



Modulators of estrogen receptor inhibit proliferation and migration of prostate cancer cells



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ABSTRACT

In the initial stages, human prostate cancer (PC) is an androgen-sensitive disease, which can be pharmacologically controlled by androgen blockade. This therapy often induces selection of androgen-independent PC cells with increased invasiveness. We recently demonstrated, both in cells and mice, that a testosterone metabolite locally synthesized in prostate, the 5 α -androstane-3 β , 17 β -diol (3 β -Adiol), inhibits PC cell proliferation, migration and invasion, acting as an anti-proliferative/anti-metastatic agent. 3 β -Adiol is unable to bind androgen receptor (AR), but exerts its protection against PC by specifically interacting with estrogen receptor beta (ER β).

Because of its potential retro-conversion to androgenic steroids, 3 β -Adiol cannot be used “in vivo”, thus, the aims of this study were to investigate the capability of four ligands of ER β (raloxifen, tamoxifen, genistein and curcumin) to counteract PC progression by mimicking the 3 β -Adiol activity.

Our results demonstrated that raloxifen, tamoxifen, genistein and curcumin decreased DU145 and PC3 cell proliferation in a dose-dependent manner; in addition, all four compounds significantly decreased the detachment of cells seeded on laminin or fibronectin. Moreover, raloxifen, tamoxifen, genistein and curcumin-treated DU145 and PC3 cells showed a significant decrease in cell migration. Notably, all these effects were reversed by the anti-estrogen, ICI 182,780, suggesting that their actions are mediated by the estrogenic pathway, via the ER β , the only isoform present in these PCs.

In conclusion, these data demonstrate that by selectively activating the ER β , raloxifen, tamoxifen, genistein and curcumin inhibit human PC cells proliferation and migration favoring cell adhesion. These synthetic and natural modulators of ER action may exert a potent protective activity against the progression of PC even in its androgen-independent status.

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1. Introduction

Prostate cancer (PC) is the most frequent malignancy and the second leading cause of cancer death in men in Europe and North America [1,2]. The androgen receptor (AR) plays critical roles in PC development and progression [3,4]. Androgen deprivation therapy (ADT), by reducing circulating androgens or by blocking their interaction with AR, is a mainstay treatment for advanced PC. However, the disease often progresses to androgen-independent PC, which is lethal and incurable. Despite this, androgens are essential for the regulation of prostate growth and functions, and intra-prostatic metabolism of testosterone to estrogens has been recently proposed to play a vital role in the regulation of prostate

gland growth [5–7]. The identification and localization of estrogen receptor β (ER β) in the prostate gland [8] suggested that locally produced testosterone metabolites with estrogenic activity may serve to balance the androgenic action in this tissue. In physiological conditions, estrogen signaling driven by ER β has been shown to negatively regulate prostate gland growth [5,9–14]. In males the aromatization of testosterone to estradiol is very low in normal prostate, because of the poor expression of the enzyme aromatase [15–17], but an alternative testosterone derived ER β ligand highly produced in prostate cells is 5 β -androstane-3 β ,17 β -Adiol (3 β -Adiol) [9,12,14,18]. This endogenous estrogenic steroid derives from the 5 α -reduction of testosterone to DHT, which is subsequently metabolized by prostatic 3 β hydroxysteroid dehydrogenase [18] to 3 β -Adiol. The local 3 β -Adiol synthesis is very high and provides large amounts of a specific endogenous ligand for ER β . We previously reported that 3 β -Adiol inhibits the migration of PC cell lines via ER β activation [19,20]. We also found that 3 β -Adiol inhibits PC cell proliferation and migration, increases cell adhesion, and reduce invasiveness “in vitro”; moreover, “in vivo”

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administration of 3 β -Adiol reduces growth of established tumors and counteracts metastasis formation [19]. The complete removal of androgens, which can be obtained using GnRH analog treatments, would also remove 3 β -Adiol, the agent that protects against PC cell proliferation and metastasis formation. For this reason, the classical androgen depletion therapy widely utilized to treat PC should be re-evaluated. Therefore, the protection of 3 β -Adiol will be preserved using AR antagonists only. However, it is known that 3 β -Adiol has no direct androgenic activity, because it is unable to bind AR, nevertheless it could be a source of androgens because of its potential retro-conversion to DHT [18,21–25]. This low level of androgens production from 3beta-Adiol could limit its potential as a candidate drug for human PC treatment. It is thus necessary to find 3beta-Adiol analogs that may act as alternative ligands for ER β . Several selective ER modulators (SERMs) have been synthesized and already approved for a wide variety of human diseases and many natural compounds (the phytoestrogens) have been shown to exert estrogenic activities. These ER β ligands, by mimicking the 3beta-Adiol activity in PC, may also prevent the development and/or be useful to treat androgen-independent PC.

