Synthesis and Evaluation of the Antiproliferative Activities of Derivatives of Carboxyalkyl Isoflavones linked to N-t-Boc-hexylenediamine

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The isoflavones biochanin A (**1a**), genistein (**1b**), and daidzein (**4**) at concentrations >20 μM inhibit cell growth of various cancer cell lines. To enhance the antiproliferative activities of these compounds, we synthesized three analogs, 2-[3-carboxy-(6-tert-butoxycarbonylamino)-hexylamino-propyl]-7,5-dihydroxy-4′-methoxyisoflavone (**3a**), 2-[3-[N-[6-(tert-butoxycarbonyl)-aminohexyl]]-caboxamidopropyl]-5,7,4′-trihydroxyisoflavone (**3b**), and 5-{2-[3-(4-hydroxy-phenyl)-4-oxo-4*H*-chromen-7-yloxy]-acetylamino}-pentyl)-carbamic acid *tert*-butyl ester (**6**). When cancer cells expressing predominantly estrogen receptor mRNA of the β- relative to α-subtype were treated with **3a**, **3b**, or **6,** DNA synthesis was inhibited in a dose-dependent manner, ranging from 15 to 3000 nmol/L, with little inhibitory effect in normal vascular smooth muscle cells. Compound **6** was the most potent one, and its antiproliferative effect in cancer cells was modulated by estrogen and by the apoptosis inhibitor Z-VADFK. When tested in vivo, compound **6** decreased tumor volume of ovarian xenografts by 50%, with no apparent toxicity. Compound **6** may be a promising agent for therapy of cancer either alone or in combination with chemotherapeutic agents.

Introduction

Epidemiological and in vitro and in vivo animal studies indicate that isoflavones present in large quantities in soybeans and soy products (e.g., genistein, daidzein) and red clover (biochanin A) are promising agents for cancer chemoprevention and inhibition of tumor progression.[1] In estrogen-sensitive cancer cell lines (e.g., breast, colon, etc.) expressing estrogen receptor ERα, ERβ, or both, these compounds at concentrations ranging from 0.1 to 20 μM can act as weak estrogens and stimulate cell growth. The affinity of most isoflavones to the two subtypes of ER is low,[2] with the exception of genistein, which shows a strong selectivity for ERβ over ERα. Both genistein and daidzein display 100-fold greater sensitivity[3] for activating transcription in transfected cells via ERβ compared to ERα.

Interestingly, isoflavones inhibit cell growth at concentrations greater than 20 μM. The mechanisms underlying the antiproliferative effects of these compounds vary significantly among the various isoflavones and the type of cell under investigation. For example, inhibition of cell proliferation by isoflavones may involve interference with signaling via the epidermal growth factor receptor kinase,[4] effects on cell cycle,[5] caspases, or transforming growth factor β signaling.[6] Several approaches have been applied in an attempt to utilize and improve the cytotoxic potency of isoflavones. These include targeting of the EGFR receptor in breast cancer cells by using a conjugate of genistein coupled to EGF, EGF-Gen,[7] generation of synthetic derivatives of chromen-4-one complexed to copper,[8] the minimal structural motif of genistein, and reducing the ketone group at position C-4 of daidzein to yield the dihydro derivative of daidzein phenoxodiol. In in vitro studies, EGF-Gen and the synthetic derivatives of the structurally modified genistein analogue, chromen-4-one complexed to copper, exerted higher cytotoxic activity than genistein with lower IC_{50} in breast cancer[7] and pancreatic cancer cells, respectively.[9] Moreover, phenoxodiol showed strong apoptotic[10] and antiangiogenic activities[11] in ovarian carcinoma in vitro and reduced tumor volume of ovarian xenografts in vivo.[12]

In a previous study, we reported that a derivative of genistein, 6-carboxymethyl genistein, behaved like a selective estrogen receptor modulator, with unique and differential effects on the vasculature, bone, and uterus.[13] As a continuation of this work, we now report the synthesis and describe the properties of isoflavone derivatives that exhibit more potent inhibitory activity on cancer cell growth than the parent isoflavones while retaining no estrogenic activity. For this purpose, we designed a new strategy whereby carboxy alkyl chains of isoflavone derivatives are lengthened by linking them covalently to N-tert-butoxycarbonyl-1,6-diamino-hexane. In this format, the N-tert-butoxycarbonyl (N-t-Boc) moiety serves as a metabolically stable group, and the long alkyl chain on the isoflavone molecule may provide steric hindrance when and if the molecule is bound to a membrane and/or nuclear receptor. This approach was applied in the preparation of N-t-Boc derivatives of carboxy alkyl genistein (Figure 1, compound **3b**), carboxy alkyl biochanin A (Figure I, compound **3a**), and 7-(O)-carboxymethyl daidzein (Figure I, compound **6**). The N-t-Boc derivatives of carboxy alkyl isoflavones thus produced show no estrogenic activity. Moreover, unlike the parent isoflavones or the carboxy alkyl isoflavones, these N-t-Boc derivatives of carboxy alkyl isoflavones inhibited DNA synthesis in vitro in a dose-dependent manner at concentrations ranging from 15 to 3000 nmol/L in a number of cancer cell types (ovarian, colon, adrenal, and prostatic cell lines) expressing mRNAs for estrogen receptors

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Table 1. Expression of mRNA's Encoding for Estrogen Receptor (ER)α and β in Estrogen Sensitive Normal and Cancer Cell Lines

cell line	ERα/ERβ ratio
320DM (colon)	1:30
H295R (adrenal)	1:1.8
A2780	1:300
MLS (ovarian)[a]	
C4–2B (prostatic)	1:70
VSMC (normal)	2.7:1

[a] These cells express mRNA only for ERβ.

