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Designed modulation of sex steroid signaling inhibits telomerase activity and proliferation of human prostate cancer cells

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ABSTRACT

The predominant estrogen-receptor (ER)-β signaling in normal prostate is countered by increased ER-α signaling in prostate cancer (CaP), which in association with androgen-receptor (AR) signaling results in pathogenesis of the disease. However CaP treatments mostly target AR signaling which is initially effective but eventually leads to androgen resistance, hence simultaneous targeting of ERs has been proposed. A novel series of molecules were designed with multiple sex-steroid receptor modulating capabilities by coalescing the pharmacophores of known anti-CaP molecules that act via modulation of ER(α/β) and/or AR, viz. 3,3′diindolylmethane (DIM), mifepristone, toremifene, tamoxifen and raloxifene. *N,N*-diethyl-4-((2-(4-methoxyphenyl)-1*H*-indol-3-yl)methyl) aniline (DIMA) was identified as the most promising structure of this new series. DIMA increased annexin-V labelling, cell-cycle arrest and caspase-3 activity, and decreased expression of AR and prostate specific antigen in LNCaP cells, *in vitro*. Concurrently, DIMA increased ER-β, p21 and p27 protein levels in LNCaP cells and exhibited ~5 times more selective binding for ER-β than ER-α, in comparison to raloxifene. DIMA exhibited a dose-dependent ER-β agonism and ER-α antagonism in classical gene reporter assay and decreased hTERT (catalytic subunit of telomerase) transcript levels in LNCaP at 3.0 μM ($P < 0.05$). DIMA also dose-dependently decreased telomerase enzyme activity in prostate cancer cells. It is thus concluded that DIMA acts as a multi-steroid receptor modulator and effectively inhibits proliferation of prostate cancer cells through ER-β mediated telomerase inhibition, by countering actions of ER-α and AR. Its unique molecular design can serve as a lead structure for generation of potent agents against endocrine malignancies like the CaP.

Introduction

Clinical and experimental researches published till date clearly demonstrate that both androgens and estrogens play a crucial role in the normal development and functioning of prostate, as well as in the pathogenesis of prostate cancer (CaP) (Ellem and Risbridger, 2007). The critical equilibrium between androgen receptor (AR) and estrogen receptor (ER) signaling in normal prostatic cells (Imamov et al., 2004) is perturbed in CaP, and therefore simultaneous targeting of both AR and ER may be required for its effective management (Johnson et al., 2010). This is also supported by the fact that besides androgen, estrogens and their receptors have also been implicated in CaP development and tumor progression (Bosland, 2005; Carruba, 2007). However, current management strategies for CaP mostly focus on disruption of signaling through the androgen receptor (AR), which is initially effective but eventually leads to androgen resistance. ER-β transcripts have been reported in normal prostatic epithelial cells while in case of CaP ER-α transcripts are also detected, and the anti-estrogen mediated inhibition of CaP cell proliferation is blocked by anti-sense ER-β RNA (Lau et al., 2000). Hence, there is apparently a significant potential for the use of ER-α antagonists and/or ER-β agonists to prevent or delay disease progression (Bonkhoff and Berges, 2009) in adjunct to androgen ablation therapy.

The plant derived 3, 3′-diindolyl methane (DIM, digested product of indole-3-carbinol, a potential anticancer component of cruciferous vegetables) functions as a strong AR antagonist (Le et al., 2003) and directly down-regulates signaling through AR (Abdelbaqi et al., 2011). DIM also acts by selectively activating ER-β (Vivar et al., 2010) which can antagonize AR action (Bektic et al., 2004) and induce targeting of CaP cells (Hussain et al., 2012). Similarly mifepristone, the well-known antiprogestin, significantly inhibits proliferation of androgen sensitive LNCaP prostate cancer cells (El Etreby et al., 2000) by behaving as a potent AR antagonist and blocking the androgen action in absence of progesterone receptors in prostate cancer (Brolin et al., 1992). Mifepristone exhibits higher AR binding affinity than hydroxyflutamide and bicalutamide (Song et al., 2004), and its anti-cancer activity is not dependent on the expression of progesterone receptors (Tieszen et al.,

