Design, synthesis and molecular docking study of thiophenyl hydrazone derivatives as tubulin polymerization inhibitors

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ABSTRACT

A series of thiophene derivatives substituted at 2, 3 and 5 positions were designed and synthesized using 2,3-disubstituted thiophene aldehyde and alcohol as key building blocks. In vitro cytotoxicity assessed against PC-3, DU145 (prostate), A549 (lung), HT29, HCT116 (colon), MCF7, MDAMB231 (breast), B16F10 (melanoma) NCI (Colorectal) cancer cell lines by conducting (MTT) assay of thiophene derivatives. Most of these synthesized compounds showed anti-cancer activity, compound 5b showed good cytotoxicity with IC50 = 2.61 ± 0.34 μM on HT29 cell line. Also, the key property of cell migration was observed while treating cells with 5b. The cell cycle arrest at G2/M phase was observed by 5b on HT29 cell line which inhibited tubulin polymerization with IC50 value of 8.21 ± 0.30 μM. Moreover, binding pose with co-crystal ligand and interaction with colchicine binding site of 5b was established by molecular docking studies. Hence this scaffold can be developed as anti-cancer agents that target tubulin polymerization.

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Introduction

Cancer is the second most leading cause of mortality globally and about 1 in 6 deaths are mainly due to cancer. There were over 1.8 million new cases in 2018. In 2021, 1,898,160 new cancer cases and 608,570 cancer deaths are projected to occur in the United States. Colorectal cancer is the third most commonly occurring cancer in men and the second most commonly occurring cancer in women.

One of the approaches for the treatment of cancer is the disruption of tubulin dynamics. The colchicine binding site is one of the most preferable pockets for potential tubulin polymerization inhibitors. Next to the colchicine binding site, vinca alkaloid site and taxane sites are regularly useful binding pockets. For cancer treatment, FDA approved drugs are paclitaxel, docetaxel, and vincristine which belong to vinca alkaloid site. As per the previous literature natural compounds incorporating heterocyclic unit display good bioactivity (Fig. 1). In addition, ample importance was given for the thiophene and furan ring derivatives in the development of anticancer agents through tubulin inhibition (Fig. 2). Both thiophene and benzene derivatives show similar activity because of its similarity in \([6 \pi(\pi)]\) electrons resonance as well as aromaticity. In comparison with benzene moiety the presence of heteroatom in the thiophene ring may improve its bioavailability and subsequently it may enhance its therapeutic value. Interestingly thiophene used as a central ring system or as a part of the fused ring system in scaffold synthesis, and replacement of aromatic rings in biologically important scaffolds with thiophene also lead

![Figure 1. Structure of some representative bioactive natural products containing thiophene, furan moieties.](image-url)
Improved bioavailability and patentability of drug candidate.

Hydrazone derivatives are biologically active molecules found in many anti-cancer drugs and may act as tubulin polymerization inhibitors. They were also identified as potent anti-tumor and tubulin depolymerization agents.

Like thiophene and hydrazone scaffolds, furan ring is also present in several natural products like furanalactones, furanoflavanoids, and furanocoumarins. Furan has unique features like less polar aromatic compound and the presence of ether oxygen gives polarity which improves hydrogen bonding acceptance. Hence furan is useful while optimizing pharmacokinetic properties especially it improves the bioavailability of poorly soluble lead molecules. Hence, the furan ring with oxygen atom improves the interaction toward the colchicine binding site of tubulin.

Inspired by the precedence of the very interesting role of all these scaffolds (arene, thiophene, hydrazone, and furan) in the drug design and development, in this article, we report the design, synthesis and biological evaluation of thiophenyl hydrazones specifically as anticancer agents.

In continuation to produce potential lead molecules as tubulin polymerization inhibitors, we portrayed relevant switches at C-2 and C-3 positions of thiophene scaffolds by conducting selective bromination and regioselective coupling reaction using Suzuki reaction.

