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SYNTHESIS OF NEW 2, 5, 6-SUBSTITUTED IMIDAZO[2,1-B][1,3,4]THIADIAZOLE DERIVATIVES AS POTENTIAL ANTICANCER AGENTS

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HIF, Imidazo[2,1-b][1,3,4]thiadiazole, PHD2, Anticancer activity, NCI-USA, Docking

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ABSTRACT: A series of 2-cyclopropylimidazo [2,1-b] [1,3,4] thiadiazole derivatives 5(a-i) have been synthesized by reacting 5-cyclopropyl-1,3,4-thiadiazol-2-amine (3) and an appropriate 2-bromo-1,2-(substituted aryl) ethanones 4(a-i). Structures of these compounds were recognized by IR, ^{1}H NMR, ^{13}C NMR spectroscopy and Mass spectrometry. Hypoxia-inducible factor (HIF) has been identified as an important cancer drug target. HIF transcription complex, which is activated by low oxygen tension, controls a diverse range of cellular processes, including angiogenesis and erythropoiesis. Here we analyzed the capacity of synthesized molecules to inhibit hypoxia-inducible factor prolyl hydroxylase (PHD2) *in-silico* as well as an *in-vitro* assay. Four compounds were granted NSC code at National Cancer Institute (NCI), USA, for anticancer activity at a single high dose (10^{-5} M) in full NCI 60 cell panel. Among the compounds tested,2-Cyclopropyl-6-(4-methoxyphenyl)-5-phenylimidazo[2,1-b][1,3,4]thiadiazole 5a (NSC D-754956/1) was found to be the most active candidate of the series at five dose level screening with a degree of selectivity toward leukemic cancer cell line.

INTRODUCTION: Cancer is a class of disease that displays uncontrolled growth, invasion, and sometimes metastasis of abnormal cells. It affects people of all age groups with the risk of most types increasing with age [1].

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Hypoxia-inducible factor (HIF) has been known as an important drug target for the treatment of cancer. HIF is a transcriptional complex that plays a key role in oxygen homeostasis in mammals and monitors a host of hypoxic response genes that regulate angiogenic, glycolytic, and erythropoietic processes [2-4].

The HIF-α regulates oxygen availability through two distinct oxygen-dependent pathways; regulation of HIF-α protein stability and regulation of HIF-α transcriptional activation, mediated by prolyl and asparaginyl hydroxylation, respectively [5].

Hydroxylation of either of the two proline residues (Pro402 and Pro564) of human HIF-α signals the ubiquitin-proteasome pathway for the degradation of HIF-α. When oxygen availability becomes limiting, HIF prolyl hydroxylase activity is reduced, resulting in the accumulation of HIF-α, which dimerizes with constitutively expressed HIF-β to stimulate expression of genes with the hypoxia-responsive element (HRE)-containing promoters **Fig. 1** [6].

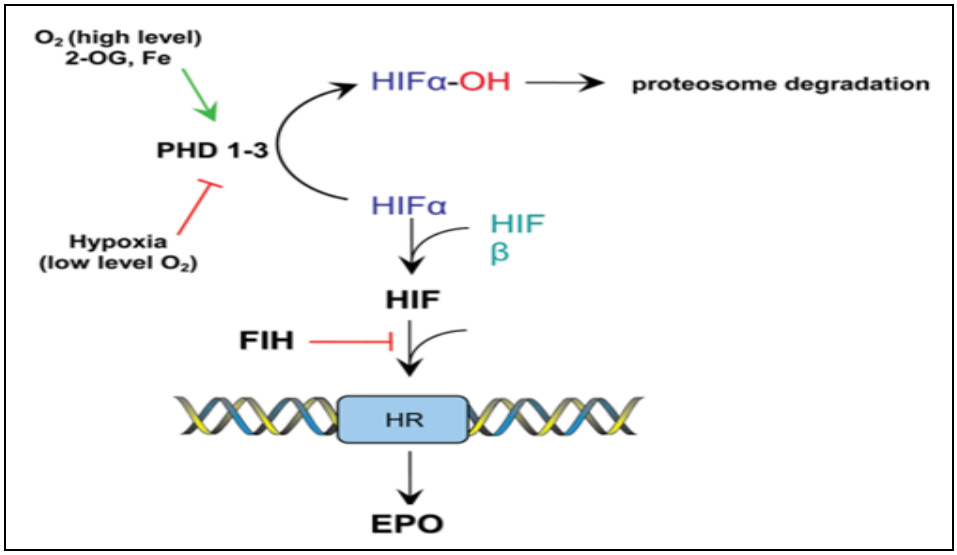


FIG. 1: ENDOGENOUS EPO UPREGULATION THROUGH THE UPREGULATION OF HYPOXIA-INDUCIBLE FACTOR

HIF prolyl hydroxylation is catalyzed by three closely related enzymes PHD1-3, whereas HIF asparaginyl hydroxylation is catalyzed by FIH (factor inhibiting HIF). Both PHDs and FIH are Fe(II)- and 2-oxoglutarate-dependent dioxygenases that use molecular oxygen as a co-substrate, and couple hydroxylation of HIF-α to the oxidative decarboxylation of 2-oxoglutarate (2-OG) and carbon dioxide [7, 8]. This gives a double control framework whereby within sight of oxygen HIF-α subunits are both inactivated and demolished, while in hypoxia, catalysis of these hydroxylations is smothered, empowering HIF-α subunits to escape von Hippel-Lindau-interceded proteolysis, enroll co-activators, and structure a beneficial transcriptional complex. This activity of hypoxia can be halfway emulated by a few operators that hinder 2-OG oxygenases, including iron chelators, change metal particles, and small molecule 2-OG analogs [9]. HIF hydroxylase inhibitors can be a prospective approach for the treatment of ischemic/hypoxic disease.

Several imidazo-fused heterocycles having anticancer activity were discovered in search of anticancer drugs [10-14]. The 2-amino-1,3.4-thiadiazole derivatives were reported to have potential anticancer activity against several transplanted animal tumors [15].

Earlier Gadad *et al*., have reported the synthesis methods and potential anticancer activity of imidazo[2,1-b][1,3,4]thiadiazoles [16]. Nalan *et al*., have reported some hydrazone derivatives of 2,6-dimethylimidazo[2,1-b][1,3,4]thiadiazole-5-carbo-hydrazide as anticancer agents against ovarian cancer cell lines OVCAR [17]. Andrew *et al*., have studied anticancer activity of some imidazo[2,1-b][1,3,4]thiadiazole guanyl hydrazones against several cancer cell lines [18]. Ibrahim *et al*., prepared 4-(3-substituted-1,2,4-triazolo[3,4-b][1,3,4] thiadi-azole-6-yl)aniline derivatives as a novel class of potential antitumor agents [19].

