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Pyrazolo[3,4-*b*]quinoxalines. A New Class of Cyclin-Dependent Kinases Inhibitors

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Abstract—Protein kinases are involved in most physiological processes and in numerous diseases. Therefore, inhibitors of protein kinases have therefore a wide therapeutic potential. While screening for inhibitors of cyclin-depent kinases (CDK's) and glycogen synthase kinase-3 (GSK-3), we identified pyrazolo[3,4-*b*]quinoxalines as sub-micromolar inhibitors of CDK1/cyclin B. A preliminary structure–activity relationship study suggests that this family of compounds can be optimized to inhibit CDK's and GSK-3. Compounds were tested for their anti-proliferative activity and the results show that several of them displayed a significant inhibitory effect on CDK1/cyclin B. The most active compound (**1**) was also tested against the brain kinases CDK5/p25 and GSK-3, and proved to be a good inhibitor of both of them. On the contrary, none of the compounds showed any activity in the CDC25 phosphatase assay. As an additional approach, affinity chromatography on immobilized pyrazolo[3,4-*b*]quinoxalines will be used to identify the intracellular targets of this family of compounds.

Introduction

Most anticancer agents which have been approved for clinical use are molecules that damage deoxyribonucleic acid (DNA), block DNA synthesis by inhibiting the nucleic acid precursors biosynthesis, alter tubulin polymerization/depolymerization or disrupt hormonal stimulation of cell growth.[1] However, there has been a recent shift of emphasis towards novel mechanistic targets that have directly arisen from the in-depth study of the underlying genetic changes related to the cancerous state. Thus, for instance, the identification of oncogenes such as *ras* which are activated in tumors, has suggested many pathways and specific molecular entities as rational targets for anticancer drug discovery.[2,3]

More recently, medicinal chemistry research has been focused on the proteins that drive and control cell cycle progression. Among them, the cyclin-dependent kinases (CDK's) play an essential role. The CDK's are a group of serine/threonine kinases which rule the transmission between successive stages of the cell cycle.[4,5] The activity of CDK's is regulated by multiple mechanisms, including binding to cyclins, which is a broad class of positive regulatory CDK-binding proteins. In addition to this, there are other different ways by which these proteins are regulated: subunit production, complex formation, (de)phosphorylation, intracellular translocations and interaction with various natural protein inhibitors. Recently, deregulations of CDK's have been proven in human primary tumors and in tumor cell lines[6] and therefore, CDK's constitute attractive targets for the development of antitumor drugs.[3,7–21] In addition, the involvement of CDK5 in Alzheimer's disease provides a new potential application for CDK inhibitors.[11]

Among the chemical agents that act selectively as CDK inhibitors are: a lactone (butyrolactone I), flavonoids (flavopiridol), indirubins, hymenialdisine and several purine derivatives (see Fig. 1). Butyrolactone I has shown antiproliferative activity for colon and pancreatic carcinoma cell lines.[22] Flavopiridol is the first CDK inhibitor that has entered clinical trials as an anticancer agent.[23] The class of purine-related derivatives comprises olomoucine,[24] roscovitine[25] and purvalanols.[26] These purine-derived compounds as well as flavopiridol have been shown to bind to the ATP-binding pocket of CDK's.[23,27]

For several years our group has been investigating new quinoxaline and quinoxaline 1,4-di-*N*-oxide derivatives

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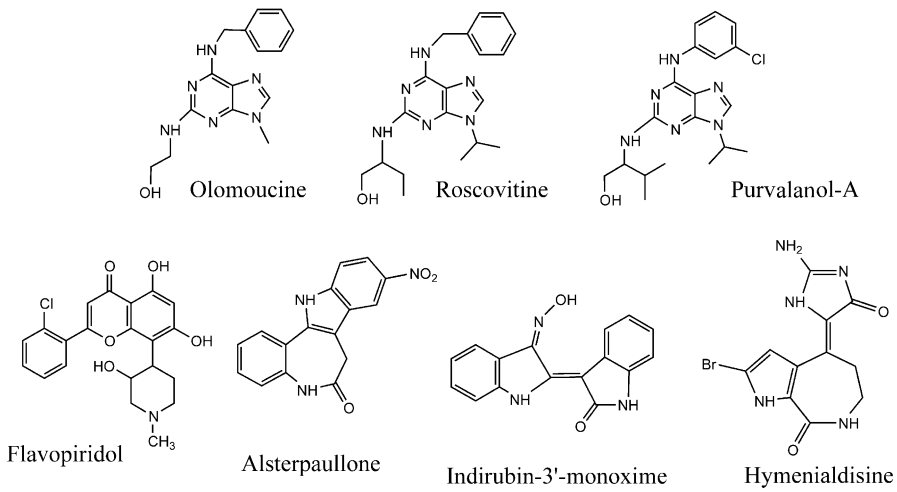


Figure 1. CDK inhibitors.

that have been shown to possess antitumoral properties.[28,29] As a continuation of this research, novel pyrazolo[3,4-*b*]quinoxalines have been synthesized and evaluated as potential CDK inhibitors. These chemical structures were chosen due to their structural similarity with some purine derivatives, such as olomoucine and roscovitine. The biological evaluation of the new compounds would provide information regarding how the quinoxaline-fused pyrazol ring may modify the antitumoral properties of previously synthesized quinoxalines and quinoxaline 1,4-di-*N*-oxides. In addition, with the attachment of different aliphatic amines to the pyrazole ring we attempted to achieve a structural similarity with well-known antitumor drugs.

