We report herein the development of a series of pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4-amine derivatives as ATP-competitive epidermal growth factor receptor (EGFR) inhibitors. The compounds were evaluated for their activity against EGFR in a panel of human cancer cell lines. The most potent compound, 5a, exhibited an IC₅₀ of 36.7 nM and selective inhibition against EGFR. The compound showed promising in vivo efficacy in a xenograft model of A-431 cells, with tumor growth inhibition of 45.5% at a dose of 30 mg/kg/day. These findings support the potential of pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4-amine derivatives as promising EGFR inhibitors for the treatment of cancer.
stitution pattern. In addition, the variation of the substitution pattern on the third pyridine ring and the exploitation of the basic pyridine nitrogen to act as a solubilizing moiety (Fig. 1).

**Results and Discussion**

**Chemistry** The 4-chloropyrido[3′,2′:4,5]thieno[3,2-α]-pyrimidine derivatives (1a, b) have been prepared as reported earlier by our group. Compounds (1a, b) have been allowed to react with a variety of substituted anilines and benzyl-
amino group respectively to afford the target compounds \((2-8a, b)\)
(Chart 1).

The structures of the targeted compounds \((2-8a, b)\) were established on the basis of their elemental analyses and spectral data (MS, IR, \(^1\)H-NMR and \(^13\)C-NMR). The \(^1\)H-NMR spectra of the target compounds \((2-5a, b)\) displayed characteristic aromatic protons, which were assigned to the aniline singlet peak at 3.99–4.20 ppm characteristic for OCH\(_3\) protons. The IR spectra showed collectively secondary amine signals of the target compounds. The IR spectra were characterized also by the appearance of multiplet signals of the target compounds. In general, the substitution on the pyridine ring was confined to small alkyl substituents such as methyl group. The targeting on the pyridine ring might enhance the physical properties of the tricyclic core structure. The substitution on the pyridine ring was confined to small alkyl substituents such as methyl group. In general, the substitution on the pyridine ring might enhance the physical properties of the tricyclic core structure. The substitution on the pyridine ring might enhance the physical properties of the tricyclic core structure.

The nature of the linking group between the tricyclic core and phenyl side chain has a great effect on the inhibitory activity. Upon increasing the length of this spacer by one carbon atom \(6a, b-8a, b\), we noticed a reduction in the anticancer activity.

In the rigid tricyclic ring system, combination of dimethyl groups on pyridine ring with dichloro substituents on the aniline ring might hinder optimum binding of the compound \(5b\) into the ATP binding site of EGFR leading to lesser inhibitory activity than that with unsubstituted pyridine ring with dichloro aniline \(5a\). On the other hand, compound \(4b\) with only one chloro substituent at the 4-position on the anilino moiety and dimethyl groups on the pyridine ring of the tricyclic scaffold compound of \(4b\) could lead to optimum binding.

### Biological Evaluation

#### Protein Kinase Inhibitory Assays

The Initial Screening Assay

The protein kinase inhibitory assays were performed at BPS Bioscience laboratories. In the initial screening assay, compounds \((3b)\) and \((4-6a, b)\) were tested at a single concentration 10\(\mu\)M over a panel of six human kinases. VEGFR-1/Flt-1 (BPS \#40223), VEGFR-2/KDR (BPS \#40301) and the wild type of EGFR (BPS \#40178) are tyrosine kinases. Cyclin-dependent kinase (CDK)5/\(\alpha\) (BPS \#40105), GSK3\(\alpha\) (BPS \#40006) and GSK3\(\beta\) (BPS \#40007) are serine/threonine kinases.

Compounds \((5a)\) exhibited kinase inhibitory activity against EGFR with 81% percentage of inhibition at 10\(\mu\)M and IC\(_{50}\)%. While compound \((4b)\) inhibited EGFR at 10\(\mu\)M with 70% percentage of inhibition. The percentages of EGFR inhibition exerted by the other tested compounds were below 60\%\(^{(36)}\) (Table 1).

