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Synthesis, Docking Study And *In Vitro* Anticancer Evaluation Of New Derivatives Of 2-(1-(2-Flouro-[1,1-Biphenyl]-4-Yl)Ethyl)-6-(Substituted Phenyl) Imidazole[2,1-B][1,3,4]Thiadiazole Derived From Flurbiprofen

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ABSTRACT

Objectives: New derivatives of 2-(1-(2-flouro-[1,1-biphenyl]-4-yl)ethyl)-6-(substituted phenyl) imidazole[2,1-b][1,3,4]thiadiazole, compounds(**2-5**), were designed and synthesized from the starting parent molecule, flurbiprofen.

Materials and Methods: Compound (**1**) was synthesized by refluxing flurbiprofen, as a starting material , with phosphorus oxychloride ($POCl_3$), and thiosemicarbazide, resulting in flurbiprofen -1,3,4 thiadiazole -2 amine derivative. The target compounds(**2-5**) were obtained by refluxing compound(**1)** with different substituted phenacyl bromides,

Results: The new compounds were characterized by FTIR, ^{1}HNMR and CHNS analysis. A molecular docking study of the synthesized compounds (**2-5**) against HIV protease indicated such compounds occupied the critical site of HIV protease pocket, and demonstrated excellent positioning of the compounds in the pocket. The best binding energy values were (-7.49, -7.22 , -7.51 and-7.12 kcal/mol) for the compounds (**2 -5),** respectively. The anticipated physio-chemical parameters(calculated logarithmic of partition cofficent(Clog p),molecular volume (MV), number of violations (*n*viol), topological surface area (TPSA), and percent of absorption(%Abs.) were computed using software Molinspiration. *In vitro* outcomes, the target compounds were assessed by cell line study for their anticancer effects. All the tested compounds showed the most plausible anticancer activity, when compared to a positive control (atazanavir), using MTT cytotoxic assay, against human prostatic tumor (PC-3), glioma cell line (LN68), and normal WRL-68 cell line.

Conclusion*:* The results revealed that compounds **2,3,4 and 5** exhibited the highest inhibitory activity against PC3 cell line at IC_{50} concentrations of 178.2, 75.09, 129.3and 110.5µg/mL, respectively, and they had moderate effects against LN299 cell line at IC_{50} concentrations 203,172.5, 169,and 157.7 µg/mL, respectively, compared to atazanavir, as a positive control against PC-3, at IC_{50}, 157.3 µg/mL, and in LN299, at IC_{50} 195.7 µg/mL. Also compound **(5)** attributed to cell cycle arrest, and induction of apoptosis. The target compounds could be considered as promising as potential anticancer drugs.

Keywords: Thiadiazole, phenacyl bromide, molecular binding, docking, HIV1 protease inhibitor.

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NTRODUCTION

Cancer is a molecularly heterogeneous disease and a major cause of death worldwide. According to the American Cancer Society, there is still an anticipated significant rise in cancer rates, particularly in Africans region. [1,2]

Cancer is the abnormal growth of cells that contribute to malignancies and death. The world's most frequently observed is female cancer [3].

Many compounds with 1,3,4-thiadiazole moiety have a wide range of therapeutic effects such as antibacterial[4], antifungal[5], anti-mycobacterial[6], anti-leshmaniasis[7], analgesic, anti-inflammatory[8], anti-psychotic[9] and anti-convulsant[9,10] activities. Compounds with the 1,3,4-thiadiazole moiety exhibited remarkable *in vitro*[11-13] and *in vivo*[14-17] anticancer activity.

Various mechanisms of action have been ascribed to the anticancer effects of 1,3,4-thiadiazole moiety, such as inhibiting DNA and RNA synthesis, in particular the considerable effects on protein synthesis[18], carbonic anhydrase inhibitor[19], phosphodiesterase-7 (PDE7) inhibitor[20], histone deacetylase inhibitor[21] or adenosine A3 receptor antagonists[22].The inhibitory effects on cancer cells proliferations, invasions and angiogenesis indicate that H IV protease inhibitors (PIs) might still benefit from therapeutic outcomes by each hematological and solid malignancy[23]. Nelfinavir and atazanavir are HIV-PIs that have already been the most effective in preventing the activation of growth factor receptors and downstream Akt.-signaling, consequently provoking endoplasmic reticulum stress (ERS), autophagy and apoptosis, including both caspse dependent and caspase independent pathways recognized through recruitment (ERS) and autophagy [24,25] .

The major aspect of protease inhibitors that could be prescribed for their use, such as anti-tumor agents, is their ability to sensitize more cancer cells to radiotherapy and chemotherapy. For example, indinavir, saquinavir

and ritonavir have been shown to improve the effects of all trans retinoic acids on acute myeloblastic leukemia[26], and ritonavir has once been shown to focus on improving the antitumor action of docetaxel on prostatic tumor cells *in vitro* and *in vivo*[27].

The present study was conducted to design and synthesize new substituted imidazole[2,1-b]-1,3,4-flurbiprofen thiadiazole derivatives, and to evaluate their antitumor effects *in vitro*, with additional validation using docking study.

MATERIALS AND METHODS

Flurbiprofen, as a racemic mixture, was purchased from Sigma-Aldrich. Reactions were monitored using thin-layer chromatography (TLC) on silica gel(60) F254 Merck (Germany) and exposed to UV_{254} nm light. FT-IR spectra were recorded using a spectrophotometer on KBr disk (v = cm^{-1}). Melting points were measured using a melting point apparatus in open capillary tubes and are uncorrected. CHNS micro-analysis was carried out using a Euro EA3000 elemental analyzer, and ^{1}HNMR spectra were measured using tetramethylsilane (TMS) as an internal standard, on Inova show Ultra shield 500MHz . The chemical shifts were expressed once as (δ, ppm), and DMSO-d_6 was utilized as a solvent.

Chemical Synthesis

Synthesis of 5-(1-(2-Fluoro-[1,1'-bi phenyl]-4-yl) ethyl)-1,3,4-thiadiazol-2-amine (1)[28, 29]

A mixture of flurbiprofen (5mmol, 1.22g),thiosemicarbazide (5mmol, 0.455g) and $POCl_3$ (5mL) were refluxed for 4h.Then, the mixture was cooled and ice water (25mL) was added gradually and the mixture of reaction was refluxed for further 3 h, then cooled and filtered. Neutralization of the filtrate with KOH solution(10%) was then performed, and the precipitate was filtered and re-crystallized from MeOH.

Brown precipitate, yield (62%),m.p (218-221 ^{0}C), R_f =0.38, FTIR (KBr, v= cm^{-1}): 3280 and 3118 str of *prim*. (NH_2); 3035 (Ar-CH) str, 2976 , 2933 and 2875 (CH) str. of *aliph*.-CH & -CH_3,1952, 1892 (Aromatic overtone/combination bands), 1622 and 1608 (Ar-C=N)str, 1514,1485,and1415 (Ar-C=C) str, 1130 (N-N)str, 766 and725 C-S str.; ^{1}H- NMR (500MHz,Methanol-$_{d4}$, δppm): 7.46-7.07 (8H,m, Ar-H), 4.45(q,1H,CH-*aliph*), NH_2(masked by Methanol$_{d4,}$solvent**)**,1.68 (d, 3H,CH_3**)**; CHNS analysis: Calcd. for ($C_{16}H_{14}FN_3S$): C, 64.19; H, 4.71; N, 14.04; S, 10.71 .Observed: C, 63.85; H, 4.81
; N, 13.80 ; S, 10.45

General method for the Synthesis of (2-5) [30,31]

An equimolar mixture (2.5 mmol,0.75 g) of compound (**1**), and each substituted phenacyl bromide,(0.498 g of phenacyl bromide; 0.584g of *p*-chloro phenacyl bromide; 0.573g of *p*-methoxy phenacyl bromide, and 0.61g of *p*-nitro phenacyl bromide) was refluxed with ethanol(15mL), and DMF (10 mL) for 18h, and monitoring the reaction with TLC. The excess of solvent was evaporated under reduced pressure, and the hydro bromide salt was separated and collected by filtration, suspended in water, and neutralized by saturated aqueous NaOAc solution(5mL) to produce the free base. The precipitate was washed with water, dried and re-crystallized from MeOH. Syntheses of the target compounds **(2-5)** are shown in Figure 1.

