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Design, synthesis of anticancer and anti-inflammatory 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio) pyrimidine-5-carbonitriles

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ABSTRACT

A novel series of 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio) pyrimidine-5-carbonitriles (**4a–i**) was synthesized and evaluated for anticancer potential against cell lines for breast cancer. Compounds **4b**, **4e**, and **4h** exhibited prominent cytotoxicity against human breast carcinoma MCF-7 cell line with GI_{50} of 2.0, 0.5, and 0.5 μM, respectively. Molecular docking study against EGFR tyrosine kinase could provide valuable insights into the plausible mechanism of action. The compounds could bind with significantly high binding affinity and their binding affinity scores could correlate well with the observed anticancer activity. Furthermore, compounds **4a**, **4c**, **4e**, **4g**, and **4i** exhibited significant inflammatory activities as well which could expand the therapeutic domain of this novel series.

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Introduction

Cancer is one of the major causes of mortality worldwide. Literature reports 10.0 million deaths and around 19.1 million new cases just in 2020.[1] Perhaps the cancer frequency will augment by above 30% in the forthcoming decades. Compared to all other types of cancer, breast cancer is communal hostile with an assessed 2.3 million new cases.[2–4] Presently for breast cancer treatment numerous therapy are existing, such as surgery, radiotherapy, teletherapy, chemotherapy, and nanotechnology techniques however, these tactics have some confines.[5,6] In recent decades cellular pathways and specific biomolecular inhibition strategies have immense significant targets in cancer therapy.[7,8] Henceforth there is a stipulation to find the anticancer compounds, which

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could act upon the multiple target site into the cancer cell.[9] Earlier reports suggested that the scaffolds anticancer compounds enhanced the exploration for probable biomolecular targets into the cancer cells.[10–12]

Body physiological response to tissue injury is inflammation, it is because of infection, physical damage, and toxin contact.[13] Chronic inflammation may lead to various diseases, such as arthritis, Alzheimer, cancer, and autoimmune disease.[14,15] Inflammatory cells have a prevailing influence on the development of the tumor, which promotes angiogenesis, and creates a favorable condition for tumor growth early in the neoplastic processes.[16] Hence for cancer preclusion and treatment targeting inflammation is one of the tactics.[17]

Long-before indole nucleus received substantial focus due to its bioactivities, such as anti-microbial,[18] anti-rheumatoid, anti-inflammatory, antioxidant, antipyretic, anticonvulsant, antidiabetic, antimalarial, analgesic, anticancer, and selective inhibitor of COX-2.[19–21,22] Literature review suggested that indole derivatives were excellent anticancer agents and induced apoptosis in pancreatic, colon, cervical, squamous cell carcinoma, prostate, and breast cancer cell lines.[23–27]

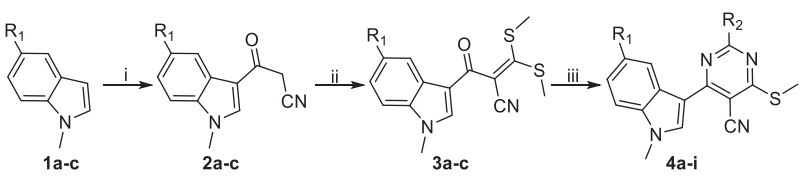
Pyrimidine is an aromatic heterocyclic compound having wide existence in nature, which is present in uracil, thymine, cytosine DNA bases, and isolated from terrestrial as well as marine plants.[28–30] Pyrimidine and its natural, as well as semisynthetic derivatives, were reported to have a broad range of pharmacological activities, such as antidiabetic, anti-inflammatory, anti-HIV, antimicrobial, anti-tubercular, cardiovascular, antioxidant, analgesic, diuretic, and anticancer.[31–34] Compared to all other bioactivities, the anticancer activity of pyrimidine and its derivatives were studied extensively. It is a potential anticancer skeleton, which showed cytotoxicity against a range of cancerous cell lines.[35–37] Pyrimidines-based molecules were reported to demonstrate the most significant cytotoxicity against breast carcinoma amongst all other cancer cell lines (Fig. 1).[38–40]

Chemical hybridization leading to the blending of multiple scaffolds is one of the effective strategies for drug discovery, to enhance the bioactivity of individual molecules.[41–44] With this objective, we have synthesized indol-pyrimidine scaffolds, as cytotoxic and anti-inflammatory agents. Moreover, the in-silico approach of molecular docking was adopted to gain an insight into their plausible anticancer activity for which Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase was used as the target protein. Furthermore, these compounds were also evaluated for potential anti-inflammatory activity.