ERα and ERβ, in which ERβ was more abundant than ERα, had little effect (for instance, vascular smooth muscle cells (VSMC)).

When tested in vivo, compound **6** was capable of reducing tumor volume by >50% in mice implanted with ovarian xenografts and did not cause death or weight loss in tumor-bearing mice.

Results

Expression of Estrogen Receptor Isoforms ERα and ERβ in Cancer and Normal Cells. Cells were grown in culture and characterized in terms of the expression of mRNAs for ERα and ERβ by real-time PCR. Colon 320DM and adrenocarcinoma H295R cells expressed mRNAs for both ERs. The ERα:ERβ ratios were 1:30 and 1:1.8 in 320DM and H295R cells, respectively. Ovarian A2780 and prostatic C4–24 cells expressed mainly mRNA for ERβ, and the ERβ:ERα ratios were 300:1 and 70:1, respectively. MLS ovarian cells expressed only ERβ mRNA. Human vascular smooth muscle cells (VSMC) expressed mRNA for both ERα and ERβ, and the ERα:ERβ ratio was 2.7:1. The results are shown in Table 1.

Effect of Isoflavone Analogues on [^{3}H]-Thymidine Incorporation in Vitro in Cancer and Normal Cells. For our initial screening of the growth inhibitory activity of the various N-t-Boc derivatives of carboxyalkyl isoflavones we chose estrogen sensitive cancer cell lines (see Table 1) expressing mRNAs for ERα and ERβ in which ERβ expression was more pronounced than ERα. Treatment of the cancer cells by 30 nM estradiol 17β (E2) stimulated DNA synthesis by 139−210%. On the other hand, E2 at 30 nM inhibited DNA synthesis in normal vascular smooth muscle cells (VSMC) (Table 2), whereas E2 in these cells stimulated DNA synthesis at 3 nM (data not shown, see ref 14).

The N-tBoc derivatives of the carboxy alkyl isoflavones (Figure 1, compounds **3a**, **3b**, and **6**) were first screened for their growth inhibitory activity using a single concentration of 3 μM (see Table 2). When all three derivatives were compared in this setting, the N-t-Boc derivative of carboxy alkyl daidzein, compound **6**, was the most potent inhibitor of growth as assessed by inhibition of DNA synthesis in these cells. Because the colon 320DM cancer cells were sensitive to the growth inhibitory effects of N-t-Boc derivatives of carboxyalkyl isoflavones (compounds **3a**, **3b**, and **6**), we compared the effects on DNA synthesis of the N-t-Boc derivative of carboxyalkyl biochanin (**3a**) and the N-t-Boc derivative of carboxyalkyl genistein (**3b**) to the carboxy alkyl analogues (compounds **2a** and **2b**) and the parent compounds (**1a** and **1b**).

Both genistein (**1b**; 30−3000 nM) and the 2-carboxy alkyl derivative of genistein (30−3000 nM; Figure 1, compound **2b**) significantly stimulated [^{3}H]-thymidine incorporation into DNA (Figure 2). On the other hand the N-t-Boc derivative of carboxy alkyl genistein (**3b**; 30−3000 nM; Figure 1, compound **3b**) elicited a significant growth inhibitory effect in these cells (Figure 2).

In the same cells, biochanin A (**1a**; 30−3000 nM) had no effect on growth. The carboxyalkyl derivative of biochanin A (30−3000 nM; Figure 1, compound **2a**) stimulated the DNA synthesis, whereas its N-t-Boc derivative (compound **3a**; 30−3000 nM) significantly inhibited DNA synthesis in these cells.

The most potent analog of the N-t-Boc isoflavone derivatives, compound **6** (15−6000 nM), induced dose-dependent decreases in [^{3}H]-thymidine incorporation into DNA in 320DM and MLS ovarian cells (Figure 3). A plateau of inhibition of DNA synthesis was reached after 72 h of incubation in both cell types (data not shown). In these cells, daidzein (**4**) and the carboxymethyl derivative (**5**) at a 3 μM concentration increased DNA synthesis by 155−259% (data not shown).

