Design, Synthesis, and Biological evaluation of 1,2,4-Oxadiazole based Novel Non-Steroidal Derivatives for the Treatment of Prostate Cancer

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Abstract

This study encompasses the synthesis and evaluation of 25 compounds (SP1-SP25) as potential inhibitors for prostate cancer, utilizing Bicalutamide as a benchmark standard. The synthesis process involves a multi-step approach, commencing with the creation of substituted trans cinnamic acids and substituted amidoximes. These components are subsequently merged to generate intermediate (E)-N'-(cinnamoyloxy)benzimidamides, leading to the final desired (E)-3-phenyl-5-styryl-1,2,4-oxadiazole molecules. Thorough characterization via spectroscopic techniques validates compound structures.

In the context of prostate cancer inhibition, compounds SP6 and SP7 manifest potency equivalent to or exceeding Bicalutamide. Meanwhile, compounds SP8, SP14, SP16, SP18, SP20, SP21, SP24, and SP25 exhibit moderate inhibitory activities. Contrarily, compound SP17 demonstrates markedly diminished potency. These findings identify candidates for further exploration, although considerations extending beyond potency, such as safety and selectivity, are pivotal in drug development. Insights into structural attributes contributing to potency provide a foundation for refinement.

The subsequent stages entail preclinical model assessment and detailed mechanistic studies to ascertain compound efficacy. Selectivity assays and toxicity evaluations are imperative to ensuring safety profiles. This investigation underscores the intricate landscape of prostate cancer treatment and underscores the need for comprehensive evaluation before clinical translation.

Keywords: Prostate Cancer, Oxadiazole, Bicalutamide, PC-3

Introduction

One of the most prevalent cancers and the second leading cause of cancer-related deaths in males worldwide is prostate cancer[1]. As per the recent reports of World Health Organization (WHO), a total of 1.41 million males are suffering from the prostate cancer while approx. 3,76,000 were died due to prostate cancer[2]. Prostate, situated below the urinary bladder, is a part of male reproductive system and has been classified as exocrine gland which have a size of a walnut with three lobes[3]. Prostate cancer or carcinoma of prostate gland is generally developed at the age of 50 years in men with the symptoms like increased frequency of urination, weak flow during urination, red coloured urine/ blood in urine, persistent lower back pain, trouble with urination & incomplete emptying of urinary bladder[4]. These are the various symptoms which can classify a man with prostate cancer. A multiple treatment options are available for the prostate cancer like radiation therapy in which high powered X-Ray damaged the DNA and kill prostate cancer cells[5], cryotherapy by which cancerous cells of prostate were damaged by freezing the tissues, hormonal therapy, removal of prostate by surgery (prostactomy) & chemotherapy[6]. Chemotherapy act by antagonizing the androgen receptors which is precursor for binding of androgens[7]. Chemotherapy is further divided in to two parts: a) Steroids based antiandrogen agents and b) non steroids based anti-androgen agents for prostate cancer treatment[8]. Few Steroidal antagonists are marketed like oxendolone[9], cyproterone[10], spironolactone[11], etc[12], but these agents have various limitations like poor oral bioavailability & pharmacokinetics[13], potential hepatotoxicity, lack of tissue selectivity, cross reaction with other steroid receptors[14] and structural modifications of steroidal ligands are limited because of its rigidity[15]. Further, many non-steroidal antagonists like flutamide, nilutamide, *R*-bicalutamide, apalutamide, etc., are available in market and more favourable for clinical applications (**Figure 1**)[16]. These agents are having various advantages over steroidal antagonists such as good tissue selectivity, favourable pharmacokinetics properties, androgen receptor specificity, lack of steroidal related side effects & it also allows more flexible structural modifications[17]. Although, non-steroidal has more advantages but still clinical applications of these agents have been limited due to its side effects like gynecomastia, hepatoxicity after long term administration. These drawbacks of currently available drugs emphasize the need for the development of new candidates with high anti-prostate cancer activity and low adverse effects[18]. Therefore, present study of this research work emphasis on

design, synthesis of novel non-steroidal heterocyclic derivatives and *in vitro* investigation of these derivatives towards androgen receptors.

