SUPPLEMENTAL DATA

Re-engineering of anticancer C-Kit kinase inhibitor promoting activity over toxicity: From *in silico* to *in vivo* models

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A) Calculation of local dehydration propensities

The local mean residence time, $\langle\tau\rangle_i$, of hydrating molecules at residue *i* is defined with respect to a spherical domain *D(i)* of 6Å-radius centered at the α-carbon of residue *i*. The actual computation of residence times is given as:

$$\langle\tau\rangle_i = \int\tau f_i(\tau)d\tau/(\int f_i(\tau)d\tau),\ \int_{0\le\tau'\le\tau} f_i(\tau')d\tau' = P_i(0)-P_i(\tau),$$
$$P_i(\tau) = \Theta^{-1}\int_{0\le t\le\Theta}[\sum_{\substack{v(t)\in U(i,t)\\ w(t+\tau)\in U(i,t+\tau)}} \delta(v(t), w(t+\tau))]dt\ ; \qquad (1)$$

where $f_i(\tau)d\tau/\int f_i(\tau)d\tau$ is the expected fraction of water molecules that remained in D(i) for a period τ and exit D(i) within a period of time in the range $(\tau, \tau+d\tau]$; $P_i(\tau)$ is the expected number of water molecules remaining in D(i) after a period τ has elapsed; v(t), w(t+τ) denote indexes labeling water molecules contained in D(i) at times t and t+τ, respectively; U(i,t), U(i,t+τ) denote collection of indexes of water molecules contained in D(i) at times t and t+τ, respectively; δ is the Kronecker symbol (δ(v(t), w(t+τ))=1 if v(t)=w(t+τ) and 0, otherwise); and the integration over variable t is carried out over the interval of sampled times (t=0 to t=Θ=10ns) after 50ns of prior equilibration (the sampling is considered exhaustive since $\langle\tau\rangle \ll \Theta$ for all residues).

Molecular dynamics. In order to determine mean residence times of hydrating molecules at protein interfaces, classical molecular dynamic (MD) simulations[1] were performed using crystal-structure coordinates of protein-inhibitor complexes. We performed simulations of an uncomplexed free form of the kinases equilibrated for 50ns after *in silico* dissociation from the crystallized complex. The initial structures were immersed in a pre-equilibrated truncated octahedral cell of TIP3P explicit water molecules[1] and Cl^- ions were added to neutralize the system using the LEAP module of AMBER, version 9[2-4]. Protein atoms were described with the parm99SB force field parameterization[5]. Water

molecules extended at least 8Å from the surface of the protein. The PMEMD module of AMBER was used to perform the simulations in the NPT ensemble, employing periodic boundary conditions. Ewald sums[6] and a 8Å distance cutoff were used for treating long-range electrostatic interactions. A SHAKE algorithm was employed to keep bonds involving hydrogen atoms at their equilibrium length[7], which allowed us to employ a 2 fs time step for the integration of Newton's equations. Constant pressure of 1 atm and temperature of 300K was maintained using the Berendsen coupling scheme[8]. The optimized systems were heated to 300 K and equilibrated for 50ns. The resulting structures were adopted as starting point for the 10ns MD runs.

References

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Figure A. 1. Hydrogen-bond matrix for the C-Kit residues in contact with imatinib (PDB.1T46). Hydrogen bonds involving dehydration-prone residues are marked in green, whereas those engaging residues not favoring de-wetting are marked in grey. Residue *i* is in contact with the ligand if an atom of the latter lies within *D(i)*.

