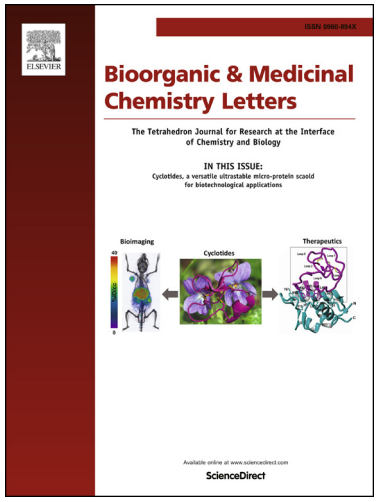

Synthesis, biological evaluation and molecular docking study of 1-amino-2-aroylnaphthalenes against prostate cancer

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Synthesis, biological evaluation and molecular docking study of 1-amino-2-aroylnaphthalenes against prostate cancer

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Abstract: A series of functionalized naphthalene was synthesized and screened against human prostate cancer cell line (PC-3). The *in vitro* antiproliferative activity of the synthesized compounds was evaluated by monitoring their cytotoxic effects against PC3 by using MTT assay. We observed that compound **5f** resulted in more than 50% cell death at 14µM. Treatment of PC-3 cells with **5f** provides apoptosis by flow cytometry. Western blotting showed decreased expression of pro-caspase 8 and 9. Our study shows that cancer cell treated with **5f** has higher concentration of reactive oxygen species as compare to untreated sample, which facilitate cancerous cell to enter apoptosis. Exact mechanism by which ROS is generated after **5f** treatment is still under study. Molecular docking study further strengthens the results obtained from *in vitro* experiments. Compound **5f** can be considered as a promising leads for anticancer agent against prostate cancer cells due to its potent cytotoxic activity and apoptotic effect.

Key words: Naphthalene; Prostate cancer; oxidative damage; apoptosis; cytotoxicity

Prostate cancer is the fifth leading cause of cancer-related deaths in men worldwide and this disease is frequently observed in males at the age between 65-74 years [1]. A report in 2014 says that approximately 24 lac men in USA suffered from prostate cancer and was the cause of their mortality [2]. Prostate cancer are androgen sensitive in the beginning which respond to anti-

hormonal therapy, later they turn out to be refractory and develop without androgen. This cancer is known as castrate resistant prostate cancer (CRPC), and difficult to treat. Recently, US Food and Drug Administration have approved various new molecules for the treatments of prostate cancer patients with castration resistant prostate cancer, but very few have provided survival benefits [3, 4]. Recently, a review have been published on drug designing for prostate cancer [4]. Thus search of new molecules with effective therapeutic properties for prostate cancer is much more required.

Various skeletons have been studied for their anticancer properties. Some of the naphthalene based molecules like naphthalenediimide (NDI) is present in various anticancer agents and they can intercalate with DNA during the interaction [5-9]. Various molecules containing 2-aminodiaryl ketones as a main or substructure showed activity as antitubulin agents. Some of the reported compounds like Phenstatin **I** and Hydroxyphenstatine (**II**) [10] and 2-aminobenzophenone analogues (**III**) act as antitubulin agents [11]. 2-Amino-1-aroylnaphthalene and 2-hydroxy-1-aroylnaphthalene were studied for their anti-proliferative activity against human cancer cell and their activity was almost comparable to the potency of colchine [12]. Molecules containing aminated phenyl aryl ketones as substructure like suitably substituted 4*H*-imidazo [1, 5-*a*] [1, 4] benzodiazepines and related compounds act as central benzodiazepine receptor (CBR) ligands (Fig. 1) [13]. Another naphthalene based compound neo-transhinlactone