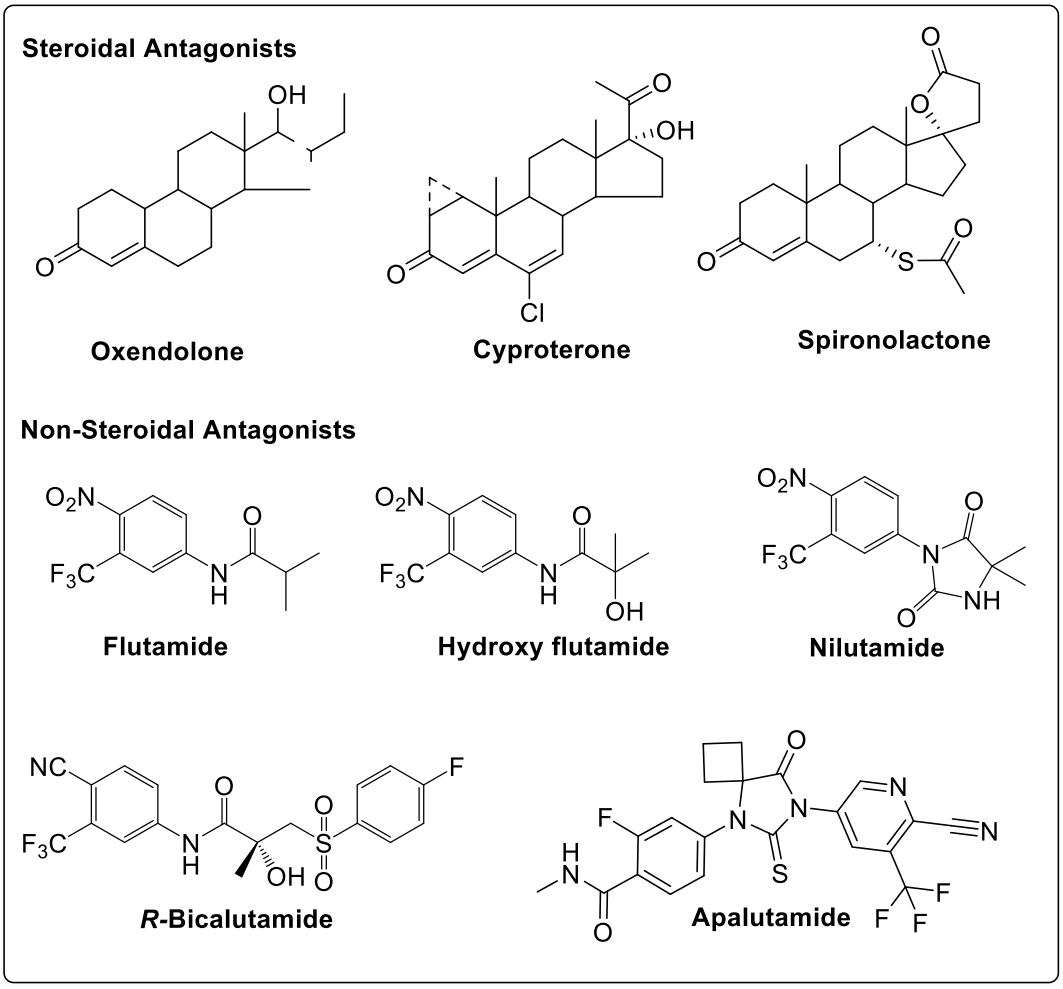


Fig 1: Marketed drugs for the treatment of prostate cancer

Rationale

Bicalutamide is one of the drugs for the treatment of prostate cancer. From the structure of bicalutamide, it was seen that **bicalutamide** contain two rings of benzene substituted with electron withdrawing groups. Moreover, from the literature survey it was found that various researchers had synthesized derivatives of bicalutamide as effective antiprostate cancer agent. For e.g., Kandil S.B. *et al*., designed and synthesized a series of bicalutamide derivatives and after evaluation it was found to be compound **4** (**Figure 2**) was the effective compound for the treatment of prostate cancer[19]. Apart from that Kandil S.B. *et al.*, also synthesized deshydroxy derivatives of bicalutamide and evaluated that compound **2** was one of potent derivatives as antiprostate cancer agent. So, target molecules were designed by keeping in mind that there

should be two rings substituted with various electron withdrawing groups (EWGs) or in other words the ring should be electron deficient[20].