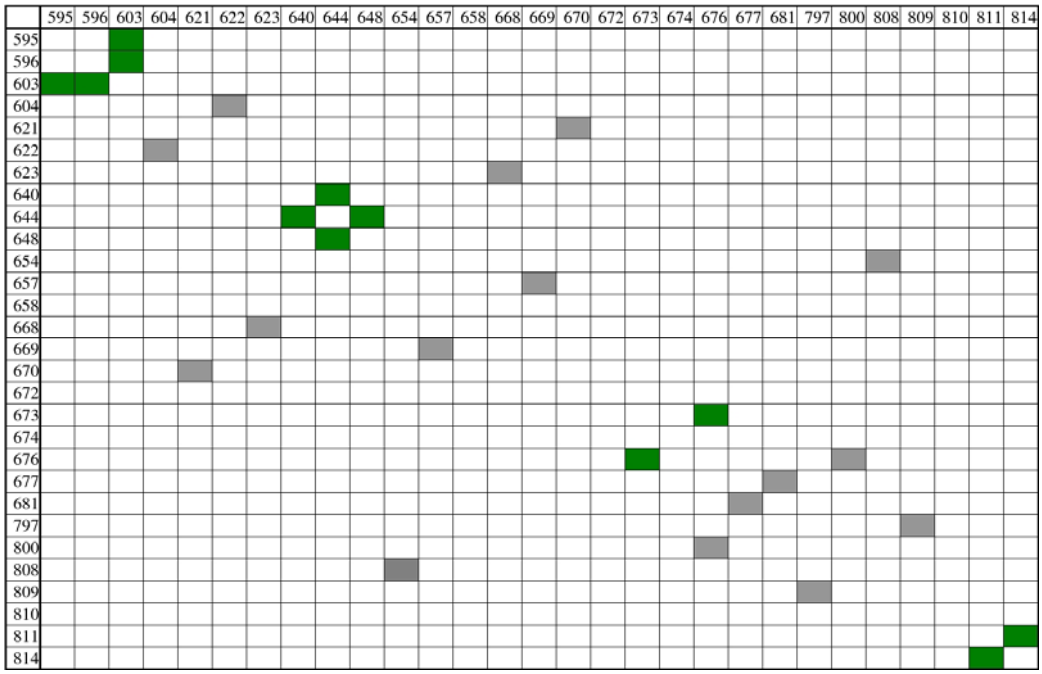


Figure A. 2. Aligned hydrogen bond matrix for Bcr-Abl (PDB.1FPU). De-wetting hot spots are marked in red.

