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Neo-tanshinlactone inspired synthesis, in vitro evaluation of novel substituted benzocoumarin derivatives as potent anti-breast cancer agents ☆

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ABSTRACT

A small library of novel benzocoumarin derivatives based on naturally occurring neo-tanshinlactone scaffold was constructed and their antiproliferative activities against breast cancer cells MCF-7 and MDA-MB-231 were evaluated. A number of derivatives showed good anti-breast cancer activity, in some cases higher to that of the reference compound tamoxifen. In particular, benzocoumarins **Bc-5**, **Bc-8** and **Bc-9** strongly inhibited the proliferation of MCF-7 cancer cell line with the IC_{50} values of 3.8, 7.9 and 6.5 μM, respectively. The compounds were capable of inducing nuclear fragmentation, cell cycle arrest and caspase dependent apoptosis in MCF-7 cell lines. In addition, these derivatives were devoid of cytotoxic effect against normal osteoblast cells. These synthetic benzocoumarins hold promises for developing safer alternative to the existing anti-breast cancer agents.

Breast cancer, a leading pandemic, affects women of all ages.[1] In hormone dependent breast carcinoma, estrogens play a critical role, stimulating cancer cell proliferation.[2,3] Since high serum levels of estrogens favor the progression of breast cancer, estrogen receptor antagonist approaches have made a huge contribution in the breast cancer chemotherapy.[4] Tamoxifen, a selective estrogen receptor modulator (SERM) has been used thoroughly in breast cancer patients for more than three decades.[5] Although tamoxifen is most widely used clinical agent, but overcoming drug resistance[6] and increase risk of uterine cancer in postmenopausal patients merits development of potent anti-breast cancer agents with novel scaffold or new mode of action.[7,8]

Natural and synthetic coumarins have been found to exhibit a variety of pharmacological activities like anti-HIV,[9] anticoagulant,[10] antibacterial,[11] antioxidant,[12] and anti-inflammatory.[13] Among the diverse biological activities of coumarin the most intriguing bioactivity is the effect against breast cancer.[14–16] Figure 1 shows the chemical structures of some potent anti-breast cancer molecules that contain a coumarin in their molecular make-up and in this regard it is worth mentioning that 667 COUMATE is in phase1 clinical trials for the treatment of hormone dependent breast cancer in postmenopausal women.[17] Furthermore, Lee and co-workers reported that Neo-tanshinlactone, a steroid-like tetracyclic natural product (Fig. 1) showed significant inhibition against two estrogen receptor positive human breast cancer cell lines and was 10-fold more potent and 20-fold more selective as compared to tamoxifen.[18] It's analog, 4-ethyl neo-tanshinlactone exhibited selective and potent in vivo anti-breast cancer activity in three different mouse models.[19] A lot of studies aimed at better understanding of SAR for tanshinlactone are continuing to emerge from Lee and co-workers[20] These encouraging studies and our long standing interest in this class of compounds[21] prompted us to design and synthesize novel benzocoumarin derivatives to explore their anti-breast cancer potential.

In order to establish chemical library with various functionalities and structural diversity, we have synthesized two different series of coumarins starting from dicarbaldehyde **Bc-2**. In the first series, we began with the synthesis of benzocoumarins and their Schiff bases derivatives, the detailed synthetic methodology of which has been previously reported by us and the reaction conditions are shown in Scheme 1.[22] Thus, the Pechmann reaction on naphthalene 1, 5-diol **1**, furnished 7-hydroxy-4-methyl-benzo(h)chromene-2-one **Bc-1** which on Duff formylation gave dicarbaldehyde adduct **Bc-2**. This dialdehyde was then condensed with various primary amines to give regioselective Schiff bases that existed in keto-enamine form. These derivatives (**Bc-1-10**) were evaluated for their in vitro anti-breast cancer activity in MCF-7 and MDA-MB-231 cell lines. Out of ten compounds

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† Biological activity.