Effect of Estrogen Antagonists on the Antiproliferative Activities of Compound 6 in Colon and Ovarian Cancer Cells. When MLS (ovarian cancer) or 320DM (colon cancer) cells were exposed to 30 nM estradiol, [^{3}H]-thymidine incorporation was significantly increased ($p < 0.01$) (Table 3). The stimulatory effect on DNA synthesis by estradiol (30 nM) in these cells was suppressed to basal levels in the presence of 0.3 μM **6** or the "absolute" ER inhibitor ICI 182780 (at 1.1 μM). However, when a 10-fold higher concentration of estradiol (300 nM), which similarly increased DNA synthesis ($p < 0.01$) in these cells, was used, compound **6** (0.3 μM) was unable to suppress the proliferative effect of estradiol (see Table 3).

Effect of General Apoptosis Inhibitor on the Antiproliferative Activities of Compound 6 in Colon and Ovarian Cancer Cells. In these experiments, we studied the effect on DNA synthesis of the combination of compound **6** with the general apoptosis inhibitor Z-VAD-FMK Me ester (Z-VADFK) in ovarian MLS and colon 320DM cancer cells. Under basal conditions, Z-VADFK alone (25 μM) had no effect on DNA synthesis in these cells, whereas **6** at 0.3 μM inhibited DNA synthesis by 26−32%. However, when the cells were exposed to **6** (0.3 μM) in the presence of Z-VADFK (25 μM), the inhibitory effect of **6** on ^{3}[H]-thymidine uptake was not discernible. When the DNA synthesis in these cells was 90% inhibited by a 10-fold higher concentration of **6** (3 μM), the general apoptosis inhibitor (Z-VADFK, 25μM) was capable of partially reversing the inhibitory effect of **6** on DNA synthesis (Table 4).

Effect of the N-t-Boc Derivative of 7-(O)-Carboxymethyldaidzein (6) In vivo in Mice Bearing MLS Xenografts. Mice bearing the MLS xenografts were IV injected every second day for 10 days with **6** (0.4 mg per injection) or vehicle (Neowater). Tumor growth in the groups receiving **6** was inhibited by >50% as compared to the tumor volume of untreated mice (Figure 4). No weight reduction or death of mice was seen in mice treated with **6.**

Discussion

The results of this study indicate that the N-t-Boc derivatives of the carboxy alkyl isoflavones, daidzein (**6**), biochanin A (**3a**), and genistein (**3b**), exhibit significant (50−90%) antiproliferative effects in vitro in a variety of estrogen-sensitive cancer cell lines (colon, adrenal, ovarian, prostatic) at a concentration range from 30 to 3000 nM. It is noteworthy that the antiproliferative effect of these compounds is more pronounced in cancer cells that preferentially express mRNA for ERβ relative to ERα. In addition, these compounds, rather distinctly at variance with their estrogenic parental compounds, did not possess estrogenic activity and showed only moderate (20−35%) antiproliferative

Table 2. Inhibition of DNA synthesis[a] by Compounds **3a**, **3b**, **6**, and estrogen (**E2**) in 320DM Colon, MLS and A2780 Ovarian, H295R Adrenal, and C4–2B Prostatic Cancer Cell Lines and Normal VSMC Cells (results are means ± SD of 8–16 incubates from 2−4 experiments and are expressed as the ratio between experimental and control (E/C) DNA synthesis in these cells)

	cells					
compd	320DM	MLS	A2780	H295R	C4–2B	VSMC
3a	0.22 ± 0.15	0.05 ± 0.23	0.64 ± 0.16	0.55 ± 0.03	0.41 ± 0.27	0.74 ± 0.05
3b	0.2 ± 0.09	N.D.	1.12 ± 0.19	0.6 ± 0.14	0.75 ± 0.07	0.78 ± 0.1
6	0.08 ± 0.01	0.03 ± 0.07	0.1 ± 0.15	0.08 ± 0.21	0.28 ± 0.2	0.73 ± 0.13
E2	1.64 ± 0.13	1.39. ± 0.03	1.42 ± 0.1	2.10 ± .10	2.10 ± 0.10	0.55 ± 0.12

[a] Compounds **3a**, **3b**, and **6** were used at 0.3 μM in the prostatic cell line C4–2B and at 3μM in all other cells. E2 was used at 30 nM throughout. N. D.: not determined.

