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Synthesis, cytotoxicity assessment and molecular docking of novel thienyl-pyrazoles as VEGFR2 Inhibitors.

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Abstract: Novel series of thienyl-pyrazole derivatives have been developed and evaluated for antiproliferative efficacy on the basis of researches demonstrating the significance of the pyrazole framework in managing cancer progression, and suppression of VEGFR-2. Three cancer cells were used to assess the antitumor efficacy of the new hits, namely; human colon (HCT-116), mammary gland (MCF-7), and prostate (PC-3) cancer cell lines. The cytotoxic screening revealed that breast and prostate tumors are very sensitive to compound **3**, displaying superior activity to the reference drug sorafenib. Compound 3 displayed 2.1, 1.2 folds the activity of sorafenib against MCF-7 (IC_{50} = 3.36 ± 0.2 µM), and PC-3 (IC_{50} = 9.58 ± 0.7 µM) cell lines, respectively. Furthermore, compound **3** showed 73% of the anticancer activity of the reference drug against HCT-116 cell line (IC_{50} = 7.41 ± 0.5 µM). The *in vitro* anti-VEGFR2 activity of the most active derivative **3** was estimated, it revealed good inhibitory efficiency comparing to sorafenib. Furthermore, molecular modeling simulation of the intriguing derivative was achieved to spotlight its binding interactions and affinity towards the VEGFR-2 active site.

Keywords: Thiophene-Pyrazole; Antitumor; MTT; VEGFR-2; Modeling simulation.

1. INTRODUCTION

One of the core characteristics of cancer is angiogenesis, a multistep process that creates new blood vessels from ones that already exist.[1] New vessels growing enable localized tumors to invade and destroy adjacent tissues, provide a route for them to reach different body parts and nourish them from a distance, permitting primary tumor to move from its site, penetrate blood circulation, and to spread to different regions of body via metastasis.[2] Angiogenesis process is composed of several stages that are affected by various receptors and growth factors. Amongst the angiogenic factors, vascular endothelial growth factor (VEGF) has a crucial function in controlling tumor angiogenesis. [3, 4] The VEGF receptor-2 (VEGFR-2) is a receptor for VEGF that promotes the survival, sprouting, migration, proliferation and division of vascular endothelial cells.[5, 6] In light of the fact that VEGFR-2 regulates the primary angiogenesis-promoting function of VEGF, inhibiting the signaling pathway of VEGFR-2 is thought to be a prime goal for the evolution of highly effective cytotoxic agents .[7, 8]

Pyrazole skeleton received much attention, due to its diverse biological applications [9, 10]; it arises as an important scaffold in preparing effective antitumor agents.[11-15] Crizotinib and Ruxolitinib were recorded as pyrazole based antitumor drugs (Figure.1), and accepted as a treatment for non-small cell lung cancer and myelofibrosis, respectively .[16-17] Furthermore, many studies identified various substituted pyrazoles as potent antitumor agents [18-20] In addition, various literatures have reported the promising inhibitory effect of pyrazole derivatives against VEGFR-2 kinase (Figure.2). [21-23] Although pyrazole is a biologically active scaffold, the type of substitution at the periphery is crucial as well.[15] Recent strategies in molecular hybridization, afforded new structures that can be obtained by hybridizing two or more scaffolds in one molecule to create a new hybrid structure for developing new drug candidate.[24] The importance of thienyl-pyrazole

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hybrid as scaffold was found to possess interesting biological applications. [25] Accordingly, based on the above information, our work is focusing on the preparation and characterization of new thienyl-pyrazole derivatives. As well as, their anticancer activities and docking study will be determined.

2. METHODS

2.1. Chemistry

All details and information regarding the used materials and different analytical techniques were provided as shown in supplementary file. The staring compounds **1** and **2** were prepared as illustrated in the reported literatures. [26-30]

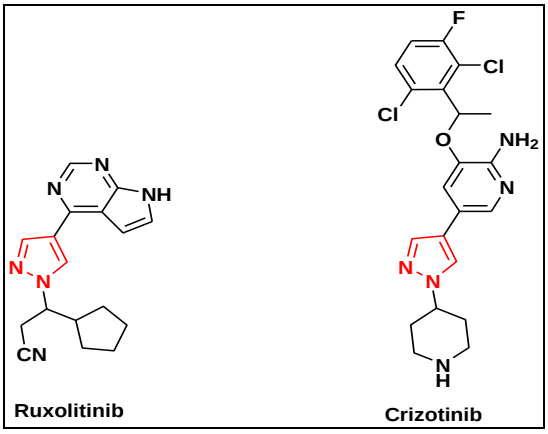


Figure 1.Pyrazole based anticancer drugs.[16-17]