2-(1-(2-Fluoro-[1,1'-biphenyl]-4-yl)ethyl)-6-phenylimidazo[2,1-b][1,3,4]thiadiazole (2)

Pinkish precipitate, yield (73%), m.p (166-168 ^{0}C), R_f = 0.66,FTIR (KBr, v =cm^{-1}): 3128 and 3028 (Ar-H) str , 2935 and 2871 (C-H) str. of *aliph*. CH & CH_3 ,1685 and 1603 Ar(C=N)str, 1581 , 1561, 1520 ,and1479 Ar(C=C) str, 1176 (N-N)str,769 and723 C-S str.

^{1}H-NMR (500MHz, DMSO-$_{d6}$, δ ppm):8.71(1H ,s, -CH=C-alkene), 7.90 (1H,m, Ar-H), 7.89-7.41 (12H,m, Ar-H), 4.81 (1H,q, CH-*aliph*.), 1.82 (3H,d, CH_3); CHNS analysis: Calcd. for ($C_{24}H_{18}FN_3S$) C,72.16; H, 4.54; N, 10.52; S, 8.03. Observed: C,71.90; H, 4.96; N,11.09; S, 7.24;

6-(4-Chlorophenyl)-2-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)imidazo[2,1-b][1,3,4]thiadiazole (3)

Yellowish precipitate, yield (77%),m.p (**170-172 ^{0}**C), R_f =0.58, FTIR (KBr, v =cm^{-1}): 3172,3060 and 3030 (Ar-H), 2979, 2935 and 2873 (C-H) str, of *aliph*. CH & CH_3, 1699 and 1612 Ar(-C=N)str , 1585,1523 , and1477 Ar(C=C) str,1167cm (N-N)str, 1090(C-Cl) str, 766 and 733 C-S str , 827 aromatic-CH bend., of *para*-Cl position.

; ^{1}H NMR (500MHz, DMSO$_{d6}$, δ ppm), 8.70(s,1H,CH=C-alkene),7.85(d,2H, Ar-H), 7.84-7.32(m,10H,Ar-H), 4.75(q,1H,CH-*aliph*), 1.75(d,3H,CH_3). CHNS analysis: Calcd. for ($C_{24}H_{17}ClFN_3S$): C,66.43; H,3.95; N,9.68; S,7.39. Observed: C, 67.00; H,4.045; N,9.54; S,7.49.

2-(1-(2-Fluoro-[1,1'-biphenyl]-4-yl)ethyl)-6-(4-methoxyphenyl)imidazo[2,1-b][1,3,4]thiadiazole (4)

Pink precipitate, yield (73%) , m.p(**169-173** ^{0}C), R_f = 0.67, IR (KBr, v =cm^{-1}): 3411 H-bonding ,3143, and 3032 (Ar-H), 2978, 2937 and 2841 (CH) str. of *aliph*. CH & CH_3, 1676 and 1603 (Ar-C=N)str, 1485,1523 and 1477 (Ar-C=C) str, 1248 *asym* (C-O) str, 1176 (N-N)str, 1026 *sym* (C-O) str, 768 ,737 and702 C-S str, 829 aromatic C-H bend., of *para*-OCH_3 position;^{1}H-NMR (500MHz,*DMSO$_{d6}$*, δ ppm): 8.53(s,1H,CH=C-alkene),7.76(d,2H,Ar-H),7.55-7.35(m,10H,Ar-H),4.75(q,1H,CH-*aliph*),3.32(s,3H,OCH_3), 1.76(d,3H,CH_3). CHNS analysis: Calcd. For(C25H20FN3OS) C,69.91; H,4.69; N,9.78; S,7.46. Observed: C,69.60; H,4.59; N,9.36; S,7.33;

2-(1-(2-Fluoro-[1,1'-biphenyl]-4-yl)ethyl)-6-(4-nitrophenyl)imidazo[2,1-b][1,3,4]thiadiazole (5)

Brown precipitate , yield (79%), m.p (**1**66-169 ^{0}C), R_f =0.69, FTIR (KBr, v=cm^{-1}): 3489 H-bonding ,3126, 3060, 3032 (Ar-H), 2993, 2937 and 2837 (CH) str. of *aliph*. CH & CH_3, 1647 and 1601 (Ar-C=N)str,1483 ,1481 and 1452 (Ar-C=C) str, 1176 (N-N)str, 766 and 737 C-S str, 1512 (NO) *asym* str, 1340(NO) *sym*.str, 831 Aromatic-CH bend., of *para* NO_2 position.

^{1}HNMR (500MHz, DMSO$_{d6}$, δ ppm): 8.94(s,1H,CH=C-alkene), 8.25(d,2H,Ar-H), 8.08(d,2H,Ar-H), 7.53-7.36 (m,8H,Ar-H), 4.78(q,1H,CH-*aliph*), 1.76(d,3H,CH_3). CHNS analysis: Calcd, for ($C_{24}H_{17}FN_4O_2S$:) C, 64.85; H, 3.86; N,12.61; S, 7.21. Observed: C, 64.47; H, 3.05; N,12.36; S, 7.33.

Biological screening

The study of MTT and HCS was carried out based on a procedure outlined earlier in the literature.[32,33]

Cytotoxic effects of the MTT assay

The MTT assay was used to estimate the cytotoxic effect of the synthesized compounds (**2-5**) against the PC-3, LN299 and WRL-68 normal cell lines, using atazanavir as a positive control.

Each of the synthesized compounds(**2-5**) was added to dimethyl sulfoxide(DMSO) to prepare a stock solution and serial dilutions (6.25-400 µg/mL). One hundred microlitres of each synthesized compound and atazanavir were added to PC-3, LN299 and WRL-68 cell lines, and the cells culture were kept in the CO_2 incubator at (37^0C) these were then incubated for 1-4 h. Once the media was removed, DMSO was added to each well to dissolve the formazan's crystals .The absorbance was read at 575 nm through the use of a Hidex Chameleon microplate reader(Lab Logic Systems- Ltd. Sheffield, United Kingdom). A small portion of the absorbance of treated cells with the tested compounds,compared to the absorbance of the untreated cells (negative control) was calculated as a percentage of cell viability. The IC_{50} values were determined for each compound[34-40].

High-content screening (HCS)

Three kits of cellomics multi-parameter cytotoxicity (Thermo Scientific, Japan) were also used to detect the independent cell health parameters in the PC-3 cell lines, a detailed previous study about this is available[41].

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed using a traditional cycle-TEST™ kit (BD Bioscience, San-Jose, CA), as previously detailed [42].

