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Discovery of dually acting small-molecule inhibitors of cancer-resistance relevant receptor tyrosine kinases EGFR and IGF-1R

Electronic supplementary information

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Experimental protocols

1. Chemistry

Commercial reagents were used without further purification. The bromo substituted benzylamines have been synthesized *via N*-benzylamine phthalimides which underwent a following reaction with hydrazine to release the corresponding amines following literature [1]. 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 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 3-nitro-2-phenoxypyridine **2***

19 g (201 mmol) of phenol were diluted in 425 mL of freshly distilled toluene. The solution was cooled down to 2 °C on an ice bath and 10.5 g (262 mmol) of a sodium hydride suspension in paraffine (60%) were added. The ice bath was removed and the mixture was stirred at room temperature until no more gas evolved from the solution. Then 25.7 g (162 mmol) of 2-chloro-3-nitropyridine **1** were added and the mixture was heated under reflux for 5.5 h under argon atmosphere. Then 300 ml of water were added and then extraction with each 200 mL of diethylether followed for four times. The organic layer was washed with both 200 mL of a saturated potassium carbonate solution and a sodium chloride solution, dried over sodium sulfate, filtered and then the diethylether was removed in vacuum. Yield 89%; orange crystals; mp 88-90 °C; ^{1}H NMR ($CDCl_3$) δ 6.96 (dd, J = 7.9, 4.9 Hz, 1H, 5-H), 6.98-

7.03 (m, 2H, 2´-, 6´-H), 7.06-7.13 (m, 2H, 3´-, 5´-H), 7.20-7.34 (m, 1H, 4´-H), 8.15 (dd, *J* = 4.9, 1.5 Hz, 1H, 6-H), 8.17 (dd, *J* = 7.9, 1.5 Hz, 4-H); MS (ESI), *m/z* = 217 [M+H].

*1.2. Formation of the 3-amino-2-phenoxypyridine **3***

30.8 g (141 mmol) of compound **2** were dissolved in 500 mL of ethylacetate. Then 3.0 g of the catalyst palladium on barium sulfate (2%) were added and the mixture was stirred for 24 h under hydrogen atmosphere and normal pressure. Then it was filtered and the solvent was removed in vacuum. Yield 98%; redish crystals; mp 99-101 °C; ^{1}H NMR ($CDCl_3$) δ 3.94 (br, 2H, *N*H_2), 6.85 (dd, *J* = 7.6, 4.9 Hz, 1H, 5-H), 7.04 (dd, *J* = 7.6, 1.6 Hz, 4-H), 7.11-7.21 (m, 3H, 2´-, 4´-, 6´-H), 7.34-7.43 (m, 2H, 3´-, 5´-H), 7.57 (dd, *J* = 4.9, 1.6 Hz, 1H, 6-H); MS (ESI), *m/z* = 187 [M+H].

*1.3. Formation of the benzo-anellated furo[2,3-b]pyridine **4***

15.0 g (81 mmol) of compound **3** were dissolved in 20 ml of acetone and the solution was dropped into 1.2 L of sulfuric acid (5%) at 35 °C under stirring. The resulting suspension was cooled down to 0 °C and then a solution of 5.6 g (81 mmol) of sodium nitrite in 120 mL of water was added dropwise under stirring. Stirring continued at the maintained temperature for 45 min. Then 60 g (980 mmol) of copper powder were added and the temperature was increased to 55 °C. After 90 min no more gas evolved from the mixture and 300 mL of chloroform were added at room temperature. The precipitate was removed and the phases were separated. Then the water phase was extracted with each 150 mL of chloroform for three times. The unified organic layer was dried over sodium sulfate, filtered and removed in vacuum. The raw product was purified by column chromatography using silica gel and an eluent mixture of cyclohexane / ethylacetate (90 / 10). Yield 22%; yellow crystals; mp 56-58

°C; ^{1}H NMR ($CDCl_3$) δ 7.34 (dd, *J* = 7.6, 5.0 Hz, 1H, 3-H), 7.39 (dd, *J* = 7.8, 7.3 Hz, 1H, 6-H), 7.53 (dd, *J* = 8.3, 7.3 Hz, 1H, 7-H), 7.64 (d, *J* = 8.3 Hz, 1H, 8-H), 7.95 (d, *J* = 7.8 Hz, 1H, 5-H), 8.27 (dd, *J* = 7.6, 1.6 Hz, 1H, 4-H), 8.45 (dd, *J* = 5.0, 1.6 Hz, 1H, 2-H); MS (ESI), *m/z* = 170 [M+H].

1.4. Formation of the 4-chloro benzo-anellated furo[2,3-b]pyridine ***5***

5.9 g (35 mmol) of compound **4** were dissolved in 60 mL of chloroform and 12.9 g (61 mmol) of *meta* chloroperbenzoic acid (mCPBA) were added in portions under stirring. The mixture was heated under reflux for 9 h. Then 80 mL of water were added at room temperature and the pH value was adjusted to 9 using a saturated potassium carbonate solution in water. After addition of 80 mL of chloroform the formed phases were separated and the water phase was extracted with each 80 mL of chloroform for six times. The unified organic layer was dried over sodium sufate, filtered and the solvent was removed in vacuum. The remaining solid was dissolved in boiling chloroform and then cooled under stirring so that the mCPBA crystallized. It was filtered off and the chloroform was removed in vacuum. Then 5,7 g of the resulting residue was dissolved in 15 mL of freshly distilled chloroform and cooled down to 0 °C. Then 52.8 mL (580 mmol) of phosphorus oyxchloride were added dropwise under stirring. Heating under reflux followed for 3.5 h. After cooling the mixture was evaporated to a reduced volume and 30 mL of chloroform were added. Then 60 mL of ice water were added and the whole mixture was neutralized with a saturated solution of sodium hydrogencarbonate in water. Then 120 mL of chloroform were added and the phases were separated. The water phase was extracted with each 90 mL of chloroform and the unified organic layer was dried over sodium sulfate, filtered and evaporated to give a solid raw product which was purified by column chromatography using silica gel and an eluent mixture of cyclohexane / ethylacetate (90 / 10). Yield 69%; white solid; mp 74-76 °C; ^{1}H NMR

($CDCl_3$) δ 7.34 (d, *J* = 5.4 Hz, 1H, 3-H), 7.53 (dd, *J* = 7.7, 7.3 Hz, 1H, 7-H), 7.58 (dd, *J* = 8.4, 7.3 Hz, 1H, 6-H), 7.66 (d, *J* = 8.4 Hz, 1H, 5-H), 8.27 (d, *J* = 7.7 Hz, 1H, 8-H), 8.34 (d, *J* = 5.4 Hz, 1H, 2-H); MS (ESI), *m/z* = 204 [M+H].

