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Design, synthesis, quantum chemical studies and biological activity evaluation of pyrazole–benzimidazole derivatives as potent Aurora A/B kinase inhibitors

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ABSTRACT

Novel pyrazole–benzimidazole derivatives have been designed and synthesized. The entire target compounds were determined against cancer cell lines U937, K562, A549, LoVo and HT29 and were screened for Aurora A/B kinase inhibitory activity in vitro. The compounds **7a**, **7b**, **7i**, **7k** and **7l** demonstrated significant cancer cell lines and Aurora A/B kinase inhibitory activities. Molecular modeling studies suggested the derivatives have bound in the active site of Aurora A kinase through the formation of four hydrogen bonds. Quantum chemical studies were carried out on these compounds to understand the structural features essential for activity. The cellular activity of **7k** was also tested by immunofluorescence.

The Aurora kinases are a family of three highly homologous serine–threonine protein kinases that play a critical role in regulating many of the processes that are pivotal to mitosis.[1] Homologues of Aurora kinases have been reported in various organisms including yeast, nematodes, fruit flies and vertebrates.[2] Humans have three classes of Aurora kinases-Aurora A, Aurora B and Aurora C, which have different functions, subcellular localization, or timing of expression and are essential to secure the correct progress of cell cycle during mitosis or meiosis.[2,3]

Human Aurora A is located at chromosome 20q13.2, which is commonly amplified in various epithelial malignant tumours, including breast, colon, bladder, ovarian and pancreatic cancers.[4–6] Overexpression of the Aurora A protein is also observed in many cancers.[7,8] The Aurora B gene maps to the 17p13.1 region of the genome that is altered in some human cancers. Both mRNA and protein levels of Aurora B are frequently increased in various human tumours, including colorectal cancers.[9–11] A gene encoding Aurora C is localized at chromosome 19q13.43, which is the region frequently deleted or rearranged in several tumour tissues. However, a distinct role for Aurora C in tumorigenesis has not been defined.[12]

A number of small molecule inhibitors of Aurora kinases have been progressed to clinical development (Fig. 1). VX-680, which is a potent inhibitor of Aurora kinases A, B and C (K_i = 0.6, 18, and 4.6 nM, respectively), is the first Aurora inhibitor to enter clinical trials.[13] AZD1152 is a dihydrogen phosphate prodrug of a pyrazoloquinazoline Aurora kinase inhibitor, and is converted rapidly to the active moiety in plasma.[14] AZD1152-HQPA is a potent and selective inhibitor of Aurora kinase B (K_i = 0.37 nM), compared with Aurora kinase A (K_i = 1368 nM), and has a high specificity over a panel of 50 other kinases.[15] MLN8237 is the Aurora A selective inhibitor, which inhibits Aurora A with an IC_{50} of 1 nM in biochemical assays and has 200-fold selectivity for Aurora A over Aurora B in cell assays.[16] AT-9283 is a multitargeted kinase inhibitor, with potent activity against Aurora kinases A and B (IC_{50} of approximately 3 nM).[17] A variety of other cancer-related protein kinases are also inhibited by AT-9283, including Abl kinase, JAK2, JAK3, Ret, and GSK3 beta. These observations have stimulated a great deal of research directed at identifying selective Aurora kinase inhibitors as anticancer agents.[17]

Based on AT-9283 as the leading compound, structure–activity relationship analysis of AT-9283, and the principals of cyclization and bioisostere, pyrimidine ring were obtained through cyclization of the urea unit, where the six-membered heterocyclic ring could act as conformation restriction group of 'pseudo-urea'. New pyrazole–benzimidazole derivatives containing pyrimidine ring had been devised and synthesized. The antitumor effects of all the newly synthesized compounds on the growth of five cell lines, human acute monocytic leukemia cell line U937, human chronic myeloid leukemia cell line K562, human non small cell lung cancer A549