Caspases -9 activity assay

Caspase Glo 9 kit (Promega Madison, WI) was used to measure the activity of caspases 9. Briefly, PC-3 cells were grown into 96-well plates, with various concentrations of compound(**5**) added for 24 h. One hundred microlitres of Glo 9 reagent was added and the cells incubated for 30 min at 25^0C, the effect of the caspase in the treated cells was determined by the degree of amino-luciferin-labeled tetra peptide cleavage, and luciferase enzyme substrate release, using a Tecan-Infinite 200 Pro micro plate readers. Furthermore, time-dependent mode experiments were performed to validate the expression degree of caspases and hence,PC-3 cells had been treated with compound (**5)** with different concentrations as shown in Table 3. Then the evaluation of the degree of expression of caspases 9 at different times was completed [43].

DNA fragmentation assay

DNA laddering assay was studied in accordance with the appropriate recommended procedure by (Su *et al*., 2005) with few modifications [44].

A total of $1^* 10^6$ cells were treated with compound**(5)**, (50, 100 and 200 µg/mL) for 48 h, and then separated by centrifugation. The DNA was once separated using commercially available kits following the manufacturer's guidelines. DNA was settled on 1.5% agarose gel (consisting of 3.0 mg/mL of ethidium-bromide in 1xTAE (Tris-acetate-EDTA) buffer, *p*H (8.25) at 90 V for 1.5 h, and the peaks were analyzed *via* UV-trans illuminator.

Statistical analysis

One-way analysis of variances (ANOVA) was used to calculate whether the variances were statistically significant. Data were calculated as the mean ± standard deviation, and statistically valid results were evaluated using Graph Pad PrismV6 (Graph Pad program, Inc.).

Molecular docking Study

Choosing the molecular targets

By comparing our tested compounds with another ligands and determination of the pharmacophoric feature that can bind with critical amino acids at the target site. The target site selection has been done by (https://www.rcsb.org/) protein data bank.The compounds were tested practically against many target sites, then good results determined the suitable protein for conducting docking studies.

Preparation of receptor for virtual screening

After choosing the protein of target site, some processes should be performed to produce imagining about molecular binding modes of the tested candidates inside the pocket of HIV protease site by using MOE 2014 algorithms, which is responsible for the encoding poly proteins gag and gag during virion maturation. The enzyme's activity is essential for the infection of the virus, which tends to make the protein an important therapeutic objective in AIDS treatment. The active binding sites were created by the co-crystallized ligand within the crystal protein (PDB codes: 1HVR). First, the water molecules were removed from the crystal protein. Then, we used the protein report to fix the crystallographic errors and unfilled valence atoms.The application of energy minimization order was used to reduce the protein energy[45] . The rigid binding site of the protein was obtained by performing fixed atom constraint. The protein critical amino acid region was defined and prepared for the molecular docking process. 2D Structures of the tested compounds were drawn utilizing Chem-Bio Draw Ultra16.0 and saved in Mol file format, from MOE 2014 algorithms, the saved file was opened, 3D structures were protonated and energy was minimized by applying RMSD of .05 kcal/mol.[46] CHARMM force field. Then, the minimized candidates were prepared for docking using a prepared ligand protocol.

RESULTS AND DISCUSSION

Biological study

MTT assay:

The compounds (**2-5**) were assessed for cytotoxicity against two tumor cell lines, PC-3 (human prostatic tumor) and LN299 (glioma cell line), and one normal cell line WRL-68 (human hepatic cell line), by 3 -(4,5-dimethyl thiazolyl-2)- 2,5- biphenyl tetrazolium bromide (MTT) assay.

The results are described as (IC_{50}), and the values are summarized in Figure 1.

. Figure 2 and Table 1 showed that compounds **(2- 5**) would have plausible cytotoxic activity against PC-3, at IC_{50} concentrations ranging from (178.2- 110.5µg/mL), and with selectivity indecies (SI)[41] ranging from(1.18-2.37) . While the treated LN299 cancer cell line at (IC_{50}) concentrations ranged from (203.0-157.7 µg/mL), showed (SI) ranging from(1.03-1.66), compared to atazanavir. Therefore, compounds **(3** and **5**) are regarded to be the most effective against cancer cell lines, PC-3, compared to LN299 cells , with relatively low cytotoxic effects towards normal cell line (WRL-68).

Multiple cytotoxic effects of compound (5) on the PC-3 cells via array scan High Content Screening (HCS)

The cytotoxic effect of compound **(5)** on PC-3 cell line over 24 h was evaluated by Array Scan High Content Screening (HCS). Six serial concentrations of compound**(5)** (200µg/mL,100µg /mL, 50µg/mL, 25µg/mL, 12.5µg/mL, and 6.25µg/mL) were prepared and utilized to determine the effects on PC-3 five-healthy cell's parameters.The changes in these five parameters were recorded as mean±SD, Figure 3 and Table 2. The most significant changes(P < 0.0001) in these five parameters were seen at concentrations of 200 µg/mL of compound **(5),** compared to the positive control (atazanavir, 200 µg /mL). At low compound (**5**) concentration (6.25 µg/mL), there were no detectable effects within the five parameters assessed, and the findings were comparable to those found for the negative control (untreated cells).

Viable cell count

It is evident from Figure 3A and Table 2 that there was a major reduction in the PC-3 viable cell count after twenty-four hours, with increasing compound **(5)** concentration. The percentage of viable cell count in PC-3 cells treated with compound**(5)** was 31.48 % (200µg /mL), 30.6 % (100µg /mL), 14.7 % (50µg /mL), 2.24% (25µg /mL), 3.39% (12.5µg /mL), and 2.25% (6.25µg /mL).Comparative study was obtained after treatment of MCF7 cells with tamoxifen[47].

Nuclear intensity

The results of PC-3 cell nuclear intensity are shown in Figure 3B, and the mean activity was significantly increased (400.7 ± 18.88, 457.3 ± 21.59 and 526. ± 39.95 , P< 0.0001) with concentrations of 50µg /mL,100µg /mL and 200µg /mL respectively, of compound (**5**), compared to (atazanavir, 200µg /mL). After PC-3 cell treatment with different concentrations of compound (**5**) ranging from (6.25-25µg /mL), no significant effects were observed, Figure 2B, and Table 2 . A comparative data were obtained after treatment of MCF7 cells with astrozole, as in a previous study[48].

Cell membrane permeability

This parameter was used to evaluate cell–complex interaction during which PC-3 cell membrane permeability, Figure 3C and Table 2, was substantially increased (evaluated by green fluorescence emission), (109.7 ± 11.72, P =0.0388), after treatment with 200 µg/mL of compound (**5**), compared to atazanavir (200µg /mL, P < 0.0001). Although such compound showed no significant changes (P > 0.4277)

in the PC-3 membrane permeability at concentrations of (6.25-100 µg/mL). Alterations in cell membrane permeability were reported to be mostly involved with ongoing toxic or apoptotic responses, and cell membrane integrity loss is a prevalent phenotype feature of noticeable cytotoxicity[49].

Mitochondrial membrane potential (MMP)

The intensity of MMP decreased with rising compound (**5**) concentration .The main intensity (182.0 ± 24.52 **)** was determined for compound (**5**) at a concentration (200µg /mL), when compared to (Atazanavir, 200µg /mL, P <0.0001).

There was a substantial reduction in the mean of the intensity of MMP activity (325.3 ± 20.74, 292 ± 31.64 , 235.3 ± 31.34 and 182.0 ± 24.52 , P <0.0001) at 25µg /mL, 50µg/ mL, 100µg /mL and 200µg /mL respectively, of compound **(5)** , compared to atazanavir, (200µg /mL), the decrease in the mean of the intensity of MMP to 93.33 ± 5.686, was observed, Figure 3D and Table 2.