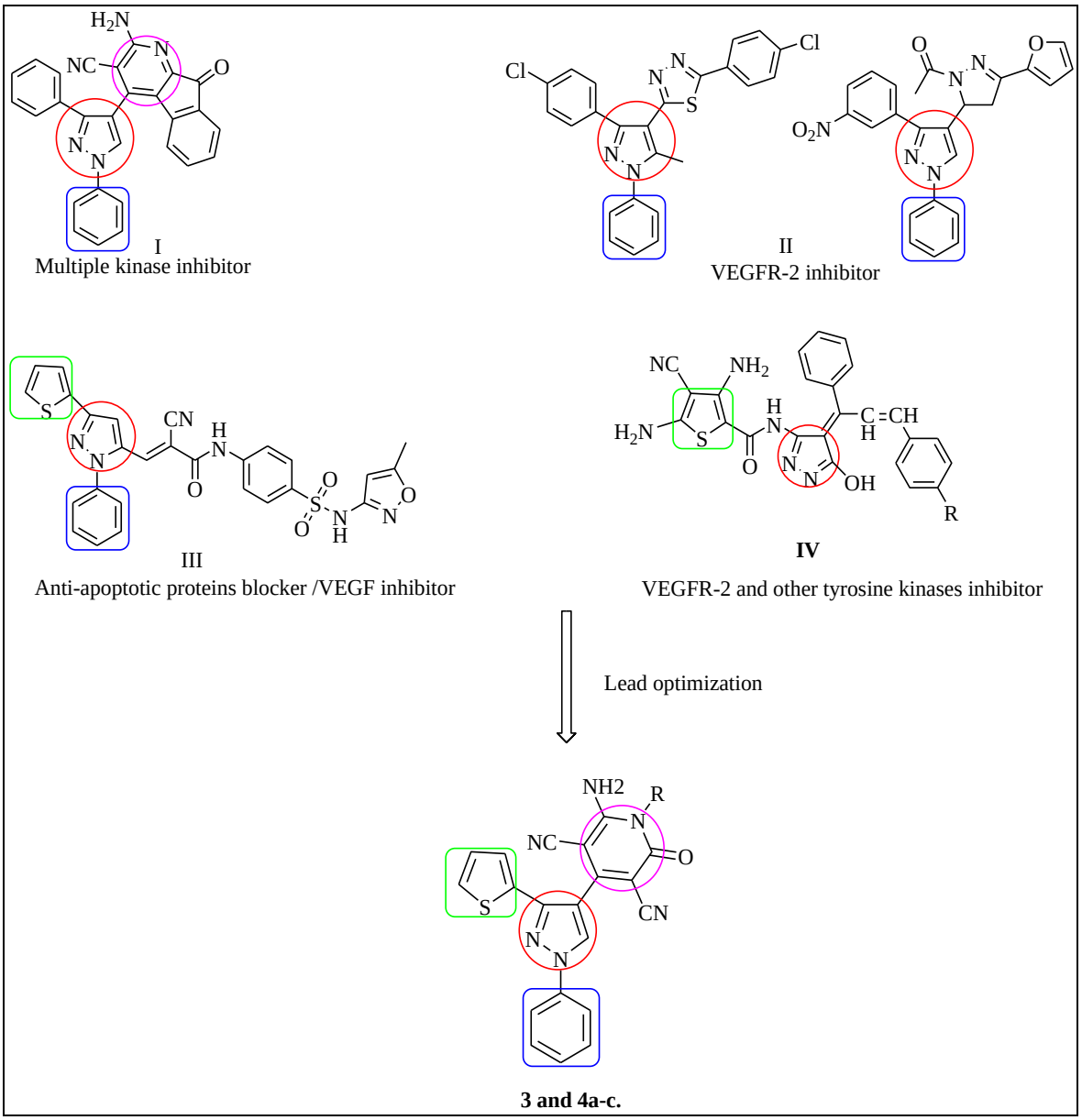


Figure 2. Some reported pyrazoles as cytotoxic agents and rational of the newly synthesized compounds. [21-23]

Synthesis of 2- ((1- phenyl-3-(thiophen-2-yl)- 1H-pyrazol-4-yl) methylene) malononitrile (2).

A mixture of compound **1** (1mmol), malononitrile (2mmol), and catalytic quantity of piperidine is heated under reflux for 3 h in ethanol followed, by pouring onto ice. The produced solid was filtered, dried, and crystallized from ethanol [27-30] **Yield** 74%; **m.p.** 178-180ºC.

Synthesis of 1, 6-diamino-2-oxo-4-(1-phenyl -3-(thiophen-2-yl)-1H-pyrazol-4-yl)-1, 2-dihydropyridine-3, 5-dicarbonitrile (3).