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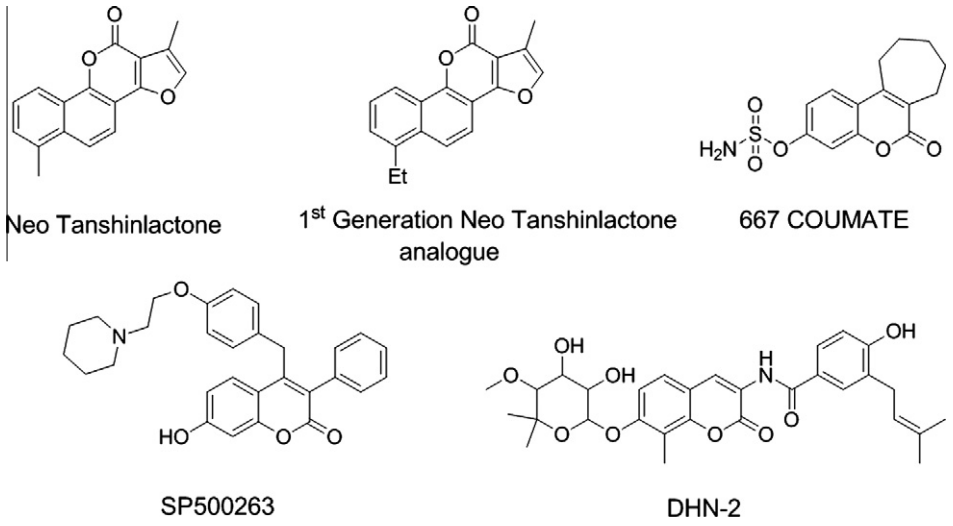
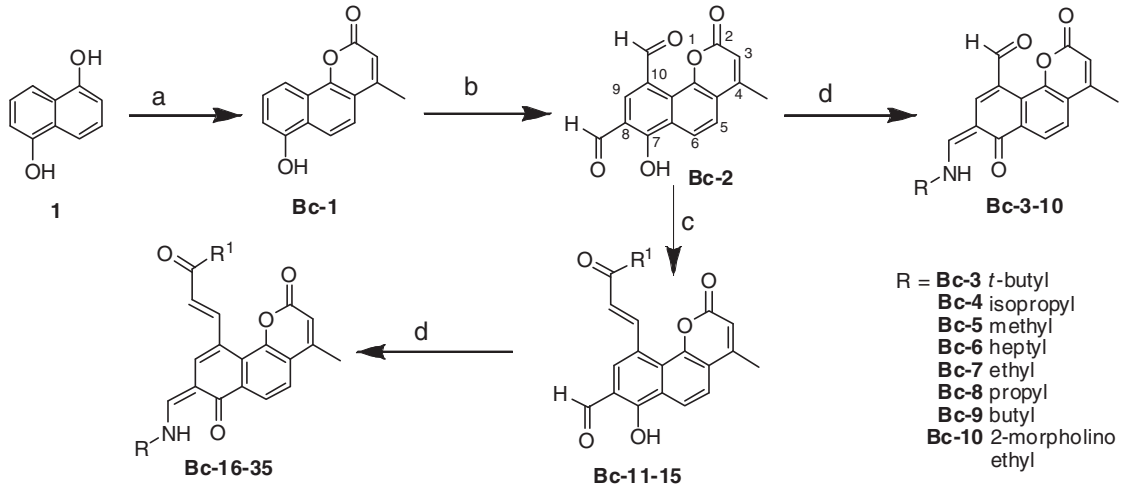


Figure 1. Examples of some potent coumarins having anti-breast cancer activity.



