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Research article

Synthesis, molecular modeling and bioevaluation of new benzimidazole derivatives as dual KSP (Kinesin Spindle Protein) and Aurora A Kinase inhibitors for antitumor activity

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Key words: Benzimidazole, Human cancer cells, Aurora A Kinase, KSP inhibition, Dioxopyrimido [2, 1-b] [1,3] Thiazine

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Abstract

A series of dioxopyrimido[2,1-b][1,3]thiazine derivatives (3a-f,6a-e) has been synthesized and their biological activities were also evaluated as potential dual KSP and Aurora A Kinase inhibitors. Bioassay tests demonstrated that most of the compounds exhibited substantial broad-spectrum antitumor activity against the seven cancer cell lines (HepG2, SNB19, HT29, K562, A549-ATCC, MDA-MB-435 and SKOV3). Moreover, all the title compounds were assayed for as dual KSP and Aurora A kinase inhibition using the MT-activated ATPase assay. Compound (6b) displayed the most potent anticancer activities, which was comparable to the positive control and Molecular modeling has been done for this compound. The results may be used for developing a new class of inhibitors having a dual roles, KSP inhibition and Aurora-A kinase inhibition for the treatment of cancer.

Introduction

One of the most important processes on the design of cancer is mitosis and its related process. So that, a proven effective strategy in cancer treatment has been to interpose with the abnormal progression of mitosis in order to stopping the cell cycle in mitosis phase and to induce apoptosis in tumor cells. Lately, much attention has been focused on the new mitotic targets such as kinesin motor proteins, polo-like kinases (PLKs), Aurora kinases.

Kinesin spindle protein (Hs Eg5 or KSP) has lately considered as a key target for selective antimitotic cancer therapy. KSP is unambiguously expressed in dividing cells, and its motor activity has been shown to be required in mitosis step for the establishment of a functional, bipolar mitotic spinder [1] So far no role for KSP outside mitosis has been found.

A band of structurally diverse KSP inhibitors have been shown to cause cell cycle arrest, differentiation and/or apoptosis of tumor cells. During the last 10 years, the first generation of KSP inhibitors, for example monastrol, [2] ispinesib [3] CK0106023[4] and ARRY-520[5] have been prepared, and many of these compounds are now go through clinical trials [6]. One of the first specific KSP inhibitor is Manstrol (Figure 1) that are acting by non-competitive reaction with microtubules (MT) through allosteric site binding. Recently, Fu *et al* has been prepared CPUYL064 that has the capability to both KSP ATPase inhibitory and anti-proliferation of hepetocelluar carcinoma cell line HepG2 cell more than Mansterol [7].

Aurora kinases are playing vital and important role in regulating many process that are crucial to mitosis, for example Aurora A kinase that are over expressed in abroad range of primary tumor and healthy cell growth and proliferation. It was reported that Aurora A kinase are over expressed in many of human tumor cell lines so that, the inhibition of Aurora- A kinase, by small molecules has attracted many of scientist in oncology drug discovery [8]. These lead to discover many of lead compounds such as compound **1a**[9] and VX-680[11] (Figure 1). By analysis the crystal structure AbI/ VX-680 they found that aminomethyl pyrazole group is responsible for anchoring to the hinge region with three ATP-type hydrogen bonds.[12] The H-bonding interactions of inhibitors with hinge region residues are observed in most of the inhibitor-Aurora complex structures and this interaction are essential for maintaining activity [8].

In viewing of KSP and Aurora A kinase are two key enzymes in the mitotic system [13] and KSP is one of Aurora A kinase substrates, so that we suggest to design a new kind of dual inhibitors by mixing of KSP inhibitors scaffold with Aurora-A kinase inhibitor's fragments.

The dual targets of KSP and Aurora-A kinase may be lead to more synergism effect by blocking multiple key components of the mitotic system compared to separate agents. With the goal of identifying such a new anti-cancer strategy, we

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devised a strategy to derivatize CPUYL064 in intensive a way to facilitate its binding to Aurora-A kinase while maintaining its KSP inhibitory activity.

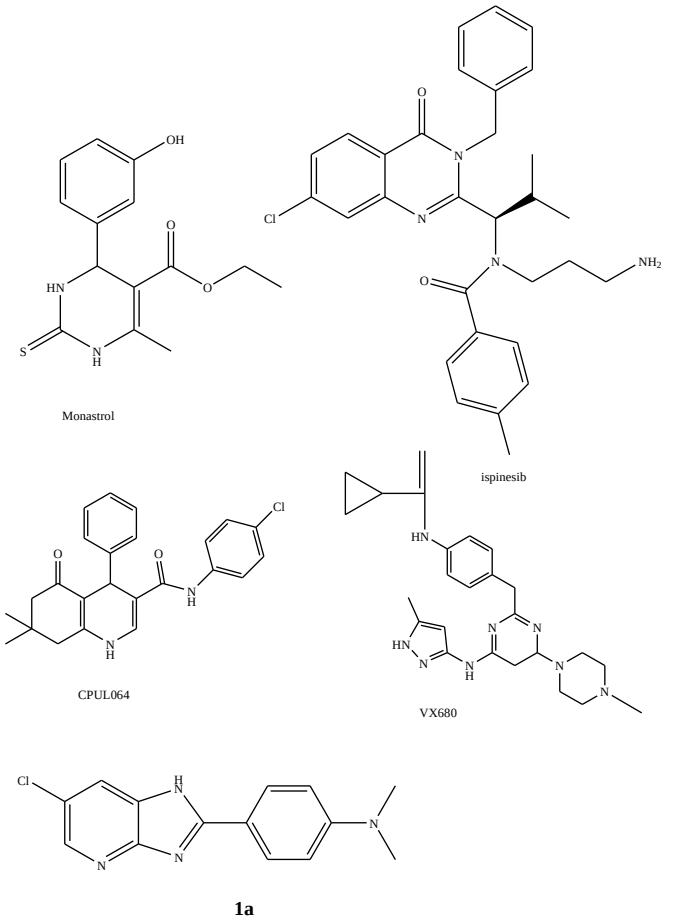


Figure 1. Structure of KSP and Aurora kinase inhibitors.

Based on the pharmacophore the binding mode analysis for KSP inhibitors, [14,15] we mapped the structure of CPUYL064 with the pharmacophore of KSP inhibitor, and we found that its consists of four chemical features (Figure 2): one aromatic ring (part 1), one hydrophobic group (part 2), a side chain (part 3) and a flexible heterocyclic ring (part 4). Substituted thiobarbturate are common feature of Aurora-A kinase inhibitor scaffolds which formatted three ATP-type hydrogen bonds to hinge residues [8,16-18].

In order to combing Aurora-A kinase inhibitory functionality into the KSP scaffold, our strategy is to apply hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide moiety as the core structure in which the thiazin ring was supposed to carry out as a hinge region-interacting group while the dihydropyridine ring maintained in CPUYL064 as a KSP scaffold [19-21].

Thus, the structure of CPUYL064 was modified by replacing the dihydropyridine group with hexahydro pyrimido[2,1-b][1,3]thiazine moiety and altering the benzene ring with different heterocyclic systems to afford the target compounds.