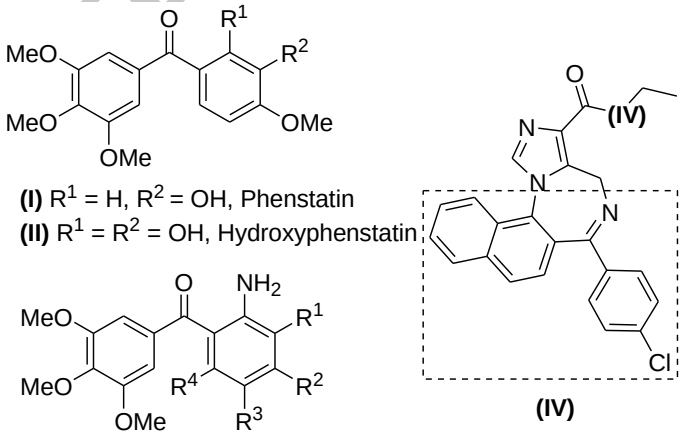
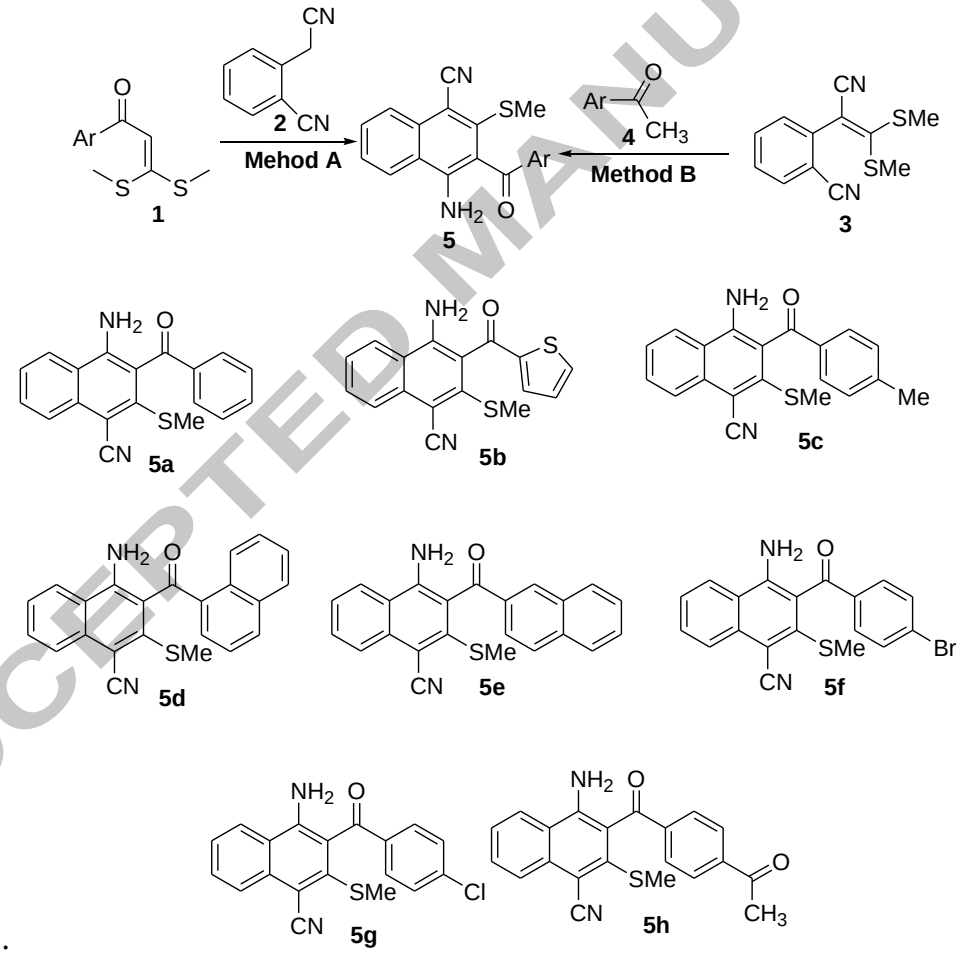


Fig. 1. Structure of phenstatin **I**, hydroxyphenstatine **II**, 2-aminobenzophenone analogues **III** 3-substituted 6-aryl-4*H*-imidazo-[1,5-*a*]benzodiazepines (**IV**)

is reported as anticancer compound and is isolated from *Salvia miltiorrhiza* [14-16]. Since naphthalene based molecules exhibit very good anticancer activity, we became interested in the synthesis of some differently functionalized naphthalene and to study their effect against prostate cancer cell line.

In order to perform the study, we have synthesized various functionalized naphthalene by two methods. Compound **1** was synthesized by reaction of acetophenone, carbon disulphide and methyl iodide under basic conditions at low temperature. Similarly, compound **3** can be synthesized by using 2-cyanomethylbenzonitrile instead of acetophenone. Under method A, we have used 4,4-bis-methylsulfanyl-arylethanone as a precursor and perform the reaction with 2-cyanomethylbenzonitrile under basic condition to achieve the desired product in good yield.



Scheme 1. Synthesis of various 4-amino-3-aroyl/heteroaroyl-2-methylsulfanylnapthalene-1-carbonitriles (**5a-h**)

While in alternative method B 2-(1-cyano-2,2-bis-methylsulfanyl-vinyl)-benzonitrile was used as a starting material. Stirring mixture of 2-(1-cyano-2,2-bis-methylsulfanyl-vinyl)-benzonitrile and acetophenone under basic conditions provided the desired functionalized naphthalenes (Scheme 1). All the synthesized compounds are characterized by spectroscopic technique and the results obtained are reported earlier [17].