1.5. Formation of the 4-chloro 6-nitro benzo-anellated furo[2,3-b]pyridine ***6***

1.0 g (4.9 mmol) of compound **5** were added in portions to 5 mL of fuming nitric acid at 0 °C. The the mixture was stirred for 3 h at the maintained temperature, poured on ice, diluted with 20 mL of water and stirring continued for 30 min. The resulting precipitate was filtered and dried within filter paper sleeves. Yield 97%; yellow solid; mp 181-186 °C; ^{1}H NMR (DMSO-d_6) δ 7.78 (d, *J* = 5.5 Hz, 1H, 3-H), 8.10 (d, *J* = 9.1 Hz, 1H, 8-H), 8.57 (d, *J* = 5.5 Hz, 1H, 2-H), 8.58 (d, *J* = 9.1 Hz, 1H, 7-H), 8.95 (s, 1H, 5- H); MS (ESI), *m/z* = 249 [M+H].

1.6. General procedure for the formation of the 4-benzylamino 6-nitro benzo-anellated furo[2,3-b]pyridines ***7a-h***

One equivalent of compound **6** and sixteen equivalents of the respective benzylamine were heated at 140 °C under reflux and argon atmosphere. Then the mixture was poured in a saturated solution of potassium carbonate (25-50 mL) and the same volume of ethylacetate was added. The phases were separated and the water phase was extracted with each the half of the former used volume of ethylaceate for three times. The unified organic layer was dried over sodium sulfate, filtered and evaporated in vacuum. The remaining residue was crystallized from alcohol or ethylacetate.

1.6.1. N-(3-Methoxybenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7a***. Yield 0.201 g (57%); yellow solid; mp 192-194 °C; ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 3H, CH_3), 4.65 (d, *J* = 5.1 Hz, 2H,

CH_2), 6.57 (d, *J* = 5.9 Hz, 1H, 3-H), 6.82 (d, *J* = 7.6 Hz, 1H, 4´-H), 7.00 (d, *J* = 7.9 Hz, 1H, 6´-H), 7.01 (s, 1H, 2´-H), 7.25 (dd, *J* = 7.9, 7.6 Hz, 1H, 5´-H), 7.88 (d, *J* = 8.9 Hz, 1H, 8-H), 7.25 (t, *J* = 5.1 Hz, 1H, *N*H), 8.01 (d, *J* = 5.9 Hz, 1H, 2-H), 8.37 (d, *J* = 8.9 Hz, 1H, 7-H), 9.46 (s, 1H, 5-H); MS (ESI), *m/z* = 350 [M+H^+]; IR (ATR): 3427, 1601, 1593, 1510, 1490, 1472, 1457, 1432, 1337, 1320, 1257, 1229, 1039, 805, 782 cm^{-1}. Anal. ($C_{19}H_{15}N_3O_4$) Calc. C 65.3, H 4.3, N 12.0; Found C 65.5, H 4.2, N 11.7.

1.6.2. N-(4-Methoxybenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7b***. Yield 0.03 g (21%); yellow crystals; mp 225-227 °C; ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 3H, CH_3), 4.96 (d, *J* = 6.0 Hz, 2H, CH_2), 6.59 (d, *J* = 5.9 Hz, 1H, 3-H), 6.90 (d, *J* = 8.3 Hz, 2H, 2´-, 6´-H), 7.36 (d, *J* = 8.4 Hz, 2H, 3´-, 5´-H), 7.89 (d, *J* = 8.9 Hz, 1H, 8-H), 7.98 (t, *J* = 6.0 Hz, 1H, *N*H)**,** 8.00 (d, *J* = 5.9 Hz, 1H, 2-H), 8.37 (d, *J* = 8.9 Hz, 1H, 7-H), 9.46 (s, 1H, 5-H); MS (ESI), *m/z* = 348 [M+H^+]; IR (ATR): 3455, 1603, 1581, 1510, 1476, 1460, 1337, 1320, 1244, 1223, 1028, 806 cm^{-1}. Anal. ($C_{19}H_{15}N_3O_4$) Calc. C 65.3, H 4.3, N 12.0; Found C 65.4, H 4.5, N 11.9.

1.6.3. N-(3-Chlorobenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7c***. Yield 0.058 g (27%); yellow crystals; mp 242-244 °C; ^{1}H NMR (DMSO-d_6) δ 4.70 (d, *J* = 6.2 Hz, 2H, CH_2), 6.58 (d, *J* = 5.9 Hz, 1H, 3-H), 7.29-7.42 (m, 3H, 4´-, 5´-, 6´-H), 7.51 (s, 1H, 2´-H), 7.90 (d, *J* = 9.1 Hz, 1H, 8-H), 8.00 (t, *J* = 5.9 Hz, 1H, *N*H), 8.03 (d, *J* = 5.9 Hz, 1H, 2-H), 8.38 (d, *J* = 9.1 Hz, 1H, 7-H), 9.45 (s, 1H, 5-H); MS (ESI), *m/z* = 354 [M+H^+]; IR (ATR): 3437, 1604, 1594, 1507, 1475, 1444, 1430, 1339, 1318, 1222, 1075, 806, 772 cm^{-1}. Anal. ($C_{18}H_{12}ClN_3O_3$) Calc. C 61.1, H 3.4, N 11.8; Found C 61.5, H 3.4, N 11.5.

1.6.4. N-(4-Chlorobenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7d***. Yield 0.075 g (21%); yellow solid; mp 256-258 °C; ^{1}H NMR (DMSO-d_6) δ 4.68 (d, *J* = 1.8 Hz, 2H, CH_2), 6.56 (d, *J* = 6.0 Hz, 1H, 3-H), 7.34-7.51 (m, 4H, benzylic H), 7.89 (d, *J* = 9.0 Hz, 1H, 8-H), 7.96-8.08 (m, 2H,

2-, *N*H)**,** 8.38 (dd, *J* = 9.0, 2.3 Hz, 1H, 7-H), 9.46 (d, *J* = 2.3 Hz, 1H, 5-H); MS (ESI), *m/z* = 354 [M+H^+]; IR (ATR): 3436, 1603, 1580, 1509, 1491, 1474, 1438, 1336, 1319, 1199, 1082, 796, 786 cm^{-1}. Anal. ($C_{18}H_{12}ClN_3O_3$) Calc. C 61.1, H 3.4, N 11.8; Found C 61.46, H 3.4, N 11.5.

