Journal of Steroid Biochemistry & Molecular Biology 126 (2011) 95–103

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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

Anti-thyroid cancer properties of a novel isoflavone derivative, 7-(O)-carboxymethyl daidzein conjugated to N-t-Boc-hexylenediamine *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 11 May 2010
Received in revised form 24 April 2011
Accepted 26 April 2011

Keywords:
Thyroid carcinoma
Estradiol
Isoflavone derivative
Apoptosis
Estrogen receptor

ABSTRACT

The incidence of thyroid cancer is up to 3 folds higher in women than in men, suggesting that estrogenic effects may be involved in the pathogenesis of this malignancy. Here, we explore whether or not human thyroid cancer cell growth can be curbed by a novel isoflavone derivative generated in our laboratory, the N-t-Boc-hexylenediamine derivative of 7-(O)-carboxymethyl daidzein (cD-tboc). With the exception of the follicular cancer cell line WRO, estrogen receptor (ER)α mRNA was only marginally expressed in cell lines derived from papillary (NPA), follicular (MRO), anaplastic thyroid carcinoma (ARO) such that the expression of estrogen receptor (ER) βmRNA was more abundant than that of ERα mRNA in these cell types. Estradiol-17β (E2; 0.03–300 nmol/l) *per se* increased proliferation in all four cell-types. The ERβ-specific agonist DPN increased [^{3}H]-thymidine incorporation in all four thyroid cancer cell lines, whereas the ERα-specific agonist PPT increased growth only in NPA and WRO. By contrast, cD-tboc, derived from the weak estrogen daidzein, did not cause cell growth and dose-dependently diminished cell growth in all four cell lines via apoptosis and not necrosis, as detected by the release of histone-DNA fragments. The cytotoxic growth inhibitory effect of cD-tboc in these cells was modulated by E2 and the general caspase inhibitor Z-VAD-FMK, and the magnitude of this salvage was cell type-and dose-dependent. When nude mice carrying ARO thyroid xenografts were treated with cD-tboc, tumor volume decreased significantly, and no apparent toxicity was observed. These results suggest that cD-tboc may be a promising agent for therapy of thyroid carcinoma either alone or in combination with existing cytotoxic drugs.

1. Introduction

The prevalence of thyroid cancer is on the rise and is 2–3 folds higher in women than in men, suggesting that estrogenic effects may be involved in the pathogenesis of this malignancy [1–4]. Furthermore, there is an age related, gender-dependent difference in the rate of thyroid cancer which is highly consistent with a role for endogenous estrogens in thyroid cancer, as female to male incidence rates are roughly equal in children, increasing sharply to ~3 by puberty. This gap is maintained until menopause, declining thereof gradually to ~1.5 by the age of 65 [3–5]. Indeed, older men appear to be more susceptible to aggressive thyroid cancer than age-matched women [6]. Tested in a number of studies, contraceptives or hormone replacement therapy appear not to confer increased risk for differentiated thyroid cancer [7]. However, other exogenous estrogen-mimetic compounds may still play a modulating role in human thyroid cancer growth: in a multiethnic population-based case-control study of thyroid cancer conducted in the San Francisco Bay Area, consumption of phytoestrogen-rich soy-based foods and alfalfa sprouts was associated with reduced risk of thyroid cancer. In particular, the isoflavones daidzein and genistein and the ligand, secoisolariciresinol appeared to confer significant reduction in the risk for thyroid cancer [8]. Apart from these

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[1] The conduction of this study is in partial fulfillment of the requirements for a PhD thesis of Meital Grafi-Cohen at the Sackler Faculty of Medicine, Tel Aviv University, Israel.

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doi:10.1016/j.jsbmb.2011.04.009

putative effects of circulating endogenous estrogens and exogenous food-derived estrogenic modulators, the thyroid gland itself may be capable of converting androgens to estrogens, as suggested by the expression of aromatase mRNA and protein in cancerous and non-cancerous human thyroid tissue [9]. Intra-thyroidal aromatization is potentially possible not only through the utilization of circulating testosterone and androstenedione, but also of the much more abundant dehydroepiandrosterone (DHEA) and its sulfated adrenal derivative DHEA-S. Because steroid sulfatase activity appears to be widespread in primate tissues [10], thyroid aromatase can potentially generate estradiol-17β (E2) from testosterone which is indirectly derived from sulfated dehydroepiandrosterone (DHEA-S). Finally, both normal and tumorous thyroid tissue express estrogen receptors [11–16]. Although both major isoforms of the estrogen receptor (ER), ERα and ERβ are reportedly expressed in thyroid cancer cells/tissue [17–19], their functional role in cell growth presently remains a matter of debate. Several types of thyroid cancer cell lines were formerly shown to display a clear growth response to E2, although, at the present time, the proposed mechanisms through which these effects are exerted are inconsistent among the various reports [20–22]. Based on experimental model in Pten−/− mice, a recent study suggests that ERα activation contributes to the increased susceptibility of females to thyroid proliferative disorders and neoplastic transformation partially via estrogenic control of p27 degradation [23]. In contrast, earlier observations that tamoxifen antagonizes proliferation and of ER-negative metastatic follicular and anaplastic thyroid cancer cells [24–26] suggested that estrogen receptor modulators can exert non-ERα or non-classical ER-mediated effects in thyroid-derived cancer cells. Our group has ongoing interest in the potential utilization of modified isoflavones as negative modulators of cancer cell survival. This interest originally stemmed from observations that the isoflavones biochanin A, genistein and daidzein at concentrations ranging from 0.1 to 20 μmol/l can act as weak estrogens and stimulate cell growth and at concentrations > 20 μmol/l inhibit cell growth of various cancer cell lines [27]. We reasoned that possibly, further modification of one or more of these common plant-derived compounds can result in agents with yet greater cytotoxic effect in cancer. Initially, 6-carboxymethyl-derivative of genistein was shown to have selective-estrogen receptor modulator (SERM) like activities [27,28] and display mixed estrogen-mimetic and estrogen antagonist effects in normal vascular, bone and uterine cells and in breast cancer MCF-7 cells. More recently, in an attempt to enhance the antiproliferative activities of these compounds, a new strategy has been applied by which 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. Using this general approach, several analogs of the original synthetic carboxymethyl derivatives were generated, one of which, 5-(27)-pentyl-carbamic acid tert-butyl ester (N-t-Boc-7-(O)-carboxymethyl daidzein [cD-tboc]), showed particular promise in terms of cytotoxicity in colon and ovarian cancer cell lines. Moreover, cD-tboc showed anti-estrogenic activity as it suppressed (at 0.3 μmol/l) the stimulatory effect on DNA synthesis exerted in these cells by estradiol-17β (E2; 30 nmol/l). One particularly appealing property of this compound was that it had little inhibitory effect in normal vascular smooth muscle cells and appeared harmless in preliminary *in vivo* experiments, in terms of animal weight and survival [27]. In the present study we assessed the response of four established human thyroid cancer cell lines to E2 and agonists and examined the possibility that cD-tboc may act as an anti-cancer agent on human thyroid cancer cells *in vitro* and *in vivo* in tumor xenografts.

2. Materials and methods

2.1. Reagents and chemicals

All reagents were of analytical grade. Chemicals, steroids and PPT were purchased from Sigma (St. Louis, MI). DPN was purchased from Tocris Bioscience (Bristol, UK). ICI was purchased from Tokris biochemicals. Methyl-[^{3}H]-thymidine (5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Anti-Pax 8 (S-20), anti TTF-1 (F-12) and blocking peptide were from Santa-Cruz (Santa-Cruz, CA). Donkey anti-rabbit peroxidase and ECL reagents from Amersham (GE Healthcare, Buckinghamshire, UK). 5-(25)-Pentyl-carbamic acid tert-butyl ester (N-t-Boc-7-(O)-carboxymethyl daidzein [cD-tboc]) was prepared as described [27].

