Neurodegenerative diseases are among the most challenging diseases with poorly known mechanism and lack of complete cure. Alzheimer’s disease (AD) in particular, is the most prevalent cause of dementia, very devastating for the patients and their families, provided that current treatments offer only modest symptomatic relief. This severe mental disorder is mostly aging-associated and represents a global health problem, since it currently affects more than 30 million people worldwide, and its incidence is predicted to rise significantly, due to the increasing average life span. In an effort to explain the pathogenesis of AD, many hypotheses have been explored, among them neurotransmitter modulation, chronic inflammation, metal-induced oxidative stress, elevated cholesterol, and glycogen synthase kinase 3 (GSK-3) implication are associated with neurodegeneration and the pathogenesis of several diseases, including diabetes type II, immune and bipolar disorders, chronic inflammation, heart failure and cancer. In AD, GSK-3 promotes neuronal death and is a linker of several diseases, including diabetes type II, immune and bipolar disorders, chronic inflammation, heart failure and cancer. In AD, GSK-3 promotes neuronal death and is a linker of several diseases, including diabetes type II, immune and bipolar disorders, chronic inflammation, heart failure and cancer.

Results and Discussion

Chemistry For the preparation of the target compounds we have used both nitroderivatives of 2-aminopicoline which were synthesized according to reported procedures. 2-Amino-5-nitropicoline (1) was diazotized and the resulting pyridinone 2 was treated with phosphorus oxychloride and provided the chloride 3. This chloride was reduced using tin chloride and the resulting aminoderivative 4 was acetylated to give the acetamide 5. Compound 5 was treated with isoamyl nitrite in the presence of potassium acetate and acetic anhydride and this reaction furnished a mixture of the corresponding 1- and 2-acyethylpyrazolo[3,4-c]pyridines, through a rearrangement of the intermediate N-nitroso compound. The acetamides were not isolated but the acetyl group was cleaved upon treatment with methanolic ammonia

Key words pyrazolopyridine; kinase inhibition; glycogen synthase kinase 3 (GSK3) αβ; purine analogue; molecular simulation
to result in the pyrazolopyridine 6. The heterocyclic compound 6 was then nitrated and the resulting 3-nitroderivative 7 was treated with methyl iodide, or 4-methoxybenzyl chloride in the presence of potassium carbonate, to provide both regio-isomers 8a, b and 9a, b, respectively, which were chromatographically separated and identified by means of two-dimensional (2D)-NMR experiments. More specifically, in the corresponding nuclear Overhauser effect (NOE) spectra we observed clear cross-peak correlation between the N1 methyl group of 8a, or the N1 methylene group of 8b with H-7, verifying unambiguously the N1 substitution. This correlation was, as expected not detected in the case of compounds 9a, b. The chlorine atom of compounds 8a and b was subsequently displaced upon treatment with cyclohexylamine to result in nitroderivatives 10a–d. This reaction was effected through a straightforward aromatic nucleophilic substitution when cyclohexylamine was used, however in the case of the weak nucleophile, aniline, the use of Buchwald–Hartwig coupling conditions was necessary. 20) Compounds 10a–d were then catalytically reduced to the 3-aminoderivatives 11a–d and subsequently converted to the corresponding chloracetamides 12a–d, which were used for the preparation of the target aminoderivatives 13a–l. The 4-methoxybenzyl group of the compounds 13g–l was then removed in acidic media and derivatives 14a–f were prepared as well.

In order to synthesize the corresponding isomeric analogues we used 2-amino-3-nitropicoline (15, Chart 2), which by a reaction sequence analogous to the above mentioned reported, corresponding to the isomer 1, was converted to the regioisomers 22a and 23, as well as 22b. Concerning the preparation of the 4-methoxybenzyl-substituted compounds, only the N1 isomer 22b was isolated, presumably due to the significant steric hindrance exerted from the 3- nitro group.

Compounds 22a and b were selected for the insertion of the 7-alkyl or arylamino substituent, consequently, they were treated with cyclohexylamine (Chart 3) and provided the 7-substituted nitroderivatives 24a and b, respectively. The nitro group of the former compounds was then reduced and the resulting amines 25a and 26a were chloracetylated to give the chloracetamides 25b and 26b, which were treated with

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**Reagents and conditions:**
- a) NaNO₂, H₂SO₄, H₂O
- b) POCl₃, 110°C
- c) SnCl₂·2H₂O, HCl(c.), 55°C
- d) Ac₂O, CH₂Cl₂, r.t.
- e) (1): AcOK, Ac₂O, isoamyl nitrite, benzene, reflux, (2): NH₃(g.), MeOH, r.t.
- f) HNO₃, H₂SO₄, 95°C
- g) (1): K₂CO₃, MeCN, 0°C, (2): CH₃I for 8a, 9a or 4-methoxybenzyl chloride for 8b, 9b, 50°C
- h) cyclohexylamine, dimethyl sulfoxide (DMSO), microwave irradiation, 60 W, 158°C for 10c or aniline, X-Phos, Pd₂dba₃, CsCO₃, toluene, reflux for 10b, d
- i) Pd/C, H₂, EtOH, 55 psi, r.t.
- j) chloroacetylchloride, Et₃N, THF, −40°C for 12a, e or 0°C for 12b, d.
- k) dimethylamine (5.6 M solution in EtOH) for 13a, d, g, j or 1-methylpiperazine for 13b, e, h, k or aniline for 13c, f, i, l, EtOH, 80°C; l) trifluoroacetic acid, 70°C.
Reagents and conditions: a) NaNO₂, H₂SO₄, H₂O; b) POCl₃, 110°C; c) SnCl₂·2H₂O, HCl(c.), 55°C; d) Ac₂O, CH₂Cl₂, r.t.; e) (1): AcOK, Ac₂O, isoamyl nitrite, benzene, reflux; (2): NH₃(g.), MeOH, r.t.; f) HNO₃, H₂SO₄, 95°C; g) (1): K₂CO₃, MeCN, 0°C, (2): CH₃I for 22a, 23 or 4-methoxybenzyl chloride for 22b, 50°C.

Chart 2

Reagents and conditions: a) cyclohexylamine, DMSO, 150°C; b) Pd/C, H₂, EtOH, 55 psi, r.t.; c) chloroacetylchloride, Et₃N, THF, −40°C; d) dimethylamine (5.6 M solution in EtOH) for 27a, 28 or 1-methylpiperazine for 27b, or aniline for 27c, EtOH, 80°C; e) trifluoroacetic acid, 70°C; f) aniline, 2-ethoxyethanol, 130°C; g) dimethylamine (5.6 M solution in EtOH) for 33a, or 1-methylpiperazine for 33b, or aniline for 33c, EtOH, 80°C.

Chart 3

Reagents and conditions: a) aniline, 1,4-dioxane, reflux; b) (1): K₂CO₃, MeCN, 0°C, (2): 4-methoxybenzyl chloride, 50°C; c) Pd/C, H₂, EtOH, 55 psi, r.t.; d) chloroacetylchloride, Et₃N, THF, 0°C; e) dimethylamine (5.6 M solution in EtOH) for 38a or 1-methylpiperazine for 38b, EtOH, 80°C; f) trifluoroacetic acid, 70°C.

Chart 4
suitable amines to result in the target derivatives 27a–c and 28 as well as to its unprotected analogue 29. On the other hand, 22a was treated with aniline, to provide compound 30. As previously reported the 7-chloropyrazolo[3,4-c]pyridine is susceptible to nucleophilic substitution, even by relatively weak arylamines. The nitroderivative 30 was finally converted in three steps to the target derivatives 33a–c.

Our attempts to substitute the chlorine atom of 22b with aniline were unsuccessful, presumably due to steric hindrance provided by the bulky 1-benzyl group. Thus we used the nitroderivative 21 (Chart 4) which was easily substituted to provide the phenylamino analogue 34. This compound was treated with 4-methoxybenzylchloride to provide selectively the corresponding N2-isomer 35, which following a procedure analogous to the described above, provided the target compounds 38a, b and 39a, b.

**Biological Evaluation**  
The kinase inhibitory activity of the new compounds was tested against a panel of protein kinases and the results concerning the derivatives found to possess IC50 values <10 µM are presented in Table 1. 6-Bromoindirubin-3'-oxime (6-BIO), a selective inhibitor of GSK-3, was used as a reference compound. It is remarkable to notice that even if the vast majority of the synthesized new compounds are not endowed with interesting inhibitory activity against most of the kinases tested, the substitution pattern around the central heterocyclic ring system plays a crucial role on the inhibitory activity when present. Note here that, the kinases affected in the tested panel are structurally related, as they are members of the same group of the kinome, the CMGC cluster (for CDK, mitogen-activated protein kinase (MAPK), GSK3 and CLK).

In general, the 7-substituted pyrazolopyridines, possessing either a phenylamino- or a cyclohexylamino-group, did not show any activity regardless the decoration of the rest of the scaffold. On the contrary, the corresponding 5-substituted analogues presented quite interesting structure–activity relationships (SARs). Among this group, compounds bearing methyl or 4-methoxybenzyl substitution at position 1, were again found inactive, with the exception of 13d, which appear to demonstrate a slight inhibitory effect (IC50 2.8 µM) against CDK5. However, the majority of the corresponding 1-unsubstituted analogues 14a–f, have shown interesting activity and various degree of specificity. More precisely, compounds 14a, c and d, e inhibited GSK3α/β with IC50 values within 0.4–1 µM and among them, 14a and d, both containing the 3-dimethylaminoacetylamino substitution are selective towards this kinase. Compounds 14b and e, bearing a 3-(4-methylpiperazin-1-yl)aminoacetylamino substitution, show inhibitory activity against CLK1 and DYRK1A with IC50 values in the range of 1.1–4.5 µM, while 14e is simultaneously active against GSK3α/β as mentioned before, being the most active analogue in the series showing IC50 value 0.4 µM.