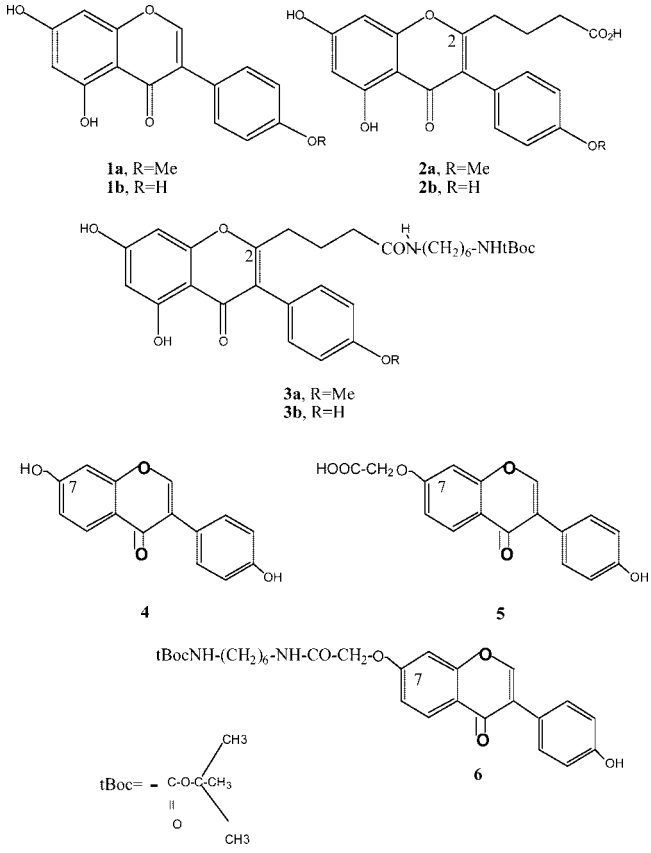

Figure 1. Schematic structures of isoflavones (**1a**, **1b**, and **4**), carboxyalkyl isoflavones (**2a**, **2b**, and **5**), and the N-t-Boc derivatives of carboxyalkyl isoflavones (**3a**, **3b**, and **6**).

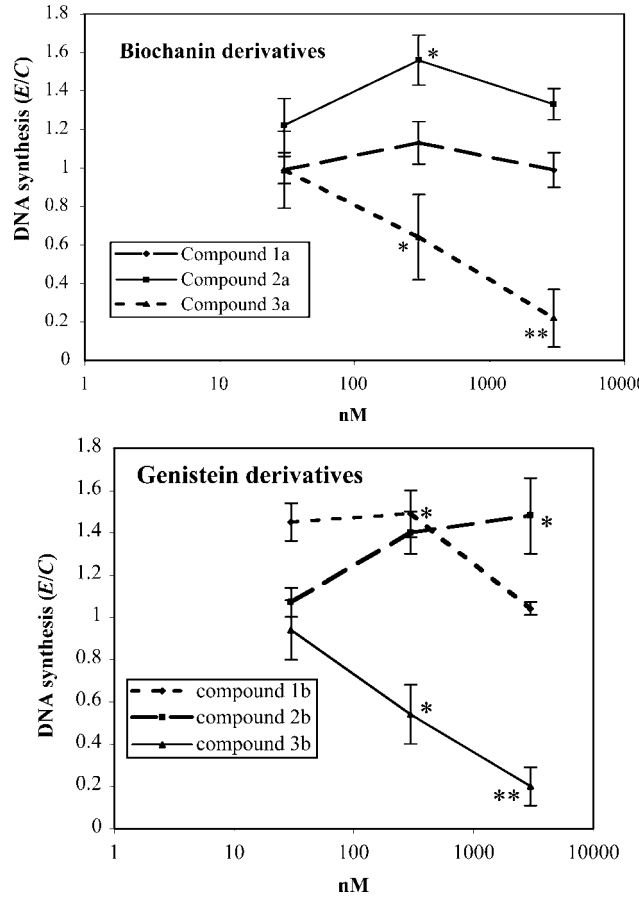

Figure 2. Effect of isoflavone derivatives on DNA synthesis in 320DM colon cancer cells. Results are means ± SD of 8–16 incubates from 2−4 experiments and are expressed as the ratio between experimental and control (E/C) DNA synthesis in these cells.

effects in normal cells (e.g., vascular smooth muscle cells) at concentration ranging from >3μM.

Because compound **6** showed growth inhibitory activities in all the cancer cell lines that we tested, it was chosen for further studies. In ovarian and colon cancer cell lines, the antiproliferative effects of compound **6** are apparently mediated via both ER-dependent and ER-independent mechanisms (see Tables 3 and 4). In these cell lines, the antiestrogenic effects and the antimitotic effect of **6** are operative, at least in part, through induction of apoptosis. When tested in vivo, compound **6** was capable of reducing tumor volume by >50% as compared to control tumor-bearing mice. Moreover, treatment of ovarian xenografts bearing mice with **6** did not cause weight loss or death of mice, suggesting that **6** was not toxic.

Ovarian cancer is often discovered in advanced stages, resulting in a five year survival rate of 20%. Although chemotherapeutic agents are widely used for the treatment of ovarian cancer, chemoresistance remains a major problem. Compound **6** may offer a novel approach to the treatment of ovarian cancer either alone or in combination with cytotoxic drugs. Interestingly, phenoxodiol, a derivative of daidzein, shows promising results in the treatment of ovarian cancer.[12]

In addition, compound **6** showed potent anticancer properties in vitro in H295R adrenocarcinoma and colon 320DM cancer cells (see Table 2). No effective treatment exists for human adrenal cancer at the present time, and colorectal cancer is one of the most common cancers, often showing poor or partial response to available chemotherapeutic agents. The efficacy of **6** in these cancer types in vitro holds promise for its potential use in vivo.