Results and discussion

Chemistry

Thiophene scaffold has diversified biological properties, which is articulated by two types of compounds one is with rigid sp² hybrid bond hydrazone linker another with rotatable sp³ hybrid bond ester linkage. By preliminary in vitro data, we found that thiophene derivatives with hydrazone linker were more potent than ester-linked analogues. C-H activation, C-C bond formation were applied as the main tools in present work to improve the medicinal chemistry profile. Significantly, the Suzuki coupling was used as the main synthetic tool for the preparation of the molecules. Thus, we followed the selective bromination and Suzuki reaction steps to simplify and execute the process. This strategy and chemical tools facilitated to obtain the required key building blocks in practical quantities.
The desired key building block was achieved in four steps (Scheme 1) (represented as in Fig. 3), which involved substituted hydrazones 5a–n, esters 6a–i were achieved in quantitative yields. Thus, we applied diversification at a late stage to obtain unprecedented druggable molecules. We used simple and commercially available thiophene-2-carboxaldehyde as a starting material. First step included selective bromination using NBS which is a convenient source of bromine radical.[13] The second step involved introducing furan at second position of thiophene ring selectively by Suzuki coupling using Pd[Ph3]4/K2CO3 as a catalyst and 2-Furanylboronic acid to obtain compound 9. To introduce phenyl ring at the third position of thiophene ring we have subjected it to bromination using Br2/AcOH and obtained compound 10 (yield 84%). Suzuki coupling was comfortably conducted to insert phenyl ring at third position of thiophene ring using Pd[Ph3]4/K2CO3 as a catalyst with 4-Chlorophenyl boronic acid to afford compound 1 (yield 82%) which is a key building block to produce hydrazone scaffolds (Fig. 3). Various substituted hydrazines were utilized to synthesize rigid sp² hydrazone linker by coupling with the key building block aldehyde 1 as shown in Table 1 (Fig. 4). To achieve another key building block, compound 1 was subjected to reduction by NaBH₄ to produce an alcohol functional group which were further converted to ester-linked scaffolds 6a–i. All the required analysis of final products 5a–n & 6a–i were characterized by using spectrometric tools like IR, ¹H NMR, ¹³C NMR and HRMS (ESI). The aldehyde key building block used for the production of hydrazone derivatives has restricted rotation linkage because of its rigid confirmation whereas the alcohol key building block used for the production of ester derivatives has free rotation.

Thus, we used various acids 4a-i to obtain ester-linked derivatives employing alcohol 2 as the key building block by using dicyclohexylcarbodiimide (DCC) and 4-
dimethylaminopyridine (DMAP).\[^{[14]}\] Mostly (DCC) is utilized for sugar and amino acid derivatives but this method has not been fully adopted for the preparation of carboxylates.

In the present work, for the esterification of carboxylic acids DMAP is used as a catalyst. We found that by addition of 10 mol % DMAP with the DCC increased speed of the reaction, in addition, there are no side products as shown in Table 2 (Fig. 5).

For the esterification process, we conducted reaction with a series of aryl, heteroaryl, and aliphatic acids and added equimolar amount of alcohol in MeCN solvent at 80°C using HClO₄-SiO₂ (1 mol %).\[^{[15]}\] We obtained excellent yields of corresponding esters and completed the reaction within a duration of 3–18hrs as illustrated in Table 2 (Fig. 5).

Table 1. Synthesis of the thiophene final products 5a-n from thiophene-2-carboxaldehyde 1.

| S.No | Product 3a-n | Time\[^{[a,b]}\] (h) | Yield\[^{[a,b]}\] (%) |
|------|--------------|------------------|------------------|
| 1    | -C₆H₅        | a                | 0.5              | 85               |
| 2    | 2-CH₃-C₆H₄   | b                | 0.5              | 84               |
| 3    | 4-CH₃-C₆H₄   | c                | 0.5              | 84               |
| 4    | 3-Cl-C₆H₄    | d                | 1                | 81               |
| 5    | 4-F-C₆H₄     | e                | 0.5              | 84               |
| 6    | 4-Br-C₆H₄    | f                | 0.5              | 81               |
| 7    | 3-NO₂-C₆H₄   | g                | 1                | 81               |
| 8    | 2,4-diCl-C₆H₄| h                | 0.5              | 77               |
| 9    | 4-CN-C₆H₄    | i                | 0.5              | 20               |
| 10   | 2-Cl-C₆H₄    | j                | 1                | 85               |
| 11   | 4-Cl-C₆H₄    | k                | 0.5              | 78               |
| 12   | 3,5-diCl-C₆H₄| l                | 1                | 87               |
| 13   | 2-Br-C₆H₄    | m                | 0.5              | 83               |
| 14   | 3-F-C₆H₄     | n                | 1                | 85               |

