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# DESIGN, SYNTHESIS, MOLECULAR DOCKING AND BIOLOGICAL EVALUATION OF SOME NEW THIOPHENE DERIVATIVES AS ANTI-CANCER AGENTS

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## ABSTRACT

Structure-based design, synthesis and biological evaluation of newly synthesized small molecules anti-cancer agents were described. On continuation of applying scaffold hopping theory, a series of substituted 2-amino, 3-cyanothiophene based on the structural features of Sorafenib was designed, synthesized and evaluated as cytotoxic agents against MCF7 and HCT 116 cell lines. Substituting the amino group of 2-aminothiophene with different pharmacophoric groups led to discovery of two potent lead compounds **IIIb** and **IIIc** with excellent cytotoxic activity against MCF7 and HCT 116 cell lines.

**KEYWORDS:** Anti-Cancer Agents; Angiogenesis; VEGFR-2; Thiophene Derivatives and Structure-based Drug Design.

## 1. INTRODUCTION

Cancer is a leading cause of morbidity and mortality worldwide with over a million cases every year.[1] The design of new compounds prototypes is a good strategy for discovery of new drugs. Designing a molecule that can selectively inhibit the proliferation of abnormal cells (target therapy) only with little or no effect on normal cells is a difficult task.[2]

Angiogenesis; the formation of new blood vessels from pre-existing ones, is a normal physiological process taking place during embryogenesis, inflammation and wound healing. Pathological angiogenesis is associated with many diseases such as cancer, where new blood vessels infiltrate tumor masses supplying them with oxygen and nutrients to enhance growth and metastasis. Hence, targeting protein kinases is an important strategy for intervention in cancer. Inhibitors are directed at the active conformation or a variety of inactive conformations. Blocking angiogenesis could be a strategy to hinder tumor growth.[3,4] Numerous growth factors are involved in angiogenesis. One of the most known angiogenic molecules is the vascular endothelial growth factor (VEGF) family members which are pivotal stimuli of physiological as well as pathological angiogenesis.[4]

Generally, VEGFR-2 inhibitors are classified according to whether they competitively bind to the ATP binding pocket in the active DFG-in motif conformation (a set of active and inactive conformations where the Phenyl residue is in contact with the C-helix of the N-terminal lobe) (type I) or the inactive "DFG-out" conformation (an inactive form where Phenyl occupies the ATP site exposing the C-helix pocket) (type II), or noncompetitively via binding outside the ATP binding pocket in the allosteric hydrophobic back pocket (type III).[5-7] Type I inhibitors act on the active "DFG-in" conformation via hydrogen bonding with the hinge region amino acid Cys919 and hydrophobic interactions in and around the adenine region.[7] Type II inhibitors such as sorafenib (Figure 1) occupy the ATP binding site and extend over the gatekeeper Val916 into the adjacent allosteric hydrophobic back pocket in the inactive "DFG-out" conformation.[7-8] Type III inhibitors bind to the inactive "DFG-out" conformation beyond the gatekeeper Val 916 exclusively to the less conservative allosteric hydrophobic back pocket outside the ATP binding pocket locking VEGFR-2 in the inactive "DFG-out" conformation. Therefore, they are expected to have superior selectivity profiles and offer new opportunities for scaffold development.[5-10]