R = **Bc-3** *t*-butyl
Bc-4 isopropyl
Bc-5 methyl
Bc-6 heptyl
Bc-7 ethyl
Bc-8 propyl
Bc-9 butyl
Bc-10 2-morpholino ethyl

R_1 = **Bc-11** phenyl
Bc-12 tolyl
Bc-13 *p*-methoxy phenyl
Bc-14 2,4-dimethyl phenyl
Bc-15 4-hydroxy phenyl

Bc-16 R = butyl, R^1 = phenyl
Bc-17 R = ethyl R^1 = phenyl
Bc-18 R = *t*-butyl, R^1 = phenyl
Bc-19 R = isopropyl, R^1 = phenyl
Bc-20 R = butyl, R^1 = tolyl
Bc-21 R = ethyl R^1 = tolyl
Bc-22 R = *t*-butyl, R^1 = tolyl
Bc-23 R = isopropyl, R^1 = tolyl
Bc-24 R = butyl, R^1 = *p*-methoxy phenyl
Bc-25 R = ethyl, R^1 = *p*-methoxy phenyl
Bc-26 R = *t*-butyl, R^1 = *p*-methoxy phenyl
Bc-27 R = isopropyl, R^1 = *p*-methoxy phenyl
Bc-28 R = butyl, R^1 = 2,4-dimethyl phenyl
Bc-29 R = ethyl, R^1 = 2,4-dimethyl phenyl
Bc-30 R = *t*-butyl, R^1 = 2,4-dimethyl phenyl
Bc-31 R = isopropyl, R^1 = 2,4-dimethyl phenyl
Bc-32 R = butyl, R^1 = 4-hydroxy phenyl
Bc-33 R = ethyl, R^1 = 4-hydroxy phenyl
Bc-34 R = *t*-butyl, R^1 = 4-hydroxy phenyl
Bc-35 R = isopropyl, R^1 = 4-hydroxy phenyl

Scheme 1. Reagents and conditions: (a) EAA, PTSA, 75 °C, 8 h; (b) (i) HMTA, TFA, 120 °C, 4 h; (ii) aq H_2SO_4, 100 °C, 1 h; (c) R^1–$COOH_3$, concd HCl, dioxane, reflux, 5 h; (d) R–NH_2, C_2H_5OH, rt, 10 min.

evaluated, three compounds showed IC_{50} in between 3.8 and 7.9 μM which was lower than tamoxifen (Table 1). It is an interesting to reveal that though all compounds were screened against both MCF-7 (estrogen receptor—positive) and MDA-MB-231

Table 1
Anticancer activity (IC_{50}, μM) of benzocoumarins in MCF-7 cells

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
Bc-1	>100	**Bc-19**	>100
Bc-2	>100	**Bc-20**	>100
Bc-3	20	**Bc-21**	>100
Bc-4	130	**Bc-22**	97
Bc-5	3.8	**Bc-23**	21
Bc-6	>100	**Bc-24**	92
Bc-7	>100	**Bc-25**	30
Bc-8	7.9	**Bc-26**	>100
Bc-9	6.5	**Bc-27**	>100
Bc-10	>100	**Bc-28**	>100
Bc-11	>100	**Bc-29**	>100
Bc-12	>100	**Bc-30**	>100
Bc-13	>100	**Bc-31**	35
Bc-14	>100	**Bc-32**	40
Bc-15	>100	**Bc-33**	85
Bc-16	100	**Bc-34**	>100
Bc-17	87	**Bc-35**	32
Bc-18	92	Tamoxifen	11.8

(estrogen receptor—negative) breast cancer cell lines, these derivatives exhibited selective and significant inhibition only in MCF-7 cell lines. Furthermore, these active compounds did not affect the viability of osteoblasts.

In the second series of compounds dicarbaldehyde **Bc-2** was used as the molecular template and an active pharmacophore for further diversification. As a free aldehyde group was present in the active compounds, for structural diversity, we wanted to introduce chalcones at this position, which are well known for their anticancer potential (e.g., Licochalcone-A, isolated from the licorice root)[23] and these compounds were synthesized by an efficient and novel method employing the reagent controlled regioselective synthesis of chalcones in aromatic dialdehydes that was recently discovered by us.[24,25]

Thus, the regioselective synthesis of chalcones from dicarbaldehyde **Bc-2** was achieved by using acid catalyzed Claisen–Schmidt reaction, by condensation with appropriate ketones (**Bc-11**-**15**), which was transformed to (**Bc-16**-**35**) by reacting them with different aliphatic amines (Scheme 1).[26] The structures of all the novel compounds were confirmed by ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry (see Supplementary data). In all the chalcones synthesized the *trans* double bond was obtained exclusively.