To a solution of 2- ((1– phenyl– 3-(thiophen -2-yl)-1H- pyrazol-4-yl) methylene) malononitrile **2** (0.01mol) in ethanol (30 ml), freshly prepared 2-cyanoacetohydrazide (0.01mol) and piperidine (catalytic amount), were added. Refluxing the reaction for 8 h, then filtering and crystallizing the resulting precipitated product from the ethanol enables us to get good yield of compound **3**.

Yield: (71%);**m.p**. 257-259ºC. **Analysis %** for $C_{20}H_{13}N_7OS$ (399.43), Calcd. (Found) C: 60.14 (60.32), H: 3.28 (3.53), N: 24.55 (24.21). **IR (KBr) (cm^{-1}):** 3335, 3180 (NH_2), 2214 (CN), 1689 (CO).**^{1}H-NMR (DMSO – d_6 - D_2O) δ (ppm**): 5.68 (s, 2H, NH_2; exchangeable with D_2O). 7.11 (t, 1H, thiophene-H_4), 7.19 (d, 1H, thiophene-H_3, *J* = 4 Hz), 7.42 (t, 1H, ph-H_4), 7.53(d, 1H, thiophene-H_5, *J* = 4 Hz),7.58 (d, 2H, ph-$H_{3,5}$,*J* = 8 Hz), 7.93(d, 2H, ph-$H_{2,6}$, *J* = 8 Hz),8.98 (s, 1H, Pyrazole-H), 10.19 (s, 2H, NH_2; exchangeable with D_2O). **^{13}C NMR** (DMSO -*d6*) *δ* (ppm): 81.70, 114.71, 115.69, 118.80, 125.93, 126.05, 126.99, 127.29, 128.27, 128.64, 129.62, 130.31, 138.58, 139.39, 146.55, 154.16, 157.48, and 170.53. **MS (m/z):** 399.41 (M^+, 24.32%), 116.12 (100%).

Generalsynthetic pathway for preparation of compounds (4a-c).

The intermediate 2- ((1-phenyl- 3-(thiophen -2-yl)-1H-pyrazol-4-yl) methylene) malononitrile**2** (0.01mol), was mixed with an equivalent amount of an appropriate 2-cyano-N-substituted phenyl acetamide (0.01mol) and piperidine (catalytic amount). Reflux heating was applied to the reaction for 8 h in absolute ethanol(30 ml), followed by filtering the resulting product before being crystallized from absolute ethanol to give the desired compounds.

6-amino-1, 2-dihydro-1-(2, 6-dimethylphenyl) -2-oxo-4-(1-phenyl-3-(thiophen-2-yl)-1H-pyrazol-4-yl) pyridine-3, 5-dicarbonitrile (4a).

Yield: (73%); **m.p.** 297-299ºC. **Analysis** % for $C_{28}H_{20}N_6OS$ (488.56), Calcd. (Found) C: 68.83 (68.48), H: 4.13 (4.35), N: 17.20 (17.54). **IR (KBr) (cm^{-1}):** 3336 (NH_2), 2196 (CN), 1620 (CO). **^{1}H-NMR (DMSO-d_6-D_2O) δ (ppm):** 2.07 (s, 3H, CH_3), 2.11(s, 3H, CH_3), 7.10-7.97 (m, 11H, Ar-H&thiophene H), 9.11 (s, 1H, Pyrazole -H). **^{13}C NMR** (DMSO-*d6*) *δ* (ppm): 15.39, 16.38, 82.78, 105.69, 114.70, 115.00, 122.31, 123.98, 125.94, 126.31, 127.27, 127.66, 127.95, 128.97, 129.61, 129.90, 131.58, 131.95, 132.26, 132.64, 133.92, 139.54, 145.87, 146.55, 146.84, 152.06, 156.49, 171.48**. MS (*m/z*)**: 488.93 (M^+, 19.71%), 184.53 (100%).

6-amino-1-(4-fluorophenyl)-1,2-dihydro-2-oxo-4-(1-phenyl-3-(thiophen-2-yl)-1H-pyrazol-4-yl) pyridine-3,5-dicarbonitrile(4b).

Yield: (69%);**m.p.** 170-172ºC. **Analysis** % for $C_{26}H_{15}FN_6OS$ (478.5), Calcd. (Found) C: 65.26 (65.52), H: 3.16 (3.40), N: 17.56 (17.18). **IR (KBr) (cm^{-1}):** 3326 (NH_2), 2205 (CN),1623 (CO).**^{1}H-NMR (DMSO-d_6-D_2O) δ (ppm):** 7.08-7.97 (m, 12H, Ar-H &thiophene-H), 8.91 (s,1H, Pyrazole-H). **^{13}CNMR** (DMSO-*d6*)*δ*(ppm): 82.09, 116.01, 116.66, 118.55, 118.99, 120.34, 120.95, 121.68, 122.31, 126.62, 126.98, 127.97, 128.25, 130.29, 130.59, 133.24, 138.86, 139.93, 143.25, 152.50, 153.86, 158.82, and 167.79. **MS (*m/z*):** 478.32 (M^+, 7.26%), 77.33 (100%).

