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Novel aspects in structure-activity relationships of profiled 1-aza-9-oxafluorenes as inhibitors of alzheimer disease-relevant kinases cdk1, cdk5 and gsk3β

Electronic supplementary information

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Experimental protocols

1. Chemistry

Commercial reagents were used without further purification. Methoxy-1,4-benzoquinone was prepared from methoxy-1,4-hydroquinone by using sodium metaperiodate as oxidizing agent according to literature [17] by stirring at rt in an aquous solution. The ^{1}H-NMR spectra (400 MHz) were measured using tetramethylsilane as internal standard. TLC was performed on E. Merck 5554 silica gel plates. The EI mass spectra were measured with an AMD 402 mass spectrometer, the ESI spectra were recorded on a Finnigan LCQ Classic mass spectrometer. IR spectra were recorded on a FT-IR spectrometer. Elemental analysis indicated by the symbols of the elements was within ± 0.4% of the theoretical values and was performed using a Leco CHNS-932 apparatus.

*1.1. Formation of the N-acetyl-1,4-dihydropyridine **2***[16]

3-Benzyloxypyridine (12.2 g, 66 mmol), copper(I) iodide (0.65 g, 3.5 mmol) and lithium chloride (0.29 g, 6.8 mmol) were added to dried THF and the solution was cooled down to -20 °C. After 15 min of stirring 4.7 mL of acetyl chloride (5.2 g, 66 mmol) was added dropwise. After additional 15 min 33 mL of a phenylmagnesium chloride solution in THF (2M) were added. The low temperature was kept for 30 min and the mixture continued stirring. After reaching rt the solution was hydrolysed using 140 mL of a solution of ammonium chloride (20%). Then extraction followed with each 150 mL of diethylether for three times. The unified organic layers were worked up as described yielding compound **2** as a yellow oil which was purified by column chromatography over silica gel using an eluent mixture of cyclohexane/ethyl acetate (60/40).

1.2. General procedure for the formation of the 4-phenyl-1-aza-9-oxafluorenes ***4a-d***

0.85 g of *N*-acetyl 1,4-dihydropyridine **2** (2.8 mmol, 1 eq.) and the corresponding quinone derivative (1.2 eq.) were dissolved in a minimum volume of dried dioxane. Then a mixture of 10 parts of dried dioxane and 1 part of perchloric acid (70%) were added reaching a final solution reaction volume of 75 mL. After 24 h of stirring at rt the added quinone partly disappeared according to tlc analysis of the reaction mixture and additional quinone (0.4 eq.) was added. The addition of portions of quinones continued until no more tlc-detectable tetrahydro derivative was present in the reaction mixture. Then ice water was added (15 mL) and the pH of the solution was adjusted to pH = 9 using sodium hydroxide solution (1M). Then the reaction mixture was extracted four times with each 50 mL of chloroform. The organic layers were unified and dried over sodium sulfate. After filtration and evaporation the remaining oil was purified by column chromatography over silica gel using an eluent mixture of cyclohexane/ethyl acetate (80/20 for compounds **4a**, **4c** and **4d** and 60/40 for compound **4b**). The collected compound-containing fractions were evaporated yielding each compound as a powder.

1.2.1. 3-Benzyloxy-7-methyl-4-phenyl-benzo[4,5]furo[2,3-b]pyridine-6-ol ***4a***

Yield 0.11 g (10%); brownish powder; mp 200-205 °C; ^{1}H NMR (DMSO-D_6) δ 9.24 (s, 1H, OH), 8.24 (s, 1H, 2-H), 7.56-7.52 (m, 5H, 4-C_6H_5), 7.40 (s, 1H, 8-H), 7.29-7.23 (m, 5H, CH_2-C_6**H**$_5$), 6.60 (s, 1H, 5-H), 5,12 (s, 2H, C**H**$_2$-C_6H_5), 2.19 (s, 3H, CH_3); MS (ESI), *m/z* = 382 [M + H^+]; IR (KBr): 3169, 3061, 2929, 2876, 1628, 1593, 1583, 1375, 1358, 1265, 1249, 1232 cm^{-1}. Anal. ($C_{25}H_{19}NO_3$) Calc. C 78.72, H 5.02, N 3.67; Found C 78.43, H 4.77, N 3.32.