2.2. Cells

We used four well-established and extensively studied human thyroid carcinoma cell lines, kindly provided by Dr. G.J.F. Juillard (University of California at Los Angeles, Los Angeles, CA): MRO 87-1 and WRO (follicular); NPA (papillary); and ARO 81-1 (anaplastic). These cell lines have TSH receptors but may have post-receptor defects [29,30]. Cells were cultured in RPMI-1640 medium, 10% charcoal-stripped FCS and antibiotics, grown to sub-confluence and then treated with various hormones or agents as indicated. Regarding the RT-PCR amplification of TTF-1 and PAX8, the method of preparation of benign human thyroid cells from tissue obtained at thyroidectomy from patients with benign colloid nodules, has been previously described in detail [30,31].

2.3. Preparation of total RNA

Total RNA from cancer cells was extracted using the TRIzol reagent (Gibco Life Technologies, Invitogen, Paisley, UK), according to the manufacturer's instructions and then subjected to reverse transcription (RT) using the Advantage RT for polymerase chain reaction (PCR) kit (Clonetech, Mountainview, CA, USA). Extracted RNA was then reverse transcribed using the RevertAid^{TM} first Strand cDNA synthesis kit from Fermentas Life Science (St. Leon-Rot, Germany).

2.4. Semi-quantitative RT PCR amplification of TTF-1 and PAX8

Semi-quantitative RT PCR amplification of TTF-1and PAX8 was performed as previously described [28]. Oligonucleotide primers for human TTF-1 and PAX8 mRNA amplification were as follows: TTF-1 5′ (sense) ATGTCGATGAGTCCAAAGCACA and TTF-1 3′ (antisense) ACCTGCGCCTGCGAGAAGAGCA; PAX8 5′ (sense) GGGGACTACAAACGCCAGAAC, and PAX8 3′ (antisense) CGGAGCTAGATAAAGAGGAAG; cyclophillin 5′ (sense) TCCTAAAG-CATACGGGTCCTGGCAT, and cyclophillin 3′ (antisense) CGCTC-CATGGCCTCCACAATATTCA. The expected human TTF-1 product from a cDNA template is 519 bp and the PAX8 product is 705 bp. The amplification reaction was carried out for 30 cycles. Each cycle consisted of 94 °C for 20 s, 64 °C for 30 s and 72 °C for 60 s. The last cycle was followed by a final 10 min elongation at 72 °C. Cyclophilin served as a house keeping gene. The PCR products were resolved on 2% E-gels (Invitrogen, Paisley, UK).

2.5. Real Time RT-PCR amplification

ER mRNA expression was quantified with an ABI 7700 Real Time PCR System using specific primer probe sets obtained from Applied Biosystems (Foster City, CA). Each Real Time PCR contained 12.5 μl TaqMan Universal PCR Master Mix, 1.25 μl. Assays-on demand Gene Expression Assay Mix for either ERα (HS00174860M1) or

ERβ (HS00230957M1), 2.5 μl nuclease free water, and 9 μl cDNA. Endogenous controls (RNAse P) were run in triplicate to assure repeatability.

2.6. RNA interference (siRNA)

siRNA was used to block ERα and ERβ. ERα siRNA, ERβ siRNA and the non-targeting siRNA (negative control) were obtained from Dharmacon (Lafayette, CO, USA).

ARO cells were seeded in 12-well plates, 2.8×10^5 cells/well, and were incubated in 1 ml normal growth medium containing serum, without antibiotics, overnight to allow cells to attach to the plates. The cells were then transfected with 100 nmol/l of siRNA using DharmaFECT (Lafayette, CO, USA), according to the manufacturer's instructions. Cells were incubated at 37 °C in a CO_2 incubator for 48 h. After incubation, cells were treated with different concentrations of cDtboc for 24 h. At the end of treatment, cells were harvested for DNA synthesis analysis.

Knock down of ERα and ERβ were determined by Real Time PCR as described.

2.7. Protein extract preparation

PBS washed thyroid cancer cells were washed, collected and lysed in lysis buffer (4 °C) [PBS (7.4), 1% Triton X-100, 0.1% SDS and "Complete" Proteinase Inhibitor Cocktail (Roche, Mannheim, Germany)]. Mild glass–glass hand homogenization (10 strokes on ice) and centrifugation [10,000 × *g*, 19 min, 4 °C] followed as described [22–33]. Protein was quantified by the method of Bradford [34] using bovine serum albumin (BSA) as a standard.

2.8. Western Blot analysis

Proteins were fractionated by SDS–PAGE and transferred from the SDS–polyacrylamide gel to a nitrocellulose membrane (Protean nitrocellulose 85, S&S, Dassel, Germany Schleicher & Schuell). The membranes were then blocked with 20 mM Tris, pH 7.6, 137 mM NaCl, containing 0.1% Tween 20 and 2% BSA (TBS-T) for 60 min at room temperature. The membranes were then washed and incubated with either the antibody alone or antibody preincubated with its blocking peptide S-20 (5 min, at 100× for anti PAX8 hybridizations, according to the manufacturer's instructions (Santa Cruz)) in 1% BSA/TBS-T (binding buffer) for 60 min. The membranes were incubated for 1 hr with either anti-TTF1 (F-12 at a 1:250 dilution in binding buffer) or anti-PAX8 (S-20 at 1:250 dilution in binding buffer). Followed by extensive washing in TBS-T, the membranes were incubated 60 min with donkey anti-goat IgG-HPR conjugate (1:20,000 in 1% BSA/TBS-T) for 60 min. Subsequently, the membranes were washed extensively with 1% BSA/TBS-T and the protein bands were then visualized by ECL plus.

2.9. Assessment of DNA synthesis

Cells were grown until sub-confluence, using conditions previously described [27,28,35] and then treated with various hormones or agents for 24 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 and aliquots were taken for counting radioactivity and [^{3}H]-thymidine incorporation into DNA was calculated.

2.10. Assessment of cell proliferation

Cells were grown until sub-confluence, as previously described [24,25,32], and then treated with various compounds for 24 h as indicated. At the end of incubation, cell proliferation was assayed using the cell proliferation kit based on XTT colorimetric assay of Biological Industries (Kibbutz Beit Haemek, Israel).

2.11. Assessment of cell death and detection of apoptosis and necrosis

Cells were grown until sub-confluence, as previously described [32] and then treated with various compounds for 24 h as indicated. At the end of incubation, photometric enzyme-immunoassay for the quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (mono-and oligo-nucleosomes) after induced cell death was assayed using Cell Death Detection ELISA plus kit from Roche kit Molecular Biochemicals. This is a "sandwich" assay constructed to identify DNA fragments through the use of two antibodies, one against histones and the second directed against DNA. Necrosis was also assayed at the end of the incubation by measuring lactic dehydrogense (LDH) activity released to the culture medium from the cytoplasm of disintegrating cells using standard commercial LDH assay (Advia Centaur, Siemens).

2.12. Assessment of creatine kinase specific activity

Cells were grown until sub-confluence, as previously described [27,35]. Cells were treated for 24 h with the various hormones as specified, and were then collected and homogenized in an extraction buffer. Supernatant extracts were then obtained by centrifugation of homogenates at 14,000 × *g* for 5 min at 4 °C in an Eppendorf micro centrifuge. Creatine kinase activity (CK) was assayed by a coupled spectrophotometric assay [27,35]. For this assay, protein was then determined by Coomasie blue dye binding using BSA as the standard.

