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Ester and carbamate ester derivatives of Biochanin A: Synthesis and in vitro evaluation of estrogenic and antiproliferative activities

Nikolas Fokialakis [a,†], Xanthippi Alexi [b,†], Nektarios Aligiannis [a], Despina Siriani [b], Aggeliki K. Meligova [b], Harris Pratsinis [c], Sofia Mitakou [a,*], Michael N. Alexis [b,*]

[a] Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimiopolis, 157 71 Athens, Greece
[b] Molecular Endocrinology Programme, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vassileos Constantinou Ave., 116 35 Athens, Greece
[c] Laboratory of Cell Proliferation & Ageing, Institute of Biology, NCSR 'Demokritos', GR-15310, Athens, Greece

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ABSTRACT

Biochanin A (BCA), a major isoflavone in red clover and many other legumes, has been reported to display estrogenic as well as cancer chemopreventive properties. Ingested BCA is known to display low bioavailability due to poor solubility, extensive metabolism and rapid clearance. Esters of bioactive isoflavones are known to increase metabolic stability and bioavailability following local rather than systemic administration. We synthesized BCA from phloroglucinol and *p*-methoxy-phenylacetic acid by a Friedel–Crafts reaction and cyclization. We also synthesized esters (**1**, **3**) and carbamate esters (**2**, **4**, **5**) at position 7 of BCA using short aliphatic chains bearing a chlorine (**1**, **2**) or a bromine atom (**3**, **4**) or long aliphatic chains without such atoms (**5**). We tested the estrogenic and antiproliferative activities of **1**–**5** and BCA using human breast and endometrial adenocarcinoma cells. We found that **5** affects MCF-7 and Ishikawa cells in a manner providing for induction of gene expression to a level similar to 17β-estradiol and BCA but, unlike both of the latter, for suppression of cell proliferation as well. In addition, **5** appeared to display higher stability compared to **1**–**4** and BCA in both MCF-7 and Ishikawa cells. The inference is that **5** may represent a safer than BCA alternative to hormone replacement therapy.

1. Introduction

Hormone replacement therapy (HRT) of postmenopausal symptoms using combined estrogen–progestin or estrogen-only regimens is associated with increased breast and endometrial cancer risk.[1,2] However, menopausal symptoms such as hot flashes, poor wound healing and vaginal dyspareunia may be treated by local rather than systemic administration of estrogen, thus limiting adverse side effects of HRT. Estrogen action is primarily mediated by two estrogen receptor (ER) subtypes, ERα and ERβ, with ERβ functioning as tumor suppressor by opposing the mitogenic action of ERα.[3] Accordingly, in human breast, malignant progression is associated with down-regulation of ERβ expression.[4] Phytoestrogens are plant derived natural products that have functional similarities with estrogen and are considered as a safe alternative to HRT.[5] Isoflavones are a major group of phytoestrogens that have been the focus of many studies. Biochanin A (BCA, 5,7-dihydroxy-4′-methoxyisoflavone), an ERβ-selective isoflavone in red clover and many other legumes, is commercially available as a nutraceutical and is known to display cancer chemopreventive properties.[6] Although BCA is thought to exert its beneficial effects predominantly through ERα and ERβ, BCA agonism of estrogen-related receptors (ERR) is also well documented.[7]

Phytoestrogen-containing dietary supplements are widely consumed for putative health benefits. However, although preclinical studies suggest that dietary intake of phytoestrogens can protect from postmenopausal symptoms and endocrine-related degenerative diseases, including endocrine-related cancer,[8] clinical trials suggest that their health benefits are inconsistent or marginal.[9–11] Pharmacokinetic studies in rats suggest that plasma BCA concentrations $\leqslant$0.01 μM are attainable with a daily oral intake of 5 mg per kg of body weight.[12] Following ingestion, BCA is known to display low bioavailability due to poor solubility, extensive metabolism and rapid clearance.[12] BCA is primarily metabolized to the isoflavone genistein and to BCA and genistein conjugates.[12,13] Esters and especially carbamate esters of bioactive phenols are known to increase bioavailability and/or metabolic stability.[14,15] Interestingly, intervention studies have shown that it is possible to introduce intact genistein ester molecules in sufficiently high concentrations by subcutaneous but not through oral administration.[16] The present study

* Corresponding authors. Tel.: +30 210 7274598; fax: +30 210 7274594 (S.M.); tel.: +30 210 7273741; fax: +30 210 7273677 (M.N.A.)
E-mail addresses: mitakou@pharm.uoa.gr (S. Mitakou), mnalexis@eie.gr (M.N. Alexis).
† These authors contributed equally to this work.

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describes the preparation of esters and carbamate esters of BCA and their biological evaluation using human breast and endometrial adenocarcinoma cells.

2. Results

2.1. Chemistry

In order to synthesize BCA, starting from phloroglucinol and the addition of *p*-methoxy phenylacetic acid, a Friedel–Crafts reaction catalyzed by boron trifluoride took place to afford the intermediate product 1-(2,4,6-trihydroxyphenyl)-2-(4-methoxyphenyl)ethanone. Then a cyclization to BCA was performed using CH_3SO_2Cl in DMF and freshly distilled $BF_3{\cdot}Et_2O$ as catalyst under argon (Scheme 1).[17] Then BCA was mixed with the corresponding acylchlorides or isocyanates in order to prepare the esters (**1**, **3**) and carbamate esters (**2**, **4** and **5**) at position 7 of BCA.