We have synthesized a number of compounds but on the basis of results obtained from molecular docking studies, we have selected this series **(5a-h)** for further studies.

Table 1: Growth inhibition values of all the compounds measured by MTT assay[a]

S.No.	Compound	Growth Inhibition (%)
1	5a	24.76
2	5b	23.36
3	5c	22.42
4	5d	15.88
5	5e	13.08
6	**5f**	**84.24**
7	5g	24.76
8	5h	63.97
9	cisplatin	87.27

[a]Results expressed are Mean of three independent experiments.

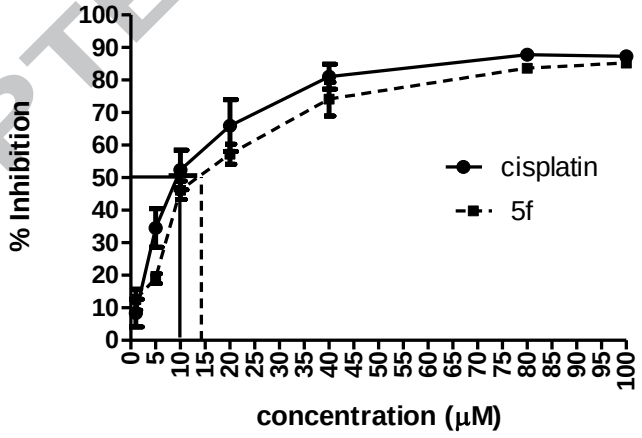
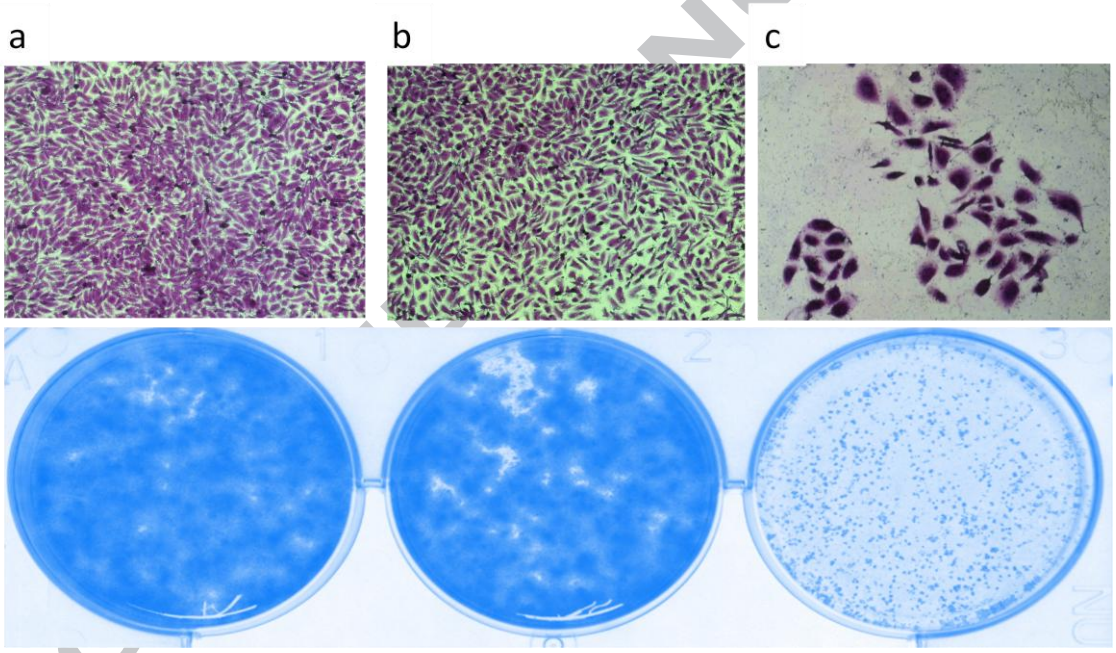


Fig 2. Cytotoxicity of **5f** and cisplatin was measured by MTT assay. PC-3 cells were treated with different concentration of Cisplatin and compound **5f (**1µM, 5µM, 10µM, 20µM, 40µM, 80µM and 100µM).

The *in vitro* antiproliferative activity of synthesized compounds mentioned in **Table 1** was evaluated by monitoring their cytotoxic effects on human prostate cancer (PC-3) cell line. In the initial screening **5f** emerged as a potential antiproliferative compound. At tested concentration (100µM) compound **5f** showed 84.24% growth inhibition. For the calculation of IC_{50} value of compound **5f**, PC-3 cells were cultured with varied concentration of **5f** solubilised in DMSO. We estimated the cytotoxicity of a known anticancer drug cisplatin also on PC-3 cells as a reference compound. Due to cytotoxicity of DMSO, its concentration was kept below 0.1% throughout the experiments and also the control reaction was set with and without DMSO. After 72 hours, the compound showed 50% cell growth inhibition at 14 µM concentration (IC_{50}), however the IC_{50} value for cisplatin was found to be 10 µM (**Fig 2**).