The idea of synthesizing these benzocoumarins was not without reason, it was envisaged that since these molecules are fluorescent (due to extended conjugation) they would enhance the anticancer activity due to their photosensitization properties.[27] However, on evaluation these compounds (**Bc-11**-**35**) they were found to be less active than parent structure. Only compounds **Bc-23**, **Bc-25**, **Bc-31**, **Bc-32** and **Bc-35** showed moderate inhibition against MCF-7 cell lines (Table 1).

Of the two different set of compounds synthesized, the first series of compounds in which the benzocoumarins were derivatized with keto-enamine Schiff bases (**Bc-3**-**10**) were more active than the other (**Bc-11**-**15**) and (**Bc-16**-**35**). Overall, within this series, **Bc-5**, **Bc-8** and **Bc-9** were found to be more effective in inhibiting cell growth of breast cancer cells, indicating in terms of SAR, that the functional group substituted at the position 10 may be size dependent and substitution at this position with bulky group's results in loss of activity. Because of high selective cytotoxic effect in MCF-7 cells and with no effect in primary osteoblast cells, we took **Bc-5**, **Bc-8** and **Bc-9** for subsequent biological experiments (see Supplementary data).[28] Furthermore, in an attempt to better characterize the cellular basis of their anti proliferative effects, the ability of these selected compounds to induce apoptosis was investigated and the results of various studies are discussed next.

Benzocoumarins induce loss of cell viability in MCF-7. Three compounds **Bc-5**, **Bc-8** and **Bc-9** induced loss of cell viability of MCF-7 cells in a concentration-dependent fashion and their activity was comparable to tamoxifen (Fig. 2). A significantly higher activity of **Bc-5** at 10, 20 and 30 μM and for **Bc-8** at 20 and 30 μM was observed as compared to tamoxifen. Particularly, **Bc-5** induced significant loss of cell viability even at 10 μM, while, **Bc-8** induced significant loss of cell viability at 20 μM that was observed as potent as tamoxifen. The calculated IC_{50} of antiproliferative activity in MCF-7 cells of the three compounds were found to be lower than tamoxifen (Table 1). Out of the three active compounds, **Bc-5** had lowest IC_{50} (Table 1).

Benzocoumarins induce increase in sub-diploid population and G0/G1 arrest. Flow cytometry of PI stained treated cell analysis for cell cycle phase distribution showed that all three compounds **Bc-5**, **Bc-8** and **Bc-9** induced significant increase in sub-diploid population (Fig. 3). Thus, the molecular events involved in cellular response to the effective compounds, were investigated and to this end, the compound **Bc-5** and **Bc-8** differed from **Bc-9** as they caused significant blockade at G_0/G_1 phase of cell cycle, whereas **Bc-9** caused G_2 arrest (Fig. 3).

Benzocoumarins induce apoptotic changes. Manual field quantitation of percent apoptotic cells based on cytoplasmic condensation, presence of apoptotic body, nuclear fragmentation and relative

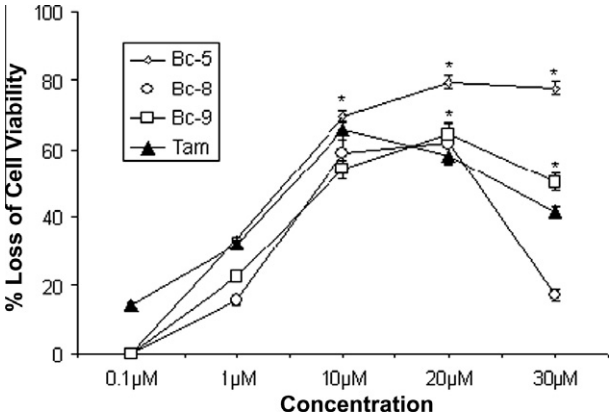


Figure 2. Loss of viability of MCF-7 cells by benzocoumarin derivatives: 1×10^5 MCF-7 cells of 70–80% confluence were incubated 16 h with increasing concentrations of benzocoumarin derivatives. Percent inhibition of cell growth (Total/Control) was determined with MTT assay. Tamoxifen was used as positive control ($n = 9$, $^{*}p < 0.05$ vs Tam).