1.2.2. 3-Benzyloxy-7-methoxy-4-phenyl-benzo[4,5]furo[2,3-b]pyridine-6-ol ***4b***

Yield 0.11 g (10%); brownish powder; mp 215-220 °C; ^{1}H NMR (DMSO-D_6) δ 9.13 (s, 1H, OH), 8.35 (s, 1H, 2-H), 7.96 (d, *J* = 8.1 Hz, 2H, 2-H and 6-H of 4-C_6H_5), 7.56-7.52 (m, 10H, 8-H, 5-H, 3-H and 4-H and 5-H of 4-C_6H_5, CH_2-C_6**H**$_5$), 5,28 (s, 2H, C**H**$_2$-C_6H_5), 3.90 (s, 3H, OCH_3); MS (ESI), *m/z* = 398 [M + H^+]; IR (KBr): 3175, 3004, 1629, 1398, 1260, 1253, 1224 cm^{-1}. Anal. ($C_{25}H_{19}NO_4$) Calc. C 75.56, H 4.82, N 3.52; Found C 75.18, H 4.65, N 3.16.

1.2.3. 3-Benzyloxy-5,7-dimethoxy-4-phenyl-benzo[4,5]furo[2,3-b]pyridine-6-ol ***4c***

Yield 0.12 g (10%); brownish powder; mp 132-135 °C; ^{1}H NMR (DMSO-D_6) δ 8.66 (s, 1H, OH), 7.98 (s, 1H, 2-H), 7.94 (d, *J* = 7.1 Hz, 2H, 2-H and 6-H of 4-C_6H_5), 7.45-7.34 (m, 5H, CH_2-C_6**H**$_5$), 7.30-7.27 (m, 3H, 3-H, 4-H and 5-H of 4-C_6H_5), 7.14 (s, 1H, 8-H), 5.30 (s, 2H, C**H**$_2$-C_6H_5), 3.95, 3.88 (2 x s, 6H, OCH_3); MS (ESI), *m/z* = 428 [M + H^+]; IR (KBr): 3535, 1636, 1598, 1385, 1362, 1265, 1234 cm^{-1}. Anal. ($C_{26}H_{21}NO_5$) Calc. C 73.06, H 4.95, N 3.28; Found C 72.82, H 4.65, N 2.95.

1.2.4. 3-Benzyloxy-4-phenyl-naphtho[1´,2´:4,5]furo[2,3-b]pyridine-6-ol ***4d***

Yield 0.17 g (15%); brownish powder; mp 219-222 °C; ^{1}H NMR (DMSO-D_6) δ 10.06 (s, 1H, OH), 8.36 (s, 1H, 2-H), 8.30 (d, *J* = 8.3 Hz, 1H, 7-H), 8.21 (d, *J* = 8.3 Hz, 1H, 10-H), 7.71 (t, *J* = 8.3 Hz, 2H, 8-H, 9-H), 7.63-7.59 (m, 5H, 4-C_6H_5), 7.32-7.28 (m, 5H, CH_2-C_6**H**$_5$), 6.63 (s, 1H, 5-H), 5.20 (s, 2H, C**H**$_2$-C_6H_5); MS (ESI), *m/z* = 418 [M + H^+]; IR (KBr): 3170, 3056, 3028, 2982, 2917, 1596, 1583, 1374, 1260, 1228 cm^{-1}. Anal. ($C_{28}H_{19}NO_3$) Calc. C 80.49, H 4.59, N 3.36; Found C 80.13, H 4.25, N 2.99.