As a result, a large number of imidazothiadiazole derivatives have been reported to possess diverse pharmacological properties such as antitubercular, cardiotonic, antibacterial, antifungal, anti-inflammatory, analgesic, anticonvulsant, diuretic, antisecretory and herbicidal activities apart from anticancer activity [20-28]. After the development of Levamisole, a known anthelmintic, several researchers explored its potential as an immuno-modulator and modified it to imidazo[2,1-b][1,3,4]thiadiazole system [29]. These imidazo[2,1-b][1,3,4]thiadiazole derivatives of the Levamisole have been reported as potential antitumor agents [30]. Later antitumor activity of 5-formyl-6-aryli-midazo[2,1-b][1,3,4]thiadiazole sulfonamides[21]

and derivatives of imidazo[2,1-b][1,3,4]thiadiazole were also reported [31-33]. Researchers elsewhere have reported other fused heterocyclic systems as HIF inhibitors along with mechanisms [34, 35]. By understanding the anticancer property of imidazo[2,1-b][1,3,4]thiadiazoles, we sought to identify suitable small molecule HIF-1α prolyl hydroxylase inhibitors based on structure-based design approach utilizing the crystal structure of PHD2 in complex with the isoquinoline inhibitor [36] **Fig. 2**.

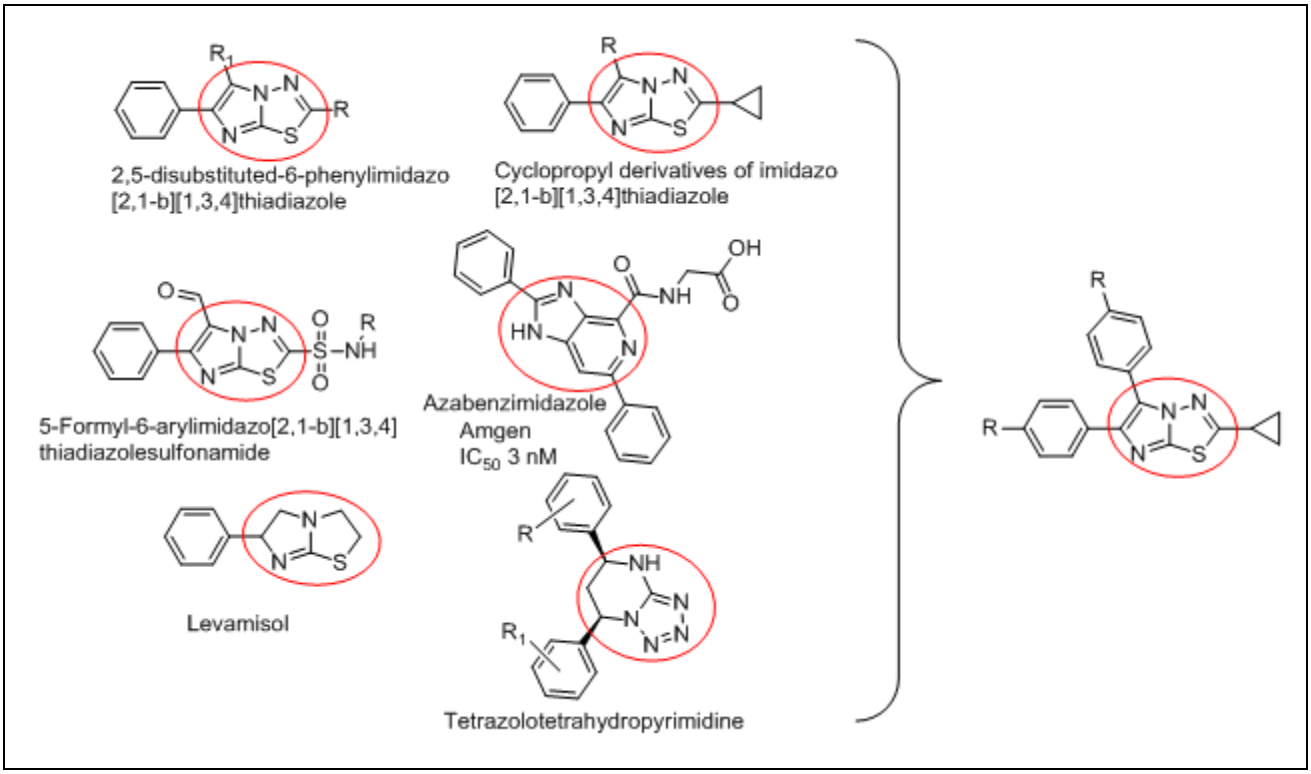
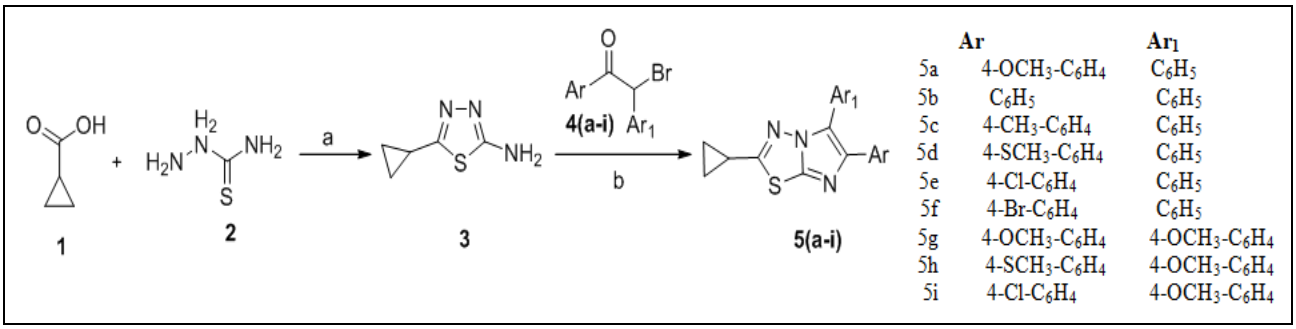


FIG. 2: DESIGN CONCEPT FOR NEW IMIDAZO[2,1-B][1,3,4]THIADIAZOLE DERIVATIVES AS ANTICANCER AGENTS

Given the above facts and an attempt to achieve new compounds with possible anticancer activities, a new series of 2-cyclopropylimidazo[2,1-b][1,3,4] thiadiazoles derivatives 5(a-i) have been designed, synthesized and screened *in-vitro* for PHD2 inhibitory activity and anticancer activity at NCI (National Cancer Institute)-USA.

RESULTS AND DISCUSSION:

Chemistry: The synthesis of imidazo[2,1-b][1,3,4]thiadiazole derivatives 5(a-i) are outlined in **Scheme 1**. The compound 5-cyclopropyl-1,3,4-thiadiazol-2-amine 3 was obtained from cyclization of commercial available compounds cyclopropane-carboxylic acid 1 and thiosemicarbazide 2 in presence $POCl_3$. The imidazo [2,1-b] [1,3,4] thiadiazole derivatives 5(a-i) were prepared by refluxing 2-bromo-1,2-(substituted aryl) ethanone 4(a-i) with 5-cyclopropyl-1,3,4-thiadiazol-2-amine 3 in dry ethanol.