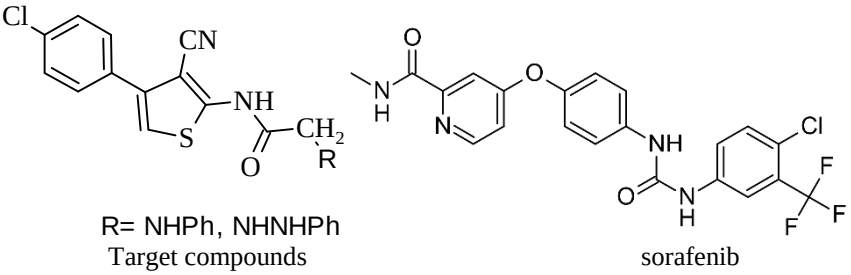



**Figure 1**

Moreover, replacing the benzene with bioisoteric thiophene ring in sorafenib (Figure 1) give us predication to increase the activity of newly synthesized compounds. The amino group of thiophene was substituted with various hydrophobic groups such as alkyl, and phenyl ring systems through a spacer or without a spacer in position 2. The aim of this suggested modification was to increase the hydrophobic nature of the newly synthesized derivatives in order to increase their binding affinity to VEGFR-2 through increasing the hydrophobic interaction with its allosteric hydrophobic back pocket. The designed novel 2-aminothiophene derivatives were synthesized and their antitumor activity against MCF-7 and HCT116 cell lines was evaluated. Molecular docking studies were performed to find out the possible binding mode of the newly synthesized compounds with VEGFR-2, and to study their interaction with the receptor hot spots (key amino acids) with the aim of explaining their VEGFR-2 inhibitory activity. The strategy of this work was the usage of the potent inhibitors' common features to design a series of potential anti-cancer of thiophene derivatives. Synthesis of thiophene derivatives was represented in **Schemes 1-2.**

## 2. RESULTS AND DISCUSSION

**2.1. Designing the Target Compounds:** By using the above findings as guidance for the molecular modeling study and by varying the substitution on 2- amino group to explore their effect on activity, and also by comparing the compounds to be synthesized to the reference compound, forty derivatives were docked in VEGFR2/KDR kinase. Only fifteen derivatives showed good binding to the receptor, good superposition and good docking scores. From these fifteen derivatives only nine compounds were synthesized and six derivatives out of these nine compounds subjected to biological screening for their antitumor activity.

Docking study showed that, the docked compounds bind to the binding site with moderate to good binding score. Moreover, they fit to the active site pocket with moderate to good interactions with the amino acids **(Table 1)** suggesting moderate to good biological activity as bioactive agents.

**Table 1: Docking data for the newly synthesized compounds and the reference compound in VEGFR-2 active site.**

| Cpd. No | No of H- bonds | Amino acid residues forming H-bonds | Binding energy score [a] |
|---|---|---|---|
| **IIIa** | 3 | Asp 1046,Glu 885,Ph 1047 | -14.06 |
| **IIIIb** | 2 | Asp 1046,Glu 885 | -14.4 |
| **IIIIc** | 2 | Asp 1046,Glu 885 | -14.9 |
| **IIId** | 2 | Asp 1046,Glu 885 | -14.15 |
| **IIIe** | 2 | Asp 1046,Glu 885 | -13.47 |
| **IIIf** | 3 | Asp 1046,Glu 885,Asp 814 | -14.6 |
| **IVa** | 4 | Asp 1046, Asp 1046, Asp 1046, Glu 885 | -14.32 |
| **IVb** | 3 | Asp 1046,Glu 885,Ph 1047 | -12.0 |
| **V** | 1 | Asp 1046 | -12.14 |
| **Sorafenib** | 4 | Asp 1046,Glu 885,Cys 919, Cys 919 | -15.92 |

[a] Binding energy score (kcal/mol): energy of interaction of the ligand and organic compounds in the active site.

Molecular docking was carried out with the aim of explaining the promising VEGFR-2 inhibitory activity of the newly synthesized compounds through investigating their binding mode and their interaction with the key amino acids in the active site of the VEGFR-2. Several crystal structures are available in the protein data bank for VEGFR-2[6], for this work we selected (PDB ID: 4ASD)[11] which has VEGFR-2 in the inactive "DFG-out" conformation co-crystallized with sorafenib as type II inhibitor. First, validation of the molecular docking protocol was performed by re-docking of the co-crystallized ligand (sorafenib) in the VEGFR-2 active site. The re-docking validation step reproduced the experimental binding pattern of the co-crystallized ligand efficiently indicating the suitability of the used protocol for the planned docking study as demonstrated by the small RMSD of 0.79 Å between the docked pose and the co-crystallized ligand (energy score (S)=-15.92 kcal/ mol) and by the capability of the docking pose to reproduce all the key interactions accomplished by the

co-crystallized ligand with the key amino acids in the active site (Glu885, Cys919 and Asp1046) **(Figure 2).**