The benzotriophene raloxifene is a SERM that has been reported to be a mixed estrogen agonist/antagonist [26,27]. Raloxifen is a safe agent for use in breast cancer prevention and female and male osteoporosis [28,29]. Raloxifen binds ER β with a lower affinity than ER α [27,30,31], but with a selective ER β activation and a prevalent ER α antagonistic action; this might also explain some effects of raloxifen on prostate cells [32]. Raloxifen induces apoptosis in both androgen-dependent (e.g., LNCaP) and -independent cell lines (e.g., PC3 and DU145; [27,30]), and caused growth inhibition of human xenografted CWR22 and CWRSA9 PC cells [33]. However, the signaling pathways involved in the control of prostate carcinogenesis by raloxifen are still unclear.

Tamoxifen is another SERM, acting as ER antagonist in mammary gland, thus widely used to treat breast cancer in premenopausal women and gynaecomastia in men [34,35]. On the contrary, Tamoxifen acts as an ER agonist in bone where it induces positive effects on bone density and has an estrogenic-agonistic effect on endometrium [36–38].

Phytoestrogens or 'dietary estrogens', are a diverse group of non-steroidal compounds naturally present in plants. Because of their structural similarity with estradiol (17 β -estradiol), they bind ER acting as estrogens or antiestrogens [39]. Phytoestrogens are divided in isoflavones, lignans and coumestrans. All compounds are contained in plants or seeds.

The best known isoflavone is genistein (4',5,7-trihydroxyisoflavone), a major constituent of soybeans and soy products, able to bind ER β [40]. Genistein is also known for its ability to inhibit tyrosine kinases thereby affecting pleiotropic cell signaling pathways to regulate cell growth [41]. Epidemiological evidence indicates that the incidence and mortality rates of PC are considerably lower in high soy-use countries (e.g.: China), compared to the Western countries [42]. The genistein serum concentration in Asian men is on average higher than that in the US population [43]. Several studies suggested that isoflavone intake may reduce PC risk [44–47], and genistein has been shown to exert anticancer effects on PC [48,49], acting on multiple molecular targets [49].

Curcumin (diferuloylmethane) is present in the spice turmeric (*Curcuma longa*) belonging to the ginger (Zingiberaceae) family. Among the several activities of curcumin [50], one is its ability to modulate the estrogen-induced ER mediated transcription [51]. Curcumin may have some anti-cancer properties related to its effect on several targets including transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and cellular signaling molecules. Curcumin may have positive effects on PC [52–55]. Curcumin (alone or in combination with

phenethyl isothiocyanate) also inhibits growth of PC3 prostate tumor xenografts in nude mice [56].

Little is known about the role of ER β in mediating the anticancer properties of these compounds in PC. In this report, we focused on the effect of treatments with both synthetic and natural modulators of ER activities on proliferation, adhesion and migration of two androgen independent prostate cancer cell lines, DU145 and PC3.

2. Materials and methods

2.1. Reagents

Raloxifen and tamoxifen were kindly donated by Siena Biotech (Siena, Italy). Genistein, curcumin, and ICI 182,780 were from Sigma–Aldrich (Milan, Italy).

2.2. Cell culture

The cell lines DU145 and PC3 were originally obtained from American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI 1640 medium (Biochrom KG, Berlin, Germany), supplemented with 5% fetal bovine serum (FBS) that was obtained from GIBCO BRL, Grand Island, NY), glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μ g/mL) in a humidified atmosphere of 5% CO₂: 95% air at 37 °C.

2.3. Cell growth studies

DU145 and PC3 cells, plated in 10-mm dishes in RPMI medium supplemented with 5% charcoal stripped-FBS (CS-FBS), were treated for 48 h with graded doses of raloxifen, tamoxifen, genistein or curcumin (0.01–1 μ M). Cells were then harvested and counted using a haemocytometer. The results of three separate experiments are presented as the mean \pm SD. Each experimental group was composed of 8 replicates.