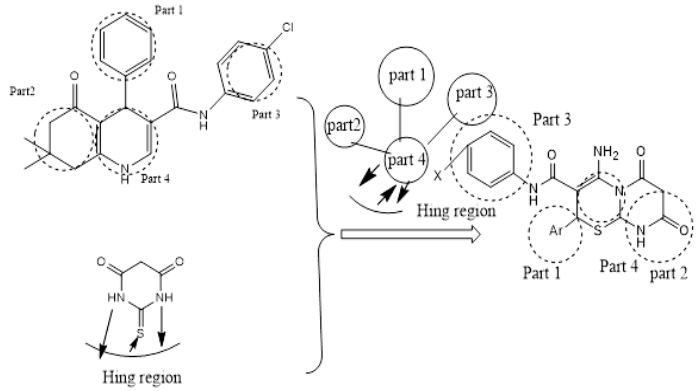


Figure 2. Modification of structure of CPuyL064 by replacing the dihydropyridine group with hexahydropyrimido [2,1-b][1,3] thiazine moiety.

Experimental

Chemistry

All chemicals and reagents used in the current study were of analytical grade. The reactions were monitored by thin layer chromatography (TLC) on Merck pre-coated silica GF254 plates.

Melting points (uncorrected) were determined on a XT4MP apparatus (Taike Corp., Beijing, China). ESI mass spectra were obtained on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra were collected on a Bruker DPX300 spectrometer at room temperature with TMS and solvent signals allotted as internal standards. Chemical shifts are reported in (δ) ppm. Elemental analyses were performed on a CHN-O-Rapid instrument, and were within 0.4% of the theoretical values.

Preparation of 4-(5-nitro-1H-benzo[d]imidazol-2-yl) aniline (1)

A mixture of 4-aminobenzoic acid (0.009 mol) and 4-nitrobenzene-1, 2-diamine (0.006 mol) was heated under reflux in hot syrupy o-phosphoric acid (50–60 mL) at 180–200 °C for 4 h. The reaction mixture was then partially cooled (to approx. 50 °C), poured on to crush ice and neutralized with 10% NaOH solution. The precipitated product was collected by vacuum filtration, washed with excess 10 % NaOH solution, and then dried and recrystallized from ethanol.

Yield 90%, m.p. 209–211°C. ^{1}H-NMR (DMSO-d_6, 500 MHz, δ ppm) 5,84 (br., 1H,NH benzimidazole, D_2O exchangeable) ; 6.67–7.82 (m, 7H, aromatic rings);13.10 (s,2H, NH_2aminophenyl, D_2O exchangeable). MS: m/z 254 .IR spectrum (KBr, cm^{-1}) υmax: υ 3470 (NH benzimidazole); υ 3374, 3206 (NH_2aminophenyl); υ 3058 (CH arom); υ 1631 (C=Nbenzimidazole);. Anal. calcd. for $C_{13}H_{10}N_4O_2$M.W. 254: C, 61.41; H, 3.96; N, 22.04. Found: C, 61.22; H, 4.11; N, 22.43.

Preparation of 2-cyano-N-(4-(5-nitro-1H-benzo[d] imidazol-2-yl) phenyl)acetamide (2)

A mixture of 4-(5-nitro-1H-benzo[d]imidazol-2-yl) aniline (1) (0.001mol) and excess ethylcyanoacetate in absolute ethanol (20mL) was boiled under reflux for 3h. The product separated upon storing the reaction mixture to cool at room temperature. Then product was collected and crystallized from ethanol.

Yield 74%, m.p. >300°C. ^{1}H-NMR (DMSO-*d*$_6$, 500 MHz, δ ppm) 3.54 (s, 2H, CH_2); 5.84 (br., 1H,NH benzimidazole, D_2O exchangeable);7.29–7.63 (m, 7H, aromatic rings).MS: m/z 321. The IR spectrum (KBr, cm^{-1}) υ max:υ 3405 (NH benzimidazole); υ 3043 (CH arom); υ 2268 (C≡Nbenzimidazole); υ1689 (C=O); υ 1625 (C=N).Anal. calcd. For $C_{16}H_{11}N_5O_3$, M.W. 321: C, 59.81; H, 3.45; N, 21.80. Found: C, 59.651; H, 3.76; N, 21.92.

General procedure for the synthesis of 4-imino-2-(substituted)-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-6,8-dioxo-4,6,7,8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3a-f)

An equimolar amount (0.01mol) mixture of cyanoacetamide compound (2) with the appropriate aldehyde and thiobarbturic acid in glacial acetic acid (50mL) was heated under reflux for appropriate time. The reaction mixture cooled at room temperature for 24h. Then the product was filtered off and crystallized from methanol.

4-amino-2-(3-methoxyphenyl)-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-6,8-dioxo-2,6,7,8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3a)

Yield 70%, m.p..>300°C.^{1}H-NMR (500MHz, DMSO-*d*$_6$, δ ppm): δ 1.87 (s, 2H ,CH_2 of pyrimidine),3.85 (s, 3H, OCH_3); 5.86(s,3H, of 3-methoxyphenyl ring); 7.03–7.82 (m, 10H, aromatic rings); 8.31 (s, 1H, NH benzimidazole exchangeable D_2O);9.83(s,1H, CH of pyrimidine); 11.51 (s, 1 H, NH pyrimidine exchangeable D_2O);12.24 (br., 2 H, NH_2, exchangeable D_2O);12.34 (s, 1H, NH amide exchangeable D_2O). MS: m/z 585. IR spectrum (KBr, cm^{-1}) υmax: υ3320, 3295 (NH_2), υ3427 (NH amid), υ3196 (NH pyrimidine), υ3127 (NH benzimidazole), υ 1786 (C=O), υ1642 (2C=O), υ1605 (C=N benzimidazole ring), υ1375 (C–S of thiazine ring). Anal.calcd. for $C_{28}H_{23}N_7O_6S$, M.W. 585: C, 57.43; H, 3.96; N, 16.74; S, 5.48. Found: C, 57.62; H, 3.67; N, 16.98; S, 5.61.

2-(2-hydroxyphenyl)-4-imino-6,8-dimethylene-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-4,6,7,8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3b)

Yield 94%, m.p. 140–143°C. ^{1}H-NMR (500MHz, DMSO-*d*$_6$, δ ppm): 1.87 (s, 2H ,CH_2 of pyrimidine), 4,80 (s, 1H, OH exchangeable D_2O); 5.94(s,1H, of hydroxyphenyl ring); 6.31 (s, 1H, NH benzimidazole exchangeable D_2O) ; 6.34 (s, 1H, NH pyrimidine exchangeable D_2O); 7.30–7.51(m, 10H, aromatic rings); 8.39 (br., 2H, NH_2, exchangeable D_2O); 10.22(s, 1H, CH of pyrimidine);13.12 (s, 1H, NH amid exchangeable D_2O).MS: m/z 571. IR spectrum (KBr, cm^{-1}) υmax: υ3750 (OH), υ3404, 3270 (NH_2), υ3200 (NH amid), υ3167 (NH pyrimidine), υ3060 (NH benzimidazole), υ1791 (C=O), υ1622 (2C=O),υ1604 (C=N benzimidazole ring), υ1373 (C–S of thiazine ring). Anal.calcd. for $C_{27}H_{21}N_7O_6S$ M.W. 571: C, 56.74; H, 3.70; N, 17.15; S, 5.61. Found: C, 56.90; H, 3.54; N, 17.32; S, 5.48.

