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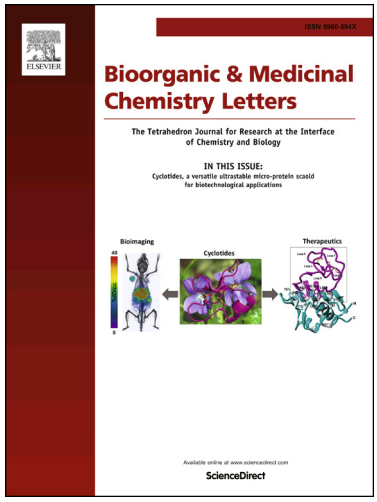

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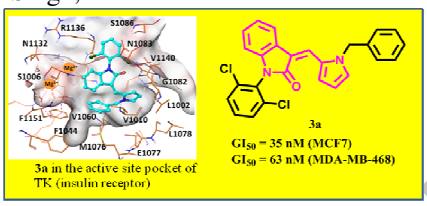

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ABSTRACT

Analysis of the crystal structure of tyrosine kinase in complexation with an ATP analogue, supplemented with the molecular docking studies of semaxanib and sunitinib in the ATP binding site of the enzyme enabled us to make design of a series of tyrosine kinase inhibitors. The combination of pyrrole and indolinone in one molecule and placement of appropriate substituent thereof made the molecule compatible for the hydrophobic sub-pocket of the enzyme. Screening of the compounds over 60 cell line panel of human tumor cell lines identified compound **3a** that exhibited GI_{50} 35 nM and 63 nM against MCF7 and MDA-MB-468 cell lines of breast cancer.

Cancer continues to be one of the major health problems and a leading cause of human suffering and deaths worldwide.[1] Population growth, increasing life expectancy and adoption of cancer associated lifestyle such as smoking are some of the specific grounds for the growing burden of cancer especially in the economically developed/developing countries. Among the various cancers, breast cancer is the most commonly diagnosed cancer in women as it rarely occurs in men. After lung cancer, breast cancer is the second leading cause of cancer deaths in women and as per the current records, in U.S. alone, 41,070 (40610 women and 460 men) people are estimated to die of breast cancer in 2017.[2] Besides the other remedial measures, chemotherapy is widely used for the treatment of majority of cancers[3] and a number of chemotherapeutic drugs such as taxol,[4] vinblastine,[5] vincristine,[6] etoposide,[7] camptothecin,[8] mitoxantrone,[9] 5-fluorouracil[10] and cisplatin[11] are in the clinical use. These drugs target cancer associated enzymes and signaling pathways. However, the economical availability and the associated side effects of these drugs are the major bottlenecks that hamper their practical applications.[12]

The signaling pathways are the critical cellular links wherein the tyrosine kinases play a pivotal role in post-translational modifications and hence in maintaining normal cellular communication.[13] Nonetheless, effected by the mutations, epidermal growth factor receptors (EGFR) and insulin growth factor receptors (IGFR); the activation of tyrosine kinases (TK) alters the signaling pathways and obstructs the regular cell functions like cell division, growth and normal cell death. Consequently, the role of tyrosine kinases are implicated in the breast cancer, prostate cancer, non-small cell lung cancer and bladder cancer making tyrosine kinases as the potential targets of anti-cancer drugs.[14]

The availability of the crystal structure of tyrosine kinase and the analysis of its ATP binding site by making use of molecular modeling studies helped to a large extent in the design of TK inhibitors.[15,16] Further exploration of the ATP binding site of IGFR-tyrosine kinase provided insight to the mode of interaction between ATP and TK. A number of H-bond interactions between the OH of sugar, N/NH of adenine and the amino acid residues were observed in the crystal coordinates of TK in complex with ATP analogue (Figure 1). The sugar and adenine template of ATP analogue were placed in the hydrophobic region constituted by L1002, V1010 and F1044 (Figure 1). Additionally, the interactions of TK inhibitors like sunitinib and semaxanib in the ATP binding site of the enzyme were also examined. It was observed that the polar region of sunitinib interacts through H-bonds whereas the hydrophobic region of both sunitinib and semaxanib is placed in the hydrophobic pocket of the enzyme (Figure 2). However, the large hydrophobic space in the active site of TK constituted by Val, Phe, and Leu residues remains unoccupied. Therefore, it is worthwhile to design TK inhibitors with large hydrophobic region so that they exhibit better interactions in the hydrophobic pocket of the enzyme. Advantageously, the hydrophobic interactions impart better reversibility to the enzyme–ligand complexation in comparison to the polar interactions.