Cytochrome-C release

After PC-3 cells were treated with compound **(5)**,the increase in cytochrome-C release intensity was a concentration-dependent ,Figure 3E and Table 2. There was a significant elevation in the mean of the intensity of cytochrome-C release (323.0 ± 10.44, 400.7 ± 18.88 , 412.0 ± 52.56, 457.3 ± 21.59 and 526. ± 39.95, P < 0.0001), at 12.5 µg/mL, 25 µg/mL, 50 µg/mL , 100 µg/mL and 200 µg/mL respectively of compound **(5)** , compared to the positive control (atazanavir, 200 µg/mL). The conclusions drawn suggested that PC-3 cells treated with compound **(5)** undergo apoptosis through cell membrane disruption and the release of cytochrome-C, since these two events are closely related to caspases activation[50].

Effect of compound (5) on PC-3 cell caspase 9

Treatment of PC-3 cell line with compound **(5)** at (50 µg/mL and 100 µg/mL),resulted in a rise in the mean activity of caspase 9 (P < 0.0001), particularly in comparison to a negative control (DMSO), as shown in Figure 4 andTable 3, Caspase 9 is a prompt to activate downstream caspases, such as 3 and/or 7, giving rise to cell apoptosis[51].

The effect of the compound)5(on cell cycle phase on PC-3 cell line

Cell cycle analysis was conducted to assess the compound's (**5**) effect on the G1, S and G2 / M cell cycles of the tumor cells of the PC-3. Cell cycle phase distribution on PC-3 cell viability was performed to identify whether the cell cycle arrest was associated with the inhibitory effect of the PC-3 cell viability of compound **(5)**.

Flow cytometry was used to detect the DNA content of the propidium iodide (PI) in the cells, as shown in Figure 5A and B. For atazanavir, as a positive control, and compound **(5),** the cell cycle distribution was analyzed 24 h after treatment of PC-3 cells at a concentration of (200µg /mL). The results are shown in Figure6, and in the G1 phase, the percentage of cells in the treated compound **(5)** was significantly increased (P < 0.001) . Also, findings in the S phase showed that treatment of cells with (200 µg/mL) of a compound **(5)** had a non-pronounced effect, in comparison to the negative control, (untreated), (P = 0.9882). At the same time, atazanavir at (200µg /mL) showed a non-significant effect on the S phase in PC-3 cells, (P = 0.4292). While, in the G2/M phase, compound**(5)** , afforded a significant decrease in the cells G2/M population, compared to the negative control, after 24h with p= 0.0138 . Atazanavir also produced a significant decrease in the cells G2/M population (P<0.0001). Similar results were shown in the previous literature[52].

From the above results, the induction of cell cycle arrest, and apoptosis is expected to be the most crucial topic in the development of anticancer drugs. [53] This indicates that the transition from G1 to S phase was blocked by compound **(5)**, mainly at a concentration of (200 µg/mL).

DNA fragmentation assay

In order to determine the inter-nucleosomal DNA fragmentation induced by the compound **(5),** a DNA laddering assay was achieved in accordance with the standard protocol outlined by Su *et al*. (2005) [43] .

DNA ladder formation was detected in the DNA samples of PC-3 cell lines treated with 50µg /mL,100µg /mL and 200µg /mL of compound **(5**) . The appearance of DNA fragmentation by treating DNA samples in comparison to DNA in untreated cells (negative control), confirmed the induction of apoptosis by compound (**5).** A clear fragmented ladder of DNA was observed in PC-3 cell line treated with various concentrations of compound (**5**) where apoptotic cells were higher when treated even at a low concentration (50µg/mL), compared to a positive control atazanavir, (200 µg/mL). However, fragmented DNA was not seen in the negative control , Figure 7. Therefore, data collected from this study confirmed that compound (**5**) against PC-3 prostate cancer cell line induced cell death through the process of apoptosis *via* DNA damage, defending the assumption that treatment with compounds (**5**) led to apoptosis instead of necrosis in PC-3 prostate cancer cells, similar study approved this assumption previously[54].

Molecular docking process

The docking method was achieved through using DOCKE R protocol.

DOCKER is a grid-based molecular docking system that involves the CHAR MM-based molecular dynamics (MD) scheme to dock ligands within the receptor binding site .

The receptor was kept rigid while the ligands were allowe d to be flexible during the refining process.

Almost every molecule was utilized to create ten different poses with the protein. The docking scores (DOCKER interaction energy) of the best-fitting poses to the active site at HIV protease [40] was then measured, as shown in Table 4.

Most of these processes are often used to estimate the suggested binding interactions, affinity, preferred orientation of each docking pose and binding free energy (ΔG) of the tested compounds to HIV protease. The estimated energies for the tested compounds were consistent with observed outcome, that displayed the candidate compounds are liable for plausible inhibitors of HIV protease,Table 4.

The key binding site of HIV protease consists of amino acids Tyr644, Ile619, Leu746, Thr692, Lys646, Glu706, Lys702 and Glu663. Figure 8 shows the binding mode of the tested compounds in 2D and 3D simulations against HIV protease binding site.

The binding mode of compound (**2**), exhibited an energy binding of –7.49 kcal/mol. The thiadiazole ring formed a hydrophobic interaction with IleA50, while AspB29 amino acid formed further hydrophobic interaction with a phenyl ring, (Figure 8A).

Compound (**3**) exhibited binding energy of –7.22 kcal/mol.The *p*-chloro phenyl ring formed a hydrophobic interaction with Gly B48, (Figure 8B).

In addition,compound's **(4)** binding mode**,** clearly indicated energy binding of – 7.51 kcal/mol.The thiadiazole ring formed a hydrogen bond with IleB50 at a distance of 2.57 ºA. Whereas, The *p*-methoxy phenyl ring formed two hydrophobic interactions With Ala A28 and Asp29,Figure 8C.

Finally,The binding mode of compound **(5),** revealed an energy binding of –7.12 kcal/mol. The *p*-nitro group formed two hydrogen bonds with ArgB8 with a distance of 2.16-2.39 ºA., Figure 8D .

***In silico* ADMET and carcinogenicity analysis**

The ADMET features of the proposed compound (**2-5**) were tested with the ADMET descriptor component of the pre-ADMET online software of small molecule protocol .

Table 6 highlights the main parameters investigated, and showed that the potential of target compounds to pass through BBB was very low. Moreover, most of the target compounds have excellent intestinal absorption, It is properly known that many medicines fail during medical assessments due to difficulties related to their absorption properties, if HIA value below < 40[55,56] . All the compounds are CYP2D6 inhibitors, and most of them are anticipated to be non-carcinogenus (on mouse and rat), and safe upon administration,Table 5

Physicochemical properties of the synthesized compounds (2-5)

The clog p values, TPSA and MV values were calculated using Cheminformatics on the Net (http://www.molinspiration.com), and are summarized in (Table 7).

The correlation between the lipophilicity of the synthesized compounds and their pharmacological activity was once determined *via* the correlation of HIV protease enzyme inhibitors with the clog p value of all the synthesized compounds. Improving this value means raising the lipid solublity feature of the examined compounds[57].

