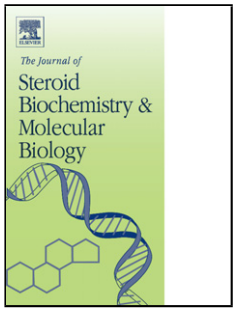

Title: Induction of targeted osteogenesis with 3-aryl-*2H*-benzopyrans and 3-aryl-*3H*-benzopyrans: Novel osteogenic agents

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Induction of targeted osteogenesis with 3-aryl-*2H*-benzopyrans and 3-aryl-*3H*-benzopyrans: Novel osteogenic agents

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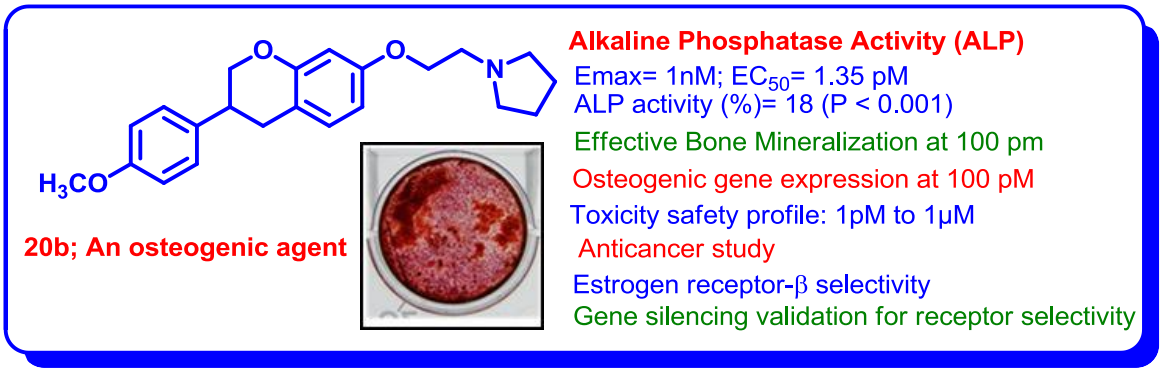
Graphical abstract



Highlights:

- 3-aryl-*2H*-benzopyran and 3-aryl-*3H*-benzopyran derivatives as novel osteogenic agents.
- **20b, 22a**, **27**and **32** showed significant osteogenic activity at EC_{50} values 1.35, 34.5, 407 and 29.5pM.
- **20b** and **32** significantly increased mineral nodule formation and the transcript levels of BMP-2, RUNX-2 and osteocalcin at 100 pM.

- **22b** showed potent anticancer activity at IC_{50} 6.5-13.2 µM in human cancer cell lines, by inducing apoptosis and arresting cell cycle at sub-G_0 phase.

Abstract; Development of target oriented chemotherapeutics for treatment of chronic diseases have been considered as an important approach in drug development. Following this approach, in our efforts for exploration of new osteogenic leads, substituted 3-aryl-*2H*-benzopyran and 3-aryl-*3H*-benzopyran derivatives (**19**, **20a-e**, **21**, **22a-e**, **26**, **27**, **28a-e**, **29**, **31a-b**, **32** and **33**) have been characterized as estrogen Receptor-β selective osteogenic (bone forming) agents. The synthesized compounds were evaluated for osteogenic activity using mouse calvarial osteoblast cells. Four compounds *viz* **20b, 22a**, **27**and **32** showed significant osteogenic activity at EC_{50} values 1.35, 34.5, 407 and 29.5pM respectively. Out of these, **20b** and **32** were analyzed for their bone mineralization efficacy and osteogenic gene expression by qPCR. The results showed that **20b** and **32** significantly increased mineral nodule formation and the transcript levels of BMP-2, RUNX-2 and osteocalcin at 100 pM concentrations respectively**.** Further mechanistic studies of **20b** and **32** using transiently knocked down expression of ER-α and β in mouse osteoblast (MOBs) showed that **20b** and **32** exerts osteogenic efficacy *via* activation of estrogen receptor-β preferentially. Additionally, compounds showed significant anticancer activity in a panel of cancer cell lines within the range of (IC_{50}) 6.54 to 27.79 µM. The most active molecule, **22b** inhibited proliferation of cells by inducing apoptosis and arresting cell cycle at sub-G_0 phase with concomitant decrease in cells at S phase.

Keywords: Estrogen, antiestrogen, SERMs, isoflavone, anticancer, antiosteoporotic.

1.0 Introduction:

The skeleton is a highly specialized and dynamic organ that undergoes continuous regeneration [1,2]. Once the skeleton attains maturity, regeneration continues in the form of a periodic replacement of old bone with new one at same location [3]. This process is known as remodelling and is responsible for complete regeneration of adult skeleton every ten years. The bone remodelling involves two highly synchronized biological processes namely osteoblastogenesis and osteoclastogenesis through osteoblast and osteoclast cells respectively [4,5]. Any imbalance in the process of remodelling which leads to exaggerated bone resorption culminates in a disorder known as osteoporosis [6a-b]. It is now established that Osteoprotegrin (OPG)/ receptor activator NFκB ligand (RANKL)/ receptor activator NFκB (RANK) system is a dominant and ultimate mediator of osteoclastogenesis [7]. Osteoprotegrin, is a member of tumour necrosis factor receptor superfamily IIB (TNF II B) and is a decoy receptor for the receptor activatror NF κB ligand (RANKL) on osteoblast/stromal cells [8]. It blocks the RANKL-RANK ligand interaction between osteoblast/stromal cells and osteoclast precursor cells thus inhibits differentiation of osteoclast precursor cell into a mature osteoclast cell and eventually bone resorption.

Furthermore, estrogen has great role in bone remodelling and management by acting as bone resorption inhibitor as well as osteogenic agent. It has been reported to stimulate production of Osteoprotegrin (OPG) and to down regulate many cytokines such as iterlukins IL-1, IL-6, TNF-α and monocyte/macrophage colony stimulating factor (M-CSF) involved in bone resorption [9,10,11a-b]. The anti-resorptive action of estrogen was further shown by Parika et.al. who concluded that estradiol treatment significantly

decreased the depth of resorption pits by disturbing the metalloproteinases and cysteine proteinases mediated organic matrix degradation [12]. The osteogenic effect of estrogen is through their involvement in stimulated differentiation and activity of osteoblast cells [13]. Osteobalst cells are reported to express both estrogen receptor-alpha (ER-α) and estrogen receptor-beta (ER-β) and their differential level of expression during human osteoblast differentiation reveal gradual increase in ER-β mRNA expression [14]. The observed differential regulation of ER is suggestive for an additional functional role of ER-β to ER-α in bone maintenance [15].

In elderly persons specially in women after menopause, the decreased level of estrogen leads to lack of adequate new bone formation but also exaggerate bone resorption leading to osteoporosis [16,17]. To compensate the deficiency of estrogen at this stage of life Estrogen replacement or hormone replacement therapies are generally recommended [18]. However, apart from beneficial effects, these options are also associated with some serious side effects especially in reproductive tissues and offer threats for estrogen dependent cancers such as breast, uterine and ovarian cancer. With the advance studies of ER-α and ER-β, it is now established that ER-α is dominant in the adult uterus and deletion of ER-β does not affect the response of uterus to estrogen [19,20]. Similarly, ER-α dominates in breast cancer tissue and is the main culprit for excess cell proliferation while ER-β is less abundant and has antagonising effect in this tissue [19,20]. These findings suggest that ligands selectively targeting ER-β may be an important therapeutic strategy for the clinical management of osteoporosis without or minimal threats for secondary complications such as breast, uterine and ovarian cancer [20]. A deeper insight

into the ligand binding pocket (LBP) of the ER-α and ER-β, reveals that although LBP of ER-α is slightly larger that ER-β but both are lined with nearly identical residues expect two amino acid differences [19-22]. The first difference is in the residues below the D-ring pocket of the binding cavity where ER-α- has Met-421 while ER-β has Ile-373 [21]. The second difference is found above the D-ring pocket where ER-α has Leu-384 while ER-β has Met-336. These differences in LBP among ER-α and ER-β imparts subtle differences between two receptor binding cavities which can be used for development of receptor isofrom selective ligands i.e. ER-α *vs* ER-β selective ligands [19,21,22]. Also, there is ample scope for structural modification in the region of D-ring pocket of cavity. A successful story based on this fact is the development of TAS-108 (SR-16,234) and SR 16,137 [23a-d].

Further, it is interesting to note that difference in the size of the binding cavities and the above mentioned differences in the amino acid residues of both receptors make the ER-α more selective and accommodative towards three dimensionally bulky molecules such as 17β-estradiol (3) and other steroids while ER-β more towards structurally cylinderical molecules such as such as daidzein (**4**), genistein (**5**), formononetin (**6**) and isoformononetin (**7**) and their reductive metabolites such as equol (**8-10**) [20-24].

Based on these observations, in the present study, we planned to develop 3-aryl-*2H*-benzopyran and 3-aryl-*3H*-benzopyran based non-steroidal structurally cylindrical molecules as novel target oriented osteogenic agent. It was proposed that these molecules will have an aryl group substituted with tertiary alkylamino group, usually present in selective estrogen receptor modulators (SERMs) in a region encompassing D-ring of the 17β-estradiol along with a phenolic or its substituent analogous to its phenolic group of

present in ring A. To achieve the objective of the project following prototypes (**11** and **12**) were designed and synthesized.