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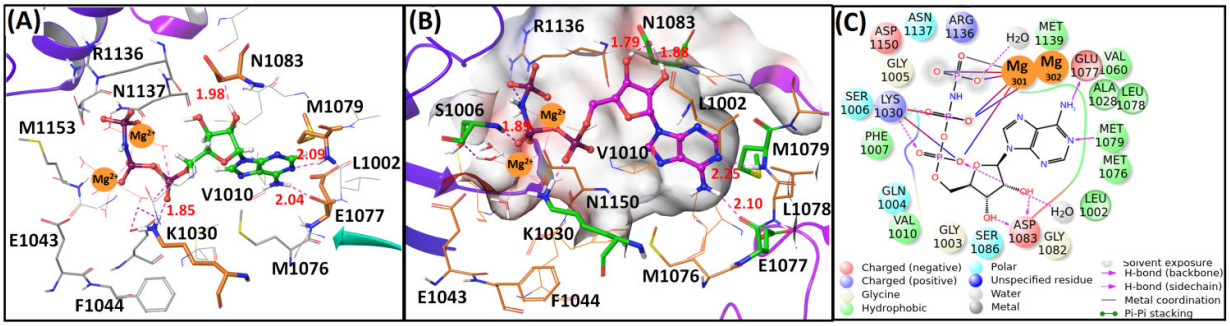


Figure 1. (A) ATP analogue in the crystal coordinates of tyrosine kinase (phosphorylated insulin receptor, pdb ID 1IR3) showing H-bond interactions (pink dotted lines) with the amino acid residues, (B) ATP analogue docked in the ATP binding site of tyrosine kinase (phosphorylated insulin receptor, pdb ID 1IR3). It is apparent that the adenine and sugar units are placed in the hydrophobic pocket made of L1002, V1010 and F1044, (C) 2D view of ATP analogue docked in tyrosine kinase binding site.

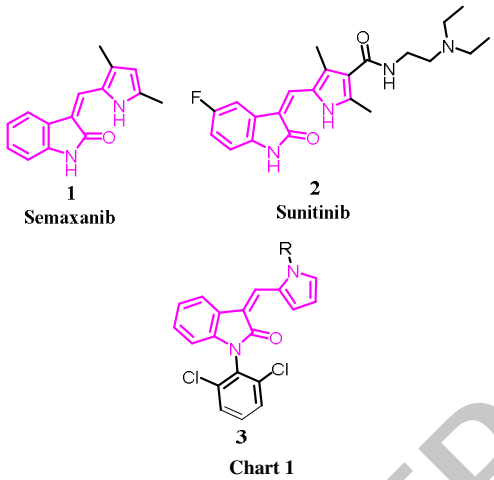
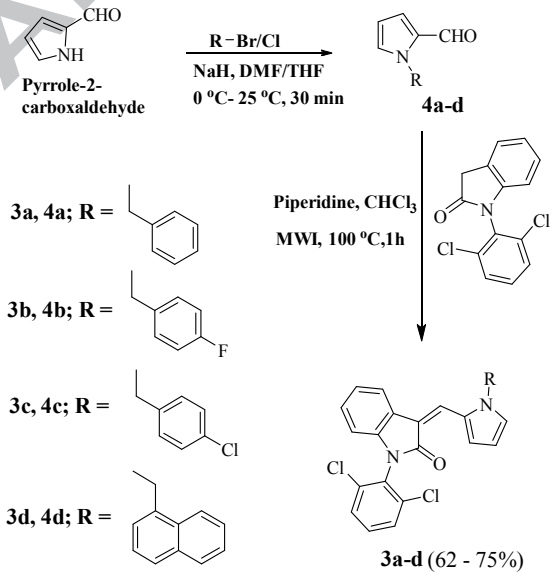