**Theoretical Calculations**  
To gain insight to the putative interaction mode between the active pyrazolopyridine derivatives 14a–f and GSK3α/β, docking calculations were performed by implementing the Glide SP algorithm and post-docking flexible minimization utilizing MacroModel. The co-crystal structure of GSK3β with indirubin was used as a template for docking calculations (pdb. id: 1UV5). The predicted binding mode of the pyrazolopyridine scaffold is depicted in Fig. 1. The inhibitors bind the active site of the kinase through a typical type-I interaction mode (Fig. 1A). Two hydrogen bonds are formed between the pyrazole nitrogen of positions 1 and 2 and the D133 carbonyl of the hinge region, while a third bond is formed between the exocyclic amide nitrogen of position 3 and the V135 backbone amide. An additional hydrogen bond between the nitrogen position 3-substituent and the sidechain of Y134 further stabilizes the complex (Fig. 1B). This interaction mode is in excellent agreement with the two basic SAR notions derived from experiment. Indeed, capping of N1 diminishes activity as one of the hydrogen bonds would be excluded while the geometry of the rest would be moderately perturbed, as evident for analogues 13a–f. Moreover, the lack of activity for analogues carrying a bulky group on position 7 can be explained by the existence of extensive steric clashes with the hinge backbone and the gatekeeper sidechain (for example, derivative 39a as compared to 14a and d as well as 39b to 14b and e). Within the series of active compounds 14a–f, the preference for a more flexible substitution in position 5 such as a cyclohexyl instead of a phenyl ring can be explained by the enhanced capacity of the

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aliphatic group to fit to the plasticity of the cavity entrance as defined by the glycine (Gly)-loop.

Conclusion

In conclusion we have designed and synthesized a number of new N-substituted (pyrazolo[3,4-c]pyridin-3-yl)acetamides, possessing a variety of substituents on the heterocyclic ring system, in order to study their kinase inhibitory profile. Among the derivatives, only compounds that bear 5-aryl or alkyl substituents and retain the pyrazole nitrogens unsubstituted, have shown inhibition of GSK3α/β, with adequate selectivity, without being cytotoxic. Docking calculations showed that the aforementioned combination of substituents can provide a rationale for the further development of this original versatile lead into highly potent and selective inhibitors of kinase GSK3α/β.

Experimental Chemistry

Melting points were determined on a Büchi apparatus and are uncorrected. 1H-NMR spectra and 2D spectra were recorded on a Bruker Avance III 600 or a Bruker Avance DRX 400 instrument, whereas 13C-NMR spectra were recorded on a Bruker Avance III 600 or a Bruker AC 200 spectrometer in deuterated solvents and were referenced to tetramethylsilane (TMS) (δ scale). The signals of 1H and 13C spectra were unambiguously assigned by using 2D-NMR techniques: 1H–1H correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), hetero-nuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC). Mass spectra were recorded with a LTQ Orbitrap Discovery instrument, possessing an Ionmax ionization source. Flash chromatography was performed on Merck silica gel 60 (0.040–0.063 mm). Analytical TLC was carried out on precoated (0.25 mm) Merck silica gel F-254 plates. The purity of all the synthesized compounds was >95% as ascertained by elemental analysis. Elemental analyses were undertaken using a PerkinElmer, Inc. PE 240C elemental analyzer (Norwalk, CT, U.S.A.) and the measured values for C, H, and N were within ±0.4% of the theoretical values.

5-Chloro-3-nitro-1H-pyrazolo[3,4-c]pyridine (7) Chloro derivative 6 (490 mg, 3.19 mmol) was dissolved into concentrated sulfuric acid (9.50 mL) at 0°C and then a mixture of concentrated sulfuric acid (0.49 mL) and nitric acid (0.49 mL) was added dropwise into this solution. This reaction mixture was heated at 95°C for 1 h. Upon cooling, it was poured into crushed ice, neutralized with an aqueous solution of NH₃ and the product was extracted with ethyl acetate (3×100 mL). The combined organic layers were dried over Na₂SO₄ and evaporated under reduced pressure to provide 600 mg of pure 7 as a yellow solid. Yield 94%, mp: 195–197°C (EtOAc). 1H-NMR (400 MHz, CDCl₃) δ: 8.10 (1H, d, J = 1.0 Hz), 9.16 (1H, d, H-7, J = 1.0 Hz). 13C-NMR (50 MHz, CDCl₃) δ: 131.3 (C-4), 131.8 (C-5), 137.1 (C-6), 141.3 (C-7). 1H–1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) were recorded under reduced pressure to provide 600 mg of pure 7 as a yellow solid. Yield 94%, mp: 195–197°C (EtOAc).

General Procedure for the Synthesis of 1-Substituted Derivatives 8a, b and 2-Substituted Derivatives 9a, b Potassium carbonate (530 mg, 3.84 mmol) was added into a solution of nitro derivative 7 (540 mg, 2.72 mmol) in anhydrous acetonitrile (20 mL) at 0°C and under argon, and this solution was stirred for 30 min, followed by dropwise addition of iodomethane (0.22 mL, 3.53 mmol) or 4-methoxybenzyl chloride (0.41 mL, 3.3 mmol). This reaction mixture was heated at 50°C for 5 h. Upon completion of the reaction, the solvent was evaporated and the residue was extracted with ethyl acetate (3×100 mL) and water (100 mL). The combined organic layers were dried over Na₂SO₄ and evaporated under reduced pressure. The crude product was then purified by column chromatography (silica gel) to provide pure compounds 8a, b and 9a, b.

5-Chloro-1-methyl-3-nitro-1H-pyrazolo[3,4-c]pyridine (8a) and 5-Chloro-2-methyl-3-nitro-2H-pyrazolo[3,4-c]pyridine (9a) These compounds were prepared according to the general procedure described above. Purification was effected using a mixture of cyclohexane–ethyl acetate (7:3, v/v) as the eluent.

Data for 8a: Yield 80%. Pale yellow solid, mp: 172°C (Et(O).) 1H-NMR (400 MHz, CDCl₃) δ: 4.34 (3H, s, CH₃), 8.16 (1H, d, H-4, J = 1.0 Hz), 8.89 (1H, d, H-7, J = 1.0 Hz).
\textsuperscript{13}C-NMR (50 MHz, CDCl\textsubscript{3}) \(\delta\): 38.0 (CH\textsubscript{2}), 114.4 (C-4), 123.3 (C-3a), 134.2 (C-7), 137.5 (C-7a), 145.9 (C-3). \textit{Anal.} Calcd for C\textsubscript{13}H\textsubscript{17}N\textsubscript{5}O\textsubscript{2}: C, 56.72; H, 3.48; N, 17.58. Found: C, 53.01; H, 3.69; N, 17.58. 

Data for 8a: Yield 7%. Yellow solid, mp: 157°C (EtOAc). \textit{1H-NMR} (400 MHz, CDCl\textsubscript{3}) \(\delta\): 1.78 (2H, m, cyclohexyl H), 2.07 (2H, m, cyclohexyl H), 3.48 (1H, m, cyclohexyl H-1\textsuperscript{d}, C-6\textsuperscript{d}), 125.3 (C-1\textsuperscript{d}), 132.4 (C-7\textsuperscript{d}), 133.8 (C-7a\textsuperscript{d}), 146.0 (C-3\textsuperscript{d}), 153.5 (C-3\textsuperscript{c}), 134.8 (C-7), 136.9 (C-7a), 145.7 (C-3), 160.4 (C-4). \textit{Anal.} Calcd for C\textsubscript{14}H\textsubscript{11}ClN\textsubscript{4}O\textsubscript{3}: C, 52.76; H, 3.48; N, 17.44. }
The eluent, providing 178–179°C (EtOAc). 1H-NMR (400 MHz, DMSO-

This compound was prepared following an analog to that described for the synthesis of 11a, starting from the nitro derivative 10b. The product was purified by column chromatography (silica gel) using a mixture of dichloroethane–ethyl acetate (5:5 up to 2:8, v/v) as the eluent, to provide pure 11b as a green solid, in 84% yield. mp: 178–179°C (EtOAc). 1H-NMR (400 MHz, CDCl3) δ: 3.75 (3H, s, CH3), 5.61 (2H, brs, D2O exch., NH), 6.78 (1H, t, H-4, J=7.2 Hz), 7.21 (3H, m, H-3', H-5', H-4'), 7.37 (2H, dd, H-2', H-6', J=8.01 Hz, J=0.9 Hz), 8.56 (2H, m, H-7, NH-aniline). 13C-NMR (50 MHz, DMSO-d6) δ: 35.1 (CH3), 97.9 (C-4), 116.2 (C-2', C-6'), 118.8 (C-4'), 121.1 (C-3a), 128.6 (C-3', C-5'), 130.4 (C-7), 134.8 (C-5'), 143.5 (C-7'), 146.4 (C-6'), 147.3 (C-3). Anal. Calcd for C16H15N3O: C, 67.96; H, 6.62; N, 19.62. Found: C, 67.83; H, 6.87; N, 19.50.