Structure–activity relationship (SAR) studies for the ability of pyridothienopyrimidine scaffold to inhibit EGFR-TKs activity revealed that both of the pyrimidine nitrogen atoms were absolutely essential for activity. Furthermore, the fusion of a third ring containing basic nitrogen such as the pyridine ring might enhance the physical properties of the tricyclic core structure. The substitution on the pyridine ring was confined to small alkyl substituents such as methyl group. In general, compounds with halogenated aniline moiety displayed higher activity than non-halogenated as it might be able to occupy the lipophilic regions in the ATP binding site of EGFR-TK.

### Measurement of Potential Enzyme Inhibitory Activity IC\(_{50}\)

The profiling data for compound \((5a)\) against EGFR showed an IC\(_{50}\) value of 36.7 nM. This IC\(_{50}\) value of compound \((5a)\) is superior to the one exerted by the standard anti-EGFR erlotinib against the wild type of EGFR, which is 486 nM (Table 1).\(^{(17)}\)

In **In Vitro** Anticancer Screening

The newly synthesized compounds were submitted to National Cancer Institute NCI, Bethesda, Maryland, U.S.A., under the Developmental Therapeutic Program DTP. The operation of this screening utilized 60 different human cancer cell lines, including leukemia, melanoma, lung, colon, brain, ovary, breast, prostate and kidney cancers. Compounds with drug-like properties are prioritized by the NCI screening service, based on computer-aided design. Six compounds were selected for the primary anticancer screening based on their ability to add diversity to the NCI small molecule compounds collection. The target compounds \((4-6a, b)\) were assigned with the following NCI codes NSC D-778769/1, NSC D-776678/1, NSC D-778770/1, NSC D-778771/1, NSC D-774742/1 and NSC D-774743/1, respectively.\(^{(18)}\)

**Primary Single High Dose (10^{-5}M)** against Full NCI 60 Cells Panel

In **In Vitro** screening the full NCI 60 cell lines panel. Results for each compound were reported as a mean graph of the percent growth of the treated cells when compared to the untreated control cells. The percentage growth of the target compounds \((4-6a, b)\) against the full 60-cell line panel are illustrated in (Table S1). After obtaining the results for the single dose assay, an analysis of the historical Development Therapeutics Program (DTP) was performed and compounds \((4b)\) (NSC D-776678/1) and \((5a)\) (NSC D-778770/1) which satisfied the predetermined threshold inhibition criteria were selected for the NCI full panel 5 doses assay (Figs. 2, 3).

**In Vitro** 5 Doses Full NCI 60 Cell Panel Assay

All the 60 cell lines, representing nine cancer subpanels, were incubated at five different concentrations (0.01, 0.1, 1, 10 and 100\(\mu\)M) of the tested compounds. The outcomes were used to create log\(_{10}\) concentration versus percentage growth inhibition curves and three response parameters (GI\(_{50}\) total growth inhibition (TGI) and LC\(_{50}\)) were calculated for each cell line. The GI\(_{50}\) value (growth inhibitory activity) corresponds to the concentration of the compound causing 50% decrease in net cell growth. The TGI value (cytostatic activity) is the concentration of the compound resulting in total growth

### Table 1. Summary of the % Inhibitory Effects of Targeted Compounds (3b) and (4-6a, b) on EGFR Activity

| Compound | EGFR | IC\(_{50}\) (nM) |
|----------|------|----------------|
| 3b       | 52\(^{a}\) | —              |
| 4a       | 48   | —              |
| 4b       | 70   | —              |
| 5a       | 81   | 36.7           |
| 5b       | 55   | —              |
| 6a       | 26   | —              |
| 6b       | 33   | —              |

\(^{a}\) % Inhibition values of compounds on EGFR activity at 10\(\mu\)M.
inhibition. The LC_{50} value (cytotoxic activity) is the concentration of the compound causing net 50% loss of initial cells at the end of the incubation period of 48h.