2.4. Adhesion assay

48-well flat-bottomed plastic plates were coated with 20 μ g/mL laminin (Chemicon International) or 20 μ g/mL fibronectin (Sigma). DU145 and PC3 cells, pre-treated for 48 h with raloxifen, tamoxifen, genistein or curcumin (1 μ M) either alone or in combination with ICI 182,780 (1 μ M), were collected and plated at 200,000 cells per well. Cells were allowed to adhere for 3 h (DU145) or 30 min (PC3) at 37 °C. At the end of the incubation, the cells were fixed in methanol, then stained with Diffquik (Biomap, Italy) and measured by absorption at 595 nm with Wallac Victor 14290 Multilabel Counter (Perkin-Elmer).

2.5. Microchemotaxis assay

Briefly, cell migration assay was performed using a 48 well-Boyden chamber (Neuroprobe, Inc) containing 8 μ M polycarbonate filters (Nucleopore, Concorezzo, Milan, Italy). Filters were coated on one side with 50 μ g/mL laminin, rinsed once with PBS, and then placed in contact with the lower chamber containing RPMI 1640 medium. DU145 and PC3 cells, pre-treated for 48 h with raloxifen, tamoxifen, genistein or curcumin (1 μ M) either alone or in combination with ICI 182,780 (1 μ M), were collected and then added in aliquots (75,000 cells/50 μ L) to the top of each chamber and allowed to migrate through coated filters for 4 h. At the end of the incubation, the migrated cells attached on the lower membrane surfaces were fixed, stained with Diffquik (Biomap, Italy) and counted in standard optical microscopy.

The results of three separate experiments of migration are presented as the mean \pm SD. Each experimental group consisted of 12

samples. The results are expressed as a percentage of migrated cells vs. control cells.

2.6. Statistical analysis

Statistical analysis, when necessary, was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. The significance is presented as * $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1. Regulation of PC cell growth by raloxifen, tamoxifen, genistein and curcumin

In the initial set of experiments, we have measured the effects of increasing concentrations of raloxifen, tamoxifen, genistein or curcumin (0.01–1 μM) on AR-negative PC cell proliferation. We performed the treatments in RPMI medium supplemented with 5% charcoal stripped-FBS (CS-FBS). 48 h after treatment, numbers of viable cells were determined in relation to untreated cells. Growth inhibition analysis revealed that proliferation of DU145 and PC3 cells was significantly reduced after 48 h of treatment with 0.1 μM of tamoxifen (decreased to 52.20% for DU145 and 58% for PC3). Maximum growth inhibition was observed using 1 μM tamoxifen, down to 50% for DU145 and 45% for PC3 (Fig. 1 panels A and B). Whereas, DU145 cells did not respond to the treatment with 0.1 μM of raloxifen, DU145 growth was significantly inhibited at the maximum dose of this SERM (1 μM ; Fig. 1 panels A and B). On the other hand, treatment with graded doses of raloxifen was able to significantly inhibit growth of PC3 cells already at 0.1 μM (71% for 0.1 μM treatment and 54% for 1 μM treatment respectively) (Fig. 1 panels A and B).

Very similar data were obtained after 48 h treatment with graded doses of genistein and curcumin (Fig. 1 panels C and D).

Indeed, genistein was able to significantly reduce the number of DU145 cells with a reduction of about 75% for 0.1 μM and 55% for 1 μM respectively. Genistein significantly decreased PC3 cells proliferation only at the maximal concentration tested (1 μM ; Fig. 1 panels C and D). Curcumin showed a trend comparable to that of genistein on DU145 cells, with a percentage of inhibition of cell proliferation that was 55% for the doses 0.1–1 μM .

Even in the case of PC3 cells, we observed antiproliferative effects only at the maximum dose of genistein and curcumin (1 mM; decreased to 62% for genistein and 48% for curcumin respectively) (Fig. 1 panels C and D).