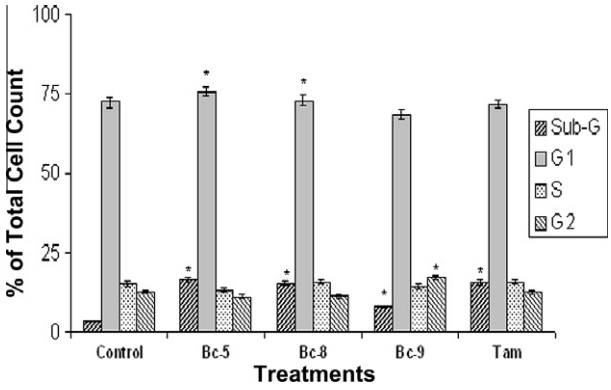


Figure 3. Effect of benzocoumarin derivatives (10 μM each) on cell cycle progression of MCF-7 cells: 5×10^5 cells/well in 6-well plates with 70–80% confluence was serum starved before treatment. After 24 h treatment in phenol red free media, cells were fixed in 70% ethanol, re-hydrated in PBS, and stained with PBS containing PI (50 μg/ml) and ribonuclease A (100 μg/ml) as described in materials and methods. Data are expressed as % of cell count in each phase of cell cycle induced by each compound. ($^{*}p < 0.05$ vs control).

fluorescence in cells treated with test compounds revealed that compound **Bc-5**, **Bc-8** and **Bc-9** treatment in MCF-7 cells resulted in significant increase in percentage of apoptotic cell (Fig. 4). Out of the three compounds, **Bc-5** induced significantly higher apoptotic changes in treated cells.

Benzocoumarins enhanced Annexin-V expression. MCF-7 cells were exposed for 24 hours to 10 μM concentration of each compounds and analyzed by flow cytometry using Annexin-V-FITC and PI. Data showed that all three compounds **Bc-5**, **Bc-8** and **Bc-9** significantly induced percentage of Annexin-V-FITC positive and PI negative cells compared with untreated controls indicating increase in the proportion of cells undergoing early stages of apoptosis (Fig. 5). Significantly higher Annexin-V positivity was observed with **Bc-5** treatment under similar condition.

Benzocoumarins induced apoptosis via caspase activation. Homogeneous caspase activity was increased in MCF-7 cells by all three compounds **Bc-5**, **Bc-8** and **Bc-9**. The caspase activity upon treatment of compound was **Bc-5**, **Bc-8** and **Bc-9** significantly high versus untreated control indicating that all these compounds activated of general caspases (Fig. 6).

Benzocoumarins do not affect viability of osteoblasts. Effect of benzocoumarins on the viability of osteoblasts was studied at 10 and 20 μM concentrations. None of these compounds showed loss of cell viability of primary osteoblasts (Fig. 7). Instead, these three compounds exhibited increase in osteoblast numbers at 10 and 20 μM concentration (Fig. 7). Our choice of osteoblast was not without reason as the onset of metastatic breast cancer often coincides with bone loss disorders. Our data showed that whereas all three benzocoumarins had no effect on the viability of osteoblasts, all could actually increase osteoblast number compared to untreated cells indicating the possibility in their involvement in osteoblast proliferation. Recently, furanocoumarin, a class of coumarin derivative, was found to promote bone formation by stimulating differentiation and mineralization of osteoblasts.[29] It is possible that these derivatives, particularly **Bc-9**, beside its cell growth promoting action, could have similar osteogenic effect which is beyond the scope of this study.