Experimental Section

Reagents. All reagents were of analytical grade. Chemicals [N-(tert-butoxycarbonyl)-1,6-diamino-hexane, carbodiimide, etc.) were purchased from Sigma (St. Louis, MO). ICI 182780 was purchased from Tokris (Shirehumpton, Bristol, U.K.). Hydroxybenzotriazole was purchased from MP Biomedicals (Aurora, OH). The apoptosis inhibitor Z-VADFMK Me ester was obtained from

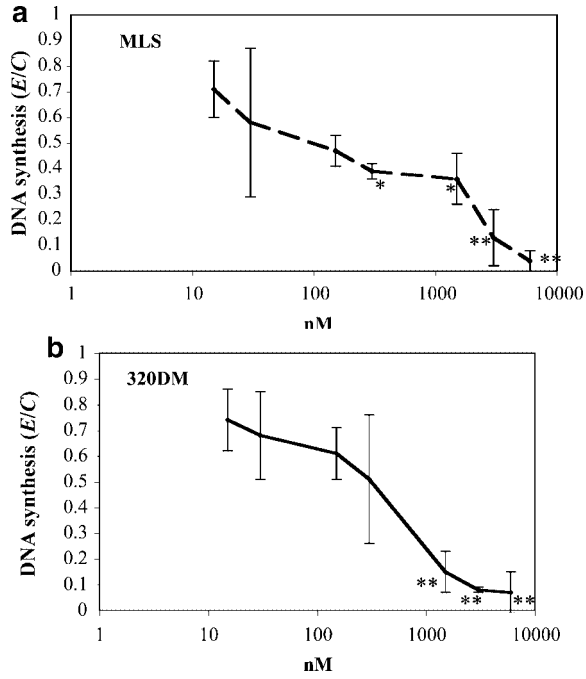

Figure 3. Dose-independent inhibition of DNA synthesis after treatment with compound **6** for 48 h (a) ovarian MLS and colon (b) 320DM cancer cells. Results are means ± SD of 8–16 incubates from 2−4 experiments and are expressed as the ratio between experimental and control (E/C) DNA synthesis in these cells.

Table 3. Modulation of the Inhibitory Effect by Compound **6** on DNA Synthesis in MLS Ovarian and 320DM Colon Cancer Cells by Antiestrogens (results are means ± SD of 8–16 incubates from 2−4 experiments and are expressed as the ratio between experimental and control (E/C) DNA synthesis in these cells)

	cells	
compd	MLS	320DM
none (control)	1 ± 0.15	1 ± 0.12
E2 (30 nM)	2.35 ± 0.06[b]	2.06 ± 0.09[b]
E2 (300 nM)	1.70 ± 0.15[a]	1.87 ± 0.18[b]
6 (300 nM)	0.61 ± 0.10[a]	0.63 ± 0.11[b]
6 + E2 (30 nM)	0.96 ± 0.24	1.08 ± 0.16
ICI 182780 (1.1 μM)	0.52 ± 0.12[b]	0.63 ± 0.08[b]
ICI 182780+ E2 (30 nM)	0.53 ± 0.11	0.63 ± 0.08[b]
ICI 182780+ E2 (300 nM)	0.95 ± 0.08	0.92 ± 0.18

[a] $p < 0.05$. [b] $p < 0.01$.

Table 4. Modulation of the Inhibitory Effect by Compound **6** on DNA Synthesis in MLS Ovarian and 320DM Colon Cancer Cells by the General Apoptosis Inhibitor Z-VADFK (results are means ± SD of 8–16 incubates from 2−4 experiments and are expressed as the ratio between experimental and control (E/C) DNA synthesis in these cells)

	cells	
treatment	MLS	320DM
control	1 ± 0.07	1 ± 0.16
Z-VADFK (25 μM)	1.06 ± 0.1	1.11 ± 0.08
6 (300 nM)	0.74 ± 0.11[b]	0.68 ± 0.14[b]
6 (300 nM) + Z-VADFK	1.32 ± 0.26	1.18 ± 0.14
6 (3 μM)	0.44 ± 0.14[a,b]	0.04 ± 0.22[a,b]
6 (3 μM) + Z-VADFK	0.76 ± 0.16	0.5 ± 0.11[b]

[a] $p < 0.05$. [b] <0.01.

Axxora (San Diego, CA); Neowater solubilization enhancer was obtained from Do-Coop Technology (Or-Yehuda, Israel). Methyl-[^{3}H]-thymidine (5 Ci/mmole)) was obtained from New England Nuclear (Boston, MA).

Cells. Gastric (320DM), ovarian A2780, and H295R adrenocarcinoma cancer cells were from ATTC (Rockville, MD) and grown according to the instructions of ATTC. Prostatic (*C*2–4B) and

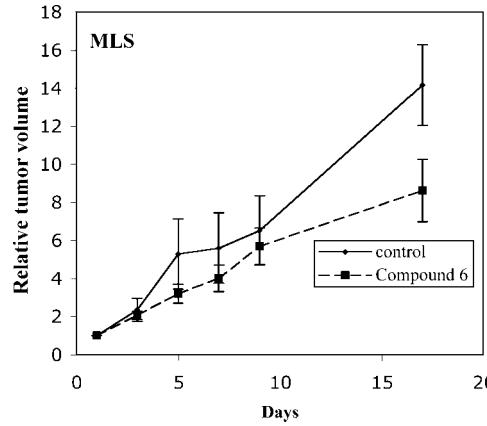

Figure 4. Effect of **6** on tumor growth of ovarian MLS xenografts. Tumor volumes were calculated using the formula length × width × height × $\pi/6$ and referenced to the values on the first day of treatment (relative tumor volume). Results are given as means ± SEM.

ovarian cancer cells MLS were obtained through the generosity of Prof. M.C. Farach-Carson, University of Delaware, and Prof. M. Neeman, Weizmann Institute, Rehovot, Israel, respectively. Cells were cultured in a MEM supplemented with 10% FCS and antibiotics. Cells were grown to subconfluence and then treated with various hormones or agents as indicated.