2.2. Assessment of estrogenic and anticancer activities of BCA and its derivatives

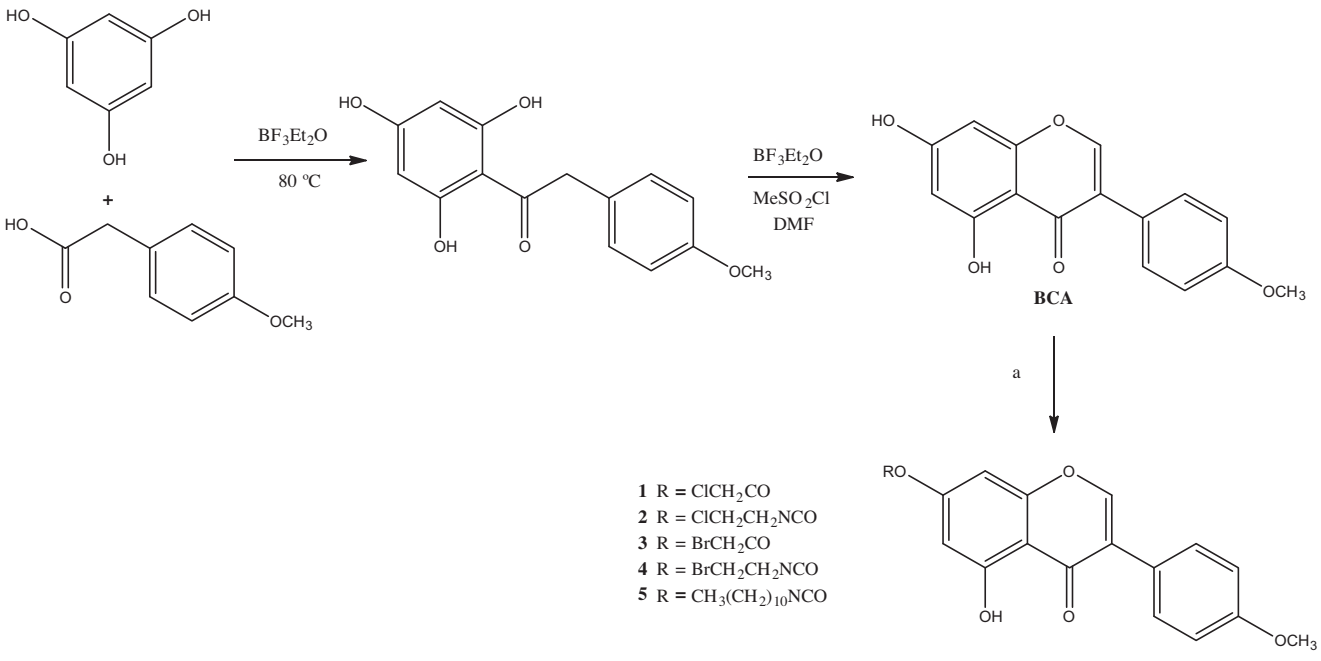
Initially we used fluorescence polarization to assess the ability of BCA and its ester (**1**, **3**) and carbamate ester (**2**, **4**, **5**) derivatives to compete with fluorescent estradiol for binding to purified recombinant human ERα and ERβ. Relative (to estradiol) binding affinity (RBA) values of BCA for ERα and ERβ were found equal to 0.24 ± 0.11 and 0.92 ± 0.16, respectively (Table 1). The RBAα and RBAβ values of **1**–**5** were found to be lower than BCA by factors of 10 on average. The previously reported RBAα and RBAβ values of the ERα-specific agonist PPT (propyl-pyrazol-triol), ERα-specific antagonist MPP (methyl-piperidino-pyrazole) and the potency-selective ERβ agonist DPN (diaryl-propionitrile) are also shown for comparison.[18,19]

Table 1
Relative ERα- and ERβ-binding affinity of BCA and its derivatives[a]

Compound	RBAα	RBAβ
Estradiol	100	100
PPT[b]	50.2 ± 2.4	1.4 ± 0.3
MPP[c]	20.2 ± 4.3	0.3 ± 0.1
DPN[d]	0.32 ± 0.04	13.4 ± 2.5
BCA[e]	0.24 ± 0.11	0.92 ± 0.16
1	0.03 ± 0.01	0.09 ± 0.02
2	0.05 ± 0.01	0.06 ± 0.01
3	0.03 ± 0.01	n.d.
4	0.05 ± 0.02	n.d.
5	0.06 ± 0.01	0.08 ± 0.02

[a] The RBA values (mean ± SEM of at least three independent experiments) of Biochanin A (BCA) and its derivatives for ERα (RBAα) and ERβ (RBAβ) were calculated by [RBA = (IC_{50} estradiol/IC_{50} test compound) × 100], where IC_{50} values are estradiol or test compound concentrations capable of inhibiting the binding of the fluorescent estrogen ES2 (1 nM) to ERα and ERβ by 50%. IC_{50} values of estradiol for ERα and ERβ were 2.22 and 3.3 nM, respectively. The RBAα and RBAβ of estradiol were set equal to 100.
[b] PPT (propyl-pyrazol-triol), ERα-specific agonist.
[c] MPP (methyl-piperidino-pyrazole), ERα-specific antagonist.
[d] DPN (diaryl-propionitrile), potency-selective ERβ agonist.
[e] BCA, Biochanin A.

(a) Reagents for compound **1**: Py, $ClCH_2COCl$,
for compound **2**: Py, $ClCH_2CH_2NCO$,
for compound **3**: Py, $BrCH_2COCl$,
for compound **4**: Py, $BrCH_2CH_2NCO$
for compound **5**: Py, $CH_3(CH_2)_{10}NCO$,



Scheme 1. Synthesis of BCA derivatives. (a) Reagents for compound **1**: Py, $ClCH_2COCl$, for compound **2**: Py, $ClCH_2CH_2NCO$, for compound **3**: Py, $BrCH_2COCl$, for compound **4**: Py, $BrCH_2CH_2NCO$ for compound **5**: Py, $CH_3(CH_2)_{10}NCO$.

The ability of **1**–**5** and BCA to induce estrogen response element (ERE)-dependent gene expression was assessed using MCF-7:D5L cells, a previously reported clone of MCF-7 cells.[20] As expected from previous findings,[20–22] treatment of estrogen-free MCF-7:D5L cells with estradiol concentrations $\geqslant$0.1 nM resulted in full (3- to 4-fold) induction of luciferase expression in a manner that was fully inhibited by the ER destabilizer fulvestrant (Fig. 1A and B). Figure 1A shows, in addition, that the ERα-specific agonist PPT displayed an induction efficacy similar to estradiol; and that the ERα-specific antagonist MPP and an equimolar combination of MPP and the potency-selective ERβ agonist DPN failed to induce luciferase activity. Interestingly, treatment of MCF-7:D5L cells with **1**–**5** or BCA induced luciferase expression to a level considerably higher than 0.1 nM estradiol in ER-dependent manner, as assessed using fulvestrant (Fig. 1B and data not shown). The rank order of luciferase induction efficacies (Table 2, columns 3 and 4) at 1 μM test compound was, **1** > **2** $\geqslant$ **3** $\geqslant$ **4** $\geqslant$ **5** $\geqslant$ BCA > E2 > vehicle (*t*-test), while at 10 μM test compound was, **1** $\approx$ **3** > **2** $\geqslant$ **4** > **5** $\geqslant$ BCA > E2 > vehicle (*t*-test). Assessment of the number of viable cells using MTT suggested that the higher luciferase induction efficacies of test compounds compared to estradiol were not due to differences in viable cell numbers (data not shown).