2.0 Chemistry:

The synthetic strategy for designed molecules **11** and **12** required 7-hydroxy-3-(4-methoxyphenyl)-*3H*-benzopyran-4-one (**18**) and 7-methoxy-3-(4-hydroxyphenyl)-*2H*-benzopyran-4-one (**29**) in large quantity. The synthesis of **18** was done through Friedel-Craft acylation reaction on resorcinol (**13**) using 4-methoxyphenyl acetic acid (**14**) in BF_3-OEt_2 at 100°C which yielded **15** in 75-80 % yield (Scheme-1) [25]. Compound **15** was subjected to alkylation reaction using benzyl bromide in presence of anhydrous potassium carbonate in dry acetone at reflux which gave corresponding benzylated derivative (**16**) in 78 % yield. Further, cyclization of **16** to get desired 7-benzyloxy-3-(4-methoxyphenyl)-*3H*-benzopyran-4-one (**17**) using paraformaldehyde in 50% NaOH solution at 150°C yielded **17** in 85% yield. Controlled catalytic hydrogenation of **17** using 10% palladium charcoal (Pd/C) in Tetrahydrofuran (THF) gave 7-hydroxy-3-(4-methoxyphenyl)-*3H*-benzopyran-4-one (**18**) in 90% yield however, under these reaction conditions, prolong reduction of **17** yielded 7-hydroxy-3-(4-methoxyphenyl)-*3H*-benzopyran (**19**) in 88% yield. Furthermore, Grignard reaction performed on **18** using methylmagnesium bromide (CH_3MgBr) in THF-OEt_2 at reflux gave **21** in 65% yield. Finally, compounds **19** and **21** were subjected to alkylation reaction using different chloroethylalkylamines in presence of anhydrous potassium carbonate in dry acetone which gave target compounds **20**(a-e) and **22**(a-e) in 77-90% yields.

The synthesis of 7-methoxy-3-(4-hydroxyphenyl)-*2H*-benzopyran-4-one (**29**) was attempted following above synthetic methodology used for synthesis of **18,** however, this approach could not yield **29** in good quantity. Therefore, an alternate approach was adopted for synthesis of **21**. In this approach, synthesis of **29** was started from deoxybenzoin (**25**), which was synthesized through Friedel-Craft acylation reaction of 3-methoxyphenol (**23**) and 4-hydroxyphenylacetic acid (**24**) in BF_3-OEt_2 at 100°C in 74% yield (Scheme-2). Compound **25** was then reacted with dimethylformamide-dimethylacetal (DMF-DMA) in dimethylformamide (DMF) at room temperature to yield 7-methoxy-3-(4-hydroxyphenyl)-benzopyran-4-one (**26**) in good yield. Controlled catalytic hydrogenation with palladium charcoal (Pd/C) in Tetrahydrofuran (THF) gave 7-methoxy-3-(4-hydroxyphenyl)-*2H*-benzopyran-4-one (**29**) in 50% yield along with **27**, however, under these reaction conditions, prolong reaction time gave 7-methoxy-3-(4-hydroxyphenyl)-*3H*-benzopyran (**27**) in 68% yield. Furthermore, Grignard reaction on **26** using methylmagnesium bromide (CH_3MgBr) in THF-OEt_2 at reflux gave compound **30** in 68% yield. Finally, compounds **27** and **30** were subjected to alkylation reaction using different chloroethylalkylamines in presence of anhydrous potassium carbonate in dry acetone gave target compounds **33**(a-e) in 81-92% yields.

2.1 Optical Resolution of 20a:

3-aryl-*3H*-benzopyran derivatives have one chiral centre at C-3 atom of bezopyran nucleus which can generates two optically active isomers. The synthetic methodology used for synthesis of 3-aryl benzopyran derivatives in this study yielded racemic mixture of products. In order to know the effect of orientation of phenyl group present at C-3 on

biological activities of 3-aryl benzopyran derivatives, pure enantiomers of 3-aryl-*3H*-benzopyran derivatives were required. For this purpose, one of the active compounds, **20a** was selected for optical resolution. The optical resolution of **20a** was achieved through its salt formation with pure enantiomers of tartaric acid in absolute methanol followed by repeated crystallization [26].

3.0 Biology

3.1 Assessment of ostegenic potential of compounds using Osteoblast differentiation assay

Generally, alkaline phosphatase (ALP), osteoclacin (OCN) and type-1 collagen are some important bone markers which are frequently used to follow the differentiation process of osteoblasts [27]. Among these bone markers, ALP and type-1 collagen are considered as early markers of osteoblastogenesis. In our study, we have used ALP as a marker of osteoblast differentiation to assess osteogenic activity in mouse calvaria derived osteoblast cells. Bone forming activity of **19, 20a-e, 21, 22a-e, 26, 27, 28a-e, 29, 32** and **33** was thus evaluated by measuring ALP activity. The mouse calvarial osteoblast cells were treated with test compounds and ALP activity was measured spectrophotometrically. Compounds **20a-e, 22a-e, 26,** and **32** showed significant increase in ALP activity compared to the control untreated cells. Since, Equol (**10**), an active metabolite of Daidzein (**4**), inhibits bone loss apparently without estrogenic activity and has a high affinity for estrogen receptor-β [34, 35, 40a], it was taken as a positive control in this study. Among the compounds under study, four compounds *viz* **20b**, **22a**, **27**, and **32** showed maximum osteogenic efficacy as assessed by ALP activity at concentrations of EC_{50} 1.35, 34.5, 407 and 29.5pM respectively, compared to the control untreated cells (Tabe-1). Considering significant ALP activity (osteogenic activity) of tested compounds,

two potential compounds, **20b** and **32** with lowest EC_{50} values**,** were further evaluated for their bone mineralization and osteogenic gene expression efficacy (discussed below). Further, it was also determined if these compounds stimulated osteogenesis via estrogen receptor mediated pathway.

3.2 In-vitro toxicity in healthy osteoblast cells:

Once the bone forming activity of these compounds was determined the next step was to confirm that these compounds were devoid of any toxicity. To exclude this possibility, compounds **20a-e, 22a-e, 26, 32** and equol (**10**) which showed significant ALP activity were also evaluated for *in-vitro* cytotoxicity using osteoblast cells in MTT assay. In this assay, the viable cells convert the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into its insoluble formazon, a purple reaction product, while dead cells remained uncoloured. In general, the toxicity results showed that compounds **20a-e, 22a-e, 26, 32** and equol (**10**) did not have any inherent toxicity for healthy osteoblast cells at doses ranging from 1pM to 1µM concentration. The cytotoxic activity results of the most active compounds *viz* **20b** and **32** have been presented in Figure 2a-b. Though **20b** at concentration 1nM exhibited a trend towards higher proliferation but statistically no significance was observed.

3.3 Mineralization efficacy of 20b and 32 in calvarial osteoblast cells:

The process of osteoblstogenesis involves differentiation of preosteoblastic cells into terminally differentiated osteoblast cells mainly in three stages *viz* proliferation, matrix development and maturation and mineralization. The most reliable indicator of

differentiation of osteoblastic precursor cells to the terminal osteoblastic phenotype is the mineralization. Therefore, **20b** and **32** which showed potential alkaline phosphatase (ALP) activity were further evaluated for their osteogenic activity in osteoblast mineralization assay. To assess the osteoblast mineralization efficacy of the compounds, calvarial osteoblast cells were cultured for 18 days in differentiation media containing 10mM β-glycerophosphate and 50 μg/ml ascorbic acid in presence or absence of **20b** and **32**. Cells were then stained with alizarin red-S and dye was extracted to quantify the extent of osteoblast mineralization. The alizarin dye stains newly formed mineralized nodes which accumulate calcium. After treatment, optical densitometry was used to quantitate mineralization of osteoblast cells by measuring alizarin extracted from stained cultures.

Both compounds exhibited significant increase in mineral nodule formation at concentrations as low as 100pM and 10nM compared to the control untreated cells (Figure 3a and b). The biological activity data showed that **20b** and **32** significantly enhanced the matrix mineralization. In fact, **20b** was even more potent than the standard drug. The percent increase in mineralization by **20b** at 100pM concentration over equol was found to be ~40% (Figure 3a-b).

3.4 Study of 20b and 32 mediated Osteoblastogenesis related osteogenic gene expression:

Bone morphogenetic proteins (BMPs) are members of transforming growth factor-β (TGF-β) superfamily which play an important role in osteoblast differentiation [28]. Among other BMPs, BMP-2 stimulates osteoblast phenotype expression such as increase in ALP activity and collagen synthesis [29]. Additionally, Runt-related transcription

factor-2 (RUNX-2) is a protein which is a transcription factor associated with osteoblast differentiation and skeletal morphogenesis [30a-b]. Further, osteoclacin (OCN) is an established late differentiation marker for bone formation [31]. Considering these markers as important indicators of bone formation, the mRNA expression levels of these markers were evaluated in presence of **20b** and **32** for further confirmation of their bone forming potential. To determine the effect of **20b** and **32** on osteogenic markers *viz* BMP-2, RUNX-2, and OCN, osteoblast cells were treated with compounds at 100pM concentration for 24 and 48h, total RNA was isolated and cDNA was synthesized. cDNA was used as a template in real time quantitative PCR. The house keeping gene GAPDH was used as the internal control in our experiments. Results showed that treatment of osteoblast cells with **20b** and **32** at 100pM concentration increased the transcript levels of BMP-2 (1.7 and 2.5 fold by **20b** at 24 and 48h respectively; 1.8 fold and 1.5 fold by **32** at 24 and 48h respectively), RUNX-2 (2.5 and 2.8 fold at 24 and 48 h respectively by **20b**; 2 fold and 1.7 fold at 24 and 48h respectively by **32**), and osteocalcin (~2 fold at 24 and 48h by **20b**; ~1.8 fold at 24 and 48h by **32**) which are the important markers of osteoblast differentiation (Figure 4a-c). Based on the mineralization and qPCR data, **20b** was found to have the best activity and was further taken up for the mode of action studies.