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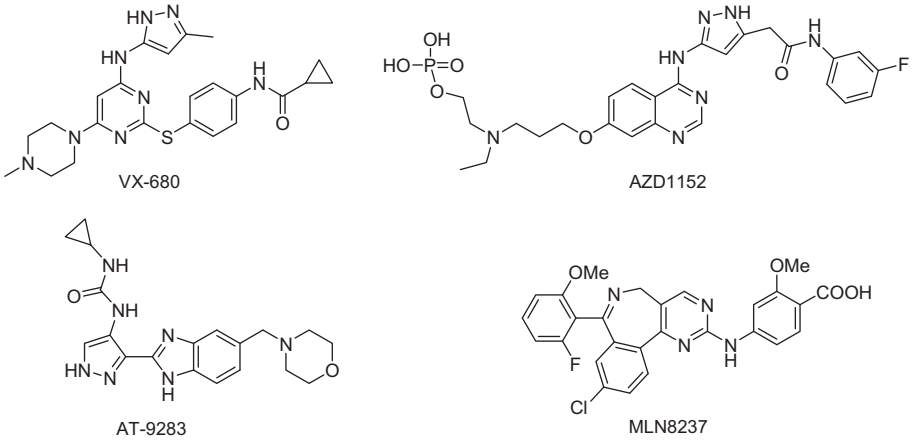


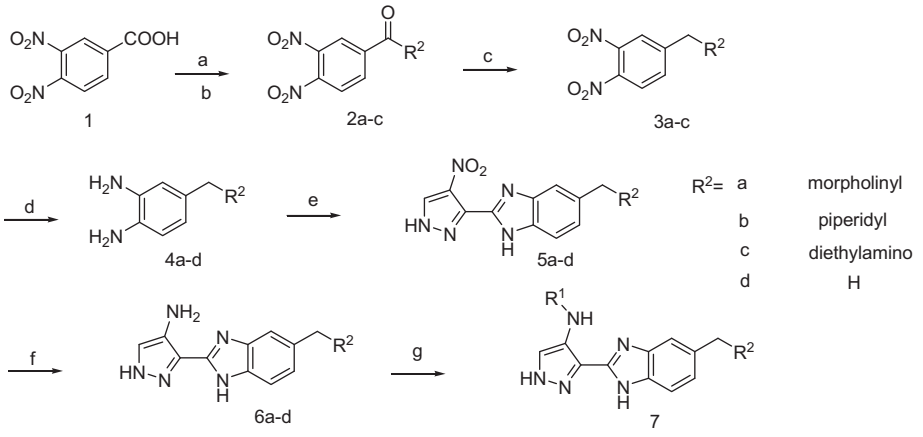
Figure 1. Chemical structures of Aurora kinase inhibitors that are currently in clinical trials.

and human colon cancer LoVo and HT29, were evaluated. Apparent growth inhibition was observed for some of the compounds, such as **7a**, **7b**, **7i**, **7k** and **7l** demonstrating potent activities against cancer cell lines U937, K562, A549, LoVo and HT29, respectively. Furthermore, the target compounds were screened for Aurora A/B kinase inhibitory activity, and the result suggested that the derivatives **7k** and **7l** have significantly Aurora A/B kinase inhibitory activity. Finally, in order to study the mechanism of action of this series of compounds, the molecular docking and quantum chemical studies were also carried out.

The preparation of the target compounds is illustrated in Scheme 1, where the starting material **1** is utilized. Treatment of 3,4-dinitrobenzoic acid **1** with $SOCl_2$ in THF, followed by secondary amine, afforded the amide **2**. Compound **3** was prepared by the reduction of the amide using $NaBH_4$ in the presence of Lewis acid $BF_3{\cdot}OEt_2$. Palladium catalyzed hydrogenation then gave the diamine **4**. The benzimidazole **5** were constructed by first coupling the diamine **4** with 4-nitro-1*H*-pyrazole-3-carboxylic acid, and followed by heating in AcOH. Subsequent palladium catalyzed hydrogenation then afforded the aminopyrazole **6**. Finally, compounds **7** were then prepared from **6**, using pyrimidine derivatives.