A



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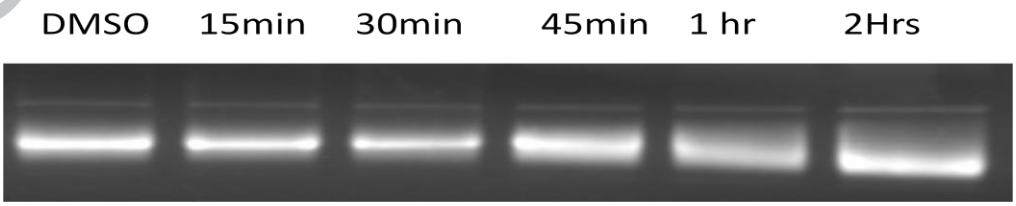


Fig. 3. (A) Colony formation assay; After 24 hours of seeding, cells were treated with 14 µM of compound **5f** and incubated for 14 days (with change of media with fresh media supplemented with compound every 48 hours). Light microscopy Nikon 6000i image was used to visualize the

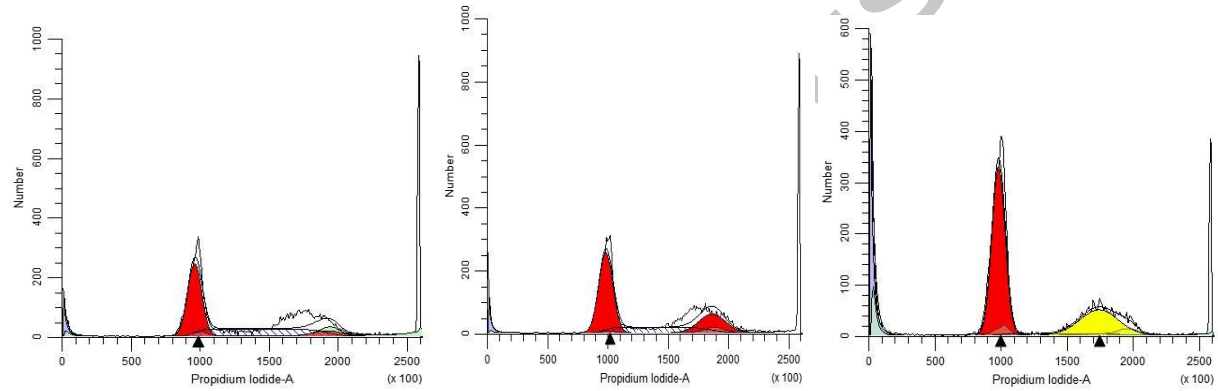
cells of a) Control; b) DMSO and c) compound 5f. GelDocX+ image of control, DMSO control and compound treated of same well plate in lower panel. (B**)** DNA fragmentation assay was performed in time dependent manner by taking 200 ng of pUC19 DNA incubated with 14 µM of compound **5f** in 15 µl reaction mixture. The fragmentation was analyzed by resolving the samples on Agarose gel.