Chart 1

The strategy of combining two or more biologically active moieties for the design of new drugs (hybrid molecules) has resulted into the development of some lead molecules with remarkable biological activity.[17] In the present study, taking into consideration the biological importance of pyrrole[18-21] and indole[22] heterocycles; particularly, in making part of anti-cancer drugs such as semaxanib and sunitinib,[23-26] we designed the conjugates of N-substituted pyrrole and indolin-2-one (**3**, Chart 1) and screened the molecules for tumor growth inhibition activity. It is worth to mention that semaxanib (**1**, chart 1) is known for effective anticancer activity against human cancer cell lines but its development was stopped due to its severe toxicity in phase-II/III studies. Modification of semaxanib to sunitinib eliminated the side effects and it was approved by U.S. Food and Drug Administration for the treatment of cancer in 2006. As per the requirement of having hydrophobic fragments in the TK inhibitors so that they better fit in the ATP binding site of the enzyme, the molecular docking of compound **3a** in the ATP binding site of TK was checked. It was apparent from the results of docking studies that the phenyl rings of the molecule get placed in the hydrophobic sub-pocket of the enzyme. Additionally, some polar interactions through the Cl were observed. Most remarkably, the indolinone moiety of compound **3a** was placed more close to the Mg^{2+} in comparison to the similar placement of sunitinib and semaxanib (Figure 2, D, E, F).

The synthesis of the compounds was accomplished by starting with N-substitution of pyrrole-2-carboxaldehye followed by condensation with indolinone. Pyrrole-2-carboxaldehyde was reacted with benzyl bromide in the presence of NaH in DMF/THF at 0 – 25 °C for 30 min to yield N-benzyl substituted compound **4a**. Compound **4a** was further treated with 1-(2,6-dichlorophenyl)-2-indolinone under microwave conditions in the presence of piperidine (catalytic amount) in $CHCl_3$ at 100 °C for 1h and compound **3a** was procured (Scheme 1). Similarly, compounds **3b-d** were synthesized by the reaction of **4b-d** with 4-fluorobenzyl chloride, 4-chlorobenzyl chloride and 1-naphthylmethyl chloride, respectively (Scheme 1).



Scheme 1

For the synthesis of compounds **3e-i**, when N-acyl substituted pyrrole-2-carboxaldehyde was treated with indolinone, the acyl substituent gets removed under the reaction conditions. Alternatively, an equimolar mixture of pyrrole-2-carboxaldehyde and 1-(2,6-dichlorophenyl)-2-indolinone was subjected to microwave irradiations in the presence of piperidine (catalytic amount) in $CHCl_3$ at 100 °C for 1h to procure compound **3e**. Compound **3e** was then treated with benzoyl chloride in the presence of NaH in DMF/THF for 30 min (0 °C- 25 °C) to synthesize compound **3f**. Using the same reaction sequence and the reaction conditions as for the preparation of **3f**; compounds **3g-i** were synthesized by reacting **3e** with 4-chlorobenzoyl chloride, 4-toulenesulfonyl chloride and 5-chlorothiophene-2-

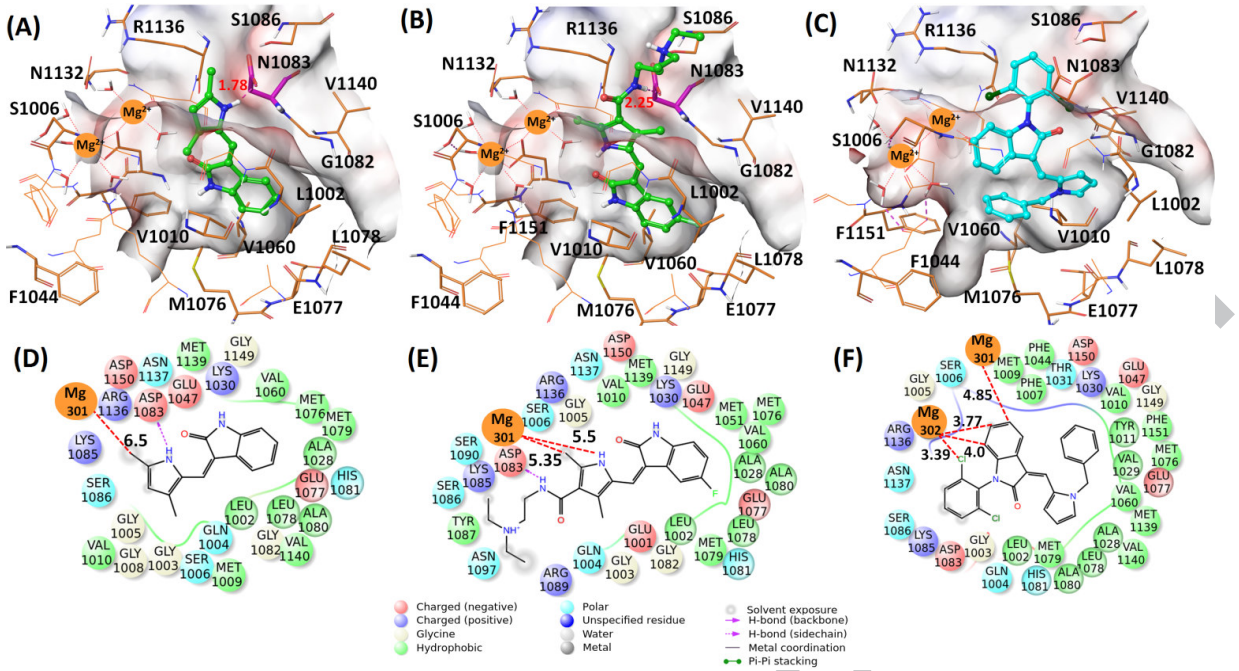
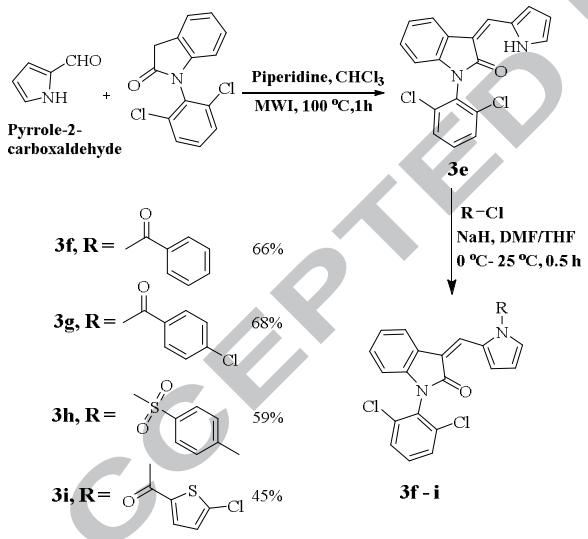


