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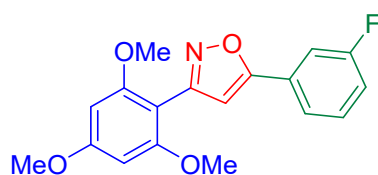
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Design, synthesis and biological evaluation of 3,5-diaryl isoxazole derivatives as potential anticancer agents

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26

IC₅₀ : 15.893 (μM) against PC3 cancer cell line
Selectivity: 7.51



Design, synthesis and biological evaluation of 3,5-diaryl isoxazole derivatives as potential anticancer agents

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ABSTRACT

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The present study was carried out in the attempt to synthesize a new class of potential anticancer agents comprising eleven compounds (**24-34**) sharing the 3,5-diarylisoxazole as a core. The chemical structure of the new synthesized compounds was established by IR, ¹H NMR, ¹³C NMR and elemental analysis. Their biological potential towards prostate cancer was evaluated by using cancer PC3 cells and non-tumorigenic PNT1a cells. Interestingly, compound **26** distinguished from others with a quite high selectivity value that is comparable to 5-FU. The binding mode of **26** towards Ribosomal protein S6 kinase beta-1 (S6K1) was investigated at a molecular level of detail by employing docking simulations based on GLIDE standard precision as well as MM-GBSA calculations.

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among men worldwide and it is the second leading cause of cancer deaths within the male population in western countries.^{1,2} 174,650 new prostate cancer cases were estimated to be diagnosed in 2019, representing approximately 10% of new cancer cases within the same period.³ Various approaches such as radical prostatectomy, radical radiotherapy, chemotherapy, and hormone ablation therapy

disease in early stage clinically localized prostate tumors.⁴ However, modern medical approaches remains to be ineffective in the treatment of advanced prostate cancer.⁵ Therefore, novel treatment strategies and tools are needed to be developed in order to enhance the success of the current diagnostic and therapeutic methods.⁶

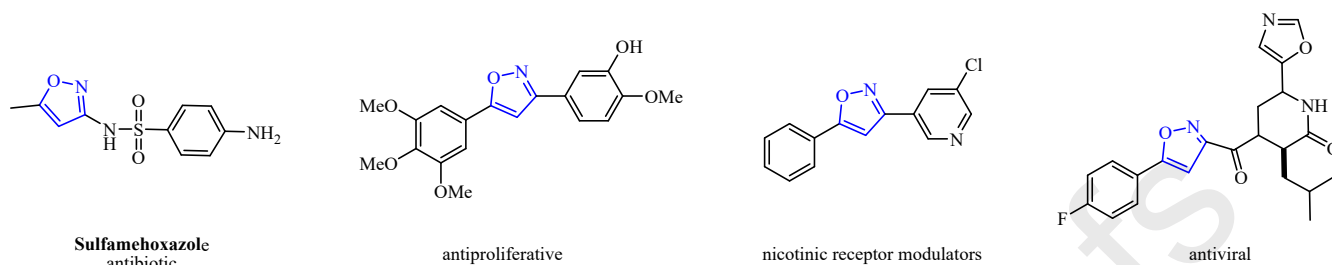


Figure 1. Some 3,5-disubstituted isoxazole based compounds with a spectrum of biological properties.

Recent developments in organic chemistry have contributed significantly to progress in many fields of science, particularly medicinal chemistry. The common characteristic of the most newly synthesized organic compounds is the presence of at least one heterocyclic ring in their structure. Thus, heterocyclic chemistry is an important tool for the search of new active substances in biological applications. One of the most interesting heterocyclic rings is isoxazole, which is a five-membered ring containing oxygen and nitrogen atoms. The synthesis of the isoxazole ring, which has a wide range of applications, is 1,3-dipolar cycloaddition to the alkenes and alkynes with nitrile oxide and the reaction of a 1,3-diketone or α,β -unsaturated ketone containing three carbons with hydroxyl amine.⁷ Isoxazole derivatives, a special unified class of compounds, exhibit antibiotic, antiproliferative, and antiviral properties and act as nicotinic receptor modulators (Fig. 1).⁸