General Procedure for the Synthesis of Derivatives 12a–d

Chloroacetyl chloride (96 µL, 1.2 mmol) and triethylamine (153 µL, 1.1 mmol) were added into a solution of the amines 11a–d (1 mmol) in anhydrous tetrahydrofuran (THF) (15 mL), under argon, at 0°C for amines 11b, c or at 0°C for amines 11a, d. The resulting solution was allowed to reach r.t. and stirring was continued for one more hour. The solvents were then vacuum-evaporated and the residue was used immediately to the next step, without any further purification.

General Procedure for the Synthesis of Derivatives 13a–l

Dimethylamine (5.6 µmol solution in ethanol, 0.89 mL, 5 mmol) or 1-methylpiperazine (0.22 mL, 2 mmol) or aniline (0.36 mL, 4 mmol) was added into a solution of the intermediate chlorides 12a–d (1 mmol) in absolute ethanol (10 mL), under argon, and the resulting solution was heated at 80°C for 4 h. Upon completion of the reaction solvents were evaporated and the residue was purified by column chromatography to provide pure amides 13a–l.

N-(5-(Cyclohexylamino)-1-methyl-1H-pyrazolo[3,4-c]-pyridine-3-yl)-2-(dimethylamino)acetamide (13a)

This compound was prepared according to the general procedure described above, upon reaction of the chloride 12a with dimethylamine (5.6 µmol solution in ethanol). The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (100:1, v/v) as the eluent to provide pure 13a as a green oil. Yield: 52%. 1H-NMR (400 MHz, CDCl3) δ: 1.30 (3H, s, cyclohexyl H), 1.40 (2H, m, cyclohexyl H), 1.61 (1H, m, cyclohexyl H), 1.72 (2H, m, cyclohexyl H), 2.02 (2H, m, cyclohexyl H), 2.39 (1H, m, cyclohexyl H), 2.37 (3H, s, OCH3), 2.14 (2H, brs, D2O exch., NH), 5.21 (2H, s, CH2), 6.32 (1H, d, H-4, J=1.0 Hz), 6.78 (2H, d, H-3', H-5', J=8.78 Hz), 7.13 (2H, d, H-2', H-6', J=8.78 Hz), 8.20 (1H, d, H-7, J=1.0 Hz). 13C-NMR (50 MHz, CDCl3) δ: 25.0 (cyclohexyl C-3', C-5'), 25.9 (cyclohexyl C-4'), 33.2 (cyclohexyl C-2', C-6'), 51.6 (cyclohexyl C-1'), 52.6 (CH3), 55.3 (OCH3), 91.2 (C-4), 114.1 (C-3', C-5'), 123.0 (C-3a), 128.8 (C-2', C-6'), 129.2 (C-1'), 131.4 (C-7), 133.9 (C-7a), 145.8 (C-3'), 151.4 (C-5), 159.2 (C-4'). Anal. Calcd for C20H25N5O: C, 68.35; H, 7.17; N, 19.93. Found: C, 68.08; H, 7.04; N, 20.22.

1-(4-Methoxybenzyl)-N2-phenyl-1H-pyrazolo[3,4-c]pyridine-3,5-diamine (11d) Ten percent Pd/C (5 mg) and triethylsilane (0.34 mL, 2.13 mmol) were added into a solution of the nitro derivative 10d (80 mg, 0.21 mmol) in anhydrous methanol (5 mL), under argon, and the resulting solution was stirred at r.t. for 12 h. The solution was filtered through a celite pad to remove the catalyst and the filtrate was evaporated to dryness. The residue was purified by column chromatography (silica gel) using a mixture of cyclohexane–ethyl acetate (8:2, v/v) as the eluent, to provide pure 11d (60 mg, 82%) as a brown solid. mp: 148°C (EtO). 1H-NMR (400 MHz, CDCl3) δ: 3.78 (3H, s, OCH3), 4.64 (2H, brs, D2O exch., NH), 5.27 (2H, s, CH2), 6.84 (2H, d, H-3', H-5', J=8.6 Hz), 7.06 (2H, m, H-4, H-4'), 7.18 (2H, d, H-2', H-6', J=8.6 Hz), 7.23 (2H, d, H-2', H-6', J=7.6 Hz), 7.32 (2H, t, H-3', H-5', J=7.6 Hz), 7.58 (1H, brs, D2O exch., NH), 8.20 (1H, s, H-7). 13C-NMR (50 MHz, CDCl3) δ: 59.0 (CH3), 55.3 (OCH3), 98.4 (C-4), 114.3 (C-3', C-5'), 120.6 (C-2', C-6'), 123.5 (C-4'), 124.2 (C-3a), 127.0 (C-7'), 128.9 (C-2', C-6'), 129.6 (C-3', C-5'), 133.2 (C-7a), 140.1 (C-1'), 146.2 (C-3), 146.5 (C-5), 159.6 (C-4'). Anal. Calcd for C16H15N3O: C, 69.55; H, 5.54; N, 20.28. Found: C, 69.29; H, 5.57; N, 20.46.

N-(5-(Cyclohexylamino)-1-methyl-1H-pyrazolo[3,4-c]-pyridine-3-yl)-2-(dimethylamino)acetamide (13a)

This compound was prepared according to the general procedure described above, upon reaction of the chloride 12a with dimethylamine (5.6 µmol solution in ethanol). The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (100:1, v/v) as the eluent to provide pure 13a as a green oil. Yield: 52%. 1H-NMR (400 MHz, CDCl3) δ: 1.22 (3H, m, cyclohexyl H), 1.40 (2H, m, cyclohexyl H), 1.61 (1H, m, cyclohexyl H), 1.73 (2H, m, cyclohexyl H), 2.05 (2H, m, cyclohexyl H), 2.42 (6H, s, N(CH2)3), 3.17 (2H, s, CH2), 3.44 (1H, m, cyclohexyl H-1'), 3.94 (3H, s, CH3–pyrazole), 6.83 (1H, s, H-4), 8.39 (1H, s, H-7), 9.55 (1H, s, D2O exch., NHCO). 13C-NMR (50 MHz, CDCl3) δ: 24.8 (cyclohexyl C-3', C-5'), 25.9 (cyclohexyl C-4'), 33.2 (cyclohexyl C-2', C-6'), 35.7 (CH2–pyrazole), 46.0 (N(CH2)3), 51.2 (cyclohexyl C-1'), 63.0 (CH3), 93.8 (C-4'), 123.9 (C-3a), 131.3 (C-7), 134.2 (C-7a), 136.9 (C-3'), 152.0 (C-5), 168.6 (CO). HR-MS (electrospray ion-
N-(5-Cyclohexylamino)-1-methyl-1H-pyrazolo[3,4-c]-pyridin-3-yl)-2-(4-methylpiperazin-1-yl)acetamide (13b) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12a with 1-methylpiperazine. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (90:1, v/v) as the eluent to provide pure 13c as a yellow solid. Yield: 35%, mp: 175–176°C (EtOAc). 1H-NMR (400 MHz, CDCl3): δ: 1.20–1.31 (3H, m, cyclohexyl H), 1.42 (2H, m, cyclohexyl H), 1.66 (1H, m, cyclohexyl H), 1.78 (2H, m, cyclohexyl H), 2.07 (2H, m, cyclohexyl H), 3.44 (1H, m, cyclohexyl H-1), 3.91 (3H, s, CH3), 4.00 (2H, s, CH3), 4.57 (1H, br, D2O exh., NH-cyclohexylamino), 6.71 (2H, d, H-2', H-6', J=7.9Hz), 6.74 (1H, s, H-4), 6.85 (1H, t, H-4', J=7.3Hz), 7.24 (2H, t, H-3', H-5'), 7.52 (7.9Hz), 8.38 (1H, s, H-7), 9.10 (1H, br, D2O exh., NHCO). 13C-NMR (50 MHz, CDCl3): δ: 24.8 (cyclohexyl C-3', C-5'), 25.9 (cyclohexyl C-4'), 33.1 (cyclohexyl C-2', C-6'), 35.7 (CH3), 49.1 (CH3), 51.2 (cyclohexyl C-1'), 93.2 (C-4'), 113.4 (C-2', C-6'), 119.5 (C-4'), 124.4 (C-3a), 129.5 (C-3a), C-131.1 (C-7a), 136.4 (C-3), 147.0 (C-1'), 151.9 (C-5), 169.2 (CO). HR-MS (ESI) m/z: Calcd for C29H25N7O3: [M+H]+ = 386.2663. Found 386.2665. Anal. Calcd for C29H25N7O3: C, 62.31; H, 8.11; N, 25.29. Found: C, 62.56; H, 8.22; N, 25.29.