All the target compounds were evaluated for their cytotoxic activity in vitro against five human tumor cell lines, representing different tumor types, U937, K562, A549, LoVo and HT29. The results of the cytotoxic studies are shown in Table 1. Most of the compounds demonstrated evident anti-proliferation effects of solid tumor cell lines (A549, LoVo and HT29) and poor anti-proliferation effects of leukemia cell lines (U937 and K562). It is noticeable that most of the compounds **7a–7m** showed moderate activity against solid tumor cell lines (A549, LoVo and HT29), while compounds **7a 7b**, **7i** and **7k** having IC_{50} values ranging from 0.30 to 0.80 μM exhibited similar potency to that of AT-9283 (IC_{50} ranging from 0.40 to 0.6 μM). When the side chain at 5-position of benzene ring was replaced by hydrogen atom, coumpounds **7e–7g** have lower activity against the cancer cell lines. We proposed that the lower cytotoxicities of these compounds could be attributed to the relatively poor membrane permeability of them, since the cytotoxic screening was carried out in vitro and the intake of the compounds from medium formed the basis of cytotoxicity effects. The introduction of small group substituent at 2-position of pyrimidine ring such as methyl, methylthio and methyl sulfonyl, could improve the activities as exemplified by analogs **7a**, **7b** and **7k**, inhibited the growth of solid tumor cell lines with IC_{50} value of 0.35–0.60 μM. However, keeping 4-(cyclopropanecarboxamido)phenylthio substituent at 2-position of pyrimidine ring produced compounds **7c** and **7d** with weak cytotoxic activities. This may be due to the steric effect of the big substituent group compared with methyl and methylthio. The compounds **7a**, **7b**, **7c** and **7k** carrying morpholine ring at the side chain, were more potent than other compounds in this series.

In order to study the mechanism of action of this series of compounds, the target compounds were screened for Aurora A/B kinase



Scheme 1. Synthesis route of target compound. Reagents and conditions: (a) $SOCl_2$/THF; (b) TEA/amine; (c) $NaBH_4/BF_3$-Et_2O; (d) Pd/C,H_2; (e) EDCI/HOBt, acetic acid and 4-nitro-1*H*-pyrazole-3-carboxylic acid; (f) Pd/C,H_2; (g) substituented pyrimidine.

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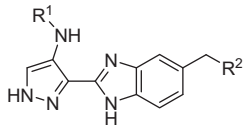
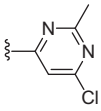
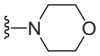
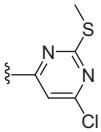
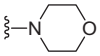
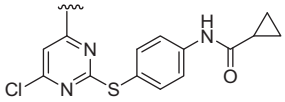
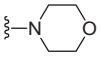
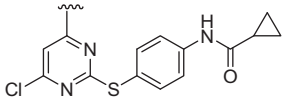
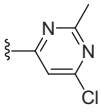
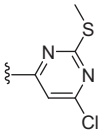
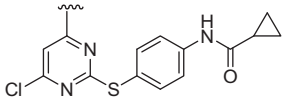
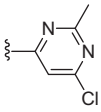
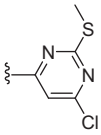
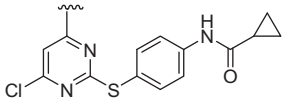
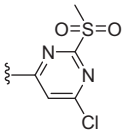
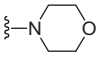
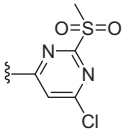
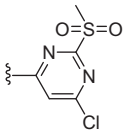
Table 1
Structure and in vitro cytotoxic activity of target compounds against cancer cells



Compd	R^1	R^2	IC_{50} (μM)[a]				
			U937	K562	A549	LoVo	HT29
7a			6.301	3.000	0.479	0.402	0.352
7b			7.703	9.012	0.441	0.422	0.599
7c			8.804	>100	0.901	0.899	0.577
7d			3.821	>100	>100	1.422	1.299
7e		H	>100	>100	0.471	0.688	0.591
7f		H	>100	>100	0.891	0.701	0.656
7g		H	>100	>100	0.831	1.228	>100
7h			>100	8.511	0.711	1.372	>100
7i			>100	2.514	0.499	0.352	0.457
7j			>100	2.002	0.831	0.361	0.798
7k			5.106	5.003	0.487	0.789	0.381
7l		H	5.117	>100	0.905	0.994	1.390