Compounds (4b) and (5a) showed a distinctive pattern of selectivity and sensitivity against different NCI cell panels regarding to sensitivity against individual cell lines, as illustrated in (Table S2), respectively. Collective dose response curves and individual dose response curves of all cell lines tested for compounds (4b) and (5a) were shown in Figs. 4–7, respectively. Compound (4b) (NSC D-776678/1) exhibited a remarkable anticancer activity against most of the tested cell lines representing nine different subpanels with GI_{50} values between 10–85 nM, except eleven cell lines namely NCI-H322M, HCC-2998, KM12, MALME-3M, SK-MEL-2, SK-MEL-28, IGROV1, OVCAR-3, OVCAR-4, TK-10 and HS 578T showed GI_{50} values >100 nM (Figs. 4, 5). The obtained data revealed an obvious sensitivity profile toward leukemia subpanel (GI_{50} value ranging from <10 to 1970 nM), least for SF-268, SF-539, SNB-19U251 and maximum for SF-295 (Table S2).

Compound (5a) (NSC D-778770/1) exhibited a remarkable anticancer activity against most of the tested cell lines representing nine different subpanels with GI_{50} values between 10–98 nM, except fifteen cell lines namely K-562, NCI-H322M, COLO 205, HCC-2998, KM12, SNB-75, SK-MEL-2, SK-MEL-28, IGROV1, OVCAR-3, OVCAR-4, SN12C, TK-10, MDA-MB-231/ATCC and HS 578T showed GI_{50} values >100 nM (Figs. 6, 7). The obtained data revealed an obvious sensitivity profile toward NSCLC subpanel (GI_{50} value ranging from <10 to 100 nM), least for A549/ATCC, HOP62, NCI-H23, NCI-H460 cell lines and maximum for NCI-H322M cell line (Table S2).

The criterion for selectivity of a compound depends upon the ratio obtained by dividing the full panel MID (the average sensitivity of all cell lines toward the test agent) by their individual subpanel MID (the average sensitivity of all cell lines of a particular subpanel toward the test agent). As per...
this criterion, compound (4b) was found to be most selective toward leukemia and CNS cancer subpanels, with selectivity ratios 2.69 and 2.40, respectively. While compound (5a) was found selective toward CNS cancer, leukaemia and NSCLC subpanels with selectivity ratios 1.40, 1.17 and 1.16, respectively. It is well known that EGFR is highly expressed in cell lines derived from CNS cancer and NSCLC.

COMPARE Analyses

COMPARE analyses were performed for compounds (4b) and (5a) in order to investigate the similarity of their cytotoxicity patterns with those of the standard anticancer agents, in the NCI database. Such analyses are based on comparing the patterns of the differential growth inhibition of the tested compounds against the cultured cancer cell lines. Thus, we can potentially gain insight into the mechanism of their growth inhibitory effects. When the growth inhibition pattern of certain compound correlates well with that of a standard agent, the Pearson’s correlation coefficient value will be greater than 0.6 (PCC >0.6). Consequently, the compound of interest might acquire the same mechanism of action.

The standard COMPARE analyses are performed at the GI50 levels. the results of COMPARE analyses indicated that compounds (4b) and (5a) demonstrate considerable correlation levels with erlotinib (NSC 718781) with PCC values 0.707 and 0.727, respectively. Such similarity in the COMPARE results could indicate a potential resemblance in the mechanism of action of compounds (4b) and (5a) with erlotinib, which is a potent ATP-competitive EGFR inhibitor.

In the light of the NCI results, the following considerations could be made. The anticancer activities of compounds (4–6a, b) were sensitive to the length of the NH spacer between the tricyclic core and the phenyl ring. This could be observed in the anticancer profiles of compounds (4, 5a, b) when compared to compounds (6a, b). The increase in the length of the NH spacer by one carbon atom led to a significant decrease in the anticancer activity. Furthermore, compounds (4b) and (5a) were the most active members among the synthesized series with mean percentage growth values of 18.51 and 31.54%, respectively. Compounds (4b) and (5a) were selective toward leukaemia, CNS cancer and NSCLC cell lines, in the 5 doses...
full NCI 60 cell panel assay.COMPARE analyses illustrated a high correlation between GI\textsubscript{50} mean graphs of compounds (4b), (5a) and the standard ATP-competitive EGFR inhibitor erlotinib with PCC values 0.707 and 0.727, respectively. Moreover, compound (5a) inhibited EGFR selectively with IC\textsubscript{50} value 36.7 nM when it was tested over a panel of six kinases.
These results might support our strategy of designing ATP-competitive EGFR inhibitors as anticancer agents.