N-(5-Cyclohexylamino)-1-methyl-1H-pyrazolo[3,4-c]-pyridin-3-yl-2-(phenylamino)acetamide (13f) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12b with aniline. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–ethanol (100:1, v/v) as the eluent to provide pure 13f as a yellow solid. Yield: 70%, mp: 180–181°C (EtOH). 1H-NMR (400 MHz, DMSO-d6): δ: 3.95 (2H, d, CH2, J=6.0Hz), 4.01 (3H, s, CH3), 6.04 (1H, t, CH3–NH–, J=6.0Hz), 6.60 (3H, m, H-2', H-4', H-6'), 6.80 (1H, t, H-4', J=7.2Hz), 7.11 (2H, t, H-3', H-5', J=7.7Hz), 7.20 (3H, m, H-3', H-5', H-4), 7.42 (2H, d, H-2', H-6', J=7.8Hz), 8.72 (1H, s, D2O exh., NH–aniline), 8.79 (1H, d, H-7, J=0.81Hz), 10.50 (1H, br, D2O exh., NHCO). 13C-NMR (50 MHz, DMSO-d6): δ: 36.1 (CH3), 47.0 (CH3), 98.9 (C-4), 112.7 (C-2', C-6'), 116.9 (C-4), 117.0 (C-2', C-6'), 119.7 (C-4'), 123.6 (C-3a), 129.1 (C-3', C-5'), 129.4 (C-3', C-5'), 132.4 (C-7), 134.7 (C-7a), 137.9 (C-3), 143.7 (C-1'), 148.3 (C-5'), 148.7 (C-1'), 169.9 (CO). HR-MS (ESI) m/z: Calcd for C39H33N7O3: [M+H]+ = 380.2193. Found 380.2200. Anal. Calcd for C39H33N7O3: C, 63.30; H, 6.64; N, 25.84. Found: C, 63.41; H, 6.79; N, 25.73.

2-(Dimethylamino)-N-(1-methyl-5-(phenylamino)-1H-pyrazolo[3,4-c]-pyridin-3-yl)-2-(dimethylamino)acetamide (13d) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12b with dimethylamine (5.6 M solution in ethanol). The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (100:2, v/v) as the eluent to provide pure 13d as a green solid. Yield: 86%, mp: 166–167°C (EtOH). 1H-NMR (400 MHz, CDCl3): δ: 2.41 (6H, s, N(CH3)2), 3.15 (2H, s, CH3), 4.00 (3H, s, CH3–pyrazole), 6.64 (1H, s, D2O exch., NH–aniline), 6.95 (1H, t, H-4', J=7.2Hz), 7.30 (4H, m, H-2', H-3', H-5', H-6'), 7.57 (1H, d, H-4, J=1.2Hz), 8.54 (1H, d, H-7, J=1.2Hz), 9.59 (1H, s, D2O exh., NHCO). 13C-NMR (50 MHz, CDCl3): δ: 35.7 (CH3–pyrazole), 46.0 (N(CH3)2), 63.0 (CH3), 98.7 (C-4), 117.8 (C-2', C-6'), 121.1 (C-4'), 123.2 (C-3a), 129.2 (C-3', C-5'), 131.5 (C-7), 135.0 (C-7a), 137.5 (C-5), 142.1 (C-1'), 147.9 (C-5'), 168.8 (CO). HR-MS (ESI) m/z: Calcd for C39H33N7O3: [M+H]+ = 373.1771. Found 373.1772. Anal. Calcd for C39H33N7O3: C, 67.73; H, 5.41; N, 22.57. Found: C, 67.94; H, 5.56; N, 22.28.

N-(5-Cyclohexylamino)-1-(4-methoxybenzyl)-1H-pyrazolo[3,4-c]-pyridin-3-yl-2-(dimethylamino)acetamide (13g) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12c with dimethylamine (5.6 M solution in ethanol). The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (90:1, v/v) as the eluent to provide pure 13g as a yellow solid. Yield: 83%, mp: 196–197°C (EtOAc). 1H-NMR (400 MHz, DMSO-d6): δ: 2.18 (3H, s, CH3–piperazine), 2.38 (4H, brs, piperazine H-3', H-5'), 2.57 (4H, brs, piperazine H-2', H-6'), 3.20 (2H, s, CH3), 4.01 (3H, s, CH3–pyrazole), 6.80 (1H, t, H-4', J=7.2Hz), 7.21 (3H, m, H-3', H-5', H-4), 7.45 (2H, d, H-2', H-6', J=8.0Hz), 8.74 (1H, s, D2O exh., NH–aniline), 8.80 (1H, d, H-7, J=1.0Hz), 10.14 (1H, brs, D2O exh., NHCO). 13C-NMR (50 MHz, DMSO-d6): δ: 35.5 (CH3–pyrazole), 45.6 (CH3–piperazine), 52.6 (piperazine C-2', C-6'), 54.5 (piperazine C-3', C-5'), 60.8 (CH3), 98.4 (C-4'), 116.5 (C-2', C-6'), 119.2 (C-4'), 123.2 (C-3a), 128.6 (C-3', C-5'), 131.8 (C-7), 134.2 (C-7a), 137.2 (C-3), 143.1 (C-1'), 147.8 (C-5'), 168.2 (CO). HR-MS (ESI) m/z: Calcd for C42H40N9O3: [M+H]+ = 380.2193. Found 380.2200. Anal. Calcd for C42H40N9O3: C, 63.30; H, 6.64; N, 25.84. Found: C, 63.41; H, 6.79; N, 25.73.
mixture of dichloromethane–methanol (100:1, v/v) as the eluent to provide pure 13g as a yellow oil. Yield: 57%. 1H-NMR (400 MHz, CDCl3) δ: 1.18 (3H, m, cyclohexyl H), 1.36 (2H, m, cyclohexyl H), 1.57 (1H, m, cyclohexyl H), 1.69 (2H, m, cyclohexyl H), 2.01 (2H, m, cyclohexyl H), 2.36 (6H, s, N(CH3)2), 3.11 (2H, s, NHCOC2H5), 3.42 (1H, m, cyclohexyl H-1′), 3.70 (3H, s, OCH3), 4.30 (1H, brs, D2O exch.), NH-cyclohexylamine, 5.32 (2H, s, CH2–pyrazole), 6.77 (2H, d, H-3′, H-5′), J = 8.7 Hz, 7.28 (1H, d, H-2′, H-6′), J = 8.5 Hz, 7.32 (2H, t, H-3′, H-5′), J = 7.5 Hz, 8.30 (1H, s, H-7), 9.05 (1H, s, D2O exch.), NHCO. 13C-NMR (50 MHz, CDCl3) δ: 24.8 (cyclohexyl C-3′, C-5′), 25.9 (cyclohexyl C-2′, C-6′), 49.3 (NHCOC2H5), 51.2 (cyclohexyl C-1′), 53.2 (CH2–pyrazole), 55.3 (OCH3), 94.1 (C-4), 113.5 (C-2′, C-6′), 114.3 (C-3′, C-5′), 119.7 (C-4′), 125.0 (C-3a), 127.9 (C-1′), 128.8 (C-2′, C-6′), 129.6 (C-3′, C-5′), 130.9 (C-7), 133.3 (C-7a), 136.9 (C-3′), 146.9 (C-1′), 151.6 (C-5), 159.5 (C-4′), 169.0 (CO). HR-MS (ESI) m/z: Calcld for C25H30N5O2: [M+H]+ = 437.2660. Found 437.2666. Anal. Calcld for C25H30N5O2: C, 66.03; H, 6.72; N, 17.34. Found: C, 66.49; H, 6.80; N, 17.12.

2-(Dimethylamino)-N-(1-(4-methoxybenzyl)-5-phenylamin-1H-pyrazolo[3,4-c]pyridin-3-yl)-1-ylacetamide (13j) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12d with dimethylamine (5.6 m solution in ethanol). The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–ethyl acetate (6:4, v/v) as the eluent to provide pure 13j as a pale green solid. Yield: 70%; mp: 143°C (Et2O). 1H-NMR (400 MHz, CDCl3) δ: 2.36 (6H, s, N(CH3)2), 3.12 (2H, s, NHCOC2H5), 3.72 (2H, s, OCH3), 5.36 (2H, s, CH2–pyrazole), 6.82 (2H, d, H-3′, H-5′), J = 8.7 Hz, 6.90 (2H, m, H-4′, NH–aniline), 7.17 (2H, d, H-2′, H-6′, J = 8.7 Hz, 7.26 (4H, m, H-2′, H-5′, H-6′), 7.59 (1H, d, H-4', J = 10.1 Hz), 8.45 (1H, d, H-7, J = 10.1 Hz), 9.69 (1H, brs, D2O exch.), NHCO. 13C-NMR (50 MHz, CDCl3) δ: 46.0 (N(CH3)2), 53.0 (CH2–pyrazole), 55.3 (OCH3), 63.0 (NHCOC2H5), 99.1 (C-4), 114.3 (C-3′, C-5′), 117.9 (C-2′, C-6′), 121.1 (C-4′), 123.8 (C-3a), 128.1 (C-1′), 128.9 (C-2′, C-6′), 129.2 (C-3′, C-5′), 131.9 (C-7), 134.5 (C-7a), 138.1 (C-3), 142.2 (C-1′), 148.1 (C-5), 159.5 (C-4′), 168.9 (CO). HR-MS (ESI) m/z: Calcld for C26H29N7O2: [M+H]+ = 431.2190. Found 431.2191. Anal. Calcld for C26H29N7O2: C, 66.96; H, 6.09; N, 19.52. Found: C, 67.11; H, 6.24; N, 19.39.