In our previous paper, fluoro and hydroxyl substituted chalcone derivatives exhibited high antiproliferative activities against A549, A498, HeLa, A375, HepG2 cancer cell lines.⁹ These results encouraged and enabled us to plan the development of new chalcone derivatives. In addition, we investigated the role of the two aromatic rings of the chalcone structure for the activity and the role of the linker group (the enone moiety) to design more rigid compounds by incorporating heterocyclic fragments. The discovery of some 3,5-diarylsubstituted isoxazole-based compounds with a spectrum of antiproliferative properties in the literature has led to better address our studies towards chalcone-like systems whose enone bridge was replaced by isoxazole linker. The preliminary anticancer screenings on prostate cancer cell lines demonstrated these new chalcone-like systems show the best cytotoxic effect on PC3 cell line. Based on this cytotoxicity screening, further studies are required to provide more insights

about the combination of heterocyclic pharmacophores in a hybrid molecule.

In this study, we aimed at preparing 3,5-diarylsubstituted isoxazoles for the construction of new drug-like molecules and at evaluating their biological activities against different cancer cell lines along with an immortalized normal prostate epithelial cell. The general structure of the synthesized compounds is shown in Figure 2.

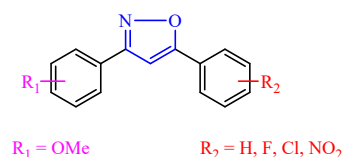
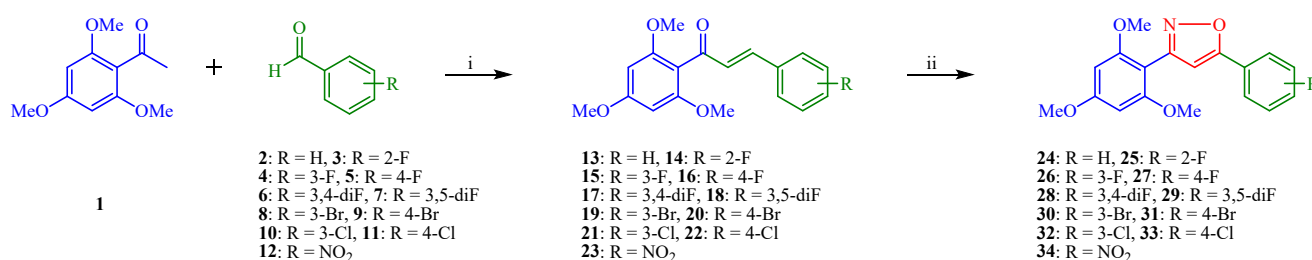


Figure 2. General structure of synthesized compounds.

The synthetic procedure for the preparation of 3,5-diarylsubstituted isoxazoles is depicted in Scheme 1. The condensation of trimethoxyacetophenone (**1**) with related benzaldehydes **2-12** in an aqueous solution of KOH afforded chalcone derivatives **13-23** in 70-90% yields through the adoption of a general synthesis protocol.⁹⁻¹⁰ Then, reaction of chalcone derivatives **13-23** with tosylhydroxylamine in the presence of K_2CO_3 and H_2O in MeOH afforded the isoxazole derivatives **24-34** in 60-78% yields.¹¹ The newly synthesized target compounds **24-34** were characterized using IR, 1H and ^{13}C NMR as well as elemental analysis. The characterization data of the target molecules were found to be compatible with their structure. The details of spectral data are given in the experimental section and spectra are provided in supplementary file.