6-amino-1,2-dihydro-2-oxo-1-phenyl-4-(1-phenyl-3-(thiophen-2-yl)-1H-pyrazol-4-yl)pyridine-3,5-dicarbonitrile(4c).

Yield: (67%);**m.p.** 200-201ºC.**Analysis** % for $C_{26}H_{16}N_6OS$ (460.51), Calcd.(Found) C: 67.81 (67.56), H: 3.50 (3.88), N: 18.25 (17.98). **IR (KBr) (cm^{-1}):** 3330 (NH_2), 2192 (CN), 1650 (CO). **^{1}H-NMR (DMSO-d_6-D_2O) δ (ppm):** 7.13-7.93 (m, 13H, Ar-H &thiophene-H), 8.98 (s, 1H, Pyrazole-H). **MS (*m/z*):** 460.70 (M^+, 25.89%), 298.89 (100%).

2.2. ***In vitro* anti-tumor evaluation**

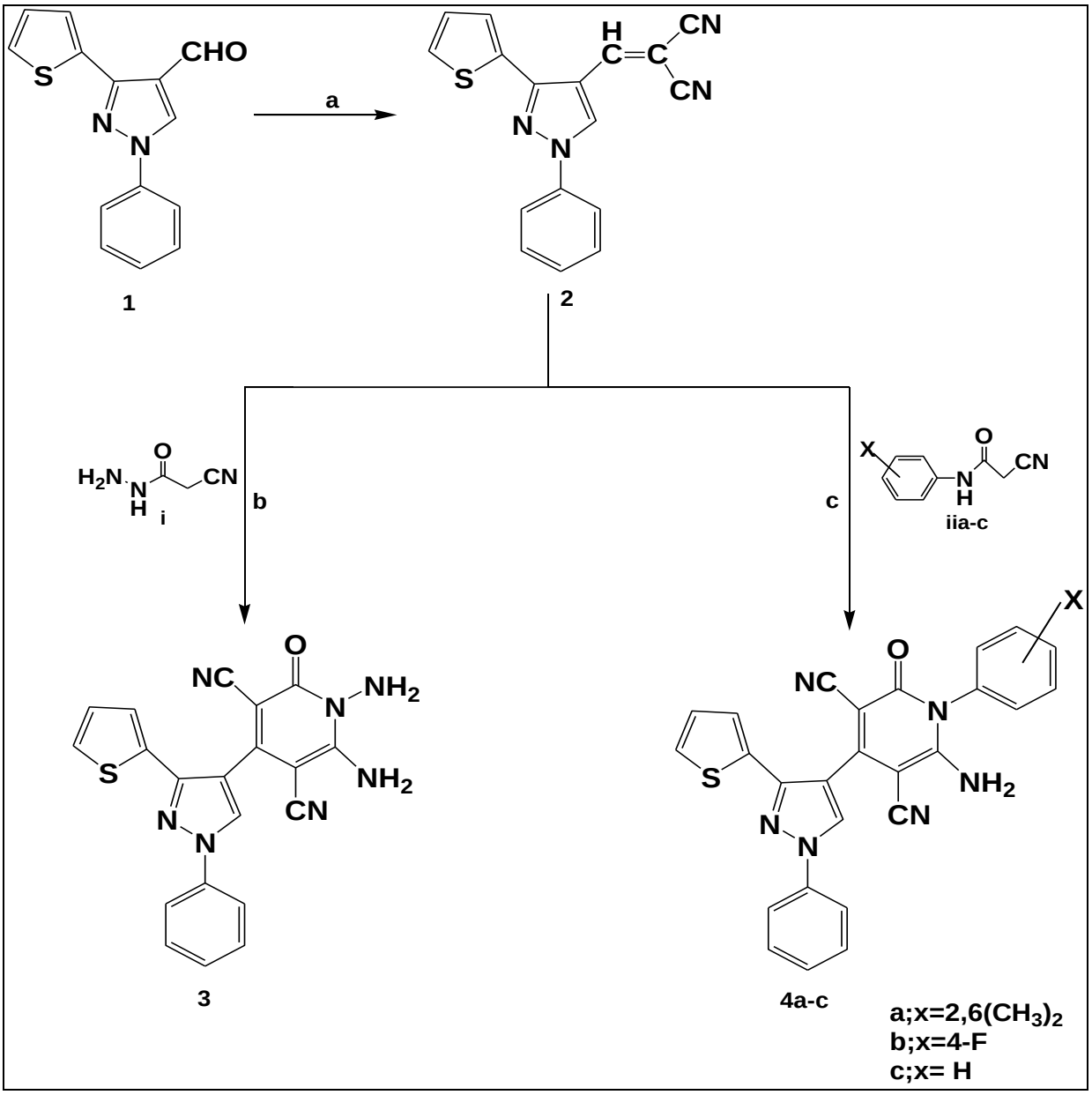
The MTT assay method was used to evaluate the tested compounds **3** and **4a-c**'s *in vitro* anti-proliferative capability (As shown in supplementary file) against HCT-116, MCF-7 and PC3 cell lines.[31, 32] Sorafenib was utilized as reference. Results were recorded in **Table 1** as 50% inhibition concentration value (IC_{50}). The safety profile of the promising derivative **3** was also tested, through determination of its cytotoxic effect on normal cells WI-38. Data was listed in **Table 1.**