Human umbilical vascular smooth muscle cells (VSMC) sensitive to estrogen served as the normal cells. These cells were grown in culture and used only at passages 1–3 when expression of smooth muscle actin was clearly demonstrable.[14]

Preparation of Total RNA. Total RNA from cancer and normal cells was extracted using the TRIzol reagent (Gibco Life Technologies) according to the manufacturer's instructions.

RT-PCR. Total RNA (1 μg) was subjected to reverse transcription using the BD Advantage one-step RT for PCR kit from BD Biosciences Clontech (Palo Alto, CA). For ERα, we used 5 μL of cDNA in the reaction mixture with the primers 5′ AATTCTGACAATCGACGCCAG 3′ (forward) and 5′ GTGCTTCAACATTCTCCCTCCTC 3′ (reverse), for 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. For ERβ, the same amount of cDNA was used with the primers 5′TGCTTTGGTTTGGGTGATTGC3′(forward) and TTTGCTTTTACTGTCCTCTGC 3′ (reverse) for 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. Plasmids pRST7-ERα and pRST7ERβ (kindly provided by Dr. D. McDonnell, Durham, NC) served as positive controls in these reactions.

Assessment of DNA Synthesis. Cells were grown until subconfluence using conditions previously described[14] and then treated with various hormones or agents for 48 h as indicated. At the end of incubation, [^{3}H]-thymidine was added for 2 h. Cells were then treated with 10% ice-cold trichloroacetic (TCA) for 5 min and washed twice with 5% TCA and then with cold ethanol. The cellular layer was dissolved in 0.3 mL of 0.3 M NaOH, aliquots were taken for counting radioactivity, and [^{3}H]-thymidine incorporation into DNA was calculated.

Statistical Analysis. The significance of differences between the mean values obtained from experimental groups and controls were evaluated by analysis of variance (ANOVA).

Synthesis of Carboxy Alkyl Isoflavones Conjugated to N-t-Boc-1,6-hexylene diamine. The N-t-Boc derivatives of carboxy alkyl isoflavones (Figure 1, compounds **3a**, **3b**, and **6**) were prepared in two steps. In the first step, carboxy alkyl derivatives of the isoflavones (Figure I, compounds **2a**, **2b**, and **5**) were synthesized using established procedures. In the second step, the carboxy alkyl isoflavones were covalently linked to N-(tert-butoxycarbonyl)-1,6-diamino-hexane. The synthetic procedures were as follows:

2-(3-Carboxypropyl)-7,5-dihydroxy-4′-methoxyisoflavone (**2a**). 2-[3-(Ethoxycarbonyl)propyl]-7,5-dihydroxy-4′-methoxyisoflavone (350 mg, 0.88 mmol), prepared according to a previous

procedure,[15] was dissolved in a mixture of EtOH (35 mL) and 10% aq. NaOH (35 mL). The solution was stirred at room temperature for 24 h. The organic solvent was then evaporated and H_2O (35 mL) was added. The mixture was cooled in an ice–water bath and the pH was adjusted to 4 with conc. HCl. The resulting mixture was left overnight in the refrigerator to complete the precipitation of the product. The solid was collected by filtration, washed with cold water, and dried under a vacuum. The resulting solid was dissolved in a small amount of EtOAc and evaporated almost completely, leaving a solid and a very small amount of EtOAc, which was removed with a pipet, carrying with it some of the impurities. The resulting pinkish solid was dried under a vacuum (170 mg, 52%) and identified as 2-(3-carboxypropyl)-7,5-dihydroxy-4′-methoxyisoflavone (**2a,** Figure 1). ^{1}H NMR (250 MHz, acetone-d_6) δ 1.98 (m, 2H), 2.33 (t, 2H, J = 7.2 Hz), 2.65 (t, 2H, J = 7.3 Hz), 6.25 (d, 1H, J = 2.1 Hz), 6.43 (d, 1H, J = 2.1 Hz), 7.00 (d, 2H, 6.8 Hz), 7.25 (d, 2H, 6.8 Hz). ESIMS (ES+) m/z 393.53 ([M + Na]$^+$, 80), 371.51 ([M + H]$^+$, 60); (ES−) m/z 369.37 ([M − H]$^-$, 100), 739.69 ([2M − H]$^-$, 25).