**Cell Cycle Studies**

IC$_{50}$ Using Sulforhodamine B (SRB) Assay

The cytotoxicity of compounds (4b) and (5a) against A549

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**Fig. 6.** Collective Dose Response Curves of Compound (5a) for All NCI 60 Cell Lines of *in Vitro* 5 Dose Assay

**Fig. 7.** Individual Dose Response Curves of Compound (5a) for All NCI 60 Cell Lines
human non-small cell lung cancer cell line was tested using SRB assay and the IC_{50} values were 0.04 and 0.03 µM, respectively (Fig. 8).

Effects of Compounds (4b) and (5a) on the Cell Cycle

DNA-flow cytometry was conducted in order to investigate the effects of the tested compounds on the cell cycle. Exposure of A549 lung cancer cells to compounds (4b) and (5a) at a concentration equal to their IC_{50} for 48h induced marked changes in the cell cycle. Exposure to compound (5a) produced significant increase in cell death as evidenced from increased accumulation of cells at Pre-G phase by 8.5 folds (p<0.001) compared to control. It also induced significant increase in the percentage of cells at G0/G1 phase by 1.4 folds (p<0.01) indicating arrest at this phase with subsequent reduction in the percentage of cells at G2/M phase by about 98% (p<0.05) compared to control. Erlotinib was previously reported to induce cell cycle arrest at G0/G1 in NSCLC cells. The pattern of cell cycle arrest induced by compound (5a) is more close to that previously reported for erlotinib in NSCLC and this further support the results from COMPARE analysis.

Effects of Compounds (4b) and (5a) on the Expression of P27kip

The significant cell cycle arrest induced by compounds (4b) and (5a) in A549 cell line was further substantiated through assessing the expression levels of the cell cycle inhibitory protein P27kip. It is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors. This family functions through the formation of heterotrimeric complexes with cyclins and
cyclin-dependent kinases (CDKs) leading to abolishing kinase activity and block of cell cycle progression through G1/S phase. The expression levels of p27 are down-regulated in different human cancers and are associated with poor prognosis. Treatment of the cells with compounds (5a) and (4b) triggered induction in the protein expression of P27kip by about 2.5 and 1.6 (p<0.05) folds compared to control as indicated by Western blotting (Fig. 10). Assessment of P27kip levels further supported the changes observed with Western blotting (Fig. 11). Downregulation of EGFR was reported to be related to the induction of P27kip levels using enzyme-linked immunosorbent assay (ELISA) for 72 h (These concentrations are equivalent to their IC50 in A549 lung cancer cells.) produced a significant reduction in the cell viability after exposure to compound (4b) by about 25% (p<0.001) compared to control and compound (5a). It is noteworthy that the viability of the normal cells was preserved after treatment with compound (5a) (Fig. 12).

Effect of the Tested Compounds (4b) and (5a) on the Viability of Normal Cells

Exposure of villous cytrophoblast placental cell line to compounds (5a) and (4b) for 72h (These concentrations are equivalent to their IC50 in A549 lung cancer cells.) produced a significant reduction in the cell viability after exposure to compound (4b) by about 25% (p<0.001) compared to control and compound (5a). It is noteworthy that the viability of the normal cells was preserved after treatment with compound (5a) (Fig. 12).

SRB Cytotoxicity Assay

Compounds (2, 3a, 3b) and (7, 8a, 8b) that were not selected by NCI screening service, were tested for cytotoxicity against 2 human cancer cell lines, namely A549-lung cancer and MCF7-breast cancer, using sulforhodamine B (SRB) assay as described by Skehan et al. Doxorubicin was used as a reference standard. The IC50 values (in µM) of the test compounds were presented in (Table 2). Compounds (3a) and (3b) showed promising activities with IC50 values <1µM against A549-lung cancer cell line, while compounds (2a) and (6b) showed moderate activities with IC50 values <10µM. The high activities of compounds (7a) and (7b) could be attributed to the presence of methoxy group at position 4 of the aniline ring, whereas the presence of benzyl CH2 linker dramatically reduced the activities of compounds (7, 8a, 8b). The previous findings highlighted compound (3b) as potential anti-proliferative agent with IC50 value 0.319 µM superior to that of the standard doxorubicin 0.411 µM against A549-lung cancer cell line (Fig. 8). However, compounds (5a) and (4b) are still the ones showing...
the highest potency against A549 among the tested series with IC50 values of 0.03 and 0.04 μM, respectively.