*1.3. General procedure for the formation of the 6-methoxy-4-phenyl-1-aza-9-oxafluorenes **4e** and **4f***

1 eq. of the corresponding 6-hydroxy-1-aza-9-oxafluorene (0.048 mmol) was dissolved in dried THF (2 mL) under argon atmosphere. The solution was cooled down to 0 °C in an ice bath. Then a 7-molar excess of sodium hydride (0.008 g, 0.34 mmol) in paraffin oil (60%) was added. After stirring for 15 min the suspension was warmed-up to rt and stirring continued. Then a 3-molar excess of methyl iodide (0.020 g, 0.14 mmol) with reference to one hydroxy function was added. After stirring for 9 h at rt the reaction mixture was poured into ice water (2 mL) and extracted with chloroform (10/10/5/5 mL) for four times. After drying over sodium sulfate the solvent was removed in vacuum and the remaining oil was purified by column chromatography over silica gel using an eluent mixture of chloroform and ethyl acetate (85/15) for **4e** and of cyclohexane and ethyl acetate (60/40) for **4f**. The collected fractions were evaporated to dryness and the residual oil was dissolved in diethyl ether from which **4e** crystallized, while **4f** crystallized from dried diethyl ether and petrol ether.

*1.3.1. 3-Benzyloxy-6-methoxy-4-phenyl-naphtho[1´,2´:4,5]furo[2,3-b]pyridine **4e***

Yield 0.20 g (95%); fawn powder; mp 173-176 °C; ^{1}H NMR ($CDCl_3$) δ 8.42 (d, J = 8.2 Hz, 1H, 10-H), 8.29 (d, J = 8.4 Hz, 1H, 7-H), 8.23 (s, 1H, 2-H), 7.69-7.54 (m, 7H, 4-C_6H_5, 8-H, 9-H), 7.34-7.22 (m, 5H, CH_2-C_6**H**$_5$), 6.53 (s, 1H, 5-H), 5.09 (s, 2H, C**H**$_2$-C_6H_5), 3.74 (s, 3H, 6-CH_3O); MS (ESI), *m/z* = 432 [M + H$^+$]; IR (KBr): 2961, 1599, 1444, 1414, 1381, 1259, 1089, 1050 cm^{-1}. Anal. ($C_{29}H_{21}NO_3$) Calc. C 80.73, H 4.91, N 3.25; Found C 80.55, H 4.85, N 3.21.

*1.3.2. 3-Benzyloxy-6-methoxy-4-phenyl-benzo[4,5]furo[2,3-b]pyridine **4f***

Yield 0.17 g (92%); fawn powder; mp 112-113 °C; ^{1}H NMR (acetone-d_6) δ 8.33 (s, 1H, 2-H), 7.66-7.57 (m, 5H, 4-C_6H_5), 7.54 (d, J = 9.0 Hz, 1H, 8-H), 7.34-7.28 (m, 5H, CH_2-C_6**H**$_5$), 7.11 (dd, J = 9.0, 2.7 Hz, 1H, 7-H), 6.69 (d, J = 2.7 Hz, 1H, 5-H), 5.20 (s, 2H, C**H**$_2$-C_6H_5), 3.63 (s, 3H, 6-CH_3O); MS (ESI), m/z = 382 [M + H^+]; IR (KBr): 2923, 1587, 1471, 1440, 1358, 1274, 1224, 1194, 1092, 1025 cm^{-1}. Anal. ($C_{25}H_{19}NO_3$) Calc. C 78.73, H 5.02, N 3.67; Found C 78.55, H 4.95, N 3.38.