2.13. Xenograft studies

Twenty female CD1 nude mice (8 weeks old) were inoculated *sc* with ARO thyroid carcinoma cells (0.5×10^6/mouse). Ten days later, when the tumors became palpable, the mice were randomized into two groups, receiving intravenously, through the tail vein, every other day either cD-tboc (0.3 mg/mouse/48 h dissolved in 0.1 ml of vehicle) (Neowater, Do-Coop Technologies Ltd., Israel) or vehicle. During treatment body weight was recorded to monitor toxicity of the treatment, and the tumors were measured every other day with an external caliper. Tumor volume was calculated using the formula length × width × height × $\pi/6$. 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 Institute of Science and all procedures were approved by the Institutional Animal Care and Use Committee, Weizmann Institute of Science.

2.14. Statistical analysis

Differences between the mean values obtained from the experimental and the control groups were evaluated by analysis of variance (ANOVA). A *p* value less than 0.05, was considered significant.

3. Results

3.1. Validation of the thyroid nature of lines used in the current study

Two common unique transcription factors associated with thyroid cells are the thyroid transcription factor 1 (TTF-1) as well as the PAX8 transcription factor. Their expression at the messenger RNA

level was compared to primary human goiter cells which served as control. Transcripts of 519 bp and 705 bp corresponding to TTF1 and PAX8 were visualized in the thyroid cancer cells as well as in the positive control (Supplementary information, Fig. 1A), but not in cultured primary human vascular smooth muscle cells or human adipocytes. As a further step in this validation, protein levels of these transcription factors were also assessed by Western blot analysis (Fig. 1B, Supplementary information) and found present in all 3 human thyroid cell lines. When the blocking peptide S-20 was added to the incubations, the protein band corresponding to PAX8 disappeared, thus indicating that they were specific for this protein. Accordingly, these 3 cell lines are of human thyroid origin.

3.2. Expression of ERα and ERβ in human thyroid cancer cells

Cells were grown in culture and characterized in terms of the expression of mRNAs for ERα and ERβ by Real Time PCR. ERα was only marginally and not consistently expressed in the three cell lines examined in this study, except for the WRO cells which expressed abundantly. On the other hand, all four cell lines expressed ERβ. Hence, there was higher abundance of ERβ relative to ERα, due to very low expression of ERα mRNA in NPA and MRO and practically undetectable expression in ARO (data not shown), except for WRO. The ratio of ERβ to ERα was > 1000:1, 228:1, 7.7:1 and 1.06:1 in ARO, MRO, NPA and WRO cells, respectively.

3.3. Effect of E2 and specific ERα and ERβ agonists on human thyroid cancer cell line proliferation

E2 elicited a dose-related increase in DNA synthesis in NPA, MRO and ARO cells as assessed by [^{3}H]-thymidine incorporation, peaking at ~150–200% of baseline levels (Fig. 1). Of particular importance is the finding that stimulation of proliferation occurs even at low concentrations of E2, at the level of ambient E2 concentrations in adult females (0.3–3 nmol/l). This contrasts with previous reports, mostly in other thyroid cancer cell lines, that 10–100 nmol/l of E2 was required for a proliferative response. Depicted in Fig. 2 is the differential response pattern of the various thyroid cancer types to selective ER activation. ARO and MRO cells responded only to the ERβ selective agonist DPN whereas NPA and WRO responded to both DPN and PPT ERα selective agonist. Raloxifene (Ral), predominantly an ERα antagonist, blocked the stimulatory effect of E2 and PPT on [^{3}H]-thymidine incorporation, but had no effect on DPN-induced DNA synthesis (Fig. 3a). Similar results were obtained when the specific activity of CK was analyzed (Fig. 3b).

3.4. Effect cD-tboc on human thyroid cancer cell growth and survival in vitro

Fig. 4a illustrates the effect imparted by different concentrations of cD-tboc on DNA synthesis in cultured NPA, MRO, ARO and WRO cells. As shown, cD-tboc at submicromolar to micromolar concentrations inhibited [^{3}H]-thymidine incorporation by 50–85%, with the largest effect seen in NPA and MRO cells and somewhat lesser, though still unequivocal inhibition observed in the anaplastic ARO and in WRO cells. In preliminary experiments, the inhibitory effect of cD-tboc on cell proliferation could be blocked by the specific ERβ antagonist, PTHPP. For example, in WRO cells, cD-tboc caused $-46 \pm 14\%$ ($p < 0.05$) reduction in basal DNA synthesis, which was blocked by PTHPP [150 nmol/l] ($+11 \pm 15\%$ compared with baseline [^{3}H]-thymidine incorporation (p = ns), but not by the ERα antagonist MMP (150 nmol/l); -44 ± 24; $p < 0.05$ compared with basal DNA synthesis). Additionally, we knocked down ERβ expression in ARO cells using ERβ siRNA, which reduced ERβ expression in these

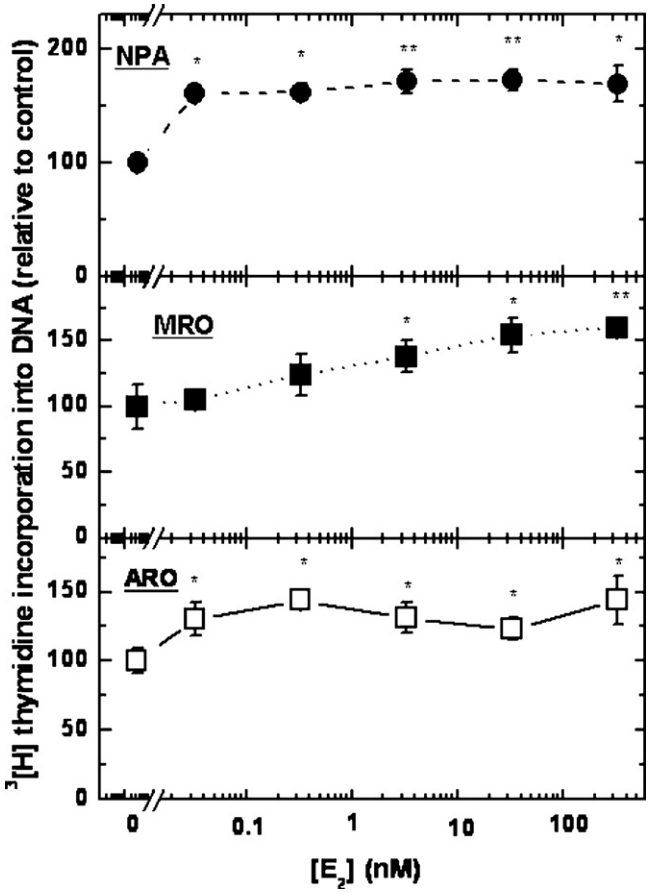

Fig. 1. Effect of increasing concentration of estradiol-17β (E2) on DNA synthesis in cultured human thyroid cancer cell lines. Cells were treated for 24 h with vehicle or E2 (0.3–300 nmol/l). Results are expressed as the mean of % of control cultures ± SEM (n = 5). *$p < 0.05$; **$p < 0.01$. Basal [^{3}H]-thymidine incorporation into DNA was, in ARO, 12,830 + 1156 dpm/well; in MRO 14,288 + 2978 and in NPA 10,653 + 1210.

cells by 98.6%. In these cells, the growth inhibitory effect of cD-tboc was markedly attenuated (Fig. 3, Supplementary data).

