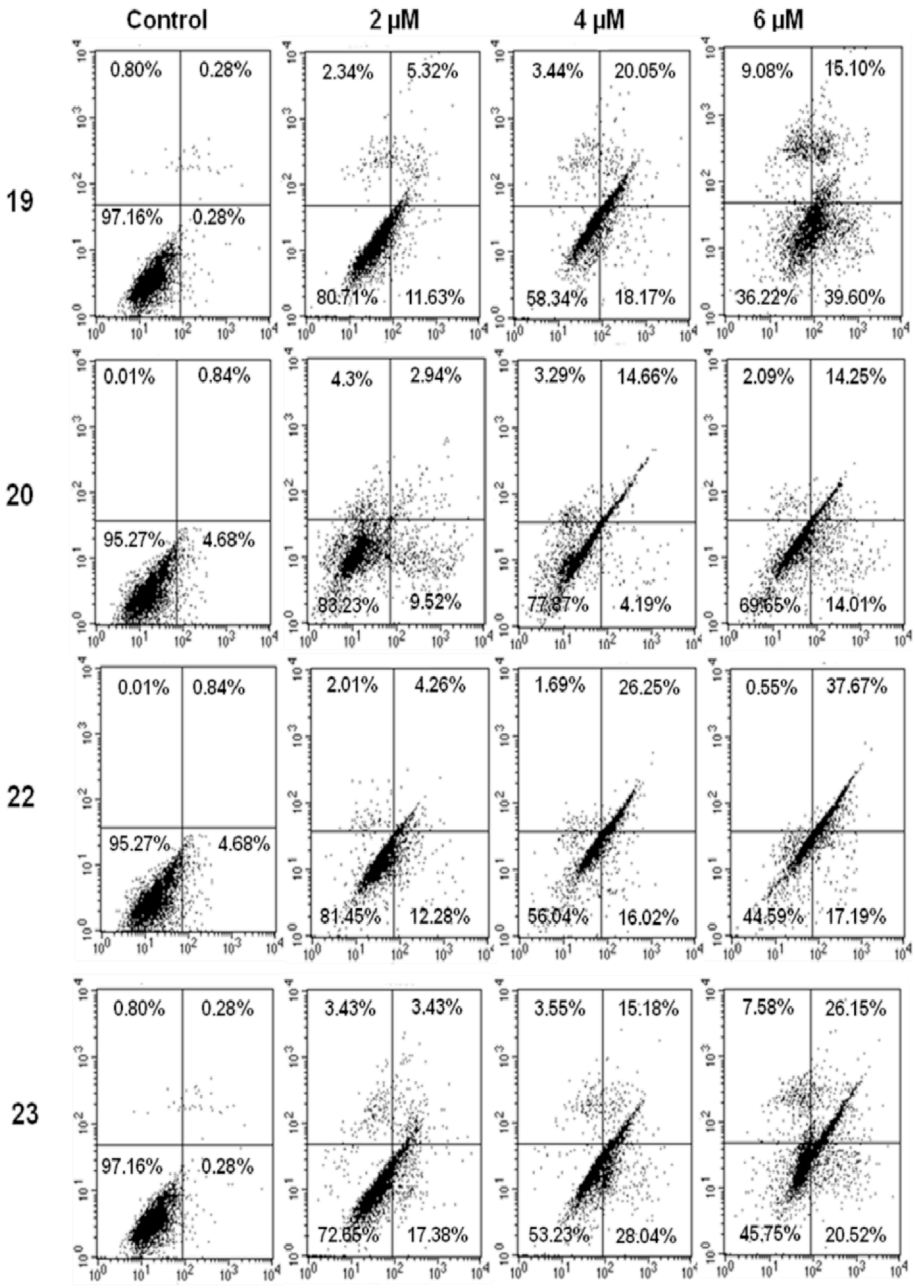


Fig. 3. Apoptosis induction by compounds **19**, **20**, **22** and **23** MDA-MB-231 cells. [After treatment for 24 h by compounds **19**, **20**, **22**, and **23** as indicated above, cells were harvested and stained by Annexin V-FITC/PI and data acquired by flow-cytometric analysis. The percentage of annexin V-FITC-negative/PI-negative (viable cells), annexin V-FITC-positive/PI-negative (early apoptotic cells), annexin V-FITC-negative/PI positive cells (late apoptotic) and annexin V-FITC-positive/PI-positive (necrosis)]

bioactive compounds on exposure for a short period of time to get an idea about the safety aspects. For safety evaluation compounds **19** and **23** were given once orally to Swiss albino mice at 5, 50, and 300 mg/kg dose.[30] No mortality was observed throughout the experimental period, there were non-significant changes in gait, posture and response of animals. Animals on gross pathological study did not show any significant changes in any of the organs studied, including their absolute and relative body-weights (Fig. 13 and Fig. 15). Blood and serum samples upon analysis showed non-significant changes in all the parameters studied like haemoglobin (Hb), Red Blood Cells (RBC), White Blood Cells (WBC), differential leukocyte count (DLC) (Fig. 14 and Fig. 16), Serum SGPT, Alkaline Phosphatase (ALP), creatinine, triglycerides, cholesterol, albumin, serum protein (Tables 4A and 4B). Therefore, the experiment showed that compounds **19** and **23** are well tolerated by the Swiss albino mice up to the dose level of 300 mg/kg bodyweight as a single acute oral dose for 7 days. However, sub-acute, chronic or sub-chronic experiments with both the compounds (**19** and **23**) are needed to be carried out for any adverse effect on repeated exposure.[31]

Safety is an essential aspect in the development of a drug candidate, nowadays referred as 'Pharmacovigilance'. It aims to enhance patient care and safety in relation to use of medicine and to support public health programmes by providing reliable, balanced information for effective assessment of risk–benefit profile of medicines.[32] Now it has been legalised and streamlined existing responsibilities for regulators and the pharmaceutical industry.

3. Conclusions

We have designed and synthesized two series of indole derivatives. These compounds were evaluated as potential antitubulin anticancer agents. Four of the derivatives (**19**, **20**, **22** and **23**) were extensively evaluated for their pharmacological effect on breast cancer. All the four compounds induced cell cycle arrest and apoptosis through multiple mechanisms by induction of ROS generation, mitochondrial membrane

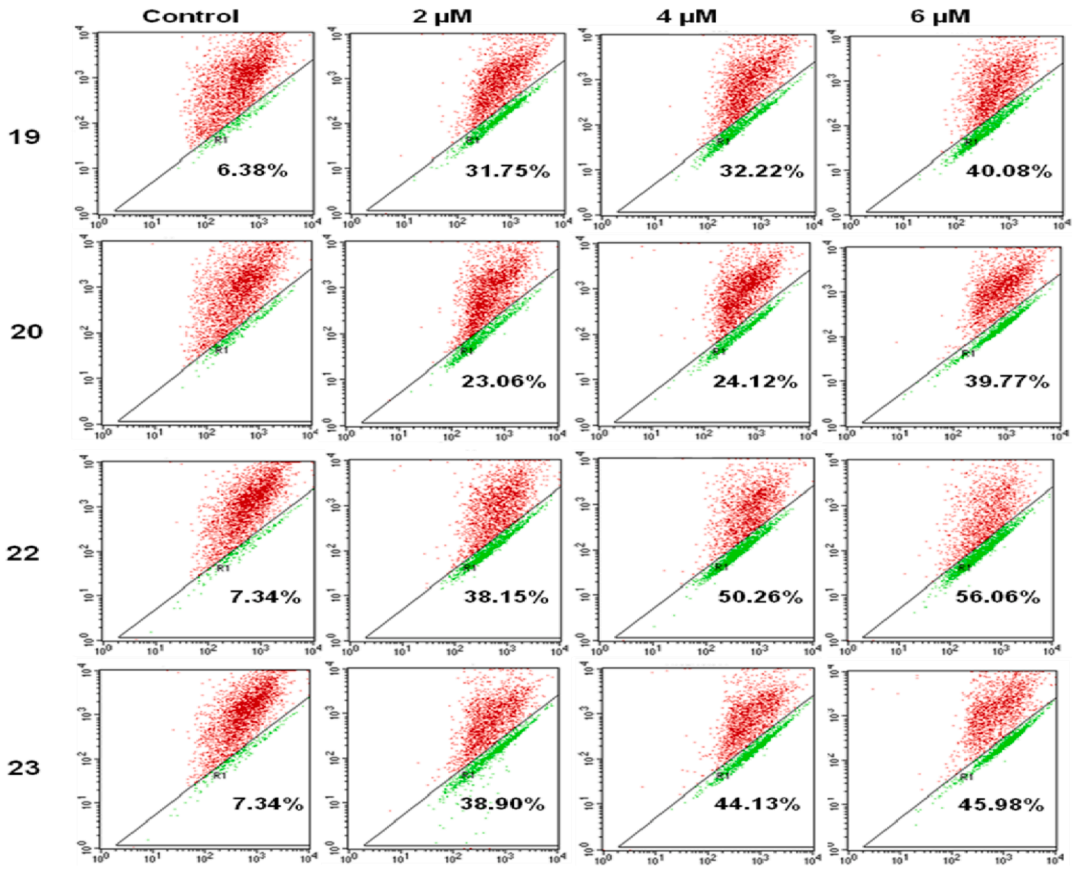


Fig. 4. Effect of compounds **19**, **20**, **22** and **23** on mitochondrial membrane depolarization in MDA-MB-231 cells. [MMP was evaluated using JC-1 dye following treatment with **19**, **20**, **22** and **23** for 24 h, by flow cytometry]