(*continued on next page*)

Table 1 (*continued*)

Compd	R^1	R^2	IC_{50} (μM)[a]				
			U937	K562	A549	LoVo	HT29
7m			>100	8.808	0.472	0.950	0.789
AT-9283			6.700	1.600	0.512	0.553	0.383

[a] Cellular proliferation was determined by MTT assay.

Table 2
Aurora A/B inhibitory activity of pyrazole–benzimidazole derivatives

Compd	IC_{50}(nM)[a]	
	Aurora A	Aurora B
7a	68.5	91.8
7b	71.9	143.9
7c	214.9	412.3
7d	124.9	191.0
7e	91.1	138.6
7f	321.3	231.3
7g	1134	NT
7h	NT[b]	1201
7i	86.1	98.8
7j	248.8	424.1
7k	28.9	2.2
7l	45.1	2.3
7m	77.9	392.3
AT-9283	3.3	2.7

[a] Typically average of at least two experiments.
[b] Not tested.

inhibitory activity (Table 2). It has been observed from Table 2 that the derivatives such as **7a**, **7i**, **7k** and **7l** with small group at 2-position of pyrimidine ring such as methyl, methylthio and methyl sulfonyl, have significantly Aurora A/B kinase inhibitory activity (IC_{50} ranging from 2.2 to 100 nM). Especially, compound **7k** and **7l** have similar inhibitory activity (IC_{50} = 2.2 and 2.3 nM, respectively) on Aurora B compared with AT-9283(IC_{50} = 2.7 nM). Although there is an extra hydrogen bond between the pyrimidine ring of the compounds and binding site, the compounds **7c**, **7g** and **7j**, which also have pyrimidine ring, have poor Aurora A/B kinase affinity due to the steric effect. In addition, the compounds that have morpholine ring at the side chain demonstrated evident Aurora A/B kinase inhibitory activity.

In vitro kinase assays revealed that **7k** inhibited the enzyme activities of Aurora A and B kinase. The cellular activity of **7k** was also tested by immunofluorescence. Histone H3, a direct substrate of Aurora B kinase, is phosphorylated on Serine 10 residue by Aurora B kinase, which becomes activated in mitosis cells.[18] Thus, detection of histone H3 phosphorylation (pHisH3) on Serine 10 reflects the activity of Aurora B kinase in cancer cells. As shown in Fig. 2, in A549 cells, the decreasing fluorescence indicated that the activity of Aurora B was inhibited by **7k** in a concentration-dependent manner.

Aurora B is a chromosomal passenger protein that is associated with the centromeres in the early stages of mitosis, later localizes to the spindle midzone and the midbody of mitosis cells. It plays a role in chromosome condensation and cytokinesis.[19] Therefore, **7k** may disturb the mitotic progression of human carcinoma cells. The morphological changes of cell nucleus were examined by using immunofluorescence staining (Fig. 3). As expected, nuclei of various sizes and shapes were also observed in **7k**-treated cells. These phenotypes are thought to be consistent with Aurora B functional repression.[20]