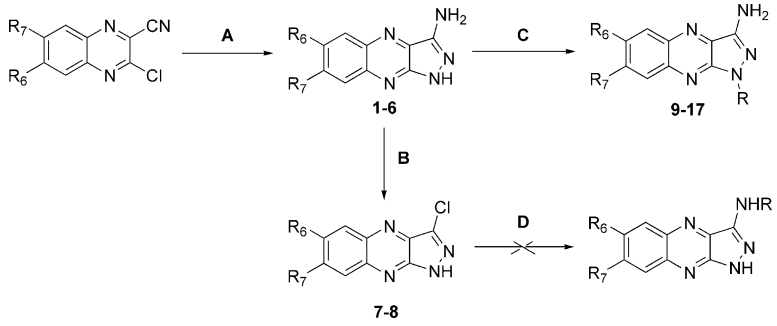
Results

Chemistry

The compounds reported in this paper were prepared according to Scheme 1. The synthesis of compounds **1** and **7** has already been reported;[30] however, in this paper, a new synthetic route for compound **7** is being described. Compounds **1**–**6** were formed from the corresponding 3-chloroquinoxaline-2-carbonitrile[31] by reaction with hydrazine hydrate under reflux. Reaction of derivatives **1** and **6** with copper(II) chloride and *tert*-butylnitrite in acetonitrile at 80 °C under nitrogen atmosphere yielded compounds **7** and **8**. Attempts to synthesize the 3-(dialkylaminoalkylamino) derivatives from the corresponding 3-chloro-1*H*-pyrazolo[3,4-*b*]quinoxalines (**7** and **8**) and *N*,*N*-dialkylaminoalkylamines always proved to be unsuccessful (Scheme 1); either the starting compounds or unmanageable mixtures were obtained in each case.

To obtain derivatives **9**–**16**, the corresponding 1*H*-pyrazolo[3,4-*b*]quinoxalines (**1**–**6**) were dissolved in chloroform and reacted with the appropriate *N*,*N*-dialkylaminoalkyl chloride in the presence of potassium carbonate. Finally, compound **17** was obtained from **1** by reaction with isonicotinic acid chloride in dichloromethane, in the presence of triethylamine which facilitates the condensation process.

With the aim of studying the influence of the substituents in positions 6 and 7 of the different pyrazolo[3,4-*b*]quinoxalines, several groups were chosen for these positions. In addition, monosubstitution as well as disubstitution were assessed. Thus, the trifluoromethyl group and chlorine were selected as withdrawing groups whereas



Scheme 1. Synthesis of compounds **1**–**17**: (A) hydrazine hydrate, reflux; (B) copper(II) chloride, *tert*-butylnitrite, acetonitrile, 80 °C; (C) *N*,*N*-dialkylaminoalkyl chloride or isonicotinic acid chloride, in the presence of potassium carbonate or triethylamine, respectively; (D) *N*,*N*-dialkylaminoalkylamine, potassium carbonate, chloroform.

methyl and methoxy were chosen as electrodonating substitutions.

As is well known, in principle, isomer-position mixtures of the 3-aminoquinoxaline-2-carbonitrile 1,4-di-*N*-oxide derivatives can be formed from the corresponding monosubstituted benzofuroxanes[32] and thus, the obtainment of isomer mixtures of the 3-aminoquinoxaline-2-carbonitrile, 3-chloroquinoxaline-2-carbonitrile and 1*H*-pyrazolo[3,4-*b*]quinoxaline derivatives would be expected. However, in the case of the compound that contains a methoxy group (**5**) and those derived from 5(6)-chlorobenzofuroxan (**6**, **8**, **10**, **13** and **15**), one of the isomers was obtained in a very low proportion and discarded during the purification processes; according to previous research carried out in our lab and literature references,[32] we assume that this corresponds to the 7-chloro isomer and, therefore, the isomer reported here would correspond to the 6-chloro. Finally, regarding compounds **2** and **4**, we were unable to separate the isomers and, thus, they were actually evaluated as the corresponding isomer–position mixture (A and B, in Experimental).

Pharmacology

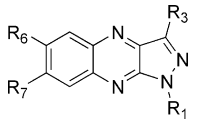
We then tested the effects of the synthesized pyrazolo[3,4-*b*]quinoxalines on two purified cell cycle regulators, the CDK1/cyclin B kinase and the CDC25 phosphatase. CDK1/cyclin B which regulates the G2/M transition was purified from M phase starfish oocytes by affinity chromatography on $p9^{CKShs1}$-sepharose and assayed with histone H1 and [^{32}P]-ATP (15 μM, final concentration) as substrates.[33] The CDC25 phosphatase regulates the phosphorylation of CDK1 and is responsible for its activation at the G2/M transition. It was expressed as a recombinant fusion protein in *Escherichia coli* and assayed with *p*-nitrophenylphosphate as a substrate.[34]

None of the compounds showed any activity in the CDC25 phosphatase assay. However, several compounds displayed a significant inhibitory effect on CDK1/cyclin B (Table 1, Fig. 2). The most active compound was further tested against the brain kinases CDK5/p25 and GSK-3. As expected from the close relationship between CDK1 and CDK5, compound **1** was a good inhibitor of CDK5 (IC_{50}: 0.4 μM). As recently suggested, many CDK inhibitors are also excellent inhibitors of the glycogen synthase kinase-3 (GSK-3).[35] This indeed turned out to be the case for compound **1** (IC_{50}: 1 μM) (Table 1, Fig. 2).