1.4. General procedure for the formation of the 3-amide-4-phenyl-1-aza-9-oxafluorenes ***6a-d***

50 Mg of 1-aza-9-oxafluorene **1** with R = COOEt (0.15 mmol) were dissolved in 10 mL of dried methanol. Then 20 mL of the respective amine solution (40%) in water were added. The mixture was left standing in the refrigerator at a temperature of -8 °C until no more starting compound was detectable by tlc. Then 30 mL of water were added and the solution was neutralized with hydrochloric acid (10%). Then extraction with 75 mL of $CHCl_3$ followed accept for derivative **6d** which was extracted with ethyl acetate for three times. After drying over sodium sulfate the solvent was removed and the remaining oil was purified by column chromatography over silica gel accept for derivatives **6a** and **6b** which crystallized under stirring from methanol (**6a**) and diethyl ether (**6b**) and cooling at a low temperature of -5 °C. The eluent mixtures for column chromatography consisted of $CHCl_3$/ethyl acetate/methanol (85:15:5) for **6c** and in a corresponding mixture relation of 85:15:30 for **6d**. The united organic layers were dried over sodium sulfate and the residue was crystallized under addition of diethyl ether.

1.4.1. 6-Hydroxy-N-methyl-4-phenyl-benzo[4,5]furo[2,3-b]pyridine-3-carboxamide ***6a***

Yield 0.031 g (65%); yellow needles; mp 285-290 °C; ^{1}H NMR (DMSO-D_6) δ 9.41 (s, 1H, OH), 8.44 (s, 1H, 2-H), 8.24 (q br, *J* = 4.7 Hz, 1H, CON**H**CH_3), 7.57-7.55 (m, 4H, 8-H, 3-H, 4-H and 5-H of 4-C_6H_5), 7.47-7.45 (m, 2H, 2-H and 6-H of C_6H_5), 6.95 (dd, *J* = 8.9, 2.5 Hz, 1H, 7-H), 6.45 (d, *J* = 2.5 Hz, 1H, 5-H), 2.58 (d, *J* = 4.7 Hz, 3H, CH_3); MS (EI), *m/z* = 318 [M]; IR (KBr): 3419, 1677, 1642, 1382, 1359 cm^{-1}. Anal. ($C_{19}H_{14}N_2O_3$) Calc. C 71.69, H 4.43, N 8.80; Found C 71.55, H 4.41, N 8.64.

1.4.2. N-Ethyl-6-hydroxy-4-phenyl-benzo[4,5]furo[2,3-b]pyridine-3-carboxamide ***6b***

Yield 0.033 g (66%); yellow powder; mp 265-268 °C; ^{1}H NMR (DMSO-D_6) δ 9.44 (s br, 1H, OH), 8.44 (s, 1H, 2-H), 8.22 (t br, *J* = 5.3 Hz, 1H, CON**H**CH_2CH_3), 7.60-7.56 (m, 4H, 8-H, 3-H, 4-H and 5-H of 4-C_6H_5), 7.51-7.47 (m, 2H, 2-H and 6-H of C_6H_5), 6.96 (dd, *J* = 8.8, 2.5 Hz, 1H, 7-H), 6.50 (d, *J* = 2.5 Hz, 1H, 5-H), 3.06 (dq, *J* = 7.2, 5.3 Hz, 2H, CONHC**H**$_2$$CH_3$), 0.84 (t, *J* = 7.2 Hz, 3H, $CONHCH_2C$**H**$_3$); MS (ESI), *m/z* = 331 [M-H$^+$]; IR (KBr): 3338, 3159, 2983, 2939, 2873, 1642, 1586, 1567, 1545, 1447, 1382, 1350, 1381, 1262, 1108 cm^{-1}. Anal. ($C_{20}H_{16}N_2O_3$) Calc. C 72.28, H 4.85, N 8.43; Found C 71.93, H 4.62, N 8.38.