3.5 Selectivity of estrogen receptor (ER) -α and β in action of compound 20b

Most of the isoflavones such as daidzein (**4**), genestein (**5**), formononetn (**6**), isoformononetin (**7**) and Equol (**10**) induce bone formation with inhibition of bone resorption and have affinity for both estrogen receptors (ER-α and β) [32-36]. Structurally all these molecules have cylindrical core structure. It is established that molecules with

cylindrical and nearly flat core nucleus have preferential affinity for ER-β isoform [37, 38]. Therefore; it was interesting to elucidate that whether **20b** exhibited its osteogenic effect through estrogen receptors and had any receptor subtype selective mode of action.

For this purpose, osteoblast cells were treated with compounds for 48h, following which lysates were prepared, electrophoresced on SDS-PAGE gel and probed with antibodies against ER-α and ER-β. It was observed that **20b** enhanced the protein expression levels of both ER-α and ER-β. However, **20b** showed selectivity towards ER-β significantly. Equol was taken as a positive control (Figure 5a-b). Based on the above observations, we propose that **20b** and its analogues exhibits osteogenic effect by enhancing osteoblast differentiation and mineralization in an estrogen receptor dependent manner.

3.6 Validation of estrogen receptor selectivity by gene silencing

In order to further validate the specific role of ER-α and ER-β in osteogenic effect of **20b**, we performed selective knockdown of ER-α and ER-β in mouse osteoblasts (MOBs). Small interfering siERα and siERβ efficiently reduced their respective protein levels thus also confirming the selectivity of knockdown of the siRNA constructs (Figure 6a). Subsequently, we determined ALP activity in the presence of selective ER siRNAs. As expected, **20b** strongly induced ALP activity and this increase was abrogated in presence of siERβ but not siERα (Figure 6b). These results showed that **20b** stimulates osteoblast function through activation of ER-β (Figure 6b).

3.7 In vitro anticancer activity

In bone remodelling process, the level of RANKL and OPG is an important and most likely determinant factor. While elevated level of OGP helps in bone formation, RANKL favours bone resorption. Some cancerous cells especially breast cancer cells are capable of imitating normal hormonal and local signals through secretion of parathyroid hormone related protein (PTHrP) which induces increased level of RANKL relative to OPG, thus promotes bone resorption and consequently increases chances of breast cancer metastasis to bone. Keeping this fact in consideration, compounds **19, 20a-e, 21, 22a-e, 26, 27, 28a-e, 29, 31a-b, 32** and **33** were further evaluated for their anticancer activity in a panel of cancer cell lines *viz* MCF-7 (ER+ breast adenocarcinoma), DLD1 (colorectal adenocarcinoma), A549 (lung carcinoma), FaDu (hypopharyngeal carcinoma), DU 145 (prostate carcinoma). These cell lines were procured from American Type Culture Collection (ATCC), Manassas, VA, USA. Out of twenty four compounds, **20a-e**, **22a-e**, **28a-e**, **31a-b** and **33** showed significant anticancer activity in the range of (IC_{50}) 6.54 to 27.79 µM concentration. The anticancer activity of **19, 20a-e, 21, 22a-e, 26, 27, 28a-e, 29, 31a-b, 32** and **33** is described by half maximal inhibitory concentration (IC_{50}) value in Table 2. In these experiments, tamoxifen (**TAM**) was used as positive control (Table 2). Compound **22a-e** and **31a-b** were found to be most active compounds among the series. The most active molecule **22b** was further taken up for cell cycle analyses and other biological experiments.

3.8 Cell division cycle study of 22b

Compound **22b** was further studied for detailed biological characterization. For this purpose, estrogen receptor (ER) positive human breast cancer cell line (MCF-7) was selected for subsequent biological assays. The effect of **22b** on cell division cycle was studied. After 24 and 48 hrs incubation with the test compound at 9 μM concentration (near IC_{50} value), **22b** showed time dependent significant accumulation of cells population at sub-G_0 phase compared to the vehicle treated controls (Figure 7a-b). This was associated with concomitant decrease in cells at S phase and marginal decrease in cell population at G_2/M phase. We further investigated ability of **22b** to trigger apoptosis in MCF-7 cells. In the present study, we monitored cleavage of Poly (ADP-ribose) polymerase (PARP) which is considered as a marker of apoptosis. Consistent with microscopic observation, Western blot analyses of MCF-7 cell lysates revealed proteolysis of PARP in **22b** treated cells confirming induction of apoptosis (Figure 8). These results are in agreement with cytotoxic activity data where significant increase in apoptotic population at Sub-G_0 was observed after **20b** treatment. Compound **22b** caused marked fragmentation of MCF-7 cellular nuclei in comparison to the vehicle control group (Figure 8). In these experiments, doxorubicin (Doxo), a known apoptosis inducer, was used as positive control.

4.0 Structure activity relationship (SAR) study

In general designed compounds belong to two different sub sets namely 3-aryl-*3H*-benzopyran (**20a-e** and **28a-e**) and 3-aryl-*2H*-benzopyran (**22a-e** and **31a-b**) derivatives. The biological activity pattern of 3-aryl-*3H*-benzopyran derivatives (**20a-e** and **28a-e**)

revealed that positions 4'and 7 of 3-aryl-*3H*-benzopyran core were optimal locations for substitution of methoxy and tertiaryaminoalkyl groups respectively to elicit bone forming activity. The reverse situation yielded compounds with devoid of any activity. Almost, similar trend of biological activity *vs* structural requirement of compounds was observed as far as their anticancer activity was concern. Additionally, a comparison of bone forming activity of **19** and **27** showed that the presence of a hydroxyl group at position 4' and a methoxy group at position 7 of 3-aryl-*3H*-benzopyran core (compound **27**) was required for bone forming activity rather reverse substituent pattern which yielded corresponding inactive compound (**19**). It is reported that daidzein (**4**) and genistein (**5**) which also incorporate 3-arylbenzopyran core fit into the ligand binding pocket of estrogen receptor in such a way that their pendent phenyl ring (ring C, figure 1) present at C-3 of benzopyranone core simulates with ring A of estradiol and benzopyranone (rings A and B) simulates with rings C and D of estradiol [20,21,37]. The similar observations were also made during SAR study of a potent 2,3-diarylbenzopyran based anti-estrogenic drug candidate, CDRI 85/287, wherein 3-arylbenzopyran, a part of the molecule, was reported to bind with ligand binding pocket as described for daidzein and geinstein [39]. Perhaps, most logistically, compounds based on 3-aryl-*3H*-benzopyran nucleus would have followed the same binding pattern within ligand binding pocket of ER. Thus, in case of compounds substituted with tertiaryaminoalkyl group at position 7 and a methoxy group at position 4', such binding pattern would have allowed the bulky tertiaryaminoalkyl group to be accommodated in the D-ring region of LBP where D-ring of estradiol fits in. However, in case of reverse substitution pattern, the bulky tertiaryaminoalkyl group would have been projected in a region where A-ring of estradiol

fits in (A-ring region of LBP) which is not a favourable condition as for ligand binding with ER and therefore compounds could not show any activity (table-1).

Further, to see the effect of orientation of aryl group present at C-3 of 3-aryl-*3H*-benzopyran nucleus, resolution of enantiomers of **20a** was made. Interestingly, while levorotatory isomer of **20a** (**33**) presented improved anticancer activity, its dextrorotatory isomer (**32**) showed potent bone forming activity as compared with racemic mixture (**20a**). Possibly, the reported enantiospecific estrogenic activity of 3-aryl-*3H*-benzopyran derivative such as S (-)-equol could have been the reason for such observed activities. [40, 41]. Surprisingly, conversion of 3-aryl-*3H*-benzopyran core to 3-aryl-*2H*-benzopyran led to compounds with significant anticancer and bone forming activities (compounds **22a-e** and **31a-b,** table 1 and 2). However, as observed in 3-aryl-*3H*-benzopyran derivatives, in this case, the impact of substitution of tertiaryaminoalkyl and methoxy groups in 3-aryl-*2H*-benzopyran derivatives was negligible. The plausible reason for such activity pattern could be that introduction of C=C bond in 3-aryl-*3H*-benzopyran core incorporated stilbene framework within the molecule which could have improved molecular affinity to estrogen receptors as reported literature [42].

Further addition of alkyl group, especially methyl in this case, to the newly generated stilbene framework resulted molecule with equal similarities of rings A and B of benzopyran with either rings A and B or rings C and D of the estradiol. Therefore, with an assumption that molecules will tend to accommodate the bulky tertiaryaminoalkyl group in ample space provided near the D-ring region of LBP, compounds based on 3-aryl-*2H*-benzopyran core had equal options to bind with ER by either A ring or C ring of 3-aryl-*2H*-benzopyran derivatives and elicited biological activity irrespective of substitution

pattern. As discussed above, the preferential ER-β affinity of most active osteogenic molecule **20b** can be ascribed to slender core structure of the molecule as described for 3-arylbenzopyran based phytoestrogens such as daidzein and equol [25, 37, 40a]. Additionally, the pyrrolidinoethyl group present at position 4' of 3-aryl-*3H*-benzopyran core in **20b** would have been well accommodated in the D-ring pocket of LBD of ER-β rather than ER-α which possibly helped in induction of ER-β selectivity of **20b**.