Discussion

We here describe a new class of kinase inhibitors with some selectivity towards cyclin-dependent kinases and glycogen synthase kinase-3. The most active compound acts with an IC_{50} of 0.6 and 1 μM on CDK1 and GSK-3. It is clear that the synthesis of more analogues is required to reach a good structure–activity relationship. Nevertheless, the first analogues which have been synthesized clearly show that even minor modifications lead to significant changes in kinase inhibitory activity. It is also evident that more work is required to reach a good view on the selectivity of this class of compounds. The initial results presented here confirm the hypothesis that most CDK inhibitors are also inhibitors of GSK-3.[35] To investigate the selectivity of pyrazolo[3,4-*b*]quinoxalines, we need to test a large panel of kinases as previously done with purines, hymenialdisine, indirubins. Alternatively, and this is the approach we will favor in the future, we would like to use affinity chromatography on immobilized pyrazolo[3,4-*b*]quinoxalines as a strategy to identify the intracellular targets of this family of compounds. This approach has been successfully used with purvalanol[36] and flavopiridol.[37]

Table 1. Effect of various compounds on the enzymatic activity of the CDK1/cyclin B kinase and the CDC25 phosphatase, assayed as described in the Experimental procedure section



Compound	R_1	R_3	R_6	R_7	CDK1/cyclin B IC_{50} (μM)	CDC25 IC_{50} (μM)
1	H	NH_2	H	H	0.6	>10
2	H	NH_2	CF_3/H	H/CF_3	4.5	>10
3	H	NH_2	CH_3	CH_3	21	>10
4	H	NH_2	CH_3/H	H/CH_3	3.5	>10
5	H	NH_2	OCH_3	H	4.8	>10
6	H	NH_2	Cl	H	4.56	>10
7	H	Cl	H	H	22	>10
8	H	Cl	Cl	H	15	>10
9	$(CH_2)_3N(CH_3)_2$ HCl	NH_2	H	H	>10	>10
10	$(CH_2)_3N(CH_3)_2$	NH_2	Cl	H	>10	>10
11	$(CH_2)_3N(CH_3)_2$	NH_2	CH_3	CH_3	>10	>10
12	$(CH_2)_2N(CH_3)_2$	NH_2	H	H	>10	>10
13	$(CH_2)_2N(CH_3)_2$	NH_2	Cl	H	>10	>10
14	$(CH_2)_2N(CH_2CH_3)_2$	NH_2	H	H	>10	>10
15	$(CH_2)_2N(CH_2CH_3)_2$	NH_2	Cl	H	>10	>10
16	$(CH_2)_2N(CH_2CH_3)_2$	NH_2	CH_3	CH_3	>10	>10
17	Pyridyl-4-carbonyl	NH_2	H	H	>10	>10

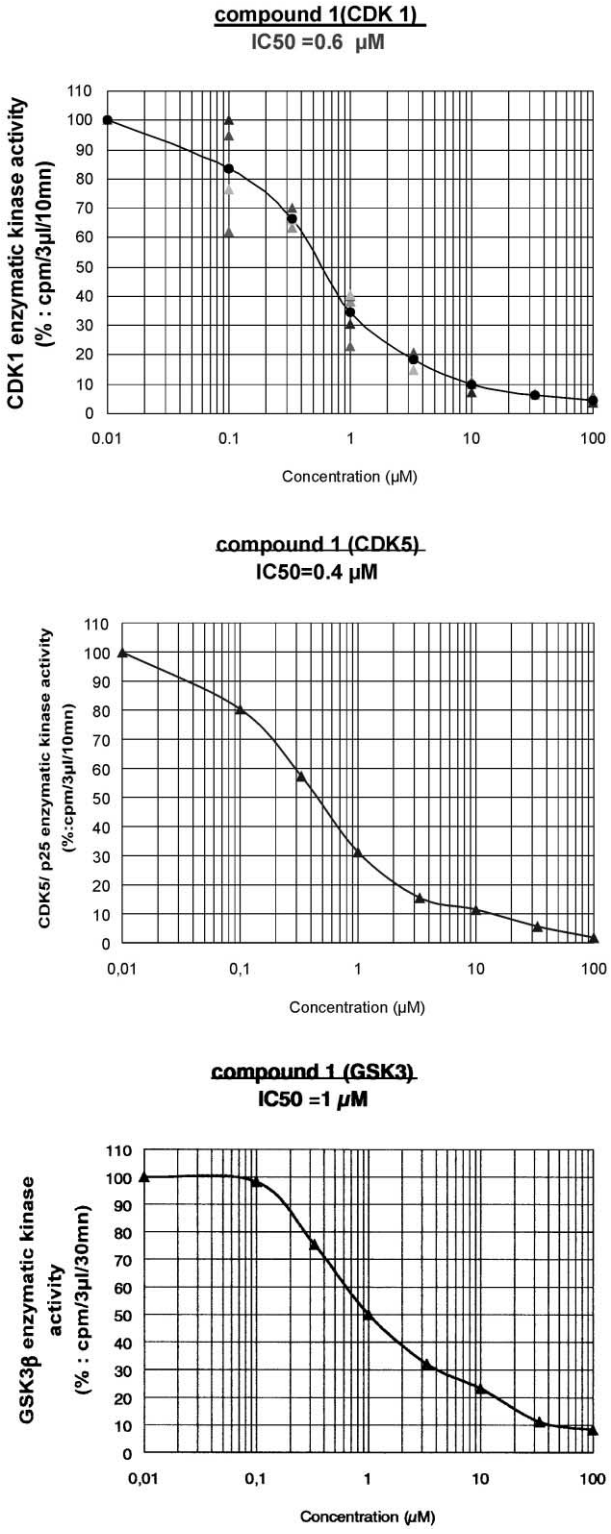


Figure 2. Compound 1 inhibits CDK1/cyclin B, CDK5/p25 and GSK-3. Dose–response curves.