The coordinate for the Aurora A (PDB ID: 2W1G) and Aurora B (PDB ID: 2VGO) structures were obtained from the RCSB Protein Data Bank. Protein structures were prepared using Glide software package. In the case of compound **7k**, the X-ray crystal structure showed the ligand sitting deeply in the ATP-binding site of Aurora A/B (Fig. 4A and B). In Aurora A, the benzimidazole motif binds in the cleft defined by Ala213, Pro214, Leu215, and Gly216. The ligand participates in hydrogen-bonding interactions with NH of Arg137 and also to the backbone NH and carbonyl of Ala213 of the protein hinge region (Fig. 4A). In addition, there have no hydrogen bond to backbone carbonyl of Glu211 and also to the backbone carbonyl of Lys162 compared with binding mode of AT-9283 with Aurora A (Fig. 4C). In Aurora B, the pyrazole ring of compound **7k** makes two hydrogen bonds to NH of Ala173 and also to the backbone carbonyl of Glu171 of the protein hinge region (Fig. 4B). As unexpected, the pyrimidine ring of the compound **7k** make two extra hydrogen bonds to the backbone NH of Lys122 compared with binding mode of AT-9283 with Aurora B (Fig. 4D). This is in agreement with the Aurora A/B kinase inhibitory activity of compound **7k** (IC_{50} of Aurora A and B is 28.9 and 2.2, respectively).

As an attempt to gain a better insight into the molecular structures of compounds under investigation, conformational analysis has been performed by use of the MM+ force field (calculations in vacuo, bond dipole option for electrostatics, Polak–Ribiere algorithm, and RMS gradient of 0.01 kcal/Å mol) as implemented in HyperChem 7.0.[21] The most stable conformer was fully optimized with DFT calculation by Gaussian 03W software[22] using the B3LYP hybrid functional[23–26] and standard 6-31G (d) basis set. The calculation results showed that the lowest energy-minimized structures of the compounds under investigation exhibited a coplanar arrangement of the benzimidazole and pyrazole moiety. Such arrangement was stabilized by intramolecular hydrogen bond between the NH group and the adjacent nitrogen atom in imidazole ring (Fig. 5).

Electronic effects have also been shown to control the pharmacological activities of drugs.[27] Quantum chemical parameters in structure–activity relationships (SAR) are reported to yield promising results for correlation of biological activity.[28–30] At the molecular level, the reactivity of a molecule is dominated by the frontier molecular orbitals (FMO), namely the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). According to the FMO concept,[31] the HOMO and LUMO of a molecule play important roles in intermolecular interactions. Extending the concept to binding in drug–receptor systems, the major contribution to binding involves the interaction between the HOMO of the drug with the LUMO of the receptor and that between LUMO of the drug with the HOMO of the receptor.[32] The extents of these stabilizing interactions are inversely related to the energy gap between the interacting orbitals. Higher HOMO energy and lower LUMO energy in the drug molecule result in larger stabilizing interactions and, hence, binding with the receptor. The orbital energies of both HOMO and LUMO and their gaps, were calculated for all the molecules and are reported in