5.0 Conclusion:

The approved therapies for osteoporosis are mainly focused on use of bone resorption inhibitors. The recent observations have revealed that bone resorption is accompanied by inhibited bone formation due to coupling of these two processes which represents main disadvantages associated with bone resorption inhibitors [42,43]. The attention has now been focused on osteogenic agents for osteoporosis treatment [44]. In our efforts, we have logically modified 3-aryl-benzopyran core as in order to develop novel osteogenic agent. The designed molecules showed significant osteogenic activity through preferential activation of estrogen receptor-β and were devoid of any inherent cytotoxicity to healthy osteobalst cells in nano to pico molar concentrations. The mechanistic studies of **20b** and **32** showed their ability to increase transcript level of important bone markers such as BMP-2, RUNX-2 and osteocalcin at 100pM concentrations. The synthesized compounds (**20a-e**, **22a-e**, **28a-e**, **31a-b** and **33**) also presented significant anticancer activity in a panel of cell lines within the range of (IC_{50}) 6.54 to 27.79 µM concentration. Effect of **22b** on cell cycle of MCF-7 cells revealed that **22b** inhibited cell proliferation by

inducing apoptosis and arresting cell cycle at sub-G_0 phase with concomitant decrease in cells at S phase.

Overall, 3-aryl-*2H*-benzopyran and 3-aryl-*3H*-benzopyran derivatives have been identified as novel structural types of potential osteognic and anticancer agents. Taken together the activity profile of these molecules, it is predictable that such 3-aryl-benzopyran based molecules could be develop as target oriented potential osteogenic agents which have minimal or no threat for development of estrogen dependent cancers or osteogenic sarcoma.

6.0 Experimental Section

Biological methods: Reagents and chemicals

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA) and all fine chemicals from Sigma Aldrich (St. Louis, MO). ECL kit was obtained from Amersham Pharmacia, USA. All antibodies for Western blot analysis were obtained from Cell Signaling Technologies (Danvers, MA).

Culture of mice calvarial osteoblasts

Mouse calvarial osteoblasts were obtained following our previously published protocol of sequential digestion. The study was conducted in accordance with current legislation of animal experiments (Institutional Animal Ethical Committee at Central Drug Research Institute). Male or female Balb/c mice of 1-2 days old were used for culture. In Brief, calvaria from neonatal mice (both sexes) were pooled. After surgical isolation from the skull and removal of sutures and adherent mesenchymal tissues, calvaria were subjected

to five sequential (10–15 min) digestions at 37ºC in a solution containing 0.1% dispase and 0.1% collagenase P. Cells released from the second to fifth digestions were pooled, centrifuged, resuspended, and plated in T-25 cm2 flasks in a-MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth medium).

Osteoblast differentiation

For the measurement of alkaline phosphatase (ALP) activity, osteoblasts at ~80% confluence were trypsinized and 2 x 10^3 cells per well were seeded in 96-well plates. Cells were treated with different concentrations of the compounds for 48 h in a-MEM supplemented with 5% Fetal bovine serum Serum, 10 mM ß glycerophosphate, 50 µg/mL ascorbic acid and 1% penicillin/streptomycin (osteoblast differentiation medium). At the end of the incubation period, the total ALP activity was measured using *p*-nitrophenylphosphate (PNPP) as a substrate and quantified colorimetrically at 405 nm. After that we quantified percentage activity of ALP. Then transformed the data using X = Log [X] and analyzed in non-linear regression (curve fit). Sigmoidal dose response was obtained by using prism software and calculated the Emax and EC_{50} values. Emax is the maximum response achievable from a drug/compound. The concentration of compound/drug at which effect (E) is 50% of Emax is termed the half maximal effective concentration and is abbreviated EC_{50}.

MTT assay

For this assay 1 × 10^4 cells/well were seeded in 100 µL DMEM, supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37°C in a CO_2

incubator. Compounds, diluted to the desired concentrations in the culture medium, were added to the wells with respect to the control. After 48 h of incubation, media were removed and to each well 100 uL MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added and plates were further incubated for 4 h. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100 µL of DMSO and the absorbance at 570 nm wavelength were recorded.

Mineralization of calvarial osteoblast cells

For mineralization studies, calvarial cells were cultured in a-MEM medium, supplemented with 10% fetal bovine serum, 50 µg/mL ascorbic acid, and 10 mM ß glycerophosphate. Cells were cultured with and without compounds for 21 day at 37^0C in a humidified atmosphere of 5% CO2 and 95% air, and the medium was changed every 48 h. After 21 d, the attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in PBS. After fixation, the specimens were processed for staining with 40 mM alizarin redS, which stains areas rich in nascent calcium. Equol was used as a positive control. For quantification of alizarin red-S staining, 800 µL of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with gentle shaking. Monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5-mL tube. After vortexing for 30 s, the slurry was over laid with 500 µL mineral oil (Sigma–Aldrich), heated to exactly 85^0C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 x *g* for 15 min and 500 µL of the supernatant was removed

to a new tube. Then 200 µL 10% (v/v) ammonium hydroxide was added to neutralize the acid. OD (405 nm) of 150 µL aliquots of the supernatant were measured in 96-well plates.

qPCR assay

Total RNA was extracted from the cultured cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 µg total RNA with the Revert Aid™ H Minus first strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). SYBR green chemistry was used for quantitative determination of the mRNAs for BMP-2, Runx-2, osteocalcin and a housekeeping gene, GAPDH, following an optimized protocol. The design of sense and antisense oligonucleotide primers was done using the Universal Probe Library (Roche Diagnostics, Indianapolis, IN). Primer sequences for BMP-2 (accession number: NM_007553.2) were 5′-CGG ACT GCG GTC TCC TAA-3′ (forward) and 5′-GGG GAA GCA GCA ACA CTA GA-3′ (reverse); for Runx2 (accession number: AF053956.1), 5′-CCC GGG AAC CAA GAA ATC-3′ (forward) and 5′-CAG ATA GGA GGG GTA AGA CTG G-3′ (reverse); for osteocalcin (accession number: L24429.1), 5′-TAA GTG GCC AGA GCC CTT AG-3′ (forward) and 5′-CAG AAA CCA CCC CAG CAC-3′ (reverse) and for GAPDH (accession number: NM_008084.2), 5′-AGC TTG TCA TCA ACG GGA AG-3′ (forward) and 5′-TTT GAT GTT AGT GGG GTC TCG-3′ (reverse).

For real-time PCR, the cDNA was amplified with Light Cycler 480 (Roche Diagnostics Pvt Ltd, Indianapolis, IN). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the Light Cycler 480 SYBER Green I Master (Roche Diagnostics Pvt. Ltd, Indianapolis, IN) to allow for quantitative detection of the PCR product in a 20 µL reaction volume. The temperature profile of the reaction

was 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 2 min, and annealing and extension at 62 °C for 30 s extension at 72 °C for 30 s. GAPDH was used to normalize differences in RNA expression.

Western blotting

Cells were grown to 60–70% confluence following which they were treated with and without compound for 48 h. Cells were lysed with cell lysis buffer (Sigma-Aldrich) with protease inhibitor cocktail (Sigma-Aldrich). Cell lysate was centrifuged at 12000 g for 15 min and supernatant was collected. Estimation of protein concentration was determined by Bradford assay. Thirty micrograms of total protein was then resolved by 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were probed with ER-a, ER-b, PARP and b-Actin antibodies (Cell Signaling Technology, Danvers, MA, USA) and then incubated with secondary antibodies conjugated with HRP (Cell Signaling Technology). Immunodetection was done using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK) using Image Quant LAS 4000 (GE Healthcare). Densitometry of blots was done using quantity 1D analysis software and gel doc imaging system.

SiRNA transfection

Twenty four hours before transfection, mice calvarial osteoblasts (MOBs) were plated in 96 well and six well plates for evaluation of ALP activity and protein expression studies respectively. The cells were then transfected with 25 nM siC, siERα or siERβ using Dharmafect I transfection reagent (Dharmacon) in reduced serum media (Opti-MEM)

following manufacturer's instructions. For ALP assay and western blotting assay, 16 h following transfection cells were treated with compound **20b** and Equol for 48 h. At the end of the incubation period, the total ALP activity was measured using *p*-nitrophenylphosphate (PNPP) as a substrate and quantified colorimetrically at 405 nm. For western blotting analysis, cells were lysed with cell lysis buffer (Sigma-Aldrich) with protease inhibitor cocktail (Sigma-Aldrich). Thirty micrograms of total protein was then resolved by 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto PVDF membranes and probed with ER-α and ER-β antibodies.

Immunodetection was done using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK) using Image Quant LAS 4000 (GE Healthcare).

Anticancer assays and other protocols:

SRB assay

In vitro anticancer potential of test molecules was determined by sulphorhidamine B (SRB) assay. Human cancer cell lines were seeded onto a 96-welled plate (@10,000 cells/well) and grown overnight at 37 °C in 5% CO_2 concentration. Serial dilutions of test compounds were added to the wells and incubated further for 48 h. Cells were then stained with SRB (0.4% w/v in 1% acetic acid, 50 µL per well) after fixing with 50% ice-cold Tri-chloroacetic acid. After a brief wash, bound dyes were dissolved in 10 mM Tris base (150 µL per well) and plates were read at 540 nm. The cancer cell lines were procured from American Type Culture Collection (ATCC), Manassas, VA, USA.

Statistical analysis

Data are expressed as mean ± S.E.M. Cell cycle data was analyzed by student's *t* test. The data obtained from remaining experiments with multiple treatments were subjected to one-way ANOVA followed by Newman–Keuls test of significance using Prism version 3.0 software.