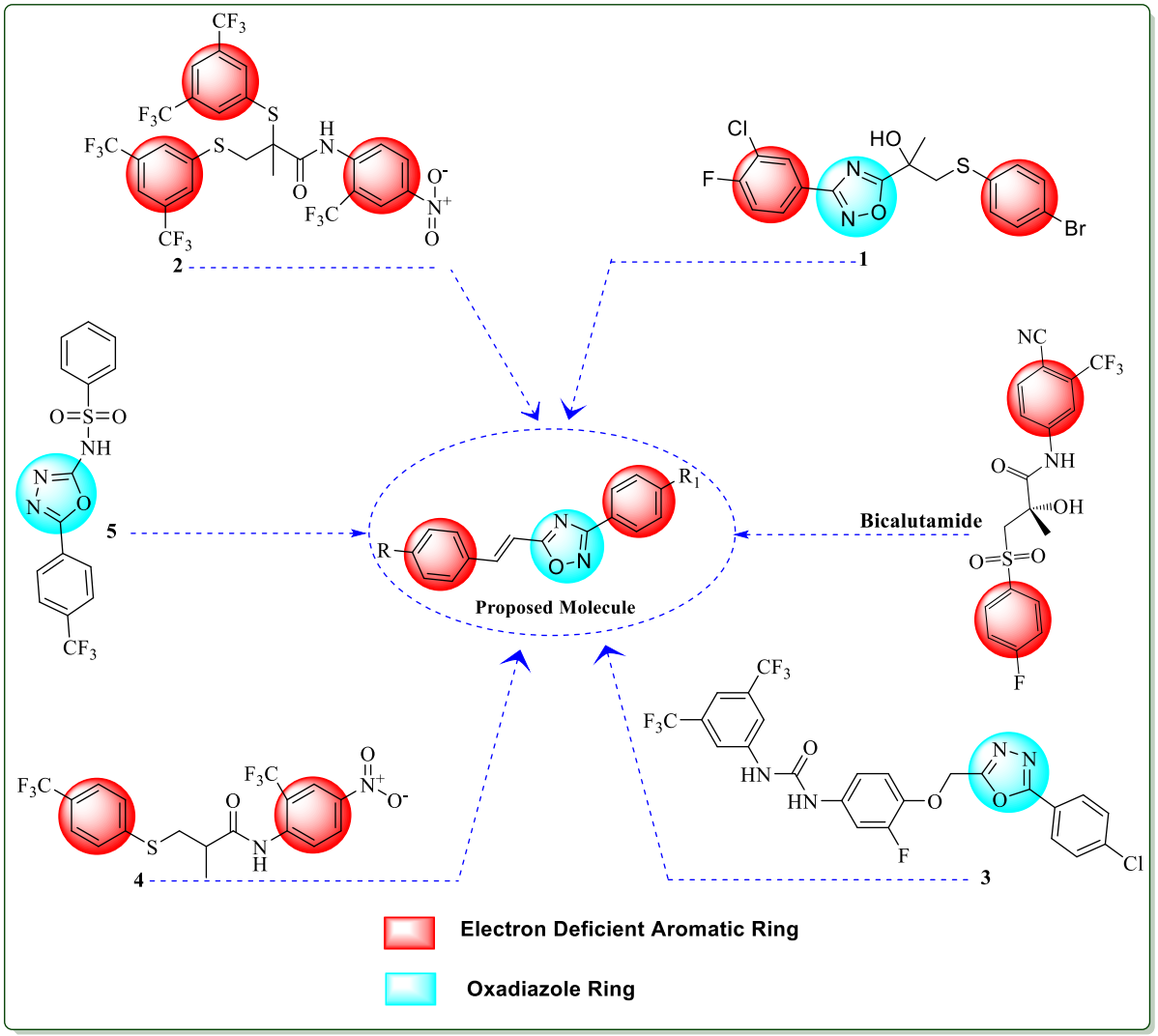
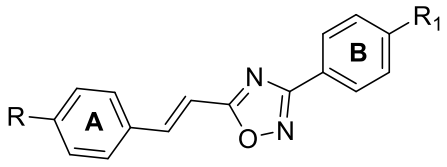


Fig 2: Rationale and designing of proposed compounds.