2.3. ***In vitro* assay of VEGFR2 inhibition**

The VEGFR2 inhibitory activity was measured by using Kinase Kit (BIO- SCIENCE, Catalog # 40,325) to compare the VEGFR-2 enzyme-inhibitory potential of the new hits to that of the standard drug sorafenib. [33] (For more details, see supplementary file).

3. RESULTS

In scheme 1,thienyl-pyrazoles derivatives 3 and 4a-c were prepared by adopting the following procedure. The corresponding intermediate 2 was reacted with 2-cyanoacetohydrazide or 2-cyano-*N*-substituted-phenylacetamide under reflux in ethanol in the presence of catalytic drops of piperidine. TLC following up proved reaction completion in a reliable yield.



Scheme 1. Synthesis of thienyl-pyrazoles3 and 4a–c.
Reagents and conditions a) malononitrile/ ethanol/ piperidine, reflux, 3h. b) 2-cyanoacetohydrazide/ piperidine/ ethanol, reflux, 8h. c) 2-cyano-*N*-substituted-phenylacetamide/ piperidine/ ethanol, reflux, 8h.

3.2. Biological evaluation

3.2.1 In vitro anticancer evaluation:

The antitumor effect of our hits was investigated as IC_{50} values towards HCT-116, MCF-7 and PC-3 cancer cells. Compound 3 displayed a potent cytotoxicity against all tested cell lines, while the derivatives 4a-b showed weak potency. Finally, thienyl-pyrazole analogue 4c showed moderate cytotoxicity against the three cells.The safety profile of the most potent derivative 3 was also estimated on normal cells WI-38, where the result displayed high margin of safety.Theresultsare tabulated in (Table1).

Table 1. *In vitro* cytotoxicity of **3**, **4a-c** and sorafenib towards HCT-116, MCF-7, PC-3 and normal fibroblast WI-38.

Comp.	IC_{50} µM			
	HCT-116	**MCF-7**	**PC-3**	**WI-38**
3	7.41±0.5	3.36±0.2	9.58±0.7	46.34±2.5
4a	57.81±3.3	49.39±2.9	64.03±3.6	ND
4b	51.27±3.0	42.07±2.6	59.21±3.4	ND
4c	34.06±2.3	28.16±2.1	33.76±2.4	ND
Sorafenib	5.47±0.3	7.26±0.3	11.53±0.9	10.65±0.8

IC50 (µM): 1 – 10 (very strong cytotoxic). 11 – 20 (strong). 21 – 50 (moderate). ND: not done.

3.3.2 In vitro assay of VEGFR-2 activity

The VEGFR-2 tyrosine kinase (VEGFR-2) assay was carried out aiming todeclarethe mechanism of cytotoxic activity observed by the synthesized analogues. The most active compound in cytotoxic assay **3**, was further evaluated to determine its inhibitory activity against our target VEGFR-2. The results were listed as 50% inhibition concentration value of the enzyme (IC_{50}) compared to sorafenib, the reference molecule **(Table2).**

Table 2. VEGFR2 inhibitory activity of compound **3** compared to sorafenib.

Comp.	IC_{50} µM± SD
3	**0.223±0.01**
Sorafenib	**0.041±0.002**

3.3 Docking study

The binding affinity of the most active derivative 3 was investigated against our target, VEGFR-2 (PDB ID: 4ASD) utilizing MOE 2014 software. The co-crystallized ligand was used as reference molecule. The output of docking studies *demonstrated* a high affinity of newly synthesized analogue 3 against the target active site compared to the reference. Compound 3 has docking score energy (-7.40 Kcal/mol) compared to sorafenib, the reference ligand (-8.52 Kcal/mol)). The resulted data are pictured in (Table 3, Fig. 3, 4).