Conclusion

We have established a rapid and efficient protocol for the synthesis of pyrazolo[3,4-*b*]quinoxalines. Here, we show that this class of compounds are inhibitors of CDK's and GSK-3, two enzymes involved in important physiological processes and possibly good screening targets to identify novel therapeutic agents. From the lead compound, more potent analogues can certainly be generated. The kinase selectivity of pyrazolo[3,4-*b*] quinoxalines remains to be investigated.

Experimental

Synthesis

Melting points were determined with a Mettler FP82 + FP80 apparatus (Greifense, Switzerland) and have not been corrected. The ^{1}H NMR spectra were recorded on a Bruker AC-200E (Rheinstetten, Germany) using TMS as the internal standard. The IR spectra were performed on a Perkin-Elmer 1600 FTIR (Norwalk, CT, USA) in KBr pellets. Elemental microanalyses were obtained on a Elemental Analyzer (Carlo Erba 1106, Milan, Italy) from vacuum-dried samples; results of analyses indicated by the symbols of the elements or functions are shown for each compound. The mass spectra were recorded on a Hewlett-Packard 5988-A instrument (Palo Alto, CA, USA) at 70 eV.

Silicagel 60 (0.040–0.063 mm) 1.09385.2500 (Merck KGaA, 64271 Darmstadt, Germany) was used for column chromatography and Alugram SIL G/UV$_{254}$ (Layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG. Postfach 101352. D-52313 Düren, Germany) was used for Thin Layer Chromatography. HPLC conditions: Column Nova Pack C18 60 A 4 µm (3.9×150 mm); mobile phase: methanol/water 80/20; flux: 1 mL/min.

Chemicals were purchased from E. Merck (Darmstadt, Germany), Scharlau (F.E.R.O.S.A., Barcelona, España), Panreac Química S.A. (Montcada i Reixac, Barcelona, España), Signa-Aldrich Química, S.A., (Alcobendas, Madrid, España), Acros Organics (Janssen Pharmaceuticalaan 3a, 2440 Geel, België) and Lancaster (Bischheim-Strasbourg, France).

General procedure for compounds 2–6

These compounds were synthesized by following the procedure used for compound **1**.[30] Yields: 20–90%.

3-Amino-6(7)-trifluoromethyl-1*H*-pyrazolo[3,4-*b*]quinoxaline (2). From 3-chloro-6(7)-trifluoromethylquinoxaline-2-carbonitrile and hydrazine hydrate. Mp 251–254 °C. IR (KBr) ν 3414, 3326, 1560, 1326, 1124 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 6.35 (s, 2H, NH_2), 7.90 (d, 1H, H_7(A) J_{7-8} = 9.0 Hz), 8.02–8.28 (m, 2H, H_8(A) + H_6(B)), 8.35–8.37 (m, 2H, H_5(B) + H_8(B)), 8.47 (s, 1H, H_5(A)), 12.54 (s, 1H, NH) ppm (A: 7-CF_3 isomer; B: 6-CF_3 isomer). MS: *m*/*z* (percentage of relative abundance): 253 ($M^{+\cdot}$, 100), 211 (15), 197 (23), 171 (47), 144 (8), 75 (20). Anal. ($C_{10}H_6F_3N_5$) C, H, N; C: calcd: 47.43; found: 46.98. H: calcd: 2.38; found: 2.46. N: calcd: 27.66; found: 27.29. HPLC: R_t = 2.79 min (A + B).

3-Amino-6,7-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoxaline (3). From 3-chloro-6,7-dimethylquinoxaline-2-carbonitrile and hydrazine hydrate. Mp 297 °C. IR (KBr) ν 3324, 1648,

1595, 1559 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.46 (s, 6H, $2CH_3$), 6.01 (s, 2H, NH_2), 7.76 (s, 1H, H_8), 7.88 (s, 1H, H_5), 12.08 (s, 1H, NH) ppm. MS: *m*/*z* (percentage of relative abundance): 213 ($M^{+\cdot}$, 100), 198 (13), 170 (9), 143 (3), 130 (4), 116 (5), 103 (6). Anal. ($C_{11}H_{11}N_5$) C, H, N. C (%): calcd: 61.95; found: 61.85. H (%): calcd: 5.19; found: 5.29. N (%): calcd: 32.84; found: 32.67.

