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Design and evaluation of novel oxadiazole derivatives as potential prostate cancer agents

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Abstract

Various 1,3,4-oxadiazole derivatives have been synthesized and their antiproliferative properties have been studied. The in vitro screening was performed against androgen dependent (LNCaP) and androgen independent (PC-3) prostate cancer cell lines. Most of the compounds showed promising activity. Among them, compounds **2d** (IC₅₀ = 0.22 and 1.3 μ M) and **2a** (IC₅₀ = 8.34 and 2.5 μ M) have shown significant activities on PC-3 and LNCaP cell lines respectively. To investigate the mechanism of cell death we performed cell apoptosis staining and cell cycle arrest assay on more sensitive PC-3 cell lines on **2d**. The results demonstrated that **2d** induced apoptosis and shifted the cells to the sub G0/G1 and S phase. Our study evidently identified the potency of compound **2d** as potential anti-prostate cancer agent.

Keywords

Azoles; 1,3,4-Oxadiazole; Prostate cancer; Apoptosis; Cell cycle

The American Cancer Society estimates that in 2016 in the United States there will be an estimated 1,685,210 new cancer cases and 595,690 cancer deaths.¹ Carcinoma of the prostate (CaP) is the second leading cause of cancer related death in men after lung cancer in the United States. If the cancer is confined to the prostate, it is often curable by surgery or radiation treatment. However, in many cases, it has already spread to other tissues, particularly the bone, at diagnosis and usually requires some form of hormone therapy to keep it in check.

Growth of CaP is primarily stimulated by androgens. Androgens bind to the androgen receptor (AR) and support the development and maintenance of normal prostate tissue. The

antiandrogens such as Bicalutamide and Enzalutamide (Fig. 1) target the AR, which is responsible for growth and progression of the disease. However, the emergence of recurrent, metastatic forms of castration-resistant/androgen-independent PCa continues to be a major challenge.² The mechanism of resistance to current antiandrogens is unclear,³ but it is known that certain mutations in the hormone binding site (HBS) of the AR will convert the drugs from receptor's antagonists to agonists.⁴⁻⁶ Part of the problem is that all known antiandrogens share the same structural motif responsible for effective AR HBS binding which may attribute to occurring resistance. Therefore, more efforts are expected to discover AR antagonists possessing novel chemical scaffolds that may partially address the problem of resistance in PCa.

Several reports recently revealed the essential features of 1,3,4-oxadiazole scaffold in medicinal chemistry to attribute various pharmacological properties. Molecular modeling and pharmacokinetic studies have also demonstrated that incorporating 1,3,4-oxadiazole moieties to drug-like molecules change their polarity, flexibility as well as metabolic profile and ability to engage in hydrogen bonding. Moreover, oxadiazoles have been widely employed as bioesters of amide and esters for a number of biological targets.^{7,8}

Derivatives of naphthalene such as naphthalimides are important aromatic heterocycles with immense pharmacological significance as they serve as core scaffold in drug designing. Naphthalimide based anticancer drugs constitute an indispensable part in the development of anticancer drugs. Naphthalimides have been reported as potent apoptosis inducers, *P*-glycoprotein inhibitors, microtubule inhibitors or glutamamide analogues. Taking into account the importance of the above considerations we investigated a new series of compounds with 1,3,4-oxadiazole scaffold as potential anticancer agents.⁹⁻¹²

Chemistry

The 2-amino-1,3,4-oxadiazole **1** was prepared from the corresponding hydrazides by reported procedure.¹³ The amide or sulfonamide derivatives **2a-e** were synthesized by using acyl or sulfonyl chlorides in pyridine following procedure reported in literature.^{14,15} (Scheme 1). The structures of the newly synthesized compounds were confirmed using IR, ¹H NMR and ¹³C NMR spectral data.