The binding pattern of all synthesized compounds accomplishes a hydrophobic interaction between the hydrophobic aromatic moiety and the hydrophobic back pocket which is lined with the hydrophobic side chains of Ile888, Leu889, Val899, Leu1019 and Ile1044 amino acids achieving the promising VEGFR-2 inhibitory activity of the newly synthesized compounds. Also most of the compounds performed further interactions with the VEGFR-2 binding site through hydrogen bonding with the side chain carboxylate of Glu885 of the C helix and/or with Asp1046 in the conserved DFG motif **(Figures 3-4).**

All synthesized compounds except **V** showed both hydrophobic interaction and the hydrogen bonding interactions with the side chain carboxylate of Glu885 and with DFG motif Asp1046 in the active site (Figures 2 -4). This binding pattern of all compounds except **IVb** and **V** have the best VEGFR-2 inhibitory activity among the newly synthesized compounds **(Table 1).**

In compound **IIIa, IIIb** and **IIIc** the presence of a para methyl, bromo or chloro substituents respectively on the aromatic ring increases the hydrophobic interaction with the allosteric hydrophobic back pocket and so increases the binding interaction as can be noticed in its docking score (14.44 kcal/mol.) as compared to that of its analogue v (12.14 kcal/mol.) **(Table 1).**

In summary, para substitution with hydrophobic groups on aromatic ring increase the hydrophobic interaction with the allosteric hydrophobic back pocket of VEGFR-2 active site. Also presence of suitable hydrogen bond donor and acceptor to interact with Glu885 and Asp1046 amino acid residues are very important.