1.4.3. 6-Hydroxy-4-phenyl-N-propyl-benzo[4,5]furo[2,3-b]pyridine-3-carboxamide ***6c***

Yield 0.017 g (33%); yellow powder; mp 226-228 °C; ^{1}H NMR (DMSO-D_6) δ 9.43 (s br, 1H, OH), 8.44 (s, 1H, 2-H), 8.24 (t br, *J* = 5.6 Hz, 1H, CON**H**$CH_2CH_2CH_3$), 7.58-7.56 (m, 4H, 8-H, 3-H, 4-H and 5-H of 4-C_6H_5), 7.51-7.48 (m, 2H, 2-H and 6-H of C_6H_5), 6.97 (dd, *J* = 8.9, 2.5 Hz, 1H, 7-H), 6.49 (d, *J* = 2.5 Hz, 1H, 5-H), 2.97 (dt, *J* = 7.1, 5.6 Hz, 2H, CONHC**H**$_2$$CH_2CH_3$), 1.30-1.20 (m, 2H, $CONHCH_2C$**H**$_2$$CH_3$), 0.69 (t, *J* = 7.4 Hz, 3H, $CONHCH_2C$**H**$_3$); MS (ESI), *m/z* = 347 [M+H$^+$]; IR (KBr): 3244, 2964, 2934, 2878, 1644,

1586, 1540, 1446, 1379, 1283, 1258, 1175 cm^{-1}. Anal. ($C_{21}H_{18}N_2O_3$) Calc. C 72.61, H 5.22, N 8.06; Found C 72.55, H 5.15, N 7.85.

1.4.4. N-2-(6-Hydroxy-4-phenyl-benzo[4,5]furo[2,3-b]pyridine-3-carboxamide)ethylamine ***6d***

Yield 0.010 g (20%); yellow crystals; mp 248-253 °C; ^{1}H NMR (DMSO-D_6) δ 8.54 (s, 1H, 2-H), 8.11 (s br, 1H, OH), 7.61-7.52 (m, 6H, 2-H, 3-H, 4-H, 5-H and 6-H of 4-C_6H_5, CON**H**$CH_2CH_2CH_3$), 7.49 (d, *J* = 8.8 Hz, 1H, 8-H), 7.03 (dd, *J* = 8.8, 2.5 Hz, 1H, 7-H), 6.58 (d, *J* = 2.5 Hz, 1H, 5-H), 3.40-3.33 (m, 2H, CONHC**H**$_2CH_2NH_2$), 3.28-3.22 (m, 2H, $CONHCH_2C$**H**$_2NH_2$), 3.01 (t, *J* = 6.5 Hz, 2H, $CONHCH_2CH_2N$**H**$_2$); MS (ESI), *m/z* = 348 [$M+H^+$]; IR (KBr): 3280, 2928, 1647, 1585, 1550, 1468, 1448, 1381, 1283, 1258, 1130 cm^{-1}. Anal. ($C_{20}H_{17}N_3O_3$) Calc. C 69.15, H 4.93, N 12.10; Found C 68.97, H 4.76, N 11.88.

2. Protein kinase affinity determination

The protein kinases were all expressed in Sf9 insect cells as human recombinant GST fusion proteins and purified by affinity chromatography using GSH-agarose. The kinase purity was finally checked by SDS-PAGE/Coomassie staining.

2..1. Assay conditions for affinity determinations

The measuring of protein kinase activity was performed in 96-well FlashPlates^{TM} in a 50 µL reaction volume. The reaction mixture consisted of 20 µL of assay buffer solution, 5 µL of ATP solution in water, 5 µL of used test compound in a 10% dmso solution and finally a premixture of each 10 µL of used substrate and enzyme solutions. The assay buffer solution contained 70 mM of HEPES-NAOH, each 3 mM of magnesium chloride and manganese(II)

chloride, 3 µM of sodium orthovanadate, 1.2 mM of DTT, 50 µg/mL of PEG20000 and finally 15 µM of [γ-^{33}P]-ATP making approximately 1.2 x 10^6 cpm per well.