3.2. Raloxifen, tamoxifen, genistein and curcumin regulation of DU145 and PC3 cell adhesion

Next, we analyzed the effects of raloxifen, tamoxifen, genistein or curcumin on cell adhesion on laminin or fibronectin matrix substrates, to mimic different extracellular adhesion conditions. To this aim, we pre-treated for 48 h DU145 and PC3 cells with raloxifen, tamoxifen, genistein or curcumin (1 μM) either alone or in combination with ICI 182,780 (1 μM); cells were then seeded on laminin or fibronectin coated plates and the adherent vs. floating cell ratios were evaluated 3 h (DU145) 30 min (PC3) after plating. The results obtained demonstrated that all four compounds significantly increased the capability of DU145 and PC3 cells to adhere to both laminin-coated and fibronectin-coated plates (Figs. 2 and 3). The effects of raloxifen and tamoxifen on the adhesion properties of DU145 and PC3 cells were analyzed in the presence or absence of the pure ER antagonist ICI 182,780 (Fig. 2, panels A–D). The pro-adhesive effects of raloxifen and tamoxifen on DU145 and PC3 cells were completely counteracted by ICI 182,780 (Fig. 2, panels A–D).

The effects of genistein and curcumin on the adhesion properties of DU145 and PC3 cells were then, analyzed in the presence or absence of the pure ER antagonist ICI 182,780 (Fig. 3, panels

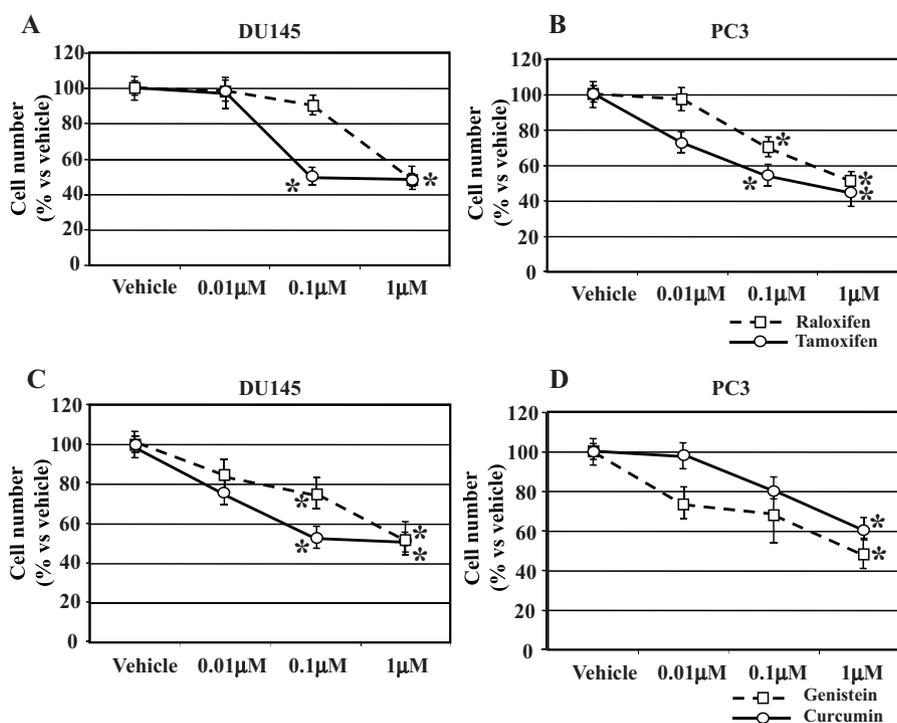


Fig. 1. Raloxifen, tamoxifen, genistein and curcumin regulation of DU145 and PC3 cell growth. Cell proliferation assays on DU145 (panels A and C) and PC3 (panels B and D) cells incubated for 48 h with graded doses (0.01–1 μM) of raloxifen, tamoxifen, genistein or curcumin. Value (cell number) are expressed as the percentage of treated vs. vehicle-treated samples. The results of three separate experiments are presented as the mean \pm SD. Each experimental group was composed of 12 replicates. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests; * $p < 0.05$ vs. vehicle.