In conclusion, in this study, among the synthesized benzocoumarins, **Bc-5**, **Bc-8** and **Bc-9** were identified to be very

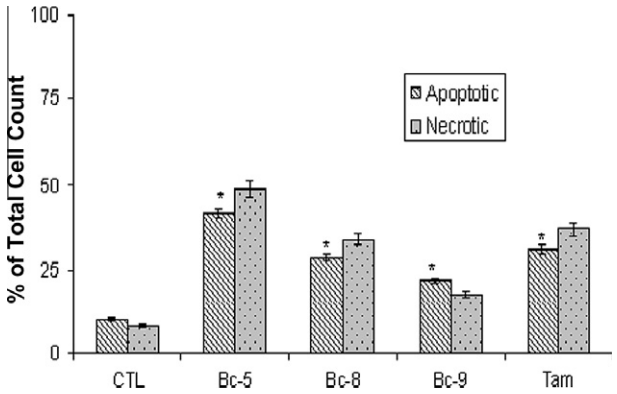


Figure 5. Quantitative determination of apoptosis by flow cytometry: MCF-7 cells (5×10^5 cells) with 70–80% confluence were incubated in DMEM supplemented with 10% FBS without (control) or with 10 μM of benzocoumarin derivatives. After 24 h, the cells were harvested, stained with Annexin-V-FITC and PI, and analyzed by flow cytometry. Data are expressed as % of apoptotic (Annexin V-FITC-positive and PI-negative) and necrotic (PI-positive) cells. ($^*p$ <0.05 vs control).

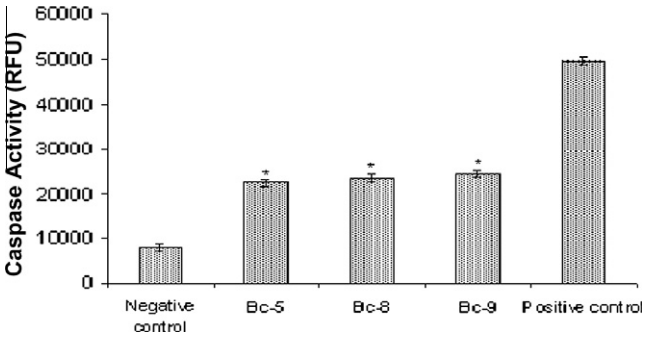


Figure 6. Benzocoumarin derivatives activate homogeneous caspase in MCF-7 cells: 1×10^4 cells/well with 70–80% confluence were grown 16 h, treated with 10 μM each compounds for 16 h. Flurometric determination of general caspase was done as described in materials and methods with an excitation filter 465 nm and emission filter 520 nm. ($^*p$ <0.05 vs negative control).