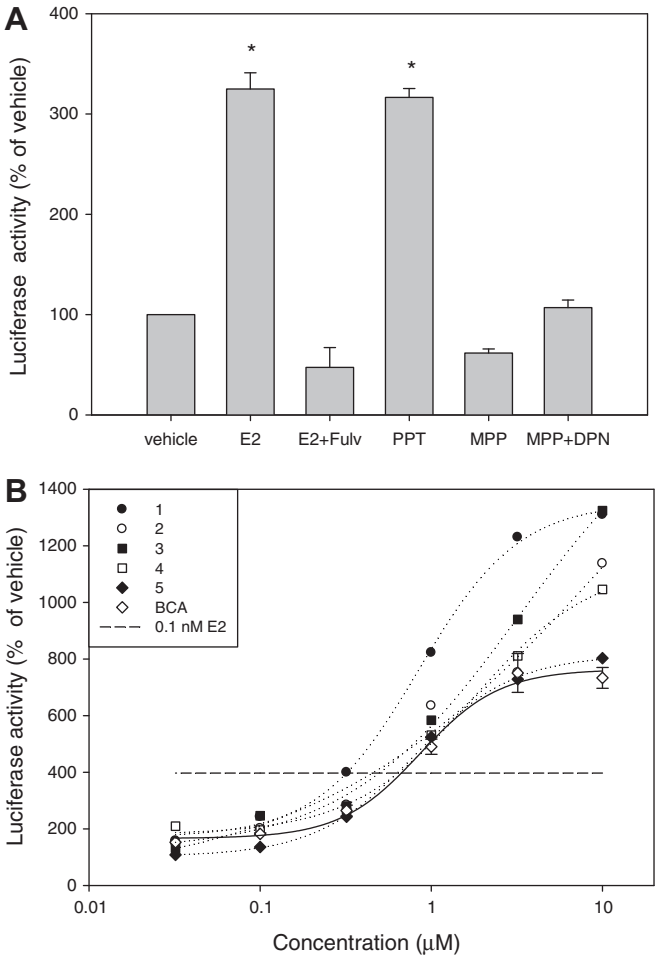


Figure 1. Induction of luciferase expression in MCF-7:D5L cells: (A) by 0.1 nM estradiol (E2) in the absence or presence of 100 nM fulvestrant, 100 nM PPT and 100 nM MPP in the absence or presence of 100 nM DPN. Data are mean ± SEM of three independent experiments carried out in triplicate. * $p \leqslant 0.05$ versus vehicle (*t*-test). (B) By increasing concentrations of Biochanin A (BCA) and its ester (**1**, **3**) and carbamate ester (**2**, **4**, **5**) derivatives. Luciferase activity is expressed as % of that of cells treated only with vehicle (compound diluent). Luciferase activity in the presence of 0.1 nM estradiol (E2) is shown for comparison. Data are mean of three independent experiments carried out in triplicate with an inter-assay variation (mean ± SEM) similar to that shown for BCA.

We also determined the potential of **1**–**5** and BCA to stimulate the proliferation of MCF-7 cells. These cells are known to cease proliferating following transfer to estrogen-free media supplemented with fetal bovine serum which has been treated with dextran coated charcoal to remove endogenous estrogens.[23] Estradiol is known to stimulate the proliferation of MCF-7 cells primarily through ERα.[24] Treatment with **1**–**4** or BCA stimulated cell proliferation to levels significantly lower (*t*-test) than 0.1 nM estradiol in an ER-dependent manner, as assessed using fulvestrant, while **5** was totally ineffective in this respect (Fig. 2 and data not shown). The rank order of proliferation stimulation efficacies (Table 2, columns 6 and 7) at 1 μM test compound was, E2 > BCA $\geqslant$ **3** $\geqslant$ **1** $\approx$ **4** $\geqslant$ **2** > **5** $\approx$ vehicle (*t*-test), while at 10 μM test compound was, E2 > BCA $\approx$ **2** $\geqslant$ **1** $\approx$ **3** $\geqslant$ **4** > **5** $\approx$ vehicle (*t*-test). We also determined the effect of **1**–**5** and BCA on the proliferation of MCF-7 cells in the presence of 0.1 nM estradiol (Fig. 3), a concentration of the hormone similar to that usually found in the plasma of postmenopausal women.[25] We observed partial suppression of estrogen stimulation of cell proliferation with 10 μM BCA, 1 and 10 μM **1** or **3** and 1 μM **4**. Notably, however, **4** at 10 μM and **5** at concentrations $\geqslant$1 μM fully suppressed the proliferation of MCF-7 cells (Fig. 3). We also tested **1**–**5** and BCA against MDA-MB-231 cells which are known to lack ERα expression and to proliferate in an estrogen-independent manner. Carbamate ester **5** inhibited by 21% the proliferation of MDA-MB-231 cells at 10 μM but was totally ineffective at 1 μM.

Finally, we determined the ability of test compounds to induce AlkP expression in Ishikawa cells. As expected from previous findings,[20,21,26] treatment of estrogen-free Ishikawa cells with estradiol at concentrations $\geqslant$0.1 nM resulted in full (~4.4-fold) induction of AlkP expression in a manner that was fully inhibited by the ER destabilizer fulvestrant (Fig. 4A and B). Figure 4A shows, in addition, that PPT induced and MPP failed to induce AlkP activity in Ishikawa cells, much like luciferase activity in MCF-7:D5L cells; however, unlike MCF-7:D5L cells, Ishikawa cells responded to the equimolar combination of MPP and DPN by inducing AlkP to a level ~70% of that of estradiol. Treatment of Ishikawa cells with increasing concentrations of **1**–**5** or BCA induced AlkP expression to a level similar (**2**, **4**) or significantly lower (**1**, **3**, **5**, BCA) than 0.1 nM estradiol in an ER-dependent manner, as assessed using fulvestrant (Fig. 4B and data not shown). The rank order of induction efficacies (Table 2, columns 9 and 10) at 1 μM test compound was, E2 > **3** $\geqslant$ **1** $\approx$ BCA $\approx$ **4** $\approx$ **2** > **5** > vehicle (*t*-test), while at 10 μM test compound was, E2 $\approx$ **2** $\approx$ **4** > **3** $\geqslant$ **5** $\geqslant$ **1** $\geqslant$ BCA > vehicle (*t*-test). We also determined the effect of **1**–**5** and BCA on the proliferation of Ishikawa cells. BCA stimulated the proliferation of Ishikawa cells at 10 μM, while **5** displayed a significant (*t*-test) inhibitory effect at the concentration of 1 as well as 10 μM (Fig. 5). In contrast, estradiol and fulvestrant failed to significantly affect the proliferation of Ishikawa cells, in accordance with previous findings.[26,27]

3. Discussion

High affinity ligand binding to ERα and ERβ largely depends on two hydroxyl groups with an O–O distance similar to estradiol (10.8 Å) or the potency-selective ERβ agonist genistein (12.1 Å).[20,28] The RBAα and RBAβ values of BCA are lower than those previously reported for genistein by factors of approx. 4 and 48, respectively.[21] The 4′-OH and the 7-OH of genistein are known to mimic the 3-OH and 17-OH of estradiol in forming H bonds with the side chains of Glu353-Arg394 (Glu305-Arg346 in ERβ) and His524 (His475), respectively.[20,28] H bond formation with Glu353 and Arg394 is presumably not possible for BCA due to the