It is beneficial to observe that the clogp values for the synthesized compounds ranged from 4.90 to 6.5 . The title compounds have shown appropriate values of TPSA [58], with better oral bioavailability suggests [59] (Table 7). Good molecular volume (MV) values were shown to determine the transport characteristics of the molecules, including the intestinal absorption.

***Pre*-metabolism analysis**

The outcomes of *pre*-metabolism study on compound (**4**) were taken as a model for predicted analysis, and showed that CYTP450 3A4 liver microsomal enzyme had a smaller effect on the tested compound, than CYP2C9 enzyme, the most metabolic response that can occur is OCH_3 de-alkylation, followed by a reduction through the hydroxyl (OH) group , with a resultant product that would be easily excreted from the human body, Table 8.

CONCLUSION

Novel compounds (**2-5**) were synthesized and confirmed by spectroscopic analysis, including FT-IR, ^{1}HNMR and CHNS . They were evaluated *in vitro* for their cytotoxic activity using MTT assay, against two cancer cell lines(PC3 and LN299) and one normal cell line (WRL-68). The target compounds **3** and **5** exhibited potential anticancer activity on PC-3 cell lines , and a moderate effect on (LN229) .Treated PC-3 tumor cells with

compound (**5**), experienced a statistically measurable cell count significant decrease, reduction of potential mitochondrial inner membrane, and a remarkable rise in total nuclear intensity, cell membrane permeability, cytochrome-C release from mitochondria , and caspase 9 activity,

In addition, compound (**5**) arrested cell cycle phase distribution at G2/M phase, and DNA ladder fragmentation was observed by the formation of apoptotic cells at a low compound (**5**)concentration, (50 µg/mL). Molecular docking studies have also shown that the target compounds may be considered as potential inhibitors of HIV protease enzyme.

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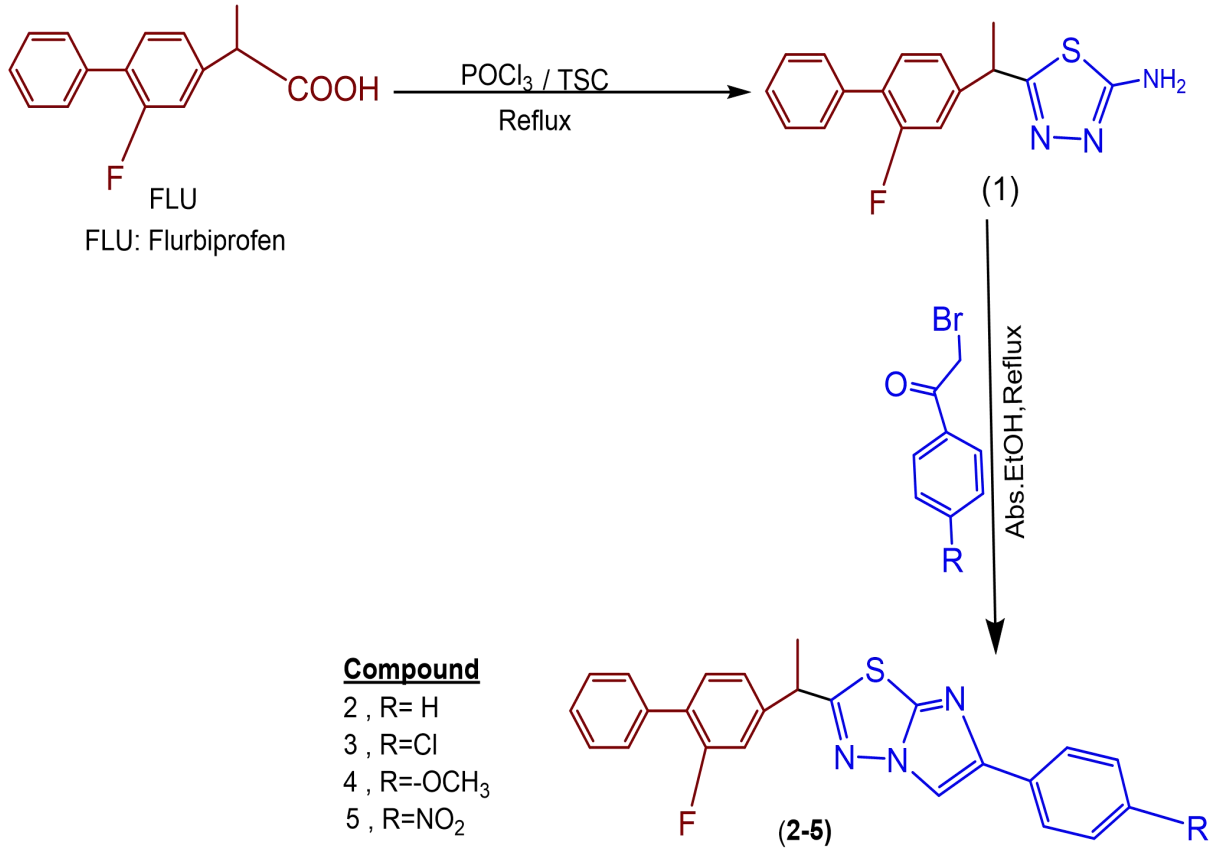


Figure1. Chemical synthesis of new compounds(**1-5**)

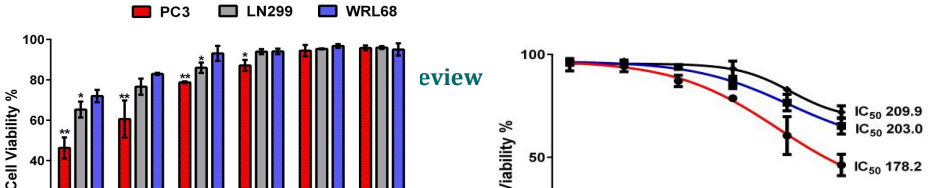


Figure2. Dose-dependent cytotoxic effect of compounds (2-5)and atazanavir on PC-3, LN299 and WRL-68 cells after 24h incubation at 37°C.

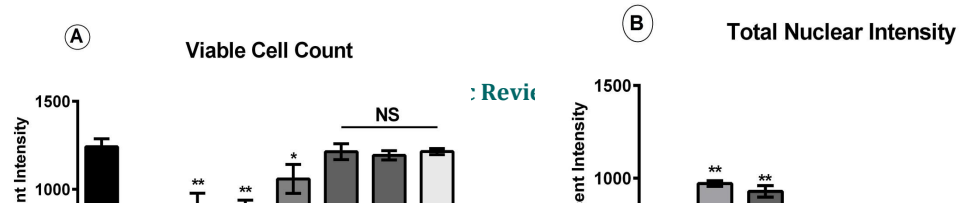


Figure3. Cytotoxicity of compound (5) on five cell health parameters by HCS.

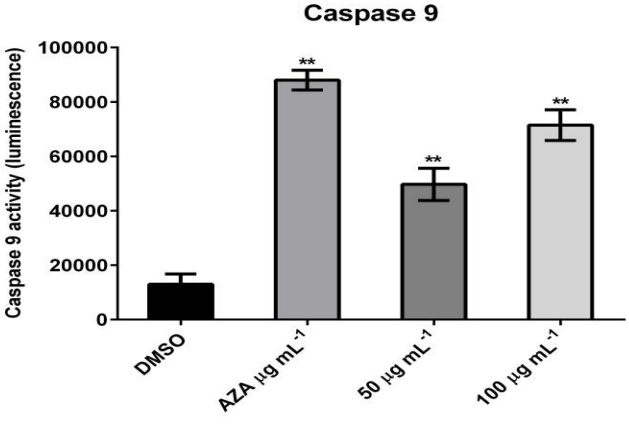


Figure 4. The Caspase-9 activity of PC-3 cells treated with 50 µg/mL and 100µg/mL of compound (5), AZA: atazanavir (positive control).