Fig. 4b shows the parallel effects of cD-tboc on cell proliferation as assessed by the XTT assay. Finally, Fig. 5 depicts actual

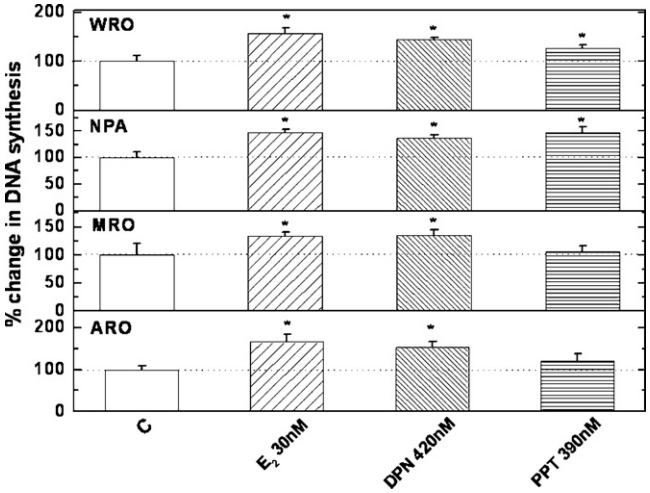

Fig. 2. The effects of estradiol-17β (E2), DPN and PPT on DNA synthesis in cultured human thyroid cancer cell lines. Cells were treated for 24 h with vehicle or E2 (30 nmol/l) or DPN (420 nmol/l) or PPT (390 nmol/l). Results are expressed as the mean of % of control cultures ± SEM (n = 5). *$p < 0.05$. Basal [^{3}H]-thymidine incorporation into DNA in ARO was 8040 + 503 dpm/well; in MRO 6349 + 503, in NPA 7325 + 532 and in WRO 8080 + 920.

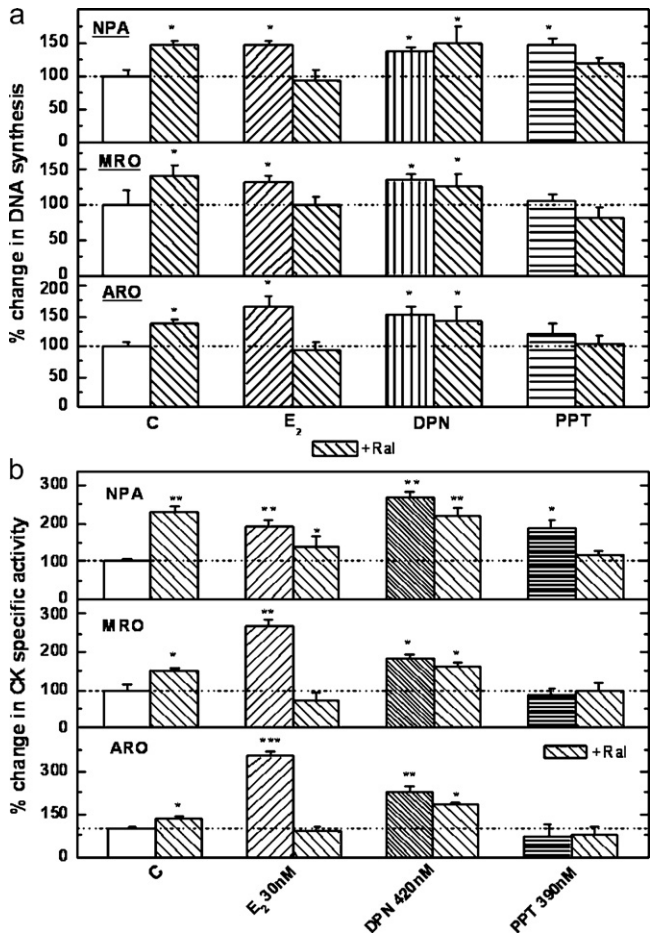

Fig. 3. The effects of raloxifene (Ral) on E2-, DPN- and PPT-induced changes on DNA synthesis (A) and creatine kinase specific activity (CK) (B) in cultured human thyroid cancer cell lines. Cells were treated for 24 h with vehicle or E2 (30 nmol/l) or DPN (420 nmol/l) or PPT (390 nmol/l) with and without raloxifene (Ral) (3 μmol/l). Results are expressed as the mean of % of control cultures ± SEM ($n = 5$). $^{*}p < 0.05$. Basal [^{3}H]-thymidine incorporation into DNA was in ARO 7536 + 220 dpm/well; in MRO 10,439 + 3888 and in NPA 7115 + 967. The basal specific activity of CK was 31 ± 0.3 nmol/min/mg protein in ARO; 21 ± 0.1 in MRO and 27 ± 0.05 in NPA.

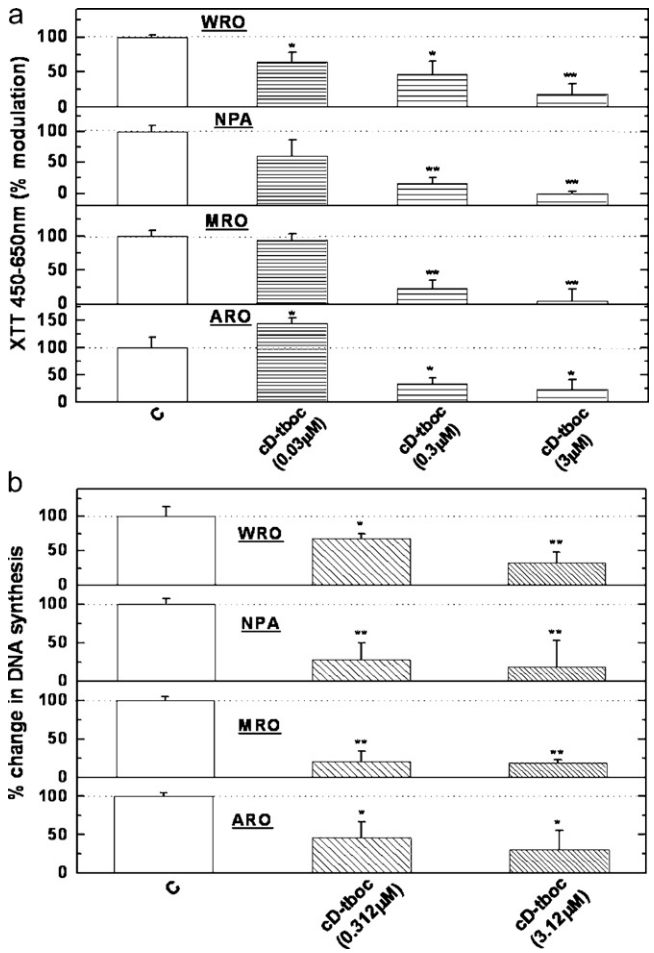

Fig. 4. Effect of cD-tboc on DNA synthesis (A) and on cell replication in cultured human thyroid cancer cell lines as measured by the XTT assay (B) in cultured human thyroid cancer cell lines. Cells were treated for 24 h with vehicle or cD-tboc (31.2, 312 and 3120 nmol/l). Results are presented relative to the control mean [^{3}H]-thymidine incorporation or relative to the mean absorbance of the control samples at a wavelength of 490 nm (after subtraction of non-specific absorbance measured at 650 nm), which is referred to as 100%, and are expressed as means ± SEM ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$. Basal [^{3}H]-thymidine incorporation into DNA was in ARO 10,356 + 550; in MRO 16,700 + 1058, in NPA 8625 + 1450 and in WRO 14,375 + 1575 dpm/well.

photographs of control and treated cells of the four thyroid cancer cell types responding to this compound. Shown photographs were obtained following 24 h of culture with vehicle control (c) as well as with the highest concentrations of applied cD-tboc (3000 nmol/l), marked as tboc a, b, c and d respectively.