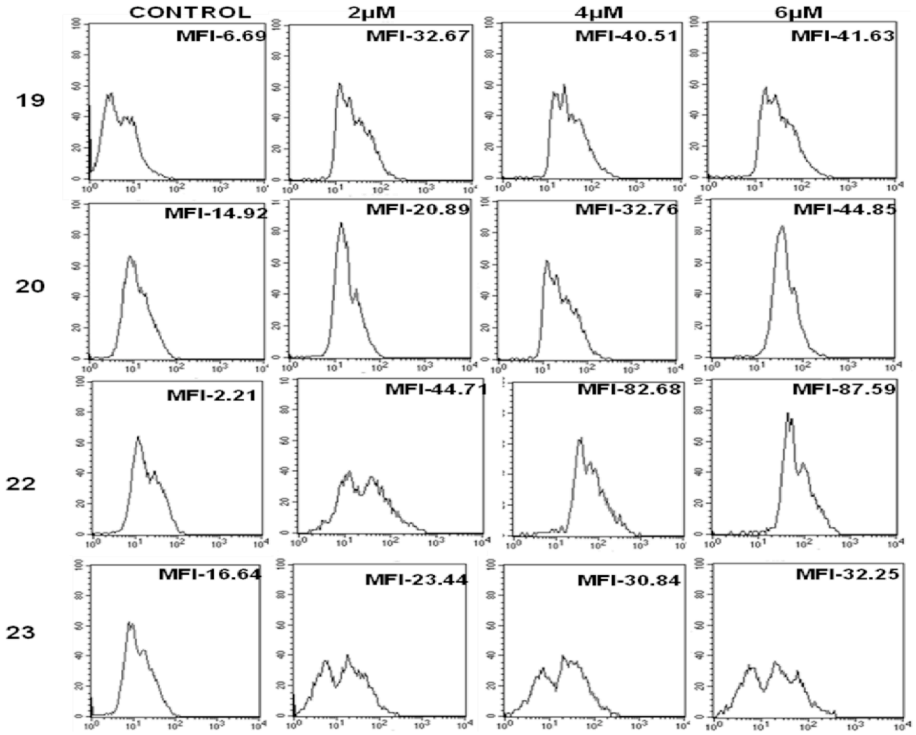


Fig. 5. Induction of ROS generation by compounds **19**, **20**, **22** and **23** in MDA-MB-231 cells. [Cells were treated with **19**, **20**, **22** and **23** for 24 h and generation of ROS is checked by DCFH-DA dye using flow cytometric analysis]

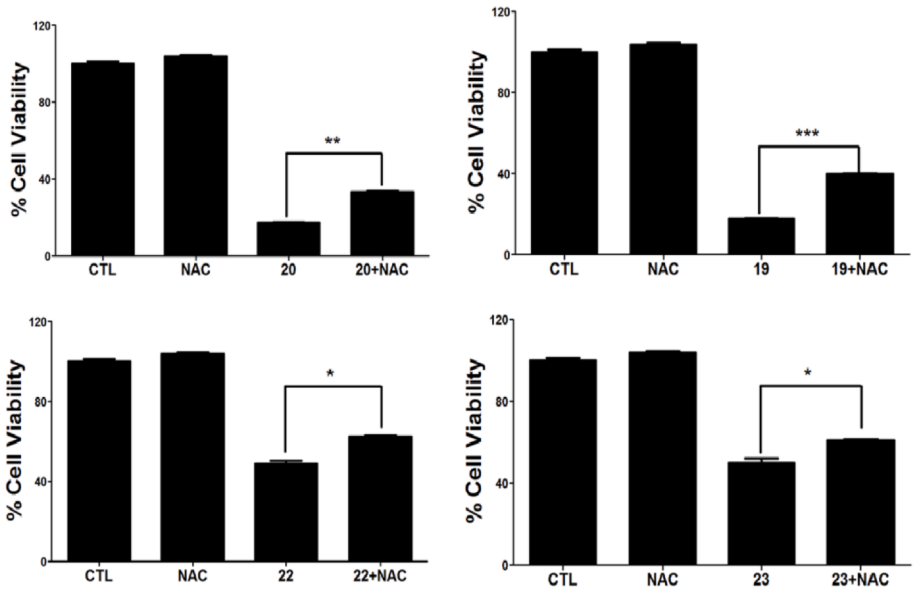


Fig. 6. Effect of compounds **19**, **20**, **22** and **23** on ROS dependent cell viability in MDA-MB-231 cells. [Cells were pre-treated with 10 mM NAC for 2 h and further treated by 4 μM of **19**, **20**, **22** and **23** for 24 h and analysed by MTT Assay. Graphical plots derived from three independent experiments and their numerical values represent mean ± S.E.M.(*P < 0.05,**P < 0.01,***P < 0.001)]

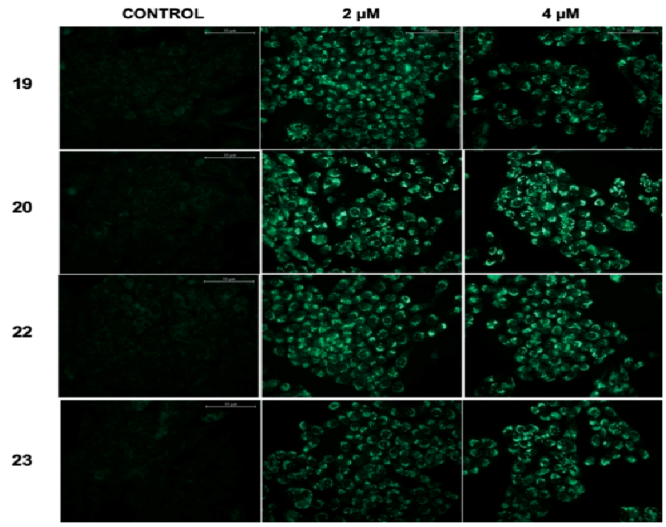


Fig. 7. Induction of autophagy by compounds **19**, **20**, **22** and **23** in MDA-MB-231 cells. [Cells were treated with **19**, **20**, **22** and **23** for 24 h and autophagosome formation is analysed by MDC staining using fluorescence microscopy.]

depolymerisation and caspase activation. All of the four compounds induced autophagy in MDA-MB-231 cells, but only compounds **19** and **20** mediated autophagy is linked with cell death. Compounds **19**, **20** and **23** also caused significant ERβ transactivation, indicating their efficacy against MCF-7 cells through estrogen receptor pathways. All the four compounds stabilized tubulin polymerization similar to paclitaxel. In *in-vivo* efficacy, all these compounds exhibited anti-tumour activity comparable or better than tested regime of paclitaxel. Both the compounds **19** and **23** were well tolerable and safe up to 300 mg/kg oral dose. Collectively, these findings suggest that these indole derivatives have potential as cancer chemotherapeutics against both types of breast cancer.

4. Experimental section

4.1. General

Melting points were determined in open glass capillaries and were uncorrected. Reagents and other chemicals were procured from Sigma-Aldrich, USA, Avra Synthesis India and Sigma-Merck India and were used without purification. Reactions were monitored on pre-coated silica gel TLC-GF_{254} aluminium sheets and visualization of compounds was done under UV light (254 nm and 365 nm) and further charring with 2% ceric sulphate-10% sulphuric acid (aqueous). Purification of compounds was done using glass column and silica gel (100–200 mesh) and characterised by ^{1}H and ^{13}C NMR, ESI-MS, and ESI-HRMS. NMR spectra were obtained with Bruker Avance-300/500 MHz spectrometer. Chemical shifts are given in δ ppm values with respect to tetramethylsilane (TMS) as an internal standard. ^{1}H–^{1}H coupling constant (J) values are given in Hz. ESI mass spectra were recorded using an APC3000 LC-MS-MS (Applied Biosystem) and High Resolution Mass (HRMS) on Agilent 6520Q-TOF after dissolving the compounds in methanol. Purity profile of potent analogues was checked on Waters UPLC system.

Human breast cancer cell lines (MDA-MB-231 and MCF-7), prostate cancer cell line (PC3), and non-malignant human embryonic kidney cell line (HEK-293) were cultured in RPMI media at 37 ^{0}C with 5% CO_2 in a humidified chamber. RPMI media was supplemented with 10% fetal bovine serum (GIBCO BRL Laboratories, New York, USA) and 1% penicillin–streptomycin solution (Sigma Chemical Co., St. Louis, MO, USA). Compounds were dissolved in DMSO (dimethyl sulfoxide) and further diluted in media so that DMSO concentration would be less than 0.001%. Unless otherwise mentioned, all the chemicals were procured from Sigma-Aldrich.