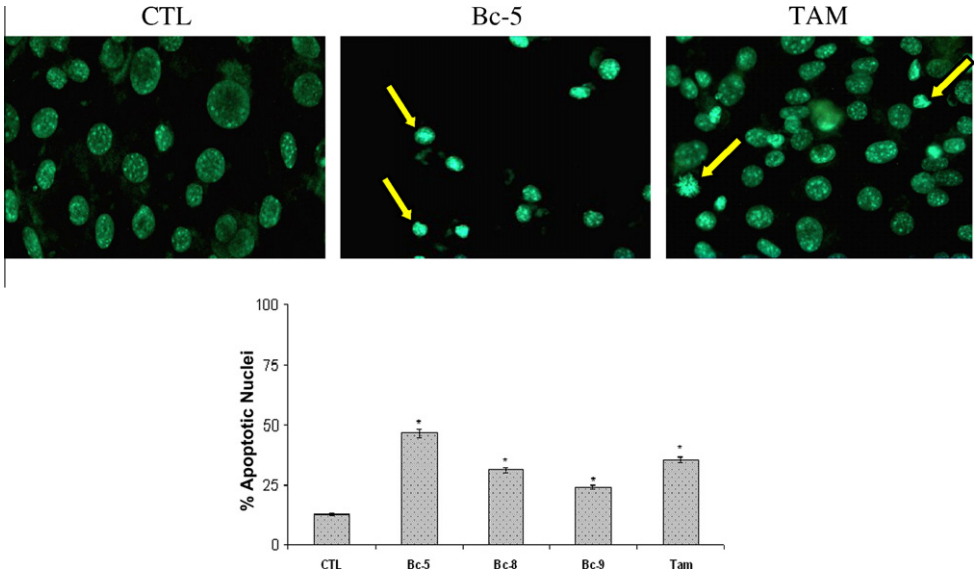


Figure 4. Benzocoumarin derivatives induce apoptosis of MCF-7 cells: (a) MCF-7 cells of 70–80% confluence were treated with 10 μM benzocoumarin derivatives for 18 h in complete growth medium. Representative photomicrographs from **Bc-5** treated cells after Hoechst 33342 staining were acquired with ×40 objective. Arrows indicate cytoplasmic condensation, apoptotic body and nuclear fragmentation. Tamoxifen was used as a positive control for apoptosis in MCF-7. (b) Quantitation of data for % of apoptotic nuclei based on the shape and intensity of Hoechst 33342 staining in cells normalized with control data. ($^*p$ <0.05 vs control).

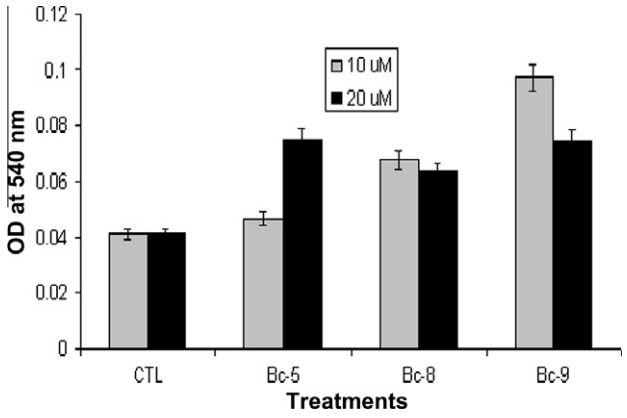


Figure 7. Effect of benzocoumarin derivatives in the proliferation of osteoblasts: Osteoblasts were cultured as described in the materials and methods. 3×10^3 cells/well were treated with 10 and 20 μM of each compound. MTT assay was performed. ($n = 9$, $^{*}p > 0.05$ vs control).

promising candidates, their excellent antiproliferative potency, together with their remarkable apoptosis-inducing activity, make them leads of great interest for further studies. Also, since these derivatives are highly fluorescent, the utility of these compounds to act as novel angiogenesis inhibitors with high selectivity over tumor cells is under investigation as it is potentially possible to inhibit and detect tumor angiogenesis simultaneously.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.040.

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26. Synthetic procedure for compounds: (a) **Bc-3**: To a mixture of **Bc-2** (2 mmol) and *tert*-butylamine (4 mmol), absolute ethanol (10 ml) was added at room temperature. After completion of the reaction solvent was evaporated and the residue was washed with hexane to afford **Bc-3** in good yield. (b) **Bc-11**: A solution of **Bc-2** (1 g, 3.54 mmol) and acetophenone (0.390 g, 3.54 mmol) in dioxane was treated with conc. HCl (5 mL) and refluxed for 5 h. Most of the excess solvent was evaporated under reduced pressure and the residue was neutralized with aq $NaHCO_3$ solution. To this residue, water (50 mL) was added and extracted 3 fold with 25 mL of $CHCl_3$. The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated, which was chromatographed over silica gel to afford compound **Bc-11**. (c) **Bc-16**: A solution of **Bc-11** (0.2 g, 0.52 mmol) and butyl amine (0.057 g, 0.78 mmol) in ethanol (10 mL) was stirred for 10 min at room temperature. After completion of the reaction solvent was evaporated and the residue was washed with hexane to afford **Bc-16**.
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