3.5. Effect of cD-tboc on the proliferative and creatine kinase specific activities of E2 in thyroid cancer cells

Whereas E2 (30 nmol/l), significantly increased [^{3}H]-thymidine incorporation and CK specific activity in all three thyroid cancer cell lines ($p < 0.01$; Tables 1a and 1b), the stimulatory effect on DNA synthesis and CK specific activity by E2 (30 nmol/l) in these cells was suppressed to basal levels in the presence of 0.3 μmol/l of cD-tboc (Tables 1a and 1b).

3.6. cD-tboc induces thyroid cancer cell death through the induction of apoptosis and not through necrosis

Measured by the Roche apoptosis assay, which, using a double antibody "sandwich" approach detects complexes containing both histone and DNA, cD-tboc elicited a ~4–20-fold increase in DNA fragmentation typical of apoptosis, in a dose- and cell-type specific manner (Fig. 6). Furthermore, when the general apoptosis inhibitor Z-VAD-FMK (1 nmol/l) was added along with cD-tboc, the inhibition of cell growth was significantly attenuated, thus indicating that cD-tboc-induced apoptosis was not an epiphenomenon, but rather one mechanism through which this compounds retards thyroid cancer cell growth (Table 2).

Table 1a
Modulation of DNA synthesis by cD-tboc in the absence or presence of estradiol-17β in ARO, MRO and NPA thyroid cancer cell lines.

Cells/compound	ARO	MRO	NPA
Control	100 ± 3	100 ± 4	100 ± 14
E2	153 ± 11	151 ± 10*	196 ± 21**
cD-tboc	57 ± 3$^{\#}$	51 ± 16$^{\#}$	58 ± 9$^{\#}$
E2 + cD-tboc	103 ± 9	126 ± 25	101 ± 13

cD-tboc was used at 0.3 μmol/l and E2 at 30 nmol/l. Results are means ± SD and expressed as the % of control of DNA synthesis in these cells.
* $p < 0.05$ E2 vs. control in ARO and MRO cells.
$^{\#}$ $p < 0.05$ cD-tboc vs. control.
** $p < 0.01$ E2 vs. control in NPO cells.

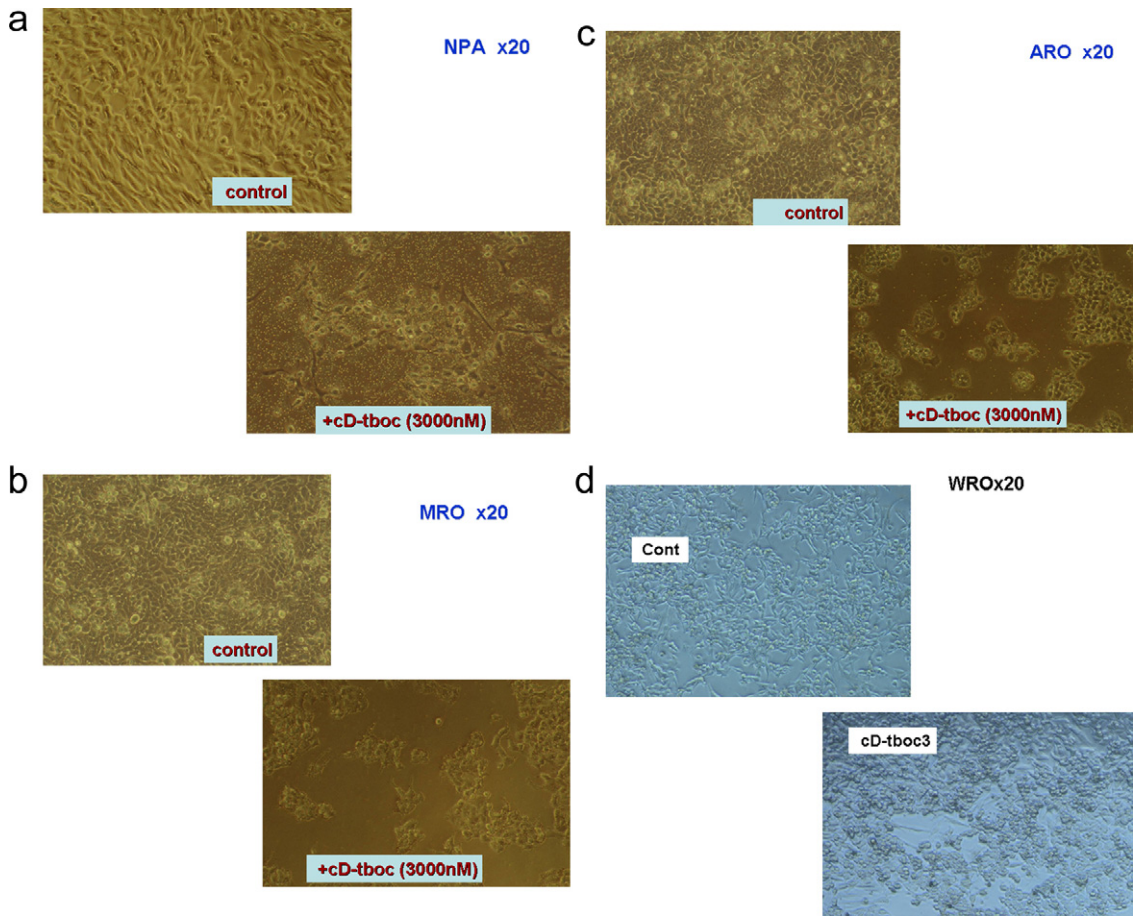

Fig. 5. Microscopic appearance of cD-tboc treated and untreated NPA (A), MRO (B), ARO (C) and WRO (D) cells in culture. The photographs are of unstained cells exposed to cD-tboc (3000 nmol/l) or its control vehicle for 24 h and are shown at 20× magnification.

Finally, thyroid cancer cell growth arrest was not the result of necrosis, since released LDH, a marker of cell necrosis, was not modified in any of the cell types responsive to cD-tboc (data not shown).

Table 1b
Modulation of CK activity by cD-tboc in the absence or presence of estradiol-17β in ARO, MRO and NPA thyroid cancer cell lines.

Cells/compound	ARO	MRO	NPA
Control	100 ± 5	100 ± 12	100 ± 3
E2	240 ± 27**	260 ± 20**	180 ± 15**
cD-tboc	67 ± 2#	57 ± 18#	60 ± 15#
E2 + cD-tboc	99 ± 10	108 ± 15	109 ± 10

cD-tboc was used at 0.3 μmol/l and E2 at 30 nmol/l. Results are means ± SD and expressed as % of control of CK activity in these cells.
# $p < 0.05$ cDtboc vs. control.
** $p < 0.01$ E2 vs. control.

Table 2
Modulation of the inhibitory effect by cD-tboc on DNA synthesis in thyroid cancer cells by the general apoptosis inhibitor Z-VADFK.