2-(4-hydroxy-3-methoxyphenyl)-4-imino-6,8-dimethylene-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-4,6,7,8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3c)

Yield 90%, m.p. 220–222°C. ^{1}H-NMR (500MHz, DMSO-*d6*, δ ppm): 1.87 (s, 2H ,CH_2 of pyrimidine),3.89 (s, 3H, OCH_3); 4,61 (s, 1H, OH exchangeable D_2O); 5.82(s,1H, of 4-hydroxy-phenyl ring); 7.34–7.82 (m, 10H, aromatic rings); 8.46 (s, 1H, NH benzimidazole exchangeable D_2O). 9.72(s,1H, CH of pyrimidine); 11.61 (s, 1H, NH pyrimidine exchangeable D_2O); 12.21 (br., 2H, NH_2, exchangeable D_2O); 12.32 (s, 1H, NH amide exchangeable D_2O); MS: m/z 601. IR spectrum (KBr, cm^{-1}) υmax: υ3752 (OH), υ3406, 3208 (NH_2), υ 3122 (NH amid), υ3167 (NH pyrimidine), υ3066 (NH benzimidazole), υ1791 (C=O), υ1645 (2C=O), υ1604 (C=N benzimidazole ring),, υ1371 (C–S of thiazine ring). Anal. calcd. for $C_{28}H_{23}N_7O_7S$ M.W. 601: C, 55.90; H, 3.85; N, 16.30; S, 5.33. Found: C, 56.04; H, 3.98; N, 16.50; S, 5.43

4-imino-6,8-dimethylene-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-2-(2-nitrophenyl)-4,6,7,8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3d)

Yield 70%, m.p. 230–233°C. ^{1}H-NMR (500MHz, DMSO-*d*$_6$, δ ppm) 1.87 (s, 2H ,CH_2 of pyrimidine); 7.34–7.87 (m, 11H, aromatic rings);8.79(s, 1H, NH benzimidazole exchangeable D_2O); 10.20(s,1H, CH of pyrimidine); 11.61 (s, 1H, NH pyrimidine exchangeable D_2O); 12.21 (br., 2 H, NH_2, exchangeable D_2O); 12.40 (s, 1H, NH amid exchangeable D_2O).MS: m/z 600 . IR spectrum (KBr, cm^{-1}) υmax: υ3412, 3236 (NH_2), υ3200 (NH amid), υ3167 (NH pyrimidine), υ3060 (NH benzimidazole), υ1791 (C=O), υ1627 (2C=O), υ1604 (C=N benzimidazole ring), υ1376 (C–S of thiazine ring). Anal.calcd. for $C_{27}H_{20}N_8O_7S$, M.W. 600: C, 54.00; H, 3.36; N, 18.66; S, 5.34. Found: C, 54.12; H, 3.36; N, 18.93; S, 5.61.

4-amino-2-(4-methoxyphenyl)-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-6,8-dioxo-2,6,7,8,9, 9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3e)

Yield 80%, m.p. 280–282°C. ^{1}H-NMR (500MHz, DMSO-d_6, δ ppm): 1.87 (s, 2H ,CH_2 of pyrimidine);3.84 (s, 3H, OCH_3); 5.85(s,3H, of 4-methoxyphenyl); 7.03–7.82 (m, 10H, aromatic rings); 8.39 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83(s,1H, CH of pyrimidine); 11.55 (s, 1 H, NH pyrimidine exchangeable D_2O); 12.25 (br., 2 H, NH_2, exchangeable D_2O); 12.35 (s, 1H, NH amide exchangeable D_2O). MS: m/z 585. IR (KBr, cm^{-1}) υmax: υ3404, 3270 (NH_2), υ3200 (NH amid), υ3167 (NH pyrimidine), υ3064 (NH benzimidazole), υ 1791 (C=O), υ1649 (2C=O) , υ1604 (C=N benzimidazole ring),υ1373(C–S of thiazine ring). Anal. calcd. for $C_{28}H_{23}N_7O_6S$ M.W. 585: C, 57.43; H, 3.96; N, 16.74;S, 5.48. Found: C, 57.21; H, 4.11; N, 16.64; S, 5.21.

4-imino-6,8-dimethylene-2-(5-methylfuran-2-yl)-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-4,6,7, 8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3f)

Yield 75%, m.p. 250–252°C. ^{1}H-NMR (500MHz, DMSO-d_6, δ ppm): 1.87 (s, 2H ,CH_2 of pyrimidine), 2.39 (s, 3H, CH_3); 7.41–7.97 (m, 9H, furan and aromatic rings);8.51 (s, 1H, NH benzimidazole exchangeable D_2O); 9.47 (s,1H, CH of pyrimidine) ;11.53 (s, 1 H, NH pyrimidine exchangeable D_2O); 12.28 (br., 2 H, NH_2, exchangeable D_2O); 12.35 (s, 1H, NH amide exchangeable D_2O). MS: m/z 559 . IR (KBr, cm^{-1}) υmax: υ3433, 3270 (NH_2), υ3200 (NH amid), υ3139 (NH pyrimidine), υ3067 (NH benzimidazole), υ1791 (C=O), υ1644 (2C=O) ,υ1604(C=N benzimidazole ring), υ1376 (C–S of thiazine ring). Anal. calcd. for $C_{26}H_{21}N_7O_6S$, M.W. 559: C, 55.81; H, 3.78; N, 17.52; S, 5.73. Found C, 56.02; H, 3.63; N, 17.31; S, 5.60

4-(1H-benzo[d]imidazol-2-yl) aniline (4)

A mixture of 4-aminobenzoic acid (0.009 mol) and *o*-phyenlenediamine (0.006 mol) was heated under reflux in hot syrupy o-phosphoric acid (50–60 mL) at 180–200 °C for 4 h. The reaction mixture was then partially cooled (to approx. 50 °C), poured on to crush ice and neutralized with 10 % NaOH solution. The precipitated product was collected by vacuum filtration, washed with excess 10 % NaOH solution, and then dried and recrystallized from ethanol.

Yield 80%, m.p. 209-211°C, blue powder, 1H-NMR spectrum of (4) showed peaks (δ ppm) 5.58 (s, 2H, NH_2, exchangeable), 7.79 -6.63 (m, 8H, aromatic rings), 12.46 (br., 1H, NH, exchangeable D_2O), MS m/z. 209, The IR (KBr, cm^{-1}) υmax :υ3430 (NH), υ3350 and υ3217 (NH_2), υ1629 (C=N). Anal. calcd. for $C_{13}H_{11}N_3$M.W. 209:C, 74.62; H, 5.30; N, 20.08. Found: C, 74.52; H, 5.41; N, 19.88

N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-oxobutanamide (5)

A mixture of 4-(1H-benzo[d]imidazol-2-yl) aniline (0.01mole) with excess ethylacetoacetate the product separated was collected and crystalized from ethanol.

Yield 74%, m.p. 250-260 °C. yellow powder ,1H-NMR (DMSO-d6, 500 MHz, δ ppm) 3.54 (s, 2H, CH2); 5,68 (br., NH benzimidazole, D_2O exchangeable);7.20–7.64 (m, 7H, aromatic rings);7.81 (br., NH phenyl). MS m/z: 293, The IR (KBr, cm^{-1}) υ max: υ3254 (NH of benzimidazole), υ 2977 (NH amide), and υ 1668 (C=O), υ1749 (C=O).Anal. calcd. For $C_{17}H_{15}N_3O_2$, M.W. 293.: C, 69.61; H, 5.15; N, 14.33;, Found: C, 69.51; H, 5.35; N, 14.53.

N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-2-(substitutedphenyl)-2,6,7,8,9,9a-hexahydro-4-methyl-6,8-dioxopyrimido[2,1-b][1,3]thiazine-3-carboxamide (6a-d)

An equimolar amount (0.01mol) mixture of N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-oxobutanamide compound (5) with the appropriate aldehyde and thiobarbturic acid in glacial acetic acid (50mL) was heated under reflux for appropriate time. The reaction mixture cooled at room temperature for 24h. Then product was filtered off and crystallized from methanol.