Table 2
Biological responses to compounds **1–5**, Biochanin A and estradiol

Compd	Luciferase expression			Cell proliferation			AlkP expression		
	EC_{50}[a] (μM)	Efficacy[b] at 1 μM	Efficacy[b] at 10 μM	EC_{50}[a] (μM)	Efficacy[b] at 1 μM	Efficacy[b] at 10 μM	EC_{50}[a] (μM)	Efficacy[b] at 1 μM	Efficacy[b] at 10 μM
1	0.14 ± 0.04	823 ± 56	1310 ± 44	0.55 ± 0.10	152 ± 11	155 ± 15	0.47 ± 0.21	314 ± 29	354 ± 4
2	0.14 ± 0.03	635 ± 40	1138 ± 27	0.68 ± 0.19	139 ± 13	166 ± 9	0.66 ± 0.14	295 ± 31	445 ± 28
3	0.18 ± 0.04	584 ± 27	1324 ± 15	0.28 ± 0.12	156 ± 14	154 ± 8	0.52 ± 0.06	332 ± 21	387 ± 29
4	0.22 ± 0.08	531 ± 29	1046 ± 34	0.42 ± 0.05	151 ± 3	145 ± 15	0.62 ± 0.07	302 ± 16	440 ± 33
5	0.32 ± 0.04	524 ± 34	803 ± 14	n-a	117 ± 10	108 ± 8	1.70 ± 0.67	203 ± 21	375 ± 20
BCA	0.30 ± 0.03	490 ± 30	733 ± 37	0.21 ± 0.08	161 ± 7	167 ± 6	0.42 ± 0.01	310 ± 6	309 ± 21
E2	19.6 ± 7.1[c]	402 ± 12	402 ± 12	6.4 ± 4.3[c]	185 ± 5	185 ± 5	24.6 ± 10.3[c]	440 ± 15	440 ± 15

n-a, non-applicable; BCA, Biochanin A; E2, estradiol.
[a] EC_{50} values (mean of three independent experiments in triplicates) are test compound concentrations required to produce 50% of the proliferative or gene expression effects of 0.1 nM estradiol.
[b] Efficacy was calculated by $[100 \times A_{\text{test compound}}]/[A_{\text{estradiol}}]$, were A is absorbance of MTT–formazan generated by proliferating MCF-7 cells or luciferase activity of MCF-7:D5L cells or alkaline phosphatase (AlkP) activity of Ishikawa cells. The A values (mean of three independent experiments) were assessed at 0.1 nM estradiol or 1 and 10 μM test compound and are expressed as % of vehicle.
[c] pM.

methylation of its 4′-OH. The lower RBA values of **1–5** compared to BCA could reflect inability of H bond formation between the 7-OH group and His524 as well. Based on previous findings with several other isoflavones,[21] the above RBA values indicate that BCA concentrations $\geqslant$0.1 μM are deemed necessary for full induction of gene expression in Ishikawa and MCF-7:D5L cells; and that the necessary concentrations of **1–5** could be even higher than BCA. Notably, however, compared to BCA **1–5** displayed considerably higher luciferase induction potencies, that is, lower EC_{50} (Table 2, column 2), than would have been predicted on the basis of their RBAα values, suggesting that their estrogenic activity was probably the result of ester hydrolysis.

We observed that luciferase expression of MCF-7:D5L cells was fully induced by the ERα-specific agonist PPT, while the equimolar combination of the ERα-specific antagonist MPP and the potency-selective ERβ agonist DPN was totally ineffective in this respect, in accordance with reports that MCF-7 cells express ERα but hardly any ERβ.[19,29] We also observed that treatment of MCF-7:D5L cells with **1–5** or BCA induced luciferase expression to a level considerably higher than 0.1 nM estradiol in ER-dependent manner. It has been reported that while estradiol is unable to bind to any one of the three subtypes of ERR (ERRα, ERRβ and ERRγ), BCA and its major metabolite genistein are ERR agonists;[27] that BCA agonism of ERR and ER occur under comparable conditions;[27] and that ERα-dependent induction of ERE-driven luciferase reporters by estradiol can be enhanced by ERR possibly through increased recruitment of co-activators to ERα.[30] In the light of these reports the data of Figure 1B are taken to suggest that, in MCF-7:D5L cells, BCA agonism of ERR enhanced isoflavone induction of luciferase through ERα, the predominant form of ER in these cells as well as in wild-type MCF-7 cells. Luciferase induction efficacies at 10 μM test compound are thought to reflect levels of free BCA inside MCF-7:D5L cells under the conditions of the assay. Hence, it appears that **2** and **4** at 10 μM released lower amounts of BCA compared to **1** and **3**, but higher amounts compared to **5**; and that metabolism and rapid efflux of BCA likely prevented the free isoflavone from mounting a luciferase response as high as **1–4**. Consequently, it appears that esters (**1**, **3**) and carbamate esters (**2**, **4**, **5**) of BCA display different stabilities in the specific cell context and hence different abilities to penetrate and remain in MCF-7:D5L cells, with the carbamate esters being more stable and therefore more effective in controlling metabolism and rapid efflux of the isoflavone. Presumably, BCA released in the cytoplasm following hydrolysis of **1–5** will be equally accessible to ER and ERR, since these nuclear receptors display intensive ligand-independent nucleocytoplasmic shuttling.[31,32]

The data of Figures 1B and 2 show that **5** induced ERE-dependent gene expression to a level similar to estradiol and BCA, but failed to stimulate the proliferation of MCF-7 cells. We have previously reported that some deoxybenzoins can exhibit a similar degree of dissociation between gene expression and cell proliferation stimulation efficacies.[20] In addition, the data of Figure 3 show that **5** fully inhibited estradiol-dependent proliferation of MCF-7 cells. This is taken to indicate that **5** displays higher ability to penetrate and remain inside MCF-7 cells, crossing cell membranes easily and partitioning more effectively in critical cellular compartments compared to the remainder test compounds. The inhibitory potency of **5** could reflect the potential of lipophilic compounds, including lipophilic esters of isoflavones,[16] to penetrate cell membranes and thus be more capable of reaching targets critical for cell proliferation and/or survival, for example, inside mitochondria. These organelles are known to accumulate lipophilic drugs.[33] Delivery of BCA to mitochondria may inhibit cell proliferation through ERβ, as already shown for resveratrol.[34] Alternatively, **1–5** may also enhance delivery of genistein, the major metabolite of BCA, to mitochondria. The intracellular level and

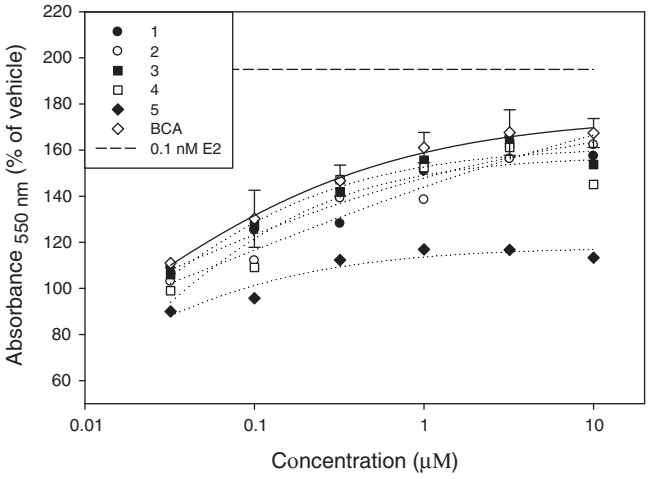


Figure 2. Stimulation of proliferation of estrogen-free MCF-7 cells by increasing concentrations of Biochanin A (BCA) and its ester (**1**, **3**) and carbamate ester (**2**, **4**, **5**) derivatives. Stimulation in the presence of 0.1 nM estradiol (E2) is shown for comparison. Absorbance of MTT–formazan (reflects the number of viable cells) is expressed as % of that of cells treated only with vehicle (compound diluent). Data are mean of three independent experiments carried out in triplicate with an inter-assay variation (mean ± SEM) similar to that shown for BCA.