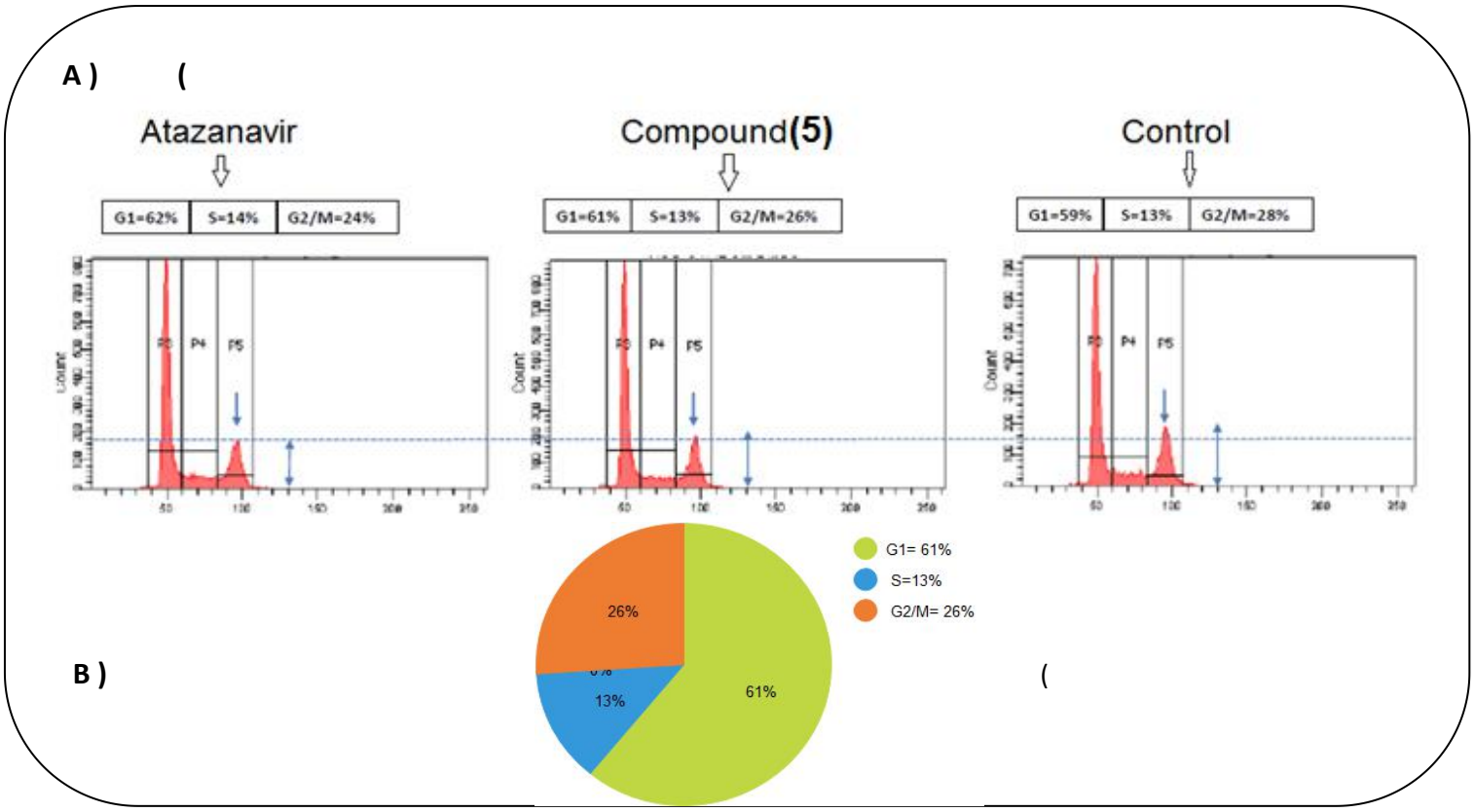


Figure 5.A: Effect of compound (5) and atazanavir (positive control) on PC-3 cell cycle phase distribution, P3:G1 , P4:S, P5: G2/M , control :untreated cell. B: Cell cytometry represents G1,S,and G2/M phases distribution of compound(5).

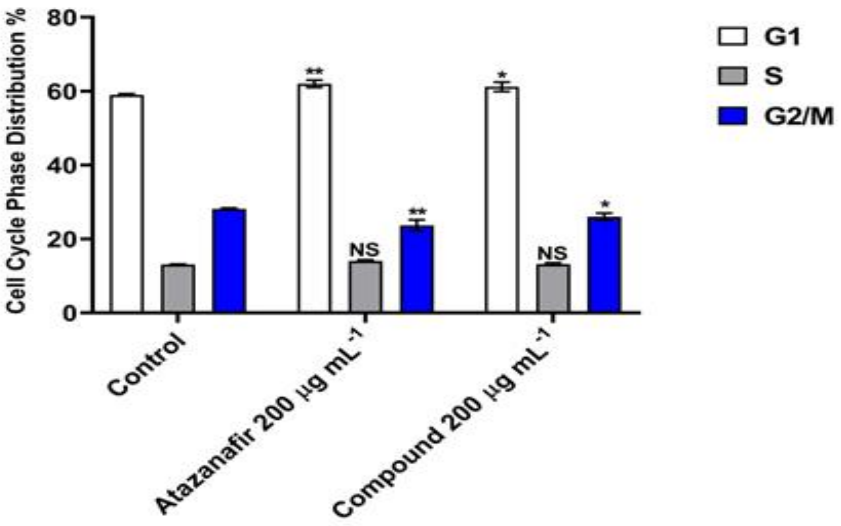


Figure6. The Relationship between DNA content (%) and treatment for G1 phase ,S and G2/M under effect of atazanavir and compound **(5)**.

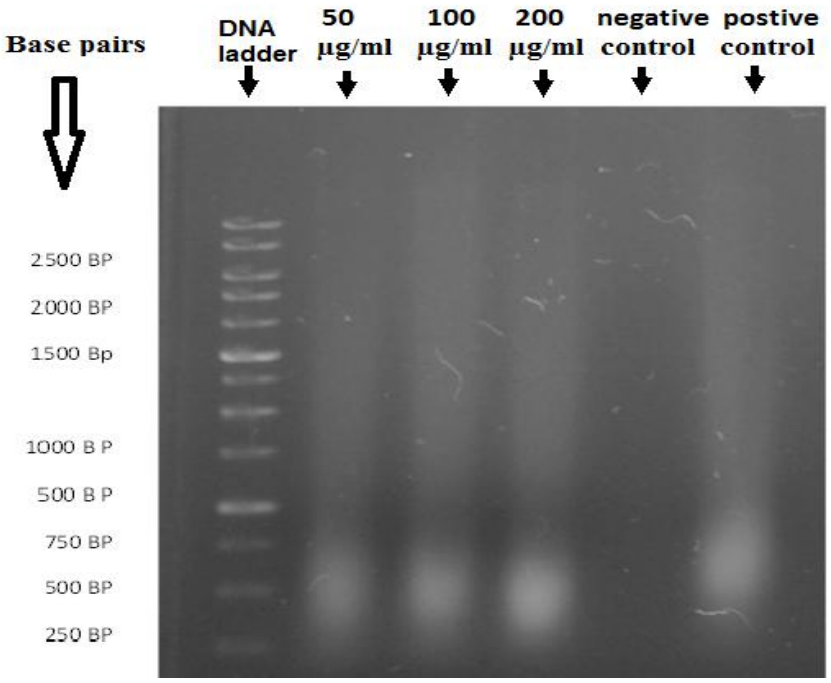


Figure 7. DNA fragmentation through degradation of nuclear DNA into inter-nucleosomal units after treatment with different concentration of compound (5), and positive control: atazanavir, negative control: untreated cell.