Result and discussion

Synthesis and characterization of 4-(1-methyl-1H-indol-3-yl)-6-(methylthio) pyrimidine-5-carbonitriles (4a–i)

In the present study, we have achieved the synthesis of the desired 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles (**4a–i**) starting from substituted 1-methyl-1*H*-indoles by chemo and regioselective cyclization in a few steps with excellent yield. Initially, 3-(1-methyl-1*H*-indol-3-yl)-3-oxopropanenitriles (**2a–c)** were synthesized by cyano acetylation of substituted (–CN, $–OCH_3$) 1-methyl-1*H*-indoles (**1a–c**)using 2-



i) $CNCH_2COOH$, $(CH_3CO)_2O$, reflux; ii) sodium tert-butoxide, CS_2, dimethyl sulfate, THF; iii) Guanidine/ acetimidamide/ benzimidamide hydrochloride, K_2CO_3, CH_3CN, reflux.

	R_1	R_2
4a	H	NH_2
4b	CN	NH_2
4c	OCH_3	NH_2
4d	H	CH_3
4e	CN	CH_3
4f	OCH_3	CH_3
4g	H	Ph
4h	CN	Ph
4i	OCH_3	Ph

Scheme 1. Synthesis of 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles.

cyanoacetic acid in acetic anhydride under reflux conditions.[45] Then, 3-(1-methyl-1*H*-indol-3-yl)-3-oxopropanenitriles (**2a–c)** on reaction with carbon disulfide in the presence of sodium tert-butoxide followed by alkylation with dimethyl sulfate converted to 2-(1-methyl-1*H*-indole-3-carbonyl)-3,3-bis(methylthio)acrylonitriles (**3a–c**).[46,47] Further, 2-(1-methyl-1*H*-indole-3-carbonyl)-3,3-bis(methylthio)acrylonitriles (**3a–c**) on cycloaddition with substituted guanidine hydrochloride under an alkaline condition in acetonitrile furnished desired 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio) pyrimidine-5-carbonitriles (**4a–i**) (Scheme 1). The products obtained were purified using the column chromatographiy using ethyl acetate in hexane. All the synthesized compounds were characterized by infrared (IR) , high-resolution mass spectrum (HRMS), proton, and carbon NMR spectra.

Anticancer activity

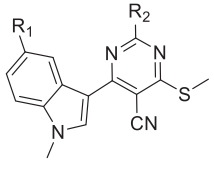
All the synthesized target molecules (**4a–i**) were screened for their anticancer potential against MCF-7, a human breast cancer cell line. The VERO African green monkey kidney epithelial cell lines were used as a control. The cytotoxicity was measured by determining the GI_{50}, TGI, and LC_{50} values, and adriamycin was treated as a positive control (Table 1). GI_{50} is a drug concentration that causes a 50% reduction in cell proliferation whereas the concentration required to kill test cells by 50% was stated as lethal concentration (LC_{50}) and cells total growth inhibition of was denoted TGI.

Among the compounds screened, **4b**, **4e**, and **4h** are found to be more potent in total growth inhibition concentration studies than other 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles. Results revealed that compound **4b**, **4e**, and **4h** have cyano group at C-5 position. Indol ring play a significant role in the activity due to which the molecule could snuggly fit into the active site of EGFR tyrosine kinase with a significantly higher binding affinity.

Compound **4h** and **4b** exhibited cytotoxicity with TGI values of 50 and 60%, respectively. 4-(1-Methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles derivatives (**4a**, **4c**, **4d**, **4f**, **4g**, **4i**) disclosed poor activity as compared to the adriamycin. Compound **4e**, **4h**, and **4b** revealed significant cytotoxicity with 50% cell growth inhibition at 0.5, 0.5, and 2.0 μM concentrations, respectively.

The lethal concentration studies indicated that all the compounds were found non-toxic. In total growth inhibition concentration studies except for **4e** (39.3 μM), other synthetic derivatives were found non-toxic against VERO cells. *In vitro* cytotoxicity

Table 1. *In vitro* anticancer screening of indole-pyrimidine scaffolds (**4a–i**) against human breast cancer cell line MCF-7[a] and normal monkey cell line VERO.