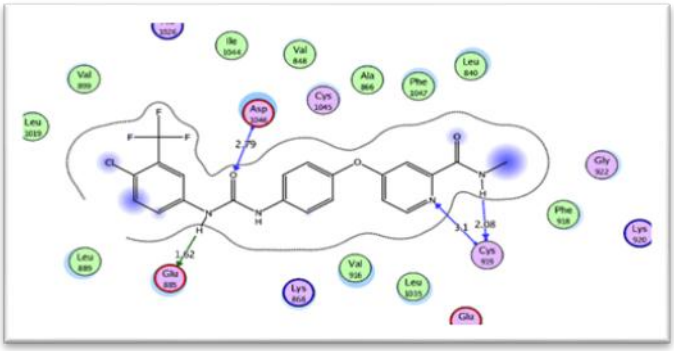
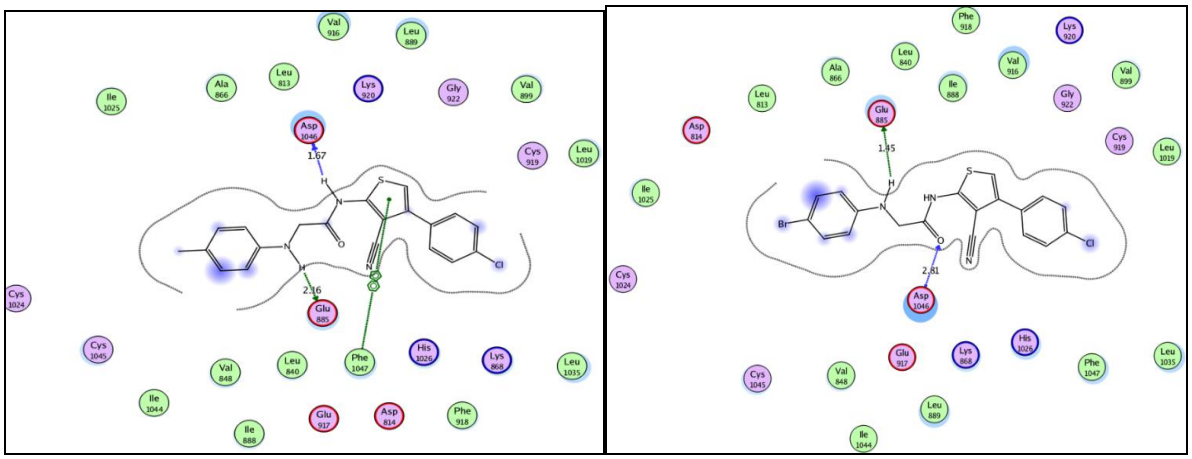
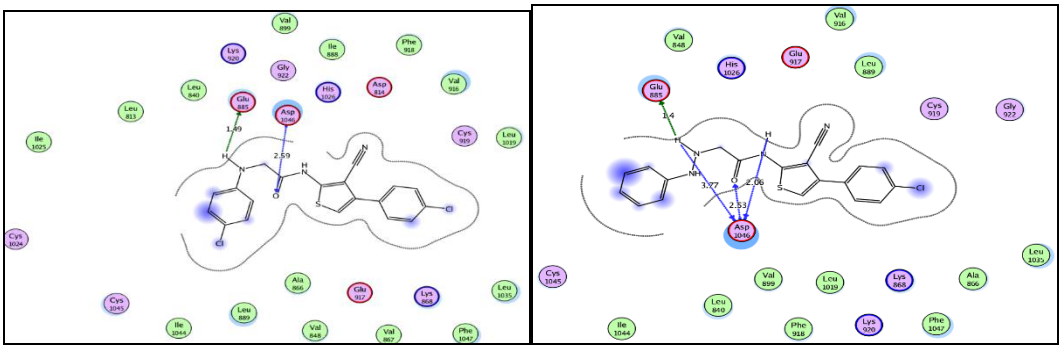


**Figure 2: 2D interaction diagram showing sorafenib docking pose interactions with the key amino acids (hot spots) in the VEGFR-2 active site. (Distances in Å).**



**(A)** **(B)**

**Figure 3: 2D diagram (A) of compound IIIa and 2D diagram (B) of compound IIIb, docked into the VEGFR-2 receptor active site. (Distances in Å).**



**(A)** **(B)**

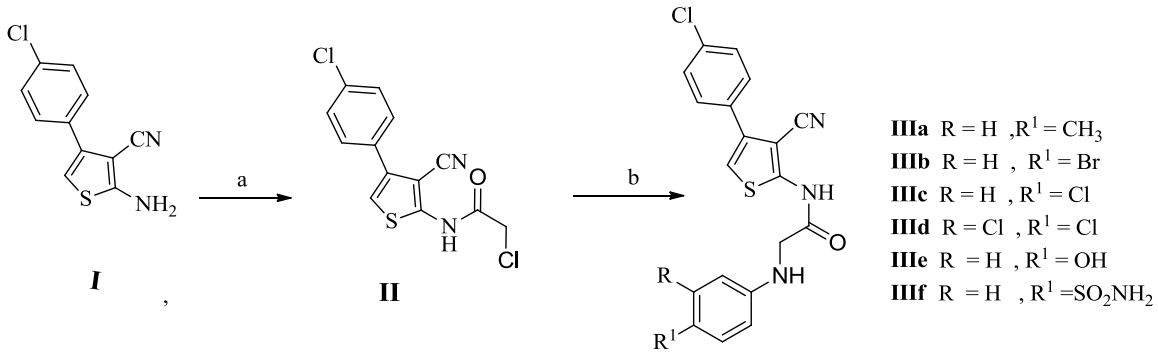
**Figure 4: 2D diagram (A) of compound IIIc and 2D diagram (B) of compound IVa, docked into the VEGFR-2 receptor active site. (Distances in Å).**