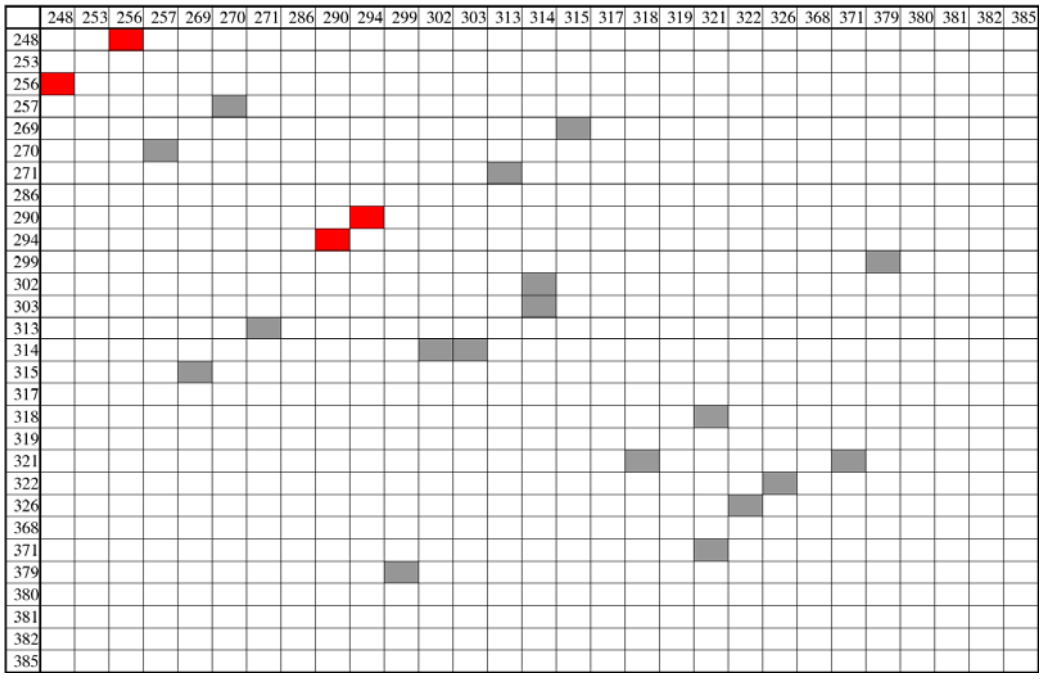
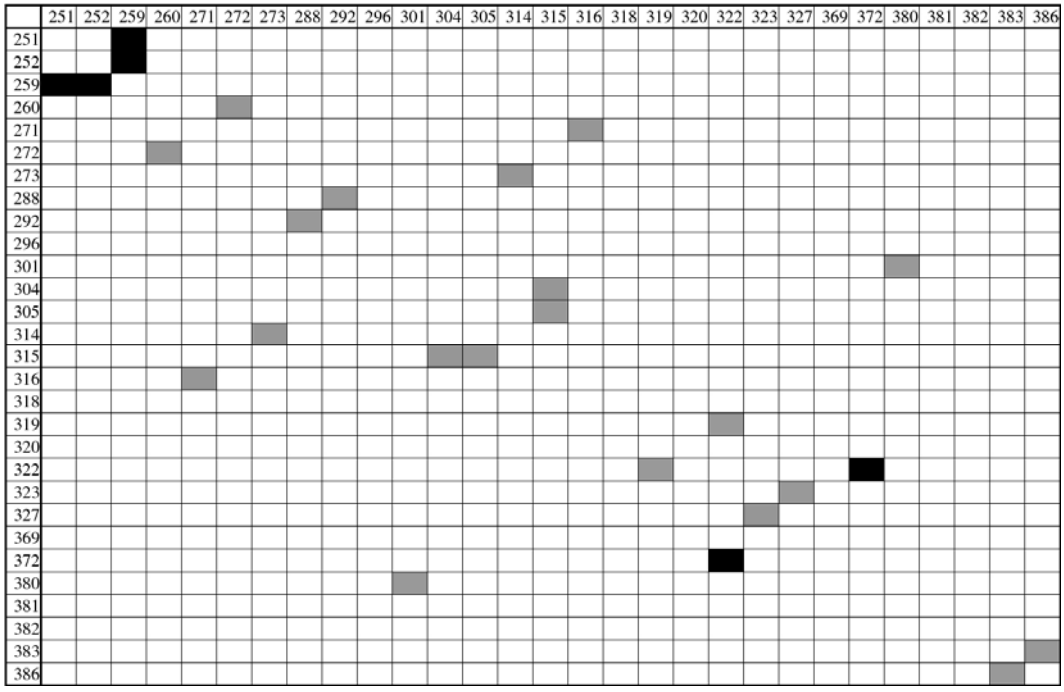


Figure A. 3. Aligned hydrogen bond matrix for Lck (PDB.3LCK). De-wetting hot spots are marked in black.