\[^{a,b}\]Reagents and conditions: aromatic hydrazines 3a-n (5.5 mmol, 1.1 eq), aldehyde 1 (5.0 mmol, 1 eq), 5.0 M NaOH (1 mmol), in MeOH (5 mL), room temperature.

All the products were fully characterized by the usual spectroscopic techniques.

Figure 4. Synthesis of the thiophene final products 5a-n from thiophene-2-carboxaldehyde 1.
Experimental protocols

General information

All chemicals, corresponding lab reagents and solvents were purchased from commercial vendors and utilized for the research work without further purification. Thin-layer chromatography (TLC) was conducted on silica gel 60-F-254 (0.5 mm) from the MERCK company. The UV light was used to visualize the spots on TLC plates. Tetramethyl silane (TMS) used as the internal standard to record the $^1$H and $^{13}$C NMR spectra analyzed by Bruker 500 MHz. Chemical shifts are denoted as parts per million (ppm) downfield from tetramethyl silane as standard. Spin ranges are designated as s (singlet), bs (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), and m (multiplet). IR spectra were evaluated in Perkin Elmer, FT-IR spectrometer using KBr and coupling constant ($J$) measured in hertz (Hz). Instrument Agilent QTOF mass
spectrometer 6540 series used for the determination of HRMS. Using silica gel 60–120 column chromatography was performed for the purification of compounds.

**General experimental procedure for the synthesis of compounds 5(a–n)**
Solvent MeOH 5 mL was taken in a round bottom flask to this hydrazine (3a-n, 5.5 mmol, 1.1 eq) was added. A key building block aldehyde compound 1 added portion-wise slowly to the reaction vessel. After stirring for 2 h, MeOH was evaporated in vacuum and recrystallized from MeOH to afford the desired thiophenyl hydrazine derivatives (5a-n) with quantitative yields.

**General experimental procedure for the synthesis of compounds 6(a-i)**

**Procedure A.** Solvent 5 ml anhydrous CH2Cl2 (in the case of sparingly soluble acids, in DMF) was taken in a round bottom flask and added carboxylic acid (4a-i, 0.5 mmol). To this reaction mixture added 30–110 mg DMAP and alcohol (2, 0.5 mmol). The reaction was stirred for 5 min at 0°C after the addition of DCC and stirred for 3 h at 20°C. Filtration was done to remove precipitated urea and filtrate evaporated by using a vacuum. Solvent CH2Cl2 was added to the residue and, removed any further precipitated urea if needed. The organic layer was washed twice with 0.5 N HCl and with saturated NaHCO3 solution and, dried with MgSO4. The solvent was evaporated by vacuum and the corresponding esters were obtained by recrystallization. To obtain products in high purity short silica gel column was used taking ethyl acetate/hexanes as eluent to obtain products (6a-i).