Scheme 1. General synthetic procedure. Reagents and conditions: (i) **1**, **2-12**, KOH, (ii) **13-23**, tosylhydroxylamine, K_2CO_3 , H_2O , MeOH

We initially investigated the effects of the compounds on the proliferation capacity of PC3 cells by employing *in vitro* cancer model and PNT1a cells as corresponding normal control. Sorted in the order of the selectivity values from higher to smaller, compounds **26**, **30**, **27**, **29**, **25**, **34**, **28**, and **24** proved to be effective and selective against cancer cells compared to normal prostate epithelial cells (Fig. 3A-D). Disappointingly, compounds **31**, **32**, and **33** lack such selectivity against cancer cells being their

observed selectivity values lower than 1. Interestingly, compound **26** distinguished from other compounds with a quite high selectivity value that is even comparable to 5-FU. Table 1 reports the selectivity of the entire pool of our 3,5-diarylsusbstituted isoxazole derivatives along with 5-FU as a reference towards both cancerous PC3 and non-tumorigenic PNT1a cells. Importantly, all the structures listed in Table 1 comply the Lipinski's rule of five as shown in Supporting Information.

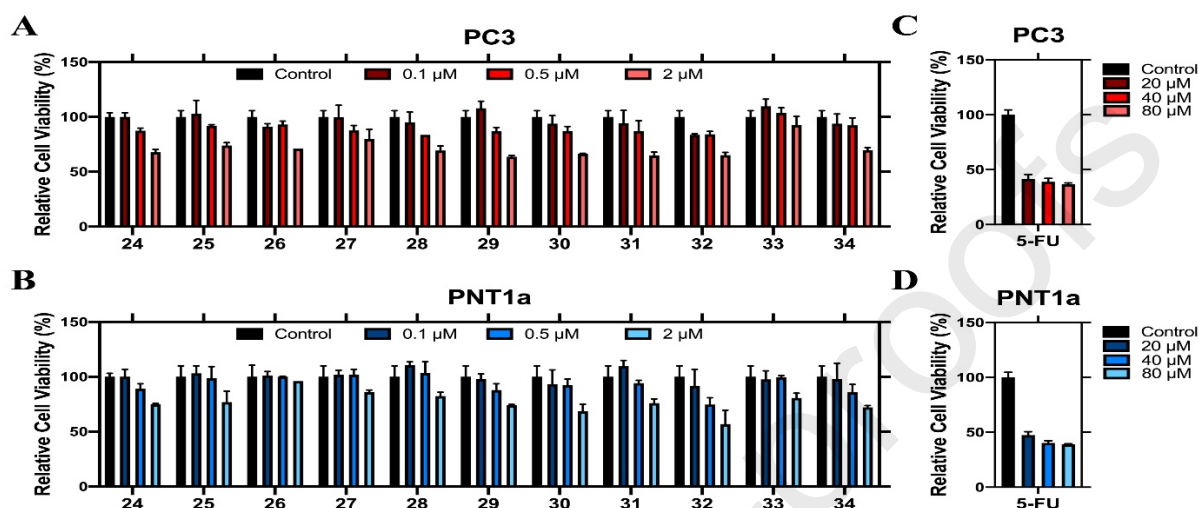


Figure 3. Relative viability of PC3 and PNT1a cells treated with diarylsusbstituted isoxazole derivatives or 5-FU as a reference.

To predict potential drug targets for these compounds, we used the PPB2 Polypharmacology Browser, which indicate several key proteins with a likely mechanistic role to explain at a molecular level the observed selective inhibitory potential against cancer cells (see Supplementary File for the list of predicted targets). To minimize the occurrence of false positive results and thus to properly prioritize the predicted targets, we excluded 1) those shared indistinctly by all the eleven compounds experimentally tested, 2) those shared by compounds with discordant selectivity values (that are greater or lower than 1 at the same time), or 3) those related to irrelevant genes considering prostate cancer pathogenesis. Interestingly, CYP1A1, CYP1A2 and CYP1B1

were predicted as putative potential targets for those compounds having experimental selectivity values greater than 1. More interestingly, compound **26**, which is provided with the highest observed selectivity value, matched 2 unique targets that are CDC like kinase 4 (CLK4) and Ribosomal protein S6 kinase beta-1 (S6K1), which did not occur in the other remaining compounds. Considering their oncogenic potential in prostate cancer as well as in other cancers, selective targeting of these 2 genes might serve as a potential tool for selective elimination of cancer cells that exclusively have higher expression for these genes compared to normal cells.