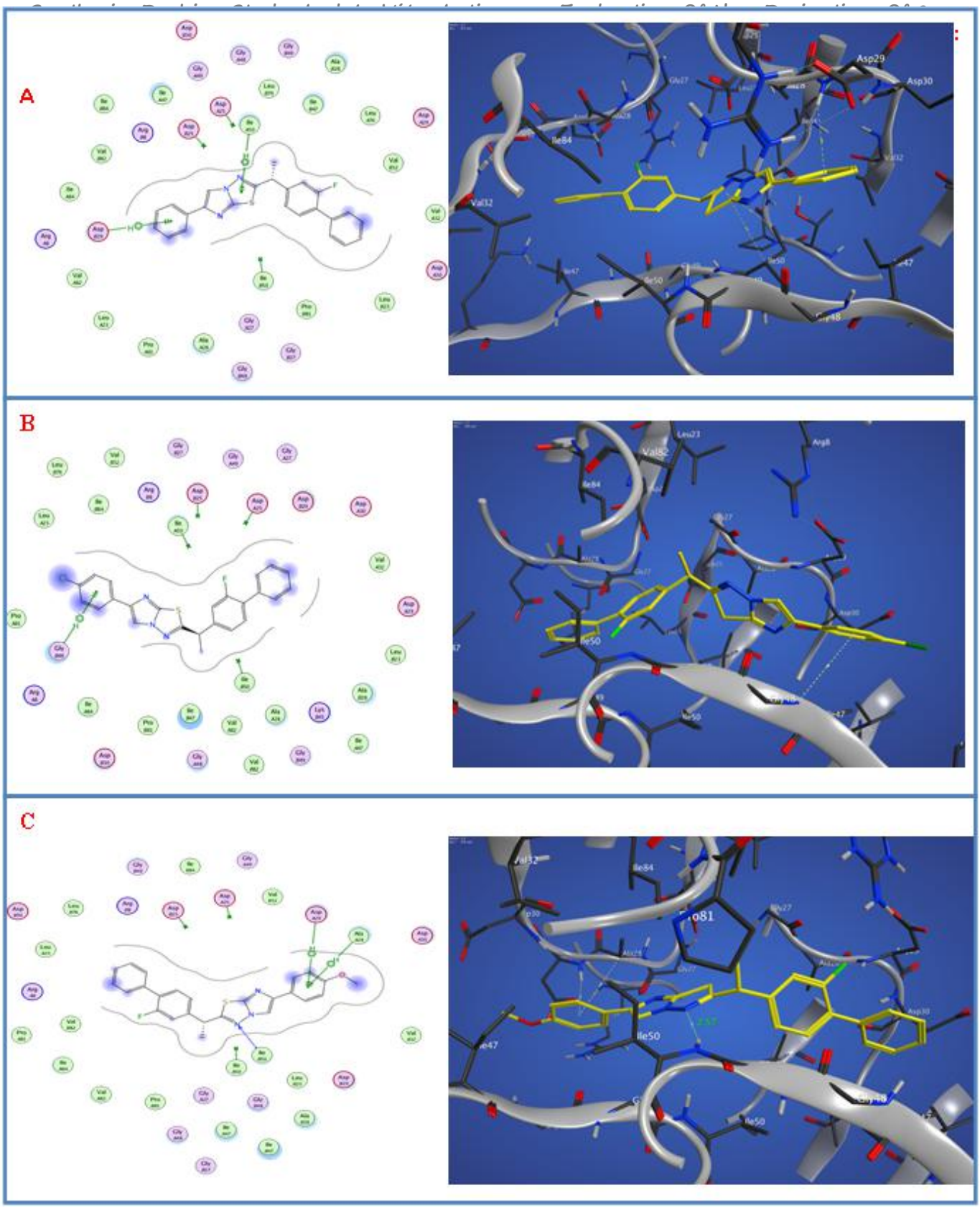

A
Asp29
Asp30
Ile84
Val32
Ile50
B
Leu21
Val82
Arg8
Ile84
Ile50
C
Val32
Ile84
Ile50
Asp30

Table 1. The IC_{50} values determined by MTT assay against PC-3 ,LN299 and normal WRL-68 Cells.

Compound No.	PC-3 IC_{50} (µg/mL)	SI*[41]	LN299 IC_{50} (µg/mL)	SI**[41]	WRL-68 IC_{50} (µg/mL)
2	178.2	1.18	203.0	1.03	209.9
3	75.09	3.89	172.5	1.69	292.3
4	129.3	1.58	169.0	1.21	203.8
5	110.5	2.37	157.7	1.66	261.6
Atazanavir	157.3	1.45	195.7	1.17	228.1

SI*= IC_{50} on normal WRL-68 cells/IC_{50} on cancer PC3;SI**:=IC_{50} on normal WRL-68 cells/IC_{50} on cancer LN299.

