Bioorganic & Medicinal Chemistry 18 (2010) 707–718

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification and characterisation of 2-aminopyridine inhibitors of checkpoint kinase 2

Stephen Hilton [a,†], Sebastien Naud [a], John J. Caldwell [a], Kathy Boxall [a], Samantha Burns [a], Victoria E. Anderson [a], Laurent Antoni [a], Charlotte E. Allen [a], Laurence H. Pearl [b], Antony W. Oliver [b], G. Wynne Aherne [a], Michelle D. Garrett [a], Ian Collins [a,*]

[a] Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK
[b] Section of Structural Biology, Chester Beatty Laboratories, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK

ARTICLE INFO

Article history:
Received 14 September 2009
Revised 16 November 2009
Accepted 27 November 2009
Available online 6 December 2009

Keywords:
CHK2
Kinase inhibitor
High-throughput screening
Crystallography

ABSTRACT

5-(Hetero)aryl-3-(4-carboxamidophenyl)-2-aminopyridine inhibitors of CHK2 were identified from high throughput screening of a kinase-focussed compound library. Rapid exploration of the hits through straightforward chemistry established structure–activity relationships and a proposed ATP-competitive binding mode which was verified by X-ray crystallography of several analogues bound to CHK2. Variation of the 5-(hetero)aryl substituent identified bicyclic dioxolane and dioxane groups which improved the affinity and the selectivity of the compounds for CHK2 versus CHK1. The 3-(4-carboxamidophenyl) substituent could be successfully replaced by acyclic ω-aminoalkylamides, which made additional polar interactions within the binding site and led to more potent inhibitors of CHK2. Compounds from this series showed activity in cell-based mechanistic assays for inhibition of CHK2.

1. Introduction

Checkpoint kinase 2 (CHK2) is a serine/threonine kinase which plays an important part in the complex signalling networks responsible for the maintenance of mammalian genomic integrity and repair of damaged DNA.[1–4] Signalling through CHK2 is activated in cells in response to DNA damage caused by external agents such as ionising radiation or genotoxic chemotherapy. Double strand breaks in DNA lead to activation of the DNA-damage transducer ataxia and telangiectasia mutated (ATM). ATM phosphorylates CHK2 on Thr68,[5,6] leading to homodimerisation of the protein and activation through *trans*-autophosphorylation[7,8] on Thr383 and Thr387 and *cis*-autophosphorylation[9,10] on Ser516. In turn, activated CHK2 phosphorylates multiple substrates that control cell cycle progression and DNA repair. In cells with a functional p53 tumour suppressor pathway, phosphorylation of HDMX by CHK2 stabilises p53, leading to a G1 cell cycle arrest and apoptotic cell death if the damaged DNA is not repaired.[11,12] Similarly, phosphorylation by CHK2 stabilizes the transcription factor E2F-1, promoting apoptosis.[13] CHK2 also signals to the Cdc25 family of phosphatases that control cell cycle progression in S, G2 and M phases through regulation of cyclin dependent kinases.[3,4] At the same time, CHK2 activates several DNA damage repair pathways. In particular, phosphorylation of BRCA1 promotes double strand break repair,[14] while phosphorylation of the transcription factor FOXM1 leads to increased expression of proteins involved in the homologous recombination and base excision repair mechanisms.[15]

There is current interest in the therapeutic potential of inhibitors of CHK2 in several distinct contexts, but especially in cancer where DNA-damaging agents remain a central component of treatment regimes.[1,2,16] The p53-mediated apoptotic response contributes to cell death in normal tissue in response to ionising radiation and chemotherapies that cause double strand DNA breaks. In contrast, many tumours lack a functional p53 pathway.[17] There is therefore the potential for CHK2 inhibition to selectively reduce p53-mediated cell death in normal tissue and suppress the side-effects of these therapies. In support of this, chk2$^{-/-}$ transgenic mice show resistance to apoptosis after exposure to ionising radiation.[18,19] Importantly, no increased tumourigenesis is seen in these CHK2-deficient animals, in contrast to p53-deficient mice. A radioprotective effect on isolated mouse thymocytes and human T-cells has been observed with selective small molecule inhibitors of CHK2.[20,21]

It is also plausible that inhibition of CHK2 alone could exert an antitumour effect. In some cancer cell lines, CHK2 is highly activated.[3,22] There is the possibility that inhibition of CHK2 activity would lead to cell death through inactivation of DNA repair pathways that are critical for the survival of the aberrant cells. Related to the inhibition of DNA repair pathways is the recent finding that replication of the human hepatitis C virus (HCV) is suppressed by

* Corresponding author. Tel.: +44 208 722 4317; fax: +44 208 722 4216.
E-mail address: ian.collins@icr.ac.uk (I. Collins).
† Present address: The School of Pharmacy, University of London, UK.

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doi:10.1016/j.bmc.2009.11.058

siRNA knockdown of CHK2 or ATM.[23] HCV infection is associated with increased genomic instability and double strand breaks in DNA.[24]

It has been suggested that selective inhibition of CHK2 in p53-deficient tumour cells may radio- or chemosensitize the cells through targeting of the G2 checkpoint.[2] CHK2 is seen to be activated in a wide range of tumour cells in response to ionizing radiation and DNA-damaging drugs. Experiments with one selective small molecule inhibitor of CHK2, VRX0466617 (**2**, Fig. 1), did not show an effect of CHK2 inhibition on the cell cycle distribution of irradiated cells, nor potentiation of the cytotoxicity of the DNA-damaging drugs cisplatin and doxorubicin.[21] However, more recently the selective CHK2 inhibitor PV1019, a cell-penetrant analogue of NSC 109555, was shown to potentiate the cytotoxicity of topotecan and camptothecin in ovarian cancer cell lines.[56] Moreover, increased cytotoxicity of the CHK2 inhibitor alone was observed in tumour cell lines with high endogenous levels of CHK2 compared to those expressing low levels of CHK2. The extent to which CHK2 inhibition can synergize with DNA-damaging drugs therefore remains unclear and is likely to depend on the interaction of cellular genomic background, DNA-damaging modality and inhibitor pharmacological profile.[1]

A limited number of small molecule inhibitors of CHK2 have been reported to date, and thus the discovery of further inhibitor chemotypes remains of interest. High-throughput screening has identified 2-arylbenzimidazole-5-carboxamides (**1**),[20,25] isothiazole carboxamidines (**2**)[21,26] and bis-guanylhydrazones (**3**)[27,56] as selective, ATP-competitive inhibitors of CHK2 (Fig. 1). Compounds that potently inhibit other kinases, particularly CHK1, in addition to CHK2 have been described, including the staurosporine analogue UCN-01,[28] an indoloazepine derivative of the natural product hymenialdisine[29] and the dual inhibitor of CHK1 and CHK2, AZD7762.[30] Few crystal structures of CHK2 complexed with small molecules are currently available in the public domain: CHK2-ADP[8] (PDB code: 2CN5), CHK2-debromohymenialdisine[8] (PDB code: 2CN8) and CHK2-NSC 109555[31] (PDB code: 2WOJ). Given the well established utility of multiple structures of protein–ligand complexes to aid in selective kinase drug design, additional characterisation of ligand binding modes to CHK2 would provide an opportunity to enhance structure-based design of new inhibitors.

We have identified 3,5-diaryl-2-aminopyridine inhibitors of CHK2 through high throughput screening of a kinase-focussed compound library. We describe efficient synthetic routes to this class of compounds and characterisation of their interaction with CHK2. We have determined the structures of representative compounds from the series bound to CHK2 to rationalise the observed structure–activity relationships.

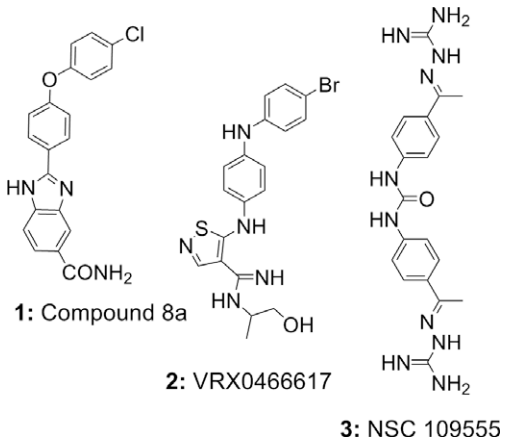


Figure 1. Structures of reported CHK2 inhibitors.