Biology: In vitro cytotoxic activity

Androgen receptor dependent (LNCaP) and androgen independent (PC-3) cell lines were selected to evaluate compounds **2a-f** for their capability to inhibit androgen mediated growth in vitro. The cytotoxic activity of the synthesized compounds was evaluated using the sulfo-rhodamine B (SRB) colorimetric assay as described previously.¹⁴ The cytotoxic activities are expressed as the median growth inhibitory concentration (IC₅₀) and are provided in Table 1. From the results, it is evident that some of the tested compounds displayed moderate growth inhibitory activity. Compound **2d** (IC₅₀ = 0.22 μM) the most potent against PC-3 cell line.

Mechanism of cell death (cell cycle arrest and apoptosis induction)

Regulation of the cell cycle and apoptosis are considered to be effective cancer therapeutic methods and most of the cytotoxic compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of cell cycle or induction of apoptosis. Therefore, it was considered of interest to understand whether the inhibition of cell growth of **2d** was on account of cell cycle arrest. In this study PC-3 cells were treated with **2d** at IC₅₀ concentration for 48 h (Fig. 2).

Apoptosis is one of the major pathways that leads to the process of cell death and is associated with chromatin condensation, nuclear shrinking and fragmented nuclei. Tumor cells often have irregular apoptotic pathways and induction of tumor cell apoptosis by natural or synthetic compounds is considered an effective therapy for cancer.^{15,16} Hence it was of interest to investigate the effects of **2d** on apoptosis inducing effect in PC-3 cancer cells (Fig. 3).

Molecular modeling studies

Molecular modeling studies were also carried out for compound **2d** which has been proved to be most active (on androgen independent cell line) and **2a** (on androgen dependent cell line). To investigate possible interactions of **2d** and **2a** with androgen receptor, we have carried out the docking studies of compound **2d** and **2a** in the active site of androgen receptor. Compounds were constructed and docked with builder tool kit of software package (Fig. 4).

Both compounds are structurally distinct from the current AR antagonists but compound **2a** still demonstrated sufficient binding to the human androgen receptor ligand binding domain (hARLBD). To understand the essential features responsible for the potency of these compounds, the receptor–ligand interaction at the binding site was examined. The hARLBD is well-characterized as a hydrophobic cavity that forms strong hydrophobic interactions with a steroidal core of androgens. In that prospective, both compounds possess hydrophobic scaffolds with one or two polar groups attached, and the docked poses of these compounds adopt similar orientations in the hARLBD compared to crystallographic ligands. As observed from the figure, no polar groups of both compounds form hydrogen bonds. The binding modes of these compounds suggest that hydrophobic interactions may be essential for the ligand coordination, while hydrogen bonding may not play a determining role.

A series of 1,3,4-oxadiazole derivatives carrying aromatic scaffolds were synthesized, characterized and evaluated for their in vitro cytotoxic effect on PC3 and LNCaP cancer cell lines. Compound **2d** bearing phenyl-1,3,4-oxadiazolyl showed cytotoxic effect in low μ M range compared to 5-FU standard. The cytotoxic effect of **2d** is associated with apoptosis induction and cell cycle arrest in sub G₀/G₁ and S phases of the cell cycle. The present investigation revealed that compound **2d** could serve as a lead in the future development of new agents that are more potent and selective in prostate cancer chemotherapy.

Biology: Chemicals

F12K medium, RPMI medium, penicillin–streptomycin antibiotic solution (100x) fetal bovine serum (FBS), trypsin–EDTA solution, phosphate-buffer solution (PBS), 50% glutaraldehyde, crystal violet, propidium iodide, rhodamine-123, IGPAL CA-630, paclitaxel were obtained from Sigma–Aldrich Company (St. Louis, MO, USA). Potassium phosphate, EDTA, D-glucose and ethanol were obtained from Thomas Scientific Company (Swedesboro, NJ, USA).