1.6.5. N-(3-Bromobenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7e***. Yield 0.208 g (52%); yellow solid; mp 215-217°C; ^{1}H NMR (DMSO-d_6) δ 4.69 (d, *J* = 6.2 Hz, 2H, CH_2), 6.59 (d, *J* = 5.9 Hz, 1H, 3-H), 7.31 (t, *J* = 7.8 Hz, 1H, 5´-H), 7.45 (d, *J* = 7.8 Hz, 2H, 4´, 6´-H), 7.65 (s, 1H, 2´-H), 7.90 (d, *J* = 9.0 Hz, 1H, 8-H), 7.99 (t, *J* = 6.2 Hz, 1H, *N*H), 8.03 (d, *J* = 5.9 Hz, 1H, 2-H), 8.38 (d, *J* = 9.0 Hz, 1H, 7-H), 9.45 (s, 1H, 5-H); MS (ESI), *m/z* = 400 [M+H^+]; IR (ATR): 3438, 1603, 1594, 1506, 1474, 1448, 1426, 1338, 1318, 1221, 997, 806, 772 cm^{-1}. Anal. ($C_{18}H_{12}BrN_3O_3$) Calc. C 54.3, H 3.0, N 10.6; Found C 54.0, H 3.0, N 10.4.

1.6.6. N-(4-Bromobenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7f***. Yield 0.059 g (25%); yellow solid; mp 252-254 °C; ^{1}H NMR (DMSO-d_6) δ 4.66 (d, *J* = 5.7 Hz, 2H, CH_2), 6.55 (d, *J* = 5.8 Hz, 1H, 3-H), 7.40 (d, *J* = 8.1 Hz, 2H, 2´-, 6´-H), 7.54 (d, *J* = 8.1 Hz, 2H, 3´, 5´-H), 7.89 (d, *J* = 9.0 Hz, 1H, 8-H), 8.01 (d, *J* = 5.8 Hz, 1H, 2-H), 8.03 (br, 1H, *N*H)**,** 8.38 (d, *J* = 9.0 Hz, 1H, 7-H), 9.46 (s, 1H, 5-H); MS (ESI), *m/z* = 398 [M+H^+]; IR (ATR): 3437, 1601, 1580, 1508, 1487, 1474, 1451, 1336, 1318, 1215, 1049, 801, 786 cm^{-1}. Anal. ($C_{18}H_{12}BrN_3O_3$) Calc. C 54.3, H 3.0, N 10.6; Found C 54.1, H 3.3, N 10.8.

1.6.7. N-(3-Aminobenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7g***. Yield 0.005 g (1.4%); yellow solid; mp 108-110 °C; ^{1}H NMR ($CDCl_3$) δ 3.49 (s, 2H, NH_2), 4.61 (d, *J* = 5.5 Hz, 2H, CH_2), 5.44 (t, *J* = 5.5 Hz, 1H, *N*H), 6.60 (d, *J* = 5.9 Hz, 1H, 3-H), 6.65 (d, *J* = 7.8 Hz, 1H, 4´-H), 6.71 (s, 1H, 2´-H), 6.79 (d, *J* = 7.7 Hz, 1H, 6´-H), 7.18 (dd, *J* = 7.8, 7.7 Hz, 1H, 5´-H), 7.69 (d, *J* = 9.0 Hz, 1H, 8-H), 8.18 (d, *J* = 5.9 Hz, 1H, 2-H), 8.39 (dd, *J* = 9.0, 2.1 Hz, 1H, 7-H),

8.64 (d, *J* = 2.1 Hz, 1H, 5-H); MS (ESI), *m/z* = 335 [M+H$^+$]; IR (ATR): 3398, 3290, 3190, 1602, 1515, 1484, 1466, 1443, 1337, 1318, 1232, 806, 767 cm^{-1}. Anal. ($C_{18}H_{14}N_4O_3$) Calc. C 64.7, H 4.2, N 17.6; Found C 64.3, H 3.8, N 17.3.

1.6.8. N-(3-Nitrobenzyl)-6-nitrobenzofuro[2,3-b]pyridine ***7h***. Yield 0.095 g (25%); brownish solid; mp 294-305 °C; ^{1}H NMR (DMSO-d$_6$) δ 4.83 (d, *J* = 6.2 Hz, 2H, CH_2), 6.62 (d, *J* = 5.9 Hz, 1H, 3-H), 7.65 (d, *J* = 7.9 Hz, 1H, 5´-H), 7.88-7.93 (m, 2H, 4´-, 6´-H), 8.03 (d, *J* = 5.9 Hz, 1H, 2-H), 8.07 (t, *J* = 6.2 Hz, 1H, *N*H), 8.12 (d, *J* = 9.0 Hz, 1H, 8-H), 8.34 (t, *J* = 2.0 Hz, 1H, 2´-H), 8.39 (dd, *J* = 9.0, 2.4 Hz, 1H, 7-H), 9.46 (d, *J* = 2.4 Hz, 1H, 5-H); MS (ESI), *m/z* = 365 [M+H$^+$]; IR (ATR): 3436, 1604, 1581, 1526, 1506, 1474, 1448, 1336, 1319, 1226, 811 cm^{-1}. Anal. ($C_{18}H_{12}N_4O_5$) Calc. C 59.3, H 3.3, N 15.4; Found C 59.4, H 3.7, N 15.4.

1.7. General procedure for the formation of the 6-amino 4-benzylamino benzo-anellated furo[2,3-b]pyridines ***8a-g***

One equivalent of the respective 6-nitro benzylamine compound **7** was suspended in 10 mL of hydrochloric acid (10%) and six equivalents of tin(II) chloride were added. The suspension was heated under reflux for 75 min and then poured into 15 mL water after cooling. The pH value was adjusted to 12 with a 10 M sodium hydroxide solution. Extraction with each 15 mL of ethylacetate followed for five times. The unified organic layer was dried over sodium sulfate and filtered. After evaporation the remaining solid was either recrystallized from chloroform or purified by column chromatography using an eluent mixture of cyclohexane and ethylacetate.