			MCF-7 (μM)		VERO (μM)					Glide energy	
Comp.	R_1	R_2	GI_{50}[d]	TGI[c]	LC_{50}[b]	GI_{50}	TGI	LC_{50}	Glide Score	(Kcal/mol)	H-bond (Å)
4a	H	NH_2	>100	>100	>100	>100	>100	>100	−7.564	−38.956	Asp831 (2.209), Lys721 (2.167), Thr766 (2.177)
4b	CN	NH_2	**2.0**	**60.0**	>100	2.0	>100	>100	−8.119	−45.169	Asp831 (2.184), Lys721 (2.236), Thr766 (2.058)
4c	OCH_3	NH_2	>100	>100	>100	>100	>100	>100	−7.504	−38.102	Asp831 (2.033), Lys721 (2.469), Thr766 (2.276)
4d	H	CH_3	>100	>100	>100	40.0	>100	>100	−7.823	−39.031	Lys721 (2.141), Thr766 (2.126)
4e	CN	CH_3	**0.5**	**99.0**	>100	2.0	39.3	>100	−8.211	−47.343	Lys721 (2.543), Thr766 (2.266)
4f	OCH_3	CH_3	>100	>100	>100	>100	>100	>100	−7.224	−37.764	Lys721 (2.441), Thr766 (2.206)
4g	H	Ph	>100	>100	>100	>100	>100	>100	−7.194	−37.223	Lys721 (2.301), Thr766 (1.867)
4h	CN	Ph	**0.5**	**50.0**	>100	7.0	>100	>100	−8.228	−47.478	Lys721 (2.660), Thr766 (2.281)
4i	OCH_3	Ph	>100	>100	>100	>100	>100	>100	−7.089	−37.286	Lys721 (2.633), Thr766 (2.388)
Adriamycin			< 0.1	40.0	>100	<0.1	10.0	>100	−8.558	−55.564	Asp776 (2.487), Asp831 (1.916)

[a]Concentrations in μM.
[b]Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) calculated from $[(Ti - Tz)/Tz] \times 100 = -50$.
[c]Drug concentration resulting in total growth inhibition (TGI) was calculated from $Ti = Tz$.
[d]Growth inhibition of 50% (GI_{50}) calculated from $[(Ti - Tz)/(C - Tz)] \times 100 = 50$.

studies indicated that **4b**, **4e** exhibited moderate cytotoxicity against VERO cell line with 2.0 μM GI_{50} values. Compound **4b** (GI_{50} 7.0 μM) showed low cytotoxicity, whereas derivatives **4a**, **4c**, **4d**, **4f**, **4g**, and **4i** are non-cytotoxic ($GI_{50} > 100$ μM) against VERO cell line.

Molecular docking

To gain mechanistic insight into the anticancer activity demonstrated by 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles (**4a–i**) investigated herein, a molecular docking study was performed against a crucial target intervening the breast cancer pathophysiology, epidermal growth factor receptor (EGFR) tyrosine kinase. Amplification or over-expression of this target has been associated with the development and progression of certain destructive types of breast cancer. Specifically, the aberrant activity of EGFR has shown to play a major role in the development and growth of tumor cells, where it is involved in numerous cellular responses including proliferation, signaling, differentiation, adhesion, migration, and survival of cancer cells. With this objective, the crystal structure of Epidermal Growth Factor Receptor Tyrosine Kinase in complex with its inhibitor was retrieved from the protein data bank (PDB) (PDB code: 1M17) and subjected to molecular docking using the standard protocol implemented in the Glide (Grid-Based Ligand Docking With Energetics) program[48,49] integrated into the Schrödinger molecular modeling package (Schrödinger, LLC, New York, NY, USA, 2018) (detail protocol is described in the Experimental section).

The in-silico binding affinity study could yield crucial information concerning the orientation of the 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles (**4a–i**) in the binding pocket of the EGFR tyrosine kinase protein. Their docking scores and the binding energy values corroborated well with the experimental anticancer potency showing a significant correlation, with an average docking score of −7.622 and Glide binding energy −40.602 kcal/mol (Table 1). Visual inspection of the binding poses indicates that these indole-pyrimidine scaffolds (Fig. 2, Figs. S5a–l) could accommodate well within the active site of EGFR tyrosine kinase protein, and the complex formed with the target enzyme was stabilized through a network of significant bonded and non-bonded interactions. A detailed analysis of the ligand-receptor interaction is elaborated for one of the most active analogs **4h** in the next section and could be visualized pictorially through Figures S5a–i for the remaining molecules in the series.