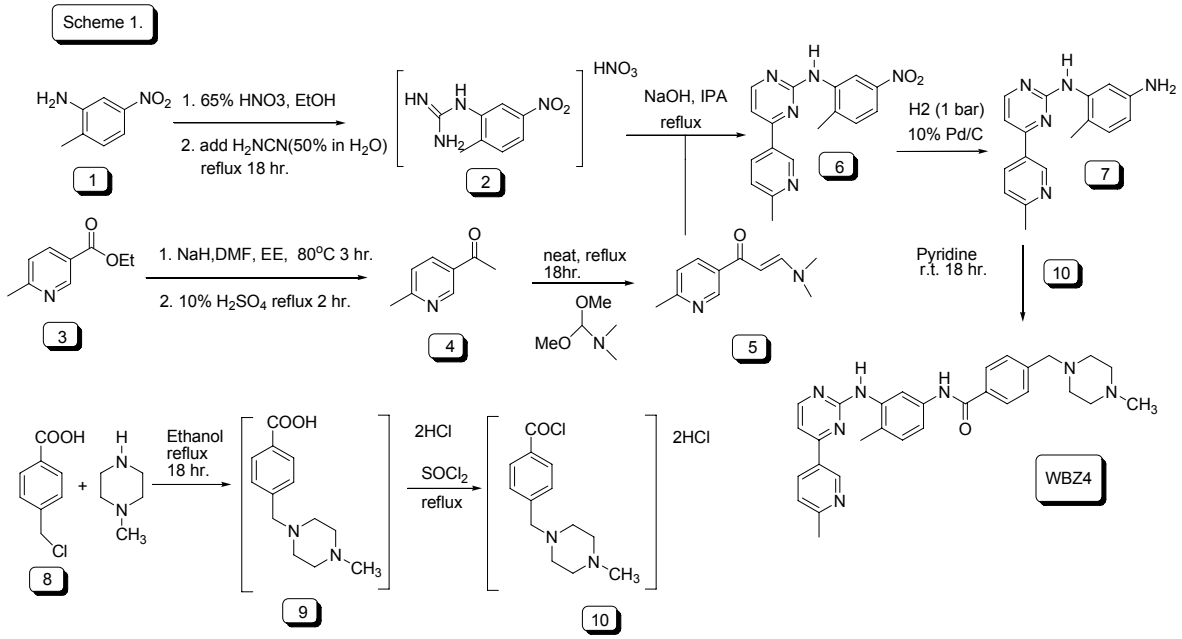


B) Molecular dynamics simulation of protein-ligand interactions

The protein-ligand interacting system is partitioned into a reaction zone and a reservoir region, and the reaction zone is further separated into a reaction region and a buffer region. The central point for partitioning was chosen to be at the substitution carbon atom on the imatinib molecule. The reaction region around the active site is defined by a sphere of radius r=14Å, the buffer region is defined by the sector: 14Å < r <16Å, and the reservoir region, by r > 16Å. All the atoms in the reservoir region were omitted. For C-Kit kinase, the final simulation system had 84 protein residues, an imatinib molecule initially positioned as in PDB entry 1T46, and 239 water molecules. For Bcr-Abl kinase (PDB.1FPU), the final simulation system had 90 protein residues, an imatinib molecule, and 300 water molecules. Atoms inside the reaction region were propagated by molecular dynamics, and atoms in the buffer region were propagated by the Langevin dynamics and harmonically restrained by forces derived from the temperature factors in crystal structures. Water molecules were confined to the active-site region by a deformable boundary potential. The friction constant in the Langevin

dynamics was 250 ps^{-1} for protein atoms and 62 ps^{-1} for water molecules. A 1fs time step was used for integrating the equations of motion. A Boltzmann distribution of initial random velocities was adopted. The system was equilibrated for 50ps at 300K, and was then followed through a 1ns-run. Five 1ns-trajectories were generated for each inhibitor-protein complexation.