1.7.1. N^4-(3-Methoxybenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8a***. Yield 0.023 g (25%); brownish solid; mp 148-150 °C; ^{1}H NMR (DMSO-d$_6$) δ 3.72 (s, 3H, CH_3), 4.58 (d, *J* = 6.0

Hz, 2H, CH_2), 4.84 (br, 2H, *N*H_2), 6.40 (d, *J* = 5.8 Hz, 1H, 3-H), 6.74 (d, *J* = 8.8 Hz, 1H, 7-H), 6.81 (d, *J* = 7.9 Hz, 1H, 6´-H), 6.97 (br, 1H, *N*H), 6.98 (s, 1H, 2´-H), 7.18-7.23 (m, 1H, 5´-H), 7.25 (d, *J* = 7.9 Hz, 1H, 4´-H), 7.29 (d, *J* = 8.6 Hz, 1H, 8-H), 7.55 (s, 1H, 5-H), 7.85 (d, *J* = 5.8 Hz, 1H, 2-H); MS (ESI), *m/z* = 320 [M+H^+]; IR (ATR): 3350, 2979, 2924, 2855, 1598, 1511, 1479, 1451, 1436, 1260, 1208, 1121, 1018, 806 cm^{-1}. Anal. ($C_{19}H_{17}N_3O_2$) Calc. C 71.5, H 5.4, N 13.2; Found C 71.72, H 5.8, N 12.8.

1.7.2. N^4-(4-Methoxybenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8b***. Yield 0.039 g (87%); green-brownish solid; mp 222-226 °C; ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 3H, CH_3), 4.58 (d, *J* = 6.1 Hz, 2H, CH_2), 4.83 (br, 2H, *N*H_2), 6.42 (d, *J* = 5.9 Hz, 1H, 3-H), 6.73 (d, *J* = 8.7 Hz, 1H, 7-H), 6.89 (d, *J* = 8.5 Hz, 2H, 2´-, 6´-H), 7.13 (t, *J* = 6.1 Hz, 1H, *N*H), 7.30 (d, *J* = 8.7 Hz, 1H, 8-H), 7.33 (d, *J* = 8.5 Hz, 2H, 3´-, 5´-H), 7.51 (d, *J* = 2.3 Hz, 1H, 5-H), 7.84 (d, *J* = 5.9 Hz, 1H, 2-H); MS (ESI), *m/z* = 320 [M+H^+]; IR (ATR): 3419, 3310, 3204, 2961, 2927, 2856, 1604, 1586, 1511, 1478, 1463, 1245, 1208, 1110, 1027, 816, 801 cm^{-1}. Anal. ($C_{19}H_{17}N_3O_2$) Calc. C 71.5, H 5.4, N 13.2; Found C 71.4, H 5.3, N 13.2.

1.7.3. N^4-(3-Chloroxybenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8c***. Yield 0.056 g (61%); pink crystals; mp 179-181 °C; ^{1}H NMR (DMSO-d_6) δ 4.62 (d, *J* = 6.2 Hz, 2H, CH_2), 4.85 (br, 2H, *N*H_2), 6.40 (d, *J* = 5.8 Hz, 1H, 3-H), 6.75 (dd, *J* = 8.6, 2.2 Hz, 1H, 7-H), 7.25 (t, *J* = 6.2 Hz, 1H, *N*H), 7.31 (d, *J* = 8.6 Hz, 1H, 8-H), 7.28-7.40 (m, 3H, 4´, 5´-, 6´-H), 7.46 (s, 1H, 2´-H), 7.52 (d, *J* = 2.2 Hz, 1H, 5-H), 7.86 (d, *J* = 5.8 Hz, 1H, 2-H); MS (ESI), *m/z* = 324 [M+H^+]; IR (ATR): 3344, 3221, 1596, 1511, 1479, 1447, 1431, 1209, 1187, 1130, 801 cm^{-1}. Anal. ($C_{18}H_{14}ClN_3O$) Calc. C 66.8, H 4.4, N 13.0; Found C 66.4, H 4.8, N 12.8.

1.7.4. N^4-(4-Chlorobenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8d***. Yield 0.023 g (51%); pink crystals; mp 238-240 °C; ^{1}H NMR (DMSO-d_6) δ 4.58 (d, *J* = 6.1 Hz, 2H, CH_2), 4.83 (br,

2H, *N*H_2), 6.36 (d, *J* = 5.8 Hz, 1H, 3-H), 6.74 (dd, *J* = 8.6, 2.2 Hz, 1H, 7-H), 7.22 (t, *J* = 6.1 Hz, 1H, *N*H), 7.30 (d, *J* = 8.6 Hz, 1H, 8-H), 7.37 (d, *J* = 8.6 Hz, 2H, 2´-, 6´-H), 7.41 (d, *J* = 8.6 Hz, 2H, 3´-, 5´-H), 7.52 (d, *J* = 2.2 Hz, 1H, 5-H), 7.83 (d, *J* = 5.8 Hz, 1H, 2-H); MS (ESI), *m/z* = 324 [M+H^+]; IR (ATR): 3332, 1602, 1511, 1480, 1459, 1199, 1126, 1092, 805 cm^{-1}. Anal. ($C_{18}H_{14}ClN_3O$) Calc. C 66.8, H 4.4, N 13.0; Found C 66.3, H 4.1, N 12.6.

1.7.5. N^4-(3-Bromobenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8e***. Yield 0.017 g (18%); white solid; mp 190-192 °C; ^{1}H NMR (DMSO-d_6) δ 4.60 (d, *J* = 6.2 Hz, 2H, CH_2), 4.83 (br, 2H, *N*H_2), 6.39 (d, *J* = 5.8 Hz, 1H, 3-H), 6.73 (dd, *J* = 8.7, 2.1 Hz, 1H, 7-H), 7.19-7.27 (m, 1H, 5´-H), 7.29 (d, *J* = 3.1 Hz, 1H, 6´-H), 7.31 (d, *J* = 3.9 Hz, 1H, 4´-H), 7.37-7.46 (m, 2H, 8-, *N*H), 7.51 (d, *J* = 2.2 Hz, 1H, 5-H), 7.59 (s, 1H, 2´-H), 7.85 (d, *J* = 5.8 Hz, 1H, 2-H); MS (ESI), *m/z* = 369 [M+H^+]; IR (ATR): 3336, 3212, 1596, 1511, 1479, 1427, 1209, 1129, 1069, 806 cm^{-1}. Anal. ($C_{18}H_{14}BrN_3O$) Calc. C 58.7, H 3.8, N 11.4; Found C 58.5, H 3.9, N 11.2.