2. Kinase-focussed library screening

A commercial library of ca. 7000 small molecules designed as potential ATP-competitive kinase inhibitors[32] was screened against purified recombinant full-length CHK2 in a high throughput AlphaScreen format.[33] A total of 76 compounds were classified as hits (>60% inhibition of CHK2 activity at 30 μM test concentration) and assessed for potential non-specific inhibition by re-screening in triplicate in the presence of 0.01% Triton X-100 detergent.[34] From this, 36 compounds showed limited reduction of activity (>50% inhibition) when the detergent was added. These compounds were counterscreened for their ability to interfere with the AlphaScreen format by conducting the assay in the presence of the inhibitors and a control phosphopeptide.[35] A reduction in the AlphaScreen signal was seen with 25 compounds, indicative of interference in the assay readout. The activity of the remaining 11 compounds was confirmed in a DELFIA assay and IC_{50} values were determined (Fig. 2, Table 1). The identities and purities of the HTS samples were confirmed by mass spectrometry and HPLC.

Interestingly, although two weakly active and chemically unrelated singleton hits were identified (not shown), the majority of the compounds (**4**–**12**) were from one well-defined class of 3,5-diaryl-2-aminopyridines. Only 2-aminopyridines bearing a 3-(4-carboxamido)phenyl (**4**–**8**) or a 3-(4-hydroxyphenyl) (**9**–**12**) substituent gave substantial CHK2 inhibition. The specificity for these

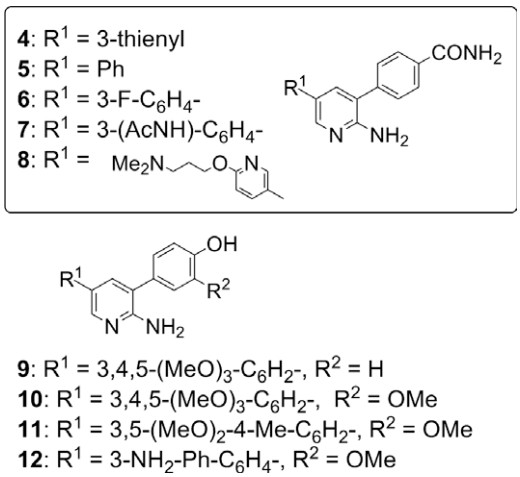
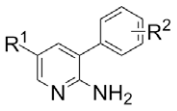


Figure 2. Structures of 2-aminopyridine hits from HTS against CHK2.

Table 1
CHK2 inhibition and calculated ligand efficiencies of 2-aminopyridine screening hits and selected analogues in the library

Compound	CHK2 IC_{50}[a] (μM)	L.E.[b]
4	0.61 (±0.12)[c]	0.40
5	2.7	0.34
6	2.1	0.34
7	2.4	0.31
8	1.8	0.27
9	2.3	0.29
10	3.0	0.27
11	4.4	0.28
12	6.3	0.31
13	>100	–
14	>100	–
15	>100	–

[a] Single determination in DELFIA assay format. Standard inhibitor staurosporine gave mean (±SEM) IC_{50} = 27 (±2.6) nM, n = 10 determinations.
[b] L.E. = ligand efficiency.[43]
[c] Mean (±SEM), n = 5 determinations.



13: R^1 = 3-thienyl, R^2 = 3-$CONH_2$
14: R^1 = Ph, R^2 = 4-$CONH(CH_2)_2OH$
15: R^1 = Ph, R^2 = 4-CH_2OH

Figure 3. Structures of selected inactive analogues in the screening set.

groups was confirmed by re-screening structurally related 2-aminopyridines in the focussed library that had not emerged as hits in the primary screen (Fig. 3, Table 1). For example, the 3-(3-carboxamidophenyl) analogue **13**, secondary amide **14**, and benzyl alcohol **15** were all inactive in the DELFIA assay. Remining of the HTS data identified a number of 2-aminopyridines that had not reached the cut-off for selection as primary hits but which possessed the 4-carboxamide and 4-phenol structural motifs and had activities weaker than the confirmed hits.

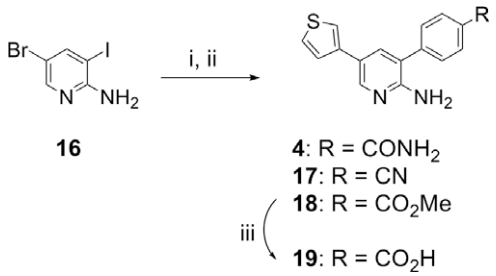
We chose to investigate the 3-(4-carboxamidophenyl)-2-aminopyridines in more detail. 3,5-Diaryl(or heteroaryl)-2-aminopyridines have been described as kinase inhibitor scaffolds in the recent patent literature,[36–42] although to our knowledge detailed descriptions of structure–activity relationships and crystallographic characterisation of the binding modes have not been reported.

The preliminary structure–activity from the HTS data indicated a specific and well-defined interaction with CHK2 for this scaffold, with a variety of substituents tolerated at C-5 of the pyridine. The calculated ligand efficiencies of these hits, ranging from 0.27 to 0.40 kcal mol^{-1} heavy $atom^{-1}$, were suitable for further progression.[43] In contrast, the activity of the 3-(4-hydroxyphenyl)-2-aminopyridines appeared reliant on the presence of the 3,4,5-trimethoxyphenyl group or similar substituents. The ligand efficiencies of this group of hits were generally lower than that of the benzamido-substituted analogues.

We used two approaches to explore the 3-(4-carboxamidophenyl)-2-aminopyridine series, initially exploiting straightforward synthetic routes to prepare a library of analogues designed to systematically define structure–activity relationships. In parallel we investigated co-crystallisation of the inhibitors with CHK2 to determine the bound conformation of the ligands.

3. Synthetic chemistry

The 2-aminopyridine **4** and close analogues were initially prepared by selective, sequential palladium-mediated cross coupling of aryl boronates to 3-iodo-5-bromo-2-pyridine **16** (Scheme 1).



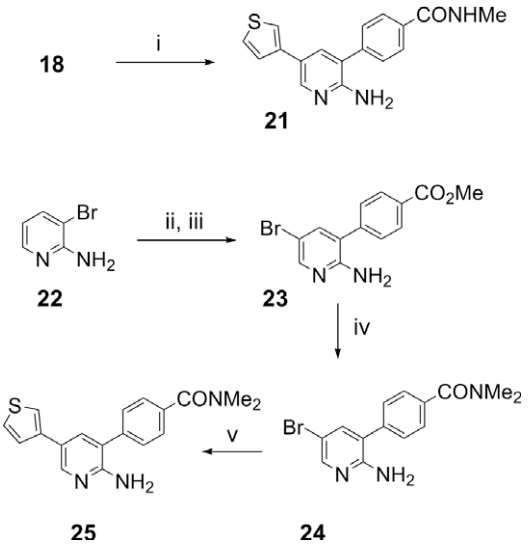
Scheme 1. Reagents and conditions: (i) $ArB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 36–68%; (ii) (3-thienyl)$B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 31–64%; (iii) NaOH aq, THF, rt 69%.

Simple nitrile (**17**) and ester (**18**) replacements for the amide functionality of **4** were introduced by incorporation of different 4-substituted arylboronates. The ester **18** was hydrolysed to yield the acid (**19**). Starting with 3,5-dibromopyridine, sequential coupling as described above gave the analogue lacking the 2-amino substituent (**20**, Table 2).

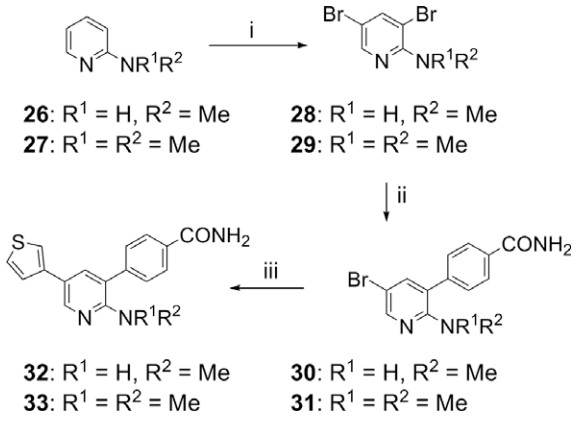
To introduce substituted amides, the benzoate **18** was directly aminated with methylamine in the presence of 1,5,7-triazabicyclo[4.4.0]dec-5-ene[44] to give **21** in excellent yield (Scheme 2). An alternative synthesis of the scaffold was developed beginning by coupling 4-(methoxycarbonyl)-benzeneboronic acid to 2-amino-3-bromopyridine **22**. Subsequent bromination of the pyridine at C-5 was achieved in high yield using 1,3-dibromo-5,5-dimethylhydantoin.[45] Reaction of the intermediate ester **23** with dimethylamine yielded **24**, which was coupled with thiophene-3-boronic acid to give **25**.