Cell line maintenance

The LNCaP and PC-3 cancer cells were grown in RPMI-1460 supplemented with 10% heat inactivated FBS, medium containing 50 units of penicillin/mL, 50 g/mL of streptomycin/mL, in T-75 cm² flasks at 37 °C in a humidified atmosphere containing 5% CO₂ incubator. The cells were maintained as ‘monolayer culture’ by serial sub-culturing.

Treatment of cells

The cells were plated at density of 1000–200 cells per well in DMEM supplemented medium, allowed to stabilize overnight in a CO₂ incubator at 37 °C. Next, the cells were treated with compounds (**2a–f**) as well as 5-Fluorouracil (5-FU) as reference compound at various concentrations in a final volume of 1 mL per well in triplicate wells for each treatment for 24 h at 37 °C in a 5% CO₂ incubator. All studies were repeated at least three times.

Cell viability and proliferation

At the end of incubation period, the cell viability was evaluated using Cell Glo assay according to previously reported method. The cells were fixed with 10% trichloroacetic acid for 1 h at 4 °C. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris–HCl for 5 min on a shaker at 1600 rpm. The optical density of each well was measured spectrophotometrically at 564 nm with an ELSA microplate reader (ChroMate-4300, FL, USA). The IC₅₀ values were calculated according to the equation for Boltzman sigmoidal concentration–response curve using the nonlinear regression fitting models. The results reported are mean of at least three separate experiments. Significant differences were analyzed according the ANOVA wherein the differences were considered to be significant at $p < 0.05$.

Cell cycle analysis (flow cytometry)

The PC-3 cells were treated with 0.2 µM of **2d** for 48 h. After treatment, the cells were washed twice with ice-cold PBS, collected by centrifugation, and fixed in ice-cold 70% (v/v) ethanol, washed with PBS, re-suspended with 0.1 mg/mL RNase, stained with 40 mg/mL PI, and analyzed by flow cytometry using FACScalibur (Becton Dickinson). The cell cycle distributions were calculated using Cell Quest software (Becton Dickinson).

Docking

Docking of **2d** and **2a** into the human androgen receptor ligand binding domain (hARLBD): for our docking studies, hARLBD (PDB: 2OZ7) was considered as it correlates very well structurally with the T877A mutant version of hAR as expressed in LNCaP cell. This mutation produces a more promiscuous binding site able to accommodate a broader range of ligands, and removal of the threonine 877 residue removes a key hydrogen bonding moiety. The ligand set comprised of our synthesized investigational oxadiazoles, hydroxyflutamide, R-bicalutamide and cyproterone acetate (CYP) which is co-crystallized with the protein in 2OZ7.pdb. Since the protein complex in the present study has bound ligand (CYP), HYBRID v3.0.1 of OEDocking¹⁶ was chosen as the appropriate docking method for our studies.

To begin with, the docking method in our current study, HYBRID was used to validate the correctness of the bound ligand (CA4) poses upon re-docking into the receptor structure. The top ten docking poses of the bound ligand were examined and superimposed with its original bound conformation in the crystal structure. The poses were identical to the original pose of the cognate ligand (C4A) in 2OZ7 crystal structure with root mean square deviation (rmsd) less than 2 Å for all poses indicating the OEDocking application's reliability as a docking tool in our modeling studies.

The ligands in the present study were sketched using Sybyl sketch Sybyl-X 1.3 Modeling suite.¹⁷ Energy was minimized for every ligand under study and was stored together as a molecule (.sdf) file. The conformer ensembles of these compounds were generated using OMEGA v2.5.1.4^{18,19} prior to docking. OMEGA ensures that low strain energy conformations were retained in the ensemble. By using Structure Preparation tool of the biopolymer module with in Sybyl-X 1.3 modeling suite, Chain A of the crystal structure 2OZ7.pdb was extracted, hydrogen atoms were added potential bumps were corrected and water molecules were removed prior to docking. Finally the structure was energy minimized using MMFF94s force fields and MMFF94 charges assigned. The water molecules in the crystal structure were removed. The resulting refined mutated Androgen receptor was used for docking the prepared ligands.