Experimental

Chemistry The structures of all tested compounds were confirmed by 1H-NMR, 13C-NMR and mass spectrometry (electron ionization (EI)-MS). The purities of the tested compounds were determined by elemental analysis. The commercial chemicals and solvents were reagent grade and used without further purification. 1H- and 13C-NMR spectra were measured in DMSO-d6 on a Varian Mercury VX-300 NMR spectrometer or Jeol LA (400 MHz for 1H-NMR, 100 MHz for 13C-NMR). Chemical shifts were reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Electron impact mass spectra were recorded on Shimadzu GCMS-QP 5050A gas chromatograph mass spectrometer (70 eV). Elemental analysis was performed at the Microanalytical Center of Cairo University, Egypt. IR spectra were measured in DMSO-d6 on a Perkin Elmer 983 spectrophotometer (KBr pellets). Values were represented in cm⁻¹. The synthetic procedure for the compounds was illustrated in (Chart 1).

General Procedures for the Synthesis of N-(Substituted Phenyl)pyrido[3',2',4',5]:4,5-thieno[3,2-d]pyrimidin-4-amines (2-5a, b) The appropriate aniline (9 mmol) was dissolved in dry tetrahydrofuran (THF) (25 mL) and Et3N (1.5 mL) under nitrogen atmosphere. Compound (1a or b) (4.5 mmol) was added slowly to the stirred solution which was then heated under reflux for 15–20 h. The reaction mixture was cooled and poured onto crushed ice. The precipitated solid was filtered off, washed with water (2×10 mL) and dried. The crude solid was purified by column chromatography using 1–2 EtOAc/hexane v/v as the eluent to give the desired compounds (2-5a, b).

N-(4-Tolyl)pyrido[3',2',4,5]:4,5-thieno[3,2-d]pyrimidin-4-amine (2a)

Yield 53%; mp 276–278°C. 1H-NMR (DMSO-d6) δ: 11.07 (1H, s), 8.71 (1H, dd, J=4.80 Hz, J=2.00 Hz), 7.93 (1H, d, J=8.00 Hz), 7.44 (1H, s), 7.16 (1H, t, J=8.00 Hz); 6.71 (4H, dd, J=8.40 Hz, J=1.2 Hz), 2.30 (3H, s). IR (KBr) cm⁻¹: 3427, 2988, 1621, 1561. MS m/z: 308.00 (M⁺, +1), 231.00. Anal. Calcd for C18H16N4S: C, 67.47; H, 5.03; N, 17.49. Found; C, 67.67; H, 5.38; N, 17.60.

N-(4-Methoxyphenyl)pyrido[3',2',4,5]:4,5-thieno[3,2-d]pyrimidin-4-amine (2b)

Yield 60%; mp> 300°C (decomp.). 1H-NMR (DMSO-d6) δ: 9.89 (1H, s), 8.88 (1H, d, J=5.00 Hz, J=2.00 Hz), 8.60 (1H, d, J=8.00 Hz), 7.99 (1H, s), 7.41 (1H, t, J=8.00 Hz); 6.64 (2H, dd, J=8.00 Hz, J=1.2 Hz), 4.37 (2H, d, J=8.00 Hz, J=1.2 Hz), 4.20 (3H, s). IR (KBr) cm⁻¹: 3395, 2907, 1643, 1461. MS m/z: 308.00 (M⁺), 231.00. Anal. Calcd for C18H18N4Os: C, 62.32; H, 3.92; N, 18.17. Found; C, 61.93; H, 3.70; N, 17.98.