To introduce alkyl substitution on the 2-amino group of **4**, the 2-alkylaminopyridines **26** and **27** were subject to bis-bromination with 1,3-dibromo-5,5-dimethylhydantoin[45] at low temperature (Scheme 3). In the case of **26**, the C-5 monobrominated product was also isolated (23%). Suzuki coupling of 4-carbamoylphenylboronic acid to the dibromopyridine **28** occurred preferentially at the C-3 halogen, analogous to the reaction of **16**, leading to the 3-bromo derivative **30** as the major product isolated after chromatography. Introduction of the 3-thienyl substituent was realised by a final Suzuki coupling to yield the 2-(methylamino)pyridine **32**. Applying the same sequence to **29** gave the 2-(dimethylamino)-pyridine **33**.

The regiochemistries of these sequential couplings were confirmed by NOE determinations for the products **32** and **33**. In **32**, NOEs were observed between the pyridyl H-6 proton (δ 8.35 ppm) and protons on the thiophene (H-2 δ 7.53 ppm; H-4 δ 7.42 ppm). No NOEs were seen between the pyridyl H-6 and protons in the 4-carbamoylphenyl group. An analogous pattern of NOEs was observed in **33**. Furthermore, reduction of the intermediate bromides **30** and **31** (H_2, Pd–C) generated the expected 2-alkylamino-3-(4-carbamoylphenyl)pyridines. Although the first Suzuki coupling is selective for the apparently more hindered 3-



Scheme 2. Reagents and conditions: (i) $MeNH_2$, 1,5,7-triazabicyclo[4.4.0]dec-5-ene, THF, 100 °C, microwave, 89%; (ii) 4-(methoxycarbonyl)-phenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 68%; (iii) 1,3-dibromo-5,5-dimethylhydantoin, DMF, rt, 75%; (iv) Me_2NH, 1,5,7-triazabicyclo[4.4.0]dec-5-ene, THF, 100 °C, microwave, 79%; (v) (3-thienyl)$B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 47%.

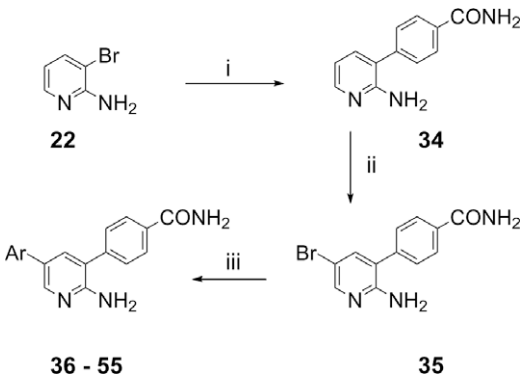


Scheme 3. Reagents and conditions: (i) 1,3-dibromo-5,5-dimethylhydantoin, CH_2Cl_2, −78 °C, 51–54%; (ii) (4-carbamoyl-phenyl)B(OH)$_2$, Pd(PPh$_3$)$_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 33–39%; (iii) (3-thienyl)B(OH)$_2$, Pd(PPh$_3$)$_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 37–38%.

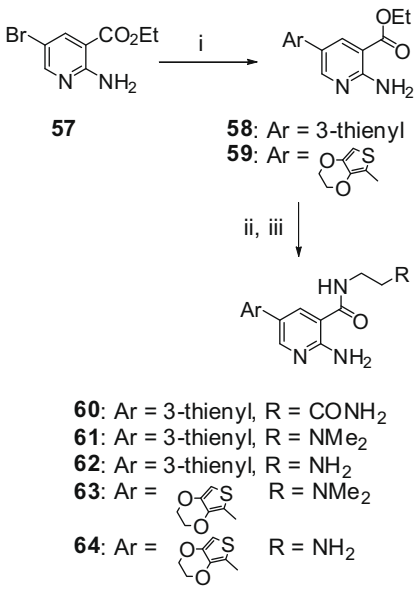
bromo substituent in **28** and **29**, this position is presumably rendered more reactive to palladium (0) insertion by steric repulsion from the adjacent substituent weakening the C3–Br bond.

A wide range of 3-(4-benzamido)-2-aminopyridines (**36–55**) were prepared by Suzuki cross coupling of 4-carboxamidophenylboronic acid to 3-bromo-2-aminopyridine **22** (Scheme 4). Bromination at C-5 of the pyridine **34** using 1,3-dibromo-5,5-dimethylhydantoin at room temperature was followed by the introduction of the second aryl substituent through palladium-catalysed cross couplings. These reactions were conveniently carried out in parallel using a microwave reactor and automated MPLC purification. In general, higher yields were obtained for the coupling of boronic acids compared to arylstannanes. The analogue bearing the amine and carboxamidophenyl groups in a 1,4 relationship (**56**, Table 2) could be prepared by applying the same reaction sequence starting from 5-bromo-2-aminopyridine, or by the route shown in Scheme 1 for compound **4** but reversing the order of coupling of the boronic acid reagents to **16**.

Ester and amide replacements for the 3-(4-carboxamidophenyl) substituent were prepared by Suzuki couplings to ethyl 5-bromo-2-aminopyridine-3-carboxylate **57** (Scheme 5). Conversion of the ethyl esters **58** and **62** to the amides was again achieved using direct amination in the presence of 1,5,7-triazabicyclo[4.4.0]dec-5-ene. The primary amines **62** and **64** were introduced using Boc-protected ethylenediamine, and the protecting group was removed by treatment with HCl in methanol.



Scheme 4. Reagents and conditions: (i) (4-carbamoylphenyl)B(OH)$_2$, Pd(PPh$_3$)$_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 31%; (ii) 1,3-dibromo-5,5-dimethylhydantoin, DMF, rt, 61%; (iii) ArB(OH)$_2$, Pd(PPh$_3$)$_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 4–62% or ArSnBu$_3$, Pd(PPh$_3$)$_4$, DMF, 130 °C, microwave, 4–16%.



Scheme 5. Reagents and conditions: (i) ArB(OH)$_2$, Pd(PPh$_3$)$_4$, Na_2CO_3 aq, DME, 135 °C, microwave, 40–59%; (ii) $RCH_2CH_2NH_2$, 1,5,7-triazabicyclo[4.4.0]dec-5-ene, THF, 100 °C, microwave, 20–71%; (iii) **62** and **64** only, 4 M HCl, MeOH, rt, 52%.

4. Results and discussion

Attempted co-crystallisation of the initial HTS hits with CHK2 to elucidate the interactions with the protein was not successful. This was attributed to the poor aqueous solubility of the compounds relative to their weak affinity for the enzyme. However based on literature precedent,[20,25,46–49] two plausible binding modes for the 3,5-diaryl-2-aminopyridines were considered (Fig. 4).

The role of the 2-aminopyridine group as a bidentate hinge-binding substituent has been shown by crystallography for several kinase inhibitors, although the orientation of the bifunctional 2-aminopyridine relative to the hinge region of the kinase varies, particularly when the 2-aminopyridine bears only one other substituent.[46–49] A general preference for placing unsubstituted amino groups facing into the interior of the ATP-binding site (towards the gatekeeper) has been noted across a wide selection of hinge-binding scaffolds.[50] Thus a likely binding mode for the 3,5-diaryl-2-aminopyridines was envisaged in

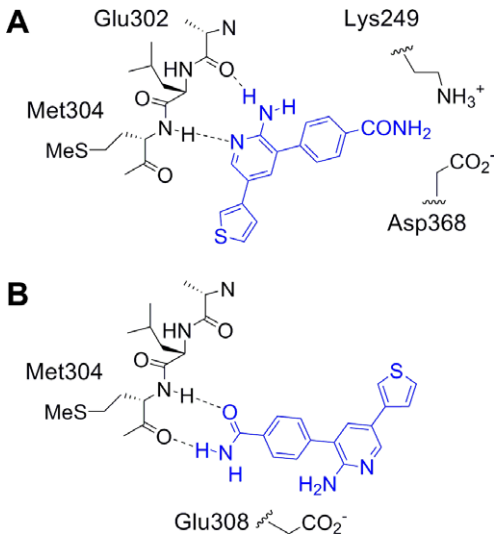


Figure 4. Possible binding modes considered for the 2-aminopyridines.

which the 2-aminopyridine bound to the hinge with the amino group oriented towards the gatekeeper residue. As a result, the 4-benzamido substituent would be directed towards the conserved lysine and DFG aspartate in CHK2 (Fig. 4A). However, an alternative binding mode has been proposed for the potent 2-arylbenzimidazole-5-carboxamides **1**, in which the carboxamide could bind to the hinge region of CHK2.[20,25] It was considered that if the 3,5-diaryl-2-aminopyridines **4–8** adopted an equivalent orientation, the 4-carboxamidophenyl functionality could serve as a hinge-binding motif while the 2-aminopyridine group would be directed into the ribose pocket (Fig. 4B). Simple overlays of the 2-aminopyridines with the structure of ADP bound to CHK2[8] indicated that the ligands might be accommodated in either of these orientations.