Further, from the literature survey it was noted that heterocyclic moieties play a major role for the treatment & prevention of various diseases especially cancer. Khatilk G.L. *et al*., designed and synthesized sulfide and sulfonyl subordinates of 1,2,4-oxadiazoles and after evaluation it was assessed that compound **1** (**Figure 2**) was the potent compound for treatment of prostate cancer[21]. Furthermore, Mochona B. *et al*., has synthesized 1,3,4-oxadiazole derivatives substituted with electron deficient rings and revealed compound **5** (**Figure 2**). as potent antiprostate cancer agent. Gamal E.D.M. *et al*., has also synthesized 1,3,4-oxadiazole derivatives (**3**, **Figure 2**). as one of the potent antiprostate cancer agent[22]. Thus, various heterocyclic moieties were chosen to replace the acyclic part of bicalutamide so that side effects given by present marketed drugs can be avoided by the proposed molecules. At last, after

considering all these findings the proposed molecule was designed by keeping electron deficient rings aside the heterocyclic moieties (**Figure 2**).

After the designing of proposed compound, various substitutions were done on both the ring. Ring **A** & **B** was substituted with Chloro, Fluoro, Bromo & Triflouro Methyl to make both the ring electron deficient. Five membered heterocyclic ring oxadiazole used for designing of compounds[23]. On the fact noticed during literature survey *para* substitution was done on Ring **A** & Ring **B**. Moreover, a vinyl bridge was introduced to attach the Ring A with heterocyclic ring and based on the above a total of 25 molecules (**SP1-SP25**) were designed (**Figure 3**)[24].



SP1-SP25

SP1: R = H; R_1 = H	**SP14**: R = Cl; R_1 = Br
SP2: R = H; R_1 = F	**SP15**: R = Cl; R_1 = CF_3
SP3: R = H; R_1 = Cl	**SP16**: R = Br; R_1 = H
SP4: R = H; R_1 = Br	**SP17**: R = Br; R_1 = F
SP5: R = H; R_1 = CF_3	**SP18**: R = Br; R_1 = Cl
SP6: R = F; R_1 = H	**SP19**: R = Br; R_1 = Br
SP7: R = F; R_1 = F	**SP20**: R = Br; R_1 = CF_3
SP8: R = F; R_1 = Cl	**SP21**: R = CF_3; R_1 = H
SP9: R = F; R_1 = Br	**SP22**: R = CF_3; R_1 = F
SP10: R = F; R_1 = CF_3	**SP23**: R = CF_3; R_1 = Cl
SP11: R = Cl; R_1 = H	**SP24**: R = CF_3; R_1 = Br
SP12: R = Cl; R_1 = F	**SP25**: R = CF_3; R_1 = CF_3
SP13: R = Cl; R_1 = Cl	

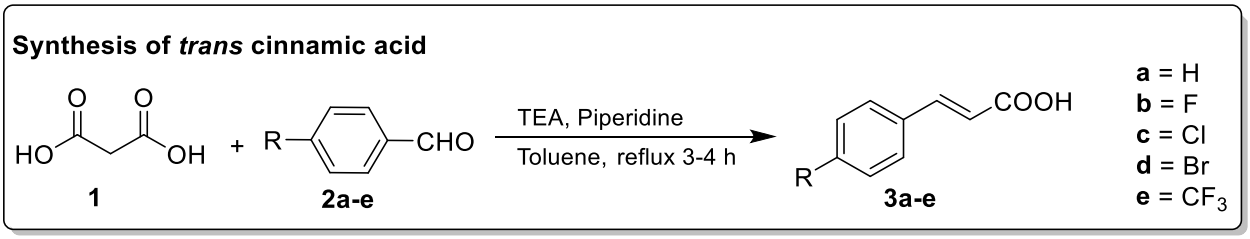
Fig 3: Structure of various designed compounds

Material & Methods

Synthetic-grade chemicals and reagents were utilized and acquired from various vendors such as BLD Pharm, Loba Chemie, and Merck, as well as local suppliers. Commercial-grade pure solvents were employed. The structural characterization of the synthesized compounds was accomplished following recrystallization and purification through column chromatography. The ^{1}H NMR and ^{13}C NMR spectra were obtained using the Avance III HD series from Bruker at frequencies of 400 MHz and 100 MHz, respectively, and the chemical shifts were recorded in parts per million (ppm). Mass spectra within the range of 0 to 700 m/z were recorded using a Gas Chromatograph-Mass Spectrometer, specifically the Shimadzu model. Melting point of the compounds were checked using capillary tube and melting point apparatus.

