RESEARCH ARTICLE

Title: Synthesis and Anticancer Activity of Novel Indole Derivatives as Dual EGFR/SRC Kinase Inhibitors

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Abstract Background: Recent studies showed that the cooperation between c-SRC and EGFR is responsible for more aggressive phenotype in diverse tumors, including glioblastomas and carcinomas of the colon, breast, and lung. Studies show that combination of SRC and EGFR inhibitors can induce apoptosis and delay the acquired resistance to chemotherapy. Therefore, such combination may lead to a new therapeutic strategy for the treatment of EGFR-mutant lung cancer. Osimertinib was developed as a third-generation EGFR-TKI to combat the toxicity of EGFR mutant inhibitors. Due to the resistance and adverse reaction of osimertinib and other kinase inhibitors, **12** novel compounds structurally similar to osimetinib were designed and synthesized.

Methods: Compounds were synthesized by developing novel original synthesis methods and receptor interactions were evaluated through a molecular docking study. To evaluate their inhibitory activities against EGFR and SRC kinase, *in vitro* enzyme assays were used. Anticancer potencies were determined using lung, breast, prostate (A549, MCF6, PC3) cancer cell lines. Compounds were also tested against normal (HEK293) cell line to evaluate their cyctotoxic effects.

Results: Although, none of compounds showed stronger inhibition compared to osimertinib in the EGFR enzyme inhibition studies, compound **16** showed the highest efficacy with an IC_{50} of 1.026 μM. It was also presented potent activity against SRC kinase with an IC_{50} of 0.002 μM. Among the tested compounds, the urea containing derivatives **6-11** exhibited a strong inhibition profile (80.12-89.68%) against SRC kinase in comparison to the reference compound dasatinib (93.26%). Most of the compounds caused more than 50% of cell death in breast, lung and prostate cancer cell lines and weak toxicity for normal cells in comparison to reference compounds osimertinib, dasatinib and cisplatin. Compound **16** showed strong cytotoxicity on lung and prostate cancer cells. Treatment of prostate cancer cell lines with the most active compound, **16,** significantly increased the caspase-3 (8-fold), caspase-8 (6-fold) and Bax (5.7-fold) levels and decreased the Bcl-2 level (2.3-fold) compared to the control group. These findings revealed that the compound **16** strongly induces apoptosis in the prostate cancer cell lines.

Conclusion: Overall kinase inhibition, cytotoxicity and apoptosis assays presented that compound **16** has dual inhibitory activity against SRC and EGFR kinases while maintaining low toxicity against normal cells. Other compounds also showed considerable activity profiles in kinase and cell culture assays.

Keywords: Anti-cancer activity, kinase inhibitors, EGFR, SRC, docking, inhibition, apoptosis

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1. INTRODUCTION

Understanding the roles of Protein Tyrosine Kinases (PTKs) in the formation of prostate, lung, breast cancers, and osteoporosis led to the development of small molecule PTK inhibitors that reached the market. This new strategy yielded a progress to combat metastatic cancers by regulating important factors such as cell growth, proliferation, invasion, and migration. Many EGFR and SRC inhibitors (geftinib, erlotinib, dasatinib, sorafenib, bosutinib, saracatinib, dacomitinib, lapatinib, lenvatinib, afatinib, osimertinib, etc.) have been used for the treatment of bone marrow, lung, prostate, and breast cancers (Fig. **1**), [1-3]. PTKs play a role in tumor formation through many pathways and inhibiting a single PTK enzyme is insufficient in terms of the effectiveness of the treatment. However, when a combination of drugs that inhibit different PTKIs is used for the treatment, there is a risk of drug-drug interaction. Another obstacle during cancer therapy is the drug resistance, which develops due to mutations and reduces the effectiveness of existing drugs [1, 4]. For these reasons, the discovery of new active molecules is important to increase the treatment efficiency of all types of cancers. The kinase inhibitors are generally classified into four different groups according to their mechanism of action. Inhibitors that compete with ATP for binding to the catalytic site of the kinases, those that prevent the activation of kinases by binding to their inactive conformations, inhibitors that bind to a region outside the catalytic site without competing with ATP, inhibitors bind to allosteric region distant from the ATP pocket that exhibit the maximum selectivity and inhibitors that make the covalent bond with the kinase active site [5-7]. Reversible inhibitors of the PTKs bind to the catalytic site of the kinases competitively with ATP. Among them, quinazoline structured compounds gefitinib (IC_{50}: 33 nM), and erlotinib (IC_{50}: 2 nM) have been reported as selective EGFR-1 tyrosine kinase inhibitors and are effectively used in the treatment of non-small cell lung cancer (NSCLC), (Fig. **1**). Another drug with a quinazoline structure Lapatinib is a reversible and selective EGFR-1 (IC_{50}:10 nM) and EGFR-2 (IC_{50}: 98 nM) receptor tyrosine kinase inhibitor and is used for the treatment of metastatic breast cancer and lung cancer [4]. Irreversible inhibitors bind to a region outside the catalytic site without competing with ATP that make a covalent bond with the kinase active site. Irreversible EGFR inhibitor afatinib (Fig. **1**) is an anilino-quinazoline derivative which was designed for the initial treatment of metastatic non-small cell lung cancer [2]. Dacomitinib (Fig. **1**) was also approved for the treatment of mutant NSCLC as an EGFR-inhibitor compound [8]. EGFR-mediated mutational activation especially a point mutation (T790M) at the gate-keeper position near the ATP binding pocket is responsible for 50% of the resistance problem and is the reason for lesser the effectiveness of first-generation TKIs such as geftinib and erlotinib [9]. Osimertinib (Fig. **1**) was discovered as third-line therapy to overcome drug resistance and EGFR activating mutations (L858R or Exon19del) or T790M mutation [10]. However, osimertinib showed some adverse effects such as diarrhea, rash, nausea, loss of appetite. Furthermore, osimertinib should be carefully used with patients with liver and renal dysfunction. The interaction details and dynamics of osimertinib binding to EGFR mutants were investigated by using the theoretical (unbiased atomic-level MD simulations) and experimental (X-ray crystallography) approaches. It was found that the indole ring of osimertinib rotates for ~180° compared to its binding pose to EGFR with wild-type gatekeeper (Thr 790) when binding to EGFR T790M. The flipped binding pose of osimertinib with T790M has been found to have a van der Waals interaction between the compound and Met 790. In this pose, it was observed that the acrylamide group of osimertinib becomes closer to the EGFR Cys797 side chain and promotes the formation of the covalent bond with EGFR [11].

Fig. (1). EGFR and SRC Inhibitors

The clarification the mechanism of protein kinase receptor interaction site and the structure-activity relationships of the compounds interacting with this site has led to the increase in the success of the studies in this field and the approval of many compounds as drugs. Several important studies were conducted to improve the clinical potency and physicochemical properties of osimertinib. For this reason, indole ring was fused at C-4 of the pyrimidine, and an aniline ring was added as a basic substituent [12]. In a study, osimertinib was modified by adding a sulfoxide side chain at the C-4 position of the aniline moiety to reduce its side effects. It was found that most of the designed compounds mentioned above presented good EGFR kinase inhibition activity and anti-proliferative activity against L858R/T790M double mutant cells [13]. Nevertheless, none of these compounds have been reported to have better activity, toxicity and pharmacokinetic profile than osimertinib.

Recent evidence presented that c-SRC modulates epidermal growth factor receptor (EGFR) activation by phosphorylation tyrosine residues on its C-terminal domain and this results in several downstream effects. Therefore, it has been claimed that the synergism between c-SRC and EGFR contributes to a more aggressive phenotype in diverse tumors, including glioblastomas and carcinomas of the colon, breast, and lung [14]. The synergistic effects of c-SRC inhibitors in combination with EGFR inhibitors were shown on dasatinib, saracatinib and bosutinib, typically tested in lung, pancreatic, colorectal and breast cancer [15]. This finding also presented that inhibition of both SFK and EGFR may be a therapeutic strategy for mutant EGFR-mutant lung cancer [16]. Among the other kinase inhibitors, osimertinib

is the most resistant against mutations and it has more tolerable side effects.