**Procedure B.** The alcohol 2 (0.5 mmol) was taken in Acetonitrile to this corresponding acid (0.5 mmol) was added and followed by HClO4-SiO2 (0.05 g, 1 mol%). The reaction was stirred at reflux condition till the completion of acid (1 to 4 h, TLC, see Manuscript Table 2). The solvent was evaporated under reduced pressure, and EtOAc (20 mL) was added to dissolve the corresponding residue and removed catalyst by filtering. The organic layer subjected to workup using saturated aq. NaHCO3 (2 × 5 mL), water (5 mL), dried (Na2SO4) and evaporated under reduced pressure using a rotary vacuum to obtain the crude product. Column chromatography was used to obtain high purity products (6a-i). The catalyst was recollected (45 mg, 90%) from cotton filter paper after applying the vacuum evaporation of the remaining solvent. Activation of the catalyst was done by heating under reduced pressure (10 mm Hg) at 100°C for 12 h. The catalyst was recycled for about four straight esterification of alcohol (2, 0.5 mmol), with corresponding acids (3a-i, 0.5 mmol). Procured quantitative yields of esters (6a-i) after 1 to 4 h by using HClO4-SiO2 (0.1 g, 0.05 mmol, 1 mol%). This general procedure was applied to carry out esterification reactions, in most cases pure products (spectral data) were obtained by a general workup. Column chromatography (60–120 mesh silica gel, EtOAc: hexane) was applied for the purification wherever necessary. All new compounds assessed by HRMS and spectral data (NMR and MS) are provided in the supplementary information.
Table 3. In vitro cytotoxicity (IC50 μM) compounds 5a-n against human cancer cell lines.

| Compound Code | PC3   | DU 145 | A549 | HT29   | HCT 116 | MCF 7   |
|---------------|-------|--------|------|--------|---------|---------|
| a             | N.A   | >20    | N.A  | 10.4±0.35 | N.A    | 9.8±0.17 |
| b             | >20   | >20    | >20  | 2.61±0.34 | 8.1±0.2 | 10.2±0.31 |
| c             | 19.5±0.26 | 18.4±0.16 | 9.1±0.4 | 10.6±0.08 | 12.1±0.09 | 13.4±0.28 |
| d             | >20   | 12.1±0.3 | N.A  | 18.9±0.19 | N.A    | 11.0±0.45 |
| e             | >20   | 10.5±0.12 | N.A  | 12.8±0.28 | N.A    | 8.9±0.51  |
| f             | >20   | 9.6±0.24 | N.A  | 9.3±0.17  | >20    | 9.1±0.22  |
| g             | N.A   | N.A    | N.A  | 14.4±0.2  | N.A    |         |
| h             | N.A   | >20    | N.A  | 16.6±0.41 | >20    | N.A      |
| i             | N.A   | N.A    | N.A  | N.A      | N.A    | N.A      |
| j             | 9.8±0.21 | N.A    | N.A  | 8.5±0.36  | 15.8±0.32 | N.A      |
| k             | N.A   | N.A    | N.A  | 8.2±0.24  | N.A    | N.A      |
| l             | N.A   | N.A    | N.A  | >20      | N.A    | N.A      |
| m             | N.A   | N.A    | N.A  | 12.4±0.56 | N.A    | 11.8±0.54 |
| N Podophyllotoxin | N.A | N.A | N.A | 10.90±0.67 | N.A    | 9.3±0.62  |

Table 4. In vitro cytotoxicity (IC50 μM) compounds against 6a-i human cancer cell lines.

| Compd Code | A549 | MCF7 | NCI  | HT29 | HCT 116 | MDAMB231 | B16F10 |
|------------|------|------|------|------|---------|-----------|--------|
| a          | >30  | >30  | >30  | >30  | >30     | >30       | >30    |
| b          | >30  | >30  | 15.78±0.17 | >30 | >30     | >30       | 17.8±0.85 |
| c          | >30  | >30  | >30  | >30  | >30     | >30       | >30    |
| d          | >30  | >30  | >30  | >30  | >30     | >30       | 10.6±0.16 |
| e          | 15.8±2.32 | >30  | >30  | >30  | >30     | >30       | >30    |
| f          | >30  | >30  | 24.45±1.80 | >30 | >30     | >30       | >30    |
| g          | >30  | >30  | >30  | >30  | >30     | >30       | >30    |
| h          | >30  | >30  | >30  | >30  | >30     | >30       | >30    |
| i          | >30  | >30  | >30  | >30  | >30     | >30       | >30    |
| PDT        | 0.09±0.008 | –    | –    | 1.25±0.03 | 1.06±0.23 | 0.024±0.006 | –      |

Spectral characterization data of compound 5b

1-((4-(4-Chlorophenyl)-5-(furan-2-yl) thiophen-2-yl) methylene)-2-(otolyl)hydrazine (5b).