The final kinase concentrations have been 3.5 nM for CDK1/cycline B, 3.3 nM for CDK5/p25, and finally 13.1 nM for GSK-3β. The substrate was RBER-CHKtide using amounts of 2000 ng/50 µL in the case of CDK1/cycline B and 1000 ng/50 µL in the case of CDK5/p25 and GSK-3β.

The reaction mixtures were incubated at 30 °C for 60 min. The reaction was stopped with 50 µL of a 2% (v/v) solution of phosphoric acid. Then the plates were aspirated and washed twice with 200 µL of water or 0.9% solution of sodium chloride. The incorporation of ^{33}Pi was determined with a microplate scintillation counter. The K_i values of protein kinase affinities have been calculated from the determined IC_{50} values using the equation: IC50 = ½ $[E_{total}] + K_i$ x $(1 + [S]/K_m)$.

3. Computational methods

3.1. Molecular docking

The inhibitor structures were built and energy minimized using MOE2010.10 (Chemical Computing Group, Inc.: Montreal, Canada, 2010) and the MMFF94 force field. The X-ray structures of cdk5 (PDB code 1UNL) and of gsk3β (PDB code 3GB2) were taken from the Protein Databank and the homology model of cdk1 was built using the closely related cdk2 X-ray structure in complex with a diaminopyrimidine inhibitor (PDB code 2FVD). Hydrogen atoms were added and the protein structure was minimized using the MMFF94 force field and position restraints on backbone atoms (force constant of 100 kcal/mol). Docking was carried out using the software GOLD 5.0 (Cambridge Crystallographic Data Centre, Cambridge, UK, 2011). Cys83(cdk5)/Leu83(cdk1)/Val135(gk3β) (located at the hinge region) were defined as

center of the binding site with a radius of 15 Å. Water molecules located at the binding pocket were considered for docking using the 'toggle' mode within GOLD. Goldscore was used as scoring function to rank all docking poses.

3.2. Moelcular dynamics simulations

The docking solutions of compounds **4d** and **4f** with cdk1, cdk5 and gsk3β were further subjected to molecular dynamics (MD) simulations. Before performing MD simulations the complexes were first prepared by assigning force field and charges for ligand (Amber Force Field (GAFF) together with AM1-BCC charges) and proteins (Amber03 force field) using the Leap module in AMBER11. The complexes were solvated by TIP3P waters and counter ions in the radius within 10 Å from the molecular surface. To relax the system two steps of energy minimization (minimization of the solvation following by minimizing the whole system) were performed. After that the position-restrained phase of MD simulation was carried out by restraining the positions of the complex with the weak force constraint (10 kcal/mol) during the first 100 ps. The temperature of the system was gradually increased from 0 to 300 K during the first few picosecond and then it was kept fix at 300 K by applying Langevin dynamics with a collision frequency of 1 ps^{-1}. In the final step free MD simulation was carried out from 100 ps to 20 ns. The NPT ensemble (a constant pressure at 1 bar and the constant temperature at 300 K) were applied for the free MD period and a time step of 2 fs with SHAKE algorithm were also applied. Particle-Mesh-Ewald (PME) method was used for calculating electrostatic interaction, while non-bonded interaction was computed by applying a cut-off radius at 10 Å.

3.3. Binding mode analysis

To investigate the stability of derived docking complexes, MD simulations of compounds **4d** and **4f** with cdk1, cdk5 and gsk3β were employed for 20 ns. The stability of the complexes was checked by plotting the root mean square deviation (RMSD) values compared to the starting structure during the simulations. As shown in Figure S1, the RMSD values of these complexes fluctuate only slightly indicating the stability of the kinase-inhibitor complex. The percent of hydrogen bond occupation during the simulation were computed in addition. As summarized in Table 1 results revealed that the interaction between the pyridine ring and Cys83 (cdk5), Leu83 (cdk1) as well as Val135 (gsk3β) remained stable. Percent hydrogen bond occupation between OH (**4d**) and the backbone CO of Ile10 of cdk1 (29.87%) and of cdk5 (37.14%) and of Ile62 of gsk3β (38.99%) were found to be lower. The calculated average distance of the hydrogen bond is 3.56 Å for cdk1, 3.50 Å for cdk5 and 3.52 Å for gsk3β. On the other hand, the distance between the oxygen atom of the methoxyl group of compound **4f** and the backbone CO of Ile10 was found to be around 4.5 Å. The distance between the 6-methoxy group of compound **4f** and the *N*H atom of side chain of Arg141 (gsk3β) was stable after 10 ns of the simulation, fluctuating in the range around 3.0 Å (Figure S2), while the distance between this atom and ε-*N* atom of Lys89 (cdk1/cdk5) was not stable during the whole simulations indicating that a hydrogen bond is unlikely for these two kinases.