In the light of findings in literature, several compounds structurally similar to osimertinib were designed and synthesized in our laboratory, which are shown in Fig. **2**. Docking studies showed that compounds at third position were considered to make flip (U shaped) binding pose and interact with EGFR binding sites similar to osimertinib [12]. Substituents in the aniline ring were chosen to obtain good interactions with the receptor site and to have better activity. In addition to these structural requirements, urea group was added to provide interaction with SRC kinase. The urea group creates a certain degree of conformational restriction and also plays an important role for aqueous solubility and permeability. Besides improving the solubility, hydrogen bonding capability with urea groups can also provide NHπ type of interaction with aromatic side chains in proteins [17]. This claim was also proved in the literature showing that the urea moiety has an important role in molecular interaction of sorafenib and lenvatinib (Figure 1) to protein kinase active site [7].

Fig. (2). General structure of targeted compounds

2. MATERIALS AND METHODS

2.1. Chemistry. All solvents and chemicals were used without further purification. The reactions were followed on TLC (Merck) using fluorescence indicator UV_{254}) in different solvent systems. Compounds were purified by using column chromatography on Merck flash silica gel 60 and the solvent mixtures were chosen in the corresponding compounds. 1H Proton and 13C NMR spectra value were obtained on a Bruker 400 using d_6-DMSO and $CDCl_3$ as solvents and data are represented in parts per million (ppm) of chemical shifts of related compounds. The purity of final compounds was proved by high performance liquid chromatography (HPLC, Schimadzu, LC 20-A) and it was observed that the purity of all compounds was 95% or higher. HPLC solvent was initiated by linearly increasing the composition of the acetonitrile/H_2O (90%: 10%) and AcCN:MeOH (50:50). Each HPLC run was set up as 20 min and 10 min were allowed for equilibration between runs. A volume of 10 µl of solutions of the test compounds in acetonitrile or MeOH as 1 mM concentration was injected into the HPLC system, and the wavelengths detected over UV spectra for each compound were used to obtain chromatograms.

2.1.1. Synthesis of 3-(2-chloropyrimidin-4-yl)-1-methyl-1*H*-indole (1a) ***Method 1 (Scheme 1)*** 2,4-Dichloropyrimidine (0.130 g, 0.87 mmol) was dissolved in 2 ml THF and $FeCl_3$ (0.141 g, 0.87 mmol) and N-methyl indole (0.926 ml, 7.4 mmol) were added sequentially at room temperature and the reaction mixture was stirred at 64 ^oC for overnight. The mixture cooled to room temperature and filtered over celite and washed with MeOH. Evaporating to dryness gave a crude compound which was purified by silica gel column chromatography (H/E: 9:1). 0.065 g Pure compound was obtained (Yield, 28.63%). LC-MS (ESI): m/z for $C_{13}H_{10}ClN_3$ [M^++1]: 244.67. ***Method 2 (Scheme 1).*** 2,4-Dichloropyrimidine (1.29 g, 8.7 mmol) was dissolved in 21 ml THF and $AlCl_3$ (1.16 g, 8.7 mmol) was added at 0^oC and stirred for 30 min. N-methyl indole (0.95 ml, 7.2 mmol) was added at room temperature and the reaction mixture was stirred at 64 ^oC for overnight. The mixture cooled to room temperature and evaporated to dryness gave a crude compound which was purified by silicagel column chromatography (H/E: 9:1). 1.02 g Pure compound was obtained (Yield, 55.14%). ***Method 3 (Scheme 1).*** Indole (0.35 g, 2.9 mmol) was dissolved in 5 ml THF and MeMgBr (1.4M in THF, 2 ml, 2.9 mmol) was added at 0^oC as dropwise and stirred for 15 min. 2,4-Dichloropyrimidine (0.447, 3.0 mmol) was added as one portion at room temperature and the mixture was stirred at rt for 16 h and then refluxed at 64 0^oC for 5h. The mixture was quenched with water and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and evaporated to dryness produced a crude compound, which was purified by column chromatography (H/E: 9.5:0.5). 0.346 g Pure compound was obtained. 3-(2-chloropyrimidin-4-yl)-1*H*-indole (0.346 g, 1.5 mmol) was dissolved in 5 ml THF and NaH (0.075 g, 3.0 mmol) was added at 0^oC as portion wise and stirred for 30 min. CH_3I (0.12 ml, 2.0 mmol) was added at room temperature and stirred for 16 h. It was quenched with water and extracted with EtOAc, dried over Na_2SO_4 and evaporated to dryness gave crude compound which was purified by column chromatography (H/E: 9.5:0.5). 0.144 g Pure compound was obtained (Overall Yield, %19.83).

2.1.2. Synthesis of 3-(6-chloro-2-(methylthio)pyrimidin-4-yl)-1-methyl-1*H*-indole (1b) ***Method 2*** (***Scheme 1***) was used to synthesize compound **1b**. 3-thiomethyl 4,6-dichloropyrimidine (1.0 g, 5.1 mmol) was dissolved in 2 ml THF and $AlCl_3$ (0.82 g, 6.2 mmol) was added at 0^oC and stirred for 30 min. N-methyl indole (0.64 ml, 5.1 mmol) was added at room temperature and the reaction mixture was stirred at 64 ^oC for overnight. The mixture cooled to room temperature and evaporated to dryness gave crude compound which was purified by silica gel column chromatography (H/E: 9:1). 1.775 g Pure compound was obtained (Yield, 72.45%). LC-MS (ESI): m/z for $C_{14}H_{12}ClN_3S$ [M^++1]: 290.81.

2.1.3. Synthesis of 4-(1-methyl-1*H*-indol-3-yl)-*N*-(4-nitrophenyl)pyrimidin-2-amine(2a)

Compound **1a** (0.283 g, 1.2 mmol) and p-nitroaniline (0.160 g, 1.2 mmol)) were dissolved in 12 ml isopropanol and p-TsOH (0.33, 1.7 mmol) was added at rt. The mixture was stirred at 105 0^oC for 5h. The precipitate was filtered and washed with isopropanol. Silica gel column chromatography (H/E: 9:1) was used to purify compounds. 0.317 g Pure compound was obtained (Yield, 40.28%). LC-MS (ESI): m/z for $C_{19}H_{15}N_5O_2$ [M^+ + 1]: 346.62.

2.1.4. 6-(1-methyl-1*H*-indol-3-yl)-2-(methylthio)-*N*-(4-nitrophenyl)pyrimidin-4-amine(2b)

Compound **1b** (0.461, 1.4 mmol) and p-nitroaniline (0.198 g, 1.4 mmol) were dissolved in 14 ml isopropanol and p-TsOH (0.327 g, 1.7 mmol) was added at rt. The mixture was stirred at 105 0^oC for 5h. The precipitate was filtered and washed with isopropanol. Silica gel column chromatography (H/E: 9:1) was used to purify compounds. 0.350 g Pure compound

was obtained (Yield, 57.66%). LC-MS (ESI): m/z for $C_{20}H_{17}N_5O_2S$ [M^++1]: 391.10.

2.1.5. Synthesis of N^1-(4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)benzene-1,4-diamine(3a)

Method 4 (Scheme 1). Compound **2a** (0.176 g, 0.51 mmol) was dissolved in 4.6 ml EtOH and 10%Pd/C (0.090 g, 0.78 mmol) and $HCOONH_4$ (0.185 g, 2.9 mmol) was added respectively. The reaction mixture was stirred at rt for 5h. The solid was filtered over celite and the filtrate was evaporated to dryness, and was purified by silica gel column chromatography (H/E:9:1). 0.149 g Pure compound was obtained (Yield, 93.25%). LC-MS (ESI): m/z for $C_{19}H_{17}N_5$ [M^++1]:316.86.

2.1.6. *N*^{1}-(6-(1-methyl-1*H*-indol-3-yl)-2-(methylthio) pyrimidin-4-yl)benzene-1,4-diamine(3b).

Method 5 (Scheme 1). Compound **2b** (0.360 g, 8.5 mmol) was dissolved in 29 ml EtOH and 5.95 ml H_2O. Fe+3 (0.333 g, 5.95 mmol) and NH_4Cl (0.182 g, 3.4 mmol) was added and the mixture was refluxed at 70-80 0^oC for 3h. It was filtered and the solvent was evaporated to dryness and gave crude compound which was purified by column chromatography (H/E 8:2). 0.103 g Pure compound was obtained (Yield, 30.83%). LC-MS (ESI): m/z for $C_{20}H_{19}N_5$ [M^++1]: 362.67.