N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-2,6,7,8,9, 9a-hexahydro-4-methyl-6,8-dioxo-2-phenylpyrimido[2,1-b][1,3]thiazine-3-carboxamide (6a)

Yield 80%, m.p. 270°C. dark brawn powder ^{1}H-NMR (500MHz, DMSO-d_6, δ ppm): 1.70 (s, 2H ,CH_2 of pyrimidine), 2.56 (s, 3H, CH_3) 7.03–7.82 (m, 10H, aromatic rings); 8.31 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83 (s,1H, CH of pyrimidine); 11.51 (s, 1 H, NH pyrimidine exchangeable D_2O);12.34 (s, 1H, NH amide exchangeable D_2O). MS: m/z 509. The IR (KBr, cm^{-1}) υmax:υ3063 (NH amid), υ2980 (NH pyrimidine), υ2931 (NH benzimidazole), υ1642 (C=O), υ1605 (C=N benzimidazole ring), υ1375 (C–S thiazine ring). Anal. calcd. For $C_{28}H_{23}N_5O_3S$, M.W. 509.:C, 66.00; H, 4.55; N, 13.74 ; S, 6.29. Found: C, 63.00; H, 4.35; N, 13.00; S, 6.00.

N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-2-(2-fluorophenyl)-2,6,7,8,9,9a-hexahydro-4-methyl-6,8-dioxopyrimido[2,1-b][1,3]thiazine-3-carboxamide (6b)

Yield 80%, m.p. >300 °C. dark brown powder 1H-NMR (500MHz, DMSO-d^6, δ ppm): 1.70 (s, 2H ,CH_2 of pyrimidine), 2.00 (s, 3H, CH_3), 7.00–7.62 (m, 9H,

aromatic rings); 8.00 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83 (s,1H, CH of pyrimidine); 11.40 (s, 1 H, NH pyrimidine exchangeable D_2O);12.33 (s, 1H, NH amid exchangeable D_2O). MS: m/z 527. IR (KBr, cm^{-1}) υ max: υ 3407 (NH amid), υ3186 (NH pyrimidine), υ3130 (NH benzimidazole), υ1640 (C=O), υ1600 (C=N benzimidazole ring), υ 1371 (C–S thiazine ring). Anal.calcd. For, $C_{27}H_{21}FN_6O_3S$, M.W. 527: C, 63.75; H, 4.20; F, 3.60; N, 13.27; S, 6.08. Found: C, 63.00; H, 4.00; F, 3.50; N, 13.00; S, 6.00.

N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-2,6,7,8,9, 9a-hexahydro-2-(4-methoxyphenyl)-4-methyl-6,8-dioxopyrimido[2,1-b][1,3]thiazine-3-carboxamide (6C)

Yield 62%, m.p. 230-240°C. yellow powder,^{1}H-NMR (500MHz, DMSO-d_6, δ ppm): 1.70 (s, 2H ,CH_2 of pyrimidine), 1.90 (s, 3H, CH_3), 3.60 (s, 3H, OCH_3); 5.21 (s, 3H of 4-methoxyphenyl ring); 7.00–7.90 (m, 10H, aromatic rings); 8.00 (s, 1H, NH benzimidazole exchangeable D_2O); 9.73 (s,1H, CH of pyrimidine); 11.40 (s, 1 H, NH pyrimidine exchangeable D_2O);12.10 (s, 1H, NH amid exchangeable D_2O). MS: m/z 539. IR (KBr, cm^{-1}) υ max: υ3427 (NH amid), υ3196 (NH pyrimidine), υ 2980 (NH benzimidazole), υ1792 (C=O), υ1647 (C=N benzimidazole ring)υ 1373 (C–S thiazine ring). Anal.calcd. For , $C_{28}H_{24}N_6O_4S$ M.W. 539: C, 64.55; H, 4.67; N, 12.98; S, 5.94, Found: C, 64.00; H, 4.00; N, 12.00; S, 5.00.

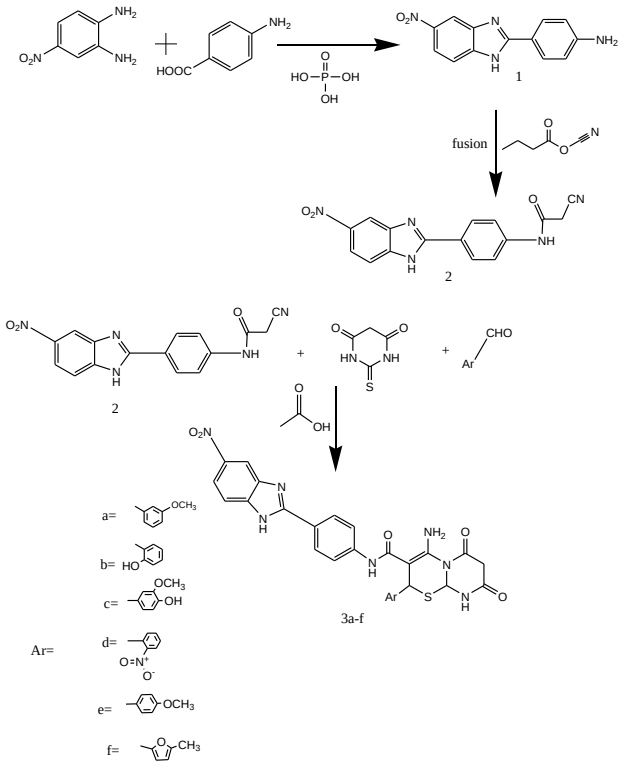
N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-2,6,7,8,9, 9a-hexahydro-4-methyl-2-(5-methylfuran-2-yl)-6,8-dioxopyrimido[2,1-b][1,3]thiazine-3-carboxamide(6d)

Yield 60%, m.p. >300 C. orange powder, ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 1.80 (s, 2H ,CH_2 of pyrimidine); 2.20 (s, 3H, CH_3);2.27 (s, 3H, $CH_3$of furan); 7.21–7.90 (m, 9H, furan and aromatic rings);8.41 (s, 1H, NH benzimidazole exchangeable D_2O); 9.40(s,1H, CH of pyrimidine) ;11.40 (s, 1 H, NH pyrimidine exchangeable D_2O); 12.35 (s, 1H, NH amid exchangeable D_2O).MS: m/z 513. The IR (KBr, cm^{-1}) υmax: υ3450 (NH amid), υ3430(NH pyrimidine), υ 2923 (NH benzimidazole), υ1696 (C=O) ,υ 1597 (C=Nbenzimidazole ring),υ 1369 (C–S thiazine ring). Anal.calcd. for $C_{27}H_{23}N_5O_4S$, M.W13: C, 63.14; H, 4.51; N, 13.64; O, 12.46; S, 6.24. Found C, 63.23; H, 4.00; N, 13.23; S, 6.00.