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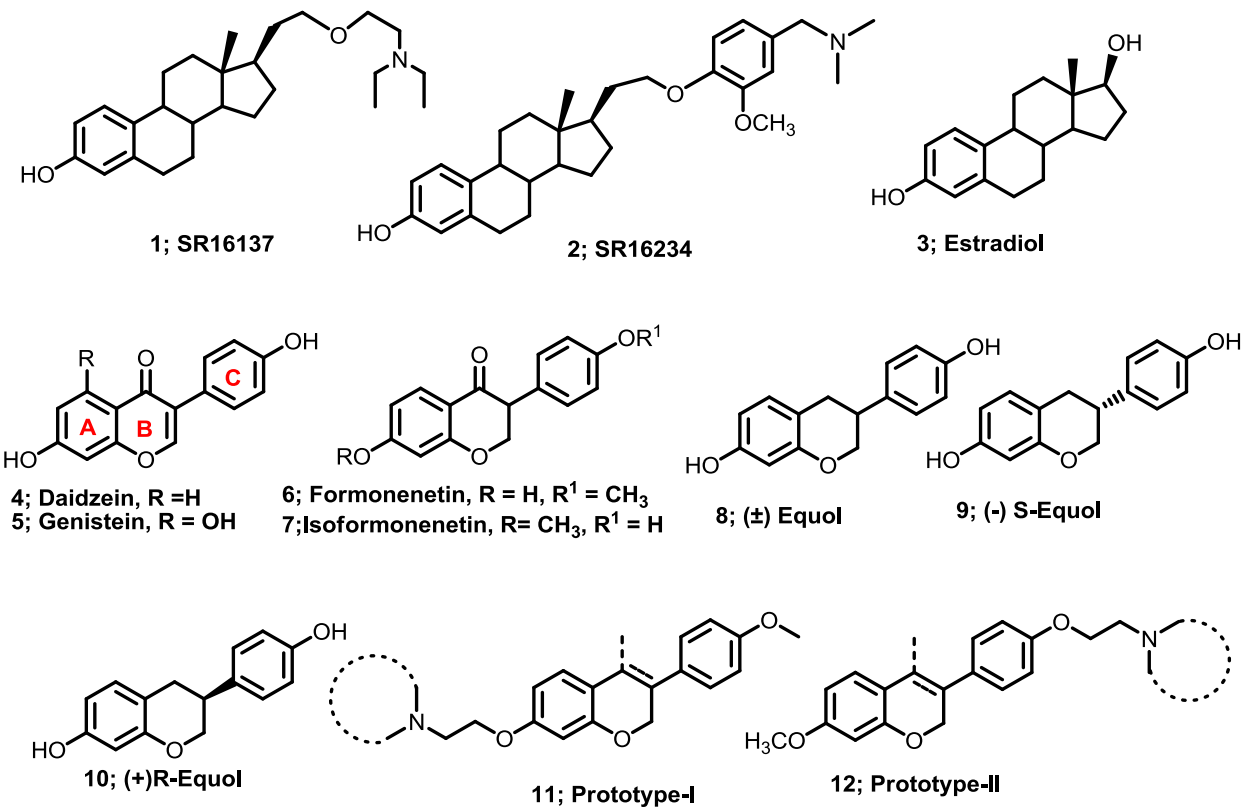


Figure 1: Steroidal estrogen, antiestrogen, isoflavone derivatives, and designed prototypes.

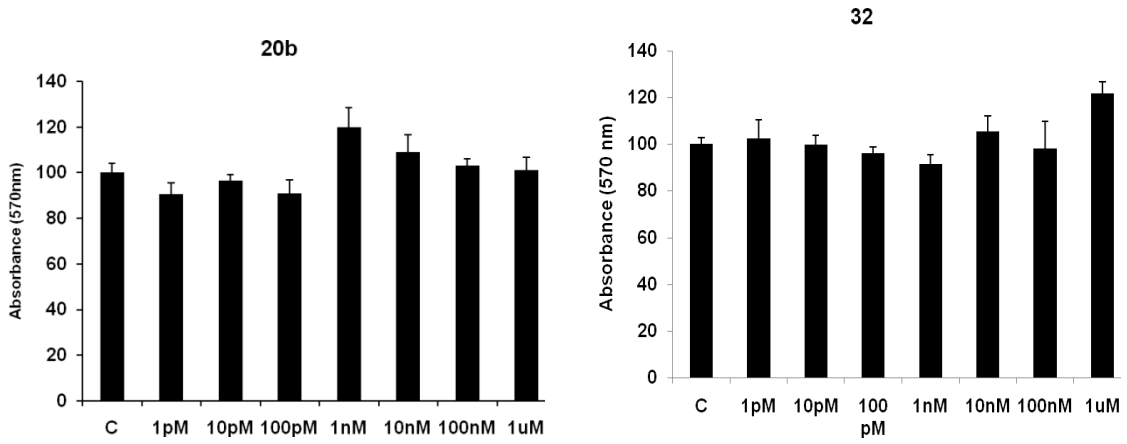
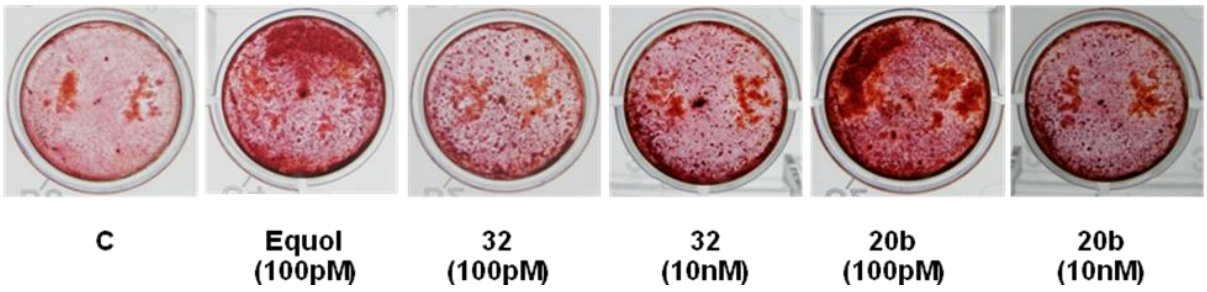


Figure 2a-b. Compounds 20b and 32 are devoid of any toxicity in osteoblast cells. Cells were cultured in osteoblast differentiation medium and treated with various concentrations of the compounds **20b** and **32**. Cell viability was assessed by MTT assay. The percent viable cells were calculated compared to untreated cells taken as control. Data represent the mean ± SEM from three independent experiments.

a



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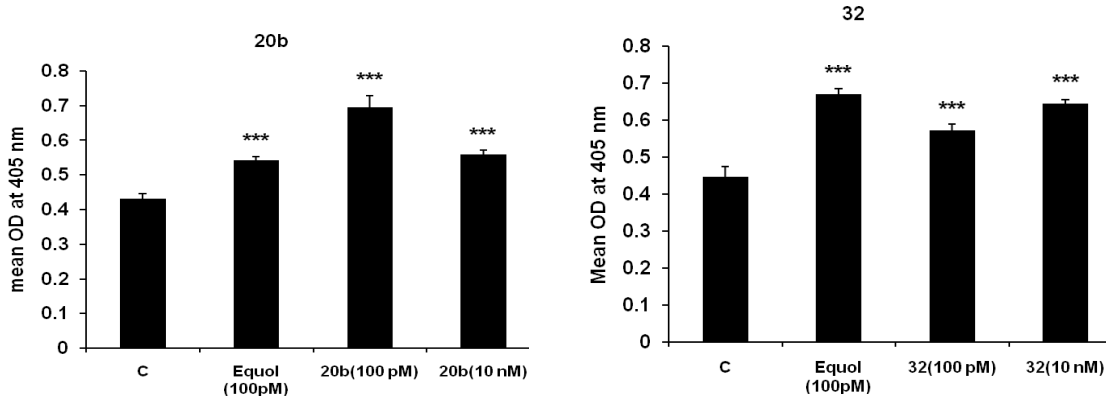


Figure 3a-b: "Compounds 20b and 32 promote mineralization". Mouse calvarial osteoblast cells were seeded in 12 well plates and treated with or without compounds, equol (100pM), **20b** (100pM and 10 nM) and **32** (100pM and 10nM), for 18 days. At the end of the incubation, cells were fixed and stained with alizarin red-S. Stain was extracted, and optical density was measured colorimetrically. Data shown as mean ±S.E.M; n=3; ***p<0.001 compared to control.