2.1.7. *N*-(4-fluoro-2-methoxy-5-nitrophenyl)-4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-amine (4a, Scheme 2). Compound **1a** (0.440 g, 1.8 mmol) was dissolved in 9 ml i-PrOH and 4-fluoro-2-methoxy-3-nitro aniline (0.336 g, 1.8 mmol) and p-TsOH (0.43 g, 2.2 mmol) were added at rt. The mixture was stirred at 105 0^oC for 5h. Yellow precipitate was collected by filtration, washed with CH_2Cl_2. 0.678 g Pure compound was obtained (Yiled,95.35%). LC-MS (ESI): m/z for $C_{20}H_{16}FN_5O_3$ [M^++1]: 394.91.

2.1.8. Synthesis of *N*-(2-methoxy-5-nitro-4-(piperazin-1-yl)phenyl)-4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-amine (4b, Scheme 2). 0.100 g compound **4a** was dissolved in 1.25 ml DMF and 0.106 g K_2CO_3 and 0.022g piperazine were added at rt respectively. The reaction mixture was stirred 30 min and then refluxed at 60 ^oC for 2h. Water was added, filtered and extracted with CH_2Cl_2. Dried over Na_2SO_4 and evaporated to dryness gave orange color oily compound which was purified by column chromatography (DCM/MetOH, 9:1). 0.110 g Pure compound was obtained (Yield, 86.21%). LC-MS (ESI): m/z for $C_{24}H_{25}N_7O$ [M^+ +1]: 461.09.

2.1.9. Synthesis of 4-fluoro-6-methoxy-*N*^{1}-(4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)benzene-1,3-diamine (5a, Scheme 2). Compound **4a** (0.321, 0.8 mmol) was dissolved in 4.0 ml EtOH and 0.8 ml H_2O. Fe^{+3} (0.320 g, 5.7 mmol) and NH_4Cl (0.176 g, 3.3. mmol) were added and stirred at 80 0^oC for overnight. The precipitate was filtered over celite and water was added. It was extracted with DCM and organic layer was extracted with 1N NaOH, dried over Na_2SO_4 and evaporated to dryness gave a 96 mg brown oily compound which was purified by silica gel column chromatography (H/E: 7:3). 0.206 g Pure compound was obtained (Yield, 69.59%). LC-MS (ESI): m/z for $C_{20}H_{18}FN_5O$ [M^+]: 363.90.

2.1.10. Synthesis of 1-(4-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-3-phenylurea (6-8) and 1-(4-((6-(1-methyl-1*H*-indol-3-yl)-(methylthio)pyrimidin-4-yl)amino)phenyl)-3-phenylurea (9-11) derivatives. 1 Eq. compound **3a** or **3b** was dissolved in 1 mmol/10 mL THF and 1 eq. TEA and 1 eq. substituted PhIS derivatives were added respectively at 0^oC and stirred for 30 min and then additional 1h at rt. Then the mixture was refluxed at 70-80^oC for 6h to overnight. The solvent was evaporated to dryness and washed with CH_2Cl_2. The precipitates of compounds **6-11** were obtained and purified by column chromatography (H/E, 1:1).

2.1.11. 1-(4-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl) amino)phenyl)-3-phenylurea (6). White powder; yield 71.0%. 1H-NMR (400.1759761 MHz, [Dδ]DMSO): δ =9.27 (s, 1H, NH); 8.58 (s, 1H, (urea NH proton); 8.51 (s, 1H, urea NH proton); 8.27 (d, 2H, pyrimidine proton); 7.71 (d, 2H, Ar-H); 7.51 (d, 1H, Ar-H); 7.45 (d, 2H, Ar-H); 7.34 (d, 3H, Ar-H); 7.27 (t, 3H, Ar-H); 7.11 (m, 1H, Ar-H); 6.95 (t, 1H, Ar-H); 3.86 (s, 3H, N-Me). 13C-NMR (125.76 MHz, [Dδ]DMSO): δ = 100.6243765 MHz ; 162.65 (C=O), 160.73, 160.54, 157.15, 153.33, 152.89, 140.55, 138.21, 136.11, 135.92, 134.44, 134.00, 133.34, 129.33, 125.93, 123.15, 122.54, 122.11, 121.48, 121.25, 120.37, 118.39, 113.21, 110.87, 107.24, 33.61 (N-$\underline{C}H_3$). LC MS (ESI): m/z for $C_{26}H_{21}N_6O$ [M^+]: 434.95. HPLC (Schimadzu, LC 20-A): UV_{max}= 286 nm, AcCN:MeOH (50:50), Rt= 5.734 min, purity found 99.94%. HRMS (ESI): m/z calculated for [M^+-1]: 433.178233 Found: 433.177030.

2.1.12. 1-(4-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl) amino)phenyl)-3-(4-(trifluoromethoxy) phenyl) urea (7). White powder; yield 42.8%. 1H-NMR (400.1759761 MHz, [Dδ]DMSO): δ =9.32 (s, 1H, NH); 8.83 (s, 1H, urea NH proton); 8.59 (m, 2H, pyrimidine and urea NH protons); 8.30 (m, 2H, pyrimidine proton and Ar-H); 7.75 (d, 2H, Ar-H); 7.59 (m, 3H, Ar-H); 7.42 (d, 2H, Ar-H); 7.28 (m, 3H, Ar-H); 7.15 (m, 2H, Ar-H); 3.87 (s, 3H, N-Me). 13C-NMR (125.76 MHz, [Dδ]DMSO): δ = 100.6243765 MHz ; 161.81 (C=O), 160.23, 156.96, 152.49, 142.38, 139.04, 137.51, 135.94, 135.71, 133.27, 132.83, 125.55, 122.40, 122.16, 121.66, 121.46, 120.65, 119.86, 119.20, 118.91, 118.56, 116.37, 112.55, 110.30, 106.84, 40.95, 33.02 (N-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{27}H_{21}F_3N_6O_2$, [M^++2]: 520.14. HPLC (Schimadzu, LC 20-A): UV_{max}= 288 nm, AcCN:H_2O (90:10), Rt= 5.368 min, purity found 96.65%. HRMS (ESI): m/z calculated for [M^++1]: 519.176182 Found: 519.17377.

2.1.13. 1-(4-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl) amino)phenyl)-3-(*p*-tolyl)urea (8). White powder; yield 55.7%. 1H-NMR (400.1759761 MHz, [Dδ]DMSO): δ =9.25 (s, 1H, NH); 8.59 (d, 1H, NH, urea NH proton); 8.49 (d, 2H, urea NH proton); 8.28 (d, 2H, pyrimidine protons); 7.70 (d, 2H, Ar-H); 7.51 (d, 1H, Ar-H); 7.37-7.31 (m, 4H, Ar-H); 7.24 (t, 1H, Ar-H); 7.13 (m, 2H, Ar-H); 7.06 (d, 2H, Ar-H); 3.86 (s, 3H, N-Me); 2.22 (s, 3H, Ar-CH_3). 13C-NMR (125.76 MHz, [Dδ]DMSO): δ = 100.6243765 MHz ; 162.55 (C=O), 162.40, 160.67, 157.32, 153.50, 153.13, 138.30, 138.09, 135.96, 135.74, 133.42, 131.10, 129.62, 126.25, 123.06, 122.67, 121.38, 120.73, 120.53, 119.49, 119.24, 118.65, 113.34, 111.00, 107.21, 33.61 (N-$\underline{C}H_3$), 20.92 (Ar-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{27}H_{24}N_6O$, [M^++2]: 450.01. HPLC (Schimadzu, LC 20-A): UV_{max}= 288 nm, AcCN:H_2O (90:10), Rt= 11.960 min, purity found 99.76%. HRMS (ESI): m/z calculated for [M^+ -1]: 447.193883, Found: 447.19305.

2.1.14. 1-(4-((6-(1-methyl-1*H*-indol-3-yl)-2-(methylthio) pyrimidin-4-yl)amino)phenyl)-3-phenylurea (9). White powder; yield 53.5%. 1H-NMR (400.1759761 MHz, [D$_6$]DMSO): δ = 9.39 (s, 1H, NH); 8.63 (s, 1H, urea NH proton); 8.57 (s, 1H, urea NH proton); 8.21-8.19 (d, 1H, pyrimidine proton); 8.10 (d, 1H, pyrimidine proton); 7.53 (t, 3H, Ar-H); 7.42 (t, 5H, Ar-H); 7.25 (m, 3H, Ar-H); 6.94 (t, 1H, Ar-H); 6.82 (s, 1H, Ar-H); 3.86 (s, 3H, N-Me); 2.55 (s, 3H, SCH_3). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 170.70 (C=O), 170.10, 160.68, 160.11, 153.36, 152.76, 140.71, 140.11, 137.78, 135.14, 134.55, 132.50, 129.52, 126.01, 122.50, 122.16, 120.97, 119.53, 118.39, 113.10, 111.31, 96.01, 67.80, 49.01, 33.71, 20.47 (N-$\underline{C}H_3$), 13.99 (S-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{27}H_{24}N_6OS$ [M^++1]: 481.75. HPLC (Schimadzu, LC 20-A): UV_{max}= 264 nm, AcCN:H_2O (90:10), Rt= 4.271 min, purity found 95.32%. HRMS (ESI): m/z calculated for [M^+ -1]: 479.165954, Found: 479.16455.