1.7.6. N^4-(4-Bromobenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8f***. Yield 0.010 g (54%); orange crystals; mp 246-248 °C; ^{1}H NMR (DMSO-d_6) δ 4.57 (d, *J* = 6.1 Hz, 2H, CH_2), 4.82 (br, 2H, *N*H_2), 6.35 (d, *J* = 5.8 Hz, 1H, 3-H), 6.73 (dd, *J* = 8.7, 2.3 Hz, 1H, 7-H), 7.22 (t, *J* = 6.1 Hz, 1H, *N*H), 7.29 (d, *J* = 8.7 Hz, 1H, 8-H), 7.35 (d, *J* = 8.4 Hz, 2H, 2´-, 6´-H), 7.50 (d, *J* = 2.3 Hz, 1H, 5-H), 7.51 (d, *J* = 8.4 Hz, 2H, 3´-, 5´-H), 7.83 (d, *J* = 5.8 Hz, 1H, 2-H); MS (ESI), *m/z* = 369 [M+H^+]; IR (ATR): 3389, 3212, 1596, 1511, 1479, 1427, 1209, 1129, 1069, 806 cm^{-1}. Anal. ($C_{18}H_{14}BrN_3O$) Calc. C 58.7, H 3.8, N 11.4; Found C 58.3, H 3.5, N 11.6.

1.7.7. N^4-(3-Aminoybenzyl)benzofuro[2,3-b]pyridine-4,6-diamine ***8g***. Yield 0.044 g (96%); yellow solid; mp 203-206 °C; ^{1}H NMR (DMSO-d_6) δ 4.44 (d, *J* = 6.0 Hz, 2H, CH_2), 4.82 (br, 2H, 3´-*N*H_2), 4.98 (br, 2H, 6-*N*H_2), 6.34 (d, *J* = 5.9 Hz, 1H, 3-H), 6.40 (d, *J* = 7.6 Hz, 1H, 4´-H), 6.51 (d, *J* = 7.6 Hz, 1H, 6´-H), 6.55 (s, 1H, 2´-H), 6.72 (dd, *J* = 8.6, 2.3 Hz, 1H, 7-H), 6.94

(t, *J* = 7.6 Hz, 1H, 5´-H), 7.09 (t, *J* = 6.0 Hz, 1H, *N*H), 7.29 (d, *J* = 8.6 Hz, 1H, 8-H), 7.51 (d, *J* = 2.3 Hz, 1H, 5-H), 7.82 (d, *J* = 5.9 Hz, 1H, 2-H); MS (ESI), *m/z* = 305 [M+H^+]; IR (ATR): 3343, 3219, 2924, 2854, 1596, 1511, 1478, 1208, 1122, 801 cm^{-1}. Anal. ($C_{18}H_{16}N_4O$) Calc. C 71.0, H 5.3, N 18.4; Found C 70.6, H 5.4, N 18.0.

1.8. Formation of the 1-(pyridine-2-yl)-1H-benzo[d](1,2,3]triazole ***9*** *[2]*

50 g (420 mmol) 1*H*-Benzotriazole were suspended in 220 mL of toluene and then 79.6 g (504 mmol) of 2-bromopyridine were added. The mixture was heated under reflux for 23 h and then poured into 1 L of ethylacetate. The precipitate was solved under addition of 100 mL of a potassium hydroxide solution (10%). The resulting phases were separated and the organic layer was washed with 300 mL of the potassium hydroxide solution for two times, dried over sodium sulfate and filtered. After evaporation of the solvent the resulting product was used without further purification. Yield 80 g (97%); yellow-white needles; mp 110-112 °C; ^{1}H NMR ($CDCl_3$) δ 7.33 (ddd, *J* = 7.5 Hz, 4.9 Hz, 1.0 Hz, 1H, 5´-H), 7.46 (ddd, *J* = 8.2 Hz, 7.0 Hz, 1.1 Hz, 1H, 5-H), 7.61 (ddd, *J* = 8.3 Hz, 7.0 Hz, 1.1 Hz, 1H, 6-H), 7.95 (ddd, *J* = 8.3 Hz, 7.5 Hz, 1.9 Hz, 1H, 4´-H), 8.13 (dt, *J* = 8.2 Hz, 1.1 Hz, 1H, 4-H), 8.31 (dt, *J* = 8.3 Hz, 1H, 3´-H), 8.62 (ddd, *J* = 4.9 Hz, 1.9 Hz, 1H, 6´-H), 8.67 (dt, *J* = 8.3 Hz, 1.1 Hz, 1H, 7-H); MS (ESI), *m/z* = 197 [M+H^+].

1.9. Formation of the 9-H-pyrido[2,3-b]indole ***10*** *[3]*

Polyphosphoric acid (29.4 g) was heated in a round flask to 170 °C. Then 11.4 g (58 mmol) of compound **9** were added under stirring for 3 h at the maintained temperature. Then 50 mL of water were added and the solution was alkalized with a 10 M potassium hydroxide solution to a pH of 10. After stirring overnight the suspension was poured into 250 mL of water, cooled

down to 0 °C in an ice bath and filtered. Precipitated disodium hydrogen phosphate was washed out with portions of water and the remaining residue was kept in under vacuum. Yield 3.6 g (36%); beige solid; mp 201-212 °C; ^{1}H NMR (DMSO-d_6) δ 7.18 (dd, *J* = 7.18 Hz, 4.8 Hz, 1H, 3-H), 7.20 (ddd, *J* = 8.0 Hz, 7.0 Hz, 1.3 Hz, 1H, 6-H), 7.43 (ddd, *J* = 8.0 Hz, 7.0 Hz, 1.2 Hz, 1H, 7-H), 7.48 (ddd, *J* = 8.2 Hz, 1.3 Hz, 0.6 Hz, 1H, 8-H), 8.14 (ddt, *J* = 8.0 Hz, 1.2 Hz, 0.6 Hz, 1H, 5-H), 8.39 (dd, *J* = 4.8 Hz, 1.6 Hz, 1H, 2-H), 8.48 (ddd, *J* = 7.7 Hz, 1.6 Hz, 0.6 Hz, 1H, 4-H), 11.74 (s, 1H, 9H); MS (ESI), *m/z* = 169 [M+H$^+$].