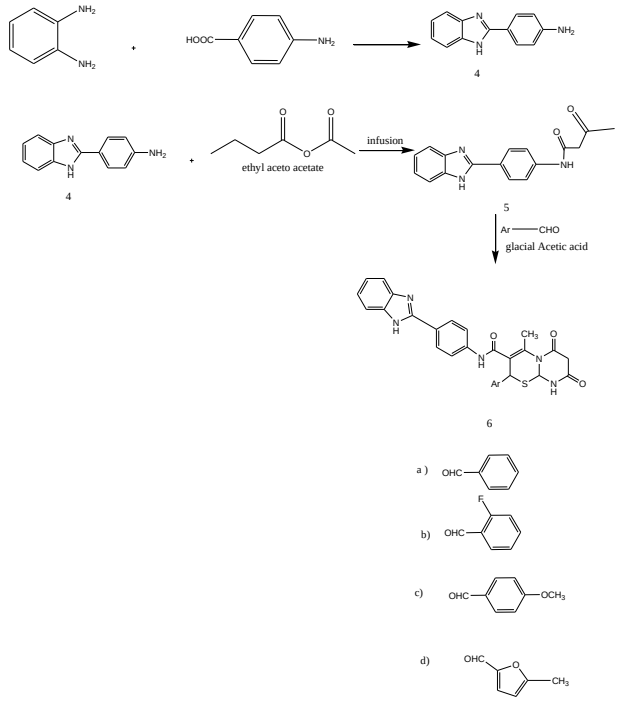
Biology

Preparation of Eg5

Coding regions were PCR amplified from a template containing full-length human Eg5. The primers used were forward 50-TATAGG GCG AAT TCC GCC ATG GCG TCG CAG CCA-30 and reverse 50-ACG GGC TGC AGC AAG CTC GAG TTT TAAACG TTC TAT-30. The region encoding residues 2–386 was subcloned into pET28a (NOVAGEN). Protein expression in Escherichia coli cells was induced with 0.5mM isopropyl b-D-I thiogalactopyranoside IPTG. Cells were harvested after 20 h of growth at 20 °C and then disrupted by sonication.



Scheme1



Scheme 2

The soluble lysate was clarified by centrifugation and applied to a SP Sepharose column (Amersham Pharmacia Biotech) in a buffer A (20mM 1,4-piperazined diethanesulfonic acid sodium salt Na-PIPES, pH 6.3; 20mM NaCl; 1mM MgCl2; 1mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N0,N0-tetraactic acid tetrasodium salt Na-EGTA). Protein was eluted with a linear gradient of 20–1000mM NaCl. Eg5 was identified by SDS–PAGE, and then applied to Mono-Q columns (Amersham Pharmacia Biotech) in a buffer B (20mM Tris–HCl, pH 8.8; 1mM $MgCl_2$; 1mM Na-EGTA). A gradient from 0 to 1000mM NaCl was used to elute Eg5. Fractions were analyzed by SDS–PAGE. The most concentrated fraction was dialyzed against ATPase buffer (20mM Na-PIPES, pH 7.5; 1mM $MgCl_2$; 1mM Na-EGTA) and then aliquoted, frozen in liquid nitrogen, and stored at -80°C [22].

ATPase activity assay

All experiments were done at room temperature. The reagents were added to wells of a 96-well clear plate and the final reaction of the assay contained 20mM PIPES, pH 7.5,5.0mM MgCl2, 1mM EGTA, 10mM paclitaxel, 0.6mM tubulin (MT), 0.5mM ATP, and 2% DMSO containing inhibitors in areaction volume of 100 mL. Reactions were started by adding ATP. The plates were incubated at 37°C for 30 min. Following incubation, the malachite-green based reagents were added to detect the release of inorganic phosphate. The plates were incubated for an additional 5 min at room temperature, and then 34% sodium citrate of 10mL was added. The absorbance at 610nm was determined using Multiskan Spectrum Micro plate Spectrophotometer (Thermo Electron Corporation). The controls without Eg5 or MTs are the background and should be subtracted from all values. The controls with MTs but without Eg5 give the nucleotide hydrolysis by MTs and should be subtracted from corresponding values with Eg5 and the same concentration of MTs. The data were analyzed using Microsoft Excel to obtain the IC50 of the test compounds [22].

***In vitro* Aurora A kinase activity assay**

To measure the inhibitory activity of Aurora A kinase by the target compounds, the HTScan R Aurora A kinase assay kit (Cell Signaling Technology) was used according to the manufacturer's instruction. Briefly, 20 ng purified recombinant human Aurora A kinase was added to a 50mL reaction mixture containing one kinase buffer and 200 mol/L cold ATP in the presence of different concentrations of tested compounds (ranging from 10–4 to 10–9 mol/L). After incubation at room temperature for 15 min, biotinylated peptide substrate (Cell Signaling Technology) was added to each reaction mixture at a final concentration of 1.5mmol/L, and the mixtures were further incubated for 30 min. A parallel control experiment was done under the same conditions without the tested compounds. The reaction was stopped by addition of 50 mmol/L ethylene diamine tetra acetic acid EDTA (pH 8). Then, 30mL reaction mixtures were transferred to a streptavidin-coated 96-well plate (PerkinElmer, Inc.) and incubated at room temperature for 60 min. After washing thrice with PBS/T, the phosphor PLK (Ser10) antibody (Cell Signaling Technology) was added to the plate for further incubation at 37°C for 60 min. After washing, HRP-labeled secondary antibody was added. After incubation at room temperature for 30 min, the plate was finally washed five times and the fluorescence signal was determined with BMG Polar star Galaxy (Germany) at 450 nm. The data were analyzed using Microsoft Excel to obtain the IC50 of the tested compounds [22].

***In vitro* anti-cancer activities**

The cytotoxicity of the newly synthesized compounds against cancer cell lines *in vitro* was performed with the MTT assay according to the Mosmann'smethod. The MTT assay is based on the reduction of the soluble 3-(4,5-methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into a blue-purple formazan product, mainly by mitochondrial reductase activity inside living cells. The cells used in cytotoxicity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2 × 104/mL) were plated in 96-well culture plates and incubated at 37°C in a 5% CO2 incubator. After 12 h, the test sample (2 μL) was added to the cells (2Ý 104) in 96-well plates and cultured at 37°C for 3 days.

The cultured cells were mixed with 20 μL of MTT solution and incubated for 4 h at 37°C. The supernatant was carefully removed from each well and 100 μL of DMSO were added to each well to dissolve the formazan crystals which were formed by the cellular reduction of MTT. After mixing with amechanical plate mixer, the absorbance of each well was measured by a microplate reader using a test wavelength of 570 nm.

The results were expressed as the IC50, which inducing a 50% inhibition of cell growth of treated cells when compared to the growth of control cells. Each experiment was performed at least 3 times. There was a good reproducibility between replicate wells with standard errors below 10% [23].

Results and Discussion

Biology

Biological rationale

A new series of anticancer compounds comprising thiazolo [3,2-a]pyrimidine derivatives 6a–q bearing a benzimidazole were prepared,some of them showed potent KSP and Aurora A kinase inhibitory activities were synthesized [22].

Also many different benzimidazole derivatives were synthesised and founded to have in vitro cytotoxic activities [24].

KSP and Aurora A kinase inhibitory activities

Some represented examples of the newly synthesised compounds were tested for their KSP and Aurora A kinase inhibitory activities, these compounds were found to have potent KSP and Aurora A kinase inhibitory activities in the following descending order 6b,6d,6a,6c,5,3f,3d,3e,3b,3c,3a and 1 (Table 1).

Its worth to mention that all these compounds were more potent than Monastrol, CPUYL064 and CK0106023.

Table 1. KSP and Aurora A kinase inhibitory activities of tested compounds.

Compounds	KSP IC_{50} (nM)	Aurora A kinase IC50 (µM)
1	8.72±0.07	13.14±0.1
3a	6.84±0.08	9.90±0.07
3b	5.66±0.05	5.89±0.05
3c	6.55±0.06	8.80±0.07
3d	4.48±0.07	4.16±0.04
3e	5.37±0.08	5.58±0.03
3f	3.99±0.06	3.77±0.06
5	3.70±0.04	3.08±0.05
6a	2.67±0.04	2.28±0.04
6b	1.12±0.02	1.25±0.03
6c	3.59±0.05	2.49±0.03
6d	2.35±0.03	1.67±0.02
Monastrol	5470	>50
CPUYL064	180	40.34
CK0106023	29	>50

The inhibitory activities against KSP were determined by measuring the MT-activated ATPase activity.