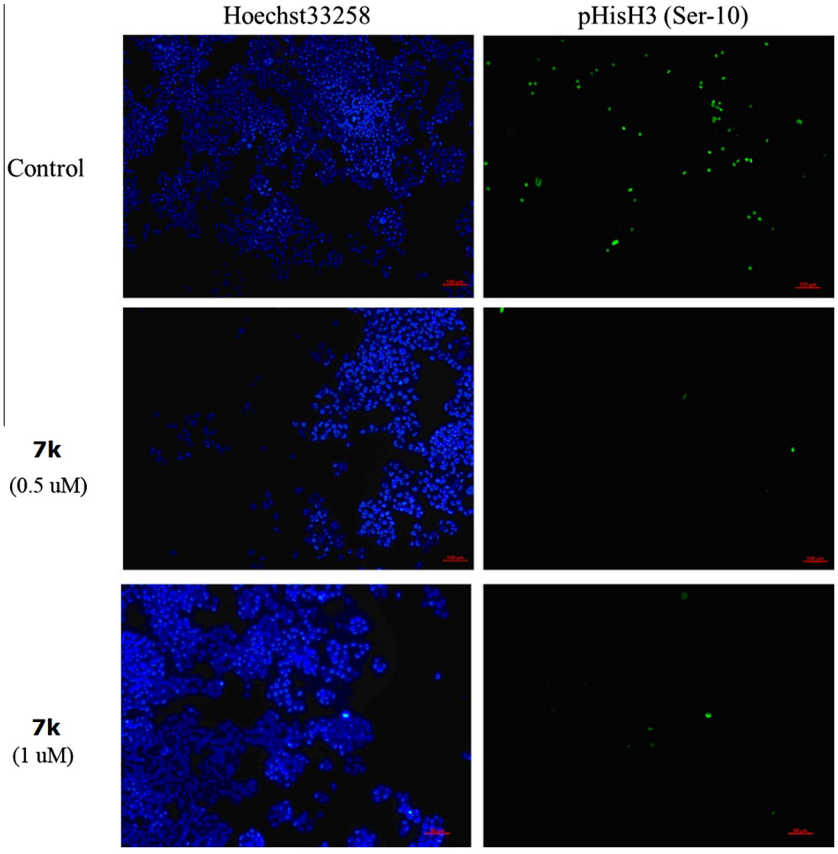


Figure 2. Analysis of the effects of AN-001 on the activity of Aurora kinases. A549 cells were treated with vehicle or **7k** at the indicated concentrations for 24 h, cells were stained to detect nucleus (blue) and phospho-H3 (green).

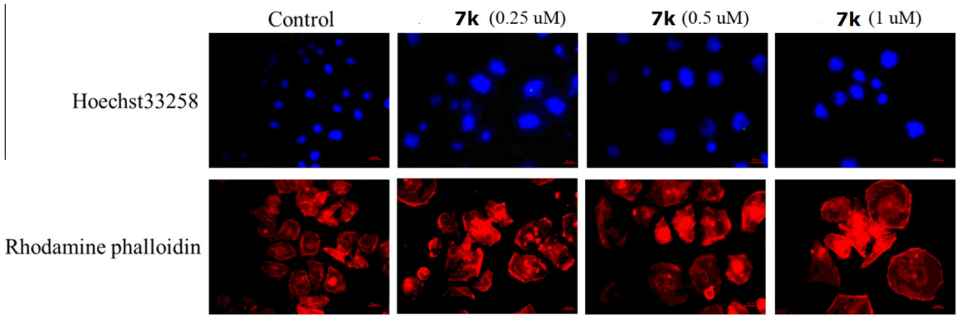


Figure 3. A549 cells treated with vehicle or **7k** were stained with Hoechst (DNA, blue) and Rhodamine phalloidin (cytoskeleton, red).

Table 3. It is remarkable that compounds **7a**, **7b, 7i** and **7k** having the lowest energy gap (ΔE) of 4.25, 4.22, 4.25 and 4.01 eV, respectively, exhibit the highest cytotoxic activity.

We also obtained a plot of the HOMO and LUMO of the molecules of each group to analyze the main atomic contributions for these orbitals. The importance of observing these plots was to determine which atoms were located at the possible sites of electronic transfer between the molecule under study and its biological target. The plots of the HOMO and LUMO of some molecules obtained from DFT calculations are displayed in Figure 6. The results illustrate that HOMO molecular orbital of **7a** is mainly located in morpholine ring, indicating the existence of a possible reactive sites; therefore, electrophilic attacks might take place on these sites. On the other hand, the LUMO of **7a** is primarily concentrated on benzimidazole and pyrazole rings in which the negatively charged polar residues of the receptor are favorable. Comparing with **7a**, the LUMO of **7e** presents similar characteristic, while the HOMO changes significantly. In this case, the HOMO is primarily located in benzimidazole, pyrazole and pyrimidine rings.

In an attempt to further understand the lower activity of compound **7e** and the higher antitumor activity of **7a**, molecular electrostatic potentials (MEPs) have been carried out for the lowest energy conformers, to examine the similarity and dissimilarity in electrostatic binding characteristic of the surface of the molecules (Fig. 7). Previous papers have used the MEP to relate the antimalarial potency of carbinolamine analogs,[33] LpxC inhibition activity of 2-aryloxazolines, aroylserines and 2-arylthiazolines,[34] and anticoagulant effect of 17β-aminoestrogens with corresponding structures.[30] Comparison of the electrostatic mappings of **7a** and **7e** show that compound **7a** possess an increased negative charge regions (in red) located on the nitrogen atoms of pyrimidine ring and the oxygen atom of morpholine ring, while the most positive