1.10. Formation of the 4-chloro-9-H-pyrido[2,3-b]indole ***11***

3.6 g (21 mmol) of compound **10** were dissolved in acetic acid and 4.4 g (45 mmol) of a 35% solution of hydrogen peroxide in water were added dropwise. The solution was heated under reflux for 5 h. Then the solution volume was reduced in vacuum and the remaining oily product was treated with a saturated potassium carbonate solution to reach a pH of 8. After stirring overnight the resulting precipitate was filtered off and dried in vacuum. After that 3.6 g (19.5 mmol) were dissolved in DMF under stirring and argon atmosphere. The solution was cooled down to 0 °C on an ice bath and then 4.2 mL (7.1 g, 46.9 mmol) of phosphoryl oxychloride were added. The whole mixture was stirred for 24 h and poured into 50 mL of water. The pH value was adjusted to 12 using a 12% solution of potassium hydroxide. After stirring for 30 min the solution was filtered and the remaining solid was dried and purified by column chromatography using silica gel and a mixture of cyclohexane and ethylacetate (80/20) to wash out the impurities and then with the eluent mixture of 50/50 to isolate the poduct **3**. Yield 2.3 g (58%); yellow-white crystals; mp 230-232 °C; ^{1}H NMR (DMSO-d_6) 7.32-7.27 (m, 1H, 6-H), 7.30 (d, *J* = 5.3 Hz, 1H, 3-H), 7.57-7.50 (m, 2H, 7-, 8-H), 8.33 (dd, *J* = 8.0 Hz, 1.3 Hz, 1H, 5-H), 8.36 (d, *J* = 5.3 Hz, 1H, 2-H), 12.16 (s, 1H, 9H); MS (ESI), *m/z* = 203 [M+H$^+$].

*1.11. Formation of the 4-chloro-6-nitro-9-H-pyrido[2,3-b]indole **12***

0.5 g (2,5 mmol) of compound **11** were added in portions to 3 mL of red fuming nitric acid at 0 °C. Then the mixture was cooled down to rt and stirred for 35 min at room temperature, poured on ice, diluted with 10 mL of water and the solution pH value was adjusted to 10 with a saturated solution of potassium carbonate in water. The suspension was left stirring over night. The resulting precipitate was filtered off and recrystallized from DMF. Yield 75%; yellow solid; mp > 360 °C; ^{1}H NMR (DMSO-d_6) δ 7.47 (d, *J* = 5.3 Hz, 1H, 3-H), 7.70 (d, *J* = 9.0 Hz, 1H, 8-H), 8.40 (dd, *J* = 9.0, 2.4 Hz, 1H, 7-H), 8.50 (d, *J* = 5.3 Hz, 1H, 2-H), 9.10 (d, *J* = 2.4 Hz, 1H, 5- H), 12.92 (s, 1H, 9-H); MS (ESI), *m/z* = 248 [M+H].

*1.12. General procedure for the formation of the 4-benzylamino 6-nitro-9-H-pyrido[2,3-b]indoles **13a-c***

One equivalent of compound **12** and fifteen equivalents of the respective benzylamine were heated at 145-160 °C under reflux and argon atmosphere until no more of compound **12** was detectable with tlc. Then 5 mL of chloroform were added at rt and the mixture was stirred for 30 min. Then the precipitate was filtered off, washed with diethyl ether and dried.

*1.12.1. N^4-(3-Methoxybenzyl)-6-nitro-9H-pyrido[2,3-b]indole-4-amine **13a***. Yield 0.149 g (43%); yellow solid; mp 228-230 °C; ^{1}H NMR (DMSO-d_6) δ 3.69 (s, 3H, CH_3), 4.62 (d, *J* = 6.1 Hz, 2H, CH_2), 6.34 (d, *J* = 5.8 Hz, 1H, 3-H), 6.79 (ddd, *J* = 8.1, 2.5, 1.1 Hz, 1H, 6´-H), 6.94-7.02 (m, 2H, 2´-, 4´-H), 7.22 (t, *J* = 8.1 Hz, 1H, 5´-H), 7.53 (d, *J* = 8.9 Hz, 1H, 8-H), 7.64 (t, *J* = 6.2 Hz, 1H, CH_2-*N***H**), 8.00 (d, *J* = 5.8 Hz, 1H, 2-H), 8.26 (dd, *J* = 8.9, 2.2 Hz, 1H, 7-H), 9.41 (d, *J* = 2.2 Hz, 1H, 5-H), 12.25 (s, 1H, 9-H); MS (ESI), *m/z* = 349 [M+H$^+$]; IR (ATR): 3433, 3086, 2973, 2885, 1600, 1585, 1508, 1487, 1475, 1456, 1312, 1280, 1269,

1038, 786, 767 cm^{-1}. Anal. ($C_{19}H_{16}NO_3$) Calc. C 65.5, H 4.6, N 16.1; Found C 65.7, H 4.8, N 15.8.

1.12.2. N^4-(3-Chlorobenzyl)-6-nitro-9H-pyrido[2,3-b]indole-4-amine ***13b***. Yield 0.186 g (53%); yellow solid; mp 346-348 °C; ^{1}H NMR (DMSO-d_6) δ 4.67 (d, *J* = 6.2 Hz, 2H, CH_2), 6.34 (d, *J* = 5.8 Hz, 1H, 3-H), 7.28 (d, *J* = 7.7 Hz, 1H, 6´-H), 7.35 (t, *J* = 7.7 Hz, 1H, 5´-H), 7.39 (d, *J* = 7.7 Hz, 1H, 4´-H), 7.48 (s, 1H, 2´-H), 7.55 (d, *J* = 8.9 Hz, 1H, 8-H), 7.67 (t, *J* = 6.2 Hz, 1H, *N*H), 8.01 (d, *J* = 5.8 Hz, 1H, 2-H), 8.26 (dd, *J* = 8.9, 2.2 Hz, 1H, 7-H), 9.41 (d, *J* = 2.2 Hz, 1H, 5-H), 12.29 (s, 1H, 9-H); MS (ESI), *m/z* = 353 [M+H^+]; IR (ATR): 3431, 2957, 1597, 1578, 1510, 1492, 1470, 1457, 1381, 1324, 1111, 786 cm^{-1}. Anal. ($C_{18}H_{13}ClN_4O_2$) Calc. C 61.3, H 3.7, N 15.9; Found C 61.5, H 3.8, N 15.5.