Chemistry

Reagents and solvents were purchased from Sigma–Aldrich Chemical Company Inc. and used as received. Melting points were determined in open capillaries on a Gallenkamp digital melting point apparatus and were uncorrected. The Infrared spectra were recorded in KBr discs using Shimadzu FT-IR 8000 spectrometer. ¹H NMR (DMSO-*d*₆) and ¹³C NMR spectra were recorded using Bruker 300 MHz spectrometer using tetramethylsilane (TMS) as internal standard. Peak multiplicities are expressed as: s, singlet; d, doublet, t, triplet; q, quartet; dd, doublet of doublet; br, broad; br s, broad singlet; m, multiplet. Thin layer chromatography was performed using precoated silica gel plates (silica gel 0.25 mm, 60G F₂₅₄).

N-(5-Phenyl-1,3,4-oxadiazol-2-yl)-benzamide (2d)

Benzoyl chloride (3.68 mL, 0.032 mol) was added dropwise into the stirred slurry of compound 2-amino-5-phenyl-1,3,4-oxadiazole (5.64 g, 0.035 mol) in 50 mL pyridine. After 2 h, the solution was poured into ice-water; and the white precipitate formed was collected and dried under vacuum. Pure product was obtained by re-crystallization from ethanol, mp 203–205 °C. Yield: 6.28 g, 74%. ¹H NMR (DMSO, 300 MHz) δ/ppm: 7.55–8.05 (m, 10 H), 12.14 (s, 1H). ¹³C NMR (DMSO, 300 MHz): δ/ppm: 165.18, 161.43, 158.14, 133.20, 132.39, 132.00, 129.70, 128.84, 128.45, 126.29, 123.59. IR (KBr): 1713, 1618, 1582, 1391, 1293, 1245, 1023, 694 cm⁻¹. HRMS (ESI⁺) calcd. for C₁₅H₁₂N₃O₂ [M+H]⁺ 266.0929, found 266.0923.

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Figure 1.
Known nonsteroidal androgen receptor antagonists (**I–IV**) and designed prototypes (**2a–f**).

Scheme 1.

Reagents and conditions: (i) pyridine, acid chloride, 0 °C, 1 h. (ii) Pyridine, sulfonyl chloride, 0 °C, 1 h.

Figure 2.

The effect of **2d** on cell cycle was analyzed with a flow cytometry: M1: Sub G0/G1 phase; M2: G0/G1 phase; M3: S phase; M4: G2/M phase. FACS analysis revealed that treatment of PC-3 cells with **2d** led to arrest at the subG0/G1 and S phase of the cell cycle. The sub-G1 population increased indicating that some cells underwent apoptosis. The drug shifted the PC3 cells to the sub G0/G1 and S phase after exposure to drugs for 48 h.

Figure 3.

Flow cytometric apoptosis staining analysis of PC-3 cells: viable (lower left), early apoptotic (lower right), late apoptotic (upper right), and necrotic cells (upper left). The population of early apoptotic, late apoptotic, and necrotic cells increase with the drug treatment. Cells were exposed to 0.2 μ M drug **2d** for 48 h treatments.

Figure 4.
(A) Compound **2d**. (B) Compound **2a** docked in active site of androgen receptor.

Table 1

Cytotoxic activity of the new compounds against LNCaP and PC-3 cancer cell lines

Compound	LNCaP	PC-3
2a	2.5 ± 0.11	8.34 ± 0.20
2b	3.2 ± 0.39	47.8 ± 0.19
2c	3.9 ± 0.26	22.2 ± 0.46
2d	1.33 ± 0.40	0.22 ± 0.10
2e	6.55 ± 0.13	11.06 ± 0.32
2f	32.33 ± 0.83	26.00 ± 0.46
5-FU	0.03 ± 0.39	0.03 ± 0.39