### 2.2. Chemistry

Knoevenagel condensation of acetophenone with malononitrile afforded 2-[1-(4-aryl)-ethylidene]-malononitrile which upon base promoted cyclization with elemental sulfur gave 2- aminothiophene-3-carbonitrile (**I**) in excellent yield.[12]

Compound **II** was prepared by treating the thiophene o-amino nitrile **I** with chloroacetyl chloride and triethyl amine to afford the corresponding N-chloroacetylated derivative **II**, **Scheme 1.** The structure of compounds **II** was confirmed by element analysis, IR, $^1$H NMR.

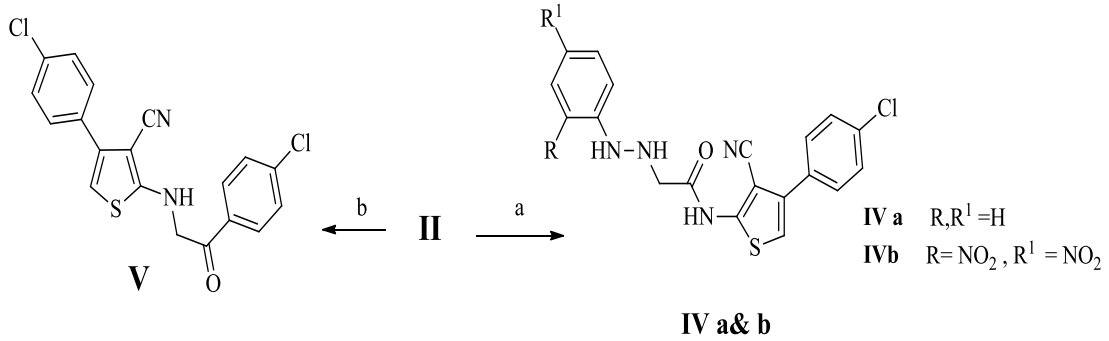
**Scheme 1**




**a-dioxan,Chloroacetylchloride,TEA**
**b-Dioxane , reflux**

N-chloroacetylated derivative **II** was in turn allowed to react with substituted aniline or with sulfonamides in dioxane and few drops of triethyl amine to give the corresponding N-phenyl aminoacetamide derivatives **IIIa-f**. On the other hand, compounds **Iva-b** were obtained by refluxing compound **II** with phenyl hydrazine or 2,4 dinitrophenyl hydrazine in ethanol. Compound **V** was obtained by reacting compound **II** with chlorophenacyl bromide in dioxane and triethyl amine. Structures of compounds **IVa-b** and **V** were confirmed using spectral data and microanalyses.

**Scheme 2**




**IV a& b**

a: Substituted phenyl hydrazine , ethanol, reflux
b: chlorophenacyl bromide, Dioxan, TEA, reflux

### 2.3. Biological studies

The synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human breast cancer cell line MCF-7 and colon cancer cell line HCT116 using Sulforhodamine B colorimetric assay (SRB assay).[13-14] The $IC_{50}$ values of the tested compounds as well as the positive controls are summarized in Table 1. From the data obtained (Table 2), it is obvious that compound **IIIc** exhibited promising activities ($IC_{50}$ = 2. 44 µg/ml against breast carcinoma cell line MCF-7.

Additionally, compounds **IIIa, IIIb** and **IVa** ($IC_{50}$= 8.2 µg/ml, 9.4 µg/ml and 13.5 µg/ml respectively) showed mild activities against MCF-7 cell line. Compound **IIIb** exhibited excellent activities ($IC_{50}$ = 0.48 µg/ml) against colon cancer cell line HCT 116.