4.2. Chemical synthesis

4.2.1. Synthesis of 5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole (3)

A mixture of L(+)-tartaric acid (1.5 g) and DMU (3.5 g) in 30:70 were heated at 90 °C to obtain a clear melt. To this melt, (174 mg, 1 mmol) of 4-methoxyphenyl hydrazine hydrochloride and 3,4,5-trimethoxyacetophenone (210 mg, 1 mmol) were added and reaction mixture was heated at 90 °C. After completion, the reaction mixture was quenched by adding water and the aqueous layer was extracted with ethyl acetate (3

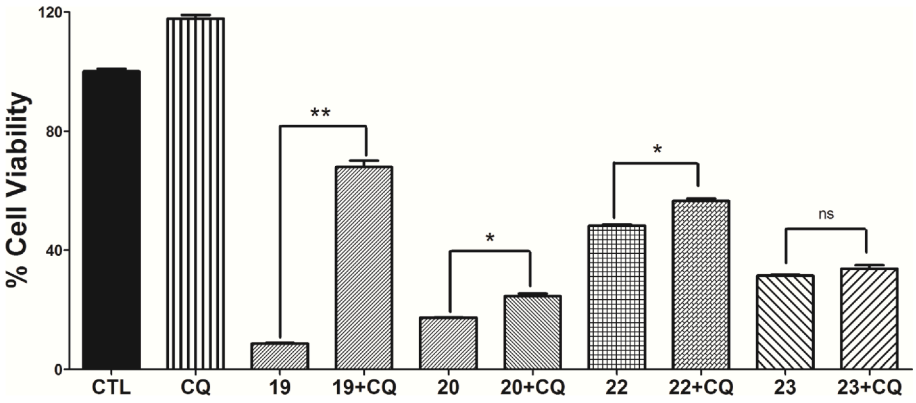


Fig. 8. Induction of autophagy mediated cell death by compounds **19**, **20**, **22** and **23** in MDA-MB-23 cells. [Cells were pre-treated with 10 mM CQ for 2 h and further treated by 4 μM of **19**, **20**, **22** and **23** for 24 h and analysed by MTT Assay. Graphical plots derived from three independent experiments and their numerical values represent mean ± S.E.M.(*P < 0.05,**P < 0.01)]

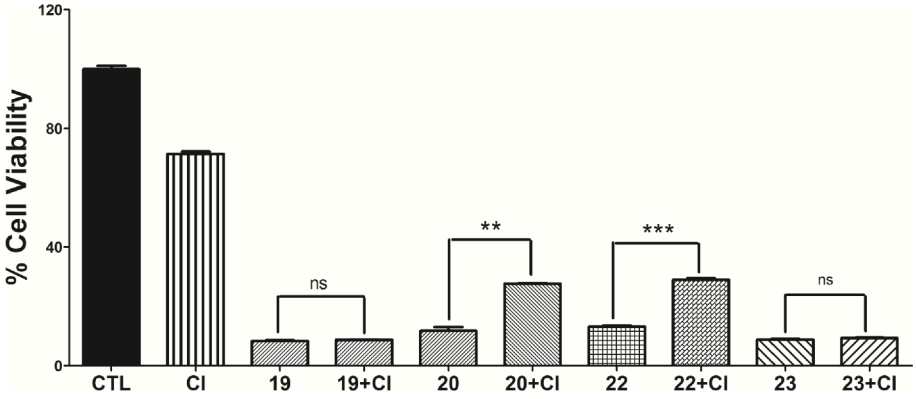


Fig. 9. Caspase mediated cell death in MDA-MB-23 cells by compounds **19**, **20**, **22** and **23**. [Cells were pre-treated with Z-VAD-FMK(CI) for 2 h followed by **19**, **20**, **22** and **23** treatment for 24 h and cell viability was assessed with MTT assay. Graphical plots derived from three independent experiments and their numerical values represent mean ± S.E.M. (**P < 0.01,***P < 0.001).]

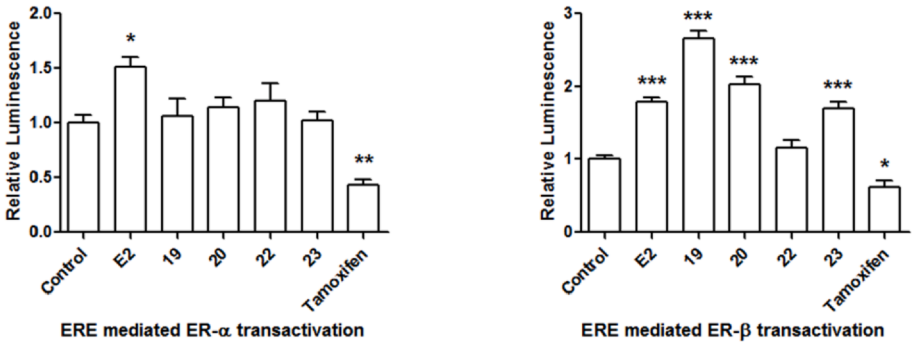


Fig. 10. Effect of compounds **19**, **20**, **22** and **23** on ERα and ERβ trans-activation. [Compounds **19**, **20**, **22** and **23** do not cause ERα trans-activation, but compounds **19**, **20** and **23** enhances ERβ trans-activation. Estradiol (E2) was used as ER (α and β) trans-activating positive control, whereas Tamoxifen (TAM) was used as negative control of ER (α and β) trans-activation. Results were described as percent of normalized relative luciferase unit (RLU). Results were expressed as mean ± SEM, n = 3. p values are *** = p < 0.001, ** = p < 0.01 and * = p < 0.05 versus control]

× 10 mL). The organic layer was washed with water (2 × 10 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude compound was purified by column chromatography over silica gel (100–200 mesh). On elution with acetone-hexane the pure product **3** was obtained as a crystalline solid (Brownish). Purity of synthesized compounds was checked by UPLC and was found to be > 95%.

Yield: 35%, brownish solid; m.p.: 168–170 °C; ESI-MS (MeOH) for $C_{18}H_{19}NO_4$, 314 $[M+H]^+$, 336.3 $[M+Na]^+$, 352.2 $[M+K]^+$; ^1H NMR ($CDCl_3$, 300 MHz): δ3.90 (s,3H,OCH_3), 3.93 (s, 3H, OCH_3), 3.94 (s, 6H, 2 × OCH_3), 6.7 (s,1H,CH, aromatic), 6.80 (s, 3H, 3xCH, aromatic), 7.10 (s, 1H, CH, aromatic), 7.30 (d, 1H, CH, aromatic, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$,75 MHz): δ 56.2, 56.6, 61.4, 100.1, 102.6, 103.1, 112.0, 112.8, 128.9, 130.1, 132.4, 138.3, 138.3, 154.1, 154.9 .

4.2.2. Synthesis of 1-(4-(benzyloxy)benzyl)-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole (5)

Compound **3** (313 mg, 1 mmol) was taken in dry DMF (10 mL) in round bottom flask and kept on ice bath to stir about 10 min. Now pre-washed sodium hydride (60 mg) was added to it and stirred. After 15 min, benzyloxybenzyl chloride (290 mg, 1.25 mmol) was added to it and reaction mixture was stirred for an hour. After completion, reaction mixture was quenched by adding cold water (5 mL) dropwise. Aqueous layer was extracted with ethyl acetate (2 × 10 mL), the combined organic layer was washed with water (3x5 mL), dried over anhydrous

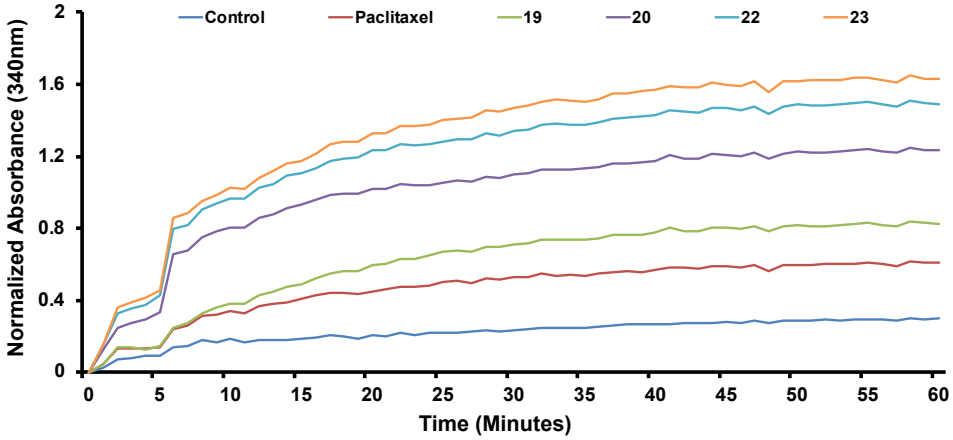


Fig. 11. Effect of compounds **19**, **20**, **22** and **23** on tubulin kinetics (tubulin polymerisation). [Compound **19**, **20**, **22** and **23** enhances tubulin polymerization in cell-free system. Paclitaxel was used as positive control. Mean absorbance obtained from duplicate reactions of each group is plotted versus time in minutes.]

Table 2
Interaction studies of compounds **19**, **20**, **22**, **23**, and paclitaxel with β-tubulin (PDB ID: 6WVR).

Name	Docking binding energy kcal/mol	Binding site residues within 4 Å region	Key amino acid residues
Paclitaxel	−8.1	GLU22, VAL23, ASP26, PHE83, LEU217, HIS229, LEU230, ALA233, PHE272, LEU275, THR276, SER277, ARG278, GLN281, PRO360, ARG369, GLY370, LEU371	**THR276**
Compound **19**	−7.9	LYS19, GLU22, VAL23, ASP26, LEU217, LEU219, ASP226, HIS229, LEU230, ALA233, SER236, PHE272, PRO274, LEU275, THR276, ARG278, GLN281, ARG320, PRO360, ARG369, LEU371	
Compound **20**	−6.8	VAL23, GLU27, LEU217, LEU219, HIS229, ALA233, PHE272, PRO274, LEU275, THR276, SER277, ARG278, GLN281, LEU286, PRO360, ARG369, GLY370, LEU371	
Compound **22**	−7.8	VAL23, ASP26, GLU27, LEU217, LEU219, HIS229, ALA233, PHE272, PRO274, LEU275, THR276, SER277, ARG278, GLN281, LEU286, PRO360, ARG369, GLY370, LEU371	
Compound **23**	−7.1	VAL23, ASP26, GLU27, LEU217, LEU219, ASP226, HIS229, LEU230, ALA233, PHE272, PRO274, LEU275, THR276, ARG278, GLN281, LEU286, PRO360, ARG369, GLY370, LEU371	

Na_2SO_4 and evaporated *in vacuo*. The crude mass was purified through column chromatography over silica gel (100–200 mesh). On elution with benzene-hexane the pure product **5** was obtained as a white crystalline solid in 45% yield. Similarly, compounds **4, 6,** and **7** were also synthesized by same reaction procedure.