2.1.15. 1-(4-((6-(1-methyl-1*H*-indol-3-yl)-2-(methylthio) pyrimidin-4-yl)amino)phenyl)-3-(4-(trifluoro-methoxy) phenyl)urea (10). White powder; yield 51.2%. 1H-NMR (400.1759761 MHz, [D$_6$]DMSO): δ = 9.40 (s, 1H, NH); 8.88 (s, 1H, urea NH proton); 8.67 (s, 1H, urea NH proton); 8.21-8.19 (d, 1H, pyrimidine proton); 8.10 (d, 1H (pyrimidine proton); 7.53 (m, 5H, Ar-H); 7.43-7.40 (d, 2H, Ar-H); 7.25 (m, 4H, Ar-H); 6.83 (s, 1H, Ar-H); 3.86 (s, 3H, N-Me); 2.55 (s, 3H, SCH_3). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 170.01 (C=O), 161.04, 159.86, 153.24, 142.82, 139.52, 138.15, 134.75, 132.22, 125.83, 122.95, 122.44, 121.80, 121.32, 119.92, 119.44, 113.05, 111.11, 95.81, 67.65, 65.49, 60.35, 33.62, 21.32, 20.37 (N-$\underline{C}H_3$), 15.66, 14.73, 14.25 (S-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{28}H_{23}F_3N_6O_2S$ [M^++1]: 565.00. HPLC (Schimadzu, LC 20-A): UV_{max}= 282 nm, AcCN:H_2O (90:10), Rt= 8.428 min, purity found 97.92%. HRMS (ESI): m/z calculated for [M -1]: 563.148253, Found: 563.14752.

2.1.16. 1-(4-((6-(1-methyl-1*H*-indol-3-yl)-2-(methylthio) pyrimidin-4-yl)amino)phenyl)-3-(4(trifluoro-methoxy) phenyl)urea (11). White powder; yield 58.4%. 1H-NMR (400.1759761 MHz, [D$_6$]DMSO): δ = 9.36 (s, 1H, NH); 8.53 (d, 2H, urea NH protons); 8.22 (d, 1H, pyrimidine proton); 8.09 (s, 1H (pyrimidine proton); 7.53 (m, 3H, Ar-H); 7.39 (d, 2H, Ar-H); 7.33 (d, 2H, Ar-H); 7.22 (m, 2H, Ar-H); 7.07 (d, 2H, Ar-H); 6.82 (s, 1H, Ar-H); 3.85 (s, 3H, N-Me); 2.48 (s, 3H, SCH_3); 2.22 (s, 3H, Ar-C$\underline{H}_3$). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 170.42 (C=O), 168.05, 160.71, 159.62, 157.82, 155.00, 152.98, 150.21, 148.12, 137.94, 137.69, 137.73, 135.08, 134.28, 132.29, 130.60, 129.60, 125.70, 122.45, 121.34, 121.07, 119.30, 118.70, 112.79, 110.95, 95.89, 78.21, 33.19 (Ar-$\underline{C}H_3$), 20.74 (N-$\underline{C}H_3$), 14.06 (S-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{28}H_{26}N_6OS$ [M^++1]: 495.00. HPLC (Schimadzu, LC 20-A): UV_{max}= 280 nm, AcCN:H_2O (90:10), Rt= 8.858 min, purity found 99.33%. HRMS (ESI): m/z calculated for [M^+ -1]: 493.181604, Found: 493.18082.

2.1.17. Synthesis of 1-(4-(5-methoxy-4-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)amino)-2-nitrophenyl)piperazin -1-yl)prop-2-en-1-one (12). Compound **4b** (0.076 g, 0.16 mmol) was dissolved in 1mmol/5 mL DCM, TEA (0.027 ml, 0.19 mmol) and acryloyl chloride (0.016 ml, 0.19 mmol) was added at 0^{o}C and stirred for 30 min and additional 12h at rt. The solvent was evaporated and crude compound was purified by column chromatography (DCM/MeOH, 9.8:0.2). 0.046 g pure compound was obtained.

2.1.18. 1-(4-(5-methoxy-4-((4-(1-methyl-1*H*-indol-3-yl) pyrimidin-2-yl)amino)-2-nitrophenyl)piperazin-1-yl)prop -2-en-1-one (12). White powder; yield 54.10%. 1H-NMR (400.1759761 MHz, [C*D*Cl$_3$]: δ = 9.69 (s, 1H, NH); 8.40-8.39 (d, 1H, pyrimidine proton); 8.26 (s, 1H, Ar-H); 8.18-8.16 (d, 1H, pyrimidine proton); 7.60 (s, 1H, Ar-H); 7.40 (m, 1H, Ar-H); 7.32 (m, 2H, Ar-H); 7.26 (s, $CDCl_3$); 7.22-7.20 (d, 1H, Ar-H); 6.59 (m, 2H, Ar-H); 6.36-6.32 (d, 1H, Ar-H); 5.76-5.73 (d, 1H, Ar-H); 3.99 (s, 3H, O-Me); 3.94 (s, 3H, N-Me); 3.91 and 3.78 (d, 4H, C$\underline{H}_2$-Piperazine); 3.09 (m, 4H, C$\underline{H}_2$-Piperazine). 13C-NMR (125.76 MHz, [C*D*Cl$_3$): δ = 100.6243765 MHz; 165.59 (C=O), 161.95, 159.05, 157.88, 152.03, 142.12, 141.86, 138.07, 136.60, 132.99, 128.16, 127.37, 125.73, 122.45, 121.39, 120.64, 116.11, 113.58, 110.18, 108.66, 102.22, 56.19, 53.52, 51.94, 46.11, 42,29, 33.52, 29.73 (N-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{27}H_{27}N_7O_4$ [M^++1]: 514.00. HPLC (Schimadzu, LC 20-A): UV_{max}= 278 nm, AcCN:H_2O (90:10), Rt= 8.910 min, purity found 98.47%. HRMS (ESI): m/z calculated for [M^+-1]: 512.205176, Found: 512.20471.

2.1.19. Synthesis of N-(2-methoxy-5-nitro-4-substituted phenyl)-4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-amine derivatives (13-15) 1 Eq. **4a** was dissolved in 1mmol/10 mL THF and 1eq. 1.4M MeMgBr in THF and 1 eq. Corresponding amine derivatives were added at 0^{o}C. The mixture was stirred at 0^{o}C for 30 min and rt for 1h. Then it was refluxed 65-70^{o}C for overnight. The precipitates were filtered and washed with EtOH. The filtrates were evaporated to dryness to obtain crude compounds **13-15**, which were purified by column chromatography (H/E, 1:1).

2.1.20. *N*-(2-methoxy-5-nitro-4-(pyrrolidin-1-yl)phenyl)-4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-amine (13). Red powder; yield 65.70%. 1H-NMR (400.1759761 MHz, [D$_6$]DMSO): δ = 8.50 (s, 1H, NH); 8.38 (d, 1H, pyrimidine proton); 8.29 (s, 2H, Ar-H and pyrimidine proton); 8.03 (s, 1H, Ar-H); 7.48 (d, 1H, Ar-H); 7.23 (t, 1H, Ar-H); 7.14 (m, 1H, Ar-H); 7.08 (t, 1H, Ar-H); 6.54 (s, 1H, Ar-H); 3.92 (s, 3H, O-Me); 3.85 (s, 3H, N-Me); 3.19 (bs, 4H, C$\underline{H}_2$-Pyrrole); 1.93 (bs, 4H, C$\underline{H}_2$-Pyrrole). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 161.89, 159.52, 158.06, 152.76, 140.71, 138.06, 133.12, 129.67, 129.52, 127.50, 125.75, 122.50, 121.57, 120.69, 120.09, 117.25, 113.70, 110.10, 107.74, 96.25, 55.94, 51.16, 33.75, 25.91 (N-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{24}H_{24}N_6O_3$ [M^++1]: 445.77. HPLC (Schimadzu, LC 20-A): UV_{max}= 284 nm, AcCN:MeOH (50:50), Rt= 6.730 min, purity found 96.55%. HRMS (ESI): m/z calculated for [M^+ +1]: 445.199362, Found: 445.19699.