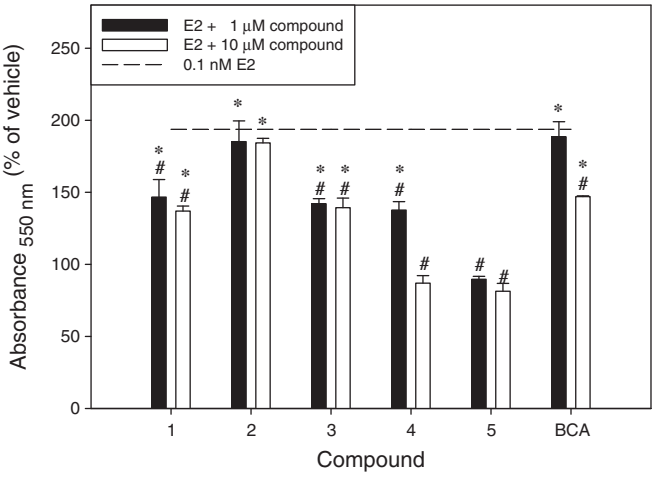


Figure 3. Effects of Biochanin A (BCA) and its ester (**1**, **3**) and carbamate ester (**2**, **4**, **5**) derivatives on the proliferation of MCF-7 cells in the presence of 0.1 nM estradiol (E2). Absorbance of MTT–formazan (reflects the number of viable cells) is expressed as % of that of cells treated only with vehicle (compound diluent). Absorbance in the presence of 0.1 nM (E2 is shown for comparison. Data are mean ± SEM of three independent experiments carried out in triplicate. * $p \leqslant 0.05$ versus vehicle, # $p \leqslant 0.05$ versus 0.1 nM E2; *t*-test.

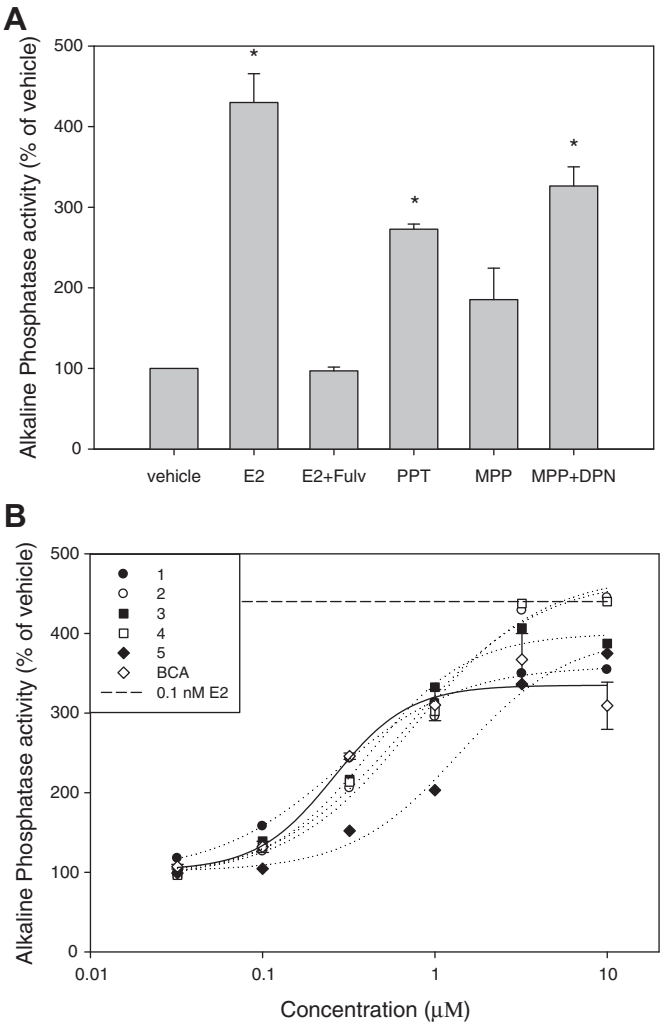


Figure 4. Induction of alkaline phosphatase expression in Ishikawa cells: (A) by 0.1 nM estradiol (E2) in the absence or presence of 100 nM fulvestrant, 100 nM PPT and 100 nM MPP in the absence or presence of 100 nM DPN. Data are mean ± SEM of three independent experiments carried out in triplicate. * $p \leqslant 0.05$ versus vehicle (*t*-test). (B) Increasing concentrations of Biochanin A (BCA) and its ester (**1**, **3**) and carbamate ester (**2**, **4**, **5**) derivatives. Alkaline phosphatase activity is expressed as % of that of cells treated only with vehicle (compound diluent). Alkaline phosphatase activity in the presence of 0.1 nM estradiol (E2) is shown for comparison. Data are mean of three independent experiments carried out in triplicate with an inter-assay variation (mean ± SEM) similar to that shown for BCA.

activity of free BCA and its major metabolites, genistein and BCA and genistein conjugates is dependent on cell-type specific metabolism. Since free hydroxyl groups are subject to conjugation, which in turn provides for rapid efflux of the conjugates, ester and in particular carbamate esters of BCA are likely to limit the level of free BCA that is available for conjugation and efflux. In addition, efflux of free BCA is likely to lack behind that of genistein. Thus, it is possible that using **1**–**5** rather than free BCA or genistein could provide for a more effective distribution of the latter between different cellular compartments and hence increase its impact on cell proliferation and viability. Notably, the mitochondria of MCF-7 cells are known to harbor functional ERβ,[35,36] the mitochondrial ERβ is said to be a vulnerability factor in these cells,[35] and genistein is a potency-selective ERβ agonist that may challenge cell viability through ERβ. That luciferase activity of MCF-7:D5L cells failed to respond to the combination of MPP and DPN cannot exclude the presence of active ERβ in the mitochondria of these cells. We also tested **1**–**5** and BCA against MDA-MB-231 cells. These cells are of the triple negative phenotype i.e. negative for ERα, progesterone receptor and HER2 but positive for ERβ.[37] We found that proliferation of MDA-MB-231 cells was only marginally inhibited by 10 μM **5**. Whether the higher sensitivity of MCF-7 cells to **5** as compared to MDA-MB-231 cells is associated with expression of ERα in the former but not the latter cells is presently unknown.

