

# Discovery of 3-alkoxyamino-5-(pyridin-2-ylamino)pyrazine-2-carbonitriles as selective, orally bioavailable CHK1 inhibitors

*Michael Lainchbury,<sup>§</sup> Thomas P. Matthews,<sup>§</sup> Tatiana McHardy,<sup>§</sup> Kathy J. Boxall,<sup>§</sup> Michael I. Walton,<sup>§</sup> Paul D. Eve,<sup>§</sup> Angela Hayes,<sup>§</sup> Melanie R. Valenti,<sup>§</sup> Alexis K. de Haven Brandon,<sup>§</sup> Gary Box,<sup>§</sup> G. Wynne Aherne,<sup>§</sup> John C. Reader,<sup>#</sup> Florence I. Raynaud,<sup>§</sup> Suzanne A. Eccles,<sup>§</sup> Michelle D. Garrett,<sup>§</sup> Ian Collins<sup>§\*</sup>*

<sup>§</sup> Cancer Research UK Cancer Therapeutics Unit, The Institute of Cancer Research, 15 Cotswold Road, Sutton, SM2 5NG U. K.

<sup>#</sup> Sareum Ltd, Unit 2A Langford Arch, London Road, Pampisford, Cambridge CB22 3FX, U. K.

## SUPPORTING INFORMATION

1. Experimental methods for the synthesis and characterization of compounds **8-19**, **21-25**, **27-35**, **37-40**, **42**, **44**, **51** and **52**.
2. Experimental methods for the determination of CHK1 and CHK2 inhibition.

3. Experimental methods for the determination of checkpoint abrogation, antiproliferative activity, and potentiation of genotoxic drug efficacy in cancer cell lines.

4. Experimental method for the determination of compound concentrations *in vivo* at selected time points following i.v. and oral dosing.

5. Kinase inhibition profile for **26**

**1. Experimental methods for the synthesis and characterization of compounds 8-19, 21-25, 27-35, 37-40, 42, 44, 51 and 52.**

*General Procedure (i): Microwave assisted Suzuki coupling of 5-iodo-2-chloropyridine intermediates to boronic acids or esters.* A mixture of the 5-iodo-2-chloropyridine intermediate (1 eq.), appropriate boronic acid or ester (1 eq.), Pd(PPh<sub>3</sub>)<sub>4</sub> (5 mol%) and Na<sub>2</sub>CO<sub>3</sub> (1.5 eq.) in MeCN (7 mL mmol<sup>-1</sup> of 5-iodo-2-chloropyridine intermediate) was heated at 100 °C in a microwave reactor for 30 min (for 4-aminopyridines) or 15 min (for 4-methoxy and 4-thiomethylpyridines). The cooled reaction mixture was absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 1-10% MeOH – CH<sub>2</sub>Cl<sub>2</sub>, gave the coupled products.

*General Procedure (ii): Sonogashira coupling of 5-iodo-2-chloropyridine intermediates to ethynyltrimethylsilane or trimethyl((2-methylbut-3-yn-2-yl)oxy)silane.* The 5-iodo-2-chloropyridine intermediate (1 eq.) was dissolved in DMF (0.9 mL mmol<sup>-1</sup> of 5-iodo-2-chloropyridine intermediate). Trimethyl(2-methylbut-3-yn-2-yloxy)silane or ethynyltrimethylsilane (1.3 eq.) were added as appropriate. PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub> (6 mol%), CuI (6 mol%) and Et<sub>3</sub>N (18 eq.) were added. The mixture was heated at 120 °C in a microwave reactor for 10 min. The mixture was cooled and evaporated to dryness. Flash column chromatography on silica gel, eluting with EtOAc – hexane, gave the coupled product.

*General Procedure (iii) Microwave assisted Buchwald coupling of 2-aminopyrazines 30, 31, 34, or 35 to 2-chloropyridines.* A mixture of the 2-chloropyridine intermediate (1 eq.), 2-aminopyrazine **30**, **31**, **34**, or **35** (1 eq.), xantphos (20 mol %), Cs<sub>2</sub>CO<sub>3</sub> (2 eq.) and Pd<sub>2</sub>dba<sub>3</sub> (10 mol %) in toluene or dioxane (10 mL mmol<sup>-1</sup> of 2-chloropyridine) was heated at 130°C in a microwave reactor for 60 min. The cooled reaction mixture was diluted with MeOH (10 mL) and absorbed onto a 2g SCX-2 acidic resin column. The column was flushed with MeOH (4 x 20 mL), followed by elution with NH<sub>3</sub> in MeOH (2 M; 4 x 20 mL). The basic eluent fractions were absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 1-10% MeOH – 1% NH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> gave the coupled product. If the product was not >95% pure due to co-elution with unreacted 2-aminopyrazine starting material, then further purification was achieved by preparative thin layer chromatography, eluting with 2-10% MeOH – 1% NH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub>.

*General Procedure (iv) Microwave assisted Buchwald coupling of 2-aminopyrazines 32 or 33 to 2-chloropyridines, followed by N-Boc deprotection.* A mixture of the 2-chloropyridine intermediate (1 eq.), 2-aminopyrazine **32** or **33** (1 eq.), xantphos (20 mol%), Cs<sub>2</sub>CO<sub>3</sub> (2 eq.) and Pd<sub>2</sub>dba<sub>3</sub> (10 mol%) in toluene or dioxane (10 mL mmol<sup>-1</sup> of 2-chloropyridine intermediate) was heated at 130°C in a microwave reactor for 60 min. The cooled reaction mixture was diluted with MeOH (10 mL) and absorbed onto a 2g SCX-2 acidic resin column. The column was flushed with MeOH (4 x 20 mL), followed by elution with NH<sub>3</sub> in MeOH (2 M; 4 x 20 mL). The basic eluent fractions were absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 1-10% MeOH – 1% NH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> gave the coupled product. The material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (140 mL mmol<sup>-1</sup> of coupled product) and CF<sub>3</sub>CO<sub>2</sub>H (35 mL mmol<sup>-1</sup> of coupled product) was added in one portion. The solution was stirred at r.t. for 30 min, then absorbed onto silica gel and solvents were removed by evaporation.

Gradient silica column chromatography, eluting with 5-20% MeOH – 1% NH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> gave the deprotected product. If the material was not >95% pure, then further purification was achieved by preparative thin layer chromatography, eluting with 2-10% MeOH – 1% NH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub>.

*(R)*-Methyl 6-((5-cyano-6-((1-(dimethylamino)propan-2-yl)oxy)pyrazin-2-yl)amino)-4-(methylamino)nicotinate (**8**) Prepared from **30** and **37** following General Procedure (iii) to give **8** (92%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.68 (s, 1H), 8.26 (s, 1H), 8.15 (d, J = 4.8, 1H), 7.03 (s, 1H), 5.50 - 5.39 (m, 1H), 3.88 (s, 3H), 2.97 (d, J = 5.0, 3H), 2.78 (dd, J = 13.4, 7.3, 1H), 2.55 (dd, J = 13.4, 4.2, 1H), 2.34 (s, 6H), 1.42 (d, J = 6.3, 4H). LC-MS (LCT, 3.5 min) Rt = 1.84 min; *m/z* (ESI) 386 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>18</sub>H<sub>24</sub>N<sub>7</sub>O<sub>3</sub> (M+H) 386.1935, found 386.1943.