Procedure for synthesis of substituted *Trans* cinnamic acids (3a-e)

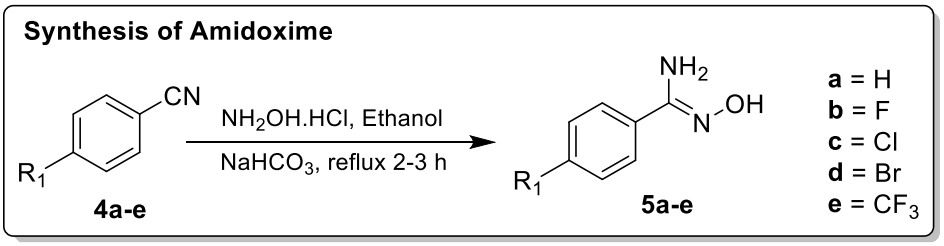
Malonic acid (4.7 mmol) and triethylamine (6.1 mmol) were mixed with 5 mL of toluene and stirred vigorously for 3-4 minutes. Subsequently, an aromatic aldehyde (4.7 mmol) and piperidine (0.8 mL) were slowly added to the mixture while maintaining vigorous stirring. Once the addition was completed, the mixture was refluxed at the reflux temperature for 3-4 hours. TLC was taken at regular intervals to continuously monitor the progress of the reaction. After the completion of the reaction, the mixture was subjected to vacuum distillation to remove the solvent and triethylamine after the complete consumption of benzaldehyde. The resulting mixture was cooled to room temperature. Next, 5 mL of a 5% w/v sodium bicarbonate solution was added and stirred for 10 minutes. The resulting reaction mixture was then washed with 10 mL of ethyl acetate, and the aqueous layer was cooled to 0°C. Finally, the acidic pH of the aqueous layer was adjusted by adding HCl. The resulting solid was filtered and dried in an oven at 60°C (**Scheme 1**).



Scheme 1: Synthesis of Cinnamic acid

Procedure for the synthesis of amidoxime (5a-e)

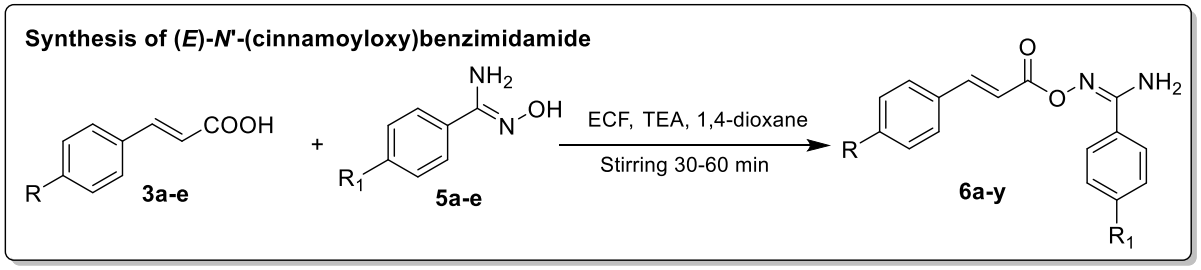
Substituted amidoximes (**5a-e**) were synthesized from the corresponding nitriles. Benzonitriles (9.69 mmol) were added to 15 mL of ethanol, along with 5 equivalents of hydroxylamine hydrochloride, and the mixture was stirred at room temperature for 15 minutes. After 15 minutes, 3 equivalents of sodium bicarbonate were added gradually, and the resulting mixture was refluxed for 2-3 hours. The progress of the reaction was monitored using TLC, and once the nitriles were completely consumed, the mixture was subjected to vacuum distillation to remove the solvent. To the resulting viscous solution, an excess amount of distilled water was added, and the mixture was partitioned with ethyl acetate. The ethyl acetate layer was separated, and the solvent was evaporated under vacuum distillation to obtain the corresponding amidoximes (**Scheme 2**).