BCA and its derivatives were also tested to determine their estrogenic and cancer chemopreventive activities using Ishikawa cells. In these cells, isoflavones induce AlkP expression through ER in a manner that is indicative of their intrinsic estrogenic activity.[38] We observed that the AlkP expression of Ishikawa cells was induced by the equimolar combination of MPP and DPN as well as by PPT, in accordance with reports that uterine epithelial cells and Ishikawa cells in particular express ERβ as well as ERα.[39,40] The AlkP induction efficacies of test compounds may be taken to

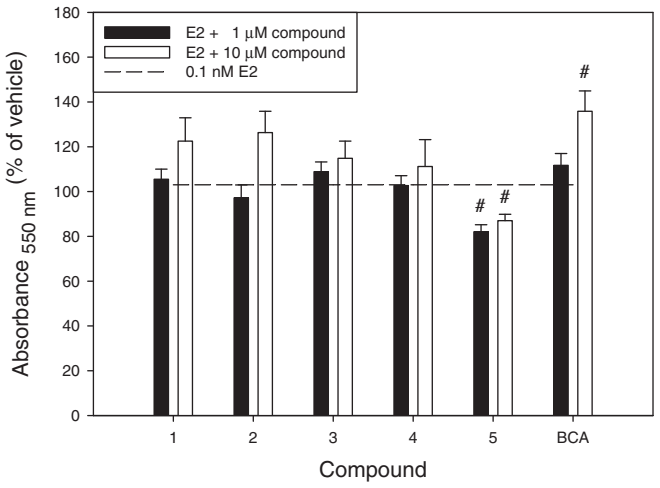


Figure 5. Effects of Biochanin A (BCA) and its ester (**1**, **3**) and carbamate ester (**2**, **4**, **5**) derivatives on the proliferation of Ishikawa cells in the presence of 0.1 nM estradiol (E2). Absorbance of MTT–formazan (reflects the number of viable cells) is expressed as % of that of cells treated only with vehicle (compound diluent). Absorbance in the presence of 0.1 nM E2 is shown for comparison. Data are mean ± SEM of three independent experiments carried out in triplicate. # $p \leq 0.05$ versus 0.1 nM E2; *t*-test.

indicate levels of free BCA inside Ishikawa cells under the conditions of the assay, as already assumed for luciferase induction efficacies in MCF-7:D5L cells. The AlkP induction efficacies of the carbamate esters (**2**, **4**) exceeded those of the respective esters (**1**, **3**). Assessment of the number of viable cells using MTT (Fig. 5) suggested that the lower AlkP induction efficacies of **1**, **3** and BCA were not due to inhibition of cell proliferation. We speculate that due to more extensive hydrolysis in Ishikawa cells compared to MCF-7 cells, **1** and **3** allowed BCA metabolism and efflux to an extent comparable to that observed with free BCA. The AlkP induction efficacy of **5** was also lower than **2** and **4** and comparable to **1**, **3** and to free BCA. However, since the induction potency of **5** was much lower than that of **1** and **3**, its low induction efficacy is probably due to limited BCA release rather than the opposite. Notably, only **5** was able to inhibit the proliferation of Ishikawa cells (Fig. 5). Again, the stability and lipophilicity of **5** could allow BCA to penetrate and remain inside Ishikawa cells to comparatively higher level, promoting isoflavone distribution to key cellular targets in Ishikawa and MCF-7 cells alike.

Our interpretation of the induction efficacy data of Figures 1B and 4B implies that carbamate esters (**2**, **4**, **5**) are better shielded from hydrolysis compared to carboxylic acid esters (**1**, **3**). The induction efficacy data of Figure 2 were not taken into account given the complexity of proliferative response which among other signaling entities and cascades is known to involve membrane ER.[41] Carbamate esters have been used in the design of prodrugs in order to protect hydroxyl groups from first-pass and systemic metabolism leading to drug clearance.[14,15,42] The therapeutic potential of a drug is reportedly increased by reducing the rate of hydrolysis of the carbamate ester prodrug. In addition, the stability of carbamate ester prodrugs is known to depend on the substitution of the carbamic acid shield.[15] Accordingly, hydrolysis of chlorinated ester **1** appeared to proceed faster than that of the brominated ester **3** in MCF-7:D5L cells, as indicated by the higher luciferase induction potency of the former compared to the latter (EC_{50} = 0.14 and 18 nM, respectively). This appears to be also the case in Ishikawa cells (EC_{50} = 0.47 and 0.52 μM, respectively). Chlorinated carbamate ester **2** appeared less stable than brominated **4** in MCF-7:D5L cells (EC_{50} = 0.14 and 0.22 μM, respectively) but not in Ishikawa cells (EC_{50} = 0.66 and 0.62 μM, respectively). Importantly, lipophilic carbamate ester **5** appeared to display higher stability compared to **1**–**4** and BCA in both MCF-7:5L and Ishikawa cells (EC_{50} = 0.32 and 1.70 μM, respectively). It should be said here that in order to experimentally support the above stability claims it would be necessary to separate free from esterified BCA by several extraction and chromatographic steps using radiolabelled BCA as internal standard and to analyze free BCA using dedicated methods and tools, similar to those developed for genistein.[16]

Taken together, Figures 1–5 suggest that **5** affects MCF-7 and Ishikawa cells in a manner providing for induction of gene expression to a level similar to estradiol and BCA and, at the same time, for suppression of cell proliferation. In contrast, estradiol and BCA stimulated the proliferation of MCF-7 cells and BCA that of Ishikawa cells. By and large, our findings indicate that **5** could be as effective as estradiol and BCA HRT wise but more effective than the free isoflavone chemoprevention wise. Since systemic administration of isoflavone esters is ineffective, while the opposite is likely the case with local administration,[16] **5** could be used to remedy post-menopausal symptoms such as vaginal dyspareunia, skin aging and poor wound healing. Skin is an established estrogen target organ that expresses both ERα and ERβ.[43,44] Notably, it was recently shown that exogenous estrogen replacement promotes cutaneous wound healing via ERβ in a preclinical model of menopause.[43]

4. Conclusions

We have synthesized two ester (**1**, **3**) and three carbamate ester derivatives (**2**, **4**, **5**) of BCA as well as the free isoflavone and evaluated their estrogenic and cancer chemopreventive activity using cell model systems of human breast and endometrial adenocarcinoma. We found that carbamate ester **5** displayed estradiol-like stimulation of gene expression as well as inhibition of proliferation of breast and endometrial cancer cells, implying that it may represent a safe alternative to HRT, especially if administered locally rather than systemically. Further investigation is needed to substantiate this hypothesis.

5. Experimental part

5.1. General chemistry methods

All chemicals were of the highest available commercial purity. Starting materials were purchased from Aldrich (analytical reagent grades) and used without further purification. All anhydrous reactions were carried out under argon atmosphere. Solvents were dried by distillation prior to use. Analytical thin-layer chromatography (TLC) was conducted on Merck silica gel 60 F_{254} and spots visualized using UV light and vanillin–sulfuric acid reagent. Liquid chromatography was performed on columns containing Si gel 60 Merck (40–63 μm). Solvent mixtures employed in chromatography are reported as volume to volume ratios. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data were recorded on a Bruker DRX400 spectrometer. COSY, HMQC, and HMBC NMR data were obtained using standard Bruker microprograms. Mass spectrometry HRMS (ESI+) were run on a Thermo Scientific LTQ Orbitrap Discovery mass spectrometer with the infusion method.