Cells/compound	ARO	MRO	NPA
Control	100 ± 8	100 ± 6	100 ± 6
Z-VADFK	79 ± 4*	118 ± 10	75 ± 16
cD-tboc	20 ± 5##	33 ± 24#	21 ± 14##
Z-VADFK + cD-tboc	50 ± 10**	65 ± 22	64 ± 15**

Results are means ± SD and expressed as % of control of DNA synthesis in these cells. cD-tboc was used at 3 μmol/l and Z-VADFK at 1 nmol/l.
* $p < 0.05$, Z-VADFK vs. control in ARO cells.
# $p < 0.05$, cD-tboc vs. control in MRO cells.
** $p < 0.01$, Z-VADFK + cD-tboc vs. control in ARO and NPA cells.

3.7. Effect of cD-tboc on ARO xenografts

Within 10 days of inoculation, 16 of the originally inoculated mice developed palpable tumors, at which time the measurement of tumor dimensions with calipers was initiated. Tumor growth in the group receiving cD-tboc ($n = 9$) was inhibited by >50% as

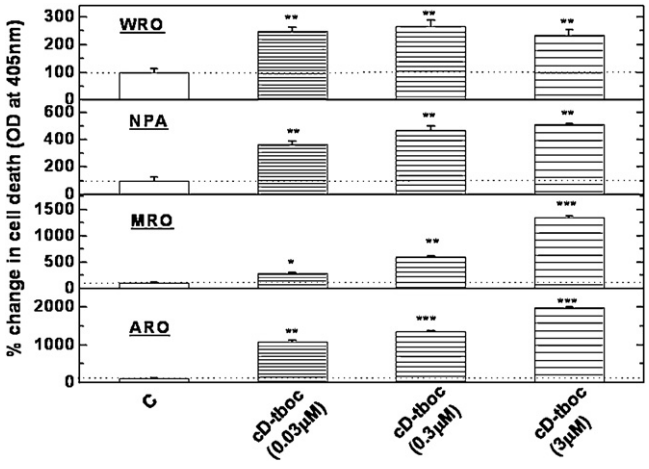

Fig. 6. Effect of cD-tboc on apoptotic cell death in cultured human thyroid cancer cell lines as assessed by the detection histone-DNA fragments. Cells were treated for 24 h with vehicle or cD-tboc (31.2, 312 or 3120 nmol/l). Results are mean values of treated relative to control cultures ± SEM ($n = 5$). *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

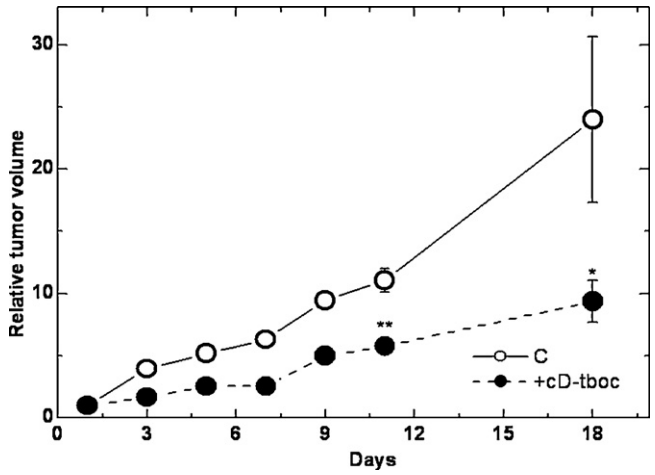

Fig. 7. Effect of cD-tboc on tumor volume in nude mice inoculated subcutaneously with ARO cells. ARO cells were prepared in culture and then inoculated (5×10^5 cells/mouse) into nude mice. Tumors were palpable within 10 days at which time they were randomized into a control group ($n = 7$) receiving vehicle or the treatment group ($n = 9$) receiving cD-tboc (0.3 mg/mouse/48 h). The average tumor volume in each group on the day of tumor measurement relative to treatment day 1 is indicated on the *y*-axis and referred to as relative tumor volume. Values are given in relative tumor volume as mean ± SEM. $^*p < 0.006$ control vs. cD-tboc treated mice on day 11 of the experiment; $^{**}p < 0.044$ vehicle control vs. cD-tboc treated mice on day 18, the end of the experiment.

compared to the tumor volume of vehicle-treated mice ($n = 7$; Fig. 7). No weight reduction or death was seen in mice treated with cD-tBoc.

4. Discussion

The key finding in the present study is that a novel N-t-Boc-hexylenediamine derivative of the 7-(O)-carboxymethyl daidzein, cD-tboc (Fig. 2, Supplementary data), can function as a potent anti-cancer agent in four different cell lines of human thyroid cancer *in vitro*. Further, tested *in vivo*, cD-tboc is also shown here to markedly retard the progression of xenografts of the ARO type, a cell line originally derived from anaplastic thyroid carcinoma. These results extend our previous experience with cD-tboc in colon and ovarian cancer cell lines [27] to human thyroid cancer cell types. Our study also underscores, but was not designed to resolve the complexity of the interactions between estrogenic compounds and the growth and survival of human thyroid cancer cells *in vitro*, consistent with earlier reports [20–22]. E2 *per se* dose-dependently stimulated cell growth in all three lines of human thyroid cancer, as assessed by [^{3}H]-thymidine incorporation and the XTT assay. However, we were unable to correlate this effect of E2 with one specific type of ER. In NPA cells, the specific ERα and ERβ agonists PPT and DPN, respectively, each elicited similar increases in cell growth, but in MRO and ARO cells only the ERβ agonist DPN was able to stimulate DNA synthesis (Figs. 2 and 3a). Of note is the finding that the selective ERα agonist PPT was also unable to increase CK activity in MRO and ARO cells, although it did raise CK activity in NPA cells (Fig. 3b). Clearly, the effects of PPT on both DNA synthesis and CK activity were then ERα-specific as they were fully blocked by raloxifene, which, in turn, was unable to block the increases in [^{3}H]-thymidine incorporation and CK activity elicited by the ERβ agonist DPN in NPA cells. Yet raloxifene *per se*, a presumed ERα antagonist or, acting *in vivo* as a mixed ERα agonist–antagonist, in itself increased both DNA synthesis and CK activity not only in NPA cells, but also in MRO and ARO cells in which the ERα selective agonist PPT had no such effects. These differences in the response to the predominantly ERα-related compounds cannot be explained by ERα mRNA expression in NPA, MRO or ARO cells, which was extremely low in all three cell types. The results should be examined on the background of previous reports on how estrogens may affect thyroid cancer cell growth, survival or cell cycle. Although E2 *per se* appears to possess pro-growth and/or survival effects in former studies, attempts to clarify E2's mode of action or the effects of other estrogen-related compounds yielded variable results. In the human thyroid carcinoma cell line HTC-TSHr, E2 increased cell proliferation through increase in cyclin D1 in a MEK-dependent manner, which could be blocked by the general ER antagonist ICI 182780 [19]. It was reported [19–21] that only activation of ERα, but not ERβ, increased cell growth in KAT5, a thyroid papillary cancer cell line, and ARO, the same anaplastic thyroid cancer cell line used in the present study. The latter finding is discordant with our observation that the ERβ agonist DPN stimulated cell growth in all three tested cell lines, including ARO. An important aspect of Zeng's study [21,22] was that the estrogenic effects in thyroid cancer cells were associated with increase in Bcl-2 and decrease in Bax, thus suggesting that E2 was operating through an anti-apoptotic pathway. Further support for antiapoptotic effects of E2 was recently provided by the findings that E2 lowered mitochondrial Bax in papillary cancer cells, followed by decreased release of cytochrome C and/or apoptosis-inducing factor (AIF) from the mitochondria to the cytosol [21]. In contrast to these presumed ERα-mediated effects, however, E2, genistein and 4-hydroxytamoxifen were recently shown to induce proliferation in human WRO, FRO and ARO cancer cell lines, in the absence of ERα, ERα transactivation or ERα translocation to the nucleus [24]. Still, in this case, MAPK activation and increased cyclin D1 expression were involved through activation of GRP30, a seven transmembrane receptor G-protein coupled orphan receptor linked to specific estrogen binding and rapid estrogen-mediated activation of adenylate cyclase [27]. The growth-curbing effects of cD-tboc in NPA, MRO, ARO and WRO cells in the present study are reminiscent of the effects of this compound in other cancer cell lines *in vitro*, including colon (320DM), ovarian (MLS and A2780) and adrenal (H295R) carcinoma, in that they are seen in cancer types which preferentially express mRNA for ERβ relative to ERα [27]. In addition, in the thyroid cancer cells tested herein, cD-tboc showed at least some anti-estrogen effects in that it was capable of inhibiting both the proliferative effect of E2 and E2-stimulated CK activity. Further, in parallel to our previous findings in MLS ovarian cancer and 320DM colon cancer cells, the cytotoxic effect of cD-tboc appeared to be accounted for, at least in part, by induction of apoptosis. Not only did cD-tboc induce a marked and dose-dependent increase in DNA fragmentation (Fig. 6) in all three cell lines, but its cytotoxic effects could be reversed by ZVAD-FK (see Table 2), the general inhibitor of caspase-dependent apoptosis. Although E2 itself is generally thought to exert anti-apoptotic effects in various estrogen-receptor expressing cancer types [35], estrogen-related metabolites can actually exert the opposite effect. For example, a recent report indicated that 2-methoxyestradiol, an endogenous metabolite of E2, induced caspase-dependent apoptosis in five out of six different human anaplastic thyroid carcinoma cell lines [36]. Daidzein, the parent compound of cD-tboc, was previously shown to act as an ER agonist, with a greater affinity for the ERβ isoform than the ERα isoform [37,38] and to induce apoptosis in prostate cancer [39]. Daidzein displays 100-fold greater sensitivity for activating transcription in transfected cells via ERβ compared to ERα [40]. Also consistent with the importance of ERβ in daidzein's actions is the report that daidzein can activate a pro-apoptotic cascade involving the cleavage of caspase-3 in human cervix epitheloid carcinoma cells cancer cells in an ERβ-dependent manner [41]. The pro-apoptotic, anticancer effects of cD-tboc in the predominantly ERβ expressing thyroid cancer lines the present report are therefore consistent with putative interaction of this new compound