Table 1. IC₅₀ and selectivity values of compounds tested on PC3 and PNT1a cells

Compounds	IC ₅₀		Selectivity
	PNT1a	PC3	PC3
24	3.40 ± 0.52	2.66 ± 0.22	1.28
25	5.21 ± 1.11	3.58 ± 0.15	1.46
26	117.33 ± 3.78	16.79 ± 6.24	6.99
27	11.89 ± 0.96	5.06 ± 0.47	2.35
28	9.16 ± 1.21	7.25 ± 2.54	1.26
29	9.51 ± 2.89	4.77 ± 1.54	1.99
30	21.81 ± 1.32	6.68 ± 1.36	3.27
31	6.89 ± 1.58	5.19 ± 0.96	1.33
32	4.49 ± 0.25	14.38 ± 5.27	0.31
33	15.74 ± 4.36	26.44 ± 7.15	0.60
34	5.58 ± 0.79	13.09 ± 2.85	0.43
5-FU	9.70 ± 0.57	1.64 ± 0.87	5.90

S6K1, docking studies were performed through GLIDE standard precision docking and MM-GBSA calculations. The redocking analysis of the cognate ligand F177 returned docking score and ΔG binding values equal to -10.439 kcal/mol and to -99.25 kcal/mol, respectively, as well as RMSD (Root Mean Square Deviation) value as small as 0.305 Å compared to the X-ray posing. As shown in Table 2, docking analysis of **26** was carried out, by returning docking score and ΔG binding values equal to -6.419 kcal/mol and -59.07 kcal/mol, respectively.

Table 2. Docking score and ΔG binding values of F177 cognate ligand and **26**.

Compound	Docking Score	ΔG binding
F177 cognate-ligand	-10.439 kcal/mol	-99.25 kcal/mol
26	-6.419 kcal/mol	-59.07 kcal/mol

Such a discrepancy in scoring when comparing **26** and F177 could be due to the different interactions established at the binding site. However, the estimation of the ligand efficiency (LE)¹², which represents a size-dependent measure for effective binding, disclosed that **26** would be a promising starting point for molecular optimization based on the observation that a smaller gap existed when comparing the LE values of **26** vs F177 that was -0.267 kcal/mol vs -0.316 kcal/mol.

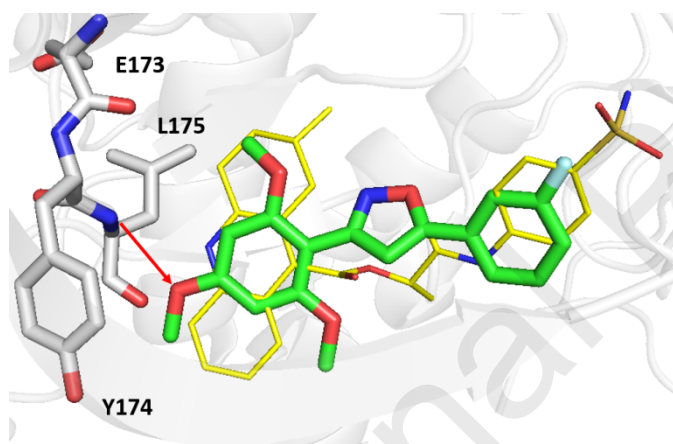


Figure 4. Zoomed in view at the binding pocket of S6K1 (PDB entry: 3WF9) whose residues are rendered in gray sticks and labeled. Green sticks and yellow wireframes represent **26** and F177 cognate ligand, respectively. Red arrows indicate hydrogen bonds.