	Ar	Ar1
5a	4-OCH3-C6H4	C6H5
5b	C6H5	C6H5
5c	4-CH3-C6H4	C6H5
5d	4-SCH3-C6H4	C6H5
5e	4-Cl-C6H4	C6H5
5f	4-Br-C6H4	C6H5
5g	4-OCH3-C6H4	4-OCH3-C6H4
5h	4-SCH3-C6H4	4-OCH3-C6H4
5i	4-Cl-C6H4	4-OCH3-C6H4

SCHEME 1: REAGENTS AND CONDITIONS: A) $POCl_3$, REFLUX FOR 4 h, 10% NaOH; B) 2-BROMO-1,2(SUBSTITUTED-ARYL)ETHANONE, DRY EtOH, REFLUX, 10% Na_2CO_3, P_2O_5

The formation of 2-aminothiadiazole 3 was established by IR spectra, which showed the presence of amino (-NH_2) band ~3200 and the absence of carbonyl stretching of carboxylic acid ~1700-1600. Structures of imidazothiadiazole derivatives were confirmed by the absence of (-NH_2) band ~3200 in IR spectra. The formation of title compound 5(a-i) was confirmed by the absence of signal for imidazole proton (H-5) in ^{1}H NMR spectra and presence of absorption bands ~1630-1600 for C=N stretch in their respective spectra. The mass spectra of these compounds further ascertained the structure of synthesized compounds.

Molecular Docking Studies: The co-crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2) with an isoquinoline inhibitor was used for docking study with the help of Maestro 9.0 molecular docking software [37]. The active site, located between the major and minor β-sheets, comprises a deep pocket.

The active site ligand **Fig. 3** binds to the Fe(II) *via* bidentate coordination through N1 of its isoquinoline ring and oxygen of the amide carbonyl, forming a *ca.* planar 5 membered chelate ring. The amide carbonyl oxygen of ligand coordinates the iron *ca.* trans to the Asp-315 side chain, and its isoquinoline nitrogen is trans to His-374 nitrogen. The identity of the three Fe(II) coordinating residues as His-313, Asp-315, and His-374 confirms predictions from mutational and sequence comparison studies [38].

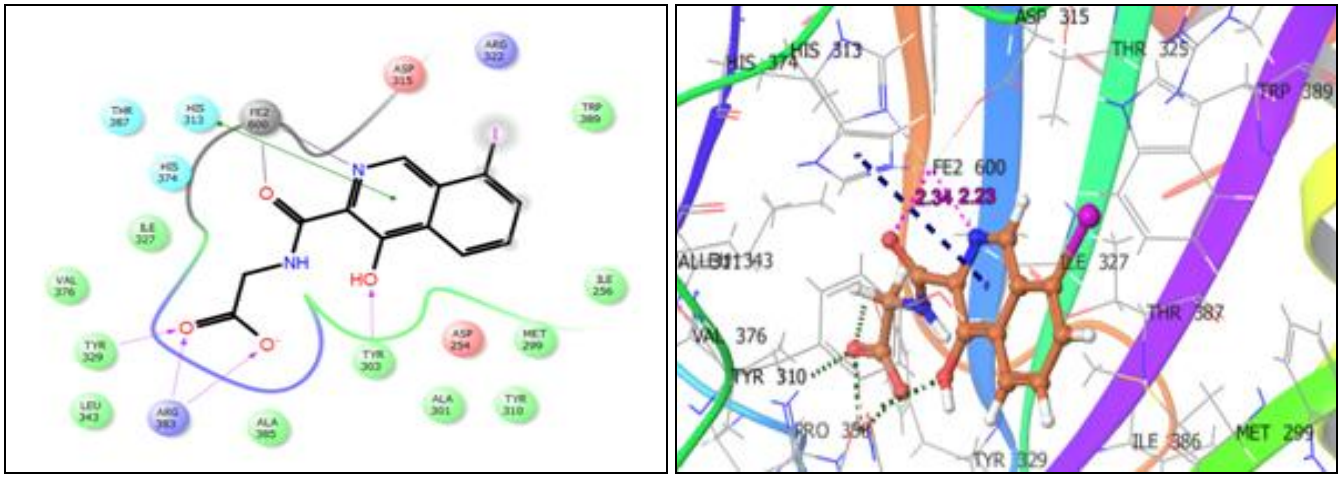


FIG. 3: BINDING MODE OF ISOQUINOLINE INTO THE BINDING SITES OF THE PHD2 ENZYME

The fused heterocyclic aromatic rings fits into active site in such a way that the polar and lipophylic amino acids wrap around the ligand with polar, pi-pi stacking, H-bond and Van der Waals interactions. Arg383, Tyr 329 hold the carboxylic group of ligand with polar interactions. The carbonyl group of amide and Nitrogen of isoquinoline is held by Heme prosthetic group. Tyr-303 interacts with hydroxyl group of the ligand with H-bond interaction.

Compounds 5a, 5c, 5e and 5f **Fig. 4-6** binds to Fe(II) through the nitrogen of imidazo ring; these compounds showed pi-pi interaction with His313 and Trp389 amino acids. In the compound, 5a methoxy group at 4th position of the phenyl ring provided an additional interaction with Arg322 substantially increased the inhibition potency. Docking score for all compounds showed in **Table 1**, the compounds 5a, 5e & 5f were showed docking score more than 4 compared to other molecules in the series but lesser than active site ligand. **Fig. 7**, is the surface representation of PHD2 with compound 5a. It is generated by MOLCAD and colored to show its lipophilic potential (LP), which ranges from brown (highest lipophilic area) to blue (highest hydrophilic area).

Initial modeling studies suggested that compounds 5a, 5e & 5f can be a potential HIF-1α prolyl hydroxylase inhibitors. Deynoux *et al*., [41] reported that hypoxia and HIF-mediated signaling play a crucial role in leukemia and leukemogenic processes. While comparing our *in-silico* results with *in-vitro* anticancer activity, we found compound 5a is selective towards leukemia cell lines with a selectivity ratio of 3.36.