3-Amino-6(7)-methyl-1*H*-pyrazolo[3,4-*b*]quinoxaline (4). From 3-chloro-6(7)-methylquinoxaline-2-carbonitrile and hydrazine hydrate. Mp 276–277 °C. IR (KBr) ν 3398, 3323, 1646, 1560 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.51 (s, 3H, CH_3), 6.09 (s, 2H, NH_2), 7.52 (dd, 1H, H_6 (B), $J_{6-5}=8.2$ Hz, $J_{6-8}=1.5$ Hz), 7.67 (dd, 1H, H_7(A), $J_{7-8}=8.8$ Hz, $J_{7-5}=1.5$ Hz) 7.77–8.02 (m, 3H, H_5(A) + H_8(A) + H_8(B)), 8.04 (d, 1H, H_5(B) $J_{5-6}=8.7$ Hz), 12.16 (s, 1H, NH) ppm (A: 7-CH_3 isomer; B: 6-CH_3 isomer) MS: *m*/*z* (percentage of relative abundance): 199 ($M^{+\cdot}$, 100), 170 (2), 156 (13), 143 (5), 116 (9), 895 (6), 77 (3). Anal. ($C_{10}H_9N_5$) C, H, N. C (%): calcd: 60.20; found: 60.18. H (%): calcd: 4.55; found: 4.65. N (%): calcd: 35.15; found: 35.24. HPLC: $R_t=2.81$ min (A + B).

3-Amino-6-methoxy-1*H*-pyrazolo[3,4-*b*]quinoxaline (5). From 3-chloro-7-methoxyquinoxaline-2-carbonitrile and hydrazine hydrate. Mp 250 °C. IR (KBr), ν 3408, 1636, 1602, 1224 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.93 (s, 3H, OCH_3), 5.95 (s, 2H, NH_2), 7.44–7.52 (m, 2H, $H_6 + H_8$), 7.90 (d, 1H, H_5, $J_{5-6}=9.1$ Hz), 12.11 (s, 1H, NH) ppm . MS: *m*/*z* (percentage of relative abundance): 215 ($M^{+\cdot}$, 100), 200 (31), 172 (26), 143 (1), 130 (12), 103 (4). Anal. ($C_{10}H_9N_5O$) C, H, N. C (%): calcd: 55.80; found: 55.90. H (%): calcd: 4.21; found: 4.30. N (%): calcd: 32.54; found: 32.85. HPLC: $R_t=2.51$ min.

3-Amino-6-chloro-1*H*-pyrazolo[3,4-*b*]quinoxaline (6). From 3,7-dichloroquinoxaline-2-carbonitrile and hydrazine hydrate. Mp >280 °C. IR (KBr) ν 3388, 3322, 1645, 1618, 1560 cm^{-1}; ^1H NMR (DMSO-d_6) δ 6.20 (s, 2H, NH_2), 7.82 (d, 1H, H_6, $J_{6-5}=9.0$ Hz), 8.01 (d, 1H, H_5, $J_{5-6}=9.2$ Hz), 8.16 (s, 1H, H_8), 12.36 (s, 1H, NH) ppm. MS: *m*/*z* (percentage of relative abundance): 219 ($M^{+\cdot}$, 100), 190 (2), 164 (7), 150 (9), 136 (8), 110 (4). Anal. ($C_9H_6ClN_5$) C, H, N. C (%): calcd: 49.21; found: 49.02. H (%): calcd: 2.75; found: 2.78. N (%): calcd: 31.88; found: 31.52. HPLC: $R_t=2.63$ min.

General procedure for compounds 7 and 8

The corresponding 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline (2.70 mmol) in acetonitrile was added to a stirred suspension of copper II) chloride (0.62 g; 4.61 mmol) in acetonitrile under reflux (80–85 °C) and under nitrogen atmosphere. A first fraction of *tert*-butyl nitrite (0.25 mL) was then added. The same amount was added every 15 min, up to a total of five times. The mixture was allowed to cool and filtered off; the solvent was removed under low pressure. The residue was then washed with plenty of dichloromethane, and the new solution was dried by removing the solvent under low pressure. The dark solid obtained was purified by flash chromatography using a mixture of dichloromethane/ethyl acetate as the mobile phase. With the fraction 80/20, the compound was separated and recrystallized from *n*-hexane/dichloromethane (yellow solid). Yields: 5–20%.

3-Chloro-1*H*-pyrazolo[3,4-*b*]quinoxaline (7). From 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline, copper(II) chloride and *tert*-butyl nitrite. Mp 225 °C (decomposition). IR (KBr) ν 3127, 3039, 1625, 1595 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.83–8.00 (m, 2H, $H_6 + H_7$), 8.16 (d, 1H, H_8, $J_{8-7}=8.5$ Hz), 8.29 (d, 1H, H_5, $J_{5-6}=8.4$ Hz), 14.30 (s, 1H, NH) ppm. MS: *m*/*z* (percentage of relative abundance): 204 ($M^{+\cdot}$, 100), 169 (16), 143 (28), 116 (21), 90 (17). Anal. ($C_9H_5ClN_4$) C, H, N. C (%): calcd: 52.84; found: 52.90. H (%): calcd: 2.44; found: 2.40. N (%): calcd: 27.38; found: 27.59.

3,6-Dichloro-1*H*-pyrazolo[3,4-*b*]quinoxaline (8). From 3-amino-6-chloro-1*H*-pyrazolo[3,4-*b*]quinoxaline, copper (II) chloride and *tert*-butyl nitrite. Mp 224–225 °C. IR (KBr) ν 3140, 1623, 1560, 873 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.96 (dd, 1H, H_7, $J_{7-5}=2.7$ Hz, $J_{7-8}=9.0$ Hz), 8.17 (d, 1H, H_8, $J_{8-7}=9.1$ Hz), 8.36 (d, 1H, H_5, $J_{5-7}=2.3$ Hz), 14.29 (s, 1H, NH) ppm. MS: *m*/*z* (percentage of relative abundance): 238 ($M^{+\cdot}$,100), 203 (15), 177 (36), 150 (29), 88 (27). Anal. ($C_9H_4Cl_2N_4$) C, H, N. C (%): calcd: 45.21; found: 44.79. H (%): calcd: 1.68; found: 1.69. N (%): calcd: 23.43; found: 23.08.