**Table 2: Results of in vitro cytotoxic activity of tested compounds and Sorafenib on breast cancer cell line (MCF-7) and colon cancer cell line (HCT 116).**

| Cpd. No | $IC_{50}$ µg/ml | |
|---|---|---|
| | MCF-7 | HCT 116 |
| **IIIa** | 8.2 | 2.34 |
| **IIIIb** | 9.4 | 0.48 |
| **IIIIc** | 2.44 | 1.89 |
| **IVa** | 13.5 | 29.9 |
| **IVb** | 38.3 | 43.3 |
| **V** | 31.4 | 46.3 |
| **Sorafenib**[15] | 4.33 | ---------- |

## 3. CONCLUSION

From the synthesized series, compounds **IIIa, IIIb, IIIc** and **IVa** showed potent cytotoxicity against MCF-7 and HCT116 cell lines in comparison to sorafenib. Compound **IIIc** was found to have promising inhibitory activity on breast cancer cell line MCF 7($IC_{50}$ = 2.44 µg/ml) in comparison to sorafenib ($IC_{50}$= 4.33 µg/ml). Molecular docking attributed their high potency to their type III inhibitors-like binding mode.

They bind to the VEGFR-2 active site through hydrogen bonding interaction with the key amino acids in VEGFR-2 active site, Glu885 and Asp1046, and their hydrophobic interaction by their 2-aminotiophenes moiety with the allosteric hydrophobic back pocket which is lined with the hydrophobic side chains of Ile888, Leu889, Val899, Leu1019 and Ile1044 amino acids The binding interaction is augmented by the presence of a ring substituent with long chain extension at position para of the aromatic ring due to its hydrophobic interaction with the hydrophobic side chains of Ile888, Leu889, Val899, Leu1019 and Ile1044 amino acids at the interface between the ATP binding site and the allosteric back pocket.

## 4. Experimental

### 4.1. Chemistry

Chemistry Melting points (mp) were determined on Stuart apparatus and the values given are uncorrected. IR spectra were determined on Shimadzu IR 8400 s spectrophotometer (KBr, cm−1). $^1$H-NMR spectra were carried out using a Mercury, a Gemini 300-BB 300 MHz and Joel (eca) 500 MHz spectrophotometers using tetramethylsilane (TMS) as internal standard. Chemical shift values were recorded in ppm on δ scale, the solvent system was benzene, chloroform and methanol with different ratios and spots were visualized using UV lamp. The docking was performed using Molecular Operating Environment (MOE 2008.10). Evaluation of the cytotoxic activity was performed at the Egyptian National Cancer Institute. Compounds **I** was synthesized according to reported method.[12]

**2-Chloro-N-[4-(4-chloro-phenyl)-3-cyano-thiophen-2-yl]-acetamide (II)**
Chloroacetyl chloride (10 mmol) was added dropwise to a solution of **I** (10 mmol) in dioxane (30 mL) and triethyl amine (1 mL). The solution continued stirring and cooling for few minutes. The reaction mixture was refluxed for 10 h then cooled and poured onto crushed ice. The crude product was filtered and crystallized from DMF/ethanol mixture in a ratio of (1:15).

Yield, 60%. mp =222–224$^{O}$C. IR (KBr, cm$^{-1}$): v =2218 (CN), 1680 (C=O). $^{1}$H NMR (DMSO) 4.01 (s, 2H, $COCH_2$), 6.80 (s, 1H, thiophene), 6.97 (d, 2H, NHAr), 6.73-8.10 (d, 2H, ArH), 9.21 (s,1H, CONH).

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-phenylamino-acetamide derivatives (IIIa-f)**
N-chloroacetylated derivatives I(2mmol) and the appropriate aniline derivatives (1.2 mmol) were dissolved in dioxane (10mL). The reaction mixture was treated with (1mL) triethyl amine, heated under reflux for 15 h. The reaction mixture was cooled and poured into crushed ice. The crude product was filtered and recrystallized from the appropriate solvent.