N-(1-(4-Methoxybenzyl)-5-(phenylamin-1H-pyrazolo[3,4-c]pyridin-3-yl)-2-(4-methylpiperazin-1-yl)acetamide (13k) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12d with 1-methylpiperazine. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (96:4, v/v) as the eluent to provide pure 13k as a yellow solid. Yield: 70%; mp: 134°C (CH2Cl2/Et2O). 1H-NMR (400 MHz, CDCl3) δ: 2.52 (4H, brs, piperazine H-3′, H-5′), 2.68 (4H, brs, piperazine H-2′, H-6′), 3.18 (2H, s, NHCOC2H5), 3.74 (3H, s, OCH3), 5.40 (2H, s, CH2–pyrazole), 6.74 (1H, brs, NH), 6.82 (2H, d, H-3′, H-5′, J = 8.7 Hz, 6.92 (1H, m, H-4′), 7.17 (2H, d, H-2′, H-6′, J = 8.7 Hz, 7.26 (4H, m, H-2′, H-5′, H-6′), 7.51 (1H, d, H-4', J = 10.1 Hz), 8.42 (1H, d, H-7, J = 10.1 Hz), 9.51 (1H, brs, D2O exch.), NHCO. 13C-NMR (50 MHz, CDCl3) δ: 45.8 (CH2–pyrazole), 53.1 (CH2–pyrazole), 53.3 (piperazine C-2′, C-6′), 54.9 (piperazine C-3′, C-5′), 55.3 (OCH3), 61.4 (NHCOC2H5), 98.7 (C-4), 114.3 (C-3′, C-5′), 118.0 (C-2′, C-6′), 121.2 (C-4′), 123.9 (C-3a), 128.0 (C-1′), 128.8 (C-2′, C-6′), 129.2 (C-3′, C-5′), 132.0 (C-7), 134.5 (C-7a), 137.8 (C-3), 142.1 (C-1′), 148.1 (C-5), 159.5 (C-4′), 168.4 (CO). HR-MS (ESI) m/z: Calcld for C25H27N7O2: [M+H]+ = 486.2612. Found
N-(1-(4-Methoxybenzyl)-5-(phenylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)-2-(phenylamino)acetamide (13i) This compound was prepared according to the general procedure described above, upon reaction of the chloride 12d with aniline. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (100:1, v/v) as the eluent to provide pure 13i as a beige solid. Yield: 58%, mp: 208–209°C (CHCl₃/CH₃OH). ¹H-NMR (400 MHz, DMSO-d₆), δ: 3.70 (3H, s, OCH₃), 3.93 (2H, brs, NHCOCH₂), 5.50 (2H, s, CH₂–pyrazole), 6.00 (1H, brs, CH₂NH), 6.58 (1H, t, H-4′, J = 7.2Hz), 6.61 (2H, d, H-2″, H-6″, J = 7.8Hz), 6.79 (1H, t, H-4, J = 7.2Hz), 6.88 (2H, d, H-3″, H-5″, J = 8.5Hz), 7.10 (2H, t, H-3″, H-5″, J = 7.5Hz), 7.19 (3H, m, H-3, H-5, H-4), 7.27 (2H, d, H-2″, H-6″, J = 8.5Hz), 7.41 (2H, d, H-2″, H-6″, J = 8.2Hz), 8.73 (1H, s, D₂O exh., NH–aniline), 8.86 (1H, s, H-7), 10.57 (1H, s, D₂O exh., NHCO), ¹³C-NMR (50 MHz, DMSO-d₆), δ: 46.4 (NHCOCH₂), 51.6 (CH₂–pyrazole), 55.0 (OCH₃), 98.7 (C-4), 112.2 (C-2″, C-6″), 113.9 (C-3″, C-5″), 116.3 (C-4″), 116.6 (C-2″, C-6″), 119.2 (C-4″), 123.5 (C-3a), 128.6 (C-3″, C-5″), 128.9 (C-1″), 129.0 (C-2″, C-6″), 131.9 (C-7), 133.6 (C-7a), 138.3 (C-13), 143.0 (C-1″), 147.9 (C-5), 148.1 (C-1″), 158.8 (C-4″), 169.4 (CO). HR-MS (ESI) m/z: Calcd for C₃₂H₂₇N₁₀O₄: [M+H]+ = 479.2190. Found 479.2194. Anal. Calcd for C₃₂H₂₇N₁₀O₄: C, 70.28; H, 5.48; N, 17.56. Found: C, 70.03; H, 5.39; N, 17.66.

General Procedure for the Synthesis of Derivatives 14a–f A solution of the compounds 13g–l (0.2 mmol) in trifluoroacetic acid (5 mL) was heated at 70°C for 96h. Then the solvent was vacuum-evaporated and the residue was neutralized with sodium bicarbonate and extracted with ethyl acetate (3x50mL). The combined organic extracts were dried (Na₂SO₄), concentrated under vacuum and the residue was purified by column chromatography to provide pure target compounds 14a–f.

N-(5-Cyclohexylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)-2-(dimethylamino)acetamide (14a) This compound was prepared according to the general procedure described above, starting from 13g. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (95:5, v/v) as the eluent to provide pure 14a as a green solid. Yield: 80%, mp: 132–133°C (Et₂O/pentane). ¹H-NMR (400 MHz, CDCl₃), δ: 1.18 (3H, t, cyclohexyl H), 1.32 (2H, m, cyclohexyl H), 1.57 (1H, m, cyclohexyl H), 1.68 (2H, m, cyclohexyl H), 2.01 (2H, m, cyclohexyl H), 2.38 (6H, s, N(CH₃)₂), 3.17 (2H, s, CH₂), 3.40 (1H, m, cyclohexyl H-1′), 4.23 (4H, s, H-4), 8.37 (1H, s, H-7), 9.62 (1H, brs, D₂O exh., NH–cyclohexylamine), 6.80 (1H, t, H-4″, J = 8.4Hz), 8.37 (1H, s, H-7), NH–cyclohexylamine), 11.62 (1H, brs, D₂O exh., NH–pyrazole). ¹³C-NMR (50 MHz, CDCl₃), δ: 24.7 (cyclohexyl C-3″, C-5″), 25.8 (cyclohexyl C-4″), 33.0 (cyclohexyl C-2″, C-6″), 46.0 (N(CH₃)₂), 51.2 (cyclohexyl C-1″), 63.0 (CH₃), 93.3 (C-3″), 123.3 (C-3a), 132.6 (C-7), 134.2 (C-7a), 138.4 (C-3), 151.8 (C-5), 169.2 (CO). HR-MS (ESI) m/z: Calcd for C₂₂H₂₄N₁₀O₂: [M+H]+ = 329.1625. Found 329.1626. Anal. Calcd for C₂₂H₂₄N₁₀O₂: C, 65.91; H, 6.64; N, 23.06. Found: C, 66.12; H, 6.77, N, 22.89.

2-(Dimethylamino)-N-(5-(phenylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)acetamide (14d) This compound was prepared according to the general procedure described above, starting from 13j. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (100:2, v/v) as the eluent to provide pure 14d as a pale green solid. Yield: 89%, mp: 194°C (EtOAc).

13C-NMR (50 MHz, DMSO-d₆), δ: 2.33 (6H, s, N(CH₃)₂), 3.16 (2H, s, CH₂), 6.78 (1H, t, H-4″, J = 7.2Hz), 7.20 (3H, m, H-3″, H-5″, H-4), 7.46 (2H, d, H-2″, H-6″, J = 7.8Hz), 8.66 (1H, d, H-7, J = 0.81Hz), 8.69 (1H, s, D₂O exh., NH–aniline), 110.4 (1H, brs, D₂O exh., NH–pyrazole). ¹³C-NMR (50 MHz, DMSO-d₆), δ: 45.2 (N(CH₃)₂), 62.1 (CH₃), 98.5 (C-4), 116.4 (C-2″, C-6″), 119.0 (C-4″), 123.0 (C-3a), 128.6 (C-3″, C-5″), 132.0 (C-7), 134.4 (C-7a), 138.6 (C-3), 143.2 (C-1″), 147.7 (C-5), 168.7 (CO). HR-MS (ESI) m/z: Calcd for C₃₀H₂₆N₁₀O₂: [M+H]+ = 311.1615. Found 311.1619. Anal. Calcd for C₃₀H₂₆N₁₀O₂: C, 61.92; H, 5.85; N, 27.08. Found: C, 62.11; H, 5.87, N, 27.17.
2-(4-Methylpiperazin-1-yl)-N-(5-(phenylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)acetamide (14e) This compound was prepared according to the general procedure described above, starting from 13k. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (100 : 5, v/v) as the eluent to provide pure 14e as a green solid. Yield: 78%, mp: 186°C (EtOAc). 1H-NMR (400 MHz, DMSO-d6): δ: 2.18 (3H, s, CH3), 2.40 (4H, brs, piperazine H-3', H-5'), 2.58 (4H, brs, piperazine H-2', H-6'), 3.20 (2H, s, CH2), 6.78 (1H, t, H-4', J=7.21Hz), 7.20 (3H, m, H-3', H-5', 7.45 (2H, d, H-2', H-6', J=8.08Hz), 8.66 (1H, s, H-7'), 8.70 (1H, s, D2O exchanged, NH–aniline), 10.12 (1H, s, D2O exchanged, NH–pyrazole). 13C-NMR (50 MHz, DMSO-d6): δ: 45.5 (CH3), 52.5 (piperazine C-2', C-6'), 54.4 (piperazine C-3', C-5'), 60.7 (CH2), 98.5 (C-4'), 116.5 (C-2', C-6'), 119.0 (C-4'), 122.8 (C-3a), 128.5 (C-3', C-5'), 132.0 (C-7a), 134.5 (C-7a), 138.6 (C-3), 143.2 (C-1'), 147.7 (C-5), 168.2 (CO). HR-MS (ESI) m/z: Calcd for C21H19ClN10O3: [M+H]+ =366.2037. Found 366.2044. Anal. Calcd for C21H19ClN10O3: C, 58.45; H, 3.64; N, 17.16. Found: C, 58.28; H, 3.69; N, 17.29.