with ERβ. However, a model by which cD-tboc simply activates ERβ to induce programmed cell death in NPA, MRO and ARO cells is difficult to accept, since the ERβ selective agonist DPN actually increased DNA synthesis in the same cell types. Alternative mechanisms must be therefore contemplated such as the possibilities that cD-tboc functions as an ERβ antagonist, merely utilizes ERβ as an anchor for some ER-unrelated effect, activates estrogen or phytoestrogen membrane receptors [42] or even operates through ER-independent pathways. Finally, the possibility of extra- or intra-thyroidal metabolism of cD-tboc to some other metabolite which is responsible for the observed growth curbing effect in this study cannot be excluded.

In conclusion, we have shown that thyroid human cancer cell lines originally derived from papillary, follicular and anaplastic carcinoma express greater abundance of ERβ relative to ERα and display increased DNA synthesis in response to estrogenic agonists. cD-tboc, a novel synthetic derivative of the phytoestrogen daidzein can arrest growth and induce apoptosis in thyroid cancer cells. These effects translate to retardation of xenografts of the anaplastic thyroid cancer cell line ARO *in vivo*. The results offer a novel tool for further testing as a means to curb presently anaplastic and incurable forms of thyroid cancer and should draw more research efforts to ER-related strategies in thyroid cancer. Studies are underway to better characterize the mechanisms enabling cD-tboc to elicit such profound effects on thyroid cancer *in vitro* and *in vivo*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.04.009.