*(R)*-Methyl 6-((5-cyano-6-(pyrrolidin-3-yloxy)pyrazin-2-yl)amino)-4-(methylamino)nicotinate (**9**) Prepared from **32** and **36** following General Procedure (iv) to give **9** (31%) as a pale yellow solid. <sup>1</sup>H NMR (500 MHz, d<sub>6</sub>-DMSO) δ 8.58 (s, 1H), 8.38 (s, 1H), 8.01 (q, *J* = 4.7 Hz, 1H), 7.22 (s, 1H), 5.62–5.51 (m, 1H), 3.81 (s, 3H), 3.18 (dd, *J* = 12.6, 5.5 Hz, 1H), 3.01–2.92 (m, 2H), 2.90 (d, *J* = 4.9 Hz, 3H), 2.85 (ddd, *J* = 10.8, 8.0, 4.8 Hz, 1H), 2.14–2.03 (m, 1H), 1.95–1.87 (m, 1H); LC-MS (3.5 min) Rt = 1.64 min; *m/z* (ESI) 370 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>17</sub>H<sub>20</sub>N<sub>7</sub>O<sub>3</sub> (M+H) 370.1622, found 370.1628.

*(R)*-Methyl 6-((5-cyano-6-(pyrrolidin-3-yloxy)pyrazin-2-yl)amino)-4-(dimethylamino)nicotinate (**10**) Prepared from **32** and **38** following General Procedure (iv) to give **10** (27%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, d<sub>6</sub>-DMSO) δ 8.56 (s, 1H), 8.35 (s, 1H), 7.20 (s, 1H), 5.68–5.59 (m, 1H), 3.81 (s, 3H), 3.39 (dd, *J* = 13, 5.2 Hz, 1H), 3.31 (brs, 1h), 3.27 (d, *J* = 13 Hz, 1H), 3.19–3.12 (m, 2H), 2.91 (s, 6H), 2.23–2.18 (m, 1H), 2.15–2.08 (m, 1H); LC-MS (3.5 min) Rt =

1.70 min;  $m/z$  (ESI) 384 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{18}H_{22}N_7O_3$  (M+H) 384.1799, found 384.1784.

*(R)*-Methyl 6-((5-cyano-6-(pyrrolidin-3-yloxy)pyrazin-2-yl)amino)-4-methoxynicotinate (**11**) Prepared from **32** and **39** following General Procedure (iv) to give **11** (27%) as a white solid.  $^1H$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  8.63 (s, 1H), 8.54 (s, 1H), 7.56 (s, 1H), 5.61 (m, 1H), 3.94 (s, 3H), 3.79 (s, 3H), 3.18–2.98 (m, 4H), 2.21–2.12 (m, 1H), 2.07–1.99 (m, 1H). LC-MS (3.5 min)  $R_t$  = 2.04 min;  $m/z$  (ESI) 371 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{17}H_{19}N_6O_4$  (M+H) 371.1462, found 371.1462.

*(R)*-Methyl 6-((5-cyano-6-(pyrrolidin-3-yloxy)pyrazin-2-yl)amino)-4-(methylthio)nicotinate (**12**) Prepared from **32** and **40** following General Procedure (iv) to give **12** (16%) as a yellow solid.  $^1H$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  8.90 (s, 1H), 8.80 (s, 1H), 7.48 (s, 1H), 5.66 (ddd,  $J$  = 7.0, 5.1, 1.9 Hz, 1H), 3.84 (s, 3H), 3.50 (dd,  $J$  = 13.3, 5.1 Hz, 1H), 3.42 (d,  $J$  = 13.3 Hz, 1H), 3.34–3.22 (m, 2H), 2.48 (s, 3H), 2.33–2.16 (m, 2H); LC-MS (3.5 min)  $R_t$  = 2.32 min;  $m/z$  (ESI) 387 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{17}H_{19}N_6O_3S$  (M+H) 387.1234, found 387.1232.

*(R)*-3-(1-(Dimethylamino)propan-2-yloxy)-5-(4-(methylamino)pyridin-2-ylamino)pyrazine-2-carbonitrile (**13**) Prepared from **41** and **30** following General Procedure (iii) to give **13** (45%) as a tan solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  10.06 (v. broad s, 1H), 8.02 (s, 1H), 7.95 (d,  $J$  = 5.9 Hz, 1H), 7.22 (d,  $J$  = 2.3 Hz, 1H), 6.26 (dd,  $J$  = 6.0, 2.2 Hz, 1H), 5.56–5.38 (m, 1H), 4.96–4.79 (m, 1H), 2.91 (d,  $J$  = 5.0 Hz, 3H), 2.81 (dd,  $J$  = 13.4, 7.1 Hz, 1H), 2.59 (dd,  $J$  = 13.4, 4.2 Hz, 1H), 2.37 (s, 6H), 1.42 (d,  $J$  = 6.3 Hz, 3H); LC-MS (3.5 min)  $R_t$  = 1.02 min;  $m/z$  (ESI) 328 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{16}H_{22}N_7O$  (M+H) 328.1880, found 328.1887.

*(R)*-5-((5-Chloro-4-(methylamino)pyridin-2-yl)amino)-3-((1-(dimethylamino)propan-2-yloxy)pyrazine-2-carbonitrile (**14**) Prepared from **42** and **30** following General Procedure (iii) to give **14** (56%) as a yellow solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.22 (s, 1H), 8.02 (brs, 1H), 7.98

(s, 1H), 7.01 (s, 1H), 5.48–5.36 (m, 1H), 4.97 (brt,  $J = 4.2$  Hz, 1H), 2.98 (d,  $J = 5.1$  Hz, 3H), 2.74 (dd,  $J = 13.3, 7.3$  Hz, 1H), 2.51 (dd,  $J = 13.3, 4.3$  Hz, 1H), 2.31 (s, 6H), 1.41 (d,  $J = 6.3$  Hz, 3H); LC-MS (3.5 min)  $R_t = 1.77$  min;  $m/z$  (ESI) 362 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{16}H_{21}ClN_7O$  (M+H) 362.1491, found 362.1492.

*(R)*-3-((1-(Dimethylamino)propan-2-yl)oxy)-5-((4-(methylamino)-5-(trifluoromethyl)pyridin-2-yl)amino)pyrazine-2-carbonitrile (**15**) Prepared from **44** and **30** following General Procedure (iii) to give **15** (66%) as a yellow solid.  $^1H$  NMR (500 MHz,  $CD_3OD$ )  $\delta$  8.60 (s, 1H), 8.16 (s, 1H), 6.98 (s, 1H), 5.59–5.53 (m, 1H), 2.94 (s, 3H), 2.82 (dd,  $J = 14, 8$  Hz, 1H), 2.51 (dd,  $J = 14, 5$  Hz, 1H), 2.33 (s, 6H), 1.42 (d,  $J = 6$  Hz, 3H). LC-MS (3.5 min)  $R_t = 1.75$  min;  $m/z$  (ESI) 396 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{17}H_{21}F_3N_7O$  (M+H) 396.1754, found 396.1764.