The lowest energy docked conformation of **4h** (Fig. 2) showed that the molecule could snuggly fit into the active site of EGFR tyrosine kinase with a significantly higher binding affinity (docking score of −8.228 and Glide binding energy −47.478 kcal/mol) engaging in a network of bonded and non-bonded interactions with the surrounding residues. A detail insight into the per-residue interactions revealed that the molecule could establish a network of significant van der Waals interactions with Asp831 (−4.187 kcal/mol), Thr830 (−2.119 kcal/mol), Thr766 (−1.295 kcal/mol), Leu764 (−2.169 kcal/mol), Met742 (−1.143 kcal/mol), Glu738 (−1.365 kcal/mol), Lys721 (−4.427 kcal/mol), Ile720 (−1.022 kcal/mol), Ala719 (−1.498 kcal/mol), and Phe699 (−2.689 kcal/mol) through the 3-(5-cyano-6-(methylthio)-2-phenylpyrimidin-4-yl)-1-methyl component while the 1H-indole-5-carbonitrile portion exhibited similar type of

interactions with Leu820 (−3.615 kcal/mol), Cys773 (−1.166 kcal/mol), Gly772 (−1.474 kcal/mol), Pro770 (−1.781 kcal/mol), Met769 (−2.098 kcal/mol), Leu768 (−2.564 kcal/mol), Val702 (−4.647 kcal/mol), Gly695 (−1.101 kcal/mol), Leu694 (−3.924 kcal/mol) lining the active site. The enhanced binding affinity of **4h** is also attributed to favorable electrostatic interactions observed with Asp831 (−1.2 kcal/mol), Lys828 (−1.261 kcal/mol), Met769 (−1.941 kcal/mol), Gln767 (−1.234 kcal/mol), Thr766 (−1.125 kcal/mol), Glu738 (−1.106 kcal/mol), and Lys721 (−3.099 kcal/mol) residues. Furthermore, two prominent hydrogen bonding interactions were also observed through pyrimidine nitrogen with Lys721 (2.660 Å) and the second with Thr766 (2.281 Å) through the nitrile function. Such hydrogen bonding interactions "anchor" the ligand to the active site of the enzyme and facilitate the steric and electrostatic interactions adding to the stability of the enzyme-inhibitor complex. Interesting introduction of a functional

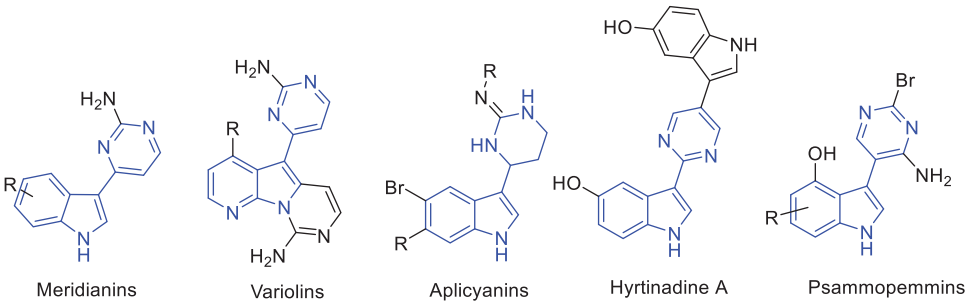


Figure 1. Structure of bioactive natural products with indolyl-pyrimidine scaffolds.

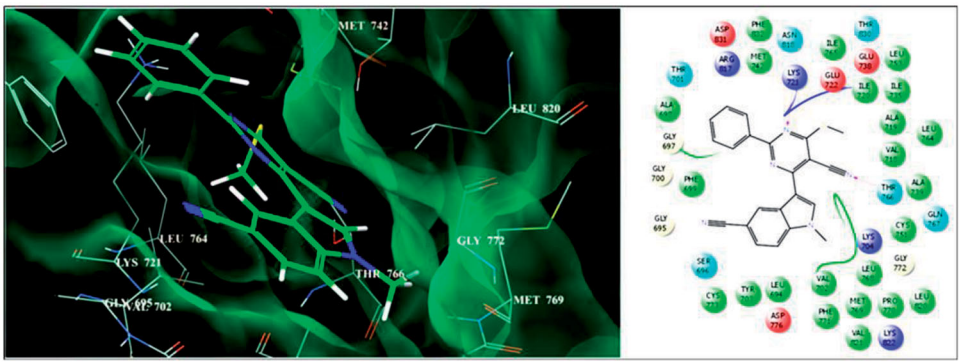


Figure 2. Binding mode of compound **4h** into the active site of EGFR tyrosine kinase (On the right side: pink lines indicate hydrogen-bonding interactions).