C) Total Synthesis of WBZ_4



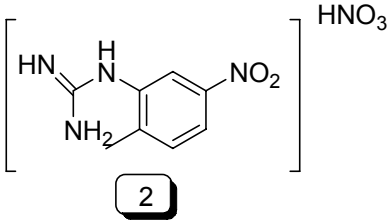
The synthesis begins with treatment of 2-methyl-5-nitroaniline (1) with 65% nitric acid in ethanol followed by the addition of cyanoamide to give the corresponding 2-methyl-5-nitroaniline-guanidine nitrate (2). One completed, the nicotinate (3) was first treated with sodium hydrate and refluxed with ethyl acetate to form methyl 6-methylnicotinylacetate. The intermediate acetate was then hydrolyzed to form 3-Acetyl-6-methylpyridine(4)[1]. The product (4) was treated with methyl dimethoxyforamide to give 3-dimethylamino-1-(3-(6-methyl-pyridyl)-2-propene-1-one (5). The nitrate salt (2) is treated with (5) and sodium hydroxide in refluxing isopropanol to give N-(2-Methyl-5-nitrophenyl)-4-(3-(6-methyl-pyridyl))-2-pyrimidine-amine (6) which is subsequently hydrogenated with 10% palladium on carbon to give N-(2-Methyl-5-aminophenyl)-4-(3-6-methyl-pyridyl)-2-pyrimidine-amine (7). The WBZ4 synthesis will consist of the reaction of α-chloro-p-toluylic acid (8) with 4-methyl-piperazine in ethanol followed by treatment with con. HCl to give the corresponding dihydrochloride 4-(4-methyl-piperazin-1-ylmethyl)-benzoic acid (9) which is subsequently treated with thionyl chloride to give the

corresponding acid chloride dihydrochloride (10). Subsequent condensation with N-(2-Methyl-5-aminophenyl)-4-(3-(6-methyl)-pyridyl)-2-pyrimidine-amine (7) in pyridine affords the imatinib analog WBZ4[2].

Material and Methods:

All chemicals and solvents were obtained from Sigma-Aldrich (Milwaukee, WI) of Fisher Scientific (Pittsburg, PA) and used without further purification. Analytical HPLC was performed on a Varian Prostar system, with a Varian Microsorb-MW C18 column (250 X 4.6 mm; 5 μ) using the following solvent system A= H_2O /0.1% TFA and B=acetonitrile/0.1% TFA. Varian Prepstar preparative system equipped with a Prep Microsorb–MWC18 column (250 X 41.4 mm; 6μ; 60 Å) was used for preparative HPLC with the same solvent systems. Mass spectra (ionspray, a variation of electrospray) were acquired on an Applied Biosystems Q-trap 2000 LC-MS-MS. UV was measured on Perkin Elmer Lambda 25 UV/Vis spectrometer. IR was measured on Perkin Elmer Spectra One FT-IR spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Brucker Biospin spectrometer with a B-ACS 60 autosampler. (600.13 MHz for ^{1}H-NMR and 150.92 MHz for ^{13}C-NMR), Chemical shifts (δ) are determined relative to d4-methanol (referenced to 3.34 ppm (δ) for ^{1}H-NMR and 49.86 ppm for ^{13}C-NMR). Proton-proton coupling constants (J) are given in Hertz and spectral splitting patterns are designated as singlet (s), doublet (d), triplet (t), quadruplet (q), multiplet or overlapped (m), and broad (br). Flash chromatography was performed using Merk silica gel 60 (mesh size 230-400 ASTM) or using an Isco (Lincon, NE) combiFlash Companion or SQ16x flash chromatography system with RediSep columns (normal phase silica gel (mesh size 230-400ASTM) and Fisher Optima TM grade solvents. Thin-layer chromatography (TLC) was performed on E.Merk (Darmstadt, Germany) silica gel F-254 aluminum-backed plates with visualization under UV (254nm) and by staining with potassium permanganate or ceric ammonium molybdate.

2-methyl-5-nitrophenyl-guanidine nitrate(2)[3]



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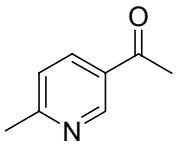
2-Methyl-5-nitroaniline (100 g, 0.657 mol) was dissolved in ethanol (250 ml), and 65% aqueous nitric acid solution (48 ml, 0.65 mol) was added thereto. When the exothermic reaction was stopped, cyanamide (41.4 g) dissolved in water (41.4 g) was added thereto. The brown mixture was reacted under reflux for 24 hours. The reaction mixture was cooled to 0° C., filtered, and washed with ethanol:diethyl ether(1:1, v/v) to give 2-methyl-5-nitrophenyl-guanidine nitrate (98

g). R_f=0.1 (Methylene chloride:Methanol:25% Aqueous ammonia=150:10:1). MS: 195.2 (M+H); ^{1}H-NMR(DMSO-d_6)=1.43(s, 3H), 6.59(s, 3H), 6.72-6.76(d, 1H), 7.21-7.27(m, 1H), 8.63-8.64(br, 1H).