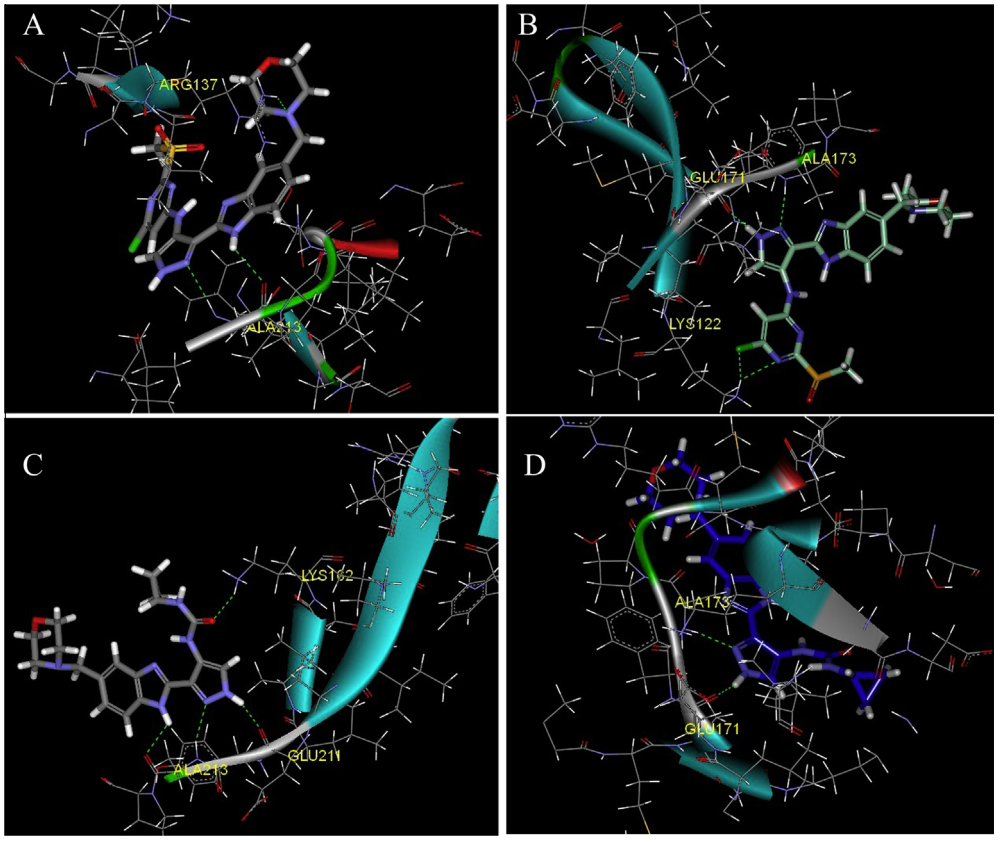


Figure 4. (A) Binding mode of compounds **7k** with Aurora A; (B) Binding mode of compounds **7k** with Aurora B; (C) Binding interaction of **AT-9283** with Aurora A; D: Binding interaction of **AT-9283** with Aurora B.

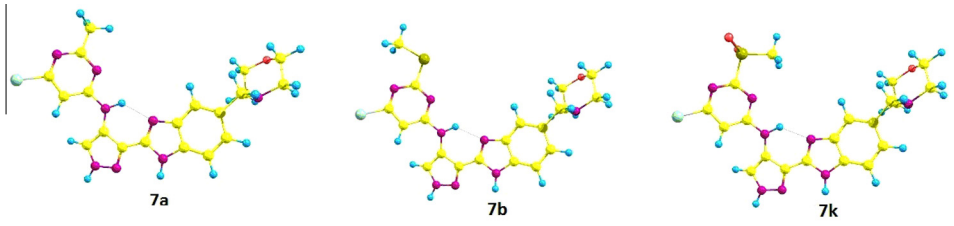


Figure 5. The optimized structures of the most active compounds **7a**, **7b** and **7k**.