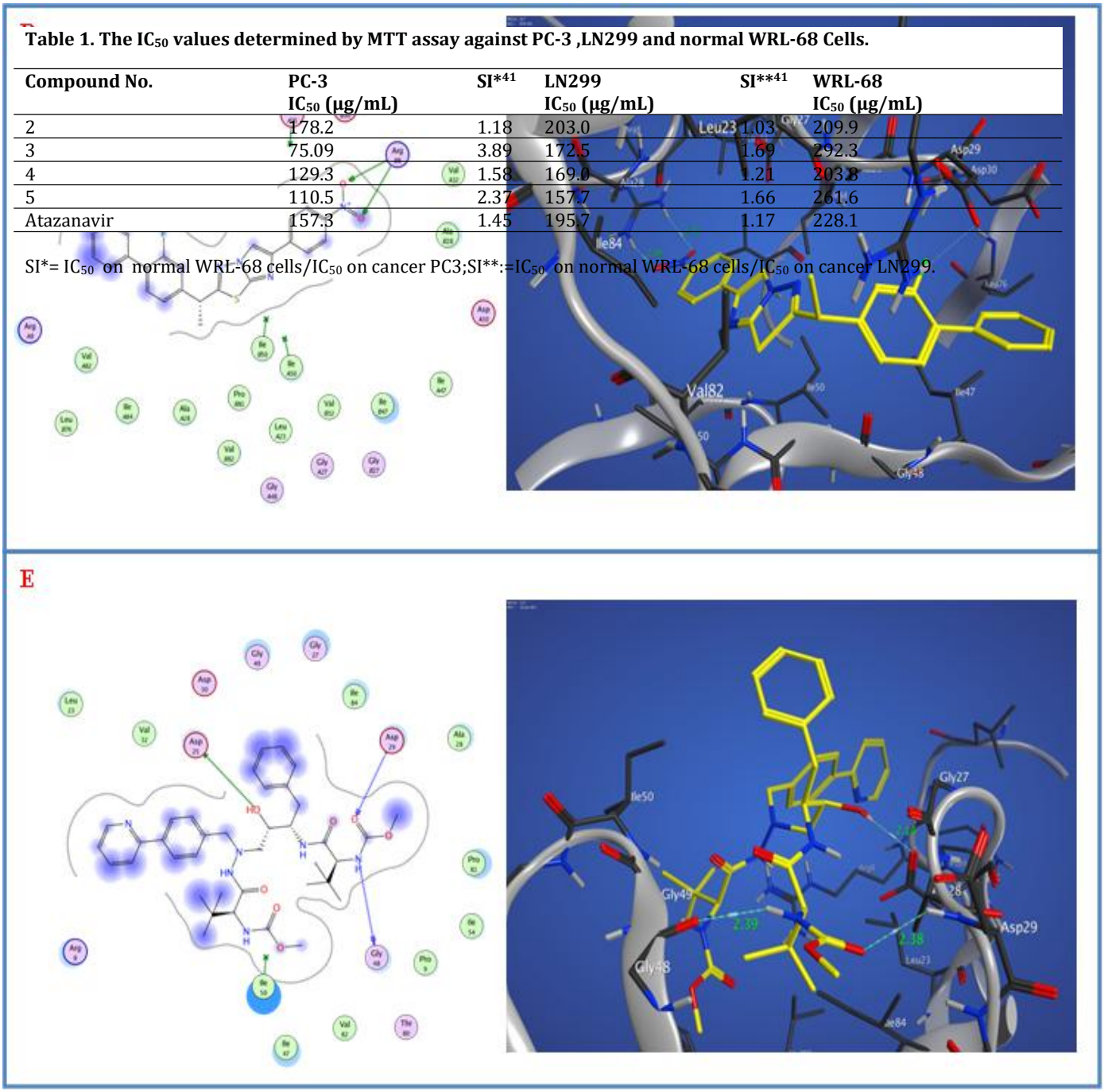


Figure 8. 2D and 3D Interaction show the binding mode, molecular surface area and length of hydrogen bonds/Å. for A: compound(2), B: compound(3), C: compound(4), D: compound(5) and E: atazanavir as potent inhibitor against HIV protease pocket

Table 2. Multiple cytotoxic effects of compound(5) on five cell parameters of PC-3 cell line via array scan high content screening (HCS).

Concentration (µg/mL)	PC-3 cell inhibition %	Nuclear intensity	Cell permeability	Mitochondrial membrane potential	Cytochrome-C release
Untreated cell (0)	0	224.3 ± 14.57	81.33 ± 6.506	451.3 ± 13.01	224.3 ± 14.57
200	31.48 **	526. ± 39.95 **	109.7 ± 11.72 *	182.0 ± 24.52 **	526. ± 39.95 **
100	30.60 **	457.3 ± 21.59**	79.33 ± 4. 041	235.3 ± 31.34 **	457.3 ± 21.59 **
50	14.70 *	400.7 ± 18.88**	62.33 ± 15.57	292 ± 31.64 **	400.7 ± 18.88 **
25	2.24	412.0 ± 52.56	73.67 ± 8.327	325.3 ± 20.74 **	412.0 ± 52.56 **

Table 4. show (ΔG) kcal/mol of tested compounds against HIV protease target site PDB ID: 1HVR

Compound NO.	Bonds NO.		Interaction with key amino acids	Score (ΔG) kcal/mol	RMSD value	E-place	Bonds length range/Å
	H.B	*pi*					
Comp (2)	0	2	IleA50-AspB29	-7.49	1.83	-90.33	-
Comp(3)	0	1	GlyB48	-7.22	2.21	-39.61	-
Comp (4)	1	2	AlaA28-AspA29-IleB50	-7.51	1.46	-107.96	2.37
Comp (5)	2	0	ArgB8	-7.12	1.69	-90.70	2.16-2.39
Atazanavir	2	0	GlyB48-AspA29-AspB25	-6.24	1.30	-90.52	2.14-2.38

12.5	3.39	323.0 ± 10.44	82.67 ± 7.371	406± 61.1	323.0 ± 10.44 **
6.25	2.25	265.0 ± 38.48	78.33 ± 7.371	392±60.8	265.0 ± 38.48
Atazanavir	50 **	702.3 ± 18.0 **	233.3 ± 20.40 **	5.686 ± 93.33 **	18.04± 7023 **

(**) Significant differences (P < 0.001)
(*) Significant differences (p ≤ 0.0388 and > 0.001

Table 5. Carcinogenicity prediction of the synthesized compounds

Compound	Ames test	Carcinogen on mouse	Carcinogen on rat	HERG inhibitor	Carcinogenicity	TA100-NA
Comp (2)	Mutagen	Positive	Negative	Low risk	0	-
Comp (3)	Mutagen	Positive	Negative	Med risk	0	-
Comp (4)	Mutagen	Negative	Negative	Med risk	0	-
Comp (5)	Mutagen	Negative	Negative	Low risk	0	-
Atazanavir	Non mutagen	Negative	Negative	Low risk	0	-

carcinogen = 1 may be
non carcinogen = 0

Table 7.The Clogp, molecular volume, topological polar surface area(TPSA)and percentage of absorption (%Abs) for the compounds(2-5).

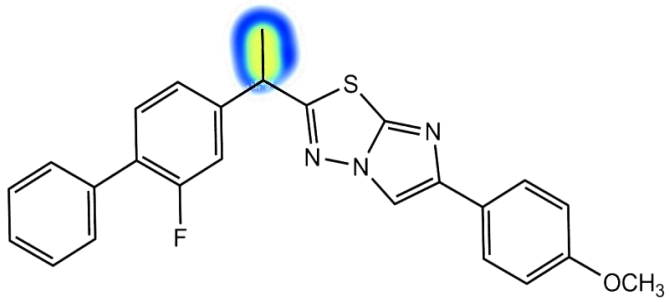
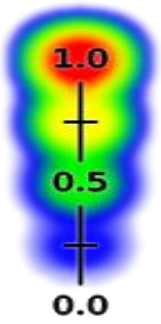
Compound NO	Clogp	MV	n-viol	TPSA	% Abs[54]
Comp (2)	5.83	348.35	1	58.4	88.8
Comp (3)	6.61	361.8	1	104.6	73
Comp (4)	5.99	373.8	1	67.6	86
Comp (5)	5.88	371	1	58.3	88.9
Atazanavir	4.02	670.23	1	171.2	50

Compound NO	BBB level	HIA	CYP450 3A4	CYP2D6	CYP2C9	PPB	Solubility level
(2)	1.71	97.03	Inhibitor	0	Inhibitor	94.5	2
Comp (3)	2.3	98.097	Inhibitor	0	Inhibitor	96.25	1
Comp (4)	1.32	97.64	Inhibitor	0	Inhibitor	93.67	1
Comp (5)	2.47	98.51	Inhibitor	0	Inhibitor	93.07	2
Atazanavir	1.89	60	Inhibitor	0	0		4

BBB: Blood-Brain Barrier; HIA: Human intestinal absorption; CYP2D6: Cytochrome P2D6, 0= Non-inhibitor; PPB: Plasma protein binding; Solubility level: (4) high sol., (3) and (2) intermediate sol., (1) less sol., (0) poor sol.

Footnote: Clogp=Calculated logarithm of partition coefficient; MV= Molecular volume; n-viol.= Number of violations; TPSA=Toplogical surface area; % Abs=Percent of absorption calculated according to the method described by Zhao *et al* [54]

Table 8. Show effect of liver microsomal enzymes on compound (4)

ENZYME	CYT. P450 enzymes effect on compound (4)	Reference scale bar
3A4		

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