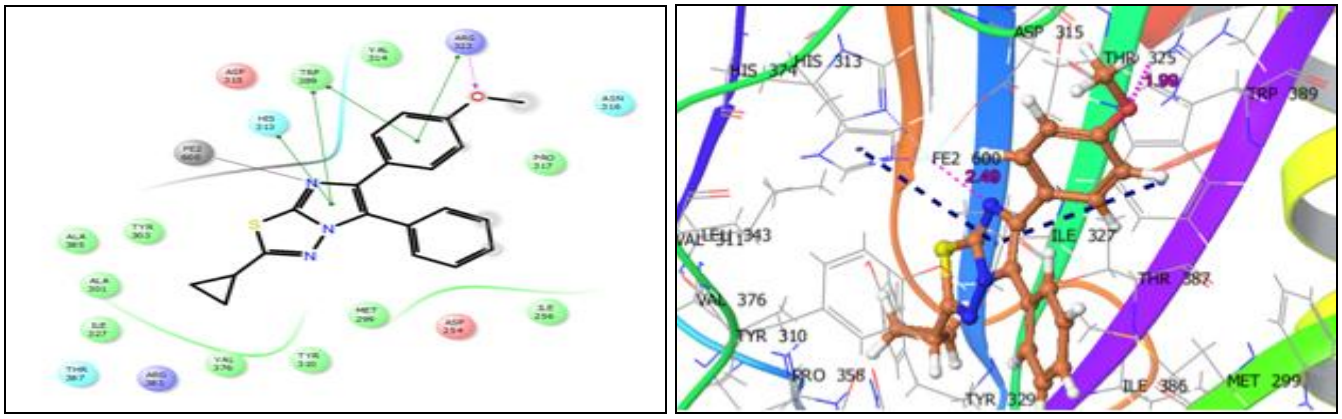


FIG. 4: BINDING MODE OF COMPOUND 5A INTO THE BINDING SITES OF PHD2 ENZYME

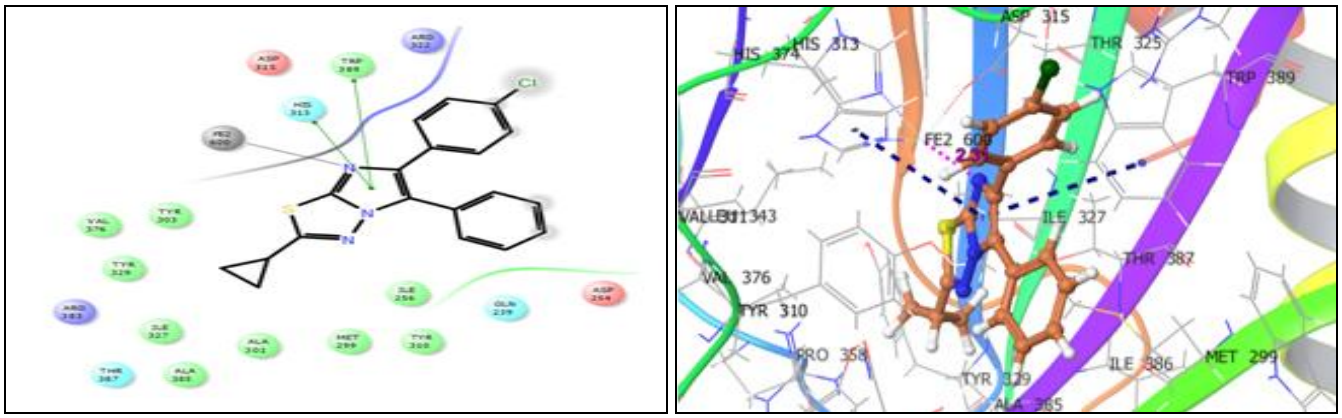


FIG. 5: BINDING MODE OF COMPOUND 5E INTO THE BINDING SITES OF PHD2 ENZYME

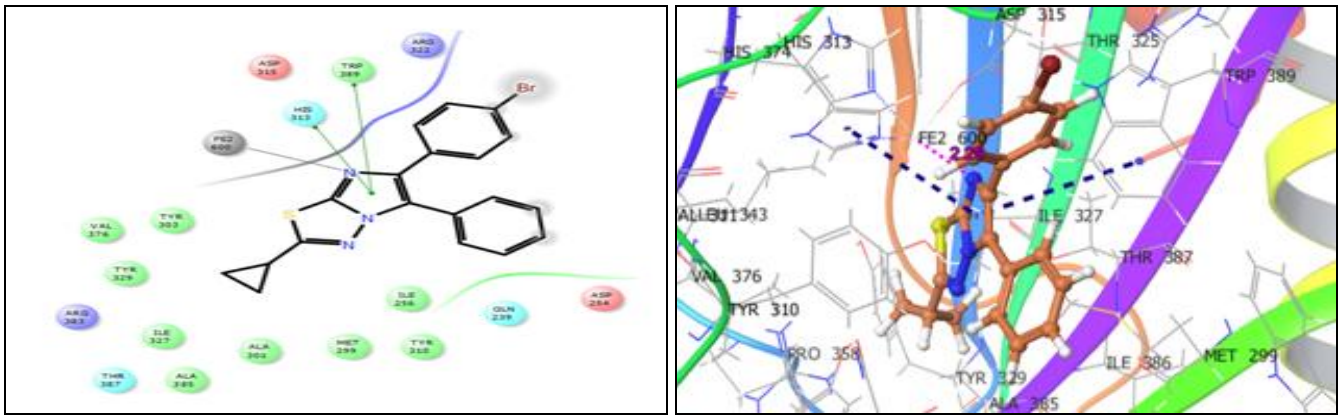


FIG. 6: BINDING MODE OF COMPOUND 5F INTO THE BINDING SITES OF PHD2 ENZYME

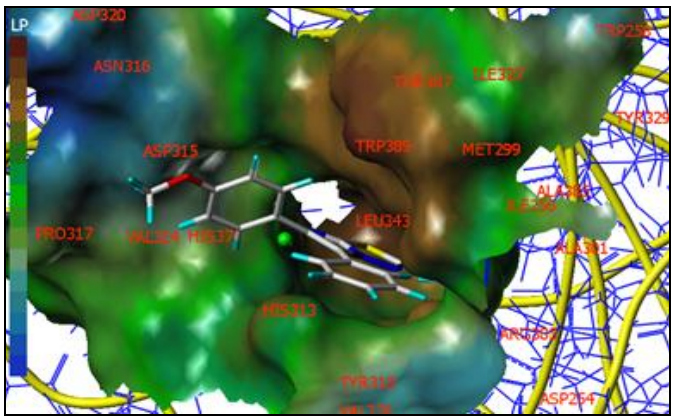


FIG. 7: SURFACE REPRESENTATIONS OF PHD2 AND COMPOUND 5A (LIPOPHILIC POTENTIAL)

TABLE 1: GLIDE DOCKING RESULTS AND INHIBITORY ACTIVITIES OF DESIGNED COMPOUNDS AGAINST THE PHD2 ENZYME

Compounds	Docking score	Glide emodel	IC_{50} µM PHD2[a]
5a	-4.113	-44.638	4.233 ± 0.12
5b	-3.598	-42.600	>40
5c	-3.913	-43.037	>40
5d	-3.482	-42.614	>40
5e	-4.375	-45.043	5.510 ± 0.03
5f	-4.181	-45.257	4.992 ± 0.009
5g	-3.162	-44.341	7.131 ± 0.43
5h	-3.584	-46-236	>40
5i	-3.693	-44.368	6.633 ± 0.004
Ligand	-7.217	-90.565	-

[a]Values are the mean of two or more separate experiment

One of the important limitations of several drugs is its pharmacokinetic profile. The maximum absorption, bioavailability, and distribution of the drug to the active site with minimum non-specific interactions, elimination of the drug after reversible interaction at the site of action with minimum toxic metabolites, and side effects are the important factors of a potential drug. Predicting the ADMET properties of the drug by virtual computational applications helps in developing not only pharmacologically effective drugs but also reduces the cost and time of production.