General procedure for compounds 9–16

A mixture of the 6 and/or 7-substituted 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline (2.25 mmol) and the corresponding *N*,*N*-dialkylaminoalkylamine (14.50 mmol) in the presence of potassium carbonate (0.5 g) was stirred in dry chloroform (30 mL) for 10 days and kept from light and moisture. The mixture was filtered off, and the residue obtained was washed with dry chloroform. The solvent was removed under low pressure and the red solid obtained was purified by flash chromatography using a mixture of dichloromethane/methanol as the mobile phase. Once the solvent was removed, the new solid was dissolved in methanol and a small amount of active carbon was added. After filtering the suspension and removing the solvent, a final red solid was obtained. Yields: 12–79%.

3-Amino-1-(3-dimethylamino-1-propyl)pyrazolo[3,4-*b*]quinoxaline hydrochloride (9). From 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline and 3-dimethylamino-1-propylamine. This compound was prepared as hydrochloride salt as follows: The red solid obtained from the column was dissolved in dichloromethane and washed with a solution of NaOH 2 N (3×100 mL) and water (3×100 mL). It was dried with anhydrous sodium sulfate and the solvent was removed under low pressure. The new residue was dissolved in ethyl acetate and three drops of hydrochloric acid 35% were added. The red precipitate which appeared was isolated by filtration. The solid was dissolved in methanol and a small amount of active carbon was added in order to remove the impurities. After filtering and removing the solvent, a red solid was obtained. Mp 238–240 °C. IR (KBr) ν 3361, 3303, 2945, 2609, 1637 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.20–2.28 (m,

2H, CH_2), 2.69 (s, 6H, $2CH_3$), 3.10 (t, 2H, CH_2), 4.31 (t, 2H, CH_2), 6.41 (s, 2H, NH_2), 7.68 (t, 1H, H_6, $J_{6-7}=7.0$ Hz), 7.84 (t, 1H, H_7, $J_{7-6}=7.3$ Hz), 8.01 (d, 1H, H_8, $J_{8-7}=8.3$ Hz), 8.16 (d, 1H, H_5, $J_{5-6}=8.3$ Hz), 10.61 (bs, 1H, HCl) ppm. MS: *m/z* (percentage of relative abundance): 270 ($M^{+\cdot}$, 40), 198 (34), 171 (5), 129 (12), 102 (15), 84 (90), 58 (100). Anal. ($C_{14}H_{18}N_6 \cdot HCl \cdot 0.25H_2O$) C, H, N. C (%): calcd: 54.01; found: 54.36. H (%): calcd: 6.31; found: 6.28. N (%): calcd: 26.99; found: 26.76.

3-Amino-6-chloro-1-(3-dimethylamino-1-propyl)pyrazolo [3,4-*b*]quinoxaline (10). From 3-amino-6-chloro-1*H*-pyrazolo[3,4-*b*]quinoxaline and 3-dimethylamino-1-propylamine. Mp 238–240 °C. IR (KBr) ν 3366, 1647, 1567, 881cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.09 (m, 2H, CH_2), 2.22 (s, 6H, $2CH_3$), 2.35 (t, 2H, CH_2) 4.40 (t, 2H, CH_2), 4.75 (s, 2H, NH_2), 7.68 (dd, 1H, H_7, $J_{7-8}=9.2$ Hz, $J_{7-5}=2.0$ Hz), 7.98 (d, 1H, H_8, $J_{8-7}=9.1$ Hz), 8.13 (d, 1H, H_5, $J_{5-7}=1.7$ Hz) ppm. MS: *m/z* (percentage of relative abundance): 304 ($M^{+\cdot}$, 3), 232 (25), 205 (4), 178 (3), 163 (10), 136 (11), 110 (1), 84 (95), 58 (100). Anal. ($C_{14}H_{17}ClN_6$) C, H, N. C (%): calcd: 55.17; found: 54.76. H (%): calcd: 5.62; found: 5.65. N (%): calcd: 27.57; found: 27.27.

3-Amino-6,7-dimethyl-1-(3-dimethylamino-1-propyl)pyrazolo[3,4-*b*]quinoxaline (11). From 3-amino-6,7-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoxaline and 3-dimethylamino-1-propylamine. Mp 150–152 °C. IR (KBr) ν 3329, 1641, 1570, 861 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.08 (q, 2H, CH_2), 2.16 (s, 6H, $2CH_3$), 2.34 (t, 2H, CH_2), 2.47 (d, 6H, $2CH_3$), 4.39 (t, 2H, CH_2), 4.64 (s, 2H, NH_2), 7.80 (s, 1H, H_8), 7.87 (s, 1H, H_5) ppm. MS: *m/z* (percentage of relative abundance): 298 ($M^{+\cdot}$, 5), 240 (32), 226 (41), 214 (10), 199 (5), 157 (10), 130 (3), 103 (10), 84 (77), 58 (100). Anal. ($C_{16}H_{22}N_6$) C, H, N. C (%): calcd: 64.40; found: 63.99. H (%): calcd: 7.43; found: 7.41. N (%): calcd: 28.16; found: 28.03.