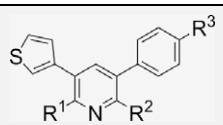
To investigate the possible roles of the carboxamide and 2-aminopyridine groups, analogues of the potent hit **6** were prepared which varied the hydrogen bonding potential of these functionalities (Table 2). Mono- or dimethylation of the 2-amino group to give **32** and **33**, respectively, led to only weakly active compounds, while removal of the amine (**20**) gave an eightfold reduction in potency. Translocation of the amine to the 6-position of the pyridine also led to a significant reduction in activity (**56**). In contrast, variation of the 4-carboxamide was better tolerated. Although translocation of the primary amide to the 3-position of the aryl ring (**13**, Fig. 3) abolished activity, the secondary and tertiary amides **21** and **25** displayed some CHK2 inhibition. Replacement of the amides by the weaker hydrogen bond accepting ester **18** or the geometrically distinct nitrile **17** was not possible, although the acid **19** retained some activity. Taken together, these results suggested a specific requirement for bidentate hydrogen bonding compatible with the 2-aminopyridine interacting with the hinge region of the kinase, with an additional requirement for an appropriate hydrogen bond acceptor group at the 4-position of the aryl ring (Fig. 4A).

With apparently limited scope for variation of the 2-amino and 3-(4-carboxamidophenyl) substituents of the pyridine, we prepared a larger number of analogues which explored changes to the 5-substituent (Table 3). Based on the model in Figure 4A, we expected that more polar heteroaromatic rings or substituents might be tolerated at this position, since this would be anticipated to be directed towards the solvent exposed surface at the opening to the ATP-binding pocket. Replacement of the thiophen-3-yl group by thiophen-2-yl (**36**) and various thiazolyl (**37–39**) or pyridyl (**40–42**) groups was possible, with the five-membered heteroaromatics generally favoured. Substitution of the prototype phenyl analogue **5** (Table 1) identified the 3-methoxyphenyl derivative **43** as more active, and a longer methoxyethylether solubilising chain (**45**) was also tolerated at this position. The 3,4-dimethoxyphenyl analogue **47** and the related 3-methoxy-4-phenol **48** gave more active compounds.

Elaboration of the dialkoxy substituent patterns to fused dioxacycles (**49–52**) identified the 1,3-benzodioxole **51** as a potent inhibitor, while a 10-fold drop in activity was observed for the ring-expanded 1,2-dihydro-1,4-benzodioxin analogue **52**. However, when the phenyl ring of **52** was replaced by a 2-thiophene, the most potent compound in this compound set was identified (**53**, CHK2 IC_{50} = 92 nM, L.E. = 0.38 kcal mol^{-1} heavy $atom^{-1}$), although with only marginally improved activity to the hit **4**. Fusion of an aromatic ring to the active thiophenes **4** or **36** to give the benzothiophenes **54** and **55** led to a complete loss of CHK2 inhibitory activity.

The addition of the potential solubilising groups led to the successful co-crystallisation of a number of the inhibitors with CHK2 (Fig. 5). The structure of **48** (PDB code 2WCT) bound to CHK2

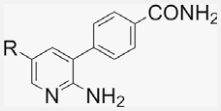
Table 2
Effect of variation of the hydrogen-bonding capacity of **6** on CHK2 inhibition

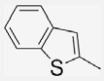


No.	R^1	R^2	R^3	CHK2 IC_{50}[a] (μM)
17	H	NH_2	CN	30%@100[b]
18	H	NH_2	CO_2Me	27%@100
19	H	NH_2	CO_2H	9.8
20	H	H	$CONH_2$	5.2
21	H	NH_2	CONHMe	6.7
25	H	NH_2	$CONMe_2$	6.1
32	H	NHMe	$CONH_2$	85
33	H	NMe_2	$CONH_2$	16%@100
56	NH_2	H	$CONH_2$	22

[a] See footnote to Table 1.
[b] % Inhibition at maximum concentration tested.

Table 3
In vitro activity of 5-aryl and 5-heteroaryl substituted 2-amino-3-(4-carboxamidophenyl)pyridines



No.	CHK2 IC_{50}[a] (μM)	CHK1 IC_{50}[b] (μM)
4 3-Thienyl	0.61 (±0.27)[c]	3.0
36 2-Thienyl	0.35	4.0
37 2-Thiazolyl	0.99	—[d]
38 4-Thiazolyl	1.1	—
39 5-Thiazolyl	0.68	2.0
40 2-Pyridyl	2.2	—
41 3-Pyridyl	2.2	—
42 4-Pyridyl	1.6	—
43 3-MeO-Ph	0.60	—
44 4-MeO-Ph	1.4	—
45 3-($MeOCH_2CH_2O$)-Ph	0.42	3.5
46 4-($MeOCH_2CH_2O$)-Ph	1.1	
47 3,4-$(MeO)_2$-Ph	0.27	2.3
48 3-(MeO)-4-(HO)-Ph	0.31	1.1
49 	1.35	—
50 	0.83	8.9
51 	0.21 (0.20, 0.22)[e]	8.5 (5.9, 11.0)[e]
52 	2.2	—
53 	0.092 (±0.018)[f]	5.2 (4.5, 5.9)[e]
54 	>100	—
55 	>10	—

[a] See footnote to Table 1.
[b] Single determination in DELFIA assay format. Standard inhibitor staurosporine gave mean (±SEM) IC_{50} = 2.1 (±0.41) nM, n = 26 determinations.
[c] Mean (±SEM), n = 5 determinations.
[d] Not determined.
[e] Mean of n = 2, individual determinations in parentheses.
[f] Mean (±SEM), n = 3 determinations.

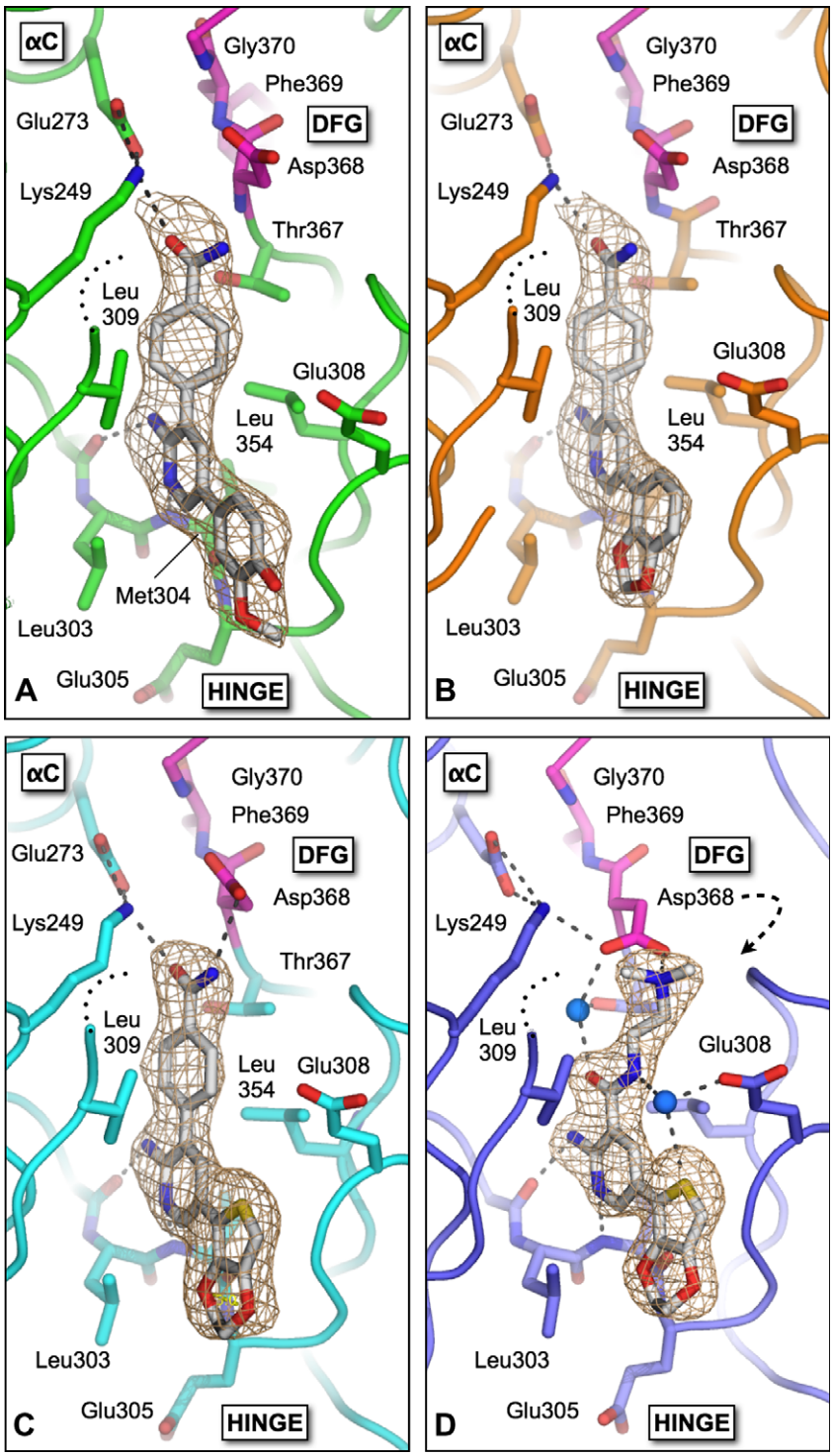
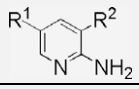
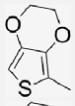
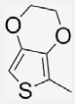