N-(4-Chlorophenyl)pyrido[3',2',4,5]:4,5-thieno[3,2-d]pyrimidin-4-amine (4a)

Yield 59%; mp 280–281°C. 1H-NMR (DMSO-d6) δ: 10.89 (1H, s), 8.00 (2H, s), 7.52 (2H, d, J=9.00 Hz), 7.09 (2H, d, J=9.00 Hz), 2.10 (3H, s), 1.92 (3H, s). 13C-NMR (DMSO-d6) δ: 159.98, 157.46, 152.51, 147.16, 146.57, 132.20, 124.19, 122.74, 121.28, 110.00, 24.03, 18.92. IR (KBr) cm⁻¹: 3438, 2919, 1665, 1465. MS m/z: 340.00 (M⁺). Anal. Calcd for C17H13ClN4S: C, 59.91; H, 3.84; N, 16.44. Found; C, 60.30; H, 4.10; N, 16.63.

N-(2,4-Dichlorophenyl)pyrido[3',2',4,5]:4,5-thieno[3,2-d]pyrimidin-4-amine (5a)

Yield 56%; mp> 300°C (decomp.). 1H-NMR (DMSO-d6) δ: 9.06 (1H, s), 8.38 (1H, s), 7.71 (1H, s), 7.55 (1H, dd, J=5.00 Hz, J=2.00 Hz), 7.47 (1H, d, J=8.00 Hz, J=2.00 Hz), 7.24 (1H, m), 6.83 (2H, m). 13C-NMR (DMSO-d6) δ: 163.33, 150.25, 136.59, 130.92, 130.81, 130.67, 130.55, 129.11, 119.64. IR (KBr) cm⁻¹: 1659, 1470. MS m/z: 345.00 (M⁺), 230.00. Anal. Calcd for C15H12Cl2N4S: C, 51.89; H, 2.32; N, 16.14. Found; C, 52.01; H, 2.54; N, 16.42.

N-(2,4-Dichlorophenyl)-7,9-dimethylpyrido[3',2',4,5]:4,5-thieno[3,2-d]pyrimidin-4-amine (5b)

Yield 63%; mp> 300°C (decomp.). 1H-NMR (DMSO-d6) δ: 9.49 (1H, s), 8.40 (1H, s), 7.69 (1H, s), 7.63 (1H, d, J=9.00 Hz), 7.41 (1H, d, J=9.00 Hz), 6.68 (1H, s), 2.89 (3H, s), 2.60 (3H, s). 13C-NMR (DMSO-d6) δ: 161.99, 158.07, 157.28, 150.43, 144.09, 129.61, 126.00, 122.00, 118.69, 20.26, 13.87. IR (KBr) cm⁻¹: 3001, 2905, 1692, 1574. MS m/z: 374.00 (M⁺), 231.00. Anal. Calcd for C17H13Cl2N4S: C, 54.41; H, 3.22; N, 14.93. Found; C, 54.79; H, 3.38; N, 15.26.

General Procedures for the Synthesis of N-(Substituted Benzyl)pyrido[3',2',4,5]:4,5-thieno[3,2-d]pyrimidin-4-amines (6–8a, b) The appropriate benzylamine (9 mmol) was dissolved in dry DCM (25 mL) and Et3N (1.5 mL) under nitrogen atmosphere. Compound (1a or b) (4.5 mmol) was added slowly to the stirred solution which was then heated under reflux for 6–9 h. The reaction mixture was cooled and poured onto crushed ice. The precipitated solid was filtered off, washed with water (2×10 mL) and dried. The crude solid was purified by column chromatography over silica gel using 1–5 EtOAc/hexane v/v as the eluent to give the desired compounds (6–8a, b).
*N-(Benzyl)pyrido[3′,2′:4,5]thieno[3,2-d]pyrimidin-4-amine (6a)*

Yield 64%; mp 214–216°C. 1H-NMR (DMSO-<d>) δ: 8.36 (1H, s), 7.71 (1H, s), 7.55 (1H, d, 3J=4.8 Hz), 7.47 (1H, d, 3J=7.8 Hz), 7.38 (6H, m), 4.91 (2H, s). 13C-NMR (DMSO-<d>) δ: 159.59, 150.97, 147.17, 146.49, 138.40, 135.68, 131.36, 130.79, 130.56, 127.70, 122.69, 121.58, 110.60, 61.62. IR (KBr) cm⁻¹: 3697, 2987, 1622, 1460. MS m/z: 369.00 (M⁺). Anal. Calcd for C₁₈H₁₅BrN₄S: C, 53.20; H, 2.79; N, 15.51.