Scheme 2: Synthesis of substituted Amidoxime

Procedure for the synthesis of substituted (*E*)-N'-(cinnamoyloxy)benzimidamide (6a-y)

Cinnamic acid (1.8 mmol), triethylamine (TEA) (3 mmol), and 1,4-dioxane (5 mL) were mixed, and ethyl chloroformate (3 mmol) was added dropwise to the mixture. The resulting reaction mixture was stirred at room temperature for 15 minutes. Subsequently, a solution of amidoxime (2.5 mmol) in 1,4-dioxane (5 mL) was added to the reaction mixture, and the resulting mixture was stirred at room temperature for 15-30 minutes. Once the reaction was complete, the reaction mixture was concentrated using vacuum distillation. It was then diluted with 25 mL of cold water, resulting in the formation of a precipitate. The precipitate was filtered, washed with cold water, and dried at room temperature. The resulting solid was purified by silica gel column chromatography eluted with ethyl acetate & *n*-hexane to yield pure compounds (**Scheme 3**).

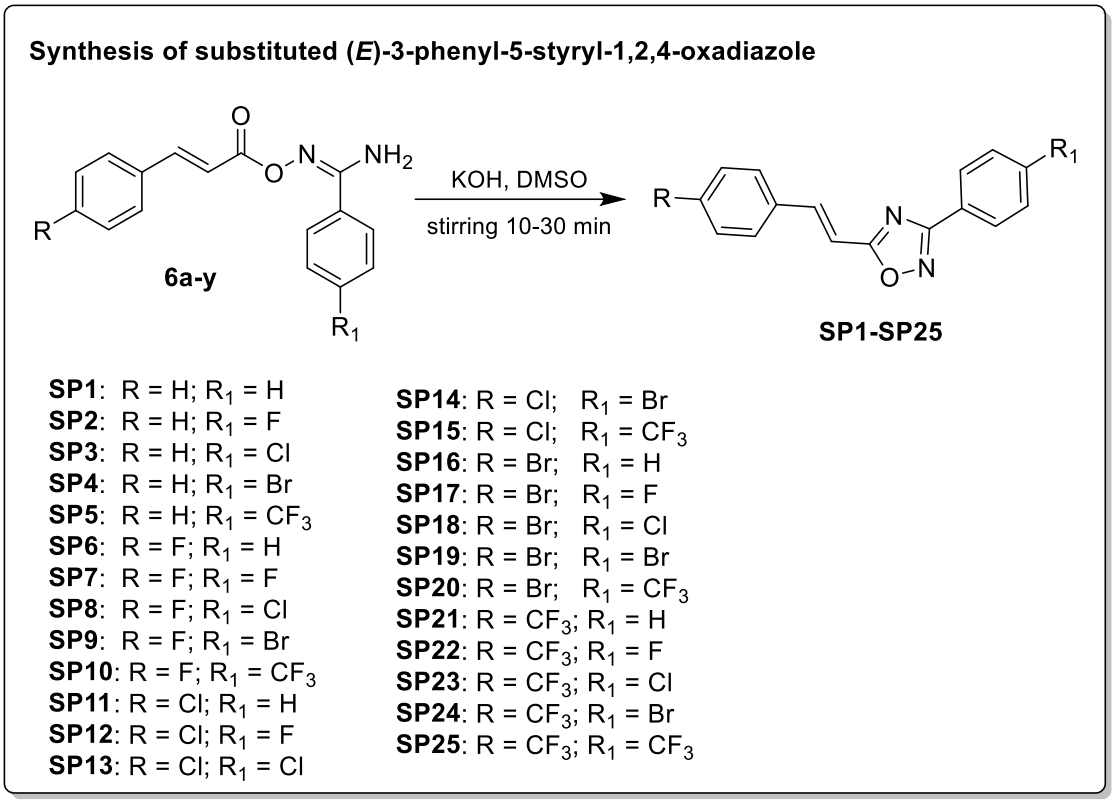


Scheme 3: Synthesis of (*E*)-N'-(cinnamoyloxy)benzimidamide

Procedure for the synthesis of substituted (*E*)-3-phenyl-5-styryl-1,2,4-oxadiazole

A suspension of potassium hydroxide (1.5 mmol) in 5 mL of dimethyl sulfoxide (DMSO) was prepared by stirring. To this suspension, (*E*)-*N*'-(cinnamoyloxy)benzimidamide (1.5 mmol) was added. The reaction mixture was stirred for 15-20 minutes, and the progress of the reaction was monitored using TLC. Upon completion of the reaction, the reaction mixture was diluted with 25 mL of cold water, resulting in the formation of a precipitate. The precipitate was filtered, washed with water, and dried at room temperature. The resulting solid was purified by

silica gel column chromatography, using a mixture of ethyl acetate and *n*-hexane as the eluent, to obtain the pure compound (**Scheme 4**).