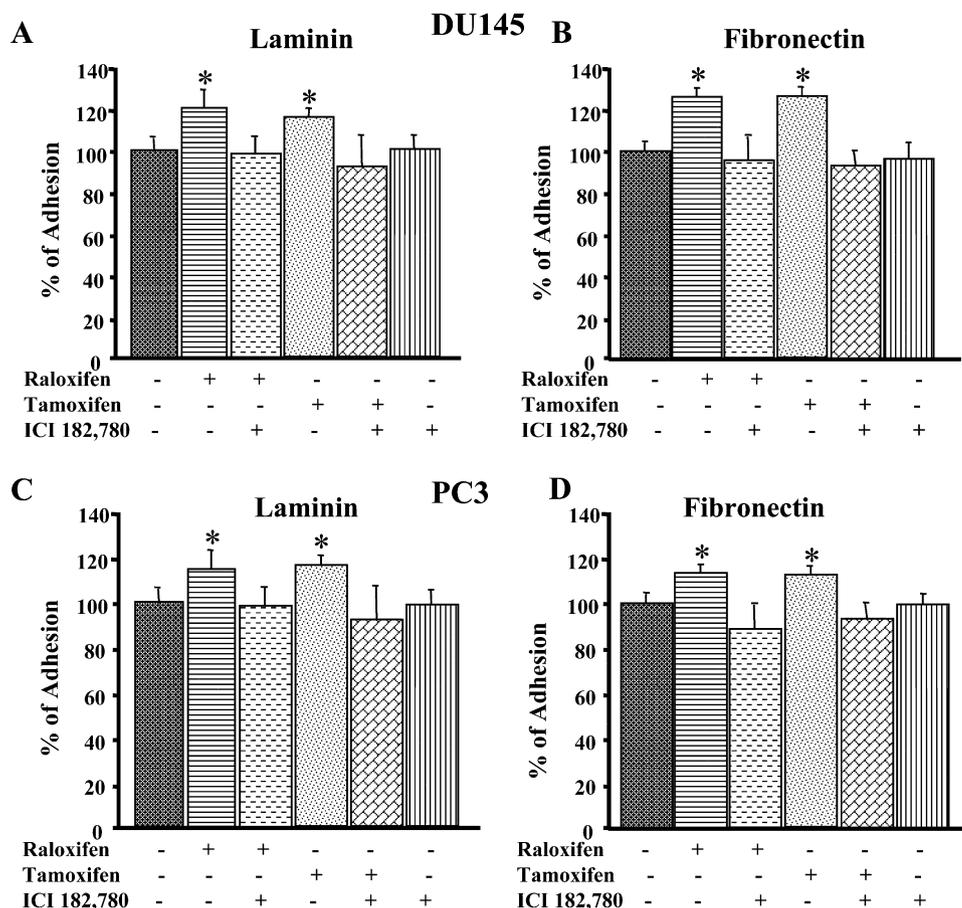


Fig. 2. Effect of raloxifen, tamoxifen, and ICI 182,780 on DU145 and PC3 cell adhesion. Adhesion assays were done using DU145 and PC3 cell lines treated or not with raloxifen (1 μ M), tamoxifen (1 μ M) and ICI 182,780 (1 μ M). Laminin-coated (panels A and C) and Fibronectin-coated (panels B and D) plates were used for assays. Data represent percentage change in the number of adherent cells relative to control cells at 3 h (DU145) and 30 min (PC3) after plating. Each experimental group was composed of 8 replicates. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. * $p < 0.05$ was considered statistically significant.

A–D). Also the pro-adhesive effects genistein and curcumin on DU145 and PC3 cells were completely counteracted by ICI 182,780 (Fig. 2, panels A–D). Very similar data have been obtained using an ER β selective antagonist (ER α agonist), R,R'-THC, at doses of 1 μ M; in fact also R,R'-THC significantly counteracted the pro-adhesive properties of genistein and curcumin on DU145 and PC3 cells estimated using the adhesion assay (data not shown).

Therefore, considering that the only ER isoform expressed in DU145 and PC3 cells is ER β [19,20], we concluded that the pro-adhesive ability of the four ligands is mediated by this receptor.

3.3. Effects of raloxifen, tamoxifen, genistein and curcumin on DU145 and PC3 cell migration

Given that detachment from extracellular matrix is required for cell migration, we tested if the pro-adhesive effects of the four ligands could be correlated with reduced migratory properties of DU145 and PC3 cells using a haptotaxis assay previously described [19,20]. Haptotaxis was evaluated in a Boyden's chamber using laminin coated membranes, because preliminary experiments had indicated that PC3 cells fail to migrate in the absence of a specific substrate (not shown). DU145 and PC3 cells pre-treated for 48 h with raloxifen, tamoxifen, genistein or curcumin (1 μ M) were characterized by a strong decrease of cell migration on laminin when compared to untreated cells (Fig. 4, panels A–D). In particular, 48 h pre-treatment with raloxifen and tamoxifen inhibited the migratory ability of DU145 and PC3 cells respectively of 30% and 25% (Fig. 4, panels A and B). In the same manner, the exposure to 1 mM

genistein and curcumin reduced the migratory activity of DU145 cells by 43% and 25%, respectively; for PC3 cells the reduction was about 40% (Fig. 4, panels C and D). The inhibitory effect of the four ligands on the motility of DU145 and PC3 cells was totally abolished by the presence of the ER antagonist ICI 182,780 (1 μ M) (Fig. 4, panels A–D).