Yield: 45%, white solid; m.p.:148–150 °C; ESI-MS (MeOH) for $C_{32}H_{31}NO_5$: 510 $[M+H]^+$,532 $[M+Na]^+$, 548 $[M+K]^+$; ^1H NMR ($CDCl_3$,300 MHz): δ3.66 (s, 6H, 2 × OCH_3), 3.90 (s, 6H, 2 × OCH_3), 5.06 (s, 2H, OCH_2), 5.31(s, 2H, N-CH_2), 6.58 (s, 1H, CH, aromatic), 6.63 (s, 2H, 2xCH, aromatic), 6.85 (bd, 2H, 2xCH, aromatic), 6.94 (d, 2H, 2xCH, aromatic, J = 8.4 Hz), 7.14 (bd, 2H, 2xCH, aromatic); ^{13}C NMR ($CDCl_3$,75 MHz): δ 47.8, 48.0, 56.2, 56.2, 56.2, 61.3, 70.4, 101.9, 102.7, 106.6, 106.6, 111.4, 112.5, 115.6, 115.6, 15.6, 127.3, 127.3, 127.3, 128.3, 128.3, 128.3, 128.3, 128.9, 131.3, 133.9, 137.3, 142.7, 153.5, 155.0, 158.2.

4.2.3. 5-Methoxy-2-(3,4,5-trimethoxyphenyl)-1,3-(4-dibenzyoloxybenzyl-indole (7)

Yield: 53%, white solid; m.p.: 149–151 °C; ESI-MS (MeOH) for $C_{46}H_{43}NO_6$: 706 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz): δ3.44 (s, 6H, 2 × OCH_3), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.04 (s, 2H, 3-CH_2), 5.03 (s, 4H, 2 × OCH_2, benzyloxy gps), 5.19 (s, 2H, *N*-CH_2), 6.38 (s, H, CH, aromatic), 6.83 (m, 8H, 8xCH, aromatic), 6.86 (d, H, CH, aromatic, J = 9 Hz), 6.97 (bd, 3H, CH, aromatic), 7.15 (s, 1H, CH, aromatic), 7.19 (bd, 10*H*, CH, aromatic).

4.2.4. Synthesis of 5-methoxy-2-(3,4,5-trimethoxyphenyl)-1-(4-hydroxy benzyl)-indole (8)

Compound **5 (**509 mg, 1 mmol) was taken in dry THF (10 mL). To this stirred solution, 10% Pd-C was added. The reaction was kept under H_2 supply through balloon for 2 h. After completion, it was filtered through celite bed (filter aid) in sintered crucible (G2), washed with acetone, organic layer was evaporated and residue was taken in ethyl acetate, washed with water, dried over anhydrous sodium sulphate. On evaporation a residue was obtained which was purified through silica gel (100–200 mesh) column using hexane–ethyl acetate as eluants. The pure product **8** was obtained as a white crystalline solid. Similarly, compound **9** was also synthesized by this procedure.

Yield: 40%, white solid; m.p.: 140–142 °C; ESI-MS (MeOH) for $C_{25}H_{25}NO_5$: 420 $[M+H]^+$; ^1H NMR (Acetone-d_6,300 MHz): δ3.76 (s, 6H, 2 × OCH_3), 3.78 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 5.39 (s, 2H, N-CH_2), 6.62 (s,1H, 3-CH, aromatic), 6.80 (bd, 5H, 5xCH, aromatic), 6.93 (d, 2H, 2xCH, aromatic), 6.98 (d 1H, CH, aromatic), 7.17 (d, 1H, CH, aromatic), 7.24 (d, 1H,CH,aromatic, J = 9 Hz), 8.01 (s, 1H,exchangeable,OH); ^{13}C NMR (Acetone-d_6, 75 MHz): δ47.3, 55.4, 55.8, 55.8, 60.1, 101.9, 102.5, 106.8, 106.8, 111.5, 112.0, 115.7, 115.7, 127.5, 127.5, 128.6, 129.1, 129.9, 132.2, 133.9, 142.5, 153.6, 153.6, 155.0, 156.9.

4.2.5. 5-Methoxy-2-(3,4,5-trimethoxyphenyl)-1,3-(4,4-dihydroxydibenzyl)-indole (9)

Yield: 56%, white solid; m.p.: 235–238 °C; ESI-MS (MeOH) for $C_{32}H_{31}NO_6$: 526 $[M+H]^+$; ^1H NMR($CDCl_3$,300 MHz): δ3.66 (s, 6H, 2 × OCH_3), 3.81(s, 6H, 2 × OCH_3), 4.05 (s,2H, 3-CH_2), 5.29 (s, 2H, *N*-CH_2), 6.63 (s, 2H, 2xCH, aromatic), 6.76 (bd, 7H, 7xCH, aromatic), 7.05 (bd, 3H, 3xCH, aromatic), 7.31(d, 1H, CH, aromatic, J = 8.1 Hz), 8.21 (s, 1H, exchangeable, OH), 8.42 (s, 1H, exchangeable, OH); ^{13}C NMR

Table 3
Details about docking studies of indole derivatives with estrogen receptor alpha and beta sub-types.

Compd code	Estrogen receptor-alpha (PDB entry 2OCF)		Estrogen receptor-beta (PDB entry 5TOA)	
	Binding affinity (Kcal/mol)	Binding pocket amino acids	Binding affinity (kcal/mol)	Binding pocket amino acids
Compound **19**	−6.5	LEU320, GLU323, ILE326, GLU353, GLY390, TRP393, ARG394, HIS398, LEU403, PRO406, GLY442, GLU443	−6.0	GLY352, ASP363, ARG364, GLU375, ASP378, MET379, LEU381, ALA382, THR383, SER385, ARG388, HIS464
Compound **20**	−6.1	LEU320, GLU323, PRO325, ILE326, LEU327, GLU353, GLY390, TRP393, ARG394, MET396, GLU397, GLY442, PHE445, LYS449	−5.5	PRO351, GLY352, ARG364, ASP378, MET379, LEU381, ALA382, THR383, SER385, ARG386, HIS464
Compound **22**	−6.9	LEU320, GLU323, PRO324, ILE326, GLU353, TRP393, ARG394, MET396, GLU397, GLN441, GLY442, GLU443	−8.0	LEU273, GLU276, PRO277, PRO278, HIS279, GLU305, MET309, VAL338, LEU339, TRP345, ARG346, ILE348, ASP349, ILE355, PRO358, HIS394, TYR397, LEU398, LYS401
Compound **23**	−6.6	GLU330, TYR331, ASP332, ARG335, PHE337, SER338, ALA340, SER341, GLY344, LEU345, ASN348, VAL534, PRO535, SER537	−6.9	LEU273, GLU276, PRO277, PRO278, HIS279, GLU305, VAL338, GLY342, TRP345, ARG346, ILE355, PRO358, TYR397, HIS394, LEU398, LYS401
β-estradiol	−10.9	GLU353, LEU384, LEU387, MET388, LEU391, ARG394, PHE404, MET421, ILE424, LEU428, GLY521, HIS524, LEU525	−11.0	LEU298, LEU301, GLU305, MET336, LEU339, MET340, LEU343, ARG346, PHE356, ILE373, ILE376, LEU380, GLY472, HIS475, LEU476

($CDCl_3$,75 MHz): δ47.3, 55.4, 55.6, 55.6, 60.1, 101.6, 108.2, 108.2, 111.3, 111.8, 112.3, 115.3, 115.3, 115.3, 127.5, 127.7, 127.7, 127.7, 129.4, 129.4, 129.4, 129.4, 129.4, 130.1, 132.8, 133.3, 139.6, 153.6, 154.6, 155.7, 156.8.

4.2.6. General procedure for the synthesis of alkyloxy alkane and alkyloxy ester derivatives

*4.2.6.1. Synthesis of 1-(4-methoxybenzyl)-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole **(10)**.* 5-Methoxy-2-(3,4,5-trimethoxyphenyl)-1(4-hydroxy benzyl)-indole **(8)** was taken in 10 mL of dry acetone and anhydrous potassium carbonate (1 g, 7.24 mmol) was added to it. To this refluxing mixture dimethylsulphate was added (0.15 mL, 1.35 mmol) and further refluxed for an hour. After completion, reaction mixture was filtered, washed with acetone and evaporated to dryness under reduced pressure. The residue was taken in ethyl acetate, washed with water and organic layer was dried over anhydrous sodium sulphate. It was evaporated to dryness to get crude compound which was purified through column chromatography over silica gel (100–200 mesh) eluting with ethyl acetate- hexane to get **10** in 84% yield (amorphous solid). Similarly, compounds **11**, **17** and **18** were also synthesized by this procedure.