Scheme 4: Synthesis of target molecule (*E*)-3-phenyl-5-styryl-1,2,4-oxadiazole

Procedure of *in vitro* study upon PC-3:

Cell Viability-

High-glucose DMEM with 10% foetal bovine serum and 5% carbon dioxide was used to cultivate PC-3 cells. The MTT test was used to determine the cytotoxicity of **SP1** to **SP25**, **MS1** to **MS15** and the standard Bicalutamide at a variety of concentrations on PC-3 cells. In brief, 96 well plates were seeded with 9×10^4 cells, and the plates were kept at 37°C and 5% CO_2 for 24 hours. After 24 hours of incubation, the cells were treated with Bicalutamide and of **SP1** to **SP25**, **MS1** to **MS15** with concentrations of 10, 50, 100, 200, 400, 600, 800, and 1000 nM of compounds. To test PC3 cell viability, 10 µL of 5mg/mL MTT in 96 well plates were applied to the cells and incubated for two hours at 37 degrees Celsius.

ROS Measurement-

A total of 1×10^5 PC-3 cells were seeded onto 12-well plates and treated with SP-6 and MS-14 to determine their effect on total ROS production. After 24 hours of treatment, plates were

treated with SP-6 at 100 nM and 200 nM, MS-14 at 150 and 300nM and Bicalutamide with concentration of 100nM, followed by supplemented with a final concentration of 10 µM of the sensitive fluorescent probe DCFH-DA, and the mixture was incubated for 30 minutes at 37°C. After the cells were stained and washed, they were analyzed using flow cytometry.

Androgen Receptor inhibition assay

Treatment with *SP-6 at 100 nM and 200 nM, MS-14 at 150 and 300nM* and Bicalutamide at 200 nM for 24 hours was performed on $1x10^5$ PC-3 cells seeded and grown in each well of 12 well plates. The cells should be harvested after 24 hours, pelleted, and the supernatant discarded. In 100 µl of 4% formaldehyde, the cell pellets should be fixed for 15 minutes. The pellet can be centrifuged and washed in 1X PBS to get rid of any remaining formaldehyde. After the cells have been permeated with cold 100% methanol, they should be centrifuged and washed twice with 1X PBS. After 30 minute incubation, resuspend the cells in 100 µl of diluted primary antibody Androgen receptor Rabbit (GTX100056). After three washes in 1X PBS, we centrifuged the samples for further five minutes at 1500×g. Following a centrifugation step and two washes in 1XPBS, the cells were incubated at room temperature with 100 µl (1:25) of diluted fluorochrome conjugated secondary antibody Alexa fluro 488 for 30 minutes. The cells were centrifuged, washed with 1X PBS, and the supernatant was thrown away. Cells were resuspended in 200-500 µl of 1X PBS for flow cytometric analysis, and the data was processed using the Flowjo V10 program.

Immunofluorescence study for AR inhibition

In a 35 mm high glass bottom dish, $1×10^5$ PC-3 cells were planted to examine the inhibition of Androgen receptor expression. After 24 hours after being exposed to 100 nM and 200 nM SP-6, 150 nM and 200 nM MS-14 and 200 nM Bicalutamide, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% triton X in 1X PBS. After 24 hours of treatment with Androgen receptor Rabbit (*GTX100056*) (1:50), the cells were washed three times with 1X PBS to remove any residual unbound primary antibody. The cells were then incubated with Anti-rabbit Alexa Fluor 488 secondary antibody (cat. 4412; 1:100) for 1 hour. Cells expression of androgen receptor was analysed, using fluorescence microscopy imaging using an Olympus CFX41.