*(R)*-5-((5-Cyclopropyl-4-(methylamino)pyridin-2-yl)amino)-3-((1-(dimethylamino)propan-2-yl)oxy)pyrazine-2-carbonitrile (**16**) 2-Cyclopropyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane was reacted with **46** following General Procedure (i) to give 2-chloro-5-cyclopropyl-*N*-methylpyridin-4-amine (49%) as a white solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  7.72 (s, 1H), 6.34 (s, 1H), 4.82 (brs, 1H), 2.85 (s, 3H), 1.37–1.34 (m, 1H), 0.85–0.82 (m, 2H), 0.49–0.46 (m, 2H); LC-MS (4 min)  $R_t = 1.23$  min;  $m/z$  (ESI) 183 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_9H_{12}ClN_2$  (M+H) 183.0684, found 183.0688. 2-Chloro-5-cyclopropyl-*N*-methylpyridin-4-amine was reacted with **30** following General Procedure (iii) to give **16** (33%) as a yellow solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.11 (s, 1H), 7.71 (s, 1H), 6.91 (s, 1H), 5.39–5.37 (m, 1H), 4.92 (brs, 1H), 2.93–2.29 (m, 3H), 2.73–2.68 (m, 1H), 2.49 (dd,  $J = 13.3, 3.9$  Hz, 1H), 2.27 (s, 6H), 1.40–1.38 (m, 1H), 1.35 (d,  $J = 6$  Hz, 3H), 0.86–0.84 (m, 2H), 0.50–0.48 (m, 2H); LC-MS (4 min)  $R_t = 1.40$  min;  $m/z$  (ESI) 368 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{19}H_{27}N_7O$  (M+H) 368.2193, found 368.2200.

*(R)*-3-((1-(Dimethylamino)propan-2-yl)oxy)-5-((5-ethynyl-4-(methylamino)pyridin-2-yl)amino)pyrazine-2-carbonitrile (**17**) Ethynyltrimethylsilane was reacted with **46** following

General Procedure (ii) give 2-chloro-*N*-methyl-5-((trimethylsilyl)ethynyl)pyridin-4-amine (50%) as a yellow powder. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.11 (s, 1H), 6.47 (s, 1H), 5.15 (brs, 1H), 2.97 (d, *J* = 5 Hz, 3H), 0.3 (9H, s); LC-MS (4 min) Rt = 3.13 min; *m/z* (ESI) 239 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>11</sub>H<sub>16</sub>ClN<sub>2</sub>Si (M+H) 239.0766, found 239.0775. 2-Chloro-*N*-methyl-5-((trimethylsilyl)ethynyl)pyridin-4-amine was reacted with **30** following General Procedure (iii) to give **17** (46%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.28 (s, 1H), 8.13 (s, 1H), 7.80 (brs, 1H), 6.94 (s, 1H), 5.45–5.40 (m, 1H), 5.21–5.19 (m, 1H), 3.49 (s, 1H), 3.00 (s, 3H), 2.76–2.73 (m, 1H), 2.54–2.52 (m, 1H), 2.34 (s, 6H), 1.43 (d, *J* = 6 Hz, 3H); LC-MS (4 min) Rt = 1.53 min; *m/z* (ESI) 352 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>18</sub>H<sub>22</sub>N<sub>7</sub>O (M+H) 352.1880, found 352.1881.

*(R)*-3-((1-(Dimethylamino)propan-2-yl)oxy)-5-((5-(3-hydroxy-3-methylbut-1-yn-1-yl)-4-(methylamino)pyridin-2-yl)amino)pyrazine-2-carbonitrile (**18**) Trimethyl((2-methylbut-3-yn-2-yl)oxy)silane was reacted with **46** following General Procedure (ii) to give 2-chloro-*N*-methyl-5-(3-methyl-3-((trimethylsilyl)oxy)but-1-yn-1-yl)pyridin-4-amine (75%) as a yellow solid <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.04 (s, 1H), 6.46 (s, 1H), 5.00 (brs, 1H), 2.94 (d, *J* = 5 Hz, 3H), 1.62 (6H, s), 0.22 (s, 9H); LC-MS (3.5 min) Rt = 2.81 min; *m/z* (ESI) 297 (M+H). 2-Chloro-*N*-methyl-5-(3-methyl-3-((trimethylsilyl)oxy)but-1-yn-1-yl)pyridin-4-amine was reacted with **30** following General Procedure (iii) to give **18** (6%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD) δ 8.46 (s, 1H), 7.95 (s, 1H), 6.92 (s, 1H), 5.59–5.55 (m, 1H), 2.97 (s, 3H), 2.85 (dd, *J* = 13, 10 Hz, 1H), 2.60 (dd, *J* = 10, 5 Hz, 1H), 2.35 (s, 6H), 1.61 (s, 6H), 1.43 (d, *J* = 6 Hz, 3H); LC-MS (3.5 min) Rt = 1.43 min; *m/z* (ESI) 410 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>21</sub>H<sub>28</sub>N<sub>7</sub>O<sub>2</sub> (M+H) 410.2299, found 410.2284.

*(R)*-3-((1-(Dimethylamino)propan-2-yl)oxy)-5-((5-(3-fluorophenyl)-4-(methylamino)pyridin-2-yl)amino)pyrazine-2-carbonitrile (**19**) 3-Fluorophenylboronic acid was reacted with **46** following

General Procedure (i) to give 2-chloro-5-(3-fluorophenyl)-*N*-methylpyridin-4-amine (79%) as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.84 (s, 1H), 7.45 (ddd, *J* = 8.0, 8.0, 6.0 Hz, 1H), 7.15–7.04 (m, 3H), 6.54 (s, 1H), 4.54 (brs, 1H), 2.85 (d, *J* = 5.1 Hz, 3H); LC-MS (3.5 min) Rt = 2.14 min; *m/z* (ESI) 237 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>12</sub>H<sub>11</sub>ClFN<sub>2</sub> (M+H) 237.059, found 237.059. 2-Chloro-5-(3-fluorophenyl)-*N*-methylpyridin-4-amine was reacted with **30** following General Procedure (iii) to give **19** (49%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.22 (s, 1H), 7.86 (s, 1H), 7.49–7.42 (m, 1H), 7.19–7.15 (m, 1H), 7.13–7.08 (m, 3H), 5.48–5.40 (m, 1H), 4.63 (q, *J* = 4.8 Hz, 1H), 2.91 (d, *J* = 5.0 Hz, 3H), 2.75 (dd, *J* = 13.4, 7.2 Hz, 1H), 2.51 (dd, *J* = 13.4, 4.4 Hz, 1H), 2.31 (s, 6H), 1.41 (d, *J* = 6.3 Hz, 3H); LC-MS (3.5 min) Rt = 1.56 min; *m/z* (ESI) 422 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>22</sub>H<sub>25</sub>FN<sub>7</sub>O (M+H) 422.2099, found 422.2109.

*(S)*-3-(1-(Dimethylamino)propan-2-yloxy)-5-(5-(1-methyl-1H-pyrazol-4-yl)-4-(methylamino)pyridin-2-ylamino)pyrazine-2-carbonitrile (**21**) Prepared from **47** and **31** following General Procedure (iii) to give **21** (31%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.18 (s, 1H), 7.87 (s, 1H), 7.60 (d, *J* = 0.8 Hz, 1H), 7.50 (d, *J* = 0.8 Hz, 1H), 7.14 (s, 1H), 5.52–5.42 (m, 1H), 4.81–4.74 (m, 1H), 4.00 (s, 3H), 2.93 (d, *J* = 5.0 Hz, 3H), 2.78 (dd, *J* = 13.4, 7.3 Hz, 1H), 2.54 (dd, *J* = 13.4, 4.3 Hz, 1H), 2.34 (s, 6H), 1.43 (d, *J* = 6.3 Hz, 3H); LC-MS (3.5 min) Rt = 1.12 min; *m/z* (ESI) 408 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>20</sub>H<sub>26</sub>N<sub>9</sub>O (M+H) 408.2255, found 408.2253.