3-Acetyl-6-methylpyridine (4)[1]



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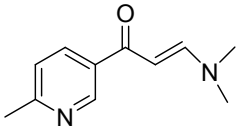
To a suspension of sodium hydride (5.2 g of a 60%, w/w, oil dispersion, 66 mmol) in toluene (80 mL) and *N*,*N*-dimethylformamide (6.6 mL) was added approximately 10% of a solution of methyl 5-methyl-nicotinate (10 g, 66 mmol) in ethyl acetate (14 mL), and the mixture was heated at 80 °C for 30 min. The remainder of the solution was added slowly over 2 h while maintaining an internal temperature of approximately 80 °C. After cooling to room temperature, the reaction mixture was diluted with water (100 ml) and thoroughly extracted with ethyl acetate (3 × 100 ml) and methylene chloride (2 × 100ml). The combined organic extracts were evaporated *in vacuo*, and the residue was heated under reflux in 10% (v/v) sulfuric acid (30 mL) for 2 h. After cooling to 0 °C, the reaction mixture was neutralized with solid K_2CO_3 and extracted with ethyl acetate (200 ml). The organic extract was dried (Na_2SO_4), filtered, and evaporated *in vacuo* to give the crude ketone as a red-orange viscous liquid. The crude product was purified with a gradient of 0-100% EtoAc in hexane to afford the desired methylketone as a clear, pale yellow, viscous liquid. 15g (10 mmol, 17%). TLC (R_f = 0.16; MS: 136.0 (M+H); ^{1}H-NMR (DMSO) δ 9.05 (d, *J* = 2.2 Hz, 1), 8.13 (dd, *J* = 8.1, 2.2 Hz, 1), 7.27 (d, *J* = 8.1 Hz, 1), 2.64 (s, 3), 2.62 (s, 3). ^{13}C NMR δ 197.48, 163.20, 149.62, 136.24, 130.06, 123.56, 27.23, 24.70.

3-dimethylamino-1-(3-(6-Methyl-pyridyl))-2-propen-1-one(5)



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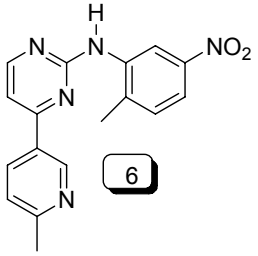
3-Acetyl-6-methyl-pyridine (1.2 g, 8.8 mmol) was added to dimethylformamide dimethylacetal (3 ml, 22 mmol), and the mixture was reacted under reflux for 18 hours. After the reaction mixture was cooled to 0° C., The solution was evaporated to dryness and a mixture of diethyl ether and hexane (3:2, v/v) (10 ml) was added and the whole mixture was stirred for 4 hours. The resulting solid was filtered and washed with a mixture of diethyl ether and hexane (10 ml, 3/2, v/v) to give 3-dimethylamino-1-(3-(4-methyl-pyridyl))-2-propen-1-one (1.5 g, 8 mmol, 90%). R_f=0.46 (Methylene chloride:Methanol=9:1). MS: 191.1 (M+H); ^{1}H NMR(DMSO) δ 8.90 (s, 1H), 8.16 (d, *J*= 7.9 Hz, 1H), 7.85 (d, *J*= 12.0 Hz, 1H),

7.35 (d, *J*= 8.0 Hz, 1H), 5.85 (d, *J*= 12.0 Hz, 1H), 3.18 (s, 3H), 2.95 (s, 3H), 2.58 (s, 3H); ^{13}C NMR δ 183.38, 158.91, 153.78, 146.11, 135.85, 131.45, 121.77, 89.36, 42.78, 34.00, 21.40.