3-Amino-1-(2-dimethylamino-1-ethyl)pyrazolo[3,4-*b*]quinoxaline (12). From 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline and 2-dimethylamino-1-ethylamine. Mp 174–178 °C. IR (KBr) ν 3294, 1651, 1572, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 6H, $2CH_3$), 2.79 (t, 2H, CH_2), 4.40 (t, 2H, CH_2), 4.67 (s, 2H, NH_2), 7.52 (t, 1H, H_6, $J_{6-7}=7.3$ Hz), 7.67 (t, 1H, H_7, $J_{7-6}=6.9$ Hz), 7.96 (d, 1H, H_5, $J_{5-6}=8.5$ Hz), 8.05 (d, 1H, H_8, $J_{8-7}=8.4$ Hz) ppm. MS: *m/z* (percentage of relative abundance): 256 ($M^{+\cdot}$, 2), 198 (2), 144 (1), 129 (2), 102 (6), 76 (1), 58 (100). Anal. ($C_{13}H_{16}N_6$) C, H, N. C (%): calcd: 60.91; found: 61.11. H (%): calcd: 6.29; found: 6.31. N (%): calcd: 32.72; found: 32.41.

3-Amino-6-chloro-1-(2-dimethylamino-1-ethyl)pyrazolo [3,4-*b*]quinoxaline (13). From 3-amino-6-chloro-1*H*-pyrazolo[3,4-*b*]quinoxaline and 2-dimethylamino-1-ethylamine. Mp 188–191 °C. IR (KBr) ν 3291, 1654, 1570, 1391, 868 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 6H, $2CH_3$), 2.89 (t, 2H, CH_2), 4.47 (t, 2H, CH_2), 4.70 (s, 2H, NH_2), 7.67 (dd, 1H, H_7, $J_{7-8}=9.3$ Hz, $J_{7-5}=2.1$ Hz), 7.97 (d, 1H, H_8, $J_{8-7}=9.3$ Hz), 8.12 (d, 1H, H_5, $J_{5-7}=1.5$ Hz) ppm. MS: *m/z* (percentage of relative abundance): 290 ($M^{+\cdot}$, 1), 232 (1), 205 (1), 178 (1), 163 (1), 135 (2), 110 (0.2), 58 (100). Anal. ($C_{13}H_{15}ClN_6$) C, H, N. C (%): calcd: 53.70; found: 53.65. H (%): calcd: 5.20; found: 5.20. N (%): calcd: 28.90; found: 28.87.

3-Amino-1-(2-diethylamino-1-ethyl)pyrazolo[3,4-*b*]quinoxaline (14). From 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline and 2-diethylamino-1-ethylamine. Mp 102–104 °C. IR (KBr) ν 3314, 2973, 1642, 1572, 752 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 6H, $2CH_3$), 2.59 (c, 4H, $2CH_2$), 2.96 (t, 2H, CH_2), 4.40 (t, 2H, CH_2), 4.68 (s, 2H, NH_2), 7.49 (t, 1H, H_6, $J_{6-7}=6.6$ Hz), 7.64 (t, 1H, H_7, $J_{7-6}=6.9$ Hz), 7.93 (d, 1H, H_8, $J_{8-7}=8.4$ Hz), 8.02 (d, 1H, H_5, $J_{5-6}=8.4$ Hz) ppm. MS: *m/z* (percentage of relative abundance): 284 ($M^{+\cdot}$, 5), 198 (4), 144 (2), 129 (4), 102 (7), 86 (100), 58 (5). Anal. ($C_{15}H_{20}N_6 \cdot H_2O$) C, H, N. C (%): calcd: 59.58; found: 59.83. H (%): calcd: 6.66; found: 7.11. N (%): calcd: 27.79; found: 27.71.

3-Amino-6-chloro-1-(2-diethylamino-1-ethyl)pyrazolo[3,4-*b*] quinoxaline (15). From 3-amino-6-chloro-1*H*-pyrazolo [3,4-*b*]quinoxaline and 2-diethylamino-1-ethylamine. Mp 152–154 °C. IR (KBr) ν 3319, 1648, 1570, 829 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.06 (t, 6H, $2CH_3$), 2.72 (c, 4H, $2CH_2$), 3.08 (t, 2H, CH_2), 4.52 (t, 2H, CH_2), 4.73 (s, 2H, NH_2), 7.69 (dd, 1H, H_7, $J_{7-8}=9.1$ Hz, $J_{7-5}=2.2$ Hz), 7.98 (d, 1H, H_8, $J_{8-7}=9.1$ Hz), 8.13 (s, 1H, H_5) ppm. MS: *m/z* (percentage of relative abundance): 318 ($M^{+\cdot}$, 1), 232 (4), 205 (2), 178 (2), 163 (3), 136 (4), 86 (100), 58 (10). Anal. ($C_{15}H_{19}ClN_6$) C, H, N. C (%): calcd: 56.51; found: 56.23. H (%): calcd: 6.00; found: 6.08. N (%): calcd: 26.36; found: 25.98.