The Qikprop module was used for the ADME studies of the selected compounds [39]. The ADME properties of the selected molecules are summarized in **Table 2**. All compounds showed ADME characteristics within the acceptable range, including very little deviation from Lipinski's rule of five. The compounds showed 100% human oral absorption profiles.

The absorbance of the drug can be indicated by Human Intestinal Absorption (HIA) and Caco-2 (QPPCaco) permeability. All the compounds that were tested for intestinal absorption have shown an excellent prediction percentage. The compounds also exhibited good permeability values in Caco-2 (QPPCaco) cells, ranging from 4616.954 to 5210.308. The partition coefficient (QPlogPo/w) and water solubility (QPlogS), critical for estimation of absorption and distribution of drugs within the body, ranged between 5.073 and 5.865 and −6.190 to −7.473. All these pharmacokinetic parameters are within the acceptable range defined for human use (see **Table 2** footnote), thereby indicating their potential as a drug-like molecule.

TABLE 2: SUMMARY OF ADME PROFILES FOR THE COMPOUNDS 5a-i AND 2G1M LIGAND

Comp.	Mol. Wt	QPlogPo/w[a]	H-bond donor	H-bond Acceptor	Violation of Lipinski's rule	QPlogS[b]	QPlog HERG[c]	QPPCaco[d]	QPPMDCK[e]	QPlog Khsa[f]	% Human Oral Absorption[g]
5a	347.434	5.073	0	3.25	1	-6.190	-4.727	4807.687	4269.061	0.864	100
5b	317.408	5.194	0	2.5	1	-6.316	-4.982	5210.308	5049.750	0.942	100
5c	331.434	5.460	0	2.5	1	-6.790	-4.762	4616.954	4102.498	1.120	100
5d	363.494	5.865	0	3	1	-7.473	-6.242	5302.077	8936.692	1.127	100
5e	351.853	5.602	0	2.5	1	-7.000	-4.880	4641.070	10000	1.054	100
5f	396.304	5.675	0	2.5	1	-7.089	-4.885	4720.495	10000	1.075	100
5g	377.460	5.321	0	4	1	-6.671	-4.866	5109.497	4942.772	0.898	100
5h	393.521	5.858	0	3.75	1	-7.405	-5.935	5196.740	8560.122	1.083	100
5i	381.879	5.734	0	3.25	1	-7.183	-4.784	5184.915	10000	1.037	100
ligand	372.119	2.181	1.25	4.5	0	-3.478	-2.873	29.064	35.508	-0.303	65.905

[a] Predicted octanol/water partition co-efficient log p (acceptable range: -2.0-6.5); [b] Predicted aqueous solubility in mol/L (acceptable range: -6.5 to 0.5); [c] Predicted IC_{50} value for blockage of HERG K+ channels (concern below -5.0); [d] Predicted Caco-2 cell permeability in nm/s (acceptable range: <25 is poor and >500 is great); [e] Predicted apparent MDCK cell permeability in nm/s; [f] Prediction of binding to human serum albumin; [g] Percentage of human oral absorption (<25% is poor and >80% is high).

In-vitro **HIF-PHD2 Activity:** Synthesized derivatives 5a-i were evaluated for inhibitory potency in PHD2 enzyme assay [40], which detects proline hydroxylation of a HIF-1α peptide **Table 1**. The compound 5a showed IC_{50} 4.23µM, which contains one of the phenyl ring with methoxy at 4th position, that of halogen-containing compounds 5e and 5f showed IC_{50} 5.51 µM and IC_{50} 4.99 µM, respectively. To increase activity, we tried to substitute both phenyl ring 4th position, but it fails (compounds 5g-i).

In-vitro **Anticancer Activity:** The tumor growth inhibition properties of the four compounds 5a, 5c-e with the NCI codes NSC D-754956/1, NSC D-754955/1, D-754953/1, and D-754952/1 respectively among the synthesized compounds 5(a-i) were screened for anticancer activity against 60 human cancer cell lines at NCI, NIH, Bethesda, Maryland, USA, under the drug discovery program of the NCI. Compound 5a (NSC D-754956/1) was further screened for 5-log dose as it has shown

profound cell growth inhibition at one dose assay against a variety of cell lines.

***In-vitro* 60 Cell Panel One Dose Assay (10^{-5} M):** All the selected compounds submitted to the National Cancer Institute (NCI) for in vitro anticancer assay were evaluated for their anticancer activity. The primary *in-vitro* anticancer assay was performed against 60 different cancer cell lines under nine different panels representing leukemia, melanoma, and cancers of lung, colon, brain, breast, ovary, kidney, and prostate. The modified MTT assay is adopted from alley *et al*.

The results obtained from the COMPARE graph of all the tested compounds represent the characteristic response to each cancer cell line like a fingerprint. Based on the preliminary criteria like minimum growth inhibition of 30 % on several cell lines (mean average) or lethal dose % results, Compound 5a (NSC D-754956/1), satisfied the pre-determined threshold inhibition criteria and was selected for NCI full panel 5 dose assay.

***In-vitro* 5 dose Full NCI 60 Cell Panel Assay and Discussion:** 5 different concentrations (0.01, 0.1, 1, 10 & 100µM) of the test compounds were tested on 60 different cancer cell lines representing nine tumor subpanels. The GI_{50}, TGI, and LC_{50} were taken into consideration as important parameters for each cell line. The GI_{50} value (growth inhibitory activity) is the concentration of test compound at which 50% of the cell growth is inhibited. The TGI value (cytostatic activity) is the concentration of the test compound at which the total growth of the cancer cell line is inhibited. The LC_{50} value (cytotoxic activity) is the lethal concentration of the test compound, at which 50% of initial cancer cells are killed. Compound under investigation 5a (NSC D-754956/1) exhibited significant anticancer activity against most of the tested cell lines representing nine different subpanels with GI_{50} values between "1.82-43.4 µM".