Figure 5. Crystal structures of **48** (A; PDB code 2WCT), **51** (B; PDB code 2WTD), **53** (C; PDB code 2WTI) and **63** (D; PDB code 2WTJ) bound to CHK2. Key interacting residues are shown for each structure, with CHK2 carbon atoms coloured green (**48**), orange (**51**), cyan (**53**) or blue (**63**), nitrogen in blue, oxygen in red, and sulphur in yellow. Water molecules involved in hydrogen-bonding networks with the compound are shown as dark blue spheres. Residues comprising the DFG motif are highlighted in magenta. Omit map (brown mesh) contoured at 1.5 σ (**48**), 2.8 σ (**51**), 4 σ (**53**), 2.5 σ (**63**).

was solved at 3.0 Å resolution and clearly showed a binding mode equivalent to that proposed in Figure 4A and consistent with the structure–activity relationships discussed above. The inhibitor occupied the ATP-binding site of CHK2 and was sandwiched between the hydrophobic side chains of Leu309 and Leu354 (Fig. 5A). The 2-aminopyridine was hydrogen bonded to the backbone carbonyl of Leu303 and the backbone amide NH of Met304. The ligand carboxamide substituent accepted a hydrogen bond from Lys249, which was also involved in hydrogen bonding to Glu273 in the αC-helix, indicative of an 'active' conformation for the kinase domain. The crystal structures of **51** (2.75 Å resolution; PDB code 2WTD) and **53** (2.5 Å resolution; PDB code 2WTI) showed

Table 4
Replacement of the 3-(4-carboxamidophenyl) group by 3-carboxy and 3-amido substituents



No.	R^1	R^2	CHK2 IC_{50}[a] (μM)	CHK1 IC_{50}[a] (μM)
58	3-Thienyl	CO_2Et	17	—
60	3-Thienyl	$CONH(CH_2)_2CONH_2$	0.41	3.4
61	3-Thienyl	$CONH(CH_2)_2NMe_2$	3.6	—
62	3-Thienyl	$CONH(CH_2)_2NH_2$	0.039[b] (0.051, 0.027)	0.78
63		$CONH(CH_2)_2NMe_2$	0.7	2.5
64		$CONH(CH_2)_2NH_2$	0.028[b] (0.028, 0.0284)	2.5

[a] See footnote to Table 1.
[b] Mean of $n = 2$, individual determinations in parentheses.

identical binding modes and key interactions to the protein (Fig. 5B and C). An additional hydrogen bond was observed between the carboxamide NH_2 group and the side chain of Asp368 in the DFG motif for the most potent compound **53**.

The dioxole and dioxin rings of **51** and **53** approached the surface defined by Leu303 and Met304 and, although partially exposed to solvent, provide a better fit to this region of the protein than an unsubstituted five- or six-membered ring. The better inhibition by the 1,3-benzodioxole **51** and the 2,3-dihydrothieno[3,4-*b*]-1,4-dioxin **53** compared to the 1,2-dihydro-1,4-benzodioxin analogue **52** can be rationalised by a preference for a coplanar orientation of the 5-substituent and the central pyridine ring. This is possibly energetically less favourable for the [6,6] bicyclic group of **52** due to a closer approach of atoms in the dioxacylic ring to the H-6 proton of the pyridine. The failure of the benzothiophenes **54** and **55** to show significant inhibition is perhaps surprising, but may reflect a mismatch between these large hydrophobic groups and their solvent exposed position.

With the exception of the C-5 substituent, the 2-aminopyridines are close mimics of ADP bound to CHK2. The compounds can also be compared to the recently determined structure of the symmetrical bis-guanylhydrazone **3** (NSC 109555; PDB code 2WOJ) bound to CHK2.[31] Here, interaction to the hinge region of the kinase was through contacts from the urea carbonyl via a single water molecule to the carbonyl of Glu302 and amide of Met304. In the present compounds this network of hydrogen bonds is replaced by direct interactions of the bifunctional 2-aminopyridines. Interestingly, the solvent exposed methyl substituent of **3** occupies a similar space to the carbon atoms of the dioxole or dioxin rings of **51** and **53**, suggesting a productive hydrophobic association with the protein in this region. The backbone of CHK2 around the ATP-binding site is similar when the bis-guanylhydrazone or 2-aminopyridines are bound, and the greatest differences in protein conformation are seen in the area around the carboxamide group of the 2-aminopyridines. For example, the carboxamide **53** interacts with both Lys249 and Asp368, whereas the larger guanylhydrazone functionality of **3** displaces and fills the space occupied by Lys249 in CHK2-ADP and interacts with Glu273 from the αC-helix. The glycine-rich loop, which coordinates the phosphate groups of ATP, is disordered in each of our structures, as in those reported for **3** and debromohymenialdisine. The conformational flexibility of this region, and the lack of direct interaction by each of the bound inhibitor molecules, is the most likely explanation for this phenomenon.

A small number of 5-(3-thienyl)- and 5-(2,3-dihydrothieno[3,4-*b*]-1,4-dioxin-5-yl)-3-carboxamido-2-aminopyridines were prepared bearing terminal hydrogen bonding functionality in an acyclic 3-substituent to replace the 3-(4-carboxamidophenyl) group (Table 4). Intramolecular hydrogen bonding between the 2-amino and 3-amido substituents was anticipated to hold the 3-substituent in a favourable position to interact with Lys249 or Asp368. Some 3-carboxamido-2-aminopyridines have been described in patents as kinase inhibitor scaffolds.[51,52]

While the intermediate ester **58** had only weak activity, the addition of a terminal amide **60** gave CHK2 inhibition comparable to the original 4-carboxamidophenyl HTS hit **4**. Substitution of the amide by the dimethylamine **61** reduced activity, but replacement of the amide by a primary amine gave a potent CHK2 inhibitor (**62**; L.E. = 0.55 kcal mol^{-1} heavy $atom^{-1}$). When the 3-thienyl group was replaced by the 2,3-dihydrothieno[3,4-*b*]-1,4-dioxin-5-yl substituent the dimethylamine **63** showed better CHK2 inhibition in this case. Affinity for the kinase was improved further for the primary amine **64** to give the most potent inhibitor identified in this study (**64**; L.E. = 0.46 kcal mol^{-1} heavy $atom^{-1}$).