*4.2.6.2. Synthesis of 1-(4-(2-(N,N'-dimethylamine-1-yl)-ethoxy)-benzyl)-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole **(12)**.* Compound **8** (419 mg, 1 mmol) was taken in dry acetone (10 mL) and potassium carbonate (1 g, 7.24 mmol) was added to it. To this refluxing mixture, *N, N'*-dimethylethyl chloride hydrochloride was added (181 mg, 1.2 mmol) and further refluxed for 2–3 h. After completion, the reaction mixture was filtered, washed with acetone and solvent was evaporated to dryness under reduced pressure. The residue was taken in ethyl acetate, washed with water, organic layer was dried over anhydrous sodium sulphate. It was evaporated to dryness to get crude mass which was purified through column chromatography by using 100–200 mesh silica gel, eluting with methanol-chloroform solvent to get pure compound as amorphous solid in 85% yield. Similarly compounds **13**–**16** and **19**–**23** were also synthesized by this procedure.

*4.2.6.3. 1,3-Bis[4-(2-(N,N'-dimethylamine-1-yl)ethoxy)benzyl]-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole **(19)**.* Yield: 86%, yellow gummy; ESI-MS (MeOH) for $C_{40}H_{49}N_3O_6$: 668 $[M+H]^+$; ESI-HRMS (MeOH) for $C_{46}H_{57}N_3O_6$ for $[M+H]^+$, cald, 668.3699, found 668.3645. ^{1}H NMR ($CDCl_3$,300 MHz): δ2.39 (s, 12H, 4 × *N*-CH_3 of chain), 2.79 (t, 4H, 2 × *N*-CH_2 of chain), 3.50 (s, 6H, 2 × OCH_3), 3.72 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 4.05 (bt, 4H, 2 × OCH_2 & 3-CH_2), 5.18 (s, 2H, *N*-CH_2), 6.40 (s, 2H, 2xCH, aromatic), 6.77 (bd, 4H, 4xCH, aromatic), 6.83 (d, 1H, CH, aromatic), 6.90 (bs, 1H, CH, aromatic), 6.93 (d, 2H, 2xCH, aromatic, J = 8.4 Hz), 7.08 (bd, 3H, 3xCH, aromatic); ^{13}C NMR ($CDCl_3$,75 MHz): δ30.0, 30.2, 45.6, 45.6, 45.6, 45.7., 47.7, 54.9, 56.1, 56.1, 56.1 ,56.2, 58.1, 61.2, 65.9, 101.8, 107.7, 107.7, 111.2, 112.0, 112.3, 114.8, 114.8, 114.8, 115.14, 115.1, 115.1, 127.3, 127.4, 129.1, 129.4, 129.4, 131.5, 132.8, 135.0, 139.7, 153.2, 153.27, 154.6,157.0,158.0.

*4.2.6.4. 1,3-Bis[4-(2-(N,N'-diethylamine-1-yl)ethoxy)benzyl]-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole **(20)**.* Yield: 83%, yellow viscous; ESI-MS (MeOH) for $C_{44}H_{57}N_3O_6$: 724 $[M+H]^+$; HRMS for $C_{44}H_{57}N_3O_6$, [M+H]+, calcd, 724.4306, obsvd 724.4306; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.15 (m, 12H, 4 × CH_3 of chain), 2.80 (bs, 8H, $(CH_2)_4$), 3.02 (bs, 4H, *N*-$(CH_2)_2$), 3.51 (s, 6H, 2 × OCH_3), 3.80 (s, 6H, 2 × OCH_3), 4.04 (bd, 6H, 3 × CH_2), 5.19 (s, 2H, 1-N-CH_2), 6.41 (s, 2H, 2xCH, aromatic), 6.79 (bs, 5H, 5xCH, aromatic), 6.92 (bd, 3H, 3xCH, aromatic), 7.10 (s, 3H, 3xCH, aromatic); ^{13}C NMR ($CDCl_3$, 75 MHz): δ10.6, 10.6, 10.8, 10.8, 32.0, 32.0, 32.0, 32.0, 34.2, 47.8, 47.8, 51.7, 56.1, 56.2 ,56.2, 61.2, 65.6, 65.9, 101.8, 107.7, 107.7, 111.9, 112.3, 114.4, 114.7, 115.0, 115.0, 116.2, 116.2, 127.5, 127.5, 129.1, 129.1,129.1, 129.4, 131.6, 132.8, 138.1, 139.7, 153.2, 153.2, 154.6, 156.8, 157.9.

*4.2.6.5. 1,3-Bis[4-(2-(piperidin-1-yl)ethoxy)benzyl]-5-methoxy-2-(3,4,5-trimethoxyphenyl)-1H-indole **(22)**.* Yield: 82%, yellow solid; m.p.: 170–173 °C; ESI-MS (MeOH) for $C_{46}H_{57}N_3O_6$: 748 $[M+H]^+$, HRMS for

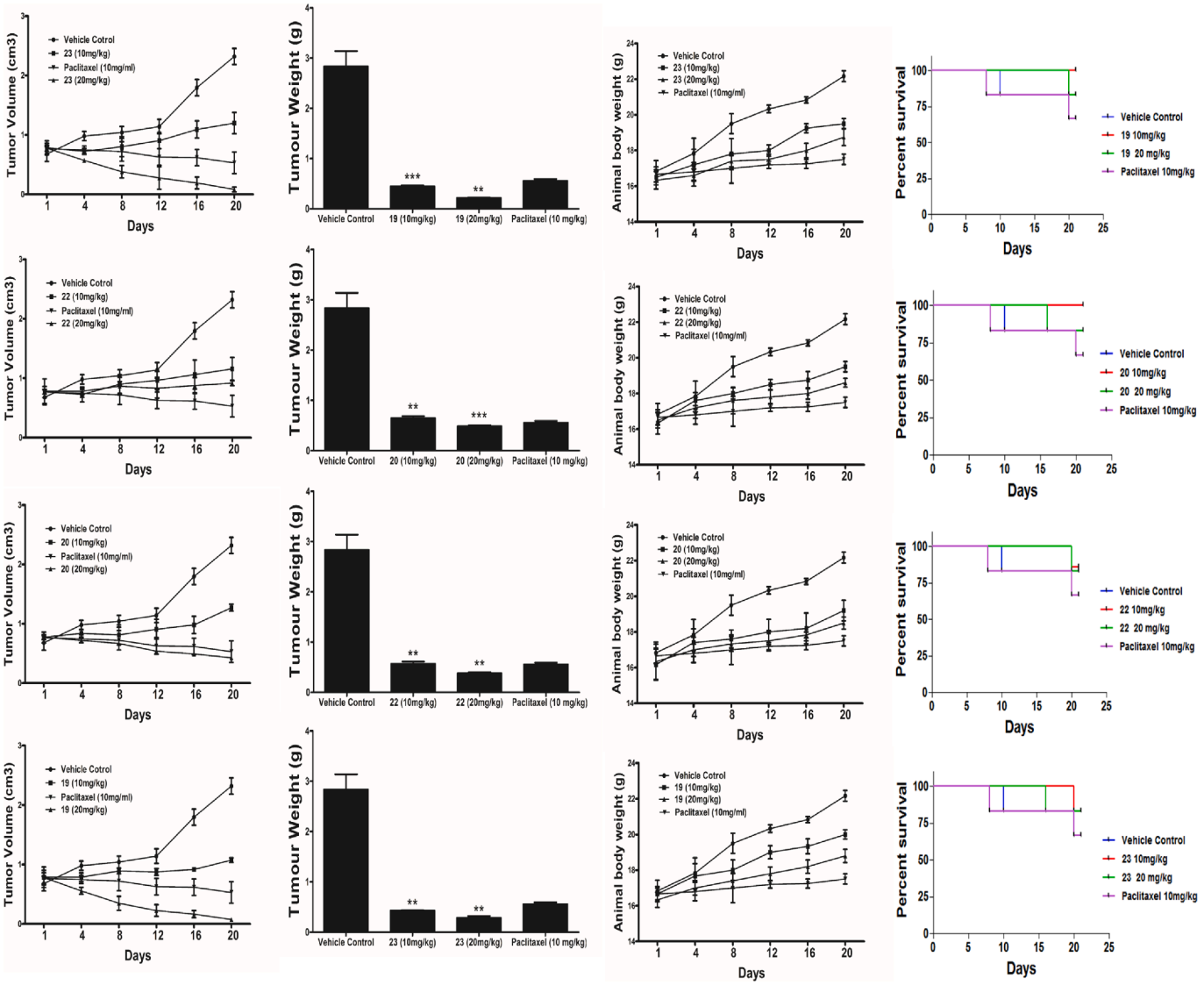


Fig. 12. Compounds **19**, **20**, **22** and **23** exhibit anti-tumour efficacy in 4 T1 orthotropic syngeneic mice mammary tumour model. [Compounds **19**, **20**, **22** and **23** at the rate of 10 mg/kg and 20 mg/kg daily as oral gavage and paclitaxel at the rate of 10 mg/kg through intravenous route twice a week were administered for 21 days. Effect of compounds **19**, **20, 22** and **23** (top to bottom) on tumour volume, tumour weight, whole body weight and animal survival (left to right) show significant anti-tumour efficacy]