In addition, given that these cells only express ER β , these findings indicate that the anti-migratory properties exerted by these four ligands are mediated by ER β .

4. Discussion

Human PC usually develops as an androgen-sensitive disease, which can be pharmacologically controlled by androgen blockade. However, this therapy often induces the selection of androgen-independent PC cells with increased invasiveness. Androgens are essential for the regulation of prostate growth and functions, and intra-prostatic metabolism of testosterone to estrogens play a vital role in the regulation of prostate gland growth [5–7]. The identification and localization of ER β in prostate gland [8] suggested that locally produced estrogenic testosterone metabolites may serve to balance the androgenic effects in this tissue. In physiological conditions, ER β -mediated estrogen signaling has been shown to inhibit prostate gland growth [5,9–14].

Recently, we found that dihydrotestosterone inhibits PC cell proliferation and migration through its conversion to 3 β -Adiol, which is unable to bind AR, but interacts specifically with ER β [19,20]. 3 β -Adiol activities are mediated by ER β and cannot be

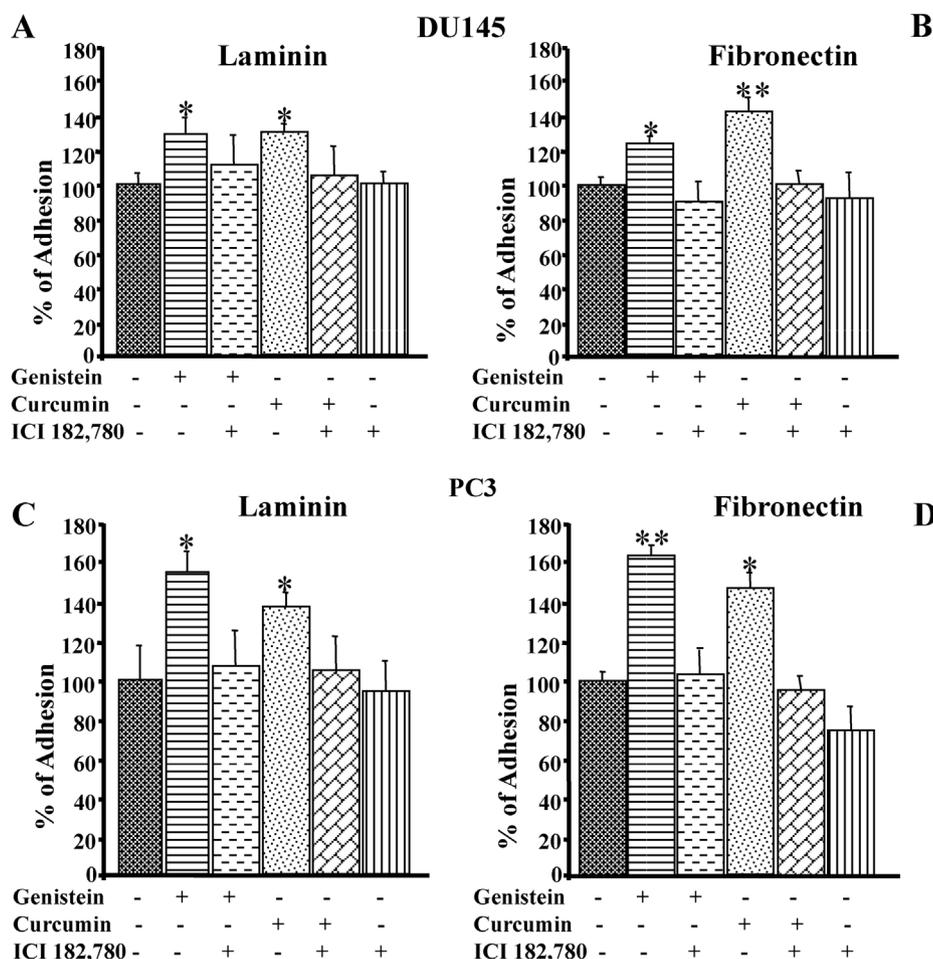


Fig. 3. Effect of genistein, curcumin, and ICI 182,780 on DU145 and PC3 cell adhesion. Adhesion assays were done using DU145 and PC3 cell lines treated or not with genistein, curcumin, and ICI 182,780 (1 μ M). Laminin-coated (panels A and C) and Fibronectin-coated (panels B and, D) plates were used for assays. Data represent percentage change in the number of adherent cells relative to control cells at 3 h (DU145) and 30 min (PC3) after plating. Each experimental group was composed of 12 replicates. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. * $p < 0.05$ was considered statistically significant.