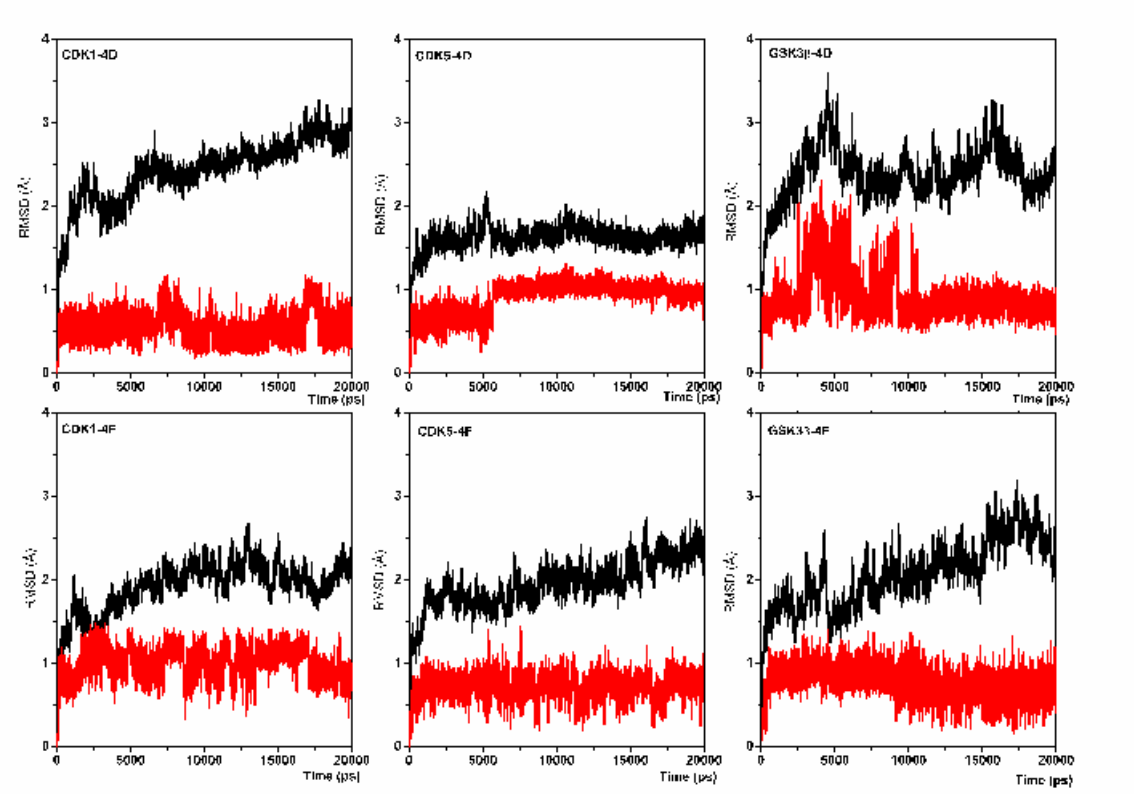

Figure S1. Root mean square deviation (RMSD) of simulated kinase-inhibitor complexes (black line - backbone atoms of protein and red line - heavy atom of inhibitors). All complexes remained stable during the whole simulation period.