In vitro anticancer activities

Some represented examples of the newly synthesised compounds were tested for theirIn vitro anticancer activities against the following cancer cell lines; MCF7 (Breast), SNB19 (CNS), HT29 (Colon), K562 (Leukemia), A549-ATCC (Lung), MDA-MB-435 (Melanoma) and SKOV3 (Ovarian).

These compounds were found to have potent in vitro anticancer activities in the following descending order 6b,6d,6a,6c,5,3f,3d,3e,3b,3c,3a and 1 (Table 2). The activity of compounds 6b is due to the present of fluorine moiety in the structure.

Chemistry

4-(5-nitro-1H-benzo[d]imidazol-2-yl) aniline (1) was prepared by the reaction of 4-aminobenzoic acid and 4-nitrobenzene-1, 2-diamine under reflux in hot syrupy o-phosphoric acid [21]. ^{1}H NMR (DMSO-d_6) δ (ppm) spectra of compound **(1)** revealed signals at δ 5.84 (br.,1H, NH benzimidazole, D_2O exchangeable); 6.67–7.82 (m, 7H, aromatic rings); 13.10 (s, 2H, NH_2aminophenyl, D_2O exchangeable). IR spectra (KBr, cm^{-1})υmax exhibited characteristic absorption bands atυ3470 (NH benzimidazole); υ 3374, υ 3206 (NH_2aminophenyl); υ 3058 (CH arom); υ 1631 (C=N).

Further treatment of compound (1) with ethylcyanoacetate in absolute ethanol gave 2-cyano-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl) phenyl)acetamide[21] (2).^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (2) revealed signals at 3.54 (s, 2H, CH_2); 5,84 (br., 1H, NH benzimidazole, D_2O exchangeable);7.29–7.63 (m, 7H, aromatic rings);7.91 (br., 1H, NH aminophenyl). IR spectra (KBr, cm^{-1}) νmaxexhibited characteristic absorption bands at υ 3405 (NH benzimidazole); 3208 (NH aminophenyl); υ 3043 (CH arom); υ 2268 (C≡N); υ 1689 (C=O); υ 1625 (C=N).

4-imino-2-(substituted)-N-(4-(5-nitro-1H-benzo[d]imidazol-2-yl)phenyl)-6,8-dioxo-4,6,7,8,9,9a-hexahydropyrimido[2,1-b][1,3]thiazine-3-carboxamide (3a-f) was synthesized via the reaction of cyanoacetamide compound (2) with the appropriate aldehyde namely (m-anisaldehyde, salicylaldehyde, vanilline, nitro benzaldehyde, *p*-anisaldehyde, methyl furfural) respectively, and 2-amino thiazole in glacial acetic acid (1). The IR spectra of the synthesized 4-imino-2-(substituted)-N-(4-(5-nitro-1H-benzo [d]imidazol-2-yl)phenyl)-6,8-dioxo-4,6,7,8,9,9a-hexahydro pyrimido[2,1-b][1,3]thiazine-3-carboxamide (3a–f) showed disappearance of the band corresponding to C≡N at υ 2286 and appearance of band at υ3315–3345 corresponding to NH_2 groups in addition to characteristic bands for each compound (see Experimental section). ^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (3a) revealed signals at): δ (ppm) 1.87 (s, 2H ,CH_2 of pyrimidine),3.85 (s, 3H, OCH_3); 5.86(s,1H, of 3-methoxyphenyl ring); 7.03–7.82 (m, 10H, aromatic rings); 8.31 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83(s,1H, CH of pyrimidine); 11.51 (s, 1H, NH pyrimidine exchangeable D_2O); 12.24 (br., 2H, NH_2, exchangeable D_2O);12.34 (s, 1H, NH amid exchangeable D_2O). IR spectra (KBr, cm^{-1}) υ max of compound (3b) exhibited characteristic absorption bands at υ3750 (OH), υ3270, 3404 (NH_2), υ3200 (NH amid), υ3167 (NH pyrimidine), υ3060 (NH benzimidazole), υ1791 (C=O), υ1622(C=O), υ1604 (C=N benzimidazole ring).

IR spectra (KBr, cm^{-1}) υ_{max} of compound (3c) exhibited characteristic absorption bands at υ3752(OH), υ3406, 3208 (NH_2), υ3122 (NH amid), υ 3167 (NH pyrimidine), υ3066(NH benzimidazole), υ1791 (C=O), υ16452(C=O) υ1604 (C=N benzimidazole ring). ^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (3d) revealed signals at δ (ppm) 1.87 (s, 2H ,CH_2 of pyrimidine); 7.34–7.87 (m, 11H, aromatic rings);8.79(s, 1H, NH benzimidazole exchangeable D_2O); 10.20(s,1H, CH of pyrimidine); 11.61 (s, 1H, NH pyrimidine exchangeable D_2O); 12.21 (br., 2 H, NH_2, exchangeable D_2O); 12.40 (s, 1H, NH amid exchangeable D_2O) see the other peak in the experimental part.

Table 2. *In vitro* anticancer activities of tested compounds.

Compound No.	IC_{50} (µM) for tested compounds against Breast Cell Lines-						
	MCF7 (Breast)	SNB19 (CNS)	HT29 (Colon)	K562 (Leukemia)	A549-ATCC (Lung)	MDA-MB-435 (Melanoma)	SKOV3 (Ovarian)
1	0.046	8.6	26.43	68.19	0.0087	44.65	0.0094
3a	0.031	0.89	14.55	62.67	0.0075	35.67	0.0073
3b	0.018	0.43	1.47	60.11	0.0044	28.57	0.0058
3c	0.021	0.56	6.36	61.45	0.0056	33.64	0.0061
3d	0.0097	0.094	0.86	34.67	0.00096	26.35	0.0052
3e	0.0098	0.21	0.94	55.67	0.00098	27.54	0.0056
3f	0.0078	0.089	0.85	22.65	0.00085	25.32	0.0051
5	0.0071	0.052	0.74	12.56	0.00077	22.78	0.0047
6a	0.0066	0.044	0.46	7.77	0.00066	13.76	0.0036
6b	0.0074	0.058	0.77	14.67	0.00084	23.46	0.0048
6c	0.0068	0.048	0.65	8.11	0.00075	14.90	0.0045
6d	0.0061	0.035	0.27	6.90	0.00056	12.45	0.0034

^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (3e) revealed signals at 1.87 (s, 2H ,CH_2 of pyrimidine);3.84 (s, 3H, OCH_3); 5.85(s,3H, of 4-methoxyphenyl); 7.03–7.82 (m, 10H, aromatic rings); 8.39 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83(s,1H, CH of pyrimidine); 11.55 (s, 1H, NH pyrimidine exchangeable D_2O); 12.25 (br., 2H, NH_2, exchangeable D_2O); 12.35 (s, 1H, NH amid exchangeable D_2O). IR spectra (KBr, cm^{-1}) υmaxexhibited characteristic absorption bands at υ3404, 3270 (NH_2), υ 3200 (NH amid), υ3167 (NH pyrimidine), υ3064 (NH benzimidazole), υ1791 (C=O),υ 1649 2(C=O), υ1604 (C=N benzimidazole ring) ^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (3f) revealed signals at δ1.87 (s, 2H ,CH_2 of pyrimidine),2.39 (s, 3H, CH_3); 7.41–7.97 (m, 9H, furan and aromatic rings);8.51 (s, 1H, NH benzimidazole exchangeable D_2O); 9.47(s,1H, CH of pyrimidine); 11.53 (s, 1 H, NH pyrimidine exchangeable D_2O); 12.28 (br, 2H, NH_2, exchangeable D_2O); 12.35 (s, 1H, NH amid exchangeable D_2O).