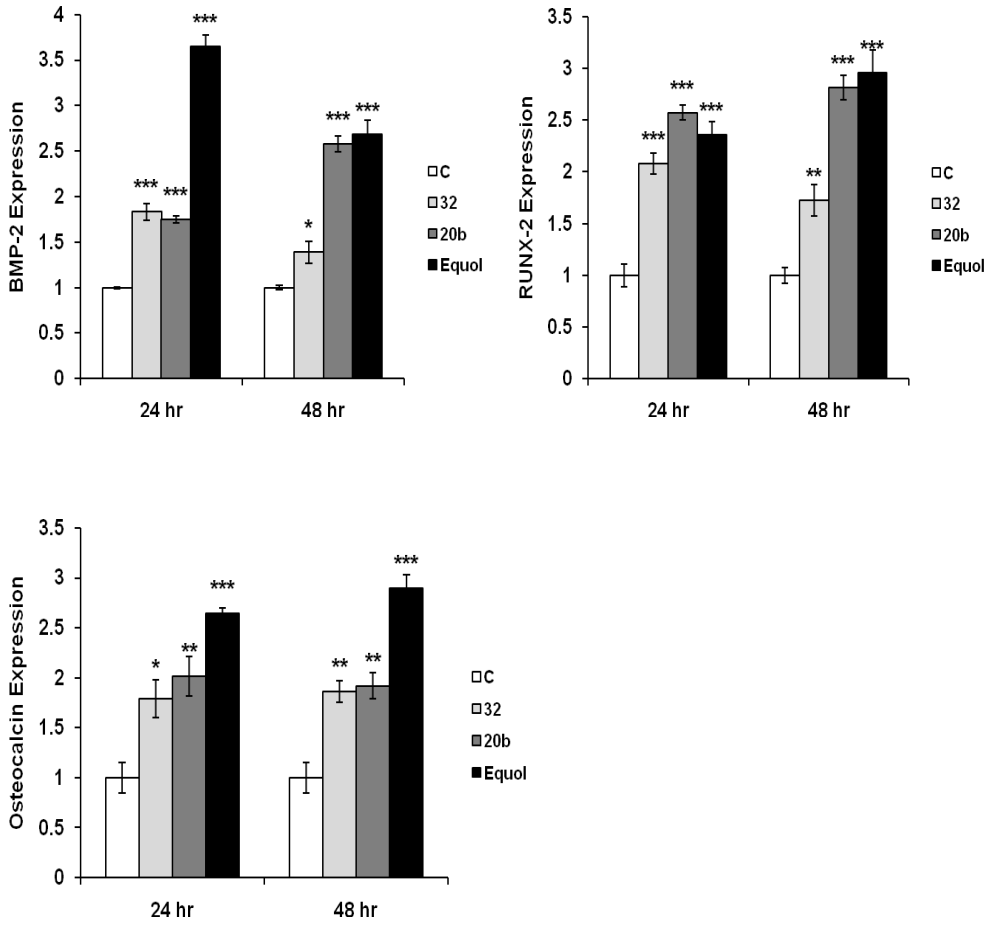


Figure 4a-c: "Compound 20b and 32 increase mRNA expression of osteogenic marker genes". Mouse calvarial Osteoblast cells were treated with compounds **20b** (100pM), **32** (100pM) and positive control **Equol** (100pM) for 24 h and 48 h. Expression of mRNA level of bone morphogenetic protein-2 (BMP-2), Runt-related transcription factor-2 (RUNX-2) and osteocalcin (OCN) were determined. qPCR data of the indicated genes represent the mean ± SEM from three independent experiments. (*)$P < 0.05$, (**)$P< 0.01$, (***)$P<0.001$ compared to control.

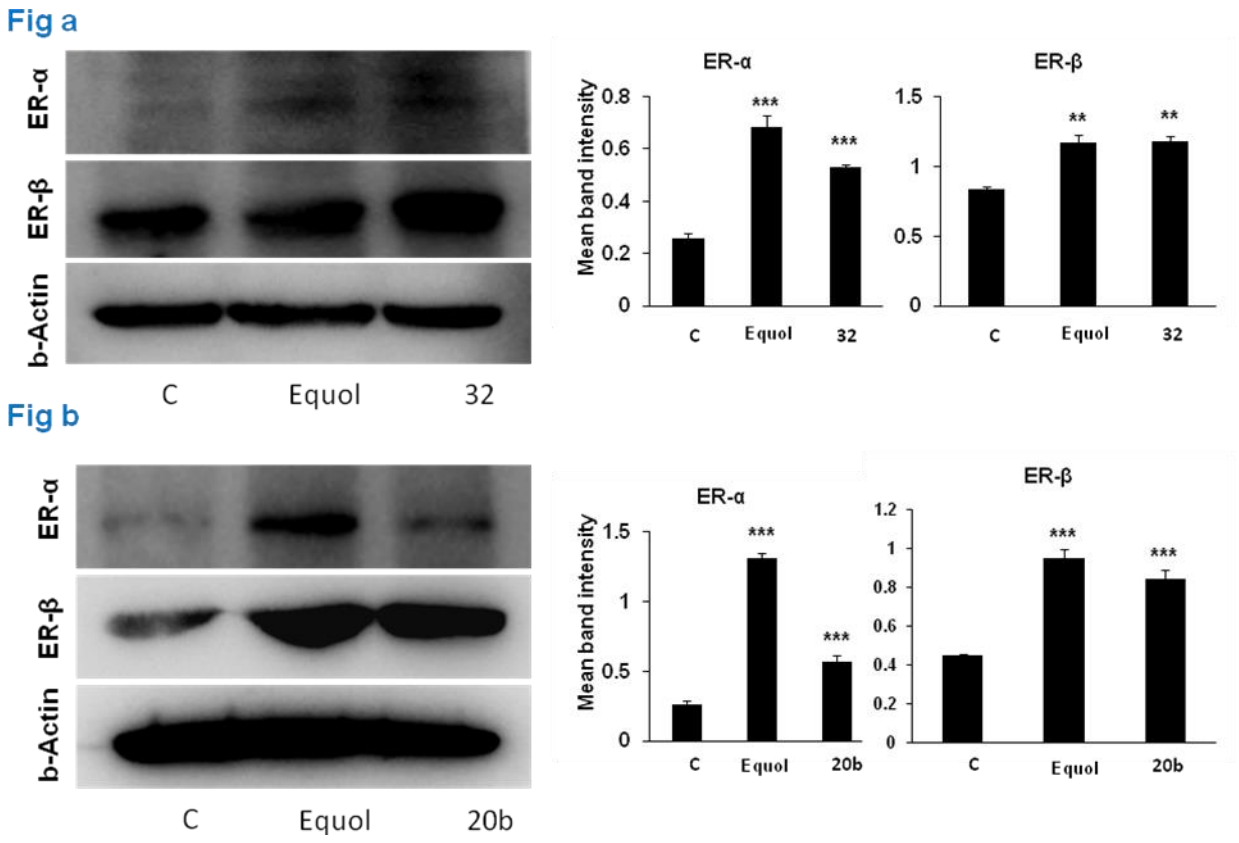


Figure 5a-b: "Compound 32 and 20b increases protein expression of Estrogen receptor-alpha (ER-α) and Estrogen receptor-beta (ER-β)". Mouse osteoblast cells were treated with or without compounds **20b** (100pM), **32** (100pM) and positive control **equol** (100pM) for 48 h time point. Protein lysates were probed with primary antibodies and these were normalized with β-actin. Graph shows the densitometric analysis (fold change) of the observed change in expression of the proteins.

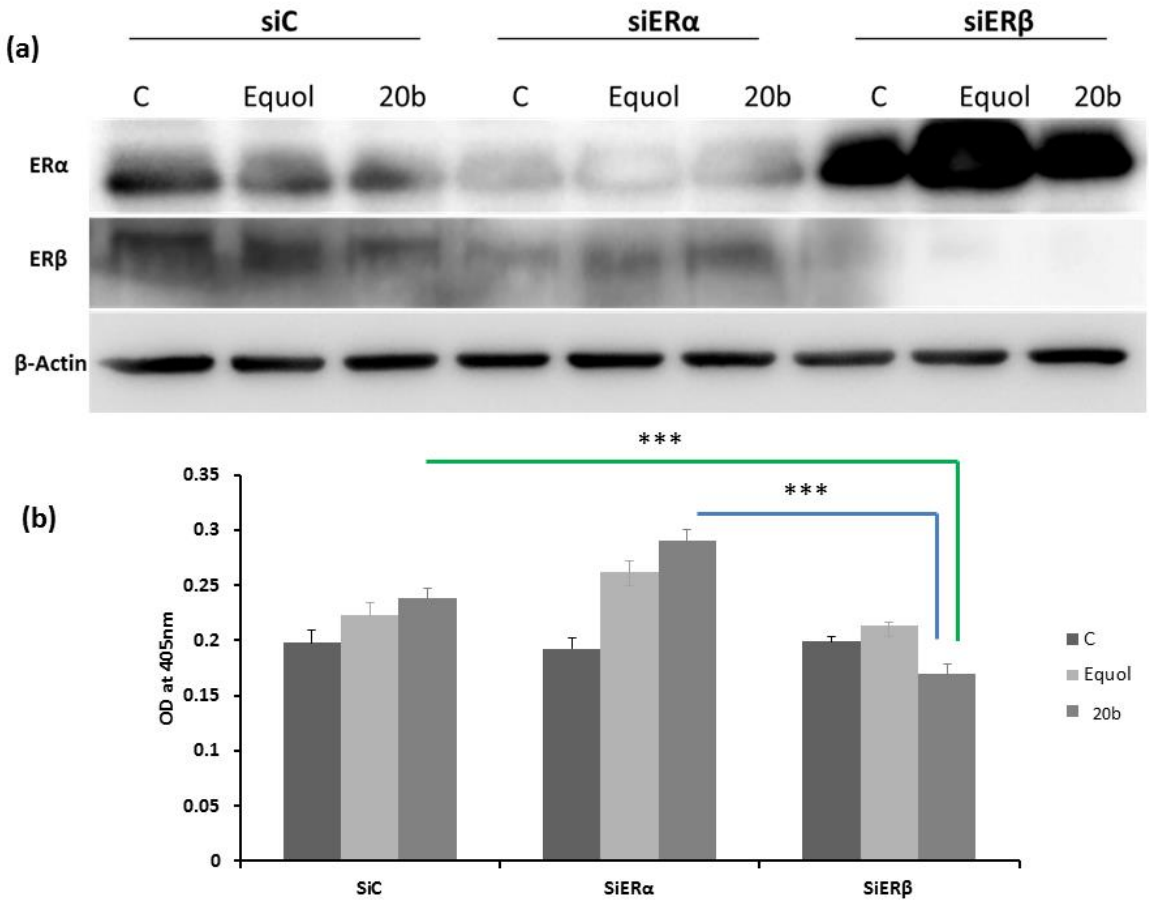
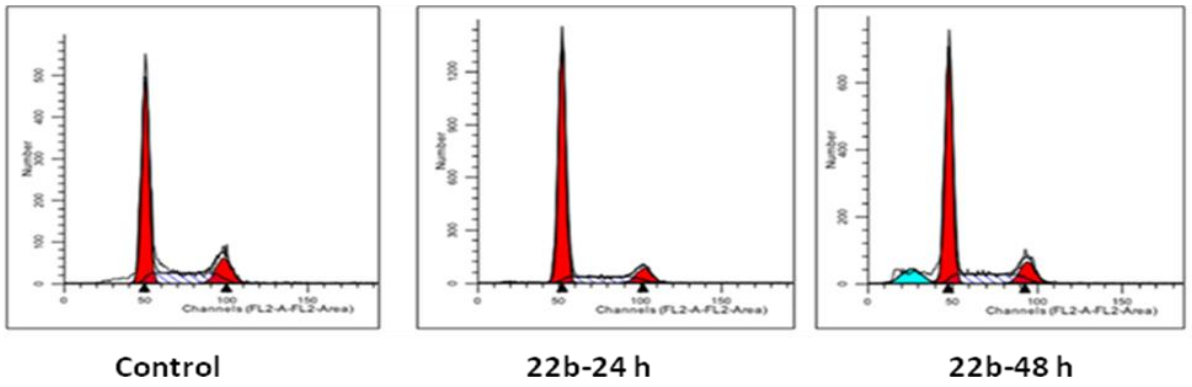