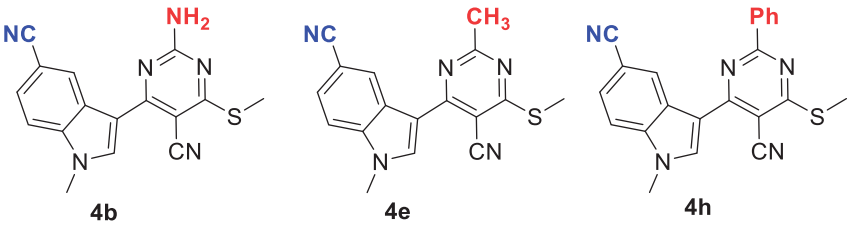


Figure 3. Active anticancer 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles.

group capable of engaging in hydrogen bonding at the R2 position of the pyrimidine ring *viz.* **4a**, **4b**, and **4c** (NH_2) resulted in an additional hydrogen bond through Asp831 residue which was missed in case of molecules lacking such group *viz.* **4d–4i**. A similar network of bonded and non-bonded interactions was established by other molecules in the series which served as the primary driving forces for mechanical interlocking of these molecules into the active site of EGFR tyrosine kinase. This information derived from this analysis is now being fruitfully utilized for the point-specific mutation around the scaffold to identify molecules with higher binding affinity and potency toward EGFR Tyrosine Kinase. 4b, 4e, and 4h (Fig. 3) more anticancer potential compared to other screened compounds in molecular docking and TGI concentration studies.

Heat-induced protein denaturation

As the protein's denaturation is a well-studied cause for inflammation.Therefore, *in vitro* anti-inflammatory activity of synthesized compouds (**4a-i**) was evaluated by using egg albumin denaturation method (Table 2). The results of anti-inflammatory screening reveal that compounds **4d**, **4e**, and **4g** displayed substantial inhibition (76.25, 80.72 and 75.10%, respectively) at 1mM concentration, compared to the positive control diclofenac sodium (90.21%). All other derivatives displayed moderate inhibition of heat-induced albumin denaturation (68.70–73.12%) except compounds **4a** and **4b** compared to the reference standard.

Antioxidant activity

It is well-documented that free radicals, such as the reactive oxygen species (ROS) are important in the pathophysiological mechanisms related to several inflammatory disorders. These free radicals were interacting with cell biomolecules, which may affect the normal physiological functions of the cells and may lead to cancer. Free radical scavenging is possible by using antioxidant therapy, which is one of the current options. Hence, we have tested all the synthetic derivatives to study their direct scavenging potential against various sensitive oxygen and nitrogen radicals, such as nitric oxide (NO), 2,2-diphenyl-2-picrylhydrazyl (DPPH), and superoxide (SOR). The results presented in Table 3indicates that most of the derivatives exhibited good to excellent activity. Utmost all the synthetic analogs exhibited substantial NO and SOR scavenging activity except **4b**

Table 2. Effect of 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles (**4a–i**) on heat-induced protein denaturation.

Entry	% inhibition (1 mM)
4a	64.37 ± 1.10
4b	56.62 ± 2.06
4c	76.25 ± 0.08
4d	73.12 ± 2.13
4e	80.72 ± 0.15
4f	71.87 ± 0.03
4g	75.10 ± 1.07
4h	68.67 ± 2.16
4i	71.80 ± 3.05
Diclofenac sodium	90.21 ± 1.75

Table 3. *In vitro* anti-oxidant activity of 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio)pyrimidine-5-carbonitriles (**4a–i**).