Table 3. Molecular docking data of compound**3** and sorafenib at the active site of VEGF

Comp. No.	Energy score (Kcal/mol)	Aminoacidresidues(bond length Aº)	Type of bond	Atoms of comp.
Sorafenib	-8.52	Glu885(1.56) Glu885(2.57) Cys919(1.76) Cys919(1.82) Asp1046(2.01) Val916	H-bond(donor) H-bond(donor) H-bond(donor) H-bond(acc) H-bond(acc) Pi-H	H(NH)(NHCONH) H(NH)(NHCONH) H(NH)($NHCH_3$) N(pyridine) O(NHCONH) Phenyl ring
3	-7.40	Glu885(2.08) Asp1046 Asp814(3.04) Arg1027(2.79) Arg1027(3.18)	H-bond(acc) Pi-H H-bond(donor) H-bond(acc) H-bond(acc)	N (Cyano group) Phenyl ring H(NH)(NNH_2) O(Pyridinone) O(Pyridinone)

4. DISCUSSION

4.1. Chemistry

Herein, the title derivatives were prepared following the sequence designated in **Scheme (1)**. **3** and **4a-c** were synthesized successfully via the reaction of arylidenemalononitrile **2,** with an equimolar amount of freshly prepared 2-cyanohydrazide for compound **3,** or synthesized 2-cyano-*N*-2-substituted-phenyl acetamide for compounds **4a-c,** in ethanol containing catalytic drops of piperidine [18, 34-36]. On the basis of spectral and elemental investigations, the aforementioned structures were identified. **I.R**, displayed the presence of absorption bands around 3336 to 3326 cm^{-1}, equivalent to NH_2, as well as sharp bands of CN

groups around 2214 to 2192 cm^{-1}, and carbonyl groups around 1689 to 1620 cm^{-1}**. ^{1}H-NMR** spectra exhibited a characteristic singlet, attributed to the Pyrazole C_5-H at the range δ 8.91 to 9.11. In addition to, a significant increase in the aromatic protons was detected for compounds **4a-c. ^{1}H-NMR** spectrum of compound **3** showed two singlets at δ 5.68, 10.19 ppm corresponding to the two NH_2 protons exchangeable with D_2O. **^{1}H-NMR** spectrum of **4a** displayed two singlets at δ2.07, 2.11 ppm representing the two methyl groups protons.**^{13}C-NMR** spectrum of **4a** exhibited a signal at δ14.9 ppm, refered to the two aliphatic carbons of CH_3 groups.

4.2 *In vitro* anticancer evaluation:

For their *in-vitro* cytotoxic activity in colon (HCT-116), breast (MCF-7), and prostate (PC-3) cancer cells, our target hits were examined. Biological activities of the tested compound were estimated by MTT evaluation method. The (IC_{50} values) were evaluated comparing to standard drug (sorafenib) **(Table1)**. Compound **3** exhibited greater cytotoxicity against all tested cell lines. Noticeably, derivative **3** (IC_{50} = 3.36 uM) displayed twice the activity of sorafenib (IC_{50} = 7.26μM) against MCF-7cell line. In addition, compound **3** was nearly equipotent to sorafenib against PC3 cell line and revealed73% of the anticancer activity of the reference drug against HCT-116 with IC_{50} values of 9.58 and 7.41 μM for the two cell lines, respectively. Concerning the effect of **3** on the normal fibroblast cells **(**WI-38), the compound recorded a high IC_{50} value of 46.34 μM, indicating remarkable selectivity towards cancer cells, and lower toxicity to normal ones **(**WI-38).

4.3 *In vitro* assay of VEGFR-2 activity

Compound **3** showing the most potent cytotoxicity against the three tested cancer cell lines was further evaluated to determine its inhibitory effect on VEGFR-2**,** aiming to affirm VEGFR-2 inhibitory activity of compound 3. According to reported methods, the activity of **VEGFR-2** was determind. Sorafenib was used as a reference in the mentioned assay. Results were recorded as 50% inhibition concentration of the enzyme (IC_{50}) **(Table 2).** Compound **3** revealed good inhibitory efficiency against **VEGFR-2** (IC_{50} = 0.223 ± 0.01μM) compared to the reference (IC_{50} = 0.041 ± 0.002μM).