*(R)*-5-((5-(1-Methyl-1H-pyrazol-4-yl)-4-(methylamino)pyridin-2-yl)amino)-3-(pyrrolidin-3-yloxy)pyrazine-2-carbonitrile (**22**) Prepared from **47** and **32** following General Procedure (iv) to give **22** (31%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, DMSO) δ 10.46 (brs, 1H), 8.50 (s, 1H), 7.89 (s, 1H), 7.84 (s, 1H), 7.60 (s, 1H), 7.04 (s, 1H), 5.84 (q, *J* = 4.7 Hz, 1H), 5.61–5.55 (m, 1H), 3.88 (s, 3H), 3.25 (dd, *J* = 12.7, 5.5 Hz, 3H), 3.07–3.00 (m, 2H), 2.93 (ddd, *J* = 10.9, 8.1, 4.7 Hz,

1H), 2.78 (d,  $J = 4.8$  Hz, 3H), 2.17–2.10 (m, 1H), 2.01–1.93 (m, 1H); LC-MS (3.5 min)  $R_t = 1.24$  min;  $m/z$  (ESI) 392 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{19}H_{22}N_9O$  (M+H) 392.1942, found 392.1945.

*(S)*-5-((5-(1-Methyl-1H-pyrazol-4-yl)-4-(methylamino)pyridin-2-yl)amino)-3-(pyrrolidin-3-yloxy)pyrazine-2-carbonitrile (**23**) Prepared from **47** and **33** following General Procedure (iv) to give **23** (47%) as a yellow solid.  $^1H$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  10.46 (brs, 1H), 8.50 (s, 1H), 7.89 (s, 1H), 7.84 (s, 1H), 7.60 (s, 1H), 7.04 (s, 1H), 5.84 (q,  $J = 4.7$  Hz, 1H), 5.61–5.55 (m, 1H), 3.88 (s, 3H), 3.25 (dd,  $J = 12.7, 5.5$  Hz, 3H), 3.07–3.00 (m, 2H), 2.93 (ddd,  $J = 10.9, 8.1, 4.7$  Hz, 1H), 2.78 (d,  $J = 4.8$  Hz, 3H), 2.17–2.10 (m, 1H), 2.01–1.93 (m, 1H); LC-MS (3.5 min)  $R_t = 1.24$  min;  $m/z$  (ESI) 392 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{19}H_{22}N_9O$  (M+H) 392.1942, found 392.1946.

*(R)*-5-((5-(1-Methyl-1H-pyrazol-4-yl)-4-(methylamino)pyridin-2-yl)amino)-3-((1-methylpyrrolidin-3-yl)oxy)pyrazine-2-carbonitrile (**24**) Prepared from **47** and **34** following General Procedure (iii) to give **24** (13%) as a yellow solid.  $^1H$  NMR (500 MHz,  $CD_3OD$ )  $\delta$  8.49 (s, 1H), 7.82 (s, 1H), 7.78 (s, 1H), 7.61 (s, 1H), 6.85 (s, 1H), 5.62–5.60 (m, 1H), 3.97 (s, 3H), 3.05–3.02 (m, 1H), 2.88 (3H, s), 2.87–2.85 (m, 2H), 2.59–2.55 (m, 1H), 2.48–2.43 (m, 1H), 2.42 (s, 3H), 2.10–2.08 (m, 1H); LC-MS (LCT, 4 min)  $R_t = 1.20$  min;  $m/z$  (ESI) 406 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{20}H_{24}N_9O$  (M+H) 406.2098, found 406.2107.

*(S)*-5-((5-(1-Methyl-1H-pyrazol-4-yl)-4-(methylamino)pyridin-2-yl)amino)-3-((1-methylpyrrolidin-3-yl)oxy)pyrazine-2-carbonitrile (**25**) Prepared from **47** and **35** following General Procedure (iii) to give **25** (22%) as an orange solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.33 (s, 1H), 7.86 (s, 1H), 7.59 (s, 1H), 7.50 (s, 1H), 6.98 (s, 1H), 5.56–5.54 (m, 1H), 4.78–4.77 (m, 1H), 4.00 (s, 3H), 3.29 (dd,  $J = 11, 6.2$  Hz, 1H), 2.92 (s, 3H), 2.80–2.78 (m, 1H), 2.74 (dd,  $J = 11, 3.5$  Hz, 1H), 2.47 (s, 3H), 2.41–2.37 (m, 1H), 2.16–2.14 (m, 1H); LC-MS (4 min)  $R_t = 1.20$

min;  $m/z$  (ESI) 406 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{20}H_{24}N_9O$  (M+H) 406.2098, found 406.2091.

*(R)*-5-((4-Methoxy-5-(1-methyl-1*H*-pyrazol-4-yl)pyridin-2-yl)amino)-3-(pyrrolidin-3-yloxy)pyrazine-2-carbonitrile (**27**) Prepared from **49** and **32** following General Procedure (iv) to give **27** (27%) as a yellow solid.  $^1H$  NMR (500 MHz, DMSO)  $\delta$  8.51 (s, 1H), 8.45 (s, 1H), 8.13 (s, 1H), 7.93 (d,  $J = 0.6$  Hz, 1H), 7.60 (s, 1H), 5.61–5.56 (m, 1H), 3.98 (s, 3H), 3.88 (s, 3H), 3.23 (dd,  $J = 12.7, 5.5$  Hz, 1H), 3.06–2.97 (m, 2H), 2.94–2.87 (m, 1H), 2.16–2.09 (m, 1H), 1.99–1.92 (m, 1H); LC-MS (3.5 min)  $R_t = 2.02$  min;  $m/z$  (ESI) 393 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{19}H_{21}N_8O_2$  (M+H) 393.1782, found 393.1789.

*(R)*-5-((4-Methoxy-5-(1-methyl-1*H*-pyrazol-4-yl)pyridin-2-yl)amino)-3-((1-methylpyrrolidin-3-yl)oxy)pyrazine-2-carbonitrile (**28**) Prepared from **49** and **34** following General Procedure (iii) to give **28** (83%) as a yellow solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.39 (brs, 1H), 8.37 (s, 1H), 8.32 (s, 1H), 7.85 (s, 1H), 7.78 (s, 1H), 7.36 (s, 1H), 5.51–5.47 (m, 1H), 4.01 (s, 3H), 3.96 (s, 3H), 3.15 (dd,  $J = 10.7, 6.3$  Hz, 1H), 2.73–2.62 (m, 3H), 2.39 (s, 3H), 2.38–2.30 (m, 1H), 2.12–2.05 (m, 1H); LC-MS (3.5 min)  $R_t = 2.01$  min;  $m/z$  (ESI) 407 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{20}H_{23}N_8O_2$  (M+H) 407.1939, found 407.1936.

*(R)*-3-((1-(Dimethylamino)propan-2-yl)oxy)-5-((5-(1-methyl-1*H*-pyrazol-4-yl)-4-(methylthio)pyridin-2-yl)amino)pyrazine-2-carbonitrile (**29**) 2-Chloro-5-(1-methyl-1*H*-pyrazol-4-yl)-4-(methylthio)pyridine **52** was reacted with **30** following General Procedure (iii) to give **29** (5%) as a yellow solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.56 (s, 1H), 8.13 (s, 1H), 7.82 (brs, 1H), 7.72 (s, 1H), 7.65 (s, 1H), 7.34 (s, 1H), 5.50–5.41 (m, 1H), 4.00 (s, 3H), 2.79 (dd,  $J = 13.4, 7.2$  Hz, 1H), 2.56 (dd,  $J = 13.4, 4.2$  Hz, 1H), 2.52 (s, 3H), 2.36 (s, 6H), 1.43 (d,  $J = 6.3$  Hz, 3H). LC-MS (3.5 min)  $R_t = 2.31$  min;  $m/z$  (ESI) 425 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_{20}H_{25}N_8OS$  (M+H) 425.1867, found 425.1864.