reproduced by the physiological estrogen, 17 β -estradiol, suggesting the existence of different pathways activated by the two ER β ligands in PC cells [19]. The anticancer activity of 3 β -Adiol has also been tested in mice in which we have topically injected PC cells into the prostate and evaluate the spreading of these cells in seminal vesicles. We found that 3 β -Adiol almost completely counteracted the invasiveness of PC cells to surrounding tissues, proving its efficacy against PC cell migration and metastatization [19].

The potential therapeutic use of 3 β -Adiol might be limited. In fact, while 3 β -Adiol has no direct androgenic activity, it could be a source of androgens by its retro-conversion to DHT [18,21–25]. It is thus necessary to find new and alternative ligands for ER β , either synthetic or natural, that could prevent the development of, and/or are useful in treating, androgen-independent PC.

It is known that the administration of estrogen-like substances or phytoestrogens, because of structural similarity with estradiol (17 β -estradiol), reduced "in vitro" proliferation of prostate cancer cell lines, and the development of prostate cancer in animals models, by activating ER β [57–59].

Thus, in the present study we analyzed the potential treatments with both synthetic (raloxifen and tamoxifen) and natural (genistein and curcumin) modulators of ER activities for the suppression of the tumoral activities of the androgen-independent PC cells DU145 and PC3, specifically proliferation, migration, invasiveness. In growth course experiments, raloxifen and tamoxifen were able to significantly inhibit proliferation of DU145 and PC3 cells

with a percentage of inhibition of about 50% at a dose of 1 μ M. To our knowledge, this is the first evidence showing that tamoxifen shows anti-proliferative activity on PC cell lines. Our results on the anti-proliferative effects of raloxifen are in agreement with the recent study of Rossi and co-worker that demonstrated that raloxifen is able to inhibit the cell cycle and to induce cell death in PC cell lines [60].

With regard to genistein and curcumin, several studies suggested that phytoestrogen intake may reduce PC risk [44–47,53–55], and genistein and curcumin has been shown to exert anticancer effects on PC [48,49,61], acting on multiple molecular targets [49].

In our study we showed that the treatment with raloxifen, tamoxifen, genistein and curcumin significantly increased the capability of DU145 and PC3 cells to adhere to both laminin-coated and fibronectin-coated plates. The effects of all these selective modulators of ER activities on the adhesion properties of DU145 and PC3 cells were analyzed in the presence or absence of the pure ER antagonist ICI 182,780. The pro-adhesive effects of raloxifen, tamoxifen, genistein and curcumin on DU145 and PC3 cells were completely counteracted by ICI 182,780. Therefore, considering that the only ER isoform expressed in DU145 and PC3 cells is ER β [19,20], we concluded that the pro-adhesive ability of the four ligands is mediated by this receptor.

Given that detachment from extracellular matrix is required for cell migration, we tested if the pro-adhesive effects of the