Concerning to sensitivity against some individual cell lines **Table 3**, the compound showed high activity against Leukemia HL-60 (TB), Colon cancer HCT-116, Melanoma MALME-3M Renal Cancer A498 with GI50 2.43, 1.82, 2.37 and 2.15µM respectively. The data obtained from the 5 dose assay revealed a noticeable sensitivity profile toward leukemic subpanel (GI_{50} value ranging from 2.43-3.66 µM). The selectivity of the test compound against specific cancer subpanel can be determined by the ratio of MID^a (the average sensitivity of all cell lines towards the test agent) and MID^b (the average sensitivity of all cell lines of a particular subpanel towards test agent). The ratio of the test compound indicating values between 3 and 6 are moderately selective against particular subpanel, whereas ratios > 6 indicate high selectivity toward the corresponding cell line [26].

The compound 5a was found to be moderate selective toward Leukemic cancer subpanel with a selectivity ratio of 3.20 and non-selective against remaining cell panel **Table 3**.

TABLE 3: NCI *IN-VITRO* TESTING RESULT OF COMPOUND 5A (NSC D-754956/1) AT FIVE DOSE LEVEL IN µM

Panel	Cell Line	GI_{50}			TGI	LC_{50}
		Conc. Per cell line	Subpanel MID^b	Selectivity ratio (MID^a :MID^b)		
Leukemia			2.99	3.20	>100	>100
	CCRF-CEM	3.66			>100	>100
	HL-60(TB)	2.43			>100	>100
	K-562	2.81			>100	>100
	MOLT-4	2.97			>100	>100
	RPMI-8226	3.22			>100	>100
	SR	2.90			>100	>100
Non-Small Cell Lung Cancer			13.37	0.71		
	A549/ATCC	7.42			>100	>100
	EKVX	5.22			>100	>100
	HOP-62	38.1			>100	>100
	HOP-92	5.59			37.9	>100
	NCI-H226	4.99			>100	>100
	NCI-H23	5.10			>100	>100
	NCI-H322M	43.4			>100	>100
	NCI-H460	5.25			>100	>100

	NCI-H522	5.31			55.4	>100
Colon Cancer			5.31	1.80		
	COLO 205	7.23			>100	>100
	HCC-2998	8.19			>100	>100
	HCT-116	1.82			>100	>100
	HCT-15	2.02			>100	>100
	HT 29	6.12			>100	>100
	KM 12	3.77			>100	>100
	SW-620	6.88			>100	>100
CNS Cancer			10.53	0.90		
	SF-268	19.3			>100	>100
	SF-295	5.04			>100	>100
	SF-539	6.78			>100	>100
	SNB-19	15.6			>100	>100
	SNB-75	12.2			51.9	>100
	U251	4.29			>100	>100
Melanoma			9.89	0.96		
	LOX IMVI	9.20			>100	>100
	MALME-3M	2.37			>100	>100
	M14	7.70			>100	>100
	MDA-MB-435	6.08			>100	>100
	SK-MEL-2	11.0			59.3	>100
	SK-MEL-28	35.6			>100	>100
	SK-MEL-5	2.17			>100	>100
	UACC-257	4.14			>100	>100
	UACC-62	4.36			>100	>100
Ovarian Cancer			15.92	0.60		
	IGROV1	17.3			>100	>100
	OVCAR-3	4.63			>100	>100
	OVCAR-4	12.7			82.1	>100
	OVCAR-5	31.9			>100	>100
	OVCAR-8	5.76			>100	>100
	NCI/ADR-RES	4.18			>100	>100
	SK-OV-3	35.0			>100	>100
Renal Cancer			9.48	0.7406		
	786-O	13.6			>100	>100
	A-498	2.37			>100	>100
	ACHN	7.06			>100	>100
	CAKI-1	9.70			>100	>100
	RXF-393	2.93			14.8	>100
	SN-12C	14.4			>100	>100
	UO-31	16.3			>100	>100
Prostate Cancer			10.71	0.9363		
	PC-3	2.23			>100	>100
	DU-145	19.1			>100	>100
Breast Cancer			5.256	1.90		
	MCF7	8.18			>100	>100
	MDA-MB-231/ATCC	6.59			>100	>100
	BT-549	4.06			>100	>100
	T-47D	4.50			73.5	>100
	MDA-MB-468	2.95			>100	>100
MID[a]		9.5808			69.5	>100

[a] MID = Average sensitivity of all cell lines in µM. [b] MID = Average sensitivity of all cell lines of particular subpanel in µM.

CONCLUSION: In this paper, we report the synthesis and anti-tumor activity of a series of 2-cyclopropylimidazo- [2,1-b] [1,3,4] thiadiazoles. These compounds were prepared by the cyclodehydration process between 5-cyclopropyl-1,3,4-thiadiazol-2-amine and an appropriate 2-bromo-1,2-(substituted aryl)ethanone.

In light of the NCI-60 results, five doses selected compound 2-cyclopropyl-6-(4-methoxyphenyl)-5-phenylimidazo [2,1-b][1,3,4]thiadiazole 5a (NSC D-754956/1) was found to be the most active candidate of the series against Leukemia HL-60 (TB), Colon Cancer HCT-116, Melanoma MALME-3M and Renal cancer A498 with GI50

2.43, 1.82, 2.37 and 2.15 µM respectively with a degree of selectivity toward Leukemic Cancer cell line based on MG MID ratio (3.36). Hypoxia is a strong signal, principally maintained by members of the HIF family. Earlier studies considered that hypoxia triggered intrinsic metabolic changes through the HIF family is significant in only solid tumors. However, recent studies have revealed the influence of HIF in leukemia cell proliferation, differentiation, and resistance to chemotherapy [41]. Hence, we carried out in vitro hypoxia-inducible factor prolyl hydroxylase (PHD2) inhibitory assay and found compound 5a with IC_{50} of 4.23 µM. In the *in-silico* study, compound 5a binds to the Fe(II) through the nitrogen of imidazo ring, pi-pi interaction with His313 and Trp389 amino acids, hydrogen bond interaction between methoxy group at 4th position of the phenyl ring and Arg322 amino acid. These preliminary studies of biological screening of the tested compounds could offer an excellent platform for further developing these class of compounds as potential anticancer agents.

EXPERIMENTAL: All Chemicals and solvents used were reagent grade. They are obtained from Sigma-Aldrich, S.D. Fine-Chem Limited, Bangalore. All the required solvents were purified and dried before use. All the chemical's purity was tested with the reference melting points. The reactions were monitored with the help of thin-layer chromatography using pre-coated aluminum sheets with GF254 silica gel, 0.2mm layer thickness (E. Merck). Melting points of the synthesized compounds were recorded on the digital Veego (VMP-MP) melting point equipment. IR spectrum was acquired on a Shimadzu Infra-Red Spectrometer (model FTIR 8400S). ^{1}H NMR, ^{13}C NMR, and mass spectra of synthesized compounds were obtained from NFDD, Rajkot University, Gujarat.