2-(Phenylamino)-N-(5-(phenylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)acetamide (14f) This compound was prepared according to the general procedure described above, starting from 13l. The product was purified by column chromatography (silica gel) using a mixture of cyclohexane–ethyl acetate (1 : 1, v/v) as the eluent to provide pure 14f as a beige solid. Yield: 84%, mp: 126°C (dec.) (EtOAc). 1H-NMR (400 MHz, CDCl3): δ: 4.00 (2H, s, CH2), 4.40 (1H, brs, D2O exchanged, CH2NH), 6.53 (1H, brs, D2O exchanged, NH–aniline), 6.71 (2H, d, H-2', H-6', J=7.81Hz), 6.85 (1H, t, H-4', J=7.21Hz), 7.00 (1H, t, H-4', J=7.1Hz), 7.23 (2H, t, H-3', H-5', J=7.71Hz), 7.28 (2H, d, H-2', H-6', J=7.71Hz), 7.33 (2H, t, H-3', H-5', J=7.61Hz), 7.49 (1H, s, H-4), 8.59 (1H, s, H-7), 9.09 (1H, s, D2O exchanged, NHCO), 9.95 (1H, brs, D2O exchanged, NH–pyrazole). 13C-NMR (50 MHz, CDCl3): δ: 49.3 (CH3), 79.7 (C-4'), 113.5 (C-2', C-6'), 118.4 (C-2', C-6'), 119.8 (C-4'), 121.7 (C-3a), 123.4 (C-3, C-5'), 129.4 (C-3', C-5'), 132.4 (C-7a), 135.1 (C-7a), 139.8 (C-3), 141.6 (C-1'), 146.6 (C-5'), 148.4 (C-1'), 169.3 (CO). HR-MS (ESI) m/z: Calcd for C21H17ClN9O3: [M+H]+ =359.1615. Found 359.1622. Anal. Calcd for C21H17ClN9O3: C, 67.01; H, 4.93; N, 17.29. Found: C, 66.89; H, 5.01; N, 23.62.

7-Chloro-3-nitro-1H-pyrazolo[3,4-c]pyridine (21) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the nitroderivative 7, upon nitration of pyrazolopyridine 20, in 94% yield. The nitroderivative 21 was obtained pure, as a yellow solid. mp: 172–173°C (EtOAc). 1H-NMR (600 MHz, DMSO-d6): δ: 8.03 (1H, d, H-4, J=5.61Hz), 8.27 (1H, d, H-5, J=5.61Hz). 13C-NMR (151 MHz, DMSO-d6): δ: 113.9 (C-4'), 122.1 (C-3a), 136.4 (C-7a), 137.5 (C-3), 141.5 (C-5), 149.6 (C-7). Anal. Calcd for C12H9ClN3O: C, 59.02; H, 3.59; N, 30.12. Found: C, 59.28; H, 3.69; N, 30.23.

7-Chloro-1-methyl-3-nitro-1H-pyrazolo[3,4-c]pyridin-7-amine (24a) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the derivative 10a, upon reaction of the chloroderivative 22a with cyclohexylamine for 2 h, in 89% yield. The product was purified by column chromatography using a mixture of cyclohexane–ethyl acetate (7 : 3 up to 6 : 4, v/v) as the eluent, to provide pure 24a as a yellow solid. mp: 171–173°C (EtOAc/Et2O). 1H-NMR (400 MHz, CDCl3): δ: 1.20–1.31 (3H, m, cyclohexyl H), 1.41–1.50 (2H, m, cyclohexyl H), 1.62–1.68 (1H, m, cyclohexyl H), 1.71–1.78 (2H, m, cyclohexyl H), 2.10–2.15 (2H, m, cyclohexyl H), 4.12–4.24 (1H, m, cyclohexyl H-1'), 4.45 (3H, s, CH3), 4.78 (1H, brs, D2O exchanged, NH), 7.33 (1H, d, H-4, J=5.81Hz), 7.95 (1H, d, H-5, J=5.81Hz). 13C-NMR (50 MHz, CDCl3): δ: 25.0 (cyclohexyl C-3', C-5'), 25.9 (cyclohexyl C-4'), 33.4 (cyclohexyl C-2', C-6'), 41.1 (CH3), 49.8 (cyclohexyl C-1'), 104.0 (C-4), 123.0 (C-3a), 128.0 (C-7a), 142.7 (C-5), 145.2 (C-3), 147.0 (C-7). Anal. Calcd for C12H16ClN3O: C, 56.72; H, 6.22; N, 25.44. Found: C, 56.93; H, 6.34; N, 25.29.

7-Chloro-1-(4-methoxybenzyl)-3-nitro-1H-pyrazolo[3,4-c]pyridin-7-amine (24b) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the derivative 10a, upon reaction of the chloroderivative 22b with cyclohexylamine for 3 h, in 70% yield. The product was purified by column chromatography using a mixture of cyclohexane–ethyl acetate (90 : 10, v/v) as the eluent, to provide pure 24b as an orange colored solid, mp: 152–154°C (CH2Cl2/Et2O). 1H-NMR (600 MHz, CDCl3): δ: 0.82–0.92 (3H, m, cyclohexyl H), 1.08–1.18 (2H, m, cyclo-
hexyl H), 1.30–1.40 (2H, m, cyclohexyl H), 1.58–1.65 (1H, m, cyclohexyl H), 1.70–1.78 (2H, m, cyclohexyl H), 1.96–2.10 (2H, m, cyclohexyl H), 3.92 (3H, s, CH₃), 3.93–3.98 (1H, m, cyclohexyl H-1'), 5.27 (2H, brs, D₂O exchange, NH), 5.62 (1H, d, D₂O exchange, NH = J = 7.3 Hz), 6.82 (1H, d, H-4, J = 5.7 Hz), 7.44 (1H, d, H-5, J = 5.7 Hz).

13C-NMR (151 MHz, CDCl₃) δ: 24.9 (cyclohexyl C-3', C-5'), 25.7 (cyclohexyl C-4'), 32.3 (cyclohexyl C-2', C-6'), 37.8 (CH₃), 49.0 (cyclohexyl C-1'), 104.0 (C-4'), 118.2 (C-3a), 128.3 (C-7a), 134.5 (C-5), 144.9 (C-3), 148.0 (C-7). HR-MS (ESI) m/z: Calcld for C₃₁H₂₉NO₅ [M+H]⁺ = 426.1713. Found 426.1715. Anal. Calcd for C₃₁H₂₉NO₅: C, 62.04; H, 6.89; N, 25.19. Found: C, 62.03; H, 6.88; N, 25.19.

N²-(Cyclohexyl-1-methyl-1H-pyrazolo[3,4-c]pyridine-3,7-diamine (25a) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the aminoderivative 11a, starting from the nitroderivative 24a (600 mg, 2.18 mmol), in 82% yield. The product was purified by column chromatography using a mixture of cyclohexane–ethyl acetate (from 1 : 1 up to 3 : 7, v/v) as the eluent, to provide pure 25a as a beige solid. mp: 144–146°C (CHCl₃/petroleum ether). 1H-NMR (400 MHz, DMSO-d₆) δ: 1.15–1.25 (3H, m, cyclohexyl H), 1.30–1.40 (2H, m, cyclohexyl H), 1.58–1.65 (1H, m, cyclohexyl H), 1.70–1.78 (2H, m, cyclohexyl H), 1.96–2.10 (2H, m, cyclohexyl H), 3.92 (3H, s, CH₃), 3.93–3.98 (1H, m, cyclohexyl H-1'), 5.27 (2H, brs, D₂O exchange, NH), 5.62 (1H, d, D₂O exchange, NH = J = 7.3 Hz), 6.82 (1H, d, H-4, J = 5.7 Hz), 7.44 (1H, d, H-5, J = 5.7 Hz).

13C-NMR (151 MHz, CDCl₃) δ: 24.9 (cyclohexyl C-3', C-5'), 25.7 (cyclohexyl C-4'), 32.3 (cyclohexyl C-2', C-6'), 37.8 (CH₃), 49.0 (cyclohexyl C-1'), 104.0 (C-4'), 118.2 (C-3a), 128.3 (C-7a), 134.5 (C-5), 144.9 (C-3), 148.0 (C-7). HR-MS (ESI) m/z: Calcld for C₃₁H₂₉NO₅ [M+H]⁺ = 426.1713. Found 426.1715. Anal. Calcd for C₃₁H₂₉NO₅: C, 62.04; H, 6.89; N, 25.19. Found: C, 62.03; H, 6.88; N, 25.19.
procedure to that described for the synthesis of the compounds 13a–l, upon reaction of the chloride 25b with aniline. The product was purified by column chromatography (silica gel) using a mixture of chloroform–methanol (100:2, v/v) as the eluent to provide pure 27 as a pale yellow solid. Yield: 51%. mp: >300°C (EtOAc). 1H-NMR (600 MHz, CDCl3) δ: 1.20–1.30 (3H, m, cyclohexyl H), 1.42–1.52 (2H, m, cyclohexyl H), 1.60–1.68 (1H, m, cyclohexyl H), 1.69–1.78 (2H, m, cyclohexyl H), 2.06–2.14 (2H, m, cyclohexyl H), 3.94 (2H, s, CH3), 4.06–4.14 (1H, m, cyclohexyl H–1′), 4.16 (3H, s, CH3), 4.62 (1H, brs, D2O exchanged, NH–cyclohexylamine), 4.83 (3H, brs, D2O exchanged, NH–aniline). 13C-NMR (50 MHz, CDCl3) δ: 24.6 (cyclohexyl C-3′, C-5′), 25.5 (cyclohexyl C-2′, C-6′), 45.1 (N(CH3)2), 48.4 (cyclohexyl C-1′), 61.9 (CH3), 104.0 (C-4′), 119.0 (C-3a), 127.8 (C-7a), 136.0 (C-5), 139.8 (C-3′), 144.4 (C-7), 168.2 (CO). HR-MS (ESI) m/z: Calcd for C16H25N6O2: [M+H]+ = 327.2084. Found 327.2087. Anal. Calcd for C16H25N6O2: C, 56.43; H, 7.75; N, 26.75.