Results and Discussion

Table 1: Biological Evaluation of the SP1-SP25 against PC-3 Cell lines

Sr. No.	**Compound Code**	**IC_{50} (nM)**
1.	SP1	887.5
2.	SP2	854.04
3.	SP3	880.55
4.	SP4	991.08
5.	SP5	1129.03
6.	SP6	238.129
7.	SP7	281.42
8.	SP8	348.72
9.	SP9	1375.35
10.	SP10	934.016
11.	SP11	1395
12.	SP12	1245.76
13.	SP13	883.35
14.	SP14	526.39
15.	SP15	883.35
16.	SP16	758.71
17.	SP17	**1645.04**
18.	SP18	556.33
19.	SP19	950.014
20.	SP20	684.77
21.	SP21	790.178
22.	SP22	1271.12
23.	SP23	950.014
24.	SP24	893.419
25.	SP25	1017.76
26.	Bicalutamide	148.359

Synthesis of 25 compounds (**SP1-SP25**) were done in four steps: Initially, various substituted *trans* Cinnamic acids (**3a-e**) were synthesized using malonic acid and various substituted benzaldehyde *via* Knoevenagel condensation while substituted amidoximes (**5a-e**) were synthesized using various substituted benzonitriles. After this, substituted intermediate (*E*)-N'-(cinnamoyloxy)benzimidamide (**6a-y**) were synthesized by clubbing both amidoximes and *trans* cinnamic acids together. And finally, desired molecules i.e. (*E*)-3-phenyl-5-styryl-1,2,4-oxadiazole (**SP1-SP25**) were synthesized by cyclizing the **6a-y** in the presence of base and DMSO. All the synthesized compounds were characterized and confirmed through spectroscopic techniques like 1^{H} & 13^{C} NMR & Mass spectroscopy.

The dataset presents a collection of compounds' IC50 values (in nM), with Bicalutamide used as the standard reference compound. Bicalutamide's IC50 value is 148.359 nM, and the IC50 values of other compounds are compared against this standard.

Higher Potency: Compounds SP6 and SP7 exhibit IC50 values of 238.129 nM and 281.42 nM, respectively, both falling within the range of Bicalutamide's potency. This suggests that these compounds are on par with, or even more potent than, Bicalutamide in inhibiting the target.

Moderate Potency: Several compounds, including SP8, SP14, SP16, SP18, SP20, SP21, SP24, and SP25, show moderate inhibitory potency with IC50 values ranging from around 348.72 nM to 1017.76 nM. While these compounds are less potent than Bicalutamide and the aforementioned highly potent compounds, they still exhibit significant inhibitory activity.

Lower Potency: Compounds such as SP1, SP2, SP3, SP4, SP5, SP9, SP10, SP11, SP12, SP13, SP15, SP19, and SP23 have IC50 values higher than Bicalutamide's, indicating that they are relatively less potent inhibitors.

Significantly Lower Potency: Compound SP17 stands out with the highest IC50 value of 1645.04 nM, suggesting it is notably less potent than Bicalutamide and most other compounds in this dataset.

Discussion:

The comparison of compounds' IC50 values to Bicalutamide's standard provides valuable insights into their potential as inhibitors. Those compounds exhibiting higher potency than Bicalutamide are promising candidates for further investigation and development. It's worth noting that while a compound might have comparable or even better potency than

Bicalutamide, other factors such as safety, selectivity, and potential side effects need to be carefully considered in the drug development process.

The structural characteristics that contribute to Bicalutamide's potency could also apply to compounds with similar or higher potencies. Analyzing the structural elements common to these compounds could offer insights into the key features required for effective inhibition.

Furthermore, conducting additional assays, such as selectivity assays against other targets and toxicity evaluations, is essential to comprehensively assess the suitability of these compounds as potential therapeutic agents.

Conclusion

In this study, 25 compounds (SP1-SP25) were synthesized in a multi-step process and evaluated as potential inhibitors for prostate cancer, using Bicalutamide as a reference standard. Compounds SP6 and SP7 demonstrated comparable or greater potency than Bicalutamide, indicating their potential as strong inhibitors. Compounds SP8, SP14, SP16, SP18, SP20, SP21, SP24, and SP25 displayed moderate inhibitory activities. Conversely, compounds like SP17 exhibited significantly lower potency. The findings suggest potential candidates for further investigation; however, factors beyond potency, such as safety and selectivity, must be considered in drug development. Insights into structural features contributing to potency provide a foundation for optimization. Moving forward, preclinical models and mechanistic studies are needed to assess compound efficacy, while selectivity assays and toxicity evaluations are crucial for safety assurance. This research underscores the complexity of prostate cancer treatment and the importance of comprehensive evaluation before clinical translation.

Conflict of Interest

The authors declared no potential conflict of interest.

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