2.1.21. N^1-(3-isopropoxypropyl)-5-methoxy-N^4-(4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)-2-nitrobenzene-1,4-diamine (14). Red powder; yield 68.10%. 1H-NMR (400.1759761 MHz, [C*D*Cl$_3$]: δ = 8.73 (s, 1H, NH); 8.64 (t, 1H, pyrimidine proton); 8.30 (s, 1H, Ar-H); 8.29-8.27 (d, 1H, pyrimidine proton); 8.06 (s, 1H, Ar-H); 7.50-7.48 (d, 1H, Ar-H); 7.21 (t, 1H, Ar-H); 7.17-7.16 (d, 1H, Ar-H); 7.06 (t, 1H, Ar-H); 6.44 (s, 1H, Ar-H); 3.94 (s, 3H, O-Me); 3.85 (s, 3H, N-Me); 1.96 (s, 1H, C$\underline{H}$); 1.88 (t, 2H, CH_2); 1.20-1.13 (m, 4H, CH_2CH_2); 1.10-1.08 (s, 6H, $(CH_3)_2$. 13C-NMR (125.76 MHz,

[D$_6$]DMSO): δ = 100.6243765 MHz; 161.95, 159.28, 157.86, 156.40, 144.09, 138.26, 133.69, 126.17, 124.75, 122.09, 121.10, 120.61, 115.28, 113.86, 110.18, 107.81, 92.78, 72.03, 65.48, 56.11, 40.82, 33.80, 31.92, 29.23 (N-$\underline{C}H_3$), 21.98, 14.19. LC-MS (ESI): m/z for $C_{26}H_{29}N_6O_4$ [M^++2]: 491.77. HPLC (Schimadzu, LC 20-A): UV_{max}= 290 nm, AcCN:H_2O (90:10), Rt= 10.836 min, purity found 97.69%. HRMS (ESI): m/z calculated for [M^++2]: 491.241227, Found: 491.23825.

2.1.22. 2-methoxy-*N*^{4}-(2-methoxyethyl)-*N*^{1}-(4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)-5-nitrobenzene-1,4-diamine (15). Red powder; yield 73.80%. 1H-NMR (400.1759761 MHz, [C*D*Cl$_3$]: δ = 8.82 (s, 1H, NH); 8.52 (t, 1H, pyrimidine proton); 8.34 (s, 1H, Ar-H); 8.31-8.30 (d, 2H, pyrimidine proton and Ar-H); 7.96 (s, 1H, Ar-H); 7.51-7.49 (d, 1H, Ar-H); 7.23 (t, 1H, Ar-H); 7.19-7.18 (d, 1H, Ar-H); 7.10 (t, 1H, Ar-H); 6.53 (s, 1H, Ar-H); 3.97 (s, 3H, O-Me); 3.86 (s, 3H, N-Me); 3.66-3.62 (m, 4H, CH_2CH_2); 3.35 (s, 3H CH_3). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 155.70, 143.80, 137.23, 134.96, 124.55, 124.03, 122.26, 121.25, 119.74, 117.92, 115.91, 109.80, 106.03, 92.29, 69.97, 58.32, 55.51, 42.33, 32.76, 30.94, 28.68 (N-$\underline{C}H_3$), 22.02, 13.40. LC-MS (ESI): m/z for $C_{23}H_{24}N_6O_4$ [M^++2]: 450.08. HPLC (Schimadzu, LC 20-A): UV_{max}= 280 nm, AcCN (%100), Rt= 6.028 min, purity found 99.15%. HRMS (ESI): m/z calculated for [M^+ +1]: 449.194277, Found: 449.19199.

2.1.23. Synthesis of *N*-(4-fluoro-2-methoxy-phenyl)-4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-amine-3-substituted urea derivatives (16-17) 1 Eq. compound **5a** was dissolved in 1 mmol/10 mL THF and 1 eq. TEA and 1 eq. substituted PhIS derivatives were added respectively at 0^{o}C and stirred for 30 min and additional 1h at rt. Then the mixture was refluxed at 70-80^{o}C for 6h. The solvent was evaporated to dryness and washed with CH_2Cl_2. The precipitates of compounds **16** and **17** were obtained and purified by column chromatography (H/E,1:1).

2.1.24. 1-(4-(dimethylamino)phenyl)-3-(2-fluoro-4-methoxy-5-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl) amino)phenyl)urea (16). White powder; yield 82.40%. 1H-NMR (400.1759761 MHz, [C*D*Cl$_3$]: δ = 8.78 (d, 1H, NH); 8.57 (s, 1H, urea NH proton); 8.47 (s, 1H, urea NH proton); 8.29-8.26 (m, 2H, pyrimidine protons); 8.18 (s, 1H, Ar-H); 7.94 (s, 1H, Ar-H); 7.48-7.46 (d, 1H, Ar-H); 7.21-7.13 (m, 5H, Ar-H); 7.07 (t, 1H, Ar-H); 7.04 (d, 1H, Ar-H); 6.68-6.66 (d, 2H, Ar-H); 3.81 (s, 3H, O-Me); 3.75 (s, 3H, N-Me); 2.81 (s, 6H, N(CH_3)$_2$). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 162.26 (C=O), 160.64, 157.89, 153.30, 147.48, 147.05, 146.05, 133.76, 132.95, 130.24, 129.60, 125.88, 125.02, 122.53, 122.10, 121.46, 120.88, 120.02, 119.81, 115.66, 113.38, 112.95, 111.08, 107.73, 100.07, 95.07, 56.16, 40.95, 33.08 (N-$\underline{C}H_3$). LC-MS (ESI): m/z for $C_{29}H_{29}FN_7O_2$ [M^++1]: 526.10. HPLC (Schimadzu, LC 20-A): UV_{max}= 268 nm, AcCN:H_2O (90:10), Rt= 8.630 min, purity found 97.43%. HRMS (ESI): m/z calculated for [M^+ +1]: 526.237225, Found: 526.23480.

2.1.25. 1-(2-fluoro-4-methoxy-5-((4-(1-methyl-1*H*-indol-3-yl)pyrimidin-2-yl)amino)phenyl)-3-(naphthalen-1-yl)urea (17). White powder; yield 48.70%. 1H-NMR (400.1759761 MHz, [C*D*Cl$_3$]: δ = 9.07 (s, 1H, NH); 8.93-8.91 (d, 1H, urea NH proton); 8.84 (s, 1H, pyrimidine proton); 8.49 (s, 1H, pyrimidine proton); 8.31-8.29 (m, 2H, Ar-H); 8.17-8.15 (d, 1H, Ar-H); 7.95 (m, 3H, Ar-H); 7.65-7.63 (d, 1H, Ar-H); 7.56 (m, 2H, Ar-H); 7.45 (m, 2H, Ar-H); 7.21-7.19 (d, 1H, Ar-H); 7-14-7.11 (m, 3H, Ar-H); 3.84 (s, 3H, O-Me); 3.64 (s, 3H, N-Me). 13C-NMR (125.76 MHz, [D$_6$]DMSO): δ = 100.6243765 MHz; 162.27 (C=O), 160.54, 158.04, 153.54, 150.74, 149.71, 147.92, 145.91, 138.11, 134.77, 134.27, 133.98, 128.95, 126.69, 126.41, 125.81, 123.60, 123.40, 122.03, 121.38, 119.80, 118.39, 115.41, 110.89, 107.70, 100.36, 100.05, 60.26, 57.23, 33.46 (N-$\underline{C}H_3$), 14.73. LC-MS (ESI): m/z for $C_{31}H_{25}FN_6O_2$ [M^++1]: 533.00. HPLC (Schimadzu, LC 20-A): UV_{max}= 280 nm, AcCN:H_2O (90:10), Rt= 7.994 min, purity found 97.52%. HRMS (ESI): m/z calculated for [M^++1]: 533.210676, Found: 533.20782.