1-Methyl-3-nitro-N-phenyl-1H-pyrazolo[3,4-c]pyridine-7-amine (30) Aniline (0.17 mL, 1.87 mmol) was added into a solution of the chlorocompound 22a (80 mg, 0.37 mmol) in 2-ethoxyethanol (2 mL), under argon, and the resulting solution was heated at 130°C for 3h. The solvent was then vacuum-evaporated and the residue was purified by column chromatography (silica gel) using dichloromethane as the eluent, to give pure 30 (60 mg, 59%) as a yellow solid. mp: 197–198°C (EtOAc). 1H-NMR (600 MHz, CDCl3) δ: 3.43 (3H, s, CH3), 7.14 (1H, t, H-4′, J=7.3 Hz), 7.28 (2H, d, H-2′, H-6′, J=7.6 Hz), 7.36 (2H, t, H-3′, H-5′, J=7.5 Hz), 7.57 (1H, brs, H-4), 7.88 (1H, brs, H-5). 13C-NMR (151 MHz, CDCl3) δ: 41.4 (CH3), 107.7 (C-4′), 119.6 (C-3a), 120.7 (C-2′, C-6′), 124.6 (C-4′), 129.9 (C-3′, C-5′), 130.6 (C-7a), 134.9 (C-5′), 140.9 (C-3), 142.6 (C-1′), 147.3 (C-7). Anal. Calcd for C24H22N6O2: C, 57.99; H, 4.12; N, 26.01. Found: C, 58.14; H, 4.22; N, 25.84.

1-Methyl-N-phényl-1H-pyrazolo[3,4-c]pyridine-3,7-diamine (31) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the compound 11a, upon hydrogenation of the nitroderivative 30. The product was purified by column chromatography (silica gel) using a mixture of cyclohexane–ethanol (from 5:5 up to 2:8, v/v) as the eluent to provide pure 31 as an orange colored solid. Yield: 75%. mp: 120–122°C (CH2Cl2/m-hexane). 1H-NMR (400 MHz, DMSO-d6) δ: 3.93 (3H, s, CH3), 5.52 (2H, s, CH2), 6.90 (1H, t, H-4′, J=7.3 Hz), 7.20 (1H, d, H-2′, H-6′, J=5.6 Hz), 7.25 (2H, t, H-3′, H-5′, J=7.8 Hz), 7.44 (2H, d, H-2′, H-6′, J=7.8 Hz), 7.60 (1H, d, H-5, J=5.6 Hz), 8.37 (1H, brs, D2O exchanged, NH). 13C-NMR (50 MHz, DMSO-d6) δ: 37.6 (CH3), 108.3 (C-4′), 118.8 (C-2′, C-6′), 119.7 (C-3a), 120.6 (C-4′), 128.4 (C-3′, C-5′), 129.8 (C-7a), 134.0 (C-5′), 141.6 (C-1′), 148.4 (C-7). Anal. Calcd for C16H19N4O: C, 65.25; H, 5.48; N, 29.27. Found: C, 65.37; H, 5.60; N, 29.03.

2-Chloro-N-(1-methyl-7-(phenylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)acetamide (32) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the compounds 12b and d, starting from the amine 31. This intermediate was used immediately to the next step, without further purification.

2-(Dimethylamino)-N-(1-methyl-7-(phenylamino)-1H-pyrazolo[3,4-c]pyridin-3-yl)acetamide (33a) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the compounds 13a–l, upon reaction of the chloride 32 with dimethylamine (5.6 mol solution in ethanol). The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (from 100:0.5 up to 100:5, v/v) as the
eluent to provide pure 33a as a beige solid, in 97% yield. mp: 140–142°C (EtOH/Et₂O). ¹H-NMR (600 MHz, DCl₃) δ: 2.44 (6H, s, N(CH₃)₂), 3.19 (2H, s, CH₃), 4.09 (3H, s, CH₃-pyrazole), 6.77 (1H, brs, D₂O exchange, NH-aniline), 7.02 (1H, t, J = 7.3 Hz), 7.21 (2H, d, H-2', J = 6.7 Hz), 7.29 (2H, t, H-3', J = 7.7 Hz), 7.45 (1H, brs, H-4'), 7.79 (1H, brs, H-5'), 9.62 (1H, brs, D₂O exchange, NHCO). ¹³C-NMR (151 MHz, CDCl₃) δ: 38.9 (CH₃-pyrazole), 46.1 (N(CH₃)₂), 63.1 (CH₁), 111.0 (C-4), 119.2 (C-2', C-6'), 122.7 (C-3a, C-4'), 129.5 (C-3', C-5'), 130.6 (C-7a), 136.6 (C-3), 138.7 (C-3'), 141.9 (C-1'), 142.3 (C-2'), 168.9 (CO). HR-MS (ESI) m/z: Calcd for C₁₇H₂₀N₆O: [M⁺+H]⁺ = 325.1771. Found 325.1773. Anal. Calcd for C₁₇H₂₀N₆O: C, 62.95; H, 6.21; N, 25.91. Found: C, 63.11; H, 6.28; N, 25.72.

-3-Nitro-4-phenyl-1H-pyrazolo[3,4-c]pyridin-7-amine (34) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of 30, upon treatment of 21 with aniline in 1,4-dioxane, at 100°C for 12h. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane–methanol (98:2, v/v) as the eluent, to provide pure 34, in 95% yield as a yellow solid. mp: 260–262°C (EtOAc). ¹H-NMR (400 MHz, DMSO-d₆) δ: 7.11 (1H, t, J = 7.1 Hz), 7.38–7.44 (3H, m, H-4', H-5'). 7.82 (2H, d, H-2', J = 7.81 Hz), 7.95 (1H, brs, H-5), 9.30 (1H, brs, D₂O exchange, NH-aniline). ¹³C-NMR (50 MHz, DMSO-d₆) δ: 104.6 (C-4), 120.0 (C-2', C-6'), 120.5 (C-3a), 123.2 (C-4'), 129.1 (C-3', C-5'), 130.1 (C-7a), 139.2 (C-3, C-1'), 142.8 (C-5), 148.8 (C-7). Anal. Calcd for C₁₃H₁₀N₆O: C, 56.47; H, 3.55; N, 27.44. Found: C, 56.66; H, 3.61; N, 27.31.

-2-(4-Methoxybenzyl)-3-nitro-4-phenyl-1H-pyrazolo[3,4-c]pyridin-7-amine (35) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the compound 22b. The product was purified by column chromatography (silica gel) using a mixture of cyclohexane–ethyl acetate–dichloromethane (from 90:10:3 up to 85:15:4, v/v/v) as the eluent, to provide pure 34 as an orange colored solid, in 45% yield. mp: 175–177°C (MeOH). ¹H-NMR (600 MHz, DCl₃) δ: 3.78 (3H, s, OCH₃), 6.04 (2H, s, CH₂), 6.87 (2H, d, H-2', J = 6.81 Hz), 7.12 (1H, t, J = 7.3 Hz), 7.29 (1H, d, H-1, J = 6.0 Hz), 7.35 (2H, d, H-3', J = 6.7 Hz), 7.41 (2H, t, H-3', J = 7.8 Hz), 7.70 (1H, brs, D₂O exchange, NH), 7.89 (2H, d, H-2', J = 7.8 Hz), 8.05 (1H, d, H-5, J = 6.01 Hz). ¹³C-NMR (151 MHz, CDCl₃) δ: 55.5 (OCH₃), 58.0 (CH₂), 104.5 (C-4), 114.5 (C-2', C-6'), 119.9 (C-2', C-6'), 122.2 (C-3a), 123.4 (C-4'), 126.4 (C-7a), 129.2 (C-3', C-5'), 129.9 (C-3', C-5'), 134.7 (C-1'), 137.3 (C-3), 139.1 (C-1'), 144.7 (C-5), 147.8 (C-7), 160.2 (C-4'). Anal. Calcd for C₁₃H₁₁N₆O: C, 63.99; H, 4.56; N, 18.66. Found: C, 64.34; H, 4.87; N, 18.33.

-2-(4-Methoxybenzyl)-N²-phenyl-2H-pyrazolo[3,4-c]pyridine-3,7-diamine (36) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the compound 11a, upon hydrogenation of the nitroderivative 34. The product was purified by column chromatography (silica gel) using a mixture of cyclohexane–ethyl acetate (from 7:3 up to 5:5, v/v) as the eluent to provide pure 35 as a brown solid, in 96% yield. mp: 54–56°C (CH₃Cl/1-n-hexane). ¹H-NMR (600 MHz, DMSO-d₆) δ: 3.71 (3H, s, OCH₃), 5.40 (2H, s, CH₂), 6.23 (2H, brs, D₂O exchange, NH), 6.87–6.91 (3H, m, H-4', H-2', H-6'), 6.95 (1H, d, H-4, J = 5.91 Hz), 7.17 (2H, d, H-3', J = 5.91 Hz), 7.25 (2H, t, H-3', J = 7.81 Hz), 7.31 (1H, d, H-5, J = 5.91 Hz), 8.04 (2H, d, H-2', J = 7.77 Hz), 8.57 (1H, brs, D₂O exchange, NH). ¹³C-NMR (151 MHz, CDCl₃) δ: 50.4 (CH₃), 55.1 (OCH₃), 106.0 (C-4), 109.3 (C-3a), 113.8 (C-2', C-6'), 118.9 (C-2', C-6'), 120.7 (C-4'), 128.2 (C-3', C-5', C-7a), 128.6 (C-3', C-5'), 128.9 (C-1'), 131.0 (C-5), 135.8 (C-3), 141.3 (C-1'), 145.8 (C-7), 158.6 (C-4'). Anal. Calcd for C₁₃H₁₁N₆O: C, 69.55; H, 5.54; N, 20.28. Found: C, 69.23; H, 5.31; N, 20.49.