Figure 6; "Compound 20b induces osteogenesis via up-regulation of ER-β". (a) Western blot analysis was performed for ER-α and ER-β from cell lysate collected at 48 h after transfection with siC, siER-α and siER-β. β-actin was used as an internal control. (b) MOBs were plated in 96 well plates. Sixteen hours following siRNA transfection, cells were treated with Equol or **20b** for 48 h. Total ALP activity was measured using *p*-nitrophenylphosphate (PNPP) as a substrate and quantified colorimetrically at 405 nm.

a



b

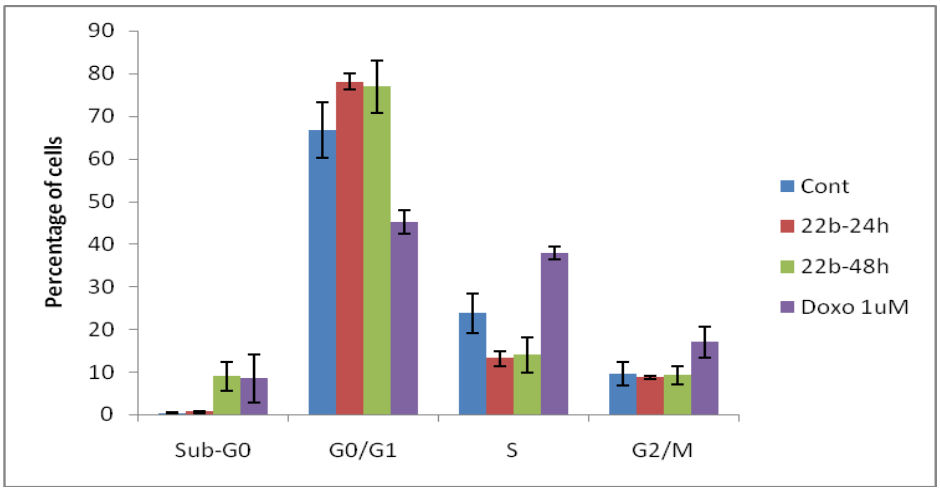


Figure 7: Effect of **22b** on cell division cycle (a) MCF-7 cells were treated with **22b** at IC_{50} concentrations for 24 h and 48 h, stained with PI and were subjected to flow cytometry. (b) Histogram showing average population cells in various phases (G1, G2, S) of cell cycle (mean ± S.E. of three independent assays analysed in Microsoft Excel). Doxorubicin (Doxo), a known apoptosis inducer, was used as positive control.

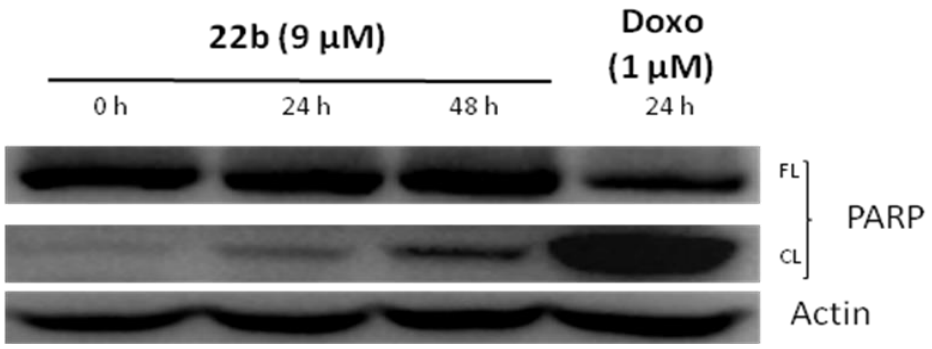
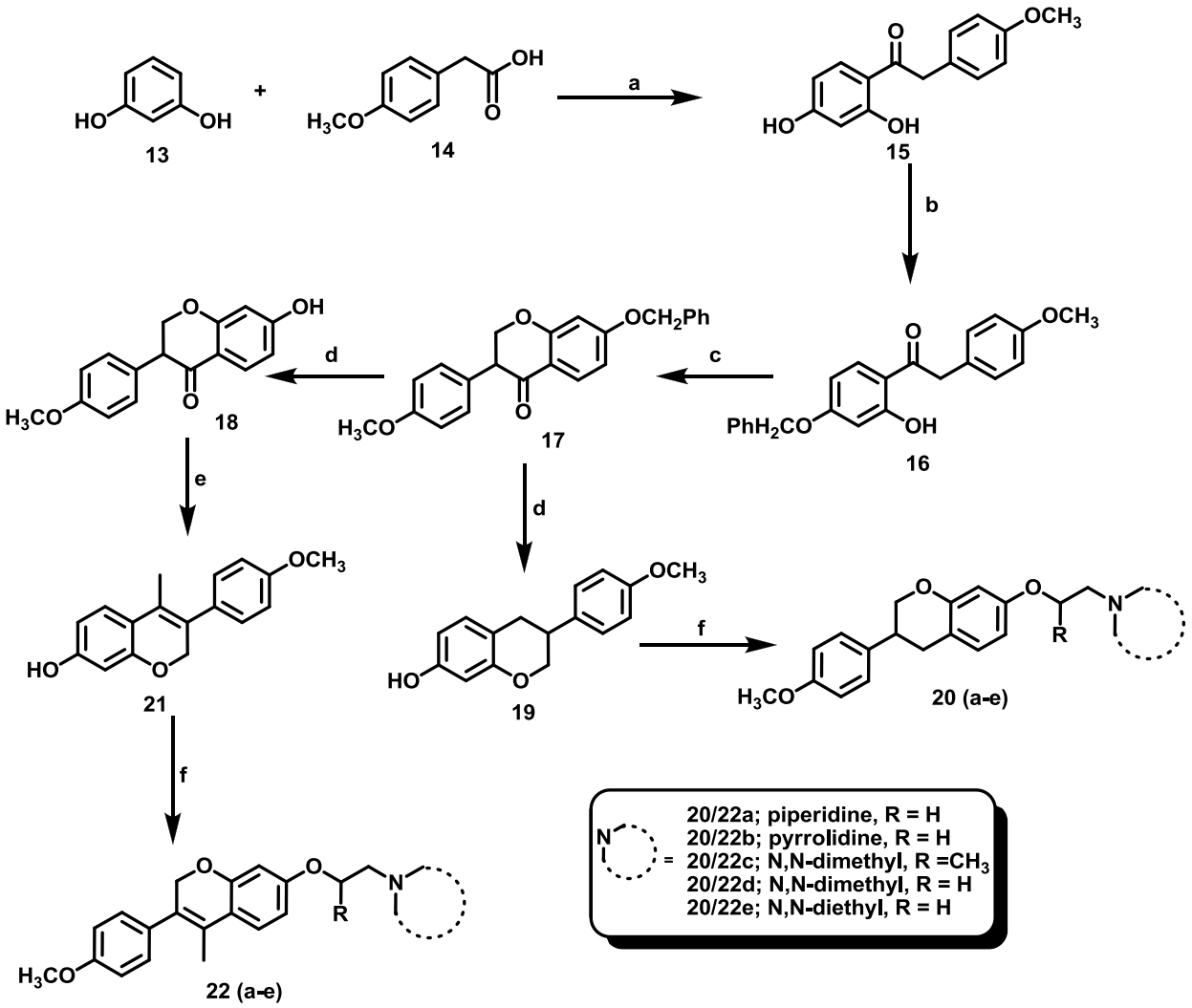
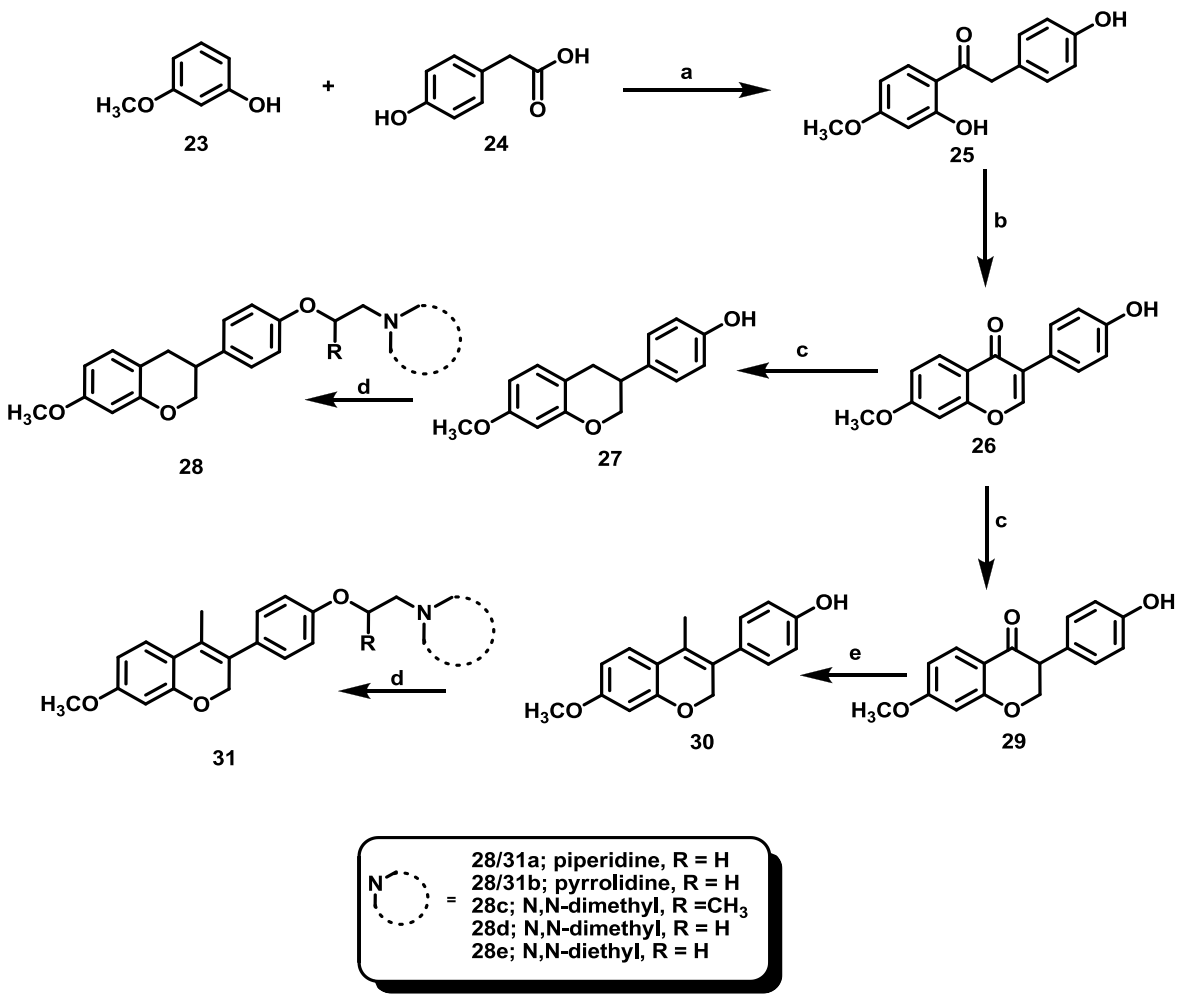