2.2. Biological Assay

2.2.1. EGFR kinase inhibition In 384-well, white flat bottom microplates, 1 µl of inhibitors or 1 µl 5% DMSO, 2 µl of EGFR protein solution and 2 µl of ATP/substrate (10 µM ATP and 2.5 µM PolyE_4Y_1) mixture were added, respectively. The mixture was incubated for 60 minutes at room temperature. To terminate the kinase reaction, 5 µl of ADP-Glo reagent (Promega) was added to the mixture and incubated for 40 minutes at room temperature. At the end of the reaction 10 µl of Kinase detection reagent (Kinase Detection Reagent, Promega) was added to the mixture, after incubation for 30 minutes at room temperature, a bioluminescence signal was detected in a microplate reader by arranging integration time as 0.5-1 second (Spectra-Max i3, Molecular Devices). The assays were performed in triplicate.

2.2.2. SRC kinase inhibitor assay In 384-well black microplates, 1 µl of 200 µM solution of the tested substances and 2 µl of ATP/substrate mixture were added onto 2 µl of SRC kinase solution. The mixture was incubated for 60 minutes at room temperature. To terminate the kinase reaction, 5 µl of ADP-Glo reagent (Promega) was added to the mixture and incubated at room temperature for 30 minutes. At the end of the period, 10 µl of Kinase detection reagent (Kinase Detection Reagent, Promega) was added to the mixture, after incubation for 30 minutes at room temperature, the bioluminescence signal was detected in the microplate reader (SpectraMax i3, Molecular Devices). **Notes on the protocol:** Assay buffer solution: 40 mM Tris, pH 7.5, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 50 µM DTT. 200 µM Stock solutions, enzyme, ATP and substrate solutions of the tested substances were prepared using assay buffer solution. Incubation conditions: room temperature, on orbital shaker (100 rpm/min). The kinase reaction contains 4 ng SRC kinase, 1 µg substrate: KVEKIGEGTYGVVYK, 100 µM ATP, 40 µM test substance and 2% DMSO.

2.2.3. Cytotoxicity Tests The cytotoxic effects of the synthesized compounds against MCF-7 (human breast cancer, ATCC® HTB-22™), A549 (human lung cancer, ATCC® CCL-185), PC3 (human prostate cancer, ATCC® CRL-1435), HEK293 (human embryonic kidney, ATCC® CRL-1573) cell lines were tested in this study.

2.2.3.1. Cell Lines and Culture Conditions "RPMI 1640 (with L-Glutamine with Phenol Red and without HEPES)" medium was used for A549 cell line and Dulbecco's Modified Eagle's Medium / High Glucose"

(DMEM/High, Gibco 41966) was used for MCF7, PC3 and HEK293 cell line. The medium was prepared by adding 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) antibiotic-antimycotic solution to sterile medium solution [18].

2.2.3.2. MTT Assay For cytotoxicity analysis, the following steps were performed, respectively [19]. First, 200 µl of 5.10^4 cells/well MCF7, 3.10^4 cells/well A549 cell suspensions were placed in each well of 96-well culture dishes. The culture dish was incubated in an incubator at 37ºC providing 5% CO_2. The effects at different concentrations of compounds were tested. The experimental group was kept in the incubator (37ºC, 5% CO_2) by applying 200 µl of media containing agents in different concentrations to the cells, and only 200 µl of the medium was used as the control group. At the end of the indicated incubation period, the medium on the cells was removed. 30 µl of MTT stock solution (0.5 mg/ml in PBS) was added and the mixture was kept in the incubator for 4 hours. 150 µl of isopropanol was added to dissolve the formazan crystals. The culture dish was shaken at room temperature on a shaker (Heidolph Unimax 1010) for solution homogeneity. The absorbance of the formed colored product at a wavelength of 570 nm was measured in a microplate reader (Spectramax i3). The results were evaluated according to the following equation. % Viability = (Experimental absorbance / Control absorbance) x 100. Experiments were carried out in 6 repetitions and 3 different periods each time.

2.2.4. Analysis of Apoptosis

2.2.4.1. Caspase-3 and Caspase-8 activity assay The experiments were performed with a commercial ELISA Kit (Elabscience, E-CK-A311, Wuhan, China) according to the manufacturer's instructions. PC3 cells were treated with active compounds. Upon incubation for 24h, cells were collected and washed with PBS (pH 7.4). Then, cells were homogenized in a 1X lysis buffer (50 mM HEPES, pH7.4, 5 nM CHAPS, 5 mM DTT). The lysate was centrifuged at 16000 x g for 20 minutes at 4°C and the supernatants were collected. The reaction was initiated by adding caspase-3 or caspase-8 substrates to each well. The microplate was incubated at 37°C for 90 minutes. The concentration of the released substrate was calculated from the absorbance values at 405 nm. All experiments were performed in triplicate [20-23].

2.2.4.2. Determination of Bcl-2 and Bax levels To determine the expression levels of apoptosis markers Bcl-2 and Bax, the compounds were first applied to PC3 cells and collected. Bcl-2 and Bax levels were estimated using commercially available ELISA kits according to the manufacturer's protocol (SunRed, 202211, Human bax; DZE201126587, Human Bcl-2; DZE201121833) [24]. All the experiments were repeated at least three times.

2.2.5. Molecular Docking Application Comparative enzyme interaction studies with osimertinib and dasatinib were performed using the Autodock vina program. For this analysis EGFR enzyme (PDB ID: 4ZAU) and SRC enzyme (PDB ID: 3QLG) were used to determine interaction by evaluation of their binding energies, RMSD (Root Mean Square Deviation; the indicator of the distance of the molecule to the effect site) values and hydrogen bonding capacities [25]. The three-dimensional structure of the compounds was drawn in ChemDraw Ultra 15.9; then, energy minimizations were done by ChemBio Ultra 15.9. Then Openbabel (version 13.1.1) was used to pdf files of compounds to pdbqt files. All water molecules were removed from the proteins, and polar hydrogens were added. The grid box was adjusted with a volumetric space of 30x30x30 for corresponding proteins. Docking analysis was visualized with MGLtools 1.5.6 version, and 10 different binding results between protein and ligand were listed. The best-interacted ligands were determined. PMW 1.5.6 molecular modelling program was used to obtain the 3D protein-ligand complex images.

3. RESULTS AND DISCUSSION

3.1. Chemistry

Compounds **1-17** are structurally similar to osimertinib and have been synthesized by using published [26, 27], and novel methods developed in our laboratory (Scheme 1 and 2). The pyrimidine substituted 1-methyl-1*H*-indole intermediate compounds (**1a** and **1b**) were synthesized by condensation of 2,4-dichloropyridine or 4,6-dichloro-2-methylsulphonyl pyrimidine and N-methyl indole and indole, respectively (Scheme 1). Two different reagents $FeCl_3$ [26], MeMgBr [27] were used for these reactions following the literature methods, however, $AlCl_3$ was used the first time for this reaction in our laboratory and the yield of this reaction was found higher than the previously reported reactions. p-Nitro anilin was added to **1a** and **1b** in the presence of p-toluene sulfonic acid to obtain intermediates **2a**, and **2b** (Scheme 1). To reduce the nitro group, Fe/NH_4Cl and $Pd\text{-}C/HCOONH_4$ were used yielding the amine intermediates (**3a**, and **3b,** Scheme 1). Compound **4a** was obtained with the reaction of **1a** and 4-fluoro-2-methoxy-3-nitro aniline (Scheme 2). Compound **4a** was reacted with piperazine in the presence of K_2CO_3/DMF to give intermediate **4b**. The resulting compound **12** was obtained from the reaction of **4b** with acryloyl chloride in CH_2Cl_2/TEA. The resulting urea derivatives (**6-11**, Scheme 1; **16-17**, Scheme 2) were obtained with the reaction of amine derivatives (**3a**, **3b** and **5a**) and substituted phenyl isocyanates in CH_2Cl_2. A Grignard reagent (MeMgBr in THF) was used for the first time in our laboratory to substitute the F group with the related amine groups to synthesize compounds **13-15** (Scheme 2). The yield of this reaction was much higher (65-70-73.80%) than the reported method in literature, which K_2CO_3 in DMF was used as a reagent [26, 27]. Therefore, it was chosen for the synthesis of compounds **13-15**. The structure of all the final compounds was confirmed using 1H NMR, 13C NMR, LC-MS and HRMS. The 13C NMR spectra of all compounds show a peak at 170.70-161.65 ppm, which indicates the presence of the carbonyl in the urea group. In the 1H-NMR spectra, NH protons in the indole ring were seen as a singlet at 9.69-8.78 ppm. In all the urea containing compounds, NH protons were seen as singlets at 8.82-8.47 ppm. Singlet peaks

at 8.59-8.09 ppm have been found in compounds which have a pyrimidine group. Indole N-methyl protons, OCH_3 and SCH_3 protons were observed at 3.94-3.64 ppm, 3.97-3.84 ppm and 2.55-2.48 ppm, respectively.