The crystal structure of **63** (PDB code 2WTJ) confirmed that the 2-aminopyridine remained anchored to the hinge region (Fig. 5D). The orientation of the 3-amido group was consistent with an intramolecular hydrogen bond from the 2-amino NH to the amide C=O. In addition, the carbonyl of the amide participated in a water mediated hydrogen bond to Asp368, which was rotated from its position in the previously determined structures. The Asp368 carboxylate functionality also interacted with the terminal dimethylamine of the ligand, expected to be protonated at physiological pH (calcd $pK_a = 8.96$). The methyl groups did not appear to interact productively with the protein, providing a rationale for the improved affinity of the unsubstituted amines **62** and **64**. A water molecule on the opposite side of the 3-amide group mediated an interaction to Glu308 not possible with the original 3-(4-carboxamidophenyl) substituted scaffold. This network of new interactions within the ATP pocket appeared to compensate for the reduction in rigidity of the 3-substituent, such that good affinity was achieved with a more polar and more flexible scaffold (**63**, L.E. = 0.34 kcal mol^{-1} heavy $atom^{-1}$).

Inhibition of the checkpoint kinase inhibitor CHK1 was investigated for selected 3,5-diaryl-2-aminopyridines with submicromolar potency for CHK2 inhibition (Tables 3 and 4). Where dual inhibitors of CHK1 and CHK2 have been examined in cellular assays, the effects of CHK1 inhibition have often been observed to dominate.[1] Selectivity for CHK2 was generally between 3 and 11-fold for the hit **4** and direct analogues, with the 3-carboxamido-2-aminopyridines **60**, **62** and **63** showing a similar pattern of inhi-

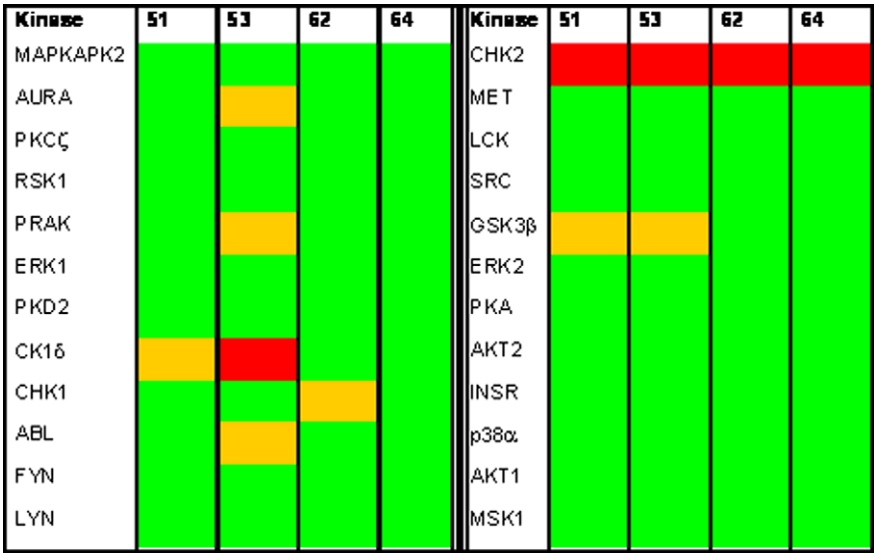


Figure 6. Selectivity of **51, 53, 62** and **64** for inhibition of a panel of 24 kinases at 1 μM concentration of test compound. Red = >70% inhibition, Orange = 70–40% inhibition, Green = <40% inhibition.

bition. More robust selectivity was seen for the benzodioxole **51** (42-fold) and 2,3-dihydrothieno[3,4-*b*]-1,4-dioxin **53** (56-fold). The combination of the 2,3-dihydrothieno[3,4-*b*]-1,4-dioxin 5-substituent with the (2-aminoethyl)amide 3-substituent in **64** gave the highest selectivity (ca. 89-fold).

The inhibition of a panel of 24 kinases was determined for compounds **51**, **53**, **62** and **64** using a microfluidic substrate phosphorylation assay[53] (Fig. 6). All compounds showed generally good selectivity for CHK2 compared to other kinases in the set, however the 3-carboxamido-2-aminopyridines **62** and **64** were notably more selective than the 3-(4-carboxamidophenyl) analogues **51** and **53**. This was reflected in the higher Gini coefficients for **62** and **64** (0.75 and 0.77, respectively) compared to **51** and **53** (0.42 and 0.46, respectively). The Gini coefficient assesses the cumulative inhibition measured for the compounds across all the kinases in the panel, with values close to 1 indicating specific inhibition of a single kinase.[54]

Compounds **51**, **53** and **64** were investigated in cellular assays. HT29 cells were treated with the topoisomerase II inhibitor etoposide to activate the DNA damage repair signalling pathway. Activation of CHK2 was shown by the appearance of Thr68 phosphorylation and a bandshift of the CHK2 protein on the electrophoresis gel. Inhibition of CHK2 was measured by assessing the ability of the compounds to inhibit the DNA-damage-induced bandshift and the specific CHK2 autophosphorylation on S516 (Fig. 7). Both **51** and **53** inhibited CHK2 activity in cells, with half maximal effects between 10–20 μM and 5–10 μM, respectively. The difference between the potency of the compounds as measured in the in vitro biochemical assay and the potency for inhibition of CHK2-specific biomarkers in cells is consistent with the increase in [ATP] between the biochemical assay and cells, taking into account the $K_{m,ATP}$ of the CHK2 enzyme.[20,55] Compound **64** also showed good cellular inhibition of CHK2 activity despite the increased polarity and hydrogen bond donor functionality in the scaffold, with half maximal effects at approximately 5 μM. Compounds **51**, **53** and **64** also showed weak antiproliferative activity in HT29 cells (GI_{50} = 27 μM, 26 μM and 28 μM, respectively), however the single agent cytotoxicity of the compounds may include non-CHK2 related effects. Notwithstanding this caveat, these compounds may prove useful for further investigating the potential for CHK2 inhibition to synergize with the antiproliferative effects of DNA-damaging agents in cancer cells.

5. Conclusion

5-(Hetero)aryl-3-(4-carboxamidophenyl)-2-aminopyridine inhibitors of CHK2 were identified from high throughput screening of a kinase-focussed compound library. Structure–activity relationships were established by rapid exploration of analogues of the hits through straightforward chemistry. X-ray crystallography of several analogues bound to CHK2 confirmed a predicted ATP-competitive binding mode for the compounds, consistent with the observed SAR. Variation of the 3-(hetero)aryl substituent identified

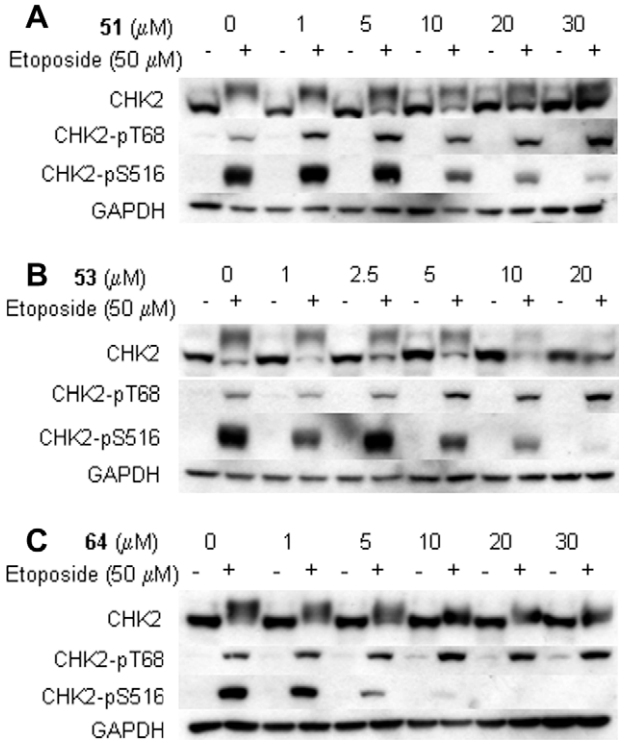


Figure 7. Western blot showing inhibition of CHK2 in HT29 cells by compounds **51** (Panel A), **53** (Panel B) and **64** (Panel C) following activation of the DNA damage response pathway by treatment with etoposide (50 μM).

bicyclic dioxins and dioxanes which gave gains in affinity for CHK2, and improved the selectivity for CHK2 versus CHK1. The 3-(4-carboxamidophenyl) substituent could be successfully replaced by an acyclic ω-aminoalkylamide which made additional polar interactions within the binding site. The 3-(carboxamido) derivatives were selective inhibitors of CHK2 over other kinases. Compounds from this series showed activity in cell-based mechanistic assays for inhibition of CHK2.