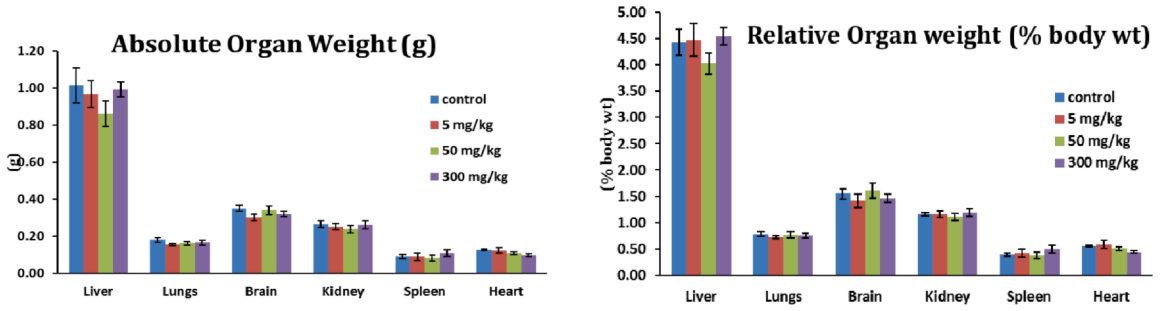


Fig. 13. Effect of compound **19** as a single acute oral dose at 5, 50, 300 mg/kg on absolute and relative organ weight in Swiss albino mice. [Animals showed non-significant changes up to 300 mg/kg single oral dose as compared to control group]

$C_{46}H_{57}N_3O_6$ for [M+H]+, calcd 748.4325, found 748.4314; ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.63 (bd,12H, 6 × CH_2 of piperidine ring), 2.59 (bs, 8H, *N*-$(CH_2)_4$ of piperidine ring), 2.81 (t,4H, *N*-$(CH_2)_2$), 3.49 (s, 6H, 2 × OCH_3), 3.73 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 4.02 (s, 2H, 3-CH_2), 4.08 (bt, 4H, 2 × OCH_2), 5.17 (s, 2H, *N*-CH_2), 6.39 (s, 2H, 2xCH, aromatic), 6.76 (bd, 5H, 5xCH, aromatic), 6.89 (bd, 2H, 2xCH, aromatic), 6.93 (d, H, CH, aromatic, J = 10 Hz), 7.07 (bd, 3H, 3xCH, aromatic); ^{13}C NMR ($CDCl_3$,75 MHz): δ25.6, 25.7, 29.5, 29.5, 29.7, 29.9, 30.0, 47.7, 55.1, 55.1, 56.1, 56.1, 56.1, 56.2, 57.9, 57.9, 61.2, 65.7, 65.8, 101.7, 107.7, 107.7, 111.2, 112.0, 112.3, 114.4, 114.8, 114.8, 115.1, 115.1, 127.4,

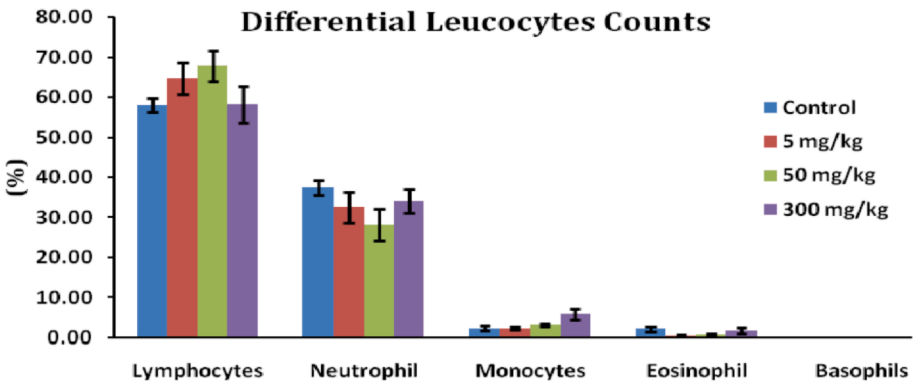


Fig. 14. Effect of compound **19** as a single acute oral dose at 5, 50, 300 mg/kg body weight on differential leucocyte count in Swiss albino mice. [Animals showed non-significant changes up to 300 mg/kg single oral dose as compared to control group]

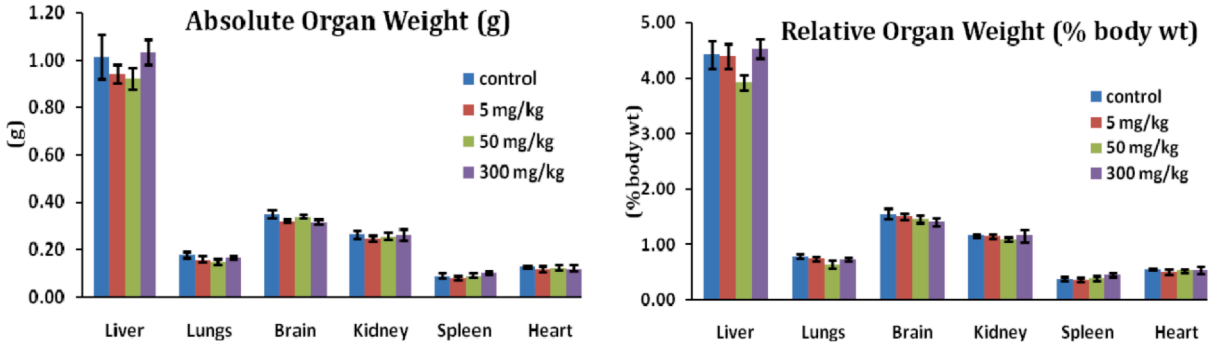


Fig. 15. Effect of compound **23** as a single acute oral dose at 5, 50, 300 mg/kg on absolute and relative organ weight in Swiss albino mice. [Animals showed non-significant changes up to 300 mg/kg single oral dose as compared to control group]

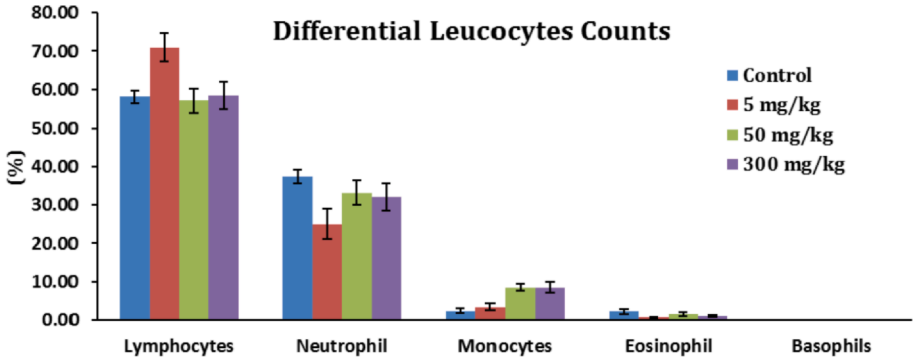


Fig. 16. Effect of compound **23** as a single acute oral dose at 5, 50, 300 mg/kg body weight on differential leucocyte count in Swiss albino mice. [Animals showed non-significant changes up to 300 mg/kg single oral dose as compared to control group]

127.4, 127.4, 127.4, 129.1, 129.4, 131.5, 134.9, 138.1, 139.7, 153.2, 153.2, 154.6, 157.0, 158.0.

*4.2.6.6. 1,3-Bis-[4-(3-dimethylaminopropyl-2-oxy)benzyl]-5-methoxy-2-(3,4,5-trimethoxy phenyl)-1H-Indole (**23**).* Yield: 89%, yellow gummy; ESI-MS (MeOH) for $C_{42}H_{53}N_3O_6$: 696 $[M+H]^+$; HRMS for $C_{42}H_{53}N_3O_6$ for [M+H]+; cacld 696.4012, found, 696.4002; ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.97 (d, 6H, 2 × CH_3 of chain), 2.47 (dd,12H, *N*-$(CH_3)_4$ of chain), 3.49 (bd, 4H, 2x*N*-CH_2), 3.50 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.04 (bd, 2H,3-CH_2), 4.04 (m, 2H, 2xCH of chain), 5.17 (s, 2H, *N*-CH_2), 6.39 (s, 2H, 2xCH, aromatic), 6.75 (m, 8H, 8xCH, aromatic),), 6.90 (bd, 2H,2xCH, aromatic); 7.10 (d, H, CH aromatic. $J = 9.6$ Hz); ^{13}C NMR ($CDCl_3$,75 MHz): δ11.9, 14.5, 18.4, 22.6, 23.0, 44.8, 45.03, 47.7, 56.1, 56.1, 56.1, 56.1, 59.1, 59.1, 59.0, 61.2, 101.9, 107.7, 107.7, 111.3, 112.99, 112.9, 114.7, 114.7, 115.0, 115.0, 116.3, 116.5, 127.6, 127.6, 127.6, 129.5, 129.59, 129.59, 131.0, 131.0, 132.0, 135.4, 139.7 , 153.3, 154.6, 156.4.

4.3. Biological evaluation

4.3.1. Cell viability assay

MTT assay was used to determine the effect of compounds on cell viability as described previously.[33] Briefly, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to determine the effect of compounds on cell viability. Cells were seeded in 96-well plate at a density of 1 × 10^4 cells/well. After 24 h of growth, cells were treated with different concentration of compounds. For inhibitor studies, MDA-MB-231 were pre-treated with 10 mM NAC, 10 mM of CQ

Table 4A
Effect of compound **19** as a single acute oral dose at 5, 50, 300 mg/kg on body weight, hematological and serum biochemical parameters in Swiss albino mice.