Table 3
Energies of both HOMO and LUMO and their gaps (in eV) calculated for all compounds

Compd	E_{HOMO}	E_{LUMO}	ΔE
7a	−5.415	−1.166	4.25
7b	−5.389	−1.167	4.22
7c	−5.599	−1.194	4.41
7d	−5.429	−1.146	4.28
7e	−5.608	−1.112	4.5
7f	−5.635	−1.113	4.52
7g	−5.599	−1.082	4.52
7h	−5.454	−1.175	4.28
7i	−5.434	−1.180	4.25
7j	−5.507	−1.097	4.41
7k	−5.569	−1.555	4.01
7l	−5.845	−1.510	4.34
7m	−5.759	−1.516	4.24

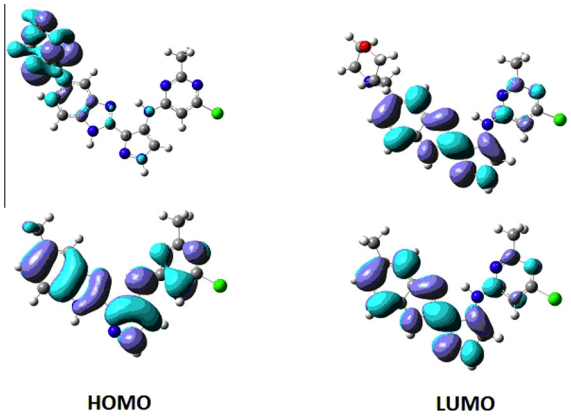


Figure 6. Plots of the HOMO and LUMO of molecules **7a** (upper) and **7e** (lower).

(in blue) on amine hydrogen atom in imidazole and pyrazole rings. This may indicate capability of hydrogen bond formation with share of nitrogen atoms of pyrimidine ring and the oxygen atom of morpholine ring or through hydrogen atom of –NH moiety. This is in agreement with the binding mode obtained from above docking study (Fig. 4). In contrast, the lower activity of compound **7e**

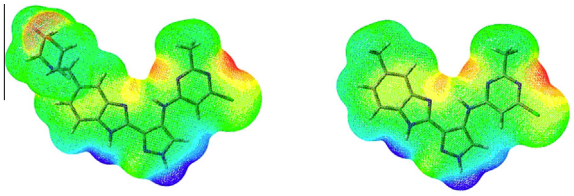


Figure 7. Molecular electrostatic potentials (MEPs) of **7a** (left) and **7e** (right), showing the most positive potential (deepest blue color), the most negative potential (deepest red color), and the intermediate potential (intermediate shades) regions.

maybe attribute to the difference in electrostatic mapping in which the red hydrogen bond area as located mainly on the nitrogen atoms of pyrimidine ring.

We designed and synthesized a series of novel pyrazole–benzimidazole derivatives which were evaluated for their in vitro antitumor activities on cancer cell lines U937, K562, A549, LoVo and HT29 and Aurora A/B kinase inhibition. The results revealed that compounds **7a**, **7b**, **7i** and **7k** demonstrated more potent antitumor activities, and the compounds **7a**, **7i** and **7l** with small group at 2-position of pyrimidine ring such as methyl, methylthio and methyl sulfonyl showed significant Aurora A/B kinase inhibitory activity. The cellular activity of **7k** was also tested by immunofluorescence, and in A549 cells, the decreasing fluorescence indicated that the activity of Aurora B was inhibited by **7k** in a concentration-dependent manner. In addition, we have studied the binding mode of compound **7k** with Aurora A crystal structure (molecular docking). The results suggested that the derivatives have bound in the active site of Aurora A kinase through the formation of four hydrogen bonds compared with AT-9283. To gain a better insight into SAR, some electronic parameters including energies and plots of HOMO and LUMO as well as plots of MEP have been obtained by quantum chemical calculation. These findings shall be useful in designing new more potent Aurora A/B kinase inhibitors. This can be achieved by incorporating such structural features into the ligands that modify the electronic properties in accordance with the results of this work.

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