6. General experimental

Starting materials and solvents were purchased from commercial suppliers and were used without further purification. Microwave reactions were carried out in a Biotage Initiator 60 microwave reactor. Organic solutions were dried over $MgSO_4$ or Na_2SO_4. Flash silica chromatography was performed using Merck Silica Gel 60 (0.025–0.04 mm). Ion exchange chromatography was performed using Isolute Flash SCX-II (acidic) or Flash NH_2 (basic) resin cartridges. Automated MPLC was performed on a Biotage SP1 Instrument using prepacked silica cartridges and UV-triggered fraction collection (254 nm). ^{1}H NMR spectra were recorded on a Bruker AMX500 instrument using an internal deuterium lock. ^{13}C NMR spectra were recorded on a Bruker AMX500 instrument at 126 MHz using an internal deuterium lock. Chemical shifts (δ) are reported relative to TMS ($\delta = 0$) and/or referenced to the solvent in which they were measured. Combined HPLC-MS analyses were recorded using a Waters Alliance 2795 separations module and Waters/Micromass LCT mass detector with electrospray ionization (+ve or −ve ion mode as indicated), and with HPLC performed using Supelco DISCOVERY C18, 50 mm × 4.6 mm or 30 mm × 4.6 mm i.d. columns, at a temperature of 22 °C with gradient elution of 10–90% MeOH/0.1% aqueous formic acid at a flow rate of 1 mL/min (run time of 4 min or 6 min as indicated). Compounds were detected at 254 nm using a Waters 2487 dual λ absorbance detector. High-resolution mass spectra were measured on an Agilent 6210 ToF HPLC-MS with a Phenomenex Gemini 3 μm C18 (3 cm × 4.6 mm i.d) column. Melting points were measured on a Leica Gallen III apparatus and are uncorrected.

7. Representative methods for the preparation of 2-amino-3,5-disubstituted-pyridines

7.1. 4-(2-Amino-5-(thiophen-3-yl)pyridin-3-yl)benzamide (4)

A mixture of **35** (0.042 g, 0.144 mmol), thiophen-3-ylboronic acid (0.020 g, 0.156 mmol), Na_2CO_3 (0.032 g, 0.30 mmol) and $Pd(PPh_3)_4$ (0.017 g, 0.015 mmol) in 1,4-dioxane (1.5 mL) and water (0.4 mL) was heated at 120 °C in a microwave reactor for 1 h. The reaction mixture was partitioned between water (20 mL) and EtOAc (20 mL). The organic layer was dried, filtered and concentrated. Preparative TLC, eluting with CH_2Cl_2/MeOH 9:1, gave **4** (0.013 g, 0.044 mmol, 31%) as a beige solid. mp 112–115 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.38 (s, 1H), 8.00–7.98 (m, 3H), 7.76 (d, J = 2.0, 1H), 7.72 (d, J = 2.0, 1H), 7.61–7.59 (m, 3H), 7.55 (d, J = 5.0, 1H), 7.36 (s, 1H), 5.81 (br s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 167.4, 155.2, 144.4, 140.7, 138.7, 135.6, 133.1, 128.5, 128.1, 126.8, 125.6, 121.2, 119.8, 118.7; LC–MS (6 min) m/z 296 $[M+H]^+$, t_R = 2.43 min, purity >95%; HRMS found $[M+H]^+$ 296.0860, $C_{16}H_{14}N_3OS$ requires 296.0852.

7.2. Methyl 4-(2-amino-5-(thiophen-3-yl)pyridin-3-yl)benzoate (18)

A mixture of **23** (0.037 g, 0.120 mmol), thiophen-3-ylboronic acid (0.017 g, 0.133 mmol), 2 M Na_2CO_3 aq (0.13 mL) and $Pd(PPh_3)_4$ (6.96 mg, 6.02 μmol) in DME (3 mL) was heated at 135 °C in a microwave reactor for 30 min. The reaction mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with hexane/EtOAc 3:1, gave **18** (0.022 g, 0.071 mmol, 59%) as a light yellow powder; mp 152–154 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.38 (d, J = 2.3, 1H), 8.17 (d, J = 8.5, 2H), 7.61–7.59 (m, 3H), 7.42 (dd, J = 3.0, 5.0, 1H), 7.38 (dd, J = 1.4, 3.0, 1H), 7.34 (dd, J = 1.4, 5.0, 1H), 4.65 (s, 2H), 3.97 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.6, 154.4, 144.8, 142.3, 138.7, 136.3, 130.5, 129.8, 128.8, 126.7, 125.7, 123.4, 120.9, 119.2, 52.3; LC–MS (6+ min) m/z 311 $[M+H]^+$, t_R = 3.38 min, purity >95%; HRMS found $[M+H]^+$ 311.0848, $C_{17}H_{15}N_2O_2S$ requires 311.0849.

7.3. 4-(2-Amino-5-(thiophen-3-yl)pyridin-3-yl)benzoic acid (19)

1 M NaOH aq (1.77 mL, 1.77 mmol) was added to a solution of **18** (0.055 g, 0.177 mmol) in THF (5 mL) at rt and the mixture was stirred for 24 h. THF was removed by evaporation and water (30 mL) was added. The solution was washed with ethyl acetate (2 × 30 mL). The aqueous phase was acidified with citric acid aq (10%) and extracted with EtOAc (3 × 20 mL). The organic extracts were dried, filtered and concentrated. Ion exchange chromatography on acidic resin gave **19** (0.036 g, 0.121 mmol, 69%) as a light brown solid; mp 286–288 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.37 (d, J = 2.3, 1H), 8.00 (d, J = 8.1, 2H), 7.76 (dd, J = 1.1, 2.9, 1H), 7.70 (d, J = 2.3, 1H), 7.59 (dd, J = 2.9, 5.0, 1H), 7.56–7.54 (m, 3H), 5.72 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 168.0, 155.4, 144.9, 140.5, 138.9, 135.2, 129.7, 128.2, 126.8, 125.6, 121.2, 119.9, 118.5 (1 × quaternary C not observed); LC–MS (4 min) m/z 297 $[M+H]^+$, t_R = 1.82 min, purity >95%; found $[M+H]^+$ 297.0688, $C_{16}H_{13}N_2O_2S$ requires 297.0692.

7.4. 4-(2-Amino-5-(thiophen-3-yl)pyridin-3-yl)-*N*-methylbenzamide (21)

A mixture of **18** (0.070 g, 0.226 mmol), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (0.010 g, 0.072 mmol), 2 M $MeNH_2$ in THF (2 mL, 4.00 mmol) and THF (1 mL) was heated in a microwave reactor at 100 °C for 60 min. Silica gel was added and solvent was evaporated. MPLC, eluting with CH_2Cl_2/EtOAc 1:1, gave **21** (0.062 g, 0.20 mmol, 89%) as a colourless solid; mp 142–144 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.38 (d, J = 2.3, 1H), 7.89 (d, J = 8.3, 2H), 7.60–7.59 (m, 3H), 7.41 (dd, J = 3.0, 5.0, 1H), 7.37 (dd, J = 1.4, 3.0, 1H), 7.34 (dd, J = 1.4, 5.0, 1H), 6.20 (br s, 1H), 4.61 (s, 2H), 3.07 (d, J = 4.8, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.6, 154.6, 145.5, 141.1, 139.0, 136.0, 134.2, 129.0, 127.7, 126.6, 125.7 123.4, 120.7, 119.0, 26.9; LC–MS (6 min) m/z 310 $[M+H]^+$, t_R = 2.63 min, purity >95%; HRMS found $[M+H]^+$ 310.1006, $C_{17}H_{16}N_3OS$ requires 310.1009.

7.5. Methyl 4-(2-amino-5-bromopyridin-3-yl)benzoate (23)

Method A: A mixture of 5-bromo-3-iodopyridin-2-amine **16** (0.113 g, 0.378 mmol), and 4-(methoxycarbonyl)phenylboronic acid (0.077 g, 0.428 mmol), 2 M Na_2CO_3 aq (0.68 mL, 1.36 mL) and $Pd(PPh_3)_4$ (0.022 g, 0.019 mmol) in EtOH (2 mL) and toluene (4 mL) was heated at 135 °C in a microwave reactor for 25 min. The reaction mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with hexane/EtOAc 3:1, gave **23** (0.058 g, 0.189 mmol, 50%) as a colourless powder. mp 157–159 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.19–8.13 (m, 3H), 7.54 (d, J = 8.3, 2H), 7.51 (d, J = 2.3, 1H), 4.63 (s, 2H), 3.97 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.5, 154.3, 148.4, 141.4, 139.8, 130.5,

130.1, 128.6, 122.3, 108.6, 52.3; LC–MS (6 min) *m/z* 308, 309 $[M+H]^+$, t_R = 4.62 min, purity = 94%; HRMS found $[M+H]^+$ 307.0078, $C_{13}H_{12}BrN_2O_2$ requires 307.0077.