Brown solid; yield: 78%; mp 164–167°C, IR (KBr): 3348, 2931, 1601, 1588, 1459, 732 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.77–7.72 (1H, m), 7.39–7.30 (5H, m), 7.11–7.05 (2H, m), 7.03–6.96 (2H, m), 6.95–6.89 (2H, m), 6.33 (1H, dd, J = 3.4, 1.8 Hz), 6.11 (1H, d, J = 3.4 Hz), 5.71 (1H, bs), 2.29 (3H, s); HRMS: m/z calcd for C₂₂H₁₇ClN₂O₂S [M + H]⁺: 393.0823; found: [M + H]⁺ 393.0826.
Pharmacology

In vitro anti-cancer activity

A series of 14 different hydrazones 5a-n and a series of 9 different ester-linked furan-2-thiophene hybrids 6a-i was synthesized. These were investigated for their cytotoxicity potential. Their cytotoxicity potentials are illustrated in Table 3.

The cytotoxic activity of the compounds 5a-n was assessed in 6 different cell lines i.e., A549 (lung cancer cells), DU145 (prostate cancer cells), PC3 (prostate cancer cells), MCF7 (breast cancer cells), HT29, HCT 116 (colon cancer cells). The IC50 values for each of these compounds were determined and illustrated in Table 3. At first hydrazone linked thiophene derivatives 5a-n were evaluated for cytotoxicity analysis in the above-mentioned cell lines. Similarly, ester-linked thiophene derivatives were also evaluated for their anti-cancer activity in 7 different cell lines and their IC50 values were determined. Their cytotoxicity potentials are illustrated in Table 4.

The compounds 5a-n showed a broad range of IC50 values ranging from 8.1 ± 0.52 μM to 19.5 ± 0.26 μM (Table 3) against the above cell lines. The most active compound 5b with 2-methyl substitution on aromatic D ring was effective on all cancer cell lines with predominant effect on HT29 and HCT116 cancer cells with IC50 values of 2.61 ± 0.34 and 8.1 ± 0.2 μM respectively. By keen observation of IC50 data, we found that most of the derivatives were not effective on A549 cells except 6c with an IC50 value of 9.1 ± 0.4 μM. Among all the compounds 5b with a 2-methyl substitution (IC50=2.61 ± 0.34 μM) and 5c with 4-methyl substitution on D ring (IC50=10.6 ± 0.48 μM) were most active against HT29 followed by HCT116 (IC50=8.1 ± 0.2 μM & IC50=12.1 ± 0.09 μM) respectively. Also compounds 5g, 5j showed good activity on HCT116 cells. Thiophene scaffolds with flexible ester linkage were not much active against HT29 and HCT116 cells.

Structure activity relationship

Compounds with substitutions 2-methyl (5b), 4-methyl (5c), 3-chloro (5d), 4-fluoro (5e), 4-bromo (5f), 2-chloro (5j), 3-fluoro (5m), 2-bromo (5n) in the D ring showed adequate biological activity evaluated for in vitro cytotoxicity studies. Based on the in vitro cytotoxicity studies, compound 5b was chosen for further understanding of its
effect on cancer cell growth inhibition and tubulin polymerization inhibition. The structure activity relationship is depicted in (Fig. 6).

Lipophilicity and conformational properties play a key role in structure-activity relationships. Hydrazone linked compounds containing 2-methyl (5b) and 4-methyl (5c) were more active when compared to the compound containing 2-methyl substitution (6b) with ester linkage. Moreover, compounds with ester linkage like the one containing 3-nitro on D ring (6d) was more active ($IC_{50} = 10.6 \pm 0.16 \mu M$) on MDAMB231 cells and those with hydrazone linkage containing 3-nitro on D ring (5g) displayed moderate activity ($IC_{50} = 16.6 \pm 0.41 \mu M$) on HCT116 cells as shown in Tables 3 and 4.

Compound 5b was selected from a series of 23 hybrids as the potential candidate based on MTT results. It showed sensitivity toward colon cancer cell line HT29.