2-[3-Carboxy-(6-tert-butoxycarbonylamino)-hexylamino-propyl]-7,5-dihydroxy-4′-methoxyisoflavone (**3a**). Compound **2a** (50 mg, 0.14 mmol) was dissolved under N_2 in a mixture of dry Cl_2CH_2 (1 mL) and dry DMF (0.25 mL). To this mixture was added hydroxybenzotriazole (26 mg, 0.17 mmol) followed by DCC (98 mg, 0.47 mmol), and the mixture was stirred for 30 min. After this time, N-(tert-butoxycarbonyl)hexylenediamine (37 mg, 0.15 mmol) was added, followed by TEA (21 μL, 0.46 mmol). The mixture was flushed with N_2, sealed, and stirred overnight at room temperature. The solid was filtered off and the filtrate was diluted with Cl_2CH_2 (20 mL) and washed with 0.1 N HCl (2 × 3 mL), H_2O (1 × 3 mL), and brine (2 × 3 mL). The organic layer was dried over Na_2SO_4 and evaporated, leaving a thick yellow oil that was purified by column chromatography using a gradient from 8:1 to 8:2 Cl_3CH:acetone. The N-t-Boc derivative of carboxy alkyl biochanin A (**3a**, Figure 1) was isolated as a colorless oil (50 mg, 66%). ^{1}H NMR (250 MHz, acetone-d6) δ 1.19–1.43 (m, 8H), 1.38 (s, 9H), 2.01 (m, 2H), 2.14 (m, 2H), 2.59 (t, 2H, J = 7.5 Hz), 2.85–3.11 (m, 4H) 3.84 (s, 3H), 6.24 (d, 1H, J = 2.1 Hz), 6.42 (d, 1H, J = 2.1 Hz), 6.99 (d, 2H, J = 8.8 Hz), 7.24 (d, 2H, J = 8.8 Hz). The purity of **3a** as checked by HPLC was 98.40% (see the Supporting Information).

2-(3-Carboxypropyl)-5,7,4′-trihydroxyisoflavone (**2b**). To a solution of 2-[3-(ethoxycarbonyl)propyl]-7,5-dihydroxy-4′-methoxyisoflavone (104 mg, 0.26 mmol), prepared as described[15] in anhydrous Cl_2CH_2 (2 mL) at −78 °C under nitrogen was slowly added 1.0 M BBr_3 in Cl_2CH_2 (2.1 mL, 2.1 mmol). The mixture was allowed to warm gradually to room temperature overnight. The mixture was then cooled in an ice bath and anhydrous MeOH (2 mL) was added slowly. The solvents were evaporated under reduced pressure, leaving a brown solid that was purified by column chromatography on silica gel (gradient elution, 8:2 Cl_2CH_2 to Cl_2CH_2/acetone 8:2) affording 2-[3-(methoxycarbonyl)propyl]-5,7,4′-trihydroxyisoflavone as a solid (72 mg, 75%). ^{1}H NMR (acetone-d_6) δ 1.99 (quintet, 2H, J = 7.3 Hz, H-2″), 2.34 (t, 2H, J = 7.3 Hz, H-3″), 2.64 (t, 2H, J = 7.3 Hz, H-1″), 3.55 (s, 3H, CO_2CH_3), 6.24 (d, 1H, J = 2 Hz, H-6), 6.41 (d, 1H, J = 2 Hz, H-8), 6.90 (d, 2H, J = 8.5 Hz, H-3′, H-5′), 7.12 (d, 2H, J = 8.5 Hz, H-2′, H-6′).

In the second step of the reaction, 10% aq. NaOH (7 mL) was added to a solution of 2-[3-(methoxycarbonyl)propyl]-5,7,4′-trihydroxyisoflavone (70 mg, 0.189 mmol) in EtOH (7 mL) and the mixture was stirred for 24 h at room temperature. The EtOH was removed under reduced pressure and H_2O (7 mL) was added. The solution was acidified to pH 3.5 with 35% HCl and the mixture was left overnight at 4 °C. The solid identified as 2-(3-carboxypropyl)-5,7,4′-trihydroxyisoflavone (**2b**, Figure 1) was collected by filtration, washed with cold water, and dried under a vacuum (53 mg, 79%). ^{1}H NMR (acetone-d_6) δ 1.98 (quintet, 2H, J = 7.3 Hz, H-2″), 2.34 (t, 2H, J = 7.3 Hz, H-3″), 2.65 (t, 2H, J = 7.3 Hz, H-1″), 6.24 (d, 1H, J = 2.1 Hz, H-6), 6.42 (d, 1H, J = 2.1 Hz, H-8), 6.90 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 7.13 (d, 2H, J = 8.6 Hz, H-2′, H-6′).