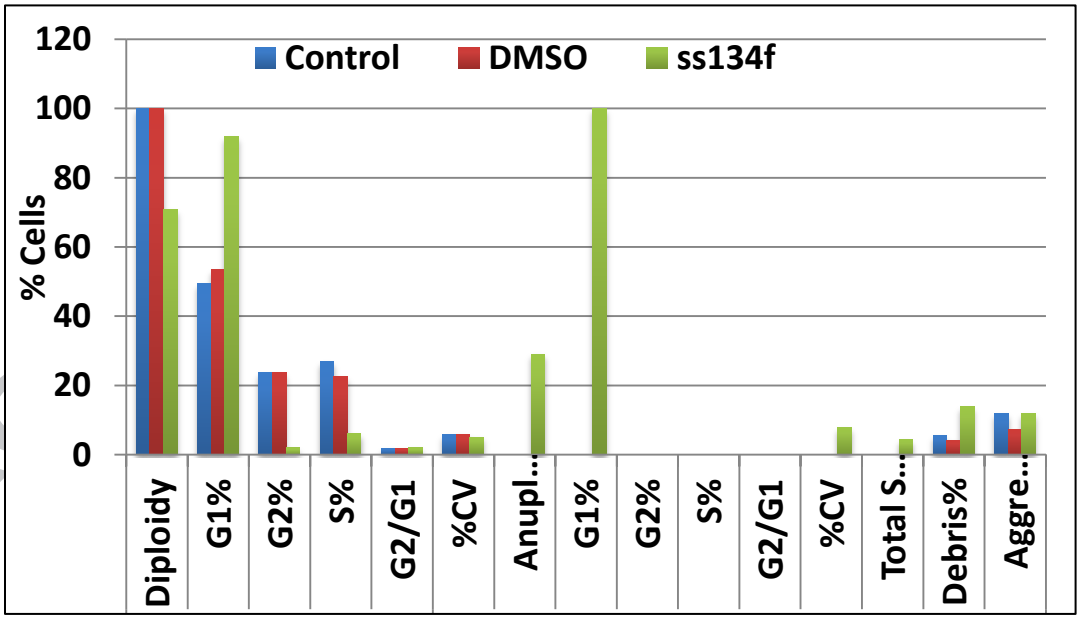
Clonogenic assay is the ability of a single cell to grow into a colony and tests every cell in the population for its ability to form tumor. For this, PC-3 cells were seeded and treated with 14µM of the compound. After 14 days, cells were washed and fixed with 3.8 % formaldehyde and stained with crystal violet dye and observed for the clonogenic ability. **Fig 3A** shows significant decrease in the clonogenic ability after drug treatment as compare to control. These observation suggests that **5f** might reduce the ability of PC-3 cells to form colony i.e. tumorogenic ability and along with cell death, cells morphology was also changed. We also noticed chromosomal aberrations indicated by large number of necrotic and apoptotic cells after treatment. To check the effect of compound on DNA, DNA cleavage assay was performed by agarose gel electrophoresis using supercoiled pUC18 plasmid DNA. DNA degradation was observed after 45 minutes of incubation with **5f** (**Fig 3B**). This assay also confirms the presence of endonuclease activity in the compound which might be due to generation of reactive oxygen species.

Encouraged from result of **5f** induced apoptosis in PC-3 cells, we further investigated the effect of the compound on cell cycle by flow cytometry after treatment of PC-3 cells. PC-3 cells were treated with 14 µM of the compound and after 48h were fixed and stained with propidium iodide. The different phases of cell cycle were analyzed by flow cytometry and it is observed that the cell cycle was arrested in G1 phase and indicate that compound has capability to induce G2/M arrest and progress to apoptosis (**Fig 4A**). To further verify its pro-apoptotic function, flow cytometry analysis was carried out after double staining with propidium iodide and annexin V-FITC. As shown in **Fig 4B**, treatment of PC-3 cell lines with 14µM of the compound resulted in significant increase in apoptosis as compared to control. Further, to confirm that **5f** treated PC-3 cells show cell death which is primarily due to apoptosis, we measured the level of apoptotic markers in treated and untreated samples by western blotting. Decreased expression of pro-caspases 8 and 9 was observed in **5f** treated samples as compared to control (**Fig 4C**), indicating that these pro-caspases are converted to caspase 8 and 9 respectively. These results suggest an enhancement in both intrinsic as well as extrinsic apoptotic pathways. The kinase Akt, an

oncoprotein correlated to cell survival and proliferation is upregulated in several cancers and is responsible for resistance to apoptotic cell death. In this study, the decreased level of phosphorylated Akt in presence of **5f** in comparison to control indicates inhibition in cell proliferation as activated Akt overcome the cell cycle arrest in G1 and G2 phases. These results collectively suggest that the compound **5f** inhibit the growth of PC-3 cell line by inducing apoptosis. The assay revealed that the rate of apoptosis increased in a time dependent manner when compared with control groups (Table 2). Naphthalene based thiosemicarbazone derivatives are also reported to cause cell death of LNCaP Prostate cancer cell line via apoptosis [18].