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-p-tolylamino-acetamide (IIIa)**
Yield, 58% (acetone). mp = 232-234$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 2219 (CN), 1693 (C=O). $^{1}$H NMR (DMSO) 2.31 (3H, s), 4.20 (s, 2H, $COCH_2$), 6.97-7.20 (m, 4H, ArH+1H, NHAr), 7.30-8.10 (m, 4H, ArH + 1H, thiophene), 9.50 (s,1H, CONH).

**2-(4-Bromo-phenylamino)-N-[4-(4-chloro-phenyl)-3-cyano-thiophen-2-yl]-acetamide (IIIb)**
Yield, 60% (ethanol). mp = 226-228$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 2220 (CN), 1690 (C=O). $^{1}$H NMR (DMSO) 4.30 (s, 2H, $COCH_2$), 6.50-7.20 (m, 4H, ArH+1H, NHAr), 7.30-8.10 (m, 4H, ArH + 1H, thiophen), 8.90 (s,1H, CONH).

**2-(4-Chloro-phenylamino)-N-[4-(4-chloro-phenyl)-3-cyano-thiophen-2-yl]-acetamide (IIIc)**
Yield, 65% (ethanol). mp = 228-230$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 2219 (CN), 1695 (C=O). $^{1}$H NMR (DMSO) 4.40 (s, 2H, $COCH_2$), 6.50-7.250 (m, 4H, ArH+1H, NHAr), 7.40-8.10 (m, 4H, ArH + 1H, thiophene), 9.0 (s,1H, CONH).

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-(3,4-dichloro-phenylamino)-acetamide (IIId)**
Yield, 63% (ethanol). mp = 223-225$^{o}$C. IR (KBr, cm$^{-1}$): v = 2221 (CN), 1689 (C=O). $^{1}$H NMR (DMSO) 4.5 (s, 2H, $COCH_2$), 6.50-7.250 (m, 2H, ArH+1H, NHAr), 7.30 (s,1H, ArH), 7.50-8.10 (m, 4H, ArH + 1H, thiophene), 9.0 (s,1H, CONH).

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-(4-hydroxy-phenylamino)-acetamide (IIIe)**
Yield, 56% (ethanol). mp = 220-222$^{o}$C. IR (KBr, cm-1): ***v*** = 3381-2918 (broad, OH), 2218 (CN), 1693 (C=O). $^{1}$H NMR (DMSO) 4.23 (s, 2H, $COCH_2$), 6.80-7.30 (m, 4H, ArH), 7.40-8.30 (m, 4H, ArH + 1H, thiophen), 9.0 (s,1H, CONH), 9.63 (s, 2H, NHAr + OH).

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-(4-sulfamoyl-phenylamino)-acetamide (IIIf)**
Yield, 50% (acetone). mp = 230-232$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 2220 (CN), 1700 (C=O). $^{1}$H NMR (DMSO) 4.15 (s, 2H, $COCH_2$), 7.0-7.250 (m, 4H, ArH+1H, NHAr), 7.30-8.0 (m, 4H, ArH + 1H, thiophene), 9.0 (s,1H, CONH), 10.95 (s,2H, $SO_2NH_2$).

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-(N'-phenyl-hydrazino)-acetamide derivatives (IVa&b)**
A mixture of **II** (1 mmol) and hydrazine hydrate (99%, 3 mL) in absolute ethanol (20 mL) was heated under reflux for 6-8 h. The reaction mixture was cooled, filtered and the precipitate was dried and crystallized from the appropriate solvent to give compounds **IVa&b** respectively.

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-(N'-phenyl-hydrazino)-acetamide (IVa)**
Yield, 70% (ethanol). mp = 180-182$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 3240 (NH), 2230 (CN), 1699 (C=O). $^{1}$HNMR (DMSO): 3.98 (s, 2H, $COCH_2$), 6.40 (s, 2H, NH), 6.60-7.0 (m, 5H, ArH), 7.20-8.0 (m, 4H, ArH + 1H, thiophene), 8.90 (s,1H, CONH).