1.12.3. N^4-(3-Bromobenzyl)-6-nitro-9H-pyrido[2,3-b]indole-4-amine ***13c***. Yield 0.232 g (90%); yellow solid; mp 342-344 °C; ^{1}H NMR (DMSO-d_6) δ 4.67 (d, *J* = 6.2 Hz, 2H, CH_2), 6.34 (d, *J* = 5.8 Hz, 1H, 3-H), 7.29 (d, *J* = 7.8 Hz, 1H, 6´-H), 7.37 (t, *J* = 7.8 Hz, 1H, 5´-H), 7.42 (d, *J* = 7.8 Hz, 1H, 4´-H), 7.54 (d, *J* = 8.9 Hz, 1H, 8-H), 7.62 (s, 1H, 2´-H), 7.66 (t, *J* = 6.2 Hz, 1H, *N***H**), 8.02 (d, *J* = 5.8 Hz, 1H, 2-H), 8.26 (dd, *J* = 8.9 Hz, 2.2 Hz, 1H, 7-H), 9.40 (d, *J* = 2.2 Hz, 1H, 5-H), 12.28 (s, 1H, 9-H); MS (ESI), *m/z* = 398 [M+H^+]; IR (ATR): 3433, 2979, 1599, 1570, 1509, 1490, 1472, 1451, 1379, 1314, 898, 782, 767 cm^{-1}. Anal. ($C_{18}H_{13}BrN_3O_2$) Calc. C 54.4, H 3.3, N 14.1; Found C 54.2, H 3.4, N 13.7.

1.13. General procedure for the formation of the 6-amino 4-benzylamino 9-H-pyrido[2,3-b]indoles ***14a-c***

One equivalent of the respective 6-amino benzylamine compound **13** was suspended in 10 mL of hydrochloric acid (10%) and six equivalents of tin(II) chloride were added. The suspension

was heated under reflux for 75 min and then poured into 20 mL of water after cooling. The pH value was adjusted to 12 with a 10 M sodium hydroxide solution. The precipitated compound was stirred for 30 min, filtered off from the solution, washed with water and finally dried.

1.13.1. N^4-(3-Methoxybenzyl)-6-amino-9H-pyrido[2,3-b]indole-4,6-diamine ***14a***. Yield 0.065 g (71%); beige solid; mp 209-211 °C; ^{1}H NMR (DMSO-d_6) δ 3.69 (s, 3H, CH_3), 4.56 (d, *J* = 6.1 Hz, 2H, CH_2), 4.60 (br, 2H, *N*H_2), 6.14 (d, *J* = 5.7 Hz, 1H, 3-H), 6.72 (d, *J* = 8.5 Hz, 1H, 7-H), 6.76 (t, *J* = 6.1Hz, 1H, *N*H), 6.78 (d, *J* = 7.9 Hz, 1H, 6´-H), 6.93-7.03 (m, 2H, 2´-, 4´-H), 7.11 (d, *J* = 8.5 Hz, 1H, 8-H), 7.22 (t, *J* = 8.1 Hz, 1H, 5´-H), 7.53 (d, *J* = 2.0 Hz, 1H, 5-H), 7.81 (d, *J* = 5.7 Hz, 1H, 2-H), 10.94 (s, 1H, 9-H); MS (ESI), *m/z* = 318 [M+H^+]; IR (ATR): 3378, 3317, 33136, 3039, 2919, 1592, 1513, 1502, 1473, 1453, 1437, 1321, 1279, 1047, 805 cm^{-1}. Anal. ($C_{19}H_{18}N_4O$) Calc. C 71.7, H 5.7, N 17.6; Found C 71.4, H 5.6, N 17.8.

1.13.2. N^4-(3-Chlorobenzyl)-6-amino-9H-pyrido[2,3-b]indole-4,6-diamine ***14b***. Yield 0.037 g (41%); beige solid; mp 250-252 °C; ^{1}H NMR (DMSO-d_6) δ 4.47 (s, 2H, *N*H_2), 4.60 (d, *J* = 6.2 Hz, 2H, CH_2), 6.11 (d, *J* = 5.7 Hz, 1H, 3-H), 6.72 (dd, *J* = 8.4, 2.0 Hz, 1H, 7-H), 6.84 (t, *J* = 6.2 Hz, 1H, *N*H), 7.11 (d, *J* = 8.4 Hz, 1H, 8-H), 7.27 (dd, *J* = 7.6, 1.8 Hz, 1H, 6´-H), 7.34 (t, *J* = 7.6 Hz, 1H, 5´-H), 7.37 (dd, *J* = 7.6, 1.8 Hz, 1H, 4´-H), 7.44 (t, *J* = 1.8 Hz, 1H, 2´-H), 7.54 (d, *J* = 2.0 Hz, 1H, 5-H), 7.82 (d, *J* = 5.7 Hz, 1H, 2-H), 10.94 (s, 1H, 9-H); MS (ESI), *m/z* = 323 [M+H^+]; IR (ATR): 3390, 3318, 3136, 3054, 2925, 1594, 1573, 1516, 1499, 1471, 1442, 1431, 1321, 1008, 796 cm^{-1}. Anal. ($C_{18}H_{15}ClN_4$) Calc. C 67.0, H 4.7, N 17.4; Found C 67.4, H 4.6, N 17.0.