The results of mass spectroscopic analysis also confirmed the structures deduced. The purity of the samples was determined by high-performance liquid chromatography (HPLC), conducted on a Shimadzu LC- 20AT series system with a C18 column (4.6 mm × 250 mm, 5 µm). The samples were eluted with three different solvent systems 90:10 acetonitrile/H_2O, 100% AcCN and 50:50 AcCN/MeOH at a flow rate of 1.0 mL/min, and was detected at a wavelength of 254 nm and maximum wavelengths of compounds. The purity of all biologically evaluated compounds is reported greater than 95%.

Scheme 1. Synthesis of compounds 6-11.

Scheme 2. Synthesis of compounds 12-17.

3.2. Biological Activities

3.2.1. EGFR kinase inhibition

The ADP-Glo^{TM} kinase assay determines kinase activity based on the quantification of the amount of ADP produced during a kinase reaction. In the present study, newly synthesized compounds were screened for their capability of inhibition on the tyrosine kinase activity of EGFR by comparing them with osimertinib. The ATP amount was measured by the luminescence using SpectraMax i3X Multimode Microplate Reader. A kinase reaction was started in the first step with EGFR and ATP reaction. The amounts of both EGFR and ATP were determined in the preliminary optimization assays. The amount of the EGFR and PolyE4Y1 substrate was used based on the recommendations of the kinase procedure [28]. In the second step, an equal volume of ADP-GloTM reagent was added to terminate the kinase reaction and deplete the remaining ATP. In the third and final step, a kinase detection reagent was added to convert the ADP produced during the kinase reaction to ATP and to determine the amount of newly synthesized ATP through a luciferase/luciferin reaction. The relative inhibition (%) of compounds were calculated compared to the control with no compounds. For screening with a small amount of enzyme and low ATP-to-ADP conversion, assay conditions were optimized to generate a high signal-to-background ratio (SB). We defined SB10 as the amount of enzyme needed to reach out signal-to-background ratio of 10. The effectiveness of inhibitors was determined by comparing the enzyme activities in the presence of the inhibitors to the maximum enzyme activity, and the results are presented in Table **1**. The data of EGFR-TK1 inhibitory activity of 12 compounds **6-17**, osimertinib and dasatinib, are shown in Fig. **3**.

Fig. (3). Kinase inhibitory activity screen of compounds towards EGFR-TK at 40 µM (**** p< 0.0001, ***p < 0.001, **p< 0.001, *p< 0.05)

Among the newly developed compounds, compound **16** showed the maximum inhibitory activity against EGFR with 86.40% inhibition, a value that is comparable to osimertinib (99.60%). However, none of the other newly developed compounds showed good potency as the reference compound osimertinib. Based on this finding, compound **16** was selected for further evaluation to determine its half-maximal inhibitory concentration (IC_{50}) value against EGFR kinase activity. The result, as presented in Fig. **4**, showed that the IC_{50} of compound **16** against EGFR-TK was 1.026±0.069 µM.

Fig. (4). Kinase inhibitory activity of compound **16** against EGFR-TK. Data represented as means ±SEM from triplicate independent experiments.

3.2.2. SRC kinase inhibition

ADP-Glo Kinase Assay (Promega) and SRC Kinase Enzyme System (Promega) kits were used to examine the SRC kinase enzyme inhibition properties of the synthesized compounds [29]. The newly synthesized compounds **6-17** were tested against the SRC protein kinase, which is another extensively studied kinase family protein. The percent inhibitory activities of the compounds at 40 µM concentration are shown in Table **1** and Fig. **5**.

Fig. (5). Kinase inhibitory activity screen of newly synthesized compounds towards SRC kinase at 40 µM (**** p< 0.0001, ***p < 0.001, **p< 0.001, *p< 0.05).

The data in Table **1** show that the compounds **6-13** and **16** have good inhibition (74.62-91.30%) against SRC kinase in comparison to the reference compound dasatinib (99.50%). Among them, the urea derivatives (**6-11**) presented stronger inhibition than non-urea derivatives. As seen in Table **1**, compound **16** exhibited the highest inhibitory activity of 91.30% against SRC kinase and its half-maximal inhibitory concentration (IC_{50}) value was calculated as 0.002027±0.000404 µM presented in Fig. **6**.

Fig. (6). Kinase inhibitory activity of compound **16** against SRC-TK. Data represented as means ±SEM from triplicate independent experiments.

Table 1. Inhibitory effects of synthesized compounds **6-17** against EGFR and SRC kinases.

3.2.3. Cytotoxic properties of the compounds

In order to determine the cytotoxic effects of the newly developed 12 compounds on normal (HEK293) cell lines, compounds at different concentrations (1, 5, 10, 20, 40, 50

μM) were added to 24-hour cultures. The % cell viability upon compound treatment on HEK293 are shown in Fig. **7**. The results showed that our compounds have less cytotoxic properties on normal cell lines compared to reference drugs cisplatin, osimertinib and dasatinib.

Fig. (7). The cytotoxicity of compounds **6-17** and the reference compounds cisplatin, dasatinib and osimertinib were evaluated using HEK293 cells. The cells were treated with the indicated compound for 24 hours (*P<0.05, **P<0.01, ***P<0.001, vertical bars show standard deviation values).

The cytotoxic effects of the synthesized compounds were also determined on MCF7, A549 and PC3 cancer cell lines, the viability tests were performed with 6x3 repetitions. The control group in the experiments was determined as the group without any agent and was considered 100% alive. Osimertinib, cisplatin and dasatinib were used as reference compounds for comparison.

One-way ANOVA (one-way ANOVA) test was applied to analyze the consistency between replications within the experimental groups to the obtained data. The significance of the groups compared to the control group was evaluated with Dunnett's and Tukey's test. The application concentrations and times of the agents were decided based on the findings obtained from the literature review [18, 19]. The fifty percent of concentration values (IC_{50}) of the agents were calculated by measuring the half-cell viability. To calculate the IC_{50} value of the agents, the sigmoidal dose-response curve ("sigmoidal dose-response variable slope") was calculated using the GraphPad Prism® 7.0 program.

To determine the cytotoxic effects of the compounds **6-17** on MCF7, A549 and PC3 cancer cell lines, different concentrations (1, 5, 10, 20, 40, 50 μM) of compounds were used. The medium of the control group, in which no substance was applied, was also refreshed. After a 24-hour incubation period, data were obtained by performing the MTT test. The %viability results of the experiments are given in Fig. **8**, Fig. **9** and Fig. **10**, respectively.

Fig. (8). The viability effect of 12 compounds (50 μM) and reference compounds cisplatin and osimertinib were evaluated on MCF7 cells for 24 hours (*P<0.05, **P<0.01, ***P<0.001, vertical bars show standard deviation values).

Fig. (9). The viability effect of 12 compounds (50 μM) and reference compounds cisplatin and osimertinib were evaluated on A549 cells for 24 hours (*P<0.05, **P<0.01, ***P<0.001, vertical bars show standard deviation values).

Fig. (10). The viability effect of 12 compounds (50 μM) and reference compounds cisplatin and osimertinib were evaluated on PC3 cells for 24 hours (*P<0.05, **P<0.01, ***P<0.001, vertical bars show standard deviation values).

As seen in Fig. **8** and **9**, most of the compounds cause more than 50% death of MCF7 and A549 cells. Compounds **6**, **7**, **9**, **12** and **13**, showed weak cytotoxic properties against both cancer cell lines. All compounds have better cytotoxic properties on PC3 cells, compounds **16** and **17** especially showed higher cytotoxic properties compared to reference compounds (Fig. **10**). The IC_{50} values of all compounds were calculated and shown in Table **2**.

Among all the compounds, **16** was found more specifically active against lung cancer cells (IC_{50}=19.18 ±0.47) compared to other compounds, and **16** also has good growth inhibition against prostate cancer cells (IC_{50}=18.19 ±0.95) and breast cancer cells (IC_{50}=24.67 ±0.98). Compound **17** showed the highest cytotoxic property (IC_{50}=17.92 ±1.02) against the prostate cancer cells. These results showed that compounds **16** and **17** are promising compounds in order to develop novel therapeutics against prostate and lung cancers. These compounds have urea substitutions at the third position of aniline ring differing from osimertinib structure. The results provide completely different profiles to obtain novel active compounds having osimertinib core structure against lung cancer and also gave hope to obtain novel compounds against prostate cancer. For this reason, structurally similar novel compounds will be designed in the future to obtain kinase inhibitors with better activity.