Parameters	Oral dose of compound **19** at mg/kg body weight as a single oral dose			
	Control	**5 mg/kg**	**50 mg/kg**	**300 mg/kg**
Body weight (gm)	22.75 ± 0.93	21.71 ± 1.00	21.31 ± 0.81	21.88 ± 0.73
Haemoglobin (gm/dL)	17.35 ± 0.30	16.59 ± 0.60	17.01 ± 0.47	15.96 ± 0.71
RBC (million/ mm^3)	9.21 ± 0.32	9.67 ± 0.74	8.72 ± 0.37	9.45 ± 0.63
WBC(thousands/ mm^3)	8.55 ± 2.71	6.73 ± 0.80	4.86 ± 0.35	5.53 ± 0.98
ALP (U/L)	123.22 ± 16.04	97.53 ± 19.48	106.88 ± 9.77	98.70 ± 13.96
SGOT (U/L)	22.94 ± 4.04	22.11 ± 2.82	21.92 ± 3.24	27.73 ± 3.16
SGPT (U/L)	12.65 ± 2.98	14.47 ± 0.84	15.07 ± 1.54	16.00 ± 1.64
Creatinine (mg/ dL)	0.11 ± 0.03	0.12 ± 0.02	0.14 ± 0.02	0.15 ± 0.03
Triglycerides (mg/ dL)	81.12 ± 7.75	63.83 ± 7.82	91.70 ± 13.60	91.20 ± 16.23
Bilirubin(mg/dL)	0.44 ± 0.06	0.30 ± 0.02	0.35 ± 0.02	0.36 ± 0.04
Cholesterol (mg/ dL)	122.05 ± 8.12	112.13 ± 6.90	121.96 ± 11.26	132.33 ± 10.57
Albumin (g/dL)	1.62 ± 0.09	1.52 ± 0.12	1.43 ± 0.23	1.94 ± 0.17
Protein (mg/ml)	2.01 ± 0.11	1.61 ± 0.16	1.48 ± 0.21	1.93 ± 0.31

Data are expressed as Mean ± SE (n = 6). SGOT: serum glutamic-oxaloacetic transaminase , SGPT: Serum glutamic pyruvic transaminase ALP: Alkaline phosphatise; gm/dL, gram per deciliter; U/L, units per litre; mg/dL, milligram per deciliter. Tukey's multiple comparison test was used to determine the significance between compound **19** and control, $P < 0.05$.

Table 4B
Effect of compound **23** as a single acute oral dose at 5, 50, 300 mg/kg on body weight, haematological and serum biochemical parameters in Swiss albino mice.

Parameters	Oral dose of compound **23** at mg/kg body weight as a single oral dose			
	Control	**5 mg/kg**	**50 mg/kg**	**300 mg/kg**
Body weight (gm)	22.75 ± 0.93	21.60 ± 0.86	23.48 ± 0.93	22.75 ± 0.63
Haemoglobin (gm/dL)	17.35 ± 0.30	15.83 ± 1.26	15.21 ± 0.37	15.54 ± 0.65
RBC (million/ mm^3)	9.21 ± 0.32	9.12 ± 0.48	8.67 ± 0.55	7.96 ± 0.44
WBC(thousands/ mm^3)	8.55 ± 2.71	6.23 ± 1.39	7.50 ± 0.82	5.60 ± 0.98
ALP (U/L)	123.22 ± 16.04	87.76 ± 8.97	84.85 ± 14.25	74.12 ± 8.11
SGOT (U/L)	22.94 ± 4.04	28.86 ± 3.88	33.28 ± 3.50	33.25 ± 2.39
SGPT (U/L)	12.65 ± 2.98	11.82 ± 1.02	18.69 ± 2.55	19.12 ± 3.65
Creatinine (mg/ dL)	0.14 ± 0.02	0.12 ± 0.04	0.12 ± 0.02	0.14 ± 0.02
Triglycerides (mg/ dL)	81.12 ± 7.75	105.82 ± 14.59	83.99 ± 8.99	86.58 ± 8.66
Bilirubin(mg/dL)	0.44 ± 0.06	0.34 ± 0.04	0.39 ± 0.02	0.36 ± 0.02
Cholesterol (mg/ dL)	122.05 ± 8.12	117.25 ± 12.55	108.65 ± 13.90	108.72 ± 11.79
Albumin (g/dL)	1.62 ± 0.09	1.48 ± 0.06	1.29 ± 0.11	1.53 ± 0.08
Protein (mg/ml)	2.01 ± 0.11	1.57 ± 0.32	1.28 ± 0.12	1.66 ± 0.24

Data are expressed as Mean ± SE (n = 6). SGOT: serum glutamic-oxaloacetic transaminase , SGPT: Serum glutamic pyruvic transaminase ALP: Alkaline phosphatise; gm/dL, gram per deciliter; U/L, units per litre; mg/dL, milligram per deciliter. Tukey's multiple comparison test was used to determine the significance between compound **19** and control, $P < 0.05$.

and 50 μM of Z-VAD-FMK for 2 h followed by treatment with 2 μM of compounds **19**, **20**, **22** and **23** for 24 h. At the end of treatment, 20 μL of MTT (5 mg/mL) was added in each well. After incubation for 3 h, media along with MTT was carefully removed. 200 μL DMSO was added to dissolve the formazan crystal and absorbance was recorded at 540 nm using ELISA plate reader. The assay was performed in triplicates having three replica of each test compound.

4.3.2. Cell cycle analysis

Cell cycle analysis was performed to evaluate effect of compounds in cell cycle progression using propidium iodide (PI) staining by flow cytometer against MDA-MB-231.[34] Briefly, MDA-MB-231 cells (1×10^6 cells) were seeded in T-25 culture flask. After 24 h of growth, cells were treated with different concentration of compounds for 24 h. At the end of treatment, all the cells including floating were harvested, washed with PBS and fixed in ice cold 70% ethanol for 1 h at 40 °C. Cells were then centrifuged, re-suspended in 300 μL PBS containing 30 μg of RNAse A and 15 μg of PI and incubated for 30 min at room temperature. Samples were acquired and analyzed by flow cytometry using FACS Calibur instrument (BD Biosciences).

4.3.3. Apoptosis analysis

Apoptosis analysis was carried out using annexinV-FITC/PI dual staining kit using flow cytometry.[33] In brief, MDA-MB-231 cells (1×10^6) were seeded in six-well plates and allowed to grow for 24 h. The medium was then replaced with medium containing different concentrations of compounds **19**, **20**, **22** and **23** and incubated for 24 h. At the end of incubation, cells were harvested by trypsinization, washed with PBS, re-suspended in binding buffer along with Annexin V-FITC and PI for 10 min in dark at room temperature. Samples were analyzed using FACS Calibur instrument (BD Biosciences).

4.3.4. Mitochondrial membrane potential (MMP) assay

MMP was measured by flow-cytometry using JC-1 (5,5,6,6-tetrachloro-1,1,3,3 tetraethylbenzimidazolecarbocyanine iodide) dye against MDA-MB-231 cells.[33] Briefly, MDA-MB-231 cells (1×10^6) were seeded in six well plates for 24 h. Cells were treated with different concentrations of compounds for 24 h. After 24 h, cells were harvested by trypsinization, washed with PBS and incubated along with 5 μg/ml JC-1 dye for 30 min in dark at room temperature. At the end of incubation, cells were re-suspended in 300 μL of PBS and analyzed using FACS Calibur instrument and FACSuite Software (BD Biosciences).

4.3.5. ROS generation assay

ROS was measured using DCFH-DA (2,7-dichlorodihydrofluorescein diacetate) dye by flow cytometer in MDA-MB-231 cell.[33] Briefly, MDA-MB-231 cells (1×10^6) were seeded in 6-well plates for 24 h. Cells were treated with different concentrations of compounds for 24 h. At the end of treatment, cells were harvested by trypsinization, fixed in cold methanol and incubated along with 30 μg/mL DCFH-DA for 30 min in dark at room temperature. At the end of incubation, cells were re-suspended in 300 μL of PBS and analyzed using FACS Calibur instrument (BD Biosciences).

4.3.6. Monodansylcadaverine (MDC) staining

MDC staining was used to detect formation of autophagic vacuoles by fluorescence microscopy in MDA-MB-231cells.[33] MDA-MB-231 (1×10^6) cells were seeded on cover slips for 24 h followed by treatment with different concentrations of compounds for 24 h. After 24 h, cells were gently washed with PBS and stained with 0.05 mM MDC for 30 min at room temperature in dark. At the end of incubation, cells were washed with PBS and samples were analyzed for autophagosome formation using Nikon ECLIPSE 80i fluorescence microscope.

4.3.7. Tubulin polymerisation assay

Tubulin polymerisation assay was performed as per reported method

using 'assay kit' from Cytoskeleton, USA.[35] Paclitaxel (Sigma, USA) was used as standard stabilizer of tubulin polymerisation. In brief, 96 well microtiter plate was kept pre-warmed and 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 it at 37 °C in the presence of our test compounds **19**, **20**, **22** and **23** with desired concentrations (10 μM). All samples were properly mixed and polymerisation was monitored in kinetic mode at 340 nm every min for 1 h using Spectramax plate reader. Paclitaxel (taxol) was used as standard stabilizer of tubulin polymerisation.