4.4. Structure activity relationship

On the basis of the outcomes of the *in vitro* antitumor assay and the VEGFR-2 inhibitory effect of our new thienyl-pyrazole compounds (Tables 1 and 2), it was noticed that hybridizing 1-phenyl-3-(thiophen-2-yl)-1H-pyrazole scaffold with a 1-amino substituted pyridine moiety exhibited a significantly higher cytotoxic activity than sorafenib. On the other hand, diminished cytotoxic potency was observed by replacement of the NH_2 group on position one of the pyridine, with a phenyl or substituted phenyl rings **4a-c.**Concerning the SAR study, we can conclude that introduction of the NH_2 group **(**compound **3)** in position 1 of the pyridine moiety produce a marked increase in the anticancer activity than phenyl substitution on the same position. However 2, 6 dimethyl or p-fluoro substituted derivatives **(4a, b)** exhibit a lower antiproliferative effect than un-substituted counterpart **(4c**). This indicates that the phenyl substitution with an electron donating or withdrawing group, impart a negative impact on the activity in regard to the un-substituted derivative.

4.5 Docking study

Considering the findings of *in vitro* VEGFR-2 kinase activity inhibition, the docking simulation of the most potent thienyl-pyrazole derivative **3**, was performed to recognize the interaction and affinity towards the essential amino acids in the VEGFR-2 binding site. The Docking study was achieved using Molecular Operating Environment MOE 2014.0901version. The crystal structure of VEGFR-2 in complex with the ligand sorafenib was downloaded from the protein databank (code: 4ASD) [23, 37]. The co-crystallized ligand, sorafenib was redocked inside the active binding site of VEGFR-2 (energy score of −8.52 kcal/mol) to perform validation process. The affinity mode of sorafenib showed that Cys919 in the binding site is linked to NH of amide moiety and nitrogen of pyridine by two hydrogen bonds. Furthermore, the urea moiety was able to form important H-bonds with Asp1046 and Glu885 **(Figure3)**. Besides, phenyl ring formed an arene-H binding with Val 916.

The promising newly synthesized compound **3** was docked into the ATP-binding site of VEGFR-2 (4ASD).

As shown in (Figure 4), the docking result of compound 3 displayed interesting binding with the ATP binding site. It formed four hydrogen bonds, one between N of 5-CN group and the key amino acid Glu885 (distance: 2.08 A), two of them between the oxygen of the pyridinone ring and Arg1027 (distance: 2.7A& 3.18 A), and the last one between nitrogen of 1-amino-1,2-dihydropyridine and Asp 814(distance: 3.04A). Moreover, phenyl ring formed arene-H binding with the essential amino acid Asp1046. The docking results were recorded in (Table 3).

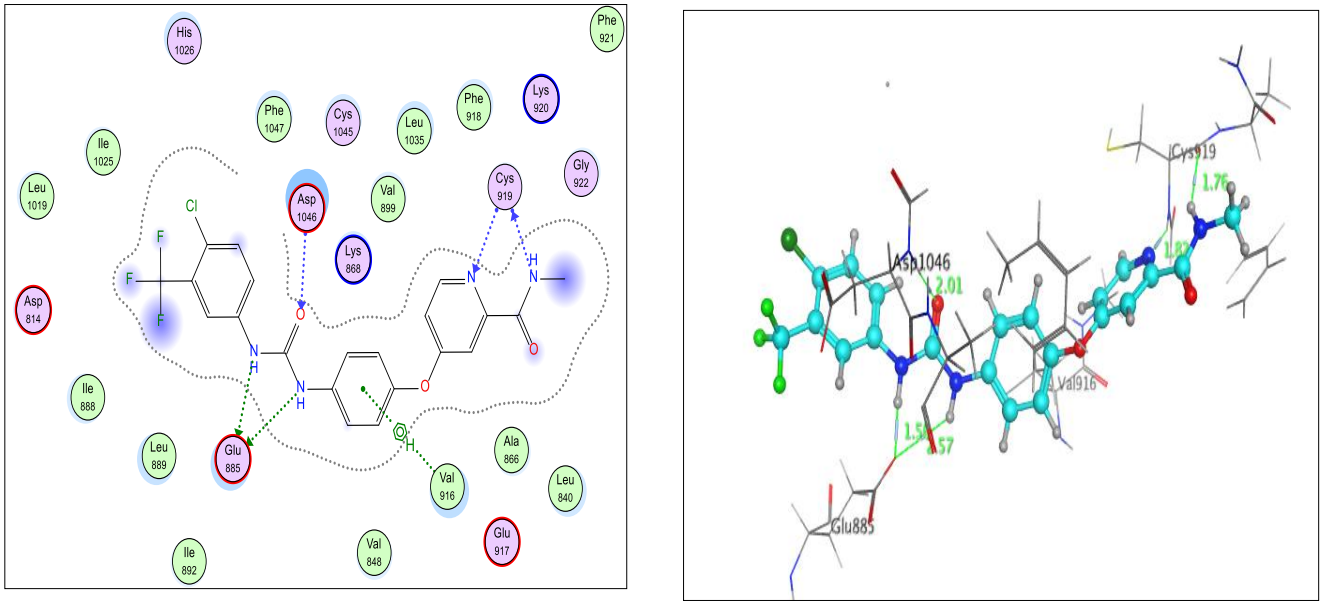


Figure 3. The proposed 2D (Left) and 3D (right) binding affinity of sorafenib at VEGFR-2 active site.