4-(1H-benzo[d]imidazol-2-yl) aniline (4) was prepared by the reaction of 4-aminobenzoic acid and *O*- phenylene diamine under reflux in hot syrupy o-phosphoric acid [21]. IR spectra (KBr, cm^{-1}) υmax of compound (4) showed absorption bands at υ3430 (NH), υ3350 and υ3217 (NH_2), υ 1629 (C=N). ^{1}H-NMR spectrum of (4) showed peaks (δ ppm) 5.58 (s, 2H, NH_2, exchangeable D_2O), 7.79-6.63 (m, 8H, aromatic rings), 12.46 (br., 1H, NH benzimidazole, exchangeable D_2O).

Further treatment of compound (4) with ethylacetoacetate in absolute ethanol gave N-(4-(1H-benzo[d]imidazol-2-yl) phenyl)-3-oxobutanamide (5) The IR spectrum (KBr, cm^{-1}) υmax showed absorption bands at υ3254 (NH of benzimidazole), υ2977 (NH amide), υ 1668 (C=O), and υ 1749 (C=O). ^{1}H-NMR (DMSO-d_6) δ ppm 3.54 (s, 2H, CH_2); 5, 68 (br., 1H, NH benzimidazole, D_2O exchangeable); 7.20–7.64 (m, 7H, aromatic rings);7.81 (br., 1H, NH phenyl). MS m/z: 293.

N-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-4-amino-2-(substituted phenyl)-2,6,7,8,9,9a-hexahydro-6,8-dioxo pyrimido[2,1-b][1,3]thiazine-3-carboxamide (6a-d) was synthesized via the reaction of oxobutan amide compound (6) with the appropriate aldehyde namely 2-floro benzaldehyde, benzaldehyde, p-anisaldehyde, and 5 methyl furfural).

The IR spectrum (KBr, cm^{-1}) υmaxN-(4-(1H-benzo [d] imidazol-2-yl)phenyl)-4-amino-2-(substitutedphenyl)-2,6, 7,8,9,9a-hexahydro-6,8-dioxopyrimido[2,1-b] [1,3] thiazine-3-carboxamide (6a-d)showed appearance of υ 1372 corresponding to C-S thiazine in addition to characteristic bands for each compound (see Experimental section).

^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (6a) revealed signals at: 1.70(s, 2H ,CH_2 of pyrimidine), 2.56 (s, 3H, CH_3) 7.03–7.82 (m, 10H, aromatic rings); 8.31 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83 (s,1H, CH of pyrimidine); 11.51 (s, 1 H, NH pyrimidine exchangeable D_2O); 12.34 (s, 1H, NH amide exchangeable D_2O).

^{1}H-NMR (DMSO-d_6) δ (ppm) spectra of compound (6b) revealed: 1.70 (s, 2H ,CH_2 of pyrimidine), 2.00 (s, 3H, CH_3), 7.00–7.62 (m, 9H, aromatic rings); 8.00 (s, 1H, NH benzimidazole exchangeable D_2O); 9.83 (s,1H, CH of pyrimidine); 11.40 (s, 1 H, NH pyrimidine exchangeable D_2O); 12.33 (s, 1H, NH amid exchangeable D_2O).

Molecular docking study

In attempts to better understand the binding affinity, docking studies with the representative compound **6b** was performed to suggest possible interaction modes of the chimeras on Aurora-A kinase (Figure 3, 4) and KSP (Figure 5).

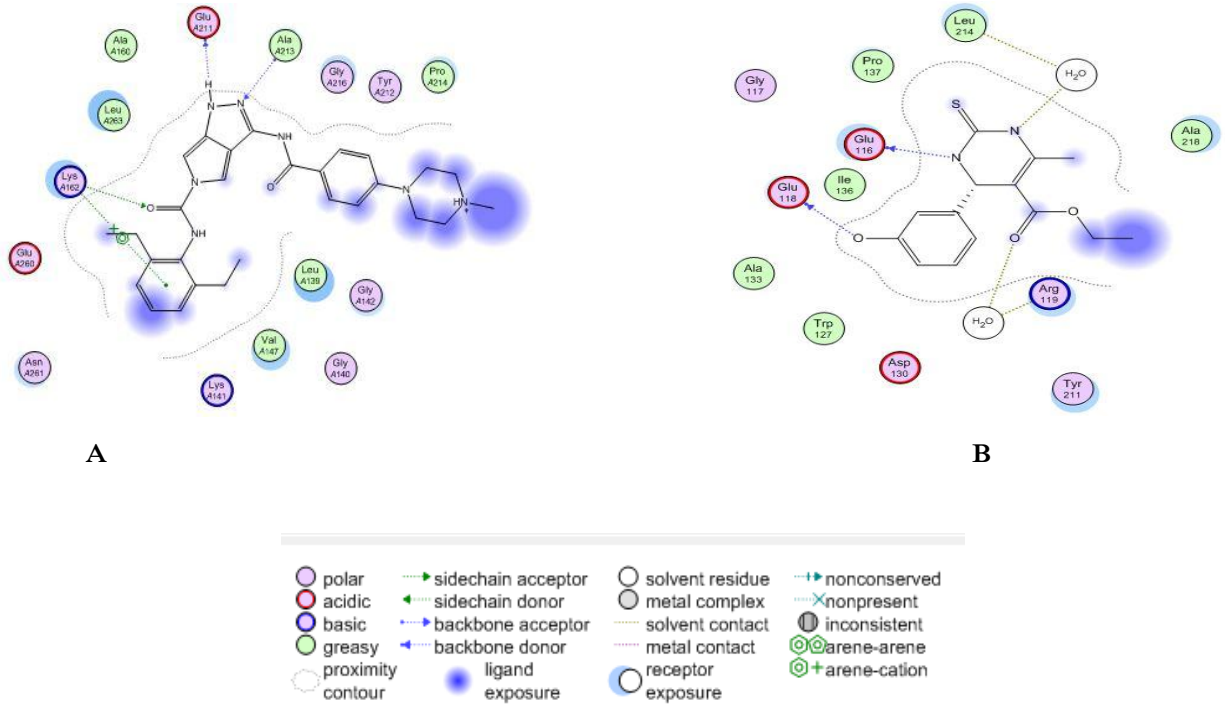


Figure 3. A) The ligand interaction and the binding mode of the native ligand (3*E*)-*N*-(2,6-diethylphenyl)-3-{[4-(4-methylpiperazin-1-yl)benzoyl]imino}pyrrolo[3,4-*c*]pyrazole-5(3*H*)-carboxamide (MPY) and exhibited one H-bond donor with GLU 211and at distance 1.95 and one H-bond acceptor with LYS 162 at distance 3.13 and one H-bond acceptor with ALA 213 at distance 2.97 shown as hatched line and it is score -12.33 kcal\mol. B) The ligand interaction and the binding mode of the native ligand Ethyl 4-(3-HydroxyphenyL)-6-Methyl-2-Thioxo- 1,2,3,4-Tetra hydropyrimidine-5-Carboxylate [Monastrol](NAT) and exhibited one H-bond donor with GLU 116 and at distance 1.91A ^{0} and one H-bond donor with GLU 118 and at distance 1.79A ^{0} and one H-bond donor with HOH 609 and at distance 2.17A ^{0} and one H-bond acceptor with HOH at distance 3.20A ^{0} and it is score -12.5688 kcal\mol.