4.3.8. *In vivo antitumor efficacy*

In vivo antitumor studies were conducted in orthotopic syngenic mice model in adult female Balb/c mice.[36] Animal studies were conducted with prior approval from the Institutional Animal Ethics Committee (IAEC), CSIR-CDRI, Lucknow, India. Adult female Balb/c mice were used to develop 4 T1 syngenic mice mammary tumor model. Briefly, 4 T1 cells (7×10^5) were injected in mammary fat pad of six week old female Balb/c mice. After one week, when the tumors become measurable, animals were randomly divided into ten groups. Compounds were dissolved in ethanol and further diluted in PBS. Different compounds were orally administered at the dose of 10 mg/kg body and 20 mg/kg of body weight daily for 21 days. 10 mg/kg of body weight of paclitaxel was given intravenous twice a week. Control group animals received vehicles only. The body weight of animals as well as tumor size were measured twice a week. The tumor volume was calculated using the formula (LW2) × 0.5, where L is the tumor length and W is the tumor width. Test compounds **19**, **20**, **22** and **23** were administered as oral gavage at the dose of 10 mg/kg and 20 mg/kg of bodyweight daily for 21 days. Paclitaxel at the dose of 10 mg/kg of bodyweight was administered intravenously twice a week for 21 days. Control group animals received vehicles only. Oral administration of compounds **19**, **20**, **22** and **23** was preferred in order to enhance safety, versatility as well as minimize pain. However, further pharmacokinetics optimization study required for maximum bioavailability. Intravenous administration of paclitaxel is widely used due its better efficacy and bioavailability.

4.3.9. *ERα and ERβ transcriptional activation assay*

Transcription activation studies were done in MDA-MB-231 cells with ERα (pSG5 mERα) and ERβ (pSG5-hER β) plasmids.[37] MDA-MB-231 cells were seeded in 24 well plate and allowed to attain a confluency of 80–90%. The cells were then transfected with 500 ng of expression vector for ERα (pSG5 mERα) and ERβ (pSG5-hER β) plasmids (generous gifts from Prof. M.G. Parker, Imperial Cancer Research Fund, London, UK) using Lipofectamine 2 LTX transfection reagent (ThermoFisher) as per manufacturer's protocol. To normalize for transfection efficiencies, pRL-SV40-luc (Promega) was used. After transfection, cells were trypsinized and seeded (2×0^4 cells/well) into the 96-well plate. Cells were incubated with E2 (100 nM), 1 μM of compounds **19**, **20**, **22** and **23** and tamoxifen 1 μM. Luciferase activity was quantified using Dual Luciferase Assay System (Promega), according to the manufacturer's protocol to detect the transcriptional activity. The firefly luciferase intensity for each sample was normalized based on transfection efficiency measured by Renilla luciferase activity. The experiments were performed intriplicate with three replicates in each.

4.3.10. *Safety studies*

Acute oral toxicity assay of compounds **19** and **23** was done as per reported method.[30,31] Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). The study and number of animals used were approved by the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India *via* CIMAP/IAEC/2016–19/01 dated 09–02-2017.

Briefly, 30 mice (15 male and 15 female) were taken and divided into four groups comprising 3 male and 3 female mice in each group weighing between 20 and 25 g. The animals were maintained at 22 ± 5 ^{0}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 provided *ad libitum* drinking water. Mice of group 1 (Group I) were kept as control and animals of groups 2, 3, and 4 (Groups II, III, & IV) were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. Test compound was dissolved in minimum volume of DMSO, diluted with 0.7% CMC (carboxymethylcellulose) and was given at 5, 50, and 300 mg/kg body weight to animals of groups 2, 3, and 4 (Groups II, III & IV) respectively once orally. 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 and there after a daily general case side clinical examination was carried out for 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. In addition to observational study, body weights were recorded and blood and serum samples were collected from all the animals on 7th day of the experiment in acute oral toxicity. The samples were analyzed for total RBC, WBC, differential leucocytes count, haemoglobin percentage and biochemical parameters like ALP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, serum protein and tissue protein activity. The animals were then sacrificed and were necropsied for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were recorded.

CRediT authorship contribution statement

Yashveer Gautam, Nandini Pathak and Dushyant Singh raghuvanshi-Chemical Synthesis Sharmistha Das and Hamidullah Khan-Cytotoxicity, cellcycle analysis, Annexin-V, ROS exp., In-vivo efficacy etc. Hina Iqbal, Pankaj Yadav and D. Chanda-Acute oral toxicity exp. Vijay Kumar Sirohi and Anila Dwivedi- Estrogen Receptor trans activation exp. Sana Khan and Feroz Phan- Docking exp. Karuna Shanker-Purity Profile Rituraj Konwar-Detailed biology exp. planning, execution Arvind S. negi-detailed chemistry exp., planning, execution.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Senior Research Fellowship to the authors (YG) from Council of Scientific and Industrial Research (CSIR) is duly acknowledged. The manuscript has CIMAP Communication No.: CIMAP/PUB/2017/25.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116252.

References

[1] Schiavon G, Smith IE. *Hematol Oncol Clin N Am.* 2013;27:715–736.
[2] daSilva JL, Nunes NCC, Izetti P, deMesquita GG, deMelo AC. *Critical Rev Oncol Hematol.* 2020;145, 102855. https://doi.org/10.1016/j.critrevonc.2019.102855.
[3] Kerru N, Gummidi L, Maddila S, Gangu KK, Jonnalagadda SB. *Molecules.* 2020;25:1909.
4 Thanikachalam PV, Maurya RK, Garg V, Monga V. *Eur J Med Chem.* 2019;180:562–612.
[5] Sravanthi TV, Manju SL. *Eur J Pharm Sci.* 2016;91:1–10.
[6] Wan Y, Li Y, Yan C, Yan M, Tang Z. *Eur J Med Chem.* 2019;183, 111691.
[7] Davies DL, Shepherd M. *The Lancet.* 1955;269:117–120.
[8] Almagro L, Fernandez-Perez F, Pedreno MA. *Molecules.* 2015;20:2973–3000.

[9] Hedner T, Edgar B, Edvinsson L, Hedner J, Persson B, Pettersson A. *Eur J Clin Pharmacol*. 1992;43:651–656.
[10] Wilkins RW, Judson WE. *New Engl J Med*. 1953;248:48–53.
11 Gruber C, Gruber D. *Curr Opin Invest Drugs London Eng*. 2004;5:1086–1093.
12 Marcin LR, Mattson RJ, Gao Q, et al. *Bioorg Med Chem Lett*. 2010;20:1027–1030.
13 Haubrich DR, Ward SJ, Baizman E, et al. *J Pharmacol Exp Ther*. 1990;255:511–522.
14 Revill P, Mealy N, Seradell N, Bolos J, Rosa E. Drug of. *Future*. 2007;32:315–322.
15 Mack GS. *Nat Biotechnol*. 2010;28:1259–1266.
16 Negi AS, Gautam Y, Alam S, et al. *Bioorg Med Chem*. 2015;23:73–389.
17 Gore S, Baskaran S, Konig B. *Org Lett*. 2012;14:4568–4571.
18 Malumbres M, Barbacid M. *Nat Rev Cancer*. 2009;9:153–166.
19 Su Z, Yang Z, Xu Y, Chen Y. *Yu Mol Cancer*. 2015;14:48.
20 Hartwell LH, Weinert TA. *Science*. 1989;246:629–634.
21 Carneiro BA, El-Deiry WS. *Nat Rev Clin Oncol*. 2020;17:395–417.
22 Dharamaraja AT. *J Med Chem*. 2017;60:3221–3240.
23 Trachootham D, Alexander J, Huang P. *Nat Rev Drug Discov*. 2009;8:579–591.
24 Kocaturk NM, Akkoc Y, Kig C, Bayraktar O, Gozuacik D, Kutlu O. *Eur J Pharm Sci*. 2019;134:116–137.
25 Boice A, Biochim Bouchier-Hayes L. Biophys Acta (BBA) – Mol. *Cell Res*. 2020;1867, 118688. https://doi.org/10.1016/j.bbamcr.2020.118688.
26 Holst F, World J. *Clin Oncol*. 2016;7:160–173.
27 Heldring N, Pike A, Andersson S, et al. *Physiol Rev*. 2007;87:905–931.
28 Chang EC, Frasor J, Komm B, Katzenellenbogen BS. *Endocrinol*. 2006;147: 4831–4842.
29 Cao YN, Zheng LL, Wang D, Liang XX, Gao F, Zhao XL. *Eur J Med Chem*. 2018;143: 806–828.
30 Chanda D, Shanker K, Pal A, et al. *J Toxicol Sci*. 2008;34:99–108.
31 Ghosh MN. In Fundamentals of Experimental Pharmacology (1st Edition). *pp156*. Kolkata: Scientific Book Agency; 1984.
32 World Health Organisation; "The SAFETY of Medicines in Public Health Programmes: Pharmacovigilance an essential tool". *ISBN 92 4 159391 1 (NLM classification: QV 771)*. Geneva: Switzerland; 2006.
33 Hamidullah Saini. KS, Ajay A, Devender N, Bhattacharjee A, Das S, Dwivedi S, Gupt MP, Bora HK, Mitra K, Tripathi RP, Konwar R. *Int J Biochem Cell Biol*. 2015;65: 275–287.
34 Chakravarti B, Akhtar T, Rai B, et al. *J Med Chem*. 2014;57:8010–8025.
35 Gautam Y, Dwivedi S, Srivastava A, et al. *RSC Adv*. 2016;6:33369–33379.
36 Khan S, Shukla S, Sinha S, Lakra AD, Bora HK, Meeran SM. *Int J Biochem Cell Biol*. 2015;58:1–16.
37 Saxena R, Chandra V, Manohar M, et al. *PLoS ONE*. 2013;8, e66246.