Figure 8: **22b** induces apoptosis in MCF-7 cells. Lysates from treated and untreated cells were subjected to immunoblotting after probing with anti-PARP antibody. Marked cleavage of PARP was observed in **22b** treated cells in time dependent manner. Doxorubicin (Doxo), a known apoptosis inducer, was used as positive control.



Scheme-1: Reagents and reaction conditions: (a) BF_3-OEt_2 at 100°C (b) benzyl bromide, anhy K_2CO_3, dry acetone, reflux (c) paraformaldehyde, 50% aq KOH, 150°C (d) 5% Pd/C, THF, room temp. (e) CH_3MgBr, dry Et_2O-THF, room temperature (f) chloroethylalkylamine hydrochloride, anhy K_2CO_3, dry acetone, reflux.



Scheme-2: Reagents and reaction conditions: (a) BF_3-OEt_2 at 100°C (b) DMF-DMA in DMF, 35°C (c) 5% Pd/C, THF, room temp. (d) chloroethylalkylamine hydrochloride, anhy K_2CO_3, dry acetone, reflux (e) CH_3MgBr, dry Et_2O-THF, room temperature.

The synthesized compounds were characterized by the use of different spectroscopic techniques *viz* NMR, IR, mass spectrometry.

Table 1. Alkaline Phosphatase Activity (ALP) of **19**, **20a-e**, **21**, **22a-e**, **26**, **27**, **28a-e**, **29**, **32** and **33**

S No.	Compound no.	E_{max} *	EC_{50} (nM)#	ALP activity (%)	ALP activity (%) compared with EQUOL
1	**19**	-	-	inactive	-
2	**20a**	100 nM	0.144.0	28 (P < 0.001)	ns
3	**20b**	1nM	0.00135	18 (P < 0.001)	ns
4	**20c**	100pM	2.27	20 (P < 0.001)	ns
5	**20d**	100pM	5.45	25 (P < 0.001)	ns
6	**20e**	10 pM	7.27	20 (P < 0.001)	ns
7	**21**	-	-	inactive	-
8	**22a**	10 pM	0.0345	18 (P < 0.001)	ns
9	**22b**	1nM	10.74	17 (P < 0.001)	ns
10	**22c**	10nM	-	15 (P < 0.01)	ns
11	**22d**	10 nM	54	19 (P < 0.001)	ns
12	**22e**	10 nM	-	14 ((P < 0.05)	ns
13	**26**	100pM	11.4	21 (P < 0.001)	ns
14	**27**	1pM	0.407	19(P < 0.001)	ns
15	**28a**	-	-	inactive	-
16	**28b**	-	-	inactive	-
17	**28c**	-	-	inactive	-
18	**28d**	-	-	inactive	-
19	**28e**	-	-	inactive	-
20	**29**	-	-	inactive	-
21	**33**	-	-	inactive	-
22	**32**	10 nM	0.0295	27 (P < 0.001)	270 (p<0.001)
23	Equol	1nM	67.0	29 (P < 0.001)	-

*E_{max} = maximum response achievable from a compound; #EC_{50} = concentration of compound at which effect (E) is 50% of Emax. Percentage of ALP activity (%) = (average ALP activity of compound / average ALP activity of control)X100; We quantified ALP activity through colorimetric assay and hence OD (optical density) has been taken for unit.

Table 2: Anticancer activity of **19, 20a-e, 21, 22a-e, 26, 27, 28a-e, 29, 31a-b, 32** and **33** in a panel of human cancer cell lines *viz* **DLD1** (colorectal adenocarcinoma)**, A549** (Lung carcinoma)**, FaDu** (hypopharyngeal carcinoma), **MCF-7** (ER+ Breast adenocarcinoma), **DU145** (prostate carcinoma) by SRB assay and values are represented as mean IC_{50} value±SE of three independent experiments. The concentration of compounds used for IC_{50} determination was 5 serial dilutions (2 fold) of the 20 or 30 µM starting concentration. The Incubation period of drug treated cells was 48h.

IC50(µM) (Mean±SE)						
S. No.	Compound No.	MCF-7	DLD1	A549	FaDU	DU145
1.	19	>30	>30	>30	>30	>30
2.	20a	27.79[b]	16.91	>30	>30[b]	>30
3.	20b	24.65[b]	17.49	28.96[b]	26.23[b]	>30
4.	20c	>30[b]	21.43	>30[b]	>30[b]	>30
5.	20d	>30	21.15[b]	>30	27.57[b]	>30
6.	20e	>30	23.54	>30	>30	>30
7.	21	>30	>30	>30	>30	>30
8	22a	8.20±1.06	7.64±2.40	14.07±1.79	13.33±2.41	14.61±1.48
9.	22b	8.38±0.66	6.54±1.81	11.53±0.25	13.14±1.77	13.24±0.84
10.	22c	12.41±0.33	8.47±1.66	15.77±2.04	13.88±0.85	13.14[b]
11.	22d	12.64±1.80	10.80±3.47	15.26±2.36	14.18±2.48	16.14[b]
12	22e	9.70±4.27	7.12±3.30	12.15[b]	10.49[b]	11.75[b]
13.	26	>20	>20	>20	>20	>20
14.	27	>20	>20	>20	>20	>20
15.	28a	>20	18.60	>20	>20	>20
16.	28b	>20	17.38	>20	>20	>20
17.	28c	>20	14.10	>20	>20	>20
18.	28d	>20	15.56	>20	>20	>20
19.	28e	>20	20	>20	>20	>20
20.	29	>20	>20	>20	>20	>20
21.	31a	14.71[b]	4.82±1.02	10.62±0.60	-	>30[b]
22.	31b	9.33± 0.48	3.23[b]	7.42±1.39	-	12.32±2.09
23.	32	>30[b]	-	>30[b]	-	>30[a]
24.	33	22.74[b]	-	23.16[b]	-	>30[a]
25.	TAM	11.80±0.33	-	12.26±0.09[a]	-	18.96±0.57[a]
26.	Equol	>30	>30	>30	>30	>30

[a,b]although the compounds had been tested thrice in this cell line, value of one assay was >50 (a) or >100 (b), therefore it could not be included in calculation of mean and SE].(-)= not done