*(R)*-5-Amino-3-((1-(dimethylamino)propan-2-yl)oxy)pyrazine-2-carbonitrile (**30**) [19] *(R)*-1-(Dimethylamino)propan-2-ol [19] (0.667 g, 6.47 mmol) was added drop wise to a suspension of NaH (60% in oil; 0.388 g, 9.71 mmol) in dioxane (16.2 mL) and stirred for 30 min. 5-Amino-3-chloropyrazine-2-carbonitrile [19] (1.00 g, 6.47 mmol) was added in one portion and the mixture was heated at 90 °C for 14 h. After cooling, water (200 mL) was added and the solution was extracted with Et<sub>2</sub>O (4 x 100 mL), dried, and the volatiles removed under vacuum. Gradient silica column chromatography eluting with MeOH: 1% NH<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub>, gave **30** (0.558 g, 2.52 mmol, 39%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 1.35 (d, *J* = 6.5 Hz, 3H) 2.37 (s, 6H), 2.52 (dd, *J* = 4.0, 13.5 Hz, 1H), 2.76 (dd, *J* = 7.5, 13.5 Hz, 1H), 5.31 (brs, 2H), 5.42–5.35 (m, 1H), 7.54 (s, 1H). LC-MS (3.5 min) Rt = 0.80 min; *m/z* (ESI) 222 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>10</sub>H<sub>16</sub>N<sub>5</sub>O (M+H) 222.1349, found 222.1357.

*(S)*-5-Amino-3-((1-(dimethylamino)propan-2-yl)oxy)pyrazine-2-carbonitrile (**31**) [19] Prepared as described for **30** using *(S)*-1-(dimethylamino)propan-2-ol [19] in place of *(R)*-1-(dimethylamino)propan-2-ol (19%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 1.35 (d, *J* = 6.2 Hz, 3H) 2.35 (s, 6H), 2.49 (dd, *J* = 4.1, 13.3 Hz, 1H), 2.76 (dd, *J* = 7.5, 13.3 Hz, 1H), 5.33–5.40 (m, 1H), 5.43 (br s, 2H), 7.52 (s, 1H); LC-MS (4 min) Rt = 1.69 min; *m/z* (ESI) 222 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>10</sub>H<sub>16</sub>N<sub>5</sub>O (M+H) 222.1349, found 222.1353.

*(R)*-*tert*-Butyl 3-((6-amino-3-cyanopyrazin-2-yl)oxy)pyrrolidine-1-carboxylate (**32**) Prepared as described for **30** using *(R)*-*tert*-butyl 3-hydroxypyrrolidine-1-carboxylate in place of *(R)*-1-(dimethylamino)propan-2-ol (43%). Characterized as a mixture of rotamers. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.61–7.58 (m, 1H), 5.49–5.44 (m, 1H), 5.35–5.30 (m, 2H), 3.69–3.65 (m, 1H), 3.63–3.50 (m, 3H), 2.28–2.11 (m, 2H), 1.46 (s, 9H); LC-MS (3.5 min) Rt = 2.67 min; *m/z* (ESI) 328 (M+Na); HRMS (ESI) *m/z* calcd. for C<sub>14</sub>H<sub>19</sub>N<sub>5</sub>NaO<sub>3</sub> (M+Na) 328.1380, found 328.1379.

*(S)-tert-Butyl 3-((6-amino-3-cyanopyrazin-2-yl)oxy)pyrrolidine-1-carboxylate (33)* Prepared as described for **30** using *(S)-tert-butyl 3-hydroxypyrrolidine-1-carboxylate* in place of *(R)-1-(dimethylamino)propan-2-ol* (56%). Characterized as a mixture of rotamers. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.61–7.58 (m, 1H), 5.49–5.44 (m, 1H), 5.35–5.30 (m, 2H), 3.69–3.65 (m, 1H), 3.63–3.50 (m, 3H), 2.28–2.11 (m, 2H), 1.46 (s, 9H); LC-MS (3.5 min) Rt = 2.67 min; *m/z* (ESI) 328 (M+Na); HRMS (ESI) *m/z* calcd. for C<sub>14</sub>H<sub>19</sub>N<sub>5</sub>NaO<sub>3</sub> (M+Na) 328.1380, found 328.1379.

*(R)-5-Amino-3-((1-methylpyrrolidin-3-yl)oxy)pyrazine-2-carbonitrile (34)* Prepared as described for **30** using *(R)-1-methylpyrrolidin-3-ol* in place of *(R)-1-(dimethylamino)propan-2-ol* (53%). <sup>1</sup>H NMR (500 MHz, d<sub>6</sub>-DMSO) δ 7.60 (brs, 2H), 7.51 (s, 1H), 5.36–5.28 (m, 1H), 2.76 (dd, *J* = 10.8, 6.1 Hz, 1H), 2.69 (ddd, *J* = 8.2, 8.2, 5.3 Hz, 1H), 2.62 (dd, *J* = 10.8, 2.8 Hz, 1H), 2.37–2.21 (m, 2H), 2.26 (s, 3H), 1.85–1.77 (m, 1H); LC-MS (3.5 min) Rt = 0.62 min; *m/z* (ESI) 220 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>10</sub>H<sub>14</sub>N<sub>5</sub>O (M+H) 220.1193, found 220.1198

*(S)-5-Amino-3-((1-methylpyrrolidin-3-yl)oxy)pyrazine-2-carbonitrile (35)* Prepared as described for **30** using *(S)-1-methylpyrrolidin-3-ol* in place of *(R)-1-(dimethylamino)propan-2-ol* (69%). <sup>1</sup>H NMR (500 MHz, d<sub>6</sub>-DMSO) δ 7.60 (brs, 2H), 7.51 (s, 1H), 5.36–5.28 (m, 1H), 2.76 (dd, *J* = 10.8, 6.1 Hz, 1H), 2.69 (ddd, *J* = 8.2, 8.2, 5.3 Hz, 1H), 2.62 (dd, *J* = 10.8, 2.8 Hz, 1H), 2.37–2.21 (m, 2H), 2.26 (s, 3H), 1.85–1.77 (m, 1H); LC-MS (3.5 min) Rt = 0.62 min; *m/z* (ESI) 220 (M+H); HRMS (ESI) *m/z* calcd. for C<sub>10</sub>H<sub>14</sub>N<sub>5</sub>O (M+H) 220.1193, found 220.1198

*Methyl 6-chloro-4-(methylamino)nicotinate (37)* MeNH<sub>2</sub> aq. (40%; 0.847 mL, 9.78 mmol) was added slowly over 5 min at 0 °C to a solution of methyl 4,6-dichloronicotinate **36** (0.40 g, 1.94 mmol) in MeCN (6 mL). The solution was stirred at 0 °C for 30 min then at r.t. for 2 h. The reaction mixture was absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 5-50% EtOAc - cyclohexane, gave **37** (278 mg, 1.39 mmol, 71%) as a white solid: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.65 (s, 1H), 8.10 (brs,

1H), 6.54 (s, 1H), 3.88 (s, 1H), 2.92 (d,  $J = 5.1$  Hz, 3H); LC-MS (3.5 min)  $R_t = 2.35$  min;  $m/z$  (ESI) 201 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_8H_{10}^{35}ClN_2O_2$  (M+H) 201.0425, found 201.0432.