A





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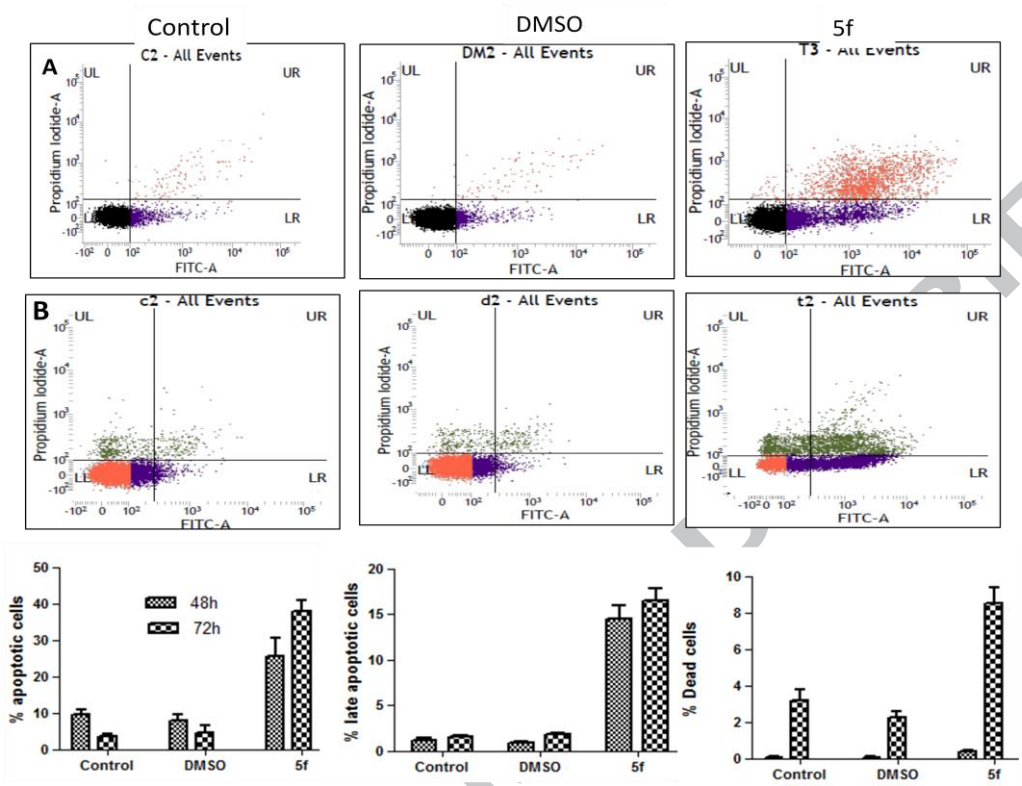

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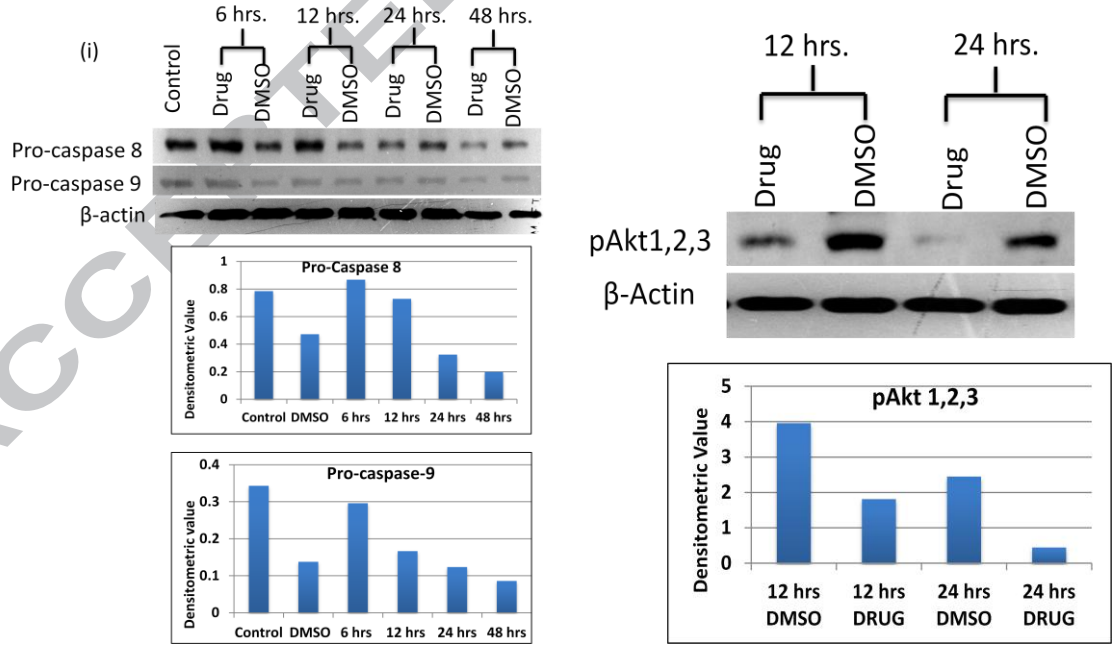


Figure 4 Cell death analysis of PC-3 cells in control, DMSO treated and 5f (14µM) treated samples. Flow cytometry was performed to detect whether cell death occurred due to Apoptosis

or Necrosis. In **Fig 4B** Y-axis shows propidium Iodide level and X-axis shows Annexin V. Panel A and B shows results obtained after 48 h and 72 h respectively after treatment with compound **5f**. Lowest panel shows the comparison between percentage changes in cell death after 48h and 72h of treatment. **Fig 4C** Immunoblotting analysis of procaspase 8 and 9 and pAkt1, 2, 3 after 20 h of treatment with compound **5f** (14µm); Samples were collected at different time points.