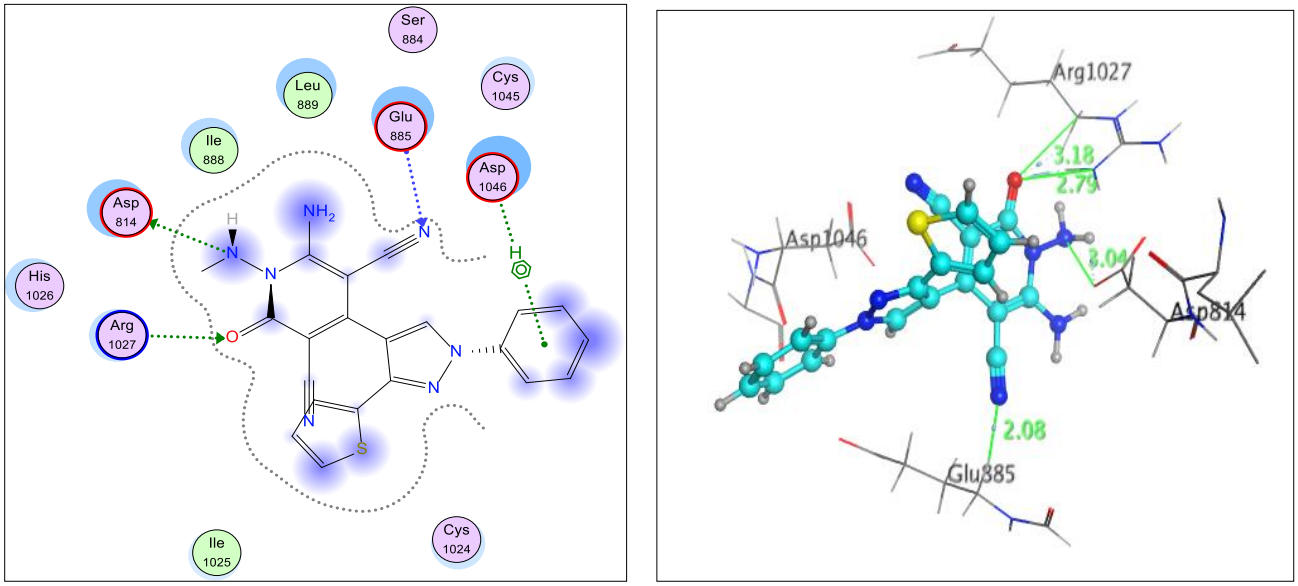


Fig 4. The proposed2D (Left) and 3D (right) binding mode of compound **3** at VEGFR-2 active site

5. CONCLUSIONS

In our work, a new series of thienyl-pyrazoles were prepared. The cytotoxic effect of the pyrazole hits was investigated against HCT-116, MCF-7, and PC-3 cancer cells. Compound 3 exhibited a prominent antitumor efficiency against MCF-7 (IC_{50}=3.36μM) superior to the reference drug sorafenib. Furthermore, compound 3 revealed 1.2 and 72% of the anticancer activity of the reference drug against PC3and HCT-116 cells, with IC_{50} values 9.58, 7.41 μM, respectively. The VEGFR-2 inhibition study revealed that compound 3 showed good inhibitory effect on VEGFR-2 (IC_{50} = 0.223μM) in comparison with sorafenib (IC_{50} = 0.041μM). The VEGFR-2 inhibitor 3 displayed excellent affinity towards VEGFR-2 active site, fitting inside the active ATP binding pocket via Pi-H contact with the essential amino acid Asp1046. As well as, H- bond formation with the essential residues Glu885, Arg1027and Asp 814.

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Author Contribution: All authors had full access to all the information and took responsibility for data integrity and data analysis accuracy. Author Hend M.A. El-Sehrawi revised spectral data, revised the manuscript and supervised the whole work. Author Yousry A. Ammar designed the chemistry part of the work and elucidated the spectral data. Author Rehab Sabour wrote andrevised the manuscript. Author Lamia H.T. Aminperformed the experimental work, wrote the manuscript. The final manuscript was read and accepted by all the contributors.

List of Abbreviations: VEGFR-2: Vascular endothelial growth factor receptor type 2, MCF-7: breast cancer cells, HCT-116: human colorectal carcinoma, PC-3: prostate cancer.

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