Table 2. IC_{50} values of 12 synthesized and reference compounds osimertinib, dasatinib and cisplatin on A549, MCF7, PC3, HEK293 cell lines

3.2.4. Effects of active compounds on apoptotic marker levels

3.2.4.1. Caspase-3 activity assay

The present study aimed to investigate the potential of synthesized compounds to induce apoptosis in PC3 cells by activating effector caspase-3, which plays a crucial role in the apoptosis pathway. Caspase-3 is activated by the stimulation of certain enzymes responsible for the initiation of caspase activation [21]. In this study, induction of apoptosis by compounds in PC3 cells was tested by ELISA method. Caspase-8 is known as the key activator of the apoptosis mechanism [30], and studies have shown that caspase-8 activation is associated with the stimulation of caspase-3 [31]. Therefore, in this study, both caspase-3 and caspase-8 enzyme activities of the synthesized compounds were evaluated to investigate their potential for inducing apoptosis. The balance between Bax and Bcl-2 levels is critical for the regulation of apoptosis, and a shift in this balance towards Bax can result in apoptosis [24]. Therefore, the observed increase in Bax levels and decrease in Bcl-2 levels induced by compound **16** may be responsible for the observed apoptotic effects in prostate tumor cells. These findings support the potential use of compound **16** as a therapeutic agent for prostate cancer treatment by targeting the Bax/Bcl-2 balance. Further studies are needed to confirm these results and explore the underlying mechanisms.

The results revealed that compound **16** significantly increased caspase-3 and caspase-8 levels by approximately 8- and 6-fold, respectively, compared to the control group (Table **3** and Fig. **11**). These findings suggest that compound **16** has the potential to induce apoptosis in PC3 cells through the activation of caspase-3 and caspase-8.

Furthermore, the significant increase in caspase-3 and caspase-8 levels observed in this study indicates the potential therapeutic significance of compound **16** in treating cancer.

Overall, the present study provides insights into the mechanism of action of synthesized compounds in inducing apoptosis and highlights the importance of caspase-3 and caspase-8 as potential targets for developing novel therapeutics against cancer. However, further studies are required to confirm the efficacy and safety of these compounds with *in vivo* studies.

Fig. (11). Caspase 3 and caspase 8 levels in PC3 cells incubated with compound **16** for 48h. (results are given as mean ± SD, n = 3, statistically significant in comparison with the control, untreated PC3 cells), ***$P < 0.0001$)

3.2.4.2. Effects of Bcl-2 and Bax proteins

Apoptosis is an important mechanism for preventing uncontrolled cell proliferation and the development of cancer. Many proteins have a role in the regulation of apoptosis, including Bax and Bcl-2 proteins, members of the Bcl-2 family. Bax is an inducer of apoptosis, while Bcl-2 is a suppressor of apoptosis [24].

In this study, the effects of synthesized compounds on Bax and Bcl-2 levels were evaluated. The results showed that compound **16** increased the level of Bax by 5.67-fold and decreased the level of Bcl-2 by 2.31-fold, compared to the control group (Table 3 and Fig. **12**). These findings suggest that compound **16** may promote apoptosis in prostate tumor cells by increasing the level of Bax and decreasing the level of Bcl-2.

Fig. (12). Bcl-2 and Bax levels in PC3 cells incubated with compound **16** for 48h. (results are given as mean ± SD, n = 3, statistically significant in comparison with the control, untreated PC3 cells), *$P < 0.05$, ***$P < 0.0001$).

Table 3. Caspase-3, Bcl-2 and Bax levels of compound **16** in PC3 cells.

3.2.5. Molecular Docking

Docking results showed that the binding energies of the EGFR enzyme interaction, the hydrogen bonding capacities, and the RMSD (Root Mean Square Deviation; an indicator of the distance of the molecule to the effect site) values of the compounds are close to osimertinib. Compounds **6** and **14** showed the covalent bond like osimertinib did with CYS797 of EGFR. The majority of other compounds made H-bonds with MET793 as osimertinib did [25]. The receptor interactions of each group of compounds, which are structurally similar to osimertinib are given in Fig. **13**. Overlapping representation was preferred in order to understand that the compounds interact similarly to the reference compound, osimertinib. As seen in Fig. **13 (A)**, compound **16** was bound to the same binding site as osimertinib by making H bond with MET793, but it interacted in different conformation. Osimertinib-like bending conformation (U-shaped) was found in compounds (**13-15**) structurally similar to osimertinib are shown in Fig. **13 (B)**. However, they did not present any hydrogen bond capabilities.

In addition, SRC enzyme interaction of compounds was evaluated by analyzing binding energies, RMSD values, hydrogen-bonding capacity, and the results were compared with dasatinib. It has been determined that the compounds have binding energy values close to dasatinib [15]. Compounds **8** and **10** showed the best interaction with MET341 amino acid, which is important in SRC interaction (Fig. **14A**). In addition, compound **16** showed good SRC enzyme interaction and also found very active in enzyme inhibition study (Fig. **14B**). However, it has slightly different binding interaction than dasatinib made.

Fig. (13). (A) Compound **16** (makes H bond with Met793) and osimertinib are shown in EGFR binding site; (B) The overlapped structures of compounds **13** (green), **14** (red), **15** (blue) and osimertinib (makes H bond with CYS797, ball and stick) are shown in EGFR binding site.

Fig. (14). (A) Compound **8** (green), **10** (red) and dasatinib (ball and stick) make H bond with Met341 are shown in SRC binding site; (B). The overlapped structures of compound **16** (cyan) and dasatinib (ball and stick) make H bond with Met341 are shown in SRC binding site.

CONCLUSION

The development of targeted cancer therapies has revolutionized the treatment of cancer, and the identification of novel compounds with dual action against EGFR and SRC enzymes is an important area of research in the field of cancer therapeutics. This study combined computational and experimental techniques to identify novel inhibitors based on indole derivatives, with the aim of developing alternative therapies to osimertinib with dual action. These compounds included an osimertinib-like structure and urea group on the aromatic ring. Molecular docking studies were used to evaluate the interaction of the compounds with the receptors, and the SAR of the compounds was investigated through in vitro enzyme inhibition, cell culture tests. The results showed that out of the **12** novel synthesized compounds, compound **16** exhibited the best inhibitory activity against EGFR-TK, which underscores the importance of small

changes in substituents and their impact on the activity of the compound. Furthermore, compound **16** was also found to have a good inhibition profile against SRC, indicating that it has dual kinase inhibition properties. This is a significant finding, as compounds that inhibit multiple targets are often more effective in therapy and can help to prevent the development of resistance. The molecular docking studies revealed that compound **16** binds to the same binding site as osimertinib, but with a different conformation. All compounds were found to bind to the active site of SRC kinase, similar to the reference compound dasatinib, conforming strong SRC enzyme inhibition profiles. Although compounds **6-17** are structurally similar to osimertinib, except compound **16**, none of them showed strong inhibition as osimertinib against EGFR. However, most urea containing compounds exhibited good inhibitory potencies against SRC kinase. This indicates that urea group plays important role for SRC enzyme interaction. The cytotoxicity assays showed that our compound is less toxic in normal cell lines in comparison to reference compounds. The cytotoxicity assays revealed that compounds **16** and **17** demonstrated strong cytotoxic activity against lung and prostate cancer cells, with lower toxicity in normal cell lines compared to reference compounds. This is an important finding since the development of more effective and less toxic cancer therapies is a critical area of research. The results of this study suggest that the osimertinib core structure with urea modification may be a promising avenue for the development of new cancer therapeutics. In conclusion, this study provides promising results in the development of novel therapeutics against lung and prostate cancers, with compound **16** identified as a potential starting compound with dual activity against EGFR and SRC kinases. The findings highlight the importance of understanding the SAR of compounds and the impact of even small changes in substituents on activity, which could lead to the development of more effective and less toxic cancer therapies. Further studies are warranted to investigate the in vivo efficacy of these compounds and their potential for clinical development.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by ethics committee of Biruni University, Istanbul, Turkey.

HUMAN AND ANIMAL RIGHTS

Neither humans nor animals were used in these studies.

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

The original documents given in the study are included in the manuscript. Detailed contributions will be provided upon request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for rights and financial issues.

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