Table 2. Apoptotic percentages of the control and compound **5f** treated groups (n=3). Results are expressed as Mean±SD.

		48 hours	72 hours
Early Apoptosis	Control	9.48± 1.46	3.61±0.66
	DMSO	7.80 ± 1.58	4.64±2.05
	5f	25.48± 5.07	37.9±3.06
Late Apoptosis	Control	1.17±0.181	1.54±0.08
	DMSO	0.82±0.14	1.80±0.15
	5f	14.52±1.48	16.46±1.30
Necrosis	Control	0.05±0.034	3.14±0.621
	DMSO	0.06±0.02	2.27±0.32
	5f	0.36±0.08	8.52±0.84

As it was observed that **5f** has apoptotic as well as necrotic effect on PC-3 cell line and showed DNA damage, we hypothesize that this could be mediated through generation of reactive oxygen species (ROS). To find the effect of this compound on generation of ROS in the cell, we estimated SOD levels in cell lysate. SOD assay was done because it is one of the indirect methods to detect the ROS level inside the cell. The result shows (**Fig. 5**) significant time dependent decrease in SOD level and after 48 hours of treatment, the SOD level in control was approximately 80% whereas in **5f** treated cells, it was approximately 50%. High SOD level in control denotes higher superoxide concentration, whereas low SOD level in treated sample reflects that superoxide concentration is less and thus the ROS level is higher as compare to untreated sample. However the exact mechanism by which ROS is generated after **5f** treatment is yet to be understood.

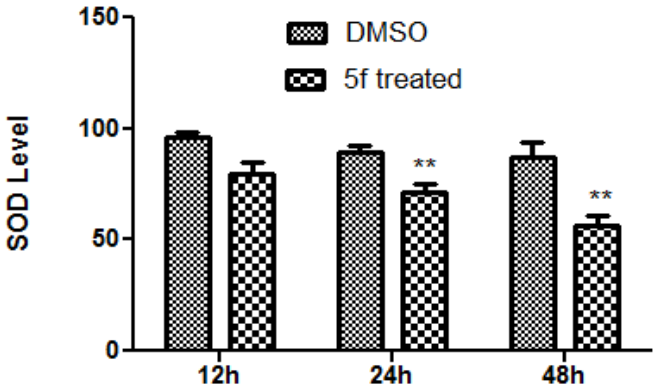


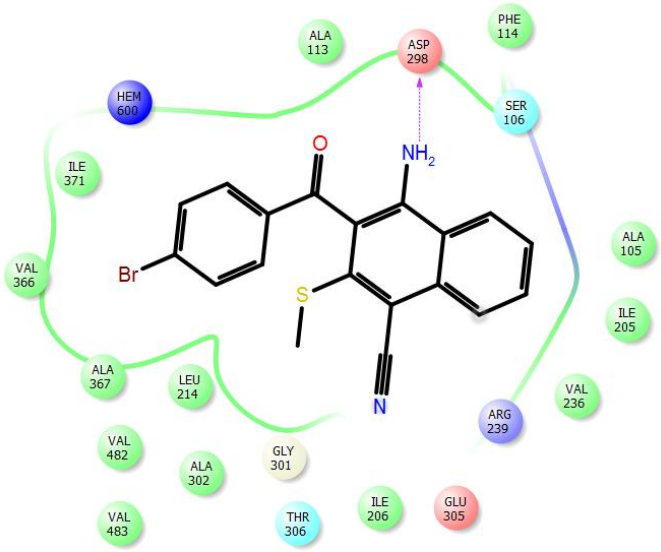
Fig. 5. SOD levels in PC-3cell lysate after treatment with compound **5f** (14µM). SOD level was measured at different time intervals (12 h, 24 h and 48 h). ($p \leq 0.05$).

The aim of the molecular docking study was to elucidate whether the newly synthesized compound **5f** as well as cisplatin, a known drug currently used in the treatment of prostate cancer modulates the cell survival by prostate cancer protein receptor cytochrome P450 17A1 (P450c17), and to study their possible mechanisms of action. Molecular docking simulation results revealed that both compounds were able to interact with the enzyme, and binds strongly with the cytochrome P450 receptor.