Figure 2. Semaxanib (A, D), Sunitinib (B, E) and Compound **3a** (C, F) docked in the ATP binding site of tyrosine kinase (phosphorylated insulin receptor, pdb ID 1IR3) showing H-bonding interactions (pink dotted lines) with the ATP binding site residues. In the 2D view (D, E, F), red dotted lines are close distances between Mg atoms and drugs.

carbonyl chloride, respectively (Scheme 2). The *Z*-Configuration at the bridged C=C bond of compounds **3a-i** was established with the help of NMR experiments (Figure 3; details of experiments for configuration assignment in the supporting information).



Scheme 2

Compounds **3a-c** and **3e-i** were tested for their tumor growth inhibitory activity over 60 human cancer cell lines at NCI, Bathesda, USA. Compounds were preliminary studied at 10^{-5} M concentration. During the single dose assay, the percent growth of tumor cells was recorded in the presence of compounds. In this primary assay, minimum growth of the tumor cells was observed in the presence of compound **3a**. Hence compound **3a** was further tested for tumor growth inhibitory screening by taking five different concentrations of the compound (10^{-4} to 10^{-8} M). The 50% growth inhibition (GI_{50}) concentration, concentration causing total growth inhibition (TGI) and concentration causing

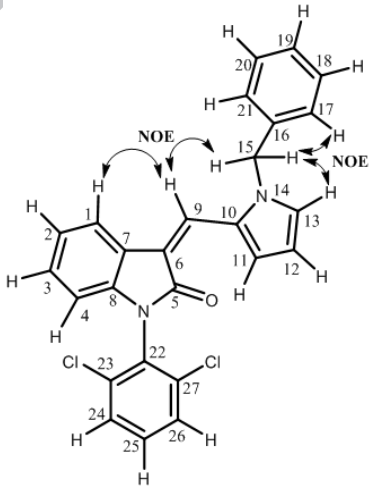


Figure 3. Structure of compound **3a** as elucidated from NMR experiments showing Z-configuration across $C_6 = C_9$. Hydrogens attached to carbons 1, 2, 3, 4; 11, 12, 13; 17, 18, 19, 20, 21 and 24, 25, 26 exhibit TOCSY within the respective groups.

50% cells death (LC_{50}) over 60 human cancer cell lines for compound **3a** are given in Table 1. The MID values (i.e mean graph midpoint) for GI_{50}, TGI, and LC_{50} of compound **3a** were 10.96, 75.85, and 93.32 µM, respectively.