N-(2-methyl-5-aminophenyl)-4-(6-methyl-pyridyl))-2-pyrimidine-amine[3]



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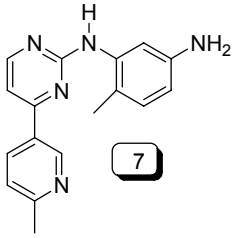
3-dimethylamino-1-(3-(6-methyl-pyridyl))-2-propen-1-one (5) (1.5 g, 8 mmol), 2-methyl-5-nitrophenyl-guanidine nitrate (2) (2 g, 8 mol), and sodium hydroxide (350 mg, 9 mmol) were dissolved in isopropanol 100 ml and reacted under reflux for 18 hours. The reaction solution was cooled to 0° C., filtered, washed with isopropanol and methanol, and dried to give N-(2-methyl-5-nitrophenyl)-4-(6-methyl-pyridyl))-2-pyrimidine-amine. The crude product. The residue was purified by silica gel chromatography using a linear gradient EtOAc-hexane to afford the product. TLC Rf =0.1 (50% EtOAc/hexane) R_f = 0.6 (Methylene chloride:Methanol=9:1). MS 322.5 (M+H).



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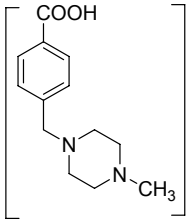
The above N-(2-methyl-5-nitrophenyl)-4-(6-methyl-pyridyl))-2-pyrimidine-amine fractions after flash chromatography were subjected to hydrogenation with 10% Palladium on active carbon 200 mg at atmosphere for 18 hour. The solution were filtered through Whatman 0.45 μm PTFE Glass filter and the solvent were evaporated to give N-(2-methyl-5-aminophenyl)-4-(6-methyl-pyridyl))-2-pyrimidine-amine (250 mg). MS: 292.2 (M+H); ^{1}H NMR($CDCl_3$) δ 8.62 (d, 1H), 8.45 (t, 1H), 8.41 (t, 1H), 7.43 (t, 1H), 7.32 (t, 1H), 7.14 (t, 1H), 6.92(m, 1H), 6.77(m, 1H), 6.34 (m, 1H), 2.42 (s, 3H), 2.15 (s, 3H); ^{13}C NMR δ 165.13, 160.52, 158.62, 149.81, 149.61, 145.54, 145.23, 137.82, 134.27, 130.99, 125.87, 118.65, 111.94, 110.97, 109.07, 20.05, 17.18.

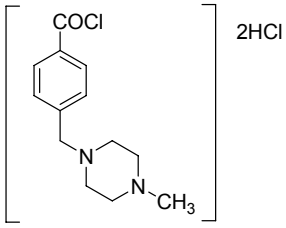
4-(4-methylpiperazinomethyl)benzoic acid dihydrochloride[4]