*Methyl 6-chloro-4-(dimethylamino)nicotinate (38)*  $Me_2NH$  (1.23 mL, 9.71 mmol) was slowly added to a stirred solution of methyl 4,6-dichloronicotinate **36** (0.40 g, 1.94 mmol) in MeCN (6 mL) at r.t. The solution was stirred at r.t. for 18 h then absorbed onto silica gel and solvents were removed by evaporation. Gradient silica gel column chromatography, eluting with 5-50% EtOAc - cyclohexane, gave **38** (335 mg, 1.56 mmol, 80%) as a white solid:  $^1H$  NMR (500 MHz, DMSO)  $\delta$  8.21 (s, 1H), 6.85 (s, 1H), 3.83 (s, 3H), 2.90 (s, 6H); LC-MS (3.5 min)  $R_t = 2.23$  min;  $m/z$  (ESI) 215 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_9H_{11}ClN_2O_2$  (M+H) 215.0582, found 215.0586.

*Methyl 6-chloro-4-methoxynicotinate (39)* Powdered NaOMe (0.136 g, 2.52 mmol) was added slowly to a stirred solution of methyl 4,6-dichloronicotinate **36** (0.40 g, 1.94 mmol) in THF (4 mL) at r.t. The reaction mixture was stirred at r.t. for 18 h, then absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 5-50% EtOAc - cyclohexane, gave **39** (218 mg, 1.08 mmol, 56%) as a white solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.71 (s, 1H), 6.92 (s, 1H), 3.97 (s, 3H), 3.90 (s, 3H); LC-MS (3.5 min)  $R_t = 2.13$  min;  $m/z$  (ESI) 202 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_8H_9ClNO_3$  (M+H) 202.0265, found 202.0269.

*Methyl 6-chloro-4-(methylthio)nicotinate (40)* NaSMe aq. (21 wt% in water; 0.650 mL, 2.141 mmol) was added to a solution of methyl 4,6-dichloronicotinate **36** (0.401 g, 1.946 mmol) in THF (4 mL) and left to stir at r.t. for 72 h. The reaction was quenched with sat.  $NH_4Cl$  aq. (10 mL) and extracted with EtOAc (2 x 10 mL). The combined organic layers were absorbed onto silica gel and solvent was removed by evaporation. Gradient silica column chromatography, eluting with 0-25% EtOAc - cyclohexane, gave **40** (236 mg, 1.084 mmol, 56%) as a colorless solid:  $^1H$  NMR (500 MHz, DMSO)  $\delta$  8.75 (s, 1H), 7.43 (s, 1H), 3.86 (s, 3H), 2.52 (s, 3H); LC-

MS (3.5 min) Rt = 2.63 min;  $m/z$  (ESI) 218 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_8H_8ClNNaO_2S$  (M+Na) 239.9856, found 239.9852.

*2,5-Dichloro-N-methylpyridin-4-amine (42)* N-Chlorosuccinimide (0.562 g, 4.21 mmol) was added to 2-chloro-N-methylpyridin-4-amine **41** (0.050 g, 3.51 mmol) and KOAc (0.688 g, 7.01 mmol) in AcOH (22 mL) and the mixture was stirred at 80 °C for 1.25 h. The mixture was cooled and solvents were removed by evaporation. The mixture was diluted with water, neutralized with sat.  $NaHCO_3$  aq. and extracted with EtOAc. The organic extracts were absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 5-15% EtOAc – cyclohexane and collecting the second eluting component ( $R_f$  = 0.31 in 25% EtOAc – cyclohexane ), gave **42** (0.083 g, 0.469 mmol, 13%) as a white solid.  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.02 (s, 1H), 6.52 (s, 1H), 4.95 (brs, 1H), 2.96 (d,  $J$  = 5 Hz, 3H); LC-MS (4 min) Rt = 2.17 min;  $m/z$  (ESI) 177 (M+H). HRMS (ESI)  $m/z$  calcd. for  $C_6H_7Cl_2N_2$  (M+H) 176.9981, found 176.9983

*2-Chloro-N-methyl-5-(trifluoromethyl)pyridin-4-amine (44)*  $MeNH_2$  in MeOH (2.0 M; 11.6 mL, 23.2 mmol) was added to 2-chloro-4-iodo-5-(trifluoromethyl)pyridine **43** (0.357 g, 1.16 mmol) and the mixture was heated at 130 °C in a microwave reactor for 1 h. Solvents were removed by evaporation. Preparative thin-layer chromatography, eluting with 20% EtOAc – hexane, gave **44** (0.077 g, 0.363 mmol, 31%).  $^1H$  NMR (500 MHz,  $d_6$ -DMSO)  $\delta$  8.17 (s, 1H), 6.90 (brs, 1H), 6.74 (s, 1H), 2.81 (d,  $J$  = 5 Hz, 3H); LC-MS (3.5 min) Rt = 1.98 min;  $m/z$  (ESI) 211 (M+H); HRMS (ESI)  $m/z$  calcd. for  $C_7H_7F_3N_2^{35}Cl$  (M+H) 211.0244, found 211.0234

*2-Chloro-5-iodo-4-(methylthio)pyridine (51)* n-BuLi (2.5M in hexanes; 0.877 mL, 2.19 mmol) was added to a solution of  $^iPr_2NH$  (0.383 mL, 2.19 mmol) in THF (2.0 mL) at -78°C and stirred for 20 min. 2-Chloro-5-iodopyridine **50** (0.50 g, 2.09 mmol) in THF (4.0 mL) was added drop wise over 15 min. A solution of MeSSMe (0.223 mL, 2.51 mmol) in THF (4.0 mL) was added

drop wise over 10 min. The reaction was warmed to r.t. over 1 h. The reaction mixture was absorbed onto silica gel and solvents were removed by evaporation. Gradient silica column chromatography, eluting with 5-25% EtOAc – cyclohexane, gave **51** (0.063 g, 0.221 mmol, 11%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.47 (s, 1H), 6.95 (s, 1H), 2.50 (s, 3H); LC-MS (3.5 min) Rt = 2.89 min; m/z (ESI) 286 (M+H); HRMS (ESI) m/z calcd. for C<sub>6</sub>H<sub>5</sub><sup>35</sup>ClINS (M+H) 285.8949, found 285.8941.

*2-Chloro-5-(1-methyl-1H-pyrazol-4-yl)-4-(methylthio)pyridine* (**52**) 1-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole was reacted with **51** following General Procedure (i) to give **52** (72%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.11 (s, 1H), 7.69 (s, 1H), 7.63 (s, 1H), 7.04 (s, 1H), 3.95 (s, 3H), 2.46 (s, 3H); LC-MS (3.5 min) Rt = 2.53 min; m/z (ESI) 240 (M+H); HRMS (ESI) m/z calcd. for C<sub>10</sub>H<sub>11</sub><sup>35</sup>ClN<sub>3</sub>S (M+H) 240.0357, found 240.0359.