In parallel to the design of the molecules as TK inhibitors and the expression of TK in breast cancer; compound **3a** showed remarkable tumor growth inhibitory activity over breast cancer cell lines MCF7 and MDA-MB-468. It showed GI_{50} 35 nM and 63 nM over MCF7 and MDA-MB-468 cell lines, respectively. Moreover, **3a** exhibited GI_{50} 0.54, 0.74 and 0.12 µM over ovarian cancer cell lines IGROV1, OVCAR-3 and OVCAR-4. Apart from these cell lines, compound **3a** also exhibited appreciable tumor growth inhibitory activity against certain cell lines of leukemia, colon cancer, non-small cell lung cancer and renal cancer (Table 2). Remarkably, compound **3a** exhibited very low toxicity to the normal cells (LC_{50} >100 µM).

Table 1. GI_{50} (μM), TGI (μM) and LC_{50} (μM) for compound **3a**.

Panel/ cell lines	3a		
	GI_{50}	TGI	LC_{50}
Leukemia			
CCRF-CEM	>100	>100	>100
HL-60(TB)	>100	>100	>100
K-562	>100	>100	>100
MOLT-4	>100	>100	>100
RPMI-8226	1.28	>100	>100
SR	>100	>100	>100
Non-small cell lung cancer			
A549/ATCC	8.31	>100	>100
EKVX	0.41	7.94	>100
HOP-62	>100	>100	>100
HOP-92	0.97	17.78	>100
NCI-H226	6.16	>100	>100
NCI-H23	39.81	>100	>100
NCI-H322M	14.79	>100	>100
NCI-H460	4.16	>100	>100
NCI-H522	>100	>100	>100
Colon cancer			
COLO 205	1.04	9.33	>100
HCC-2998	0.87	>100	>100
HCT-116	3.38	>100	>100
HCT-15	0.37	>100	>100
HT-29	6.45	>100	>100
KM12	0.38	>100	>100
SW-620	>100	>100	>100
CNS cancer			
SF-268	>100	>100	>100
SF-295	>100	>100	>100
SF-539	>100	>100	>100
SNB-19	>100	>100	>100
SNB-75	5.24	>100	>100
U251	>100	>100	>100
Melanoma			
LOX IMVI	>100	>100	>100
MALME-3M	3.16	>100	>100
M14	>100	>100	>100
MDA-MB-435	>100	>100	>100
SK-MEL-2	2.23	>100	>100
SKMEL-28	>100	>100	>100
SK-MEL-5	2.13	>100	>100
UACC-62	2.08	>100	>100
Ovarian Cancer			
IGROV1	0.54	>100	>100
OVCAR-3	0.74	>100	>100
OVCAR-4	0.12	>100	>100
OVCAR-5	3.89	>100	>100
OVCAR-8	>100	>100	>100
NCI/ADR-RES	>100	>100	>100
SK-OV3	>100	>100	>100
Renal Cancer			
786-0	>100	>100	>100
A498	>100	>100	>100
ACHN	8.31	>100	>100
CAKI-1	8.31	>100	>100
RXF 393	>100	>100	>100
SN 12C	>100	>100	>100
TK-10	0.64	>100	>100
UO-31	3.16	>100	>100
Prostate Cancer			
PC-3	6.91	>100	>100
DU-145	>100	>100	>100
Breast Cancer			
MCF7	0.035	>100	>100
MDA-MB-231/ATCC	5.88	>100	>100
HS 578T	>100	>100	>100
BT-549	>100	>100	>100
T47D	0.21	1.86	>100
MD-MB-468	0.063	0.28	1.38
Mean	**10.96**	**75.85**	**93.32**

Table 2. GI_{50} (μM), TGI (μM) and LC_{50} (μM) for compound **3a** over selected cell lines.

Cell Line	GI_{50} (μM)
Leukemia	
RPMI-8226	1.28
Non-Small cell Lung cancer	
EKVX	0.41
HOP-92	0.97
Colon cancer	
COLO 205	1.04
HCC-2998	0.87
HCT-15	0.37
KM 12	0.38
Ovarian cancer	
IGROV1	0.54
OVCAR-3	0.74
OVCAR-4	0.12
Renal cancer	
TK-10	0.64
UO-31	3.16
Breast cancer	
MCF7	0.035
T-47D	0.21
MDA-MB-468	0.063

Therefore, from a series of rationally designed pyrrole – indole hybrids, we were able to identify a highly potent molecule that was capable to inhibit the growth of breast cancer cells. Compound **3a** exhibited GI_{50} for breast cancer cells in the nM range; 35 nM and 63 nM, respectively for MCF7 and MDA-MB-468 cell lines. Further studies on compound **3a** using the animal models and the compound – TK interaction studies are underway.

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Supplementary Material

Experimental data, NMR spectra, HRMS and IR spectra of the compounds.

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