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2011). On the other hand, anti-estrogen tamoxifen inhibits proliferation of androgen refractory CaP cells (PC-3 and DU-145) and the growth of CWR22 CaP xenograft in nude mice (Bosland, 2005), and raloxifene inhibits human CaP growth in mouse xenograft models through ER-β mediated action with potential of disease stabilization in clinical situation (Shazer et al., 2006). Toremifene, a structure related to tamoxifen and potent ER-α antagonist, is reported to inhibit the proliferation of PC-3 cells (Kawashima et al., 2004) and tumor development in the transgenic adenocarcinoma of mouse prostate (TRAMP) model (Raghow et al., 2002). Clinical studies have indicated that toremifene could be a promising SERM for prevention of CaP (Price et al., 2006) and alleviation of skeletal and other complications associated with androgen deprivation therapy (Smith et al., 2013). Thus ER-β agonism and/or ER-α antagonism is seemingly desirable for CaP management.

With this background, a novel series of molecules were designed with multiple sex-steroid receptor (ER-α/β and AR) modulating capability by coalescing the crucial chemical moieties of 3, 3′diindolyl methane (DIM), mifepristone, toremifene, tamoxifen and raloxifene. The properly oriented chemical scaffolds of these established compounds created a novel series of endocrine modulators with potential against prostate cancer cells. The present study mechanistically evaluates DIMA (the most promising compound of this series) in comparison to some of its parent molecule(s) for its anti-proliferative activity against CaP cells.

Material and methods

Test compounds and reagents. A series of compounds were synthesized in-house by the onco-medicinal chemist authors (SS, AKB and AK) and assayed for anti-proliferative activity against PC3 cell line (Table 1). Amongst these, *N,N-d*iethyl-4-((2-(4-methoxyphenyl)-1*H-i*ndol-3-yl) *m*ethyl) *a*niline (DIMA) was picked up as 'most promising' on the basis of its activity towards LNCaP/PC3/DU145 cell lines, safety and ease of synthesis. Toremifene, DPN and PPT were purchased from Sigma-Aldrich, USA. 'Annexin-V-FLUOS' staining kit was purchased from Roche, USA, and CaspGLOW active caspase-3 kit was from ebiosciences. Transfections were carried out by Lipofectamine® LTX and plus reagents from Invitrogen. QIAprep® Spin Miniprep kit (Qiagen), Dual Luciferase® Reporter Assay System (Promega Corporation, USA) and TRAPeze telomerase detection kit (Millipore) were used for the assay. All culture media, MTT and other reagents were from Sigma-Aldrich, USA.

Design strategy and synthesis of DIMA compounds. The series were synthesized with a view to coalesce the crucial chemical pharmacophores of diindolylmethane (DIM), mifepristone, raloxifene, tamoxifen and toremifene as shown in Fig. 1A and the synthesis scheme is presented as Fig. 1B.

General chemistry. A multi-component reaction of indoles, formaldehydes, and tertiary aromatic amines is described for the synthesis of dialkylaminoarylated indoles using silica-supported perchloric acid ($HClO_4$–SiO_2) as an inexpensive and highly efficient catalyst (Fig. 1B). The key features of this multi-component reaction are operational simplicity, mild reaction conditions, regioselectivity, and recycling of catalyst.

General experimental procedure. A mixture of indole derivatives (1.0 mmol), *N,N*-dialkylaniline (1.0 mmol) and $HClO_4$–SiO_2 (2 mol %) was stirred in MeOH (5 ml) at room temperature for 5 min. Thereafter formaldehyde (formalin solution) 1.0 mmol, 0.086 ml was added drop wise to the stirred solution. The reaction was monitored by TLC. The products precipitated from the reaction mixture. The precipitate was filtered off, dissolved in hot MeOH and the catalyst was removed by hot filtration. The filtrate was kept at room temperature and the resulting crystallized product was collected by filtration, and washed with cold EtOH to get the crystallized product (Kumar et al., 2009).