## 2. Experimental methods for the determination of CHK1 and CHK2 inhibition.

CHK1 kinase activity was measured in a microfluidic assay that monitored the separation of a phosphorylated product from its substrate. The assay was run on an EZ Reader II (Caliper Life Sciences Ltd, Runcorn, UK) using separation buffer (#760367 Caliper LS) containing CR-8 (500nM, #760278, Caliper LS). An ECHO<sup>®</sup> 550 (Labcyte Inc<sup>™</sup>) acoustic dispenser was used to generate duplicate 8 pt dilution curves directly into 384 polypropylene assay plates (Greiner Bio-One, Gloucestershire, UK). For each compound a 50 μM stock concentration in 100% DMSO was used. The total amount of DMSO dispensed per well was 250 nL to give a final assay concentration of 2.5% DMSO and compound concentrations in the range 0.5-1000 nM. To this assay plate, 6 μL CHK1 (2 nM final concentration, in-house protein preparation), 2 μL peptide 10 (5-FAM-KKKVSRSGLYRSPSPENLNRPR-COOH, 1.5 μM final concentration, #760354

Caliper LS) and 2  $\mu$ L ATP (90  $\mu$ M final concentration) all diluted in kinase buffer (HEPES 50 mM,  $\text{NaN}_3$  0.02%, BSA 0.01%, sodium orthovanadate 0.1 mM, DTT 1 mM,  $\text{MgCl}_2$  2 mM, Tween20 0.1%) were added. The plate was sealed and centrifuged (1 min, 1000 rpm) before incubation for 1 h at room temperature. The reaction was stopped by the addition of separation buffer (90  $\mu$ L). The plate was read on an EZ Reader II, using a 12-sipper chip (#760404, Caliper LS) with instrument settings of -1.5 psi and 1750  $\Delta$ V. The percentage conversion of product from substrate was generated automatically and the percentage inhibition was calculated relative to blank wells (containing no enzyme and 2.5% DMSO) and total wells (containing all reagents and 2.5% DMSO).  $\text{IC}_{50}$  values were calculated in GraphPad Prism5 using a non linear regression fit of the log (inhibitor) vs response with variable slope equation.

CHK2 kinase activity was measured in a DELFIA® assay that monitored phosphorylation of a CDC25C peptide using a specific phospho antibody. The enzyme reaction was carried out in 96-well polypropylene plates (Greiner). The reaction mix (total volume 25  $\mu$ L) contained enzyme and peptide mix (15  $\mu$ L) (containing CHK2, prepared in-house, 1 nM; Biotin-KKKVSRSGLYRSPSPENLNRPR, 1  $\mu$ M), ATP (30  $\mu$ M, 5  $\mu$ L) and either DMSO (2.5%) or test compound (5  $\mu$ L) diluted to give a range of concentrations (0-100  $\mu$ M in 2.5% DMSO, final concentrations) in assay buffer (40 mM HEPES (pH7.4), 40 mM KCl, 2 mM  $\text{MgCl}_2$ , 10 mM DTT and 0.02% Tween 20). The reaction mixture was incubated for 30 min at room temperature and stopped by the addition of buffer (125  $\mu$ L) containing 40 mM EDTA, 0.05% Tween 20, 0.1% BSA in TBS (10x concentrate, Sigma). An aliquot (100  $\mu$ L) of the reaction mix was transferred to a black neutravidin-coated 96-well plate (Perbio) and incubated for 1 h on a shaker (Titertek, Flow Laboratories) at room temperature. The plates were washed four times with wash buffer (25 mM Tris (pH 8), 150 mM NaCl and 0.1% Tween 20) (WellWash4, Thermo Life Sciences) and incubated for 1 h as before with antibody mix (100  $\mu$ L) consisting of anti-

phospho CDC25C (diluted 1/4000 equivalent to 0.35 nM-1.25 nM, #9528, Cell Signalling Technology) and europium-labelled anti rabbit IgG, (0.3 µg/mL, AD0105, PerkinElmer Life Sciences) diluted in DELFIA assay buffer (PerkinElmer Life Sciences). The plates were washed a further four times with wash buffer before the addition of enhancement solution (100 µL/well, PerkinElmer Life Sciences). The plate was read on a EnVision™ 2103 multilabel counter (PerkinElmer Life Sciences) using a time-resolved measurement mode reading fluorescence at 615 nM. IC<sub>50</sub> values were calculated in GraphPad Prism5 using a non linear regression fit of the log (inhibitor) vs response with variable slope equation.

### **3. Experimental methods for the determination of checkpoint abrogation, antiproliferative activity, and potentiation of genotoxic drug efficacy in cancer cell lines.**

Checkpoint abrogation by CHK1 kinase inhibitors in combination with genotoxic agents was assessed using a europium based ELISA assay designed to quantify the number of cells trapped in mitosis after treatment with a genotoxic agent (to induce G2 arrest) followed by a test compound in combination with nocodazole to abrogate this arrest. HT29 cells were seeded at 104 cells per well into 96 well plates in a volume of 160 µL and left to attach for 36 h. Etoposide (10 mM stock in DMSO) was diluted in medium to 250 µM and then 40 µL was added to appropriate wells to give a final concentration of 50 µM and incubated for 1 h. This treatment had previously been optimised to induce a G2 arrest in 80% of cells 16 h following treatment. After genotoxic drug exposure, the medium was removed and replaced with fresh medium (160 µL). Cells were either untreated (untreated control or etoposide pre-treatment alone), exposed to nocodazole following etoposide pre treatment or nocodazole alone (100 ng/mL final concentration), or exposed to increasing concentrations of test compound (200 µM – 0.01 nM final concentration) in combination with nocodazole (100 ng/mL final concentration). Test compounds were added in

40  $\mu$ L using quadruplicate wells for each dose. After 21 h exposure, the medium was removed and cells were fixed in 4% formaldehyde in PBS (pH 7.4, pre-cooled to 4°C) for 30 min at 4°C, followed by 100% MeOH (pre-cooled to -20°C) for 10 min at ambient temperature. Wells were washed with PBS and blocked with 5% dried milk (Marvel) in Tris-buffered saline (TBS, pH 7.4) at 37°C for 30 minutes. Each well was washed three times with water containing 0.1% Tween20. Primary antibody (MPM-2, Upstate cat# 05-368, 1  $\mu$ g/mL in 5% milk in TBS) was added to each well and incubated overnight with shaking at 4°C. Primary antibody was removed and wells were washed with water containing 0.1% Tween20. The secondary antibody (europium labelled anti-mouse, Perkin-Elmer cat# AD0124, 333 ng/mL in assay buffer, Perkin-Elmer cat# 1244-111) was added to each well and incubated at 37°C for 1 h. Each well was washed with water containing 0.1% Tween20 and treated with enhancement solution (Perkin-Elmer cat# 1244-105). Europium emissions were counted on a Wallac, Victor2 counter (Perkin-Elmer, Bucks UK). Appropriate controls were included and results were expressed as the concentration of test compound required to allow 50% of cells to enter mitosis (Checkpoint abrogation IC<sub>50</sub>).