5.2. Synthesis of 5,7–dihydroxy-3-(4-methoxyphenyl)-4*H*-chromen-4-one (Biochanin A, BCA)

Synthesis of BCA was carried out as described by Wahala and Hase.[17] Briefly phloroglucinol (0.050 mol) and *p*-methoxy phenylacetic acid (0.050 mol) were dissolved into freshly distilled $BF_3{\cdot}Et_2O$

under argon. The mixture was stirred and heated at 80 °C on an oil-bath. The corresponding intermediate product 1-(2,4,6-trihydroxyphenyl)-2-(4-methoxyphenyl)ethanone was isolated by washing of the collected material thoroughly with aq $NaHCO_3$ and ethyl acetate. The residue was purified by column chromatography on silica gel using a mixture of dichloromethane and methanol. In a solution of the intermediate product (450 mg, 2 mmol) in DMF (6 mL), freshly distilled $BF_3{\cdot}Et_2O$ (6.3 mL) was added under argon. The mixture was heated to 50 °C, and a solution of methanesulfonyl chloride (1 mL) in dry DMF (1.5 mL) was added slowly. After reaction at 80 °C, for 1 h, the mixture was cooled to room temperature and poured into a large volume of ice-cold aq sodium acetate (12 g/100 mL). Then the mixture was extracted with ethyl acetate, and the organic layer was dried and concentrated. The residue was purified by column chromatography on silica gel using a mixture of dichloromethane and methanol.

5.3. Synthesis of BCA derivatives

For the preparation of the esters and the carbamide esters of BCA, in a solution of BCA (100 mg, 0.35 mmol) in 20 mL ether which was cooled to 0 °C, 0.8 mL of pyridine and 0.50 mL of the corresponding acylchlorides or isocyanates were added. After the completion of the reaction in room temperature, the organic solvents were evaporated and the residue was chromatographed on a silica gel column chromatography using as mobile phase a gradient CH_2Cl_2/MeOH mixture, to afford the relative compound.

5.3.1. Biochanin A (BCA)

Yield 72%. ^{1}H NMR (600 MHz, $CDCl_3$, δ ppm): 13.0 (1H, s, OH), 8.22 (1H, s, H-2), 7.54 (2H, d, J = 8.0 Hz, H-2′/H-6′), 7.01 (2H, d, J = 8,0 Hz, H-3′/H-5′), 6.42 (1H, d, J = 1.5 Hz, H-8), 6.30 (1H, d, J = 1.5 Hz, H-6), 3.89 (3H, s, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 181.6 (C-4), 165.0 (C-7), 163.9 (C-5), 161.7 (C-4′), 159.0 (C-8a), 154.5 (C-2), 131.1 (C-2′, C-6′), 124.2 (C-3) 123.8 (C-1′), 114.5 (C-3′, C-5′), 106.2 (C-4a), 100.0 (C-6) 94.5 (C-8), 55.5 (OCH_3). HRMS (ESI+) m/z 285.0756 $[M+H]^+$, calculated for 285.0763, $C_{16}H_{13}O_5$.

5.3.2. 5-Hydroxy-3-(4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 2-chloroacetate (1)

Yield 68%. ^{1}H NMR (600 MHz, $CDCl_3$, δ ppm): 12.9 (1H, s, OH), 7.99 (1H, s, H-2), 7.48 (2H, d, J = 8.0 Hz, H-2′/H-6′), 7.01 (2H, d, J = 8,0 Hz, H-3′/H-5′), 6.82 (1H, d, J = 1.5 Hz, H-8), 6.65 (1H, d, J = 1.5 Hz, H-6), 4.38 (2H, s, CH_2), 3.87 (3H, s, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 180.9 (C-4), 164.5 (C-7), 162.9 (C-5), 160.1 (C-4′), 156.9 (C-8a), 153.4 (C-2), 130.1 (C-2′, C-6′), 124.2 (C-3) 122.3 (C-1′), 114.2 (C-3′, C-5′), 109.8 (C-4a), 105.2 (C-6), 100.5 (C-8), 55.3 (OCH_3), 40.7 ($ClCH_2CO$). HRMS (ESI+) m/z 361.0482 $[M+H]^+$, calculated for 361.0479, $C_{18}H_{14}ClO_6$.

5.3.3. 5-Hydroxy-3-(4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 2-chloroethylcarbamate (2)

Yield 81%. ^{1}H NMR (600 MHz, $CDCl_3$, δ ppm): 12.8 (1H, s, OH), 7.92 (1H, s, H-2), 7.43 (2H, d, J = 8.0 Hz, H-2′/H-6′), 6.98 (2H, d, J = 8.0 Hz, H-3′/H-5′), 6.81 (1H, d, J = 1.5 Hz, H-8), 6.61 (1H, d, J = 1.5 Hz, H-6), 5.51 (1H, t, J = 5.5 Hz, CH_2NH), 3.83 (3H, s, OCH_3), 3.67 (2H, q, J = 5.5 Hz, CH_2NH), 3.51 (2H, t, $ClCH_2$). ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 181.2 (C-4), 165.4 (C-7), 164.0 (C-5), 160.0 (C-4′), 158.0 (C-8a), 156.5 (OCONH), 153.8 (C-2), 131.0 (C-2′, C-6′), 124.0 (C-3) 123.5 (C-1′), 114.0 (C-3′, C-5′), 109.2 (C-4a), 105.3 (C-6) 100.0 (C-8), 55.0 (OCH_3), 43.5 (CH_2NH), 42.0 (CH_2Cl). HRMS (ESI+) m/z 389.0648 $[M+H]^+$, calculated for 389.0666, $C_{19}H_{16}ClNO_6$.

5.3.4. 5-Hydroxy-3-(4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 2-bromooacetate (3)

Yield 58%. ^{1}H NMR (600 MHz, $CDCl_3$, δ ppm): 13.0 (1H, s, OH), 7.99 (1H, s, H-2), 7.42 (2H, d, J = 8 Hz, H-2′, H-6′), 6.98 (2H, d, J = 8 Hz, H-3′, H-5′), 6.74 (1H, d, J = 1.5 Hz, H-8), 6.57 (1H, d, J = 1.5 Hz, H-6), 4.05 (2H, s, CH_2), 3.81 (3H, s, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 181.2 (C-4), 169.7 (CH_2COO), 164.9 (C-7), 163.4 (C-5), 158.5 (C-8a), 158.0 (C-4′), 154.0 (C-2), 130.0 (C-2′, C-6′), 123.7 (C-3) 123.1 (C-1′), 115.4 (C-3′, C-5′), 108.9 (C-4a), 105.7 (C-6) 100.5 (C-8), 55.8 (OCH_3), 25.5 (CH_2). HRMS (ESI+) m/z 404.9967 $[M+H]^+$, calculated for 404.9974, $C_{18}H_{14}BrO_6$

5.3.5. 5-Hydroxy-3-(4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 2-bromooethylcarbamate (4)