Synthesis of 5-cyclopropyl-1,3,4-thiadiazol-2-amine (3): A mixture of cyclopropanecarboxylic acid (I) (0.05 mol), thiosemicarbazide (II) (0.05 mol) and $POCl_3$ (13 ml) was heated at 75 °C for 0.75 h. After cooling down to room temperature, water was added. The reaction mixture was refluxed for 4 h. After cooling, the mixture was neutralized to pH 7 by the dropwise addition of 50% NaOH solution under stirring. The precipitate was filtered and crystallized from ethanol.

Yield 90%; mp 214-216 °C; IR (KBR)υ_{max}1632.80 cm^{-1} (C=N stretch), 3272.34 cm^{-1} (N-H stretch), 3098.75 cm^{-1} (C-H stretch), 1197.83 cm^{-1} (C-N stretch), 691.50 cm^{-1} (C-S-C);^{1}H NMR (DMSO-d_6) δ ppm: 1.16-2.36 (m, 5H, cyclopropyl), 6.32 (s, 2H, NH_2); ^{13}C NMR (DMSO-d_6) δ ppm: 167.08, 158.2, 12.7, 10.3.

Synthesis of 2-cyclopropyl-5,6-diarylsubstituted imidazo[2,1-b][1,3,4]thiadiazole 5(a-i): A mixture of 2-amino-5-substituted-1,3,4-thiadiazole (3) (10 mmol) and an appropriate α- bromo- 1- (4”-substituted)phenyl- 2- (4’- substituted) phenyl- 1-ethanone 4(a-i) (10 mmol) in dry ethanol (150 mL) was heated to reflux on a water bath for 6–8 h, phosphorus pentoxide (3 mmol) was added, and refluxing was continued for another 4–6 h.

The reaction mixture was cooled overnight at room temperature. Excess of solvent was removed under reduced pressure and the solid hydrobromide separated was filtered, washed with cold ethanol, and dried.

The neutralization of hydrobromide salts with a cold aqueous solution of Na_2CO_3 yielded the corresponding free bases 5(a–i), which was purified by recrystallization from dry ethanol. Further, the compounds were purified by column chromatography using 200–400 mesh silica gel and eluted either with ethyl acetate/hexane (2:8) or chloroform/hexane (1:9) as mobile phase.

2-Cyclopropyl-6-(4-methoxyphenyl)-5-phenyli-midazo [2,1-b] [1,3,4] thiadiazole (5a): Yield 75.60%; mp 110-114 °C; IR (KBR)υ_{max} 3037.00 cm^{-1} (Ar C-H stretch), 2839.31 cm^{-1} (Ali C-H stretch), 1600.01 cm^{-1} (C=N), 1174.96 cm^{-1} (Ar C-N), 1419.66 cm^{-1} (Ar C=C), 698.25 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16-2.36 (m, 5H, cyclopropyl), 8.4 (d, 2H, aryl H), 7.98 (d, 2H, aryl H), 7.26-7.66 (m, 3H, aryl H), 6.95 (d, 2H, aryl H), 3.73 (s, 3H, 4”-OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 167.08, 158.55, 140.91, 135.32, 129.77, 129.22, 128.12, 127.49, 126.83, 125.68, 122.09, 114.17, 55.7, 55.2, 12.7, 11.9, 10.3; Mass (EI) *m/z*, 348.00 (m + 1).

2-Cyclopropyl-5,6,diphenylimidazo[2,1-b][1,3,4] thiadiazole(5b): Yield 80.01%; mp 303-307 °C; IR (KBR)υ_{max} 3111.83 cm^{-1} (Ar C-H stretch), 2889.48 cm^{-1} (Ali C-H stretch), 1682.95 cm^{-1} (C=N),

1199.11 cm^{-1} (C-N), 1428.74 cm^{-1} (Ar C=C), 694.40 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16-2.36 (m, 5H, cyclopropyl), 7.23-7.25 (d, 4H, aryl H), 7.14-7.22 (m, 4H, aryl H), 6.95 (d, 2H, aryl H); ^{13}C NMR (DMSO-d_6) δ ppm: 167.1, 136.0, 133.1, 129.5, 129.3, 128.8, 127.5, 122.0, 12.7, 10.3; HRMS (EI) *m/z* calcd for $C_{19}H_{15}N_3S$: 317.0987; found: 317.0988.

2-Cyclopropyl-5-phenyl-6-p-tolyimidazo[2,1-b] [1,3,4]thiadiazole(5c): Yield 55.78%; mp 235-240 °C; IR (KBR)υ_{max}3109.3583 cm^{-1} (Ar C-H stretch), 2902.0083 cm^{-1} (Ali C-H stretch), 1631.8383 cm^{-1} (C=N), 1174.6983 cm^{-1} (C-N), 1455.34 (Ar C=C), 694.4083 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.18-2.39 (m, 5H, cyclopropyl), 7.45 (d, 2H, aryl H), 7.36 (d, 2H, aryl H), 7.14-7.32 (m, 3H, aryl H), 6.97 (d, 2H, aryl H), 2.14 (s, 3H, 4"-CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 167.02, 138.4, 136.0, 133.1, 130.1, 129.6, 129.3, 128.8, 127.5, 127.4, 122.0, 12.7, 10.3; HRMS (EI) *m/z* calcd for $C_{20}H_{17}N_3S$: 331.1143; found: 331.1145.

2-Cyclopropyl-6-(4-(methylthio)phenyl)-5-phenylimidazo [2,1-b] [1,3,4]thiadiazole (5d): Yield 67.56%; mp 116-120 °C; IR (KBR)υ_{max} 3052.45 cm^{-1}(Ar C-H stretch), 2915.50 cm^{-1} (Ali C-H stretch), 1602.90 cm^{-1} (C=N), 1188.19 cm^{-1} (C-N), 1436.05 cm^{-1} (Ar C=C), 704.04 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.17-2.37 (m, 5H, cyclopropyl), 7.47 (d, 2H, aryl H), 7.34 (d, 2h, aryl H), 7.12-7.32 (m, 3H, aryl H), 6.83 (d, 2H, aryl H), 2.47 (s, 3H, 4"-CH_3);^{13}C NMR (DMSO-d_6) δ ppm: 167.05, 136.4, 136.0, 133.1, 129.5, 129.3, 128.5, 127.7, 127.5, 127.3, 122.1, 16.4, 14.9, 10.7; HRMS (EI) *m/z* calcd for $C_{20}H_{17}N_3S_2$: 363.0864; found: 363.0869.