As shown in Figure 4, the aromatic rings of **26** are nicely superimposed to the sulfomoylphenylamino ring and methyltetrahydroacridine ring of F177, which faces the hinge region of the protein.¹³

In addition, F177 and **26** share the same hydrophobic contacts with the following residues: L97, G98, K99, G100, V105, A121, L172, E173, Y174, L175 and M225. Furthermore, **26** established a hydrogen bond through its *para*-methoxyl engaging the backbone nitrogen atom of L175 in the hinge region, likewise F177 cognate ligand.

CYP1A1 and CYP1A2 demonstrated to not be present in normal prostate samples.¹⁴ However, they were reported to be expressed in PC3 cells.¹⁵ Besides, CYP1B1 has been shown to have oncogenic properties in prostate cancer.^{16,17} Considering our

compounds tested might be associated with expression of those genes exclusively in cancer cells.

CLK4 is one of the critical components of pre-mRNA splicing, which is quite important for cellular functions.¹⁸ In a recent study, inhibition of CLK4 proteins was postulated as a novel anticancer strategy, which aimed at selective depletion of cancer-relevant proteins after turnover.¹⁹ Interestingly, its inhibition resulted in the depletion of another putative target for **26**, that is S6K1.¹⁹

S6K1 is one of the downstream targets of PI3K/Akt/mTOR signaling pathway, which is particularly involved in mRNA translation, ribosome biogenesis, proliferation, metabolism, development, aging and malignancies like cancer.²⁰ One of the critical negative regulators of this signaling pathway PTEN, is lost in almost 80% of prostate cancers, resulting in constitutive activation of PI3K/Akt/mTOR signaling pathway.²¹ PC3 cells used in our tests are known to be null for PTEN, therefore have constitutively active PI3K/Akt/mTOR signaling, which is not the case for PNT1a cells.²² Recent studies also pointed the hyperactive status of S6K1 in PC3 cells due to active PI3K/Akt/mTOR signaling.^{20,22,23} Most importantly, **26** did not kill DU-145 prostate cancer cells, which are wild type for PTEN (Supplementary Figure 1). This might explain selective targeting of cancer cells with active PI3K/Akt/mTOR signaling by **26**. Interestingly, in a recent study, a S6K1 inhibitor PF-4708671 was demonstrated to particularly reduce migration and proliferation of PC3 cell line, but not DU-145 prostate cancer cells that carry wild type PTEN alleles.²⁴ Docking studies conducted on **26** proved its potential as S6K1 inhibitor being able to interact at the level of the hinge region of S6K1 protein likewise F177 cognate ligand and other inhibitors.¹³

In summary, the novel (2,4,6-trimethoxyphenyl)isoxazole compounds were synthesized in two steps, formation of the corresponding chalcones by a well-known standard method and condensation with TsNHOH under strongly basic conditions in good yields and purities. The preliminary anticancer screenings on prostate cancer cell lines demonstrated that the chalcone with a fluoro substituted show the best cytotoxic effect on PC3 cell line. Based on this preliminary cytotoxicity screening, further studies are required to provide more insights about the combination of heterocyclic pharmacophores in a hybrid molecule and their structure-activity-relationship in prostate cancer therapy. Also, Multi-fingerprint Similarity Search algorithm (MuSSel)^{25,26} was used to find other potential targets. In this respect, protein tyrosine phosphatase 1B (PTP1B) is the first target for 10 out of 11 molecules. PTP1B is related with obesity and type 2 diabetes mellitus and PTP1B was also found involved with breast, pancreas gastric, ovarium cancer and prostate cancer in recent years.²⁷ These result may be very helpful us for forward research. Further synthetic work on similar structures containing heterocyclic hybrids are currently in progress for generating new targeted libraries for biological screenings.

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