9

To a well-stirred suspension consisting of 17.1 g. (0.10 mole) of α-chloro-p-toluylic acid in 150 ml. of absolute ethanol under a nitrogen atmosphere at room temperature (~20° C.), a solution consisting of 44.1 g. (0.44 mole) of N-methylpiperazine dissolved in 50 ml. of ethanol was added dropwise. The resulting reaction mixture was refluxed for a period of 16 hours and then cooled to room temperature. The cooled reaction mixture was concentrated in vacuo and the thus obtained residue partitioned between 100 ml. of diethyl ether and 100 ml. of 3N aqueous sodium hydroxide. The separated aqueous layer was then washed three times with 100 ml. of diethyl ether, cooled in an ice-water bath and subsequently acidified with concentrated hydrochloric acid. The resulting solids were filtered and air-dried, followed by trituration with 150 ml. of boiling isopropyl alcohol and stirring for a period of two minutes. After filtering while hot and drying the product there were obtained 9.4 g. (35%) of pure 4-(6-methylpiperazinomethyl)benzoic acid dihydrochloride as the hemihydrate, m.p. 310°-312° C. MS: 235.1 (M+H); ^{1}H NMR(D_2O) δ 8.04 (d, *J*= 8.21 Hz, 2H), 7.59 (d, *J*= 8.21 Hz, 2H), 3.50 (s, 2H), 3.63 (br, 8H), 2.97 (s,3H); ^{13}C NMR δ 170.18, 133.13, 131.91, 130.90, 60.22, 50.61, 48.77, 43.25.

4-(4-methylpiperazinomethyl)benzoyl chloride dihydrochloride[4]



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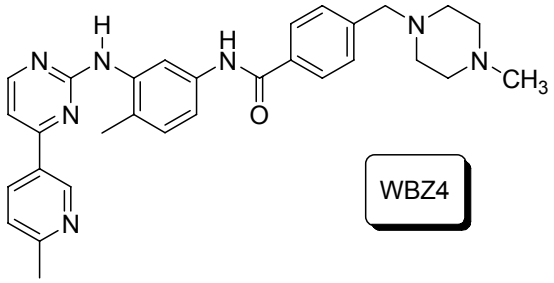
To 20 g. (0.065 mole) of 4-(4-methylpiperazinomethyl)benzoic acid dihydrochloride under a nitrogen atmosphere, there were added 119 ml. of thionyl chloride (194 g., 1.625 mole) to form a beige-white suspension. The reaction mixture was refluxed for 24 hours and then cooled to room temperature (~20° C.). The resulting suspension was filtered, and the recovered solids were washed with diethyl ether and dried to ultimately afford 17.0 g. (81%) of pure 4-(4-methylpiperazinomethyl)benzoyl chloride dihydrochloride.

N-{5-[4-(4-methyl piperazine methyl)-benzoylamido]-2-methylphenyl}-4-[3-(4-methyl)-pyridyl]-2-pyrimidine amine (free base).



A mixture of N-(2-methyl-5-aminophenyl)-4-(6-methyl-pyridyl))-2-pyrimidine-amine (7) 250 mg (0.85 mmol) and 4-(4-methylpiperazinomethyl)benzoyl chloride dihydrochloride (10) 325 mg (1 mmol) were stirred in 20 ml anhydrous pyridine at 20 ^{o}C for 18 hours. The reaction mixture was concentrated in vacuum. The residue was subjected to silica gel chromatography using 5% Methanol (7M NH_3) in DCM. MS: 508.4 (M+H); ^{1}H NMR(DMSO) δ 10.18 (s,1H), 9.15 (d, *J*= 2.1 Hz, 1H), 8.96 (s, 1H), 8.47 (d, *J*= 5.1 Hz, 1H), 8.37 (dd, *J*= 5.1, 2.1 Hz, 1H), 8.05 (d, *J*= 2.1 Hz, 1H), 7.90 (d, *J*= 8.61 Hz, 2H), 7.48 (dd, *J*= 8.24, 2.1 Hz, 1H), 7.41 (d, *J*= 8.61 Hz, 2H), 7.20 (d, 1H), 7.19 (d, 1H), 3.52 (s, 2H), 2.52 (s, 3H), 2.50 (s, 8H), 2.21 (s,3H), 2.15 (s,3H); ^{13}C NMR δ 165.12, 161.61, 161.02, 160.18, 159.17, 147.48, 142.00, 137.73, 137.06, 134.50, 133.64, 129.89, 129.34, 128.51, 127.48, 122.98, 117.08,116.55, 107.05, 61.50, 54.59, 52.48,45.65,23.91,17.57.

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