The role of compound 5b on apoptosis was determined by Annexin V binding assay. Cell cycle analysis was carried out by flow cytometry and it was found that 5b resulted...
in G2/M arrest. In addition, we have also correlated molecular docking studies on microtubule inhibition with the cell cycle data.

**Cell cycle analysis**

Most of the cytotoxic molecules show growth inhibition by cell cycle arrest at a specific phase. Flow cytometry analysis was used to check the effect of compound 5b on cell cycle.\[^{18,19}\] Anti-cancer agents inhibit the proliferation of cancer cells by arresting cell cycle at a specific checkpoint. HT29 cells were treated with 5b for 24 hours and stained with propidium iodide. Cells treated with 5b exhibited a dose-dependent increase in the G2/M population. The results indicated that untreated control cells showed 18.04% of cells in the G2/M phase whereas 5b showed 26.18% at 2.5 μM, 30.18% at 5 μM, 31.92% at 10 μM, and 33.5% at 20 μM. Podophyllotoxin was used as a standard which resulted in a rise in G2/M population from 18.04% in control to 41.16% at 400 nM. Hence treating of HT29 cells with compound 5b clearly showed G2/M arrest in cell cycle analysis using flow cytometry (Fig. 7).

** Annexin V binding assay**

Annexin V-FITC/propidium iodide staining assay was conducted on HT29 cells to check the percentage of apoptosis induced by compound 5b\[^{20,21}\]. Cells were treated with 5b in a dose-dependent manner for 24 hours using concentrations of 2.5 μM, 5 μM, 10 μM respectively followed by Annexin V-FITC/propidium iodide staining and flow cytometry (Fig. 8). Compound 5b treated HT29 cells demonstrated a rise in the total percentage of apoptotic cells, dead cells from 22.29% (control) to 25.15% (2.5 μM), 31.08% (5.0 μM) and 50.60% (10.0 μM) respectively (early and late apoptotic cells—Annexin V +ve cells). Depending on the above results we found an increase in apoptotic cells upon treating HT29 cells with compound 5b (Fig. 8).
In vitro tubulin polymerization

A fluorescence-based in vitro tubulin polymerization assay was performed according to the manufacturer’s protocol (BK011, Cytoskeleton, Inc.). Tubulin polymerization was measured by a time-dependent increase in fluorescence at 37°C for 1 hour. Fluorescence emission at 420 nm (excitation wavelength is 360 nm) was measured by
using a Spectramax multimode plate reader (Thermo scientific Inc). The IC$_{50}$ value was defined as the drug concentration required to inhibit 50% of tubulin assembly compared to control (Fig. 9). Compound 5b displayed considerable tubulin polymerization inhibition with IC$_{50}$ of 8.21 ± 0.30 μM compared to the reference compound nocodazole (Fig. 10).

**Acridine orange-ethidium bromide (AO-EB) staining**

Live, apoptotic and dead cells were determined by acridine orange/ethidium bromide staining followed by fluorescent microscopy. Versatile fluorescent agents such as acridine orange/ethidium bromide dyes are used to identify dead and apoptotic cells.

The experiment was conducted by treating cells with compound 5 at concentrations 2.5 μM, 5 μM, 10 μM for 48 hours. Live cells stain with acridine orange appears green in color while dead cells stain with ethidium bromide appears red when visualized by fluorescent microscopy. In addition, early and late apoptotic cells can also be determined by this assay. Thick orange spots due to EtBr stain indicated late-stage apoptosis. Green to dark green colored areas represents chromatin condensation, fragmentation and cell shrinkage which is a sign of early-stage apoptosis. These results confirm that compound 5b is responsible for cancer cell death in HT29 cell lines (Fig. 11).