The docking results for compound **5f** showed high binding affinity docking score indicated by -7.136 with the anticancer target cytochrome P450 receptor and also showed the formation of a H-bond of 2.0Å with acidic amino acid residue Aspartic acid 298 (Fig. 6A). Within the **5f**-cytochrome P450 complex, the chemical nature of binding site residues within a radius of 4 Å was basic (polar, hydrophobic, positive charged), for example, Arg-239 (Arginine); acidic (polar, negative charged), for example, Glu-305 (Glutamic acid) and nucleophilic (polar, hydrophobic), for example, Thr-306 and Ser-106 (Threonine); aromatic (hydrophobic), for example, Phe-114 (Phenylalanine); aliphatic (hydrophobic), for example, Gly-301(Glycine), Leu-214 (Leucine), Val-236, Val-482, Val-483, Val-366 (Valine), Ile-205, Ile-206, Ile-371(Isoleucine); aromatic (hydrophobic), for example, Phe-114 (Phenylalanine);aliphatic hydrophobic, for example, Ala-113, Ala-105, Ala-302, Ala-367 (Alanine); as a result, the bound compound showed a strong hydrophobic interaction with anticancer target protein cytochrome P450 receptor, thus leading to more stability and activity of this compound (Fig. 6B). The docking results for the control compound cisplatin with the anticancer target protein cytochrome P450 receptor showed a low

binding affinity docking score, indicated by a -3.687 without any H-bond (hydrogen bond) formation. Thus, the docking procedure of Glid software (Maestro10.7, Schrödinger) in reproducing the experimental binding affinity seems reliable, and therefore predicted as true positive.

A



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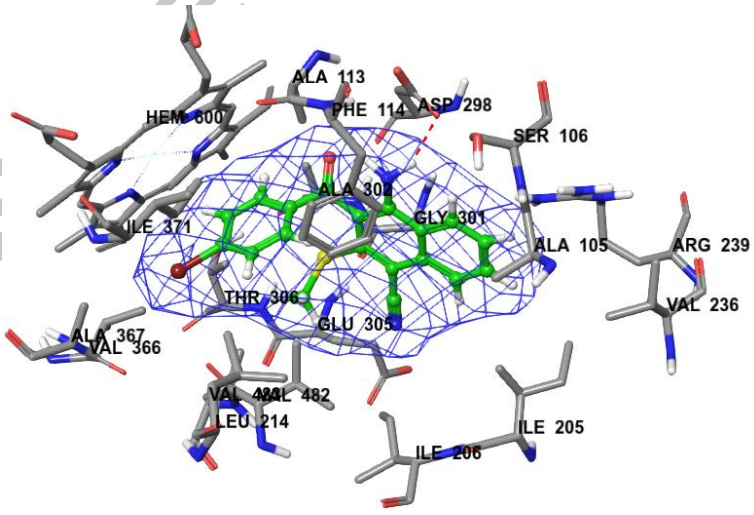

Fig. 6A: 2D Ligand interaction diagram of compound **5f** with anticancer target protein cytochrome P450 using Maestro (Schrödinger) program with the essential amino acid residues at the binding site are tagged in circles. **Fig. 6B:** The 3D docking pose showing interaction for compound **5f** binding model with anticancer target protein cytochrome P450. **5f** docked on to

cytochrome P450 (PDB: 3RUK) with high binding affinity, as indicated by a docking score of -7.136.

In conclusions, we have synthesized and studied eight naphthalene functionalized with amino and aroyl group and screened them for antiproliferative potential against prostate cancer (PC-3) cell line. MTT assay indicated that, out of eight compounds tested, compound **5f** showed 50% of cell death on PC-3 cells. To understand the mechanism of action Flow cytometry analysis was performed which showed that compound **5f** causes apoptosis of PC-3 cells. We also studied the effect of this compound for ROS production in the cell, and used indirect methods to detect the ROS level inside the cell. We observed significant time dependent decrease in SOD level in **5f** treated cells indicate the ROS level is higher. We have also performed the molecular docking study of compound **5f** exhibit high binding affinity docking score about -7.136 with the anticancer target cytochrome P450 receptor, which suggest that further modification may provide the lead molecule.

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Conflict of Interest

The Authors declare no competing interests.

Supporting Information

^{1}H and ^{13}C spectra for all the reported compounds are provided in the SI.

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Graphical abstract

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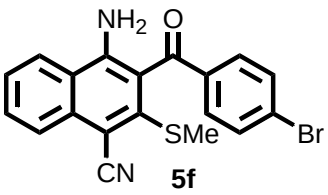
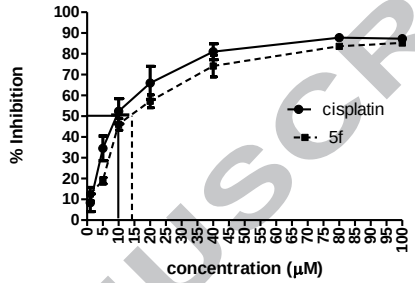


5f

- IC_{50} value for **5f** = 14 µM
- IC_{50} value for cisplatin = 10 µM



- Cytotoxicity of **5f** and cisplatin was measured by MTT assay