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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 synthetized in prostate, the 5α-androstane-3β, 17β-diol (3β-Adiol), inhibits PC cell proliferation, migration and invasion, acting as an anti-proliferative/anti-metastatic agent. 3β-Adiol is unable to bind androgen receptor (AR), but exerts its protection against PC by specifically interacting with estrogen receptor beta (ERβ).

Because of its potential retro-conversion to androgenic steroids, 3β-Adiol cannot be used "in vivo", thus, the aims of this study were to investigate the capability of four ligands of ERβ (raloxifen, tamoxifen, genistein and curcumin) to counteract PC progression by mimicking the 3β-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 ERβ, the only isoform present in these PCs.

In conclusion, these data demonstrate that by selectively activating the ERβ, raloxifen, tamoxifen, genistein and curcumin inhibit human PC cells proliferation and migration favoring cell adesion. 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.

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 (ERβ) 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 ERβ 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 ERβ ligand highly produced in prostate cells is 5β-androstane-3β,17β-Adiol (3β-Adiol) [9,12,14,18]. This endogenous estrogenic steroid derives from the 5alpha-reduction of testosterone to DHT, which is subsequently metabolized by prostatic 3β hydroxysteroid dehydrogenase [18] to 3β-Adiol. The local 3β-Adiol synthesis is very high and provides large amounts of a specific endogenous ligand for ERβ. We previously reported that 3β-Adiol inhibits the migration of PC cell lines via ERβ activation [19,20]. We also found that 3β-Adiol inhibits PC cell proliferation and migration, increases cell adhesion, and reduce invasiveness "in vitro"; moreover, "in vivo"

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administration of 3β-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 3β-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 3β-Adiol will be preserved using AR antagonists only. However, it is known that 3β-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 ERβ. 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 ERβ ligands, by mimicking the 3beta-Adiol activity in PC, may also prevent the development and/or be useful to treat androgen-independent PC.

The benzotiophene 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 ERβ with a lower affinity than ERα [27,30,31], but with a selective ERβ activation and a prevalent ERα 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 (17β-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 ERβ [40]. Genistein is also known for its ability to inhibit tyrosine kinases thereby affecting pleotropic 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 ERβ 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 μg/mL) in a humidified atmosphere of 5% CO_2: 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 μM). Cells were then harvested and counted using a haemocytometer. The results of three separate experiments are presented as the mean ± SD. Each experimental group was composed of 8 replicates.

2.4. Adhesion assay

48-well flat-bottomed plastic plates were coated with 20 μg/mL laminin (Chemicon International) or 20 μg/mL fibronectin (Sigma). DU145 and PC3 cells, pre-treated for 48 h with raloxifen, tamoxifen, genistein or curcumin (1 μM) either alone or in combination with ICI 182,780 (1 μM), 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 μM polycarbonate filters (Nucleopore, Concorezzo, Milan, Italy). Filters were coated on one side with 50 μg/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 μM) either alone or in combination with ICI 182,780 (1 μM), were collected and then added in aliquots (75,000 cells/50 μL) 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 ± SD. Each experimental group consisted of 12