**Figure 11.** AO/EB staining of compound 5b in HT29 cells: Prominent morphological changes like membrane blebbing, condensed nuclei, apoptotic body formation was clearly evident in cells in a concentration dependent manner.
Molecular docking

Molecular docking simulations were carried out to rationalize our experimental values and to explore molecular interactions between synthesized derivatives and beta tubulin. Podophyllotoxin is a beta tubulin inhibitor that disrupts tubulin dynamics, which prevents cell division and leads to apoptosis in cancer cells. Hence Podophyllotoxin is taken as a standard in the present work. The X-ray crystal structure of beta-tubulin was obtained from protein data bank (PDB ID: 3E22). GLIDE 5.6 version of Schrodinger suite was employed for docking the newly synthesized derivatives into active site of tubulin crystal structure (PDB ID: 3E22).

From the molecular docking simulation study, it was observed that the standard Podophyllotoxin (Fig. 12) showed only one hydrophobic interaction with Lys 352 at active site of beta-tubulin with a dock score of $-4.45 \text{ kcal/mol}$ and Binding energy of $-63.60 \text{ kcal/mol}$. The newly synthesized derivatives exhibited hydrogen bond interactions with Lys352, Cys241, Asn349, Asn258. The dock scores and binding energies of these derivatives ranged from $-4.95 \text{ kcal/mol}$ to $-7.05 \text{ kcal/mol}$ and $-63.64 \text{ kcal/mol}$ to $-90.96 \text{ kcal/mol}$ respectively. The top-ranked conformation of compound $5b$ was well lodged at the colchicine binding site of the tubulin (Figu 13). A more detailed analysis of compound $5b$ with tubulin protein was represented in (Fig. 13). Compound $5b$ has shown two key hydrogen bond

Figure 12. 2D & 3D Ligand interaction diagrams of standard, Podophyllotoxin.

Figure 13. 2D & 3D Ligand interaction diagrams of the most potent compound $5b$ (maroon color stick) at the active site of tubulin (PDB ID: 3E22). The red dashed line represents hydrogen bonds.
interactions, hydrogen bond interactions with Asn 349 and Asn 258. The 4-Chlorophenyl and furan ring was favorably lodged in the hydrophobic cavity. Moreover, docked compound 5b and co-crystallized ligand confirmed that compound 5b also occupies the active site in a similar fashion as that of co-crystallized ligand (Fig. 14). depicts the superimposition of the best-docked pose of compound 5b and crystallized ligand at the colchicine binding site of tubulin. Compound 5b exhibited a dock score of $-5.83 \text{kcal/mol}$ and binding energy value of $-83.18 \text{kcal/mol}$ which are greater when compared to that of the standard podophyllotoxin (Figs. 12–14). Based on modeling results, we are anticipating that our synthesized compound 5b has shown prominent anti-proliferative activity through inhibiting the tubulin protein. The dock scores of the synthesized derivatives along with the standard and their binding free energies are tabulated in Table 5.\textsuperscript{[28,29]} (Supplementary info).

All these synthesized hydrazone and ester derivatives were then evaluated for their ADME properties by employing Qikprop module in Schrodinger.\textsuperscript{[30]} Their values were found to be in the acceptable range as depicted in Table 6 (Supplementary info).

**Conclusion**

The unique hydrazone, furan-2-thiophene derivatives can be evaluated as anti-cancer lead molecules. The preliminary cytotoxicity evaluation of thiophene derived library identified lead molecule 5b with IC_{50} value of $2.61 \pm 0.34 \text{µM}$ toward HT29 cancer cell line. To accelerate further in detail, we conducted Annexin V binding assay and cell cycle analysis which supported the apoptosis in HT29 cells induced by compound 5b. The docking studies support that our best active compound 5b showing relatively good interactions, dock score, binding free energy values with the beta tubulin inhibitor (PDB ID:3E22) with their predicted ADME properties which were found to be in significantly acceptable range. Thus, combining all these results it shows the biological

![Figure 14. Superimposition of podophyllotoxin ligand (green), best docked pose of compound 5b (purple) at the active site of tubulin (PDB ID: 3E22).](image-url)
importance of hydrazone, furan-2-thiophene derivatives especially lead molecule 5b and can be developed as anti-mitotic anti-cancer agents.

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Disclosure statement

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Supplementary information

Full experimental detail, 1H and 13C NMR spectra etc material can be found in the “Supplementary information”.

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