2-[3-[N-[6-(tert-Butoxycarbonyl)-aminohexyl]]-caboxamidopropyl]-5,7,4′-trihydroxyisoflavone (**3b**). A solution of 2-(3-carboxypropyl)-5,7,4′-trihydroxyisoflavone (**2b**; 25 mg, 0.07 mmol) in dry Cl_2CH_2 (0.51 mL) and dry DMF (0.13 mL) under nitrogen was treated with 1-Hydroxybenzotriazole hydrate (HOBt) (13.5 mg, 0.088 mmol) followed by *N*,*N*′-dicyclohexylcarbodiimide (DCC) (50.81 mg, 0.25 mmol). The flask was sealed, and the mixture was stirred at room temperature for 30 min. t-N-Boc-1,6-hexanediamine hydrochloride (19.2 mg, 0.076 mmol) was added followed by triethylamine (11 μL, 0.079 mL). The flask was flushed with nitrogen and sealed, and the mixture was stirred overnight. The solid was filtered off and the filtrate was diluted with Cl_2CH_2 (20 mL) and washed with 0.1 M HCl (2 × 1.6 mL), H_2O (1.6 mL), and brine (2 × 1.6 mL); it was then dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (gradient elution, 8:1 Cl_2CH_2:acetone to 7:3 Cl_2CH_2:acetone), affording compound **3b** (Figure 1; N-t-Boc derivative of carboxy alkyl genistein) as a white foam (30 mg, 77%). ^{1}H NMR (acetone-d_6) δ 1.1–1.5 (m, 8H, 4× CH_2), 1.40 (s, 9H, *t*-Bu), 1.99 (m, 2H, H-2″), 2.05 (m, 2H, H-3″), 2.60 (t, 2H, J = 7.1, H-1″), 2.95 (m, 4H, 2× CH_2-N), 6.23 (d, 1H, J = 2.1 Hz, H-6), 6.41 (d, 1H, J = 2.1 Hz, H-8), 6.90 (d, 2H, J = 8.6 Hz, H-3′, H-5′), 7.13 (d, 2H, J = 8.6 Hz, H-2′, H-6′). MS (ES+) m/z (%) 577 [(M$^+$ + Na), 100], 1131 [(2M$^+$ + Na), 27]; MS (ES−) m/z (%) 553 [(M$^+$ − H), 100], 1108 [(2M$^+$ − H), 27]. The purity of **3b** as checked by HPLC was 98.91 (see the Supporting Information).

5-{2-[3-(4-Hydroxy-phenyl)-4-oxo-4H-chromen-7-yloxy]-acetyl-amino}-pentyl)-carbamic acid tert-butyl ester (**6**). 7-(O)-Carboxymethyl daidzein) (**5,** Figure 1)), prepared as described elsewhere,[16] (117 mg, 0.37 mmol) was dissolved in dry dichloromethane (2.8 mL) under N_2. Dry DMF (1.2 mL) was added followed by DCC (272 mg, 1.32 mmol) and HOBt (73 mg, 0.48 mmol). The flask was flushed with N_2, sealed, and stirred at room temperature. After 1 h, N-tBoc-1,6-hexanediamine hydrochloride (103 mg, 0.41 mmol) was added followed by triethylamine (0.06 mL, 43.5 mg, 0.43 mmol). The flask was flushed with N_2, sealed, and stirred overnight at room temperature. The solid was removed by filtration and the filtrate was diluted with dichloromethane (55 mL); washed with 0.1 M HCl (2 × 9 mL), H_2O (1 × 9 mL), brine (2 × 9 mL); and dried over Na_2SO_4. The solvents were evaporated under reduced pressure, leaving a residue that was purified by column chromatography on silica gel using a gradient of 8:1 to 8:2 dichloromethane: acetone. Compound **6** (Figure 1), the N-t-Boc derivative of carboxy alkyl daidzein, was isolated as a colorless oil (90 mg, 48%). ^{1}H NMR (250 MHz, Cl_3CD) δ 1.20–1.80 (m, 8H, 4× CH_2), 3.10 (m, 2H, CH_2−N), 3.40 (m, 2H, CH_2−N), 4.60 (s, 2H), 6.85 (d, 1H, J = 2.1 Hz), 6.90 (AA′BB′, 2H), 7.05 (dd, 1H, J = 8.3 Hz, J = 2.1 Hz), 7.45 (AA′BB′, 2H), 7.95 (s, 1H), 8.25 (d, 1H, J = 8.3 Hz). MS (ES+) m/z (%) 533 [(M + Na$^+$), 100], 1044 [(2M + Na$^+$), 35]; MS (ES−) m/z (%) 545 [(M − H) + Cl$^-$adduct, 100]. The purity of **6** as determined by HPLC was 97.70% (see the Supporting Infomation).

Xenograft Studies. Female CD1 nude mice (8 weeks old) were inoculated sc with MLS ovarian carcinoma cells (1.5 × 10^6/mouse). Ten days later when the tumors were palpable, the mice were randomized into 8−10 mice per group. The various groups were IV treated every other day with compound **6** (0.4 mg/mouse in 0.1 mL of vehicle) or vehicle (Neowater). During treatment, body weight was recorded to monitor toxicity of the treatment, and the tumors were measured with an external caliper. Tumor volume was calculated using the formula length × width × height × π/6. Statistical significance was assessed using student's t test, and differences were considered significant at $p < 0.05$. All animals were handled according to the policies set by the Veterinary Animal Services, Weizmann Institite of Science, and all procedures were approved by the Institutional Animal Care and Use Committee, Weizmann Institute of Science.

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Supporting Information Available: Table of analytical data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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