Cell cultures. Androgen sensitive human prostate cancer (LNCaP) cells were procured from National Center for Cell Science, Pune, India, and androgen refractory human prostate cancer cells (PC-3 and DU145), ER negative breast cancer MDAMB cell line and normal prostate cell line RWPE-1 were procured from American Type Cell Culture (ATCC, USA). Human embryonic kidney (HEK-293) cells were from Institute's repository. LNCaP cells were grown in RPMI-1640 media (Sigma-Aldrich, USA) supplemented with 15% FBS (Life Technologies Inc); PC3 and DU-145 cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1; Sigma-Aldrich, USA); MDAMB and HEK-293 cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin-G sodium and 100 μg/ml streptomycin sulphate; and RWPE cells were grown in keratinocyte serum free media (Life technologies Inc). All cell cultures were maintained in a humidified atmosphere of 5% CO_2/95% air, at 37 °C.

Plasmids. pSG5 2xERE-luc, pSG5-ERα and pSG5-ERβ were generous gifts from Prof. M.G. Parker, Imperial Cancer Research Fund, London, UK. pRL-luc was obtained from Promega, USA.

Cell proliferation assays. Cells were seeded in 96-well plates at a density of 2×10^4 cells/well, allowed 24 h for attachment and treated (in triplicates) with serial dilutions of DIMA, Toremifene or Mifepristone (40.0–1.25 μM) for 48 h at 37 °C in 5% CO_2 atmosphere. The compounds were dissolved in dimethylsulfoxide (DMSO) and diluted with culture medium to required concentrations before adding to the cells. Final concentration of DMSO was not more than 0.05%. Controls were treated with DMSO (0.05% in culture medium). After 48 h incubation in a CO_2 incubator, 10 μl of 5 mg/ml MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zolium bromide)] was added to the cells. After a further incubation of 3–4 h, the formazan crystals formed in viable cells were dissolved in DMSO and absorbance was measured at 540 nm using a microplate reader (Microquant, Bio-Tek, USA).

Annexin-V/propidium iodide labeling for detection of apoptosis. Dual fluorescent labelling with fluorescein isothiocyanate (FITC)–annexin-V and propidium iodide (PI) was used to study the externalization of phosphatidylserine on LNCaP cell surface (apoptotic cells) and the cell-membrane permeability (necrotic cells), respectively (Zhang et al., 1997). Cells seeded in 6-well plates (1×10^5/well) were incubated for 24 h at 37 °C in 5% CO_2. Thereafter the culture medium was replaced with fresh medium containing DIMA or Toremifene at a final concentration of 3.0 μM and 6.0 μM (in triplicates), and incubated for another 48 h. Control wells contained only culture medium. After incubation, the cells were harvested, washed with PBS, pelleted at 200 ×*g* and re-suspended in 100 μl of Annexin-V-Fluorescein solution containing PI for labelling cells with the fluorescent probes (Annexin-V-FLUOS staining kit for apoptosis, Roche Diagnostics, Cat no. # 11828681001) followed by incubation at ~25 °C for 10 to 15 min in dark. The staining profile of cells positive for Annexin-V and PI was determined in a flow cytometer (Model FACS Calibur, BD Biosciences, USA) equipped with an argon laser (488 nm) used for excitation. Analysis was made using Cell-Quest software and threshold signals for normal cells.

Cell cycle analysis. LNCaP cells were treated with DIMA or Toremifene at 3.0 μM concentration for 48 h as detailed above. After treatment, the cells were harvested and fixed in 75% ethanol, overnight at 4 °C. The fixed cells were washed with PBS, incubated with RNase A (20 μg/ml) and stained with PI (50 μg/ml) for 15 min, washed and analyzed on a Flow Cytometer. The percentage of cells with DNA content representing different phases of cell cycle were analysed by ModFit LT 3.0 software (Verity Software House).