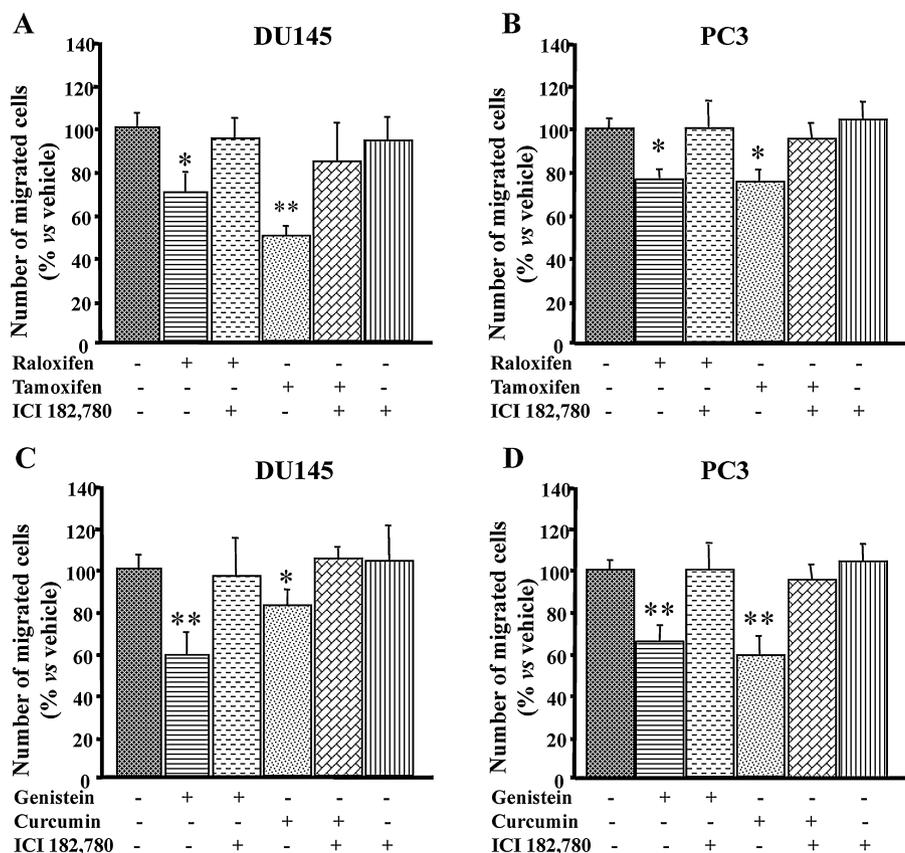


Fig. 4. Effects of raloxifen, tamoxifen, genistein, curcumin and ICI 182,780 on cell migration. DU145 (panels A and C) and PC3 (panels B and D) cells were treated with raloxifen, tamoxifen, genistein, curcumin and ICI 182,780 (1 μ M) for 48 h; cells were then detached, and transferred to Boyden's Chamber. The results of three separate experiments are presented as the mean \pm SD. Each experimental group was of 12 samples. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison tests. The results are expressed as a percentage of migrated cells vs. control cells (vehicle). * $p < 0.05$ was considered statistically significant.

four ligands could be correlated with reduced migratory properties of DU145 and PC3 cells using a haptotaxis assay previously described [19,20]. DU145 and PC3 cells pre-treated for 48 h with raloxifen, tamoxifen, genistein or curcumin (1 μ M) were characterized by a strong decrease of cell migration on laminin when compared to untreated cells. The inhibitory effect of the four ligands on the motility of DU145 and PC3 cells was totally abolished by the presence of the ER antagonist ICI 182,780 (1 μ M). These findings indicate that in addition to the pro-adhesive effects discussed above, the anti-migratory properties exerted by these four ligands are also mediated by ER β . In fact, this is the only ER isoform present in the cells we selected for this study. Furthermore, the observation that ICI 182,780 blocks specifically the action of genistein and curcumin on PC cells, strongly suggests that, in spite of their wide-spread effects [41,51], the two natural compounds can directly interact with ER β , and this receptor may have a dominant role as some sort of point of convergence in the many paths regulating growth, adhesion and migration.

Our data are in line with other studies showing that genistein and curcumin were able to inhibit migration of human PC cell lines [62–64]. However, while the previous studies associated the effects exerted by genistein seemed to be an inhibition of the activation of Focal Adhesion Kinase (FAK) and mitogen-activated kinase (MAPK) p38-heat shock protein 27 that mediates detachment and cellular migration respectively [64], we clearly showed that the protective effects of phytoestrogens on PC cells are mediated by ER β activation.

These effects of genistein were also confirmed in an "in vivo" mouse model orthotopically implanted with human PC cells [63]. Feeding mice dietary genistein before implantation led to blood concentrations similar to those measured in genistein-consuming

men. Moreover, genistein decreased metastases by 96% [63]. Also curcumin exhibits a suppressive effect on PC cell invasion, tumor growth, and metastasis via down-regulating action of matrilysin, a membrane-anchored serine protease with oncogenic roles in tumor formation and invasion.

In conclusion, our data demonstrate that raloxifen, tamoxifen, genistein and curcumin inhibit human PC cells proliferation and migration favoring cell adhesion. Therefore, these modulators of ER activities are effective agents against human PC progression, since the estrogenic effect of these compounds may be protective against PC invasion and metastasis.

Conflict of interest

No potential conflicts of interest were disclosed.

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