References

[1] B.L. Sprague, S. Warren Andersen, A. Trentham-Dietz, Thyroid cancer incidence and socioeconomic indicators of health care access, Cancer Causes Control 19 (2008) 585–593.
[2] A. Lubina, O. Cohen, M. Barchana, I. Liphshiz, I. Vered, S. Sadetzki, A. Karasik, Time trends of incidence rates of thyroid cancer in Israel: what might explain the sharp increase, Thyroid 16 (2006) 1033–1040.
[3] B.E. Henderson, R.K. Ross, M.C. Pike, J.T. Casagrande, Endogenous hormones as a major factor in human cancer, Cancer Res. 42 (1982) 3232–3239.
[4] A. Fassina, M. Rupolo, M.R. Pelizzo, D. Casara, Thyroid cancer in childrenand adolescents, Tumori 80 (1994) 257–262.
[5] J. Waterhouse, C. Muir, K. Shanmugaratnam, J. Powell, Cancer Incidence In Five Continents, vol. 4, IARC, Lyons, France, 1982, pp. 185–198.
[6] N. Rukhman, A. Silverberg, Thyroid cancer in older men, Aging Male 19 (2010 Nov) (Epub ahead of print).
[7] S. Franceschi, A. Fassina, R. Talamini, A. Mazzolini, S. Vianello, E. Bidoli, G. Cizza, C. La Vecchia, The influence of reproductive and hormonal factors on thyroid cancer in women, Rev. Epidemiol. Sante Publique 38 (1990) 27–34.
[8] P.L. Horn-Ross, K.J. Hoggatt, M.M. Lee, Phytoestrogens and thyroid cancer risk: the San Francisco Bay Area thyroid cancer study, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 43–49.
[9] W.J. Mack, S. Preston-Martin, L. Bernstein, D. Qian, M.M. Xiang, Reproductive and hormonal risk factors for thyroid cancer in Los Angeles County females, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 991–997.
[10] C. Martel, M.H. Melner, D. Gagne, J. Simard, F. Labrie, Widespread tissue distribution of steroid sulfatase, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD), 17 beta-HSD 5 alpha-reductase and aromatase activities in the rhesus monkey, Mol. Cell. Endocrinol. 104 (1994) 103–111.
[11] L. Dalla Valle, A. Ramina, S. Vianello, A. Fassina, P. Belvedere, L. Colombo, Potential for estrogen synthesis and action in human normal and neoplastic thyroid tissues, J. Clin. Endocrinol. Metab. 83 (1998) 3702–3709.
[12] S.R. Money, W. Muss, W.L. Thelmo, O. Boeckl, W. Pimpl, H. Kaindl, P. Sungler, J. Kirwin, H. Waclawicek, B.M. Jaffe, et al., Immunocytochemical localization of estrogen and progesterone receptors in human thyroid, Surgery 106 (1989) 975–978.
[13] Y. Hiasa, H. Nishioka, Y. Kitahori, K. Yane, S. Nakaoka, M. Ohshima, N. Konishi, K. Nishii, M. Kitamura, T. Matsunaga, Immunohistochemical analysis of estrogen receptors in 313 paraffin section cases of human thyroid tissue, Oncology 50 (1993) 1323–1326.
[14] H. Inoue, K. Oshimo, H. Miki, M. Kawano, Y. Monden, Immunohistochemical study of estrogen receptors and the responsiveness to estrogen in papillary thyroid carcinoma, Cancer 72 (1993) 1364–1368.
[15] K. Yane, Y. Kitahori, N. Konishi, K. Okaichi, T. Ohnishi, H. Miyahara, T. Matsunaga, J.C. Lin, Y. Hiasa, Expression of the estrogen receptor in human thyroid neoplasms, Cancer Lett. 84 (1994) 59–66.
[16] K.H. van Hoeven, C.J. Menendez-Botet, E.W. Strong, A.G. Huvos, Estrogen and progesterone receptor content in human thyroid disease, Am. J. Clin. Pathol. 99 (1993) 175–181.
[17] C. Egawa, Y. Miyoshi, K. Iwao, E. Shiba, S. Noguchi, Quantitative analysis of estrogen receptor-alpha and -beta messenger RNA expression in normal and malignant thyroid tissues by real-time polymerase chain reaction, Oncology 61 (2001) 293–298.
[18] A. Kumar, C.M. Klinge, R.E. Goldstein, Estradiol-induced proliferation of papillary and follicular thyroid cancer cells is mediated by estrogen receptors alpha and beta, Int. J. Oncol. 36 (2010) 1067–1080.
[19] W. Kawabata, T. Suzuki, T. Moriya, K. Fujimori, H. Naganuma, S. Inoue, Y. Kinouchi, K. Kameyama, H. Takami, T. Shimosegawa, H. Sasano, Estrogen receptors (alpha and beta) and 17beta-hydroxysteroid dehydrogenase type 1 and 2 in thyroid disorders: possible in situ estrogen synthesis and actions, Mod. Pathol. 16 (2003) 437–444.
[20] D. Manole, B. Schildknecht, B. Gosnell, E. Adams, M. Derwahl, Estrogen promotes growth of human thyroid tumor cells by different molecular mechanisms, J. Clin. Endocrinol. Metab. 86 (2001) 1072–1077.
[21] Q. Zeng, G.G. Chen, A.C. Vlantis, C.A. van Hasselt, Oestrogen mediates the growth of human thyroid carcinoma cells via an oestrogen receptor-ERK pathway, Cell Prolif. 40 (2007) 921–935.
[22] Q. Zeng, G. Chen, A. Vlantis, G. Tse, C. van Hasselt, The contributions of oestrogen receptor isoforms to the development of papillary and anaplastic thyroid carcinomas, J. Pathol. 214 (2008) 425–433.
[23] V.G. Antico-Arciuch, M. Dima, X.H. Liao, S. Refetoff, A. Di Cristofano, Cross-talk between PI3K and estrogen in the mouse thyroid predisposes to the development of follicular carcinomas with a higher incidence in females, Oncogene 29 (2010) 5678–5686.
[24] A. Vivacqua, D. Bonofiglio, L. Albanito, A. Madeo, V. Rago, A. Carpino, A.M. Musti, D. Picard, S. Ando, M. Maggiolini, 17beta-estradiol, genistein, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the g protein-coupled receptor GPR30, Mol. Pharmacol. 70 (2006) 1414–1423.
[25] T. Hoelting, A.E. Siperstein, Q.Y. Duh, O.H. Clark, Tamoxifen inhibits growth, migration, and invasion of human follicular and papillary thyroid cancer cells in vitro and in vivo, J. Clin. Endocrinol. Metab. 80 (1995) 308–313.
[26] T. Kishino, M. Watanabe, M. Kimura, I. Sugawara, Anti-proliferative effect of toremifene and tamoxifen on estrogen receptor-lacking anaplastic thyroid carcinoma cell lines, Biol. Pharm. Bull. 20 (1997) 1257–1260.
[27] F. Kohen, B. Gayer, T. Kulik, V. Frydman, N. Nevo, S. Katzburg, R. Limor, O. Sharon, N. Stern, D. Somjen, Synthesis and evaluation of the antiproliferative activities of derivatives of carboxyalkyl isoflavones linked to N-t-Boc-hexylenediamine, J. Med. Chem. 50 (2007) 6405–6410.
[28] D. Somjen, Y. Amir-Zaltsman, B. Gayer, T. Kulik, E. Knoll, N. Stern, L.J. Lu, L. Toldo, F. Kohen, 6-Carboxymethyl genistein: a novel selective oestrogen receptor modulator (SERM) with unique, differential effects on the vasculature, bone and uterus, J. Endocrinol. 173 (2002) 415–427.
[29] X.P. Pang, J.M. Hershman, M. Chung, A.E. Pekary, Characterization of tumor necrosis factor-alpha receptors in human and rat thyroid cells and regulation of the receptors by thyrotropin, Endocrinology 125 (1989) 1783–1788.
[30] Z. Kraiem, O. Sadeh, M. Yosef, Iodide uptake and organification, triiodothyronine secretion, cyclic AMP accumulation and cell proliferation in an optimized system of human thyroid follicles cultured in collagen gel suspended in serum-free medium, J. Endocrinol. 131 (1991) 499–506.
[31] Z. Kraiem, O. Sadeh, M. Yosef, A. Aharon, Mutual antagonistic interactions between the thyrotropin (adenosine 3′,5′-monophosphate) and protein kinase C/epidermal growth factor (tyrosine kinase) pathways in cell proliferation and differentiation of cultured human thyroid follicles, Endocrinology 136 (1995) 585–590.
[32] M. Kitazono, Y. Chuman, T. Aikou, T. Fojo, Adenovirus HSV-TK construct with thyroid-specific promoter: enhancement of activity and specificity with histone deacetylase inhibitors and agents modulating the camp pathway, Int. J. Cancer 99 (2002) 453–459.
[33] G. Weisinger, R. Limor, Y. Marcus-Perlman, E. Knoll, F. Kohen, V. Schinder, M. Firer, N. Stern, 12S-lipoxygenase protein associates with alpha-actin fibers in human umbilical artery vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 356 (2007) 554–560.
[34] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[35] D. Somjen, N. Stern, E. Knoll, O. Sharon, B. Gayer, T. Kulik, F. Kohen, Carboxy derivatives of isoflavones as affinity carriers for cytotoxic drug targeting in adrenocortical H295R carcinoma cells, J. Endocrinol. 179 (2003) 395–403.
[36] P. Roswall, S. Bu, K. Rubin, M. Landstrom, N.E. Heldin, 2-Methoxyestradiol induces apoptosis in cultured human anaplastic thyroid carcinoma cells, Thyroid 16 (2006) 143–150.
[37] G.G. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, J.A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta, Endocrinology 138 (1997) 863–870.

[38] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (1998) 4252–4263.
[39] F. Cao, T.Y. Jin, Y.F. Zhou, Inhibitory effect of isoflavones on prostate cancer cells and PTEN gene, Biomed. Environ. Sci. 19 (2006) 35–41.
[40] D.M. Harris, E. Besselink, S.M. Henning, V.L. Go, D. Heber, Phytoestrogens induce differential estrogen receptor alpha- or beta-mediated responses in transfected breast cancer cells, Exp. Biol. Med. (Maywood) 230 (2005) 558–568.
[41] P. Totta, F. Acconcia, F. Virgili, A. Cassidy, P.D. Weinberg, G. Rimbach, M. Marino, Daidzein-sulfate metabolites affect transcriptional and antiproliferative activities of estrogen receptor-beta in cultured human cancer cells, J. Nutr. 135 (2005) 2687–2693.
[42] D. Somjen, F. Kohen, M. Lieberherr, B. Gayer, E. Schejter, S. Katzburg, R. Limor, O. Sharon, E. Knoll, G.H. Posner, A.M. Kaye, N. Stern, Membranal effects of phytoestrogens and carboxy derivatives of phytoestrogens on human vascular and bone cells: new insights based on studies with carboxy-biochanin A, J. Steroid Biochem. Mol. Biol. 93 (2005) 293–303.