**N-[4-(4-Chloro-phenyl)-3-cyano-thiophen-2-yl]-2-[N'-(2,4-dinitro-phenyl)-hydrazino]-acetamide (IVb)**
Yield, 66% (ethanol). mp = 140-142$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 3330 (NH), 2225 (CN), 1690 (C=O). $^{1}$HNMR (DMSO): 4.10 (s, 2H, $COCH_2$), 6.60 (s, 2H, NH), 7.20-8.0 (m, 4H, ArH + 1H, thiophene), 8.10-8.50 (m, 2H, ArH), 8.60 (s,1H, ArH) ,9.10 (s,1H, CONH).

**4-(4-Chloro-phenyl)-2-[2-(4-chloro-phenyl)-2-oxo-ethylamino]-thiophene-3-carbonitrile (V)**
An equimolar amount (2mmol) of compound **II** and 2-Bromo-1-(4-chlorophenyl)-ethanone (1.2 mmol) were dissolved in dioxane (10mL). The reaction mixture was treated with (1mL) triethyl amine, heated under reflux for 15 h respectively. The reaction mixture was cooled and poured into crushed ice. The crude product was filtered and recrystallized from the appropriate solvent.

Yield, 49% (ethanol). mp = 190-192$^{o}$C. IR (KBr, cm$^{-1}$): ***v*** = 3330 (NH), 2220 (CN), 1696 (C=O). $^{1}$HNMR (DMSO): 4.10 (s, 2H, $COCH_2$), 6.60 (s, 1H, thiophene), 7.20-8.0 (m, 6H, ArH), 8.10-8.50 (d, 2H, ArH), 8.9 (s,1H, NH).

**4.2. Docking studies**
The X-ray crystallographic structure of VEGFR-2 co-crystallized with sorafenib as inhibitor (PDB ID: 4ASD)

was downloaded from the protein data bank http:// www.rcsb.org/.[16] Compounds were built using Molecular Operating Environment (MOE) molecule builder. The structures were energy minimized by Merck Molecular force field (MMff 94x). Hydrogen atoms and partial charges were added to the system using protonate 3D application. Compounds were grouped in databases, docked using the MOE Dock tool and default settings were used. Poses were generated by superposition of ligand atom triplets and triplets of points in the receptor binding site in a systematic way. Poses generated by the placement methodology were scored using an available method implemented in MOE. Poses resulting from the placement stage was then subjected to MMFF 94x energy minimization. For each docked compound only one pose was selected based on number of binding interactions, superposition with the original ligand, docking score and the formed H-bonds lengths (Table 1).

### 4.3. Cytotoxic activity

The antitumor activity was determined for the newly synthesized compounds in Egyptian National Cancer Institute (NCI) for *in vitro* detection of $IC_{50}$ of their antitumor activity. The breast tumor cell line (MCF-7) was obtained frozen in liquid nitrogen (-180 $^0$C) from the American Type Culture Collection (ATCC) and was maintained in the National Cancer Institute, Cairo, Egypt, by serial sub culturing. All chemicals used in this study are of high analytical grade. They were obtained from (either Sigma-Aldrich or Bio-Rad). The cytotoxicity of the test compounds was determined using.

SRB assay applying the method of Skehan et al.[17] These testing procedures were carried out at pharmacology lab at Cancer Biology Unit in Egyptian National Cancer Institute. The Cells were plated in 96-multiwell plate (104 cells/well) for 24 h before treatment with the compounds to allow the attachment of cells to the wall of the plate. Different concentrations of each compound (0, 1, 2.5, 5 and 10 mg/mL) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37 ºC and in atmosphere of 5% CO2. After 48 h, cells were fixed, washed, and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was plotted to get the survival curve of each tumor cell line, The $IC_{50}$ value was calculated using sigmoidal dose response curve-fitting models (Graph Pad, Prizm software incorporated), each concentration was repeated 3 times.

## ACKNOWLEDGEMENT

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