*Assay Protocols*

The assays for VEGFR-2/KDR, EGFR, CDK5/p25, GSK3α and GSK3β were performed using kinase-GloTM Plus luminescence kinase assay kit (Promega #V3772) and ADP-GloTM kinase assay kit (Promega #V9101). The IC₅₀ determination was carried out where quality control testing was routinely performed on each of the targets to insure compliance to acceptable standards. ATP was purchased from PerkinElmer, Inc. and ADP-GloTM was purchased from Promega. All the other materials were of standard laboratory grade.

*Biochemical Evaluation*

**Protein Kinase Inhibitory Assays**

The protein kinase inhibitory assays were carried out by BPS Bioscience at single dose concentration of 10µM. VEGFR-1/Flt1 (BPS #40223), VEGFR-2/KDR (BPS #40301), the wild type of EGFR (BPS #40187), CDK5/p25 (BPS #40105), GSK3α (BPS #40006), GSK3β (BPS #40007) served as enzymes sources. While GSK3β (BPS), Histone H1 (NEB #M2501S), Poly(Glu, Tyr) sodium salt, (4:1, Glu: Tyr) (Sigma #P7244) served as the standardized substrate, in addition to kinase-GloTM Plus luminescence kinase assay kit (Promega #V3772) and ADP-GloTM kinase assay kit (Promega #V9101). The IC₅₀ determination was carried out where quality control testing was routinely performed on each of the targets to insure compliance to acceptable standards. ATP was purchased from PerkinElmer, Inc. and ADP-GloTM was purchased from Promega. All the other materials were of standard laboratory grade.
Data Analysis

Kinase activity assays were performed in duplicate at each concentration. The luminescence data was analyzed using the computer software, graph pad Prism. The difference between luminescence intensities in the absence of kinase (Lut) and in the presence of kinase (Luc) was defined as 100% activity (Luc−Luc). Using luminescence signal (Lu) in the presence of the compound, % activity was calculated as: % activity=[(Lu−Lu)/(Lu−Luc)×100%, where Lu=the luminescence intensity in the presence of the compound (all percent activities below zero were set to 0%). % Inhibition was calculated as: % inhibition=100−(%−% activity.15)

Measurement of Potential Enzyme Inhibitory Activity IC50

IC50 value for compound (5a) against EGFR was estimated by generating a graph of log inhibitor versus normalized response with variable using the prism software.15

Evaluation of Anticancer Activity against a Panel of Sixty Human Cancer Cell Lines

The structures of the final compounds were submitted to National Cancer Institute “NCI,” Bethesda, Maryland, U.S.A., and all the submitted compounds were selected on the basis of the degree of structure variation and computer modeling techniques for evaluation of their cytotoxic activity. The screening is based on the evaluation of all compounds against the full NCI 60 cell lines panel representing leukemia, non-small cell lung cancer, melanoma, colon cancer, CNS cancer, breast cancer, ovarian cancer, renal cancer and prostate cancer at a single dose of 10−5 M. The output from the single dose screen is reported as a mean graph.21,28,29

Assay Protocol

The human cancer cell lines of the cancer-screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96 well microtiter plates in 100 mL at plating densities ranging from 5000 to 40000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37°C, 5% CO2, 95% air, well maintained at 37°C in a humidified atmosphere containing 5% CO2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line are fixed in situ with trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs are solubilized and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration and stored frozen prior to use. The drug concentration resulting in total growth inhibition (TGI) is calculated from [Ti−Tz]/(C−Tz)×100=50 which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment.21,28,29