1.13.3. N^4-(3-Bromobenzyl)-6-amino-9H-pyrido[2,3-b]indole-4,6-diamine ***14c***. Yield 0.044 g (48%); white solid; mp 219-212 °C; ^{1}H NMR (DMSO-d_6) δ 4.60 (d, *J* = 6.8 Hz, 2H, CH_2),

4.70 (br, 2H, *N*H_2), 6.13 (d, *J* = 5.7 Hz, 1H, 3-H), 6.73 (dd, *J* = 8.5, 2.0 Hz, 1H, 7-H), 6.89 (t, *J* = 6.8 Hz, 1H, *N*H), 7.13 (d, *J* = 8.5 Hz, 1H, 8-H), 7.27 (t, *J* = 7.8 Hz, 1H, 5´-H), 7.41 (dd, *J* = 7.8, 1.9 Hz, 2H, 4´-, 6´-H), 7.55 (d, *J* = 2.0 Hz, 1H, 5-H), 7.58 (t, *J* = 1.9 Hz, 1H, 2´-H), 7.83 (d, *J* = 5.7 Hz, 1H, 2-H), 10.99 (s, 1H, 9-H); MS (ESI), *m/z* = 368 [$M+H^+$]; IR (ATR): 3384, 3314, 3135, 3053, 2924, 1593, 1569, 1516, 1499, 1471, 1440, 1428, 1321, 849, 796 cm^{-1}. Anal. ($C_{18}H_{15}BrN_4$) Calc. C 58.9, H 4.1, N 15.3; Found C 58.7, H 3.9, N 14.9.

2. Protein kinase inhibition determination

The protein kinases were all expressed in baculovirus Sf9 insect cells as human recombinant GST fusion proteins and purified by affinity chromatography using GSH-agarose. The kinase identity was confirmed by mass spectrometry using LC-ESI-MS/MS technique.

2. 1. Assay conditions for inhibition determinations

The measuring of protein kinase activity was performed in 96-well FlashPlates™ in a 50 µL reaction volume. The reaction mixture consisted of 20 µL of assay buffer solution, 5 µL of an ATP solution in water, 5 µL of the 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 pH 7.5, each 3 mM of magnesium chloride and manganese(II) chloride, 3 µM of sodium orthovanadate, 1.2 mM of DTT, 50 µg/mL of PEG_{20000} and finally 15 µM of [γ-^{33}P]-ATP making approximately 7 x 10^5 cpm per well.

The final kinase concentration had been 10 ng/50 µL for EGFR-wt and IGF-1R. In the screen the final kinase concentrations had been 5 ng/50 µL (EGFR-L858R), 10 ng/50µL (EGFR-T790M), 100 ng/50 µL (HER2), 40 ng/50 µL (HER4), 25 ng/50 µL (VEGFR2) and 100 ng/50 µL (VEGFR3). The used substrate was $Poly(Glu,Tyr)_{4:1}$ in a concentration of 125 ng/50 µL.

Final kinase concentration for JAK2, 3, TIE-2 and PDGFRβ had been 100 ng, 200 ng, 10 ng and 100 ng each in 50 µL. The used substrate was Poly(Ala,Glu,Lys,Tyr)$_{6:2:5:1}$ in a concentration of 125 ng/50 µL. Finally, GSK-3β had been used in a concentration of 50 ng/50 µL and the substrate had been RBER-CHKtide in a concentration of 1000 ng/50 µL.

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 a 0.9% solution of sodium chloride. The incorporation of ^{33}Pi was determined with a microplate scintillation counter. Ten different inhibitor concentrations were measured in a range of 3 nM to 100 µM. The residual activity (%) and the IC_{50} values were finally calculated. From the IC_{50} values the affinity constants K_i were determined using the equation: $IC_{50} = 1/2\ [E_t] + K_i \times (1 + [S] / K_m)$.

3. Molecular docking studies

The 3D structures of the kinases EGFR, and IGF-1R were taken from the Protein databank. We took from the available X-ray structures the ones which were co-crystallized with an inhibitor structurally most similar to the benzo-anellated furo- and pyrrolo[2,3-b]pyridines under study (2ITY for EGF, 3QQU for IGF-1R). The protein structures were prepared as follows: water molecules and bound small molecules were deleted, hydrogen atoms were added and the resulting structures were minimized using the MMF94 force field and the conjugate gradient method until a gradient of 0.01 kcal/mol was reached. We used the docking program GOLD 5.2 (Cambridge Crystallographic Data Centre, Cambridge UK) to dock all molecules into the ATP binding site of EGFR and IGF-1R. For all docking runs the program default settings were used. The binding site for all kinases was defined on the gatekeeper residue with a radius of 15 Å. Goldscore was chosen as fitness function. For each molecule 30 docking runs were performed. A protein hydrogen bond constraint was defined

to the conserved backbone NH of the hinge region (EGFR: Met793, IGF-1R: Met1082). The resulting solutions were clustered on the basis of the heavy atom RMSD values (1 Å). First, we tested whether the docking program is able to reproduce the binding mode of the bound ligands as observed in the X-ray structures of EGFR and IGF-1R, respectively (PDB ID 2ITY and 3QQU). The RMSD values between the top-ranked docking solution and the crystal structure of the inhibitors were found to be below 1 Å (0.71 and 0.48 Å, respectively, demonstrating the usability of the applied docking program for reproducing the experimentally determined structures of the kinase inhibitor complexes.

4. Cellular growth inhibiution assay [4,5]

Cells were inoculated into 96 well microtiter plates in 100 mL at plate densities ranging from 5000 to 40.000 cells per well depending from the individual cell doubling time. Then the cell-containing plates were incubated at 37 °C under an air atmosphere containing 5% of carbon dioxide and with a relative humidity for 24 h. After that time two plates of each cell line were fixed in situ with TCA as control before drug addition. Then the drug containing dmso stock solutions were used and mixed with the cell culture medium containing 50 mg/mL of gentamicin. Aliquots of 100 mL of the respective dilution were added to the preincubated plates reaching a final drug concentration of 10 µM. The plates were incubated again under the preincubation conditions as described for 48 h. Then cells were fixed under addition of 50 mL of a cold 50% solution of TCA reaching a final concentration of 10% TCA. Incubation continued for 60 min at 4 °C. The supernatant was discarded and the plates were washed with water for five times and dried at air. Then a sulforhodamine (SRB) solution (0.4%) in acetic acid (1%) was added to each well and plates were incubated again for 10 min. The unbound dye was removed by washing for five times with an acetic acid solution (1%) and the plates were dried at air. The bound stain was solubilized with a 10 µM solution of trizma base and

the absorbance was measured with a plate reader at a wavelength of 515 nm. The growth inhibition measured as lowered net protein increase after SRB staining was determined in relation to the untreated control cells.

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