	% inhibition (1mM)			
Entry	DPPH	NO	SOR	H_2O_2
4a	52.94 ± 1.18	67.21 ± 0.62	83.73 ± 0.43	34.31 ± 1.75
4b	30.63 ± 0.23	35.89 ± 1.44	65.85 ± 2.56	39.35 ± 0.54
4c	45.29 ± 2.50	54.91 ± 0.37	95.93 ± 0.78	29.82 ± 2.64
4d	29.41 ± 0.68	64.75 ± 0.66	92.90 ± 2.85	24.95 ± 0.13
4e	30.90 ± 1.24	61.53 ± 0.45	82.92 ± 1.16	42.91 ± 3.78
4f	47.05 ± 1.45	37.70 ± 2.13	88.61 ± 4.45	24.95 ± 0.83
4g	35.29 ± 0.38	68.03 ± 1.38	74.79 ± 1.90	25.53 ± 0.91
4h	36.36 ± 1.10	62.82 ± 0.53	75.60 ± 0.67	45.75 ± 1.22
4i	44.70 ± 0.79	62.29 ± 4.87	76.92 ± 3.48	29.62 ± 2.61
AA	44.18 ± 0.54	42.63 ± 1.22	74.07 ± 2.89	47.17 ± 0.42

AA: Ascorbic acid (at 15 μg/mL); data represent mean of two replicates.

and **4f**. Compounds **4a**, **4d–f**, **4g–h, 4i** showed greater inhibition of NO radicals (>54.91%) compared to the positive control ascorbic acid (42.63%). However, only **4b** and **4f** showed poor activity with 35.89 and 37.70% inhibition, respectively. Compounds **4a**, **4d–h**, **4i** showed significant SOR scavenging activity with >74.79% inhibition compared to the standard drug ascorbic acid (74.07%). However, **4b** displayed moderate SOR scavenging activity. DPPH scavenging activity results suggested that only a few derivatives were found to be more active, **4a** (52.94%), **4c** (45.29%), **4f** (47.05%), and **4i** (44.70%) compared to the ascorbic acid (44.18%). The results of H_2O_2 radical scavenging studies showed that except compounds **4e** and **4h** all other derivatives were inactive compared to the ascorbic acid.

Conclusion

In conclusion, new 4-(1-methyl-1*H*-indol-3-yl)-6-(methylthio) pyrimidine-5-carbonitriles **4a-i** were synthesized with excellent yield (90–96%). All the synthesized compounds exhibited potent anticancer activity against breast cancer cell line, which was significantly altered with the substitution of indole and pyrimidine. Compounds **4b**, **4e**, and **4h** showed prominent cytotoxicity against MCF-7, whereas these derivatives exhibited weak cytotoxicity against normal VERO cell line. Furthermore, molecular docking study could provide valuable mechanistic insight of the binding mode and affinity toward EGFR tyrosine kinase which is a crucial target intervening in breast carcinoma. In addition, compound **4e** was found to be an effective anti-inflammatory agent and **4a**, **4c**, and **4i** were exhibited potential DPPH , NO, and SOR radical scavenging activity. This synthetic approach can be explored for the synthesis of new indole-pyrimidine based anticancer drugs.

Experimental

General procedure for the preparation of 2-(1-methyl-1H-indole-3-carbonyl)-3,3-bis(methylthio)acrylonitrile (3a–c)

To a stirred suspension of freshly prepared sodium *tert*-butoxide (3.0 mmol) in dry THF (7 mL) at 0 °C, a solution of substituted 3-(1-methyl-1*H*-indol-3-yl)-3-oxopropanenitrile (1.0 mmol) and carbon disulfide (1.2 mmol) in dry THF (5 mL) was added

through a pressure equalizer funnel, and the mixture was vigorously stirred at 0 °C for 1 h. To this suspension, a solution of dimethyl sulfate (1.2 mmol) in dry THF (5 mL) was carefully added dropwise during 10 min at 0 °C, and the reaction mixture was allowed to stir at 0 °C for 1 h. After completion of the reaction (TLC; hexane/EtOAc, 8:2), the mixture was diluted with ice water. A light-yellow solid was collected with filtration followed by water washing. The crude solid was purified by recrystallization with ethanol or dichloromethane-hexane mixture.

General procedure for the synthesis of indole-pyrimidine scaffolds (4a–i)

A mixture of 2-(1-methyl-1*H*-indole-3-carbonyl)-3,3-bis(methylthio)acrylonitrile **3a–d** (1.0 mmol), guanidine hydrochloride (1.2 mmol), anhydrous K_2CO_3 (1.5 mmol), and acetonitrile (10 mL) was heated at reflux for 12 h. After cooling, the reaction mixture was poured into ice water. The white solid obtained was filtered, washed with water, and recrystallized from ethanol to obtain pure compound **4a–i**.

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Disclosure statement

The authors declare no competing interests.

Ethical approval

All authors have agreed on the final version of this paper.

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