Cytotoxicity Test

Cell Culture

A549 human lung cancer cells and MCF-7 human breast cancer cells were grown in RPMI-1640 medium, supplemented with 10% heat inactivated fetal bovine serum (FBS), 50 units/mL of penicillin and 50 g/mL of streptomycin and maintained at 37°C in a humidified atmosphere containing 5% CO2. The cells were maintained as “monolayer culture” by serial subculturing.27

SRB Cytotoxicity Assay

The cytotoxicity was determined using SRB method as described by Skehan et al.27 Exponentially growing cells were collected using 0.25% Trypsin–ethylenediaminetetraacetic acid (EDTA) and seeded in 96-well plates at 1000–2000 cells/well in RPMI-1640 supplemented medium. After 24 h, cells were incubated for 72 h with various concentrations of the tested compounds. Following 72 h treatment, the cells will be fixed with 10% trichloroacetic acid for 1 h at 4°C. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris·HCl for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was
measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, U.S.A.). The IC_{50} values were calculated according to the equation for Boltzmann sigmoidal response curve using the nonlinear regression fitting models (Graph Pad, Prism Version 5).

DNA-Flow Cytometry Analysis

A549 cells at a density of 2×10^5 cells were exposed to the tested compounds at their IC_{50} concentration for 48 h. The cells were collected by trypsinization, washed in phosphate buffered saline (PBS) and then fixed in ice-cold absolute alcohol. Thereafter, cells were stained using Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, U.S.A.) according to the manufacturer’s instructions. Cell-cycle distribution was determined using a FACS Calibur flow cytometer (BD Biosciences).

Western Blot Analysis

Primary antibody against p27^{kip} (Cell Signaling, Danvers, MA, U.S.A.) was used to assess the protein expression of this marker in the tested cells as described previously. Cells were seeded, cultured and exposed to the tested agent for 72 h. Whole-cell protein lysates were prepared according to standard protocol using RIPA buffer (Cell Signaling). Protein (50 μg) was loaded per well of a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel using electrophoresis buffer (0.192 M glycine, 25 mM Tris and 0.1% SDS). After electrophoresis, the gel was transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using transfer buffer (0.192 M glycine, 25 mM Tris, 0.025% SDS and 10% methanol). Membranes were blocked in TBS-T with 5% BSA and incubated overnight with the primary antibody (1:1000) then incubated with secondary horseradish peroxidase (HRP)-linked antibody (1:5000). Development was done using OptiChemiluminescent substrate (Abcam plc, Cambridge, MA, U.S.A.).

Assessment of the Effect of Compounds (4b) and (5a) on P27^{kip} Using ELISA

The effects of the tested compounds on the protein levels of P27^{kip} were further confirmed using enzyme-linked immunosorbent assay kit from Abcam according to the manufacturer’s instructions. Protein content of the cell lysates was assessed using BCA kit (Biovision, Inc., Milpitas, CA, U.S.A.) according to the manufacturer’s instructions. Protein content of the cell lysates were assessed using BCA kit from Abcam plc according to the manufacturer’s instructions. Protein content of the cell lysates were assessed using BCA kit from Abcam plc according to the manufacturer’s instructions.

Conclusion

EGFR is considered as a key enzyme in the regulation of cellular metabolism, growth and proliferation. Most EGFR inhibitors are discovered to date act as ATP-competitive inhibitors. Inspired by the scaffolds of the previously reported EGFR inhibitors, a novel series of pyrido[3,2-d]pyrimidin-4-amine derivatives bearing substituted anilines and benzylamines at position 4 were designed and synthesized. The rational of our study was based on the investigation of novel ATP-competitive EGFR inhibitors. The biological studies highlighted a novel EGFR inhibitor (5a) with IC_{50} value 36.7 nM. This compound showed a significant cell growth inhibition against leukemia, CNS cancer and NSCLC cell lines that overexpress EGFR, with GI_{50} values around 10 nM. The standard COMPARE analyses demonstrated a considerable correlation between compound (5a) and erlotinib with PCC value 0.727. It is worth noting that the identification of this lead compound could help in the design of clinically useful ATP-competitive EGFR inhibitors.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.

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