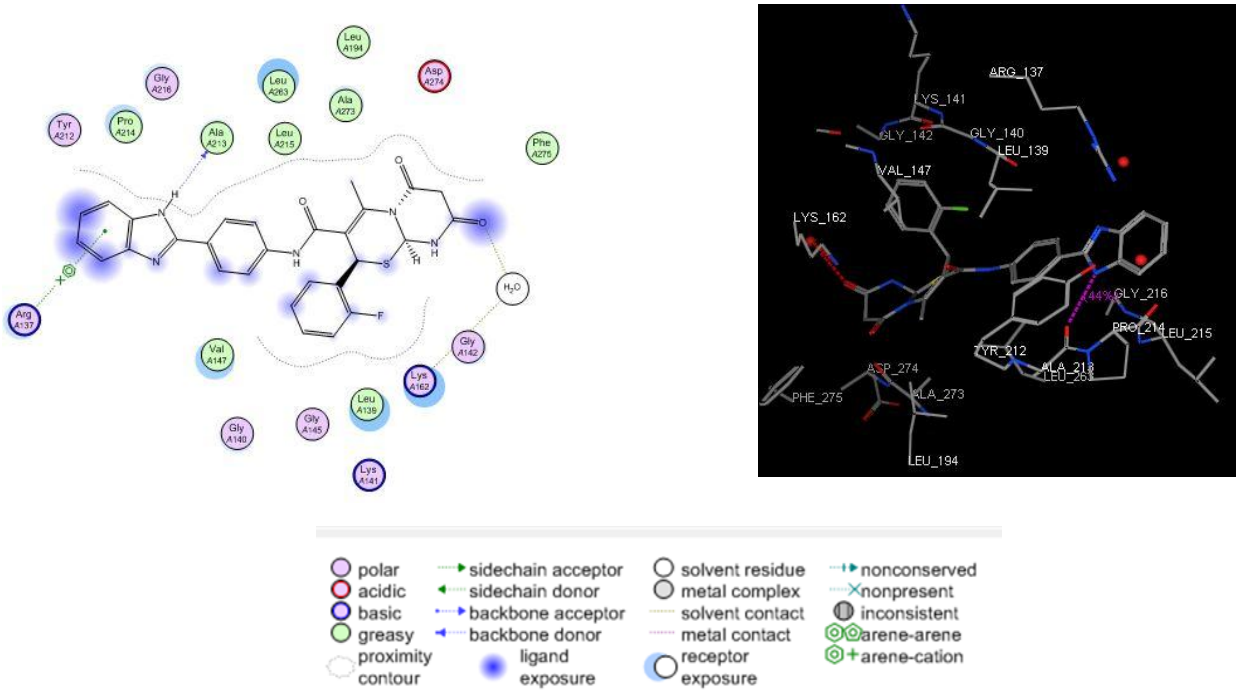


Figure 4. Model of compound 6b bound to the Aurora-A kinase. Docking simulation was performed using the program MOE and the crystal structure of Aurora-A kinase (PDB code 2BMC). For clarity, only interacting residues are displayed. (a) Compound 6b is depicted by line. (b) The Aurora-A kinase is represented by molecular surface.

The binding affinity of the synthesized compounds 18-21 into Aurora Kinase receptor

For molecular docking calculation, firstly, the protein structure (pdb: 2BMC, 1Q0B) was separated from the inhibitor then followed by refinement using molecular minimization with adding hydrogen.

Docking calculation was carried out using the slandered default variable for the MOE Program, the binding affinity was measured by RMSD values, Hydrogen bond, binding free energy (S-score, k.cal/mol). All the prepared compound were docked into the same groove of the co-crystallized ligand MPY, NAT respectively. The compounds which gave the highest docking score based on the H-bonding interaction, RMSD from the native ligand, and the binding free energy. As shown in Figure 4, compound **6b** interacted with Arora A kinase receptor, and formed one hydrogen bond donor with ALA 213 with distance 1.96A^0, one hydrogen bond acceptor with HOH 2011 with distance 2.72A^0 with score -13.303 k. cal/mol. In addition, figure 5, compound **6b** interacted with the KSP receptor through two hydrogen bond acceptor with HOH 630,HOH 675 at distance 2.82, 2.39A^0 respectively, one hydrogen bond donor with HOH630 with distance 2.82A^0 score-13.2120 k. cal/mol.

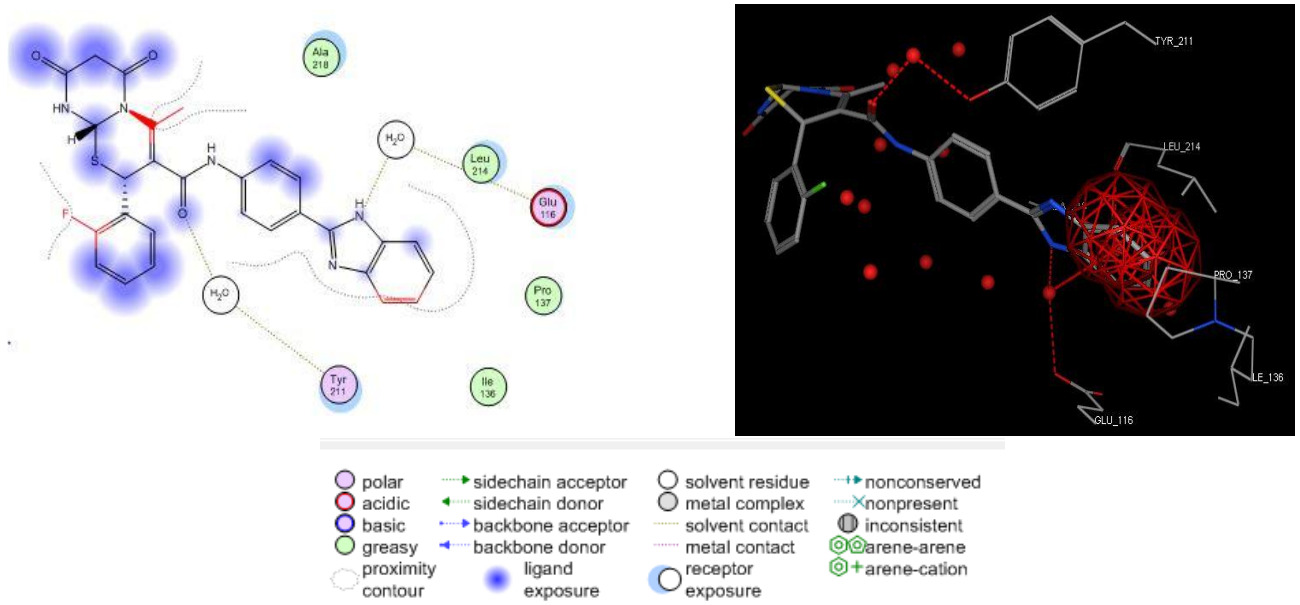


Figure 5. Model of compound 6b bound to the KSP. Docking simulation was performed using the program MOE and the crystal structure of KSP (PDB code 1Q0B). For clarity, only interacting residues are displayed. (a) Compound 6b is depicted by line. (b) The KSP is represented by molecular surface.

Conclusion

The most active synthesized compound from the dioxopyrimido[2,1-b][1,3] thiazine derivatives (3a-f, 6a-e) against the seven tested cell lines was 6b comparable to the standard drug.

The correlation between KSP inhibition and Aurora A kinase inhibition and suppression of tumor growth is validated as an important cancer target. We have been able to synthesis and evaluate a series of benzimidazoles carrying a dioxopyrimido thiazine moiety as dual inhibitors of KSP and Aurora A Kinase.

All the compounds identified through this approach were highly potency KSP and Aurora A inhibitory.

The derivative 6b can serve as lead compound for further development, as it showed potent dual KSP and Aurora A Kinase inhibition in addition to its *in vitro* cytotoxic activity against the seven cell lines.

Acknowledgements

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