6-(4-Chlorophenyl)-2-cyclopropyl-5-phenylimidazo [2,1-b] [1,3,4] thiadiazole (5e): Yield 79.06%; mp 224-226 °C; IR (KBR)υ_{max} 3121.89 cm^{-1} (Ar C-H stretch), 2829.67 cm^{-1} (Ali C-H stretch), 1632.80 cm^{-1} (C=N), 1161.19 cm^{-1} (C-N); 1432.19 cm^{-1} (Ar C=C), 604.70 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.17-2.36 (m, 5H, cyclopropyl), 7.44 (d, 2H, aryl H), 7.35 (d, 2H, aryl H), 7.13-7.33 (m, 2H, aryl H), 6.88 (d, 2H, aryl H); ^{13}C NMR (DMSO-d_6) δ ppm: 167.05, 136.2, 134.3, 133.3, 131.2, 129.4, 129.3, 128.9, 128.8, 127.5, 12.7, 10.2; HRMS (EI) *m/z* calcd for $C_{19}H_{14}ClN_3S$: 351.0597; found: 351.0593.

6-(4-Bromophenyl)-2-cyclopropyl-5-phenylimidazo [2,1-b] [1,3,4] thiadiazole (5f): Yield 75.50%; mp 320-322 °C; IR (KBR)υ_{max} 3121.89 cm^{-1} (Ar C-H stretch), 2889.46 cm^{-1} (Ali C-H stretch), 1682.95 cm^{-1} (C=N), 1212.30 cm^{-1} (C-N), 1428.74 cm^{-1} (Ar C=C), 694.40 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16-2.36 (m, 5H, cyclopropyl), 7.45 (d, 2H, aryl H), 7.36 (d, 2H, aryl H), 7.14-7.31 (m, 3H, aryl H), 6.83 (d, 2H, aryl H); ^{13}C NMR (DMSO-d_6) δ ppm: 167.0, 136.5, 133.1, 132.2, 132.1, 129.7, 129.5,128.6, 127.5, 122.0, 16.4, 10.4; HRMS (EI) *m/z* calcd for $C_{19}H_{14}BrN_3S$: 395.0092; found: 395.0096.

2-Cyclopropyl-5,6-bis(4-methoxyphenyl)imidazo [2,1-b][1,3,4]thiadiazole (5g): Yield 72.07%; mp 221-223 °C; IR (KBR)υ_{max}3116.11 cm^{-1}(Ar C-H stretch), 2900.07 cm^{-1} (Ali C-H stretch), 1654.98 cm^{-1} (C=N), 1272.10 cm^{-1} (C-N), 1431.23 cm^{-1} (Ar C=C), 691.50 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16-2.35 (m, 5H, cyclopropyl), 7.46 (d, 2H, aryl H), 7.34 (d, 2H, aryl H), 7.12-7.32 (m, 3H, aryl H), 6.85 (d, 2H, aryl H), 3.73 (s, 6H, 4"-CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 167.1, 160.7, 136.0, 129.5, 128.5, 125.4, 122.0, 114.8, 55.9, 16.7, 10.1; HRMS (EI) *m/z* calcd for $C_{21}H_{19}N_3O_2S$: 377.1198; found: 377.1196.

2- Cyclopropyl- 5- (4- methoxyphenyl)- 6- (4-(methylthio) phenyl) imidazo [2,1-b] [1,3,4] thiadiazole (5h): Yield 58.27%; mp 250-252 °C; IR (KBR)υ_{max} 3137.32 cm^{-1} (Ar C-H stretch), 2897.18 cm^{-1} (Ali C-H stretch), 1664.62 cm^{-1} (C=N), 1275.95 cm^{-1} (C-N), 1501.63 cm^{-1} (Ar C=C), 672.21 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.15-2.36 (m, 5H, cyclopropyl), 7.43 (d, 2H, aryl H), 7.33 (d, 2H, aryl H), 7.10-7.32 (m, 2H, aryl H), 6.82 (d, 2H, aryl H), 3.72 (s, 3H, 4"-OCH_3), 2.47 (s, 3H, 4"-SCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 167.2, 160.8, 136.4, 136, 129.3, 128.8, 127.6, 127.3, 122.2, 55.7, 16.5, 14.9, 10.3; HRMS (EI) *m/z* calcd for $C_{21}H_{19}N_3OS$: 393.0970; found: 393.0973.

6-(4-Chlorophenyl)-2-cyclopropyl-5-(4-methoxyphenyl) imidazo [2,1-b] [1,3,4] thiadiazole (5i): Yield 67.33%; mp 292-294 °C; IR (KBR)υ_{max} 3098.75 cm^{-1} (Ar C-H stretch), 2782.41 cm^{-1} (Ali C-H stretch), 1663.59 cm^{-1} (C=N), 1275.95 cm^{-1} (C-N), 1523.32 cm^{-1} (Ar C=C), 688.5 cm^{-1} (C-S-C); ^{1}H NMR (DMSO-d_6) δ ppm: 1.16-2.36 (m, 5H,

cyclopropyl), 7.44 (d, 2H, aryl H), 7.37 (d, 2H, aryl H), 7.14-7.32 (m, 2H, aryl H), 6.84 (d, 2H, aryl H), 3.74 (s, 3H, 4"-OCH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 167.0, 160.5, 136.1, 134.3, 133.1, 129.4, 128.9, 128.5, 122.0, 55.9, 16.4, 10.2; HRMS (EI) *m/z* calcd for $C_{20}H_{16}ClN_3OS$: 381.0703; found: 381.0707.

Molecular Modeling Studies: The molecular docking studies were performed with the help of a Maestro 9.0 docking software (Schrodinger Inc. USA) [37] on the Cellular Oxygen Sensing: Crystal Structure of Hypoxia-Inducible Factor Prolyl Hydroxylase (PHD2). The 3D structure of PHD2 for the study was downloaded from the Protein Data Bank (PDB ID: 2G1M) [36]. The downloaded structure was further refined for ideal docking results. The PDB enzyme structure was thoroughly analyzed for missing atoms, bonds and/or contacts. All the residues and water molecules except ligand from the enzyme structure were removed manually. A builder molecule was used to construct the ligand molecules and then to obtain a stable structure; the energy of the molecule was also minimized using LigPrep.

With the help of a grid box, the active sites were generated on the biopolymer. Using standard precision (SP) protocol, all molecules were docked on a biopolymer. The ADME characteristics of all selected compounds predicted using the QikProp module, to obtain an understanding of solubility and absorption levels of the compounds [39]. Descriptors like QPlogPo/w, QPlogS, QPPCaco, rule of 5, and % human oral absorption were calculated using this module.

***In-vitro* HIF-PHD2 Activity:** HIF-PHD2 activity was performed as per the procedure reported by Frohn *et al*. [40]

Anticancer Screening Methodology:

***In-vitro* Anticancer Screening at NCI-USA:** The *in-vitro* anticancer screening of 60 cell lines for one dose assay and five dose assays were adopted from alley *et al*. [42-44]

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