Table 1. Percentage of H-bond occupancy between inhibitor and protein.

complex	OH – CO Ile10 (cdk1/cdk5) OH – CO Ile62 (gsk3β)	N (pyridine)-NH Leu83 (cdk1) / N (pyridine)-NH Cys83 (cdk5) / N (pyridine)-NH Val135 (gsk3β)
cdk1/**4d**	29.87	86.94
cdk1/**4f**	-	94.66
cdk5/**4d**	37.14	90.43
cdk5/**4f**	-	95.79
gsk3β/**4d**	38.99	93.10
gsk3β/**4f**	-	96.26

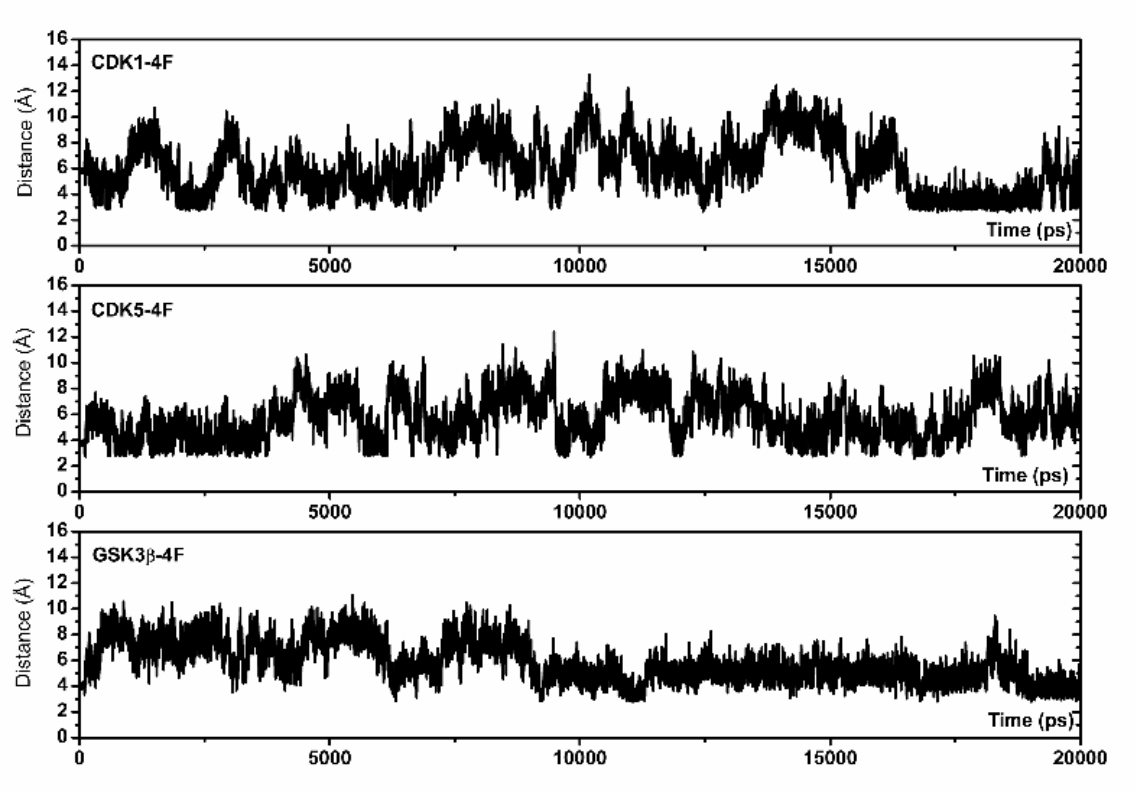

Figure S2. Distance between the 6-methoxy group of compound **4f** and the ε-*N* of Lys89 (cdk1/cdk5) and the *N*H of Arg141 (gsk3β) during the MD simulation.