Compound cytotoxicity and the ability of CHK1 inhibitors to enhance SN38 (the active metabolite of the topoisomerase I inhibitor irinotecan) and gemcitabine (an antimetabolite) cytotoxicity was assessed using a 96 h sulforhodamine B assay (SRB, Sigma cat# S9012). HT29 or SW620 cells were seeded at 1.6 to 3.2 x 10<sup>3</sup> cells per well in 96-well plates in a volume of 160  $\mu$ l medium and allowed to attach for 36 h prior to treatment. For cytotoxicity assays, CHK1 inhibitors (10 mM stock in DMSO) were serially diluted in medium from a starting concentration of 250  $\mu$ M and then 40  $\mu$ L was added to appropriate wells in quadruplicate to give a final concentration range of 50-0.1  $\mu$ M (10 concentrations). Genotoxic agents (SN38, LKT laboratories cat#C0154, and gemcitabine, Lilly “Gemzar”; 10 mM stock in DMSO) were serially

diluted in medium from a starting concentration of 2  $\mu\text{M}$  and 40  $\mu\text{L}$  was added to each well in quadruplicate to give final concentrations from 200-0.39 nM (10 concentrations). Cells were incubated for 96 h (four doublings) at 37 °C in a humidified 5%  $\text{CO}_2$  environment and then fixed and stained with SRB. Appropriate controls were included and results were expressed as the concentration of test compound required to inhibit cell growth by 50% relative to untreated controls (SRB  $\text{IC}_{50}$ ). Potentiation assays involved adding a fixed SRB  $\text{IC}_{50}$  concentration of either gemcitabine or SN38 in a volume of 20  $\mu\text{L}$  of medium (10x final concentration), to each well in quadruplicate and mixing for 1 min. CHK1 inhibitor (10 mM stock) was serially diluted from a starting concentration of 50  $\mu\text{M}$  in medium and 20  $\mu\text{L}$  was added per well in quadruplicate to give a final concentration range of 5-0.039  $\mu\text{M}$  (8 concentrations). After mixing for 1 min the cells were incubated at 37 °C in a humidified atmosphere for 96 h (four doublings) prior to fixing and SRB staining. Untreated and genotoxic alone treated controls were included and results were expressed as the concentration of CHK1 inhibitor required to inhibit cell growth by 50% (potentiation  $\text{IC}_{50}$ ). The potentiation index (PI) was used as a measure of the ability of the CHK1 inhibitor to enhance SN38 or gemcitabine cytotoxicity and was the ratio of the SRB  $\text{IC}_{50}$  versus potentiation  $\text{IC}_{50}$  (ie,  $\text{PI} = \text{SRB } \text{IC}_{50} / \text{Potentiation } \text{IC}_{50}$ ).

**4. Experimental method for the determination of compound concentrations *in vivo* at selected time points following i.v. and oral dosing.**

Female BALB/c mice (6 weeks old) (Charles River UK Ltd, Margate, UK) were kept in a controlled environment with food and sterilized water available *ad libitum*. Animals weighed 20 ( $\pm 2$ ) g at the time of experiment. All procedures were conducted in accordance with the local and national guidelines for animal experimentation. Dosing solutions were prepared by dissolving the compounds in 10% DMSO and 5% Tween20 in 85% saline. The compounds were administered i.v. and p.o., individually. Animals were warmed before receiving a single i.v. bolus injection into a lateral tail vein. Oral administration was by oral gavage. Blood was collected at selected time points (1 h and 6 h after dosing) by cardiac puncture under anesthesia into heparinized syringes, transferred to micro centrifuge tubes, and centrifuged at  $4500 \times g$  for 2 min to obtain plasma. Quantitative analysis was performed by high performance liquid chromatography tandem mass spectrometry on a triple quadrupole instrument (Agilent 6410) using multiple reaction monitoring of selected transitions with olomoucine used as internal standard. Quantitation was performed against a standard curve ranging from concentrations of 2-1000 nM in the matrix measured. Quality controls were included at the level of 25, 250 and 750 nM. If required, samples were diluted in the matrix of interest.

## 5. Kinase inhibition profile for **26**

**26** was tested at 1  $\mu$ M (140 enzymes) and 10  $\mu$ M (121 enzymes) for inhibition of human kinases in a radiometric protein phosphorylation assay (MRC Protein Phosphorylation Unit, Dundee University, UK). Assays were conducted in the presence of ATP at the approximate  $K_{m,ATP}$  for each kinase. Results are quoted as % control kinase activity remaining (100% = no inhibition, 0% = complete inhibition).

Table S1: Kinase inhibition profile of **26**

Kinase	% Activity remaining		Kinase	% Activity remaining		Kinase	% Activity remaining		Kinase	% Activity remaining	
	1 $\mu$ M	10 $\mu$ M		1 $\mu$ M	10 $\mu$ M		1 $\mu$ M	10 $\mu$ M		1 $\mu$ M	10 $\mu$ M
MKK1	80	38	PKC $\gamma$	90	80	AMPK	12	14	TBK1	71	55
MKK2	76	57	PKC $\zeta$	121	79	AMPK (hum)	5	n.d. <sup>a</sup>	PIM1	72	22
MKK6	71	91	PKD1	106	38	MARK1	95	80	PIM2	98	77
ERK1	96	71	STK33	59	16	MARK2	111	57	PIM3	58	17
ERK2	101	80	MSK1	86	41	MARK3	98	69	SRPK1	89	88
ERK5	86	85	MNK1	94	90	MARK4	105	83	EF2K	94	87
JNK1	104	93	MNK2	98	112	BRSK1	11	53	EIF2AK3	101	93
JNK2	98	79	MAPKAP-K2	112	104	BRSK2	23	14	HIPK1	95	107
JNK3	113	97	MAPKAP-K3	96	80	MELK	109	62	HIPK2	89	82
p38a MAPK	102	85	PRAK	78	97	NUAK1	29	7	HIPK3	96	111
p38b MAPK	96	92	CAMKKb	103	71	SIK2	80	n.d.	CLK2	95	50
p38g MAPK	89	109	CAMK1	92	95	SIK3	89	n.d.	PAK2	100	70
p38d MAPK	87	73	SmMLCK	80	103	TSSK1	72	n.d.	PAK4	87	70
ERK8	101	11	PHK	86	46	CK1 $\gamma$ 2	101	n.d.	PAK5	93	61
RSK1	4	14	DAPK1	95	98	CK1 $\delta$	76	22	PAK6	77	43

RSK2	9	34	CHK1	17	6	CK2	110	87	MST2	67	30
PDK1	76	76	CHK2	81	31	TTBK1	98	n.d.	MST3	89	n.d.
PKBa	98	87	GSK3b	90	52	TTBK2	100	n.d.	MST4	83	42
PKBb	81	67	CDK2- Cyclin A	114	91	DYRK1A	91	79	GCK	102	56
SGK1	79	22	CDK9- Cyclin T1	84	n.d.	DYRK2	100	100	MAP4K3	45	n.d.
S6K1	55	85	PLK1	88	104	DYRK3	98	79	MAP4K5	46	n.d.
PKA	104	78	Aurora A	84	48	NEK2a	58	64	MINK1	90	67
ROCK 2	92	40	Aurora B	42	53	NEK6	97	96	MEKK1	92	99
PRK2	87	86	TLK1	95	93	IKKb	96	86	MLK1	92	48
PKCa	115	102	LKB1	96	64	IKKe	78	84	MLK3	68	69
TESK1	110	n.d.	WNK1	95	75	JAK2	71	41	EPH-B4	96	88
TAO1	87	75	ULK1	60	n.d.	SYK	93	63	FGF-R1	107	39
ASK1	95	67	ULK2	42	n.d.	ZAP70	104	103	HER4	101	94
TAK1	97	53	TGFBR1	122	n.d.	TIE2	102	90	IGF-1R	100	73
IRAK1	17	9	Src	81	72	BRK	98	91	IR	98	84
IRAK4	61	25	Lck	60	20	EPH-A2	59	24	IRR	103	89
RIPK2	97	71	CSK	83	94	EPH-A4	73	41	TrkA	13	28
OSR1	115	106	YES1	93	39	EPH-B1	100	71	DDR2	95	n.d.
TTK	81	74	ABL	83	58	EPH-B2	110	63	VEG-FR	90	31
MPSK1	91	91	BTK	88	60	EPH-B3	68	23	PDGFRA	60	n.d.

<sup>a</sup> n.d. = not determined