Yield 63%. ^{1}H NMR (600 MHz, $CDCl_3$, δ ppm): 13.0 (1H, s, OH), 7.92 (1H, s, H-2), 7.42 (2H, d, J = 8.0 Hz, H-2′/H-6′), 6.97 (2H, d, J = 8.0 Hz, H-3′/H-5′), 6.80 (1H, d, J = 1.5 Hz, H-8), 6.60 (1H, d, J = 1.5 Hz, H-6), 5.52 (1H, t, J = 5.5 Hz, CH_2NH), 3.83 (3H, s, OCH_3), 3.69 (2H, q, J = 5.5 Hz, CH_2NH), 3.52 (2H, t, $BrCH_2$). ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 181.0 (C-4), 165.0 (C-7), 163.2 (C-5), 160.0 (C-4′), 157.1 (C-8a), 156.4 (OCONH), 153.5 (C-2), 130.0 (C-2′, C-6′), 124.1 (C-3) 123.9 (C-1′), 114.5 (C-3′, C-5′), 109.2 (C-4a), 105.0 (C-6) 100.5 (C-8), 55.6 (OCH_3), 43.2 (CH_2NH), 32.3 (CH_2Br). HRMS (ESI+) m/z 433.0170 $[M+H]^+$, calculated for 433.0161, $C_{19}H_{16}BrNO_6$.

5.3.6. 5-Hydroxy-3-(4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl undecylcarbamate (5)

Yield 65%. ^{1}H NMR (600 MHz, $CDCl_3$, δ ppm): 12.8 (1H, s, OH), 7.98 (1H, s, H-2), 7.48 (2H, d, J = 8 Hz, H-2′, H-6′), 7.01 (2H, d, J = 8 Hz, H-3′, H-5′), 6.86 (1H, d, J = 1.5 Hz, H-8), 6.62 (1H, d, J = 1.5 Hz, H-6), 5.10 (1H, t, J = 5.5 Hz, CH_2NH), 3.83 (3H, s, OCH_3). 3.82 (3H, s, OCH_3), 3.32 (2H, q, J = 1.5 Hz, CH_2NH), 1.58–1.25 (18H, m, CH_2), 0.91 (3H, t, J = 1.5 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 181.2 (C-4), 162.2 (C-7), 159.8 (C-5), 156.9 (C-8a), 156.5 (C-4′), 153.2 (C-2), 153.0 (NHCOO), 130.1 (C-2′, C-6′), 123.9 (C-3) 122.6 (C-1′), 114.1 (C-3′, C-5′), 109.1 (C-4a), 104.9 (C-6), 100.4 (C-8), 55.3 (OCH_3), 41.3 (CH_2NH), 31.9 (CH_2CH_2NH), 29.7–29.2 (5 CH_2), 22.7 (CH_3CH_2), 26.7 ($CH_3CH_2CH_2$), 14.1 (CH_3CH_2). HRMS (ESI+) m/z 482.2550 $[M+H]^+$, calculated for 482.2543, $C_{28}H_{36}NO_6$.

5.4. Biological evaluation

5.4.1. Cells, cell culture and fine chemicals

If not stated otherwise, fine chemicals and culture media were from Sigma–Aldrich (Steinheim, Germany). Fulvestrant (ICI182,780), DPN (diaryl-propionitrile), MPP (methylpiperidino-pyrazole) and PPT (propyl-pyrazol-triol) were from Tocris Bioscience (Bristol, UK). Fetal bovine serum (FBS) was from Invitrogen Corporation (Carlsbad, USA). Ishikawa human endometrial adenocarcinoma cells, MCF-7 and MDA-MD-231 human breast adenocarcinoma cells and MCF-7:D5L cells, a clone of MCF-7 cells stably transfected with the estrogen-responsive reporter plasmid pERE-Gl-Luciferase, were maintained in culture as already described.[20,45] DCC-FBS, that is, FBS that was treated with 10% dextran-coated charcoal (DCC) to remove endogenous steroids was prepared as already described.[23]

5.4.2. Test compound effects on luciferase expression of MCF-7:D5L cells

The effects of BCA and its derivatives (hereafter collectively referred to as test compounds) on the regulation of estrogen response element (ERE)-dependent luciferase gene expression of MCF-7:D5L cells were assessed as already described.[20] Briefly,

the cells were plated in 96-well microculture plates at a density of 12,000 cells per well in MEM devoid of phenol-red and supplemented with 1 μg/mL insulin and 5% DCC-FBS. Increasing concentrations (up to 10 μM) of test compounds or vehicle (DMSO to a final concentration of 0.1%) were administered to the cells 72 h after plating and luciferase expression was assessed 16 h later using a Safire II microplate reader (Tecan). Cells treated with 0.1 and 1 nM 17β-estradiol (estradiol) or only with vehicle served as controls.

5.4.3. Test compound effects on alkaline phosphatase expression of Ishikawa cells

Test compound regulation of AlkP expression of Ishikawa cells was assessed as already described.[20] Briefly, the cells were plated in 96-well microculture plates at a density of 12,000 cells per well in phenol red-free MEM supplemented with 1 μg/mL insulin and 5% DCC-FBS. Increasing concentrations (up to 10 μM) of test compounds or vehicle (DMSO to a final concentration of 0.1%) were administered to the cells 24 h after plating and AlkP activity was assessed 72 h later using a Safire II microplate reader. Cells treated with 0.1 and 1 nM estradiol or only with vehicle served as controls.

5.4.4. Test compound effects on the proliferation of endocrine cancer cells

Compound effects on the proliferation of Ishikawa, MDA-MD-231 and MCF-7 cells were assessed as already described,[20] with minor modifications. Briefly, MCF-7 cells were plated in 96-flat-bottom-well microplates at a density of 8000 cells per well in phenol red-free MEM supplemented with 1 μg/mL insulin and 5% DCC-FBS. Increasing concentrations (up to 10 μM) of test compounds or vehicle (DMSO to a final concentration of 0.1%) were administered to the cells 24 h after plating and relative numbers of viable cells were determined 72 h later by monitoring MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion to formazan at 550 and 690 nm (reference wavelength correcting for background absorbance). The difference in optical density at 550 and 690 nm, as determined using a Safire II plate reader, was taken to measure of the number of viable cells. Cells treated with 0.1 and 1 nM estradiol or only with vehicle served as controls.

5.4.5. Relative (to estradiol) binding affinity of test compounds for ERα and ERβ

The binding affinities of test compounds relative to that of fluorescent estradiol (relative binding affinity, RBA) for purified recombinant ERα and ERβ were assessed using fluorescence polarization assay kits (P2698 and P2700, Invitrogen) and a Beacon 2000 Fluorescence Polarization Reader (Invitrogen) as previously described.[21,22]

5.4.6. Statistics

The statistical significance of differences in cell proliferation and gene (luciferase and AlkP) expression was determined using independent samples *t*-test. Differences were considered significant for values of $p \leqslant 0.05$.

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Supplementary data

Supplementary data (NMR spectra of all compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.03.012.

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