-2-Chloro-4-(2-(4-methoxybenzyl)-2-(phenylamino)-2H-pyrazolo[3,4-c]pyridin-3-yl)-2-(phenylamino)-2H-pyrazolo[3,4-c]pyridin-3-yl)-2(phenylamino)-pentane-2,4-dione (37) This compound was prepared following an analogous synthetic procedure to that described for the synthesis of the compounds 12b and d, starting from the amine 36. This intermediate was used immediately to the next step, with no further purification.
Data for 38a: Yield 98%. Beige solid, mp: 183–185°C (CHCl3/EtO). 1H-NMR (600 MHz, CDCl3) δ: 2.32 (6H, s, N(CH3)2), 3.11 (2H, s, NHCOCH3), 3.76 (2H, s, OCH3), 5.45 (2H, s, CH2-pyrazole), 6.83–6.87 (3H, m, H-4, H-2', H-6'), 7.02 (1H, t, H-4', J = 7.3 Hz), 7.12 (2H, d, H-3', H-5', J = 8.58 Hz), 7.35 (2H, t, H-3', H-5'), J = 7.81 Hz), 7.68 (1H, d, H-5, J = 6.1 Hz), 7.87 (2H, d, H-2', H-6', J = 7.90 Hz), 8.70–9.40 (1H, brs, D2O exchange, NHCO), 13C-NMR (151 MHz, CDCl3) δ: 46.1 (N(CH3)2), 54.3 (CH2-pyrazole), 55.4 (OCH3), 62.8 (NHCOCH3), 104.7 (C-4), 114.4 (C-2', C-6'), 118.1 (C-3a), 119.4 (C-2', C-6'), 122.2 (C-4'), 127.1 (C-7a), 128.0 (C-1'), 128.7 (C-3', C-5'), 129.0 (C-3'), 131.6 (C-3), 136.6 (C-5'), 140.1 (C-1'), 147.3 (C-7), 159.7 (C-4'), 169.5 (CO). HR-MS (ESI) m/z: Calcd for C19H24N7O: [M+H]+ = 431.2190, Found 431.2198. Anal. Calcd for C19H24N7O: C, 66.96; H, 6.09; N, 19.52. Found: C, 67.17; H, 6.18; N, 19.44.

Data for 38b: Yield 97%. White solid, mp: 159–160°C (EtOAc/Et2O). 1H-NMR (600 MHz, CDCl3) δ: 2.30–2.40 (4H, brs, piperazine H-3', H-5'), 2.55–2.59 (4H, brs, piperazine H-2', H-6'), 3.17 (2H, s, NHCOCH3), 3.77 (3H, s, OCH3), 5.49 (2H, s, CH2-pyrazole), 6.79 (1H, d, H-4, J = 6.1 Hz), 6.86 (2H, d, H-2', H-6', J = 8.78 Hz), 7.02 (1H, t, H-4', J = 7.4 Hz), 7.07 (2H, d, H-3', H-5', J = 8.71 Hz), 7.35 (2H, t, H-3', H-5', J = 8.01 Hz), 7.58 (1H, brs, D2O exchange, NH-aniline), 7.70 (1H, d, H-5, J = 6.1 Hz), 7.88 (2H, d, H-2', H-6', J = 7.6 Hz), 9.00 (1H, brs, D2O exchange, NHCO). 13C-NMR (151 MHz, CDCl3) δ: 46.0 (CH3), 53.6 (piperazine C-2', C-6'), 54.3 (CH2-pyrazole), 55.0 (piperazine C-3', C-5'), 55.4 (OCH3), 61.3 (NHCOCH3), 104.4 (C-4), 114.6 (C-2', C-6'), 118.2 (C-3a), 119.3 (C-2', C-6'), 122.3 (C-4'), 127.3 (C-7a), 127.9 (C-1'), 128.3 (C-3', C-5'), 129.0 (C-3', C-5'), 136.2 (C-3), 137.0 (C-5), 140.1 (C-1'), 147.3 (C-7), 159.7 (C-4'), 169.0 (CO). HR-MS (ESI) m/z: Calcd for C19H24N7O2: [M+H]+ = 431.2190, Found 486.2612. Anal. Calcd for C19H24N7O2: C, 66.78; H, 6.43; N, 19.20. Found: C, 67.05; H, 6.54; N, 19.98.

Theoretical Calculations The crystal structure of human GSK3β (pdb id: 1UIVS) was utilized for docking calculations using the modules of Schrodinger Small-Molecule Drug Discovery Suite 2016.21 Protein preparation was performed by the corresponding routine as implemented in Maestro. Prior to calculations, the studied compounds were prepared in terms of correct protonation states, tautomers and stereoisomers using the LigPrep routine of Maestro. Rigid docking was performed using the Glide-SP algorithm. The Van der Waals atom radii scaling was set to 0.8 for both the protein and docked ligands. The best poses were redocked by utilizing a flexible representation of the active site residues in a 5 Å sphere around the ligand using MacroModel. The OPLS2005 force-field, an implicit GB/SA water solvent model and the TNGC minimization algorithm were used.

Kinase Activity Evaluation Kinase activities were assayed for each protein kinase in the appropriate buffers (listed below), with either a protein or a peptide as substrate in the presence of 15μM [γ-33P] ATP (3000 Ci/mmol; 10 μCi/mL) in a final volume of 30 μL following an assay previously described.27 Controls were performed with appropriate dilutions of dimethylsulfoxide. Full-length kinases are used unless specified. Peptide substrates were obtained from Proteogenix (Oberhausenbergs, France).

Buffers: (A) 10 mM MgCl2, 1 mM ethylene glycol bis(2-aminoethyl) ether-N,N',N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 25 mM Tris–HCl pH 7.5, 50 μg/mL heparin, 0.15 mg/mL of BSA +0.23 mg/mL of DTT (B) 60 mM β-glycerophosphate, 30 mM p-nitrophosphophosphate, 25 mM MOPS (pH 7), 5 mM EGTA, 15 mM MgCl2, 1 mM DTT, 0.1 mM sodium orthovanadate (C) 25 mM MOPS pH 7.2, 12.5 mM β-glycerophosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.25 mM DTT (D) 25 mM MOPS pH 7.5, 10 mM MgCl2 (E) Tris 50 mM pH 7.5, 20 mM MgCl2, 2 mM MnCl2 (R) 5 mM MOPS pH 7.2, 2.5 mM β-glycerophosphate, 4 mM
MgCl₂, 2.5 mm MnCl₂, 1 mm EGTA, 0.4 mm EDTA, 50 µg/mL BSA, 0.05 mm DTT

Each protein kinase was tested as follow: 

**HsRIPK3** (human, recombinant, expressed in Sf9 insect cells infected with 6xHIS; **HsRIPK3** specific baculoviruses) was assayed in buffer R with 0.1 µg/µL of MBP (Myelin Basic Protein) as substrate.

**HsHaspin-kd** (human, kinase domain, amino acids 470 to 798, recombinant, expressed in bacteria) was assayed in buffer H with 0.007 µg/µL of Histone H3 (1–21) peptide (ARTKQTASTGKAPKRQLA) as substrate.

**HsCDK5p25** (human, recombinant, expressed in bacteria) was assayed in buffer B, with 0.8 µg/µL of histone H1 as substrate.

**HsAuroraB** (human, recombinant, expressed by baculovirus in Sf9 insect cells, SignalChem, product #A31-10G) was assayed in buffer D with 0.22 µg/µL of the following peptide: RRKHAAIGSpAYSITA as CK1-specific substrate.

**SscGSK3β** (glycogen synthase kinase-3, porcine brain, native, affinity purified) was assayed in buffer A with 0.001 µg/µL of GS-1 peptide, a GSK-3-selective substrate (YRAVPPSPSLRHSPIQSpEDEEE, “Sp” stands for phosphorylated serine).

**SscCK1δc** (casein kinase 1δc, porcine brain, native, affinity purified) was assayed in buffer B, with 0.022 µg/µL of the following peptide: RRKHAAGIGSpAYSITA as CK1-specific substrate.

**RnDYRK1A-kd** (Rattus norvegicus, amino acids 1 to 499 including the kinase domain, recombinant, expressed in bacteria, DNA vector kindly provided by Dr. W. Becker, Aachen, Germany) was assayed in buffer A with 0.033 µg/µL of the following peptide: KKSIGRSLSPIMTEQ as substrate.

**MmCLK1** (from Mus musculus, recombinant, expressed in bacteria) was assayed in buffer A with 0.027 µg/µL of the following peptide: GRSSRSSRRSRRSRSRRSRSR.

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**Conflict of Interest** The authors declare no conflict of interest.

**References**