3-Amino-6,7-dimethyl-1-(2-diethylamino-1-ethyl)pyrazolo[3,4-b]quinoxaline (16). From 3-amino-6,7-dimethyl-1*H*-pyrazolo[3,4-*b*]quinoxaline and 2-diethylamino-1-ethylamine. Mp 136–137 °C. IR (KBr) ν 3313, 1646, 1573, 865 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 6H, $2CH_3$), 2.46 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.58 (c, 4H, $2CH_2$), 2.93 (t, 2H, CH_2), 4.41 (t, 2H, CH_2), 4.63 (s, 2H, NH_2), 7.78 (s, 1H, H_8), 7.85 (s, 1H, H_5) ppm. MS: *m/z* (percentage of relative abundance): 312 ($M^{+\cdot}$, 4), 226 (4), 213 (3), 199 (1), 157 (3), 130 (1), 103 (3), 86 (100), 58 (8). Anal. ($C_{17}H_{24}N_6$) C, H, N. C (%): calcd: 65.35; found: 65.65. H (%): calcd: 7.74; found: 8.11. N (%): calcd: 26.89; found: 26.52.

Procedure for 3-amino-1-(pyridyl-4-carbonyl)pyrazolo [3,4-*b*]quinoxaline (17). A mixture of 3-amino-1*H*-pyrazolo[3,4-*b*]quinoxaline (0.2 g; 1.08 mmol), isonicotinic acid chloride (0.23 g; 1.65 mmol) and triethylamine (0.5 mL) in dichloromethane (20 mL) was kept from light and moisture, and stirred for 6 h. After filtering and washing the residue with dichloromethane, a yellow solid was obtained. Mp 246–248 °C. IR (KBr) ν 3362, 1699, 1638, 1560 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.15 (s, 2H, NH_2), 7.79 (d, 2H, 2CH pyridine, $J=5.3$ Hz), 7.88–8.05 (m, 2H, H_6+H_7), 8.17 (d, 1H, H_5, $J_{5-6}=8.1$ Hz), 8.28 (d, 1H, H_8, $J_{8-7}=8.3$ Hz), 8.84 (d, 2H, 2CH pyridine, $J=5.5$ Hz) ppm. MS: *m/z* (percentage of relative abundance): 290 ($M^{+\cdot}$, 84), 261 (2), 106 (100), 78 (40). Anal. ($C_{15}H_{10}N_6O$) C, H, N. C (%): calcd: 62.06; found: 62.40. H (%): calcd: 3.47; found: 3.55. N (%): calcd: 28.95; found: 29.39.

Kinase assays

Biochemical reagents. Sodium *ortho*-vanadate, EGTA, EDTA, RNAse A, Mops, β-glycerophosphate, phenylphosphate, sodium fluoride, glutathione-agarose, dithiothreitol (DTT), bovine serum albumin (BSA), nitrophenyl phosphate, leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S) were obtained from Sigma Chemicals. [γ-^{32}P]-ATP (PB 168) was obtained from Amersham.

The GS-1 peptide (YRRAAVPPSPSLSRHSSPHQSpE DEEE) was synthesized by the Peptide Synthesis Unit, Institute of Biomolecular Sciences, University of Southampton, Southampton SO16 7PX, UK.

Buffers. Homogenization buffer. 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM MgC l_2, 1 mM DTT, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg leupeptin/mL, 10 μg aprotinin/mL, 10 μg soybean trypsin inhibitor/mL and 100 μM benzamidine.

Buffer A. 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris–HCl pH 7.5, 50 μg heparin/mL.

Buffer C. Homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors.

Tris-buffered saline-Tween-20 (TBST). 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20.

Kinase preparations and assays. Kinases activities were assayed in Buffer A or C (unless otherwise stated), at 30 °C, at a final ATP concentration of 15 μM. Blank values were subtracted and activities calculated as pmoles of phosphate incorporated for a 10-min incubation. The activities are usually expressed in% of the maximal activity, that is in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide. In a few cases phosphorylation of the substrate was assessed by autoradiography after SDS-PAGE (see below).

CDK1/cyclin B was extracted in homogenization buffer from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on p9^{CKShs1}-sepharose beads, from which it was eluted by free p9^{CKShs1} as previously described.[33,35] The kinase activity was assayed in buffer C, with 1 mg histone H1/mL, in the presence of 15 μM [γ-^{32}P] ATP (3000 Ci/mmol; 1 mCi/mL) in a final volume of 30 μL. After 10 min incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto 2.5×3 cm pieces of Whatman P81 phosphocellulose paper, and, 20 s later, the filters were washed five times (for at least 5 min each time) in a solution of 10 mL phosphoric acid/L of water. The wet filters were counted in the presence of 1 mL ACS (Amersham) scintillation fluid.

CDK5/p25 was reconstituted by mixing equal amounts of recombinant mammalian CDK5 and p25 expressed in *E. coli* as Glutathione-*S*-transferase (GST) fusion proteins, and purified by affinity chromatography on glutathione-agarose (vectors kindly provided by Dr. J. H. Wang) (p25 is a truncated version of the p35, the 35 kDa CDK5 activator). Its activity was assayed in buffer C as described for CDK1/cyclin B.

GSK-3β was expressed in and purified from insect Sf9 cells.[38] It was assayed, following a 1/100 dilution in 1 mg BSA/mL 10 mM DTT, with 5 μL, 40 μM GS-1 peptide as a substrate, in buffer A, in the presence of 15 μM [γ-^{32}P] ATP (3,000 Ci/mmol; 1 mCi/mL) in a final volume of 30 μL. After 30 min incubation at 30 °C, 25-μL aliquots of supernatant were spotted onto P81 phosphocellulose papers and treated as described above.

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