Method B: A mixture of 3-bromopyridin-2-amine **22** (1.61 g, 9.31 mmol), 4-(methoxycarbonyl)phenylboronic acid (1.84 g, 10.2 mmol), 2 M Na_2CO_3 aq (7.0 mL, 14 mmol) and $Pd(PPh_3)_4$ (0.538 g, 0.465 mmol) in DME (8 mL) was heated at 135 °C in a microwave reactor for 40 min. The reaction mixture was partitioned between water (50 mL) and EtOAc (3 × 50 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with an EtOAc/hexane gradient, gave methyl 4-(2-aminopyridin-3-yl)benzoate (1.45 g, 6.35 mmol, 68%) as a colourless solid; mp 127–129 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 (d, *J* = 8.3, 2H), 8.08 (dd, *J* = 1.7, 5.0, 1H), 7.54 (d, *J* = 8.3, 2H), 7.37 (dd, *J* = 1.7, 7.4, 1H), 6.75 (dd, *J* = 5.0, 7.4, 1H), 4.67 (s, 2H), 3.94 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.6, 155.6, 148.0, 142.9, 137.8, 130.3, 129.5, 128.7, 120.7, 114.5, 52.2; LC–MS (4 min) *m/z* 229 $[M+H]^+$, t_R = 1.34 min, purity >95%; HRMS found $[M+H]^+$ 229.0977, $C_{13}H_{13}N_2O_2$ requires 229.0972. 1,3-Dibromo-5,5-dimethylhydantoin (0.197 g, 0.689 mmol) was added to a solution of methyl 4-(2-aminopyridin-3-yl)benzoate (0.262 g, 1.15 mmol) in CH_2Cl_2 (10 mL) at −78 °C and the resulting mixture was stirred for 2 h. The mixture was concentrated and purified by MPLC, eluting with hexane/EtOAc 3:1, to give **23** (0.264 g, 0.860 mmol, 75%) as a pale yellow solid.

7.6. 4-(2-Amino-5-(thiophen-3-yl)pyridin-3-yl)-*N,N*-dimethylbenzamide (25)

A mixture of **23** (0.199 g, 0.648 mmol), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (0.042 g, 0.302 mmol), 2 M Me_2NH in THF (2 mL, 37.7 mmol) and THF (1 mL) was heated in a microwave reactor at 100 °C for 1 h. Silica was added and solvent was evaporated. MPLC, eluting with an EtOAc/hexane gradient, gave 4-(2-amino-5-bromopyridin-3-yl)-*N,N*-dimethylbenzamide **24** (0.163 g, 0.509 mmol, 79%) as a colourless solid; mp 219–221 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13 (d, *J* = 2.4, 1H), 7.55–7.48 (m, 5H), 4.62 (br s, 2H), 3.15 (br s, 3H), 3.04 (br s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.9, 154.5, 148.1, 139.8, 138.0, 136.4, 128.6, 128.0, 122.6, 108.5, 39.6, 35.4; LC–MS (6 min) *m/z* 319 $[M+H]^+$, t_R = 3.41 min, purity = 90%; HRMS found $[M+H]^+$ 320.0397, $C_{14}H_{15}BrN_3O$ requires 320.0393. A mixture of **24** (0.093 g, 0.290 mmol), thiophen-3-ylboronic acid (0.041 g, 0.320 mmol), 2 M Na_2CO_3 aq (0.68 mL, 1.36 mmol) and $Pd(PPh_3)_4$ (0.017 g, 0.015 mmol) in DME (3.0 mL) was heated at 135 °C in a microwave reactor for 30 min. The reaction mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The combined organic extracts were dried, filtered and concentrated. MPLC, eluting with CH_2Cl_2/EtOAc 1:1, gave **25** (0.044 g, 0.136 mmol, 47%) as a colourless solid. mp 126–128 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.34 (d, *J* = 2.0, 1H), 7.63–7.46 (m, 5H), 7.44–7.30 (m, 3H), 4.73 (s, 2H), 3.14 (s, 3H), 3.05 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.0, 154.8, 145.2, 139.2, 139.0, 136.0, 128.8, 128.0, 126.5, 125.7, 123.3, 120.9, 118.9, 39.6, 35.4 (1 × quaternary C not observed); LC–MS (6 min) *m/z* 324 $[M+H]^+$, t_R = 2.70 min, purity >95%; HRMS found $[M+H]^+$ 324.1165, $C_{18}H_{18}N_3OS$ requires 324.1165.

7.7. 3,5-Dibromo-*N*-methylpyridin-2-amine (28)

1,3-Dibromo-5,5-dimethylhydantoin (5.56 g, 19.5 mmol) was added to a solution of 2-(methylamino)pyridine **26** (2.00 mL, 19.5 mmol) in CH_2Cl_2 (10 mL) at −78 °C and the mixture was stirred for 2 h. The mixture was filtered and concentrated. The residue was mixed with CH_2Cl_2 (50 mL) and satd $NaHCO_3$ aq (50 mL). Solid sodium thiosulfate and water (50 mL) were added and the organic phase was separated. The organic extract was dried, filtered and concentrated. MPLC, eluting with an EtOAc/hexane gradient, gave **28** (2.63 g, 9.89 mmol, 51%) as a colourless solid; mp 47–49 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (d, *J* = 2.1, 1H), 7.70 (d, *J* = 2.1, 1H), 5.04 (br s, 1H), 3.01 (d, *J* = 4.9, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.0, 147.3, 140.9, 105.6, 105.9, 28.8; LC–MS (4 min) *m/z* 265, 267, 269 $[M+H]^+$, t_R = 2.54 min, purity >95%; HRMS found $[M+H]^+$ 264.8975, $C_6H_7Br_2N_2$ requires 264.8971.

Also isolated, 5-bromo-*N*-methylpyridin-2-amine: colourless solid (1.0 g, 23%), mp 72–74 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (d, *J* = 2.5, 1H), 7.49 (dd, *J* = 2.5, 8.8, 1H), 6.30 (d, *J* = 8.8, 1H), 4.66 (br s, 1H), 2.90 (d, *J* = 5.1, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.1, 148.7, 139.7, 107.6, 106.8, 29.1; LC–MS (4 min) *m/z* 187, 189 $[M+H]^+$, t_R = 0.78 min, purity >95%; HRMS found $[M+H]^+$ 186.9864, $C_6H_8BrN_2$ requires 186.9865.

7.8. 4-(5-Bromo-2-(methylamino)pyridin-3-yl)benzamide (30)

A mixture of **28** (0.309 g, 1.16 mmol), 4-carbamoylphenylboronic acid (0.211 g, 1.28 mmol), Na_2CO_3 aq (2 M, 1.16 mL) and $Pd(PPh_3)_4$ (0.067 g, 0.058 mmol) in DME (3 mL) was heated at 135 °C in a microwave reactor for 30 min. The reaction mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with hexane/EtOAc 5:1, gave 4-(5-bromo-2-(methylamino)pyridin-3-yl)benzamide (0.140 g, 0.457 mmol, 39%) as a colourless solid; mp 85–87 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20 (d, *J* = 2.4, 1H), 7.93 (d, *J* = 8.3, 2H), 7.47 (d, *J* = 8.3, 3H), 7.36 (d, *J* = 2.4, 1H), 6.15 (s, 2H), 4.55 (s, 1H), 2.92 (d, *J* = 4.9, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.7, 154.7, 148.1, 140.4, 138.8, 133.2, 129.1, 128.4, 122.8, 106.8, 28.7; LC–MS (6 min) *m/z* 306 $[M+H]^+$, t_R = 3.25 min, purity >95%; HRMS found $[M+H]^+$ 306.0240, $C_{13}H_{13}BrN_3O$ requires 306.0237.

7.9. 4-(2-(Methylamino)-5-(thiophen-3-yl)pyridin-3-yl)benzamide (32)

A mixture of **30** (0.172 g, 0.562 mmol), 3-thiopheneboronic acid (0.072 g, 0.562 mmol), Na_2CO_3 aq (2 M, (0.562) and $Pd(PPh_3)_4$) (0.032 g, 0.028 mmol) in DME (3 mL) was heated in a microwave reactor at 135 °C for 35 min. The mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with hexane/EtOAc 1:1, gave **32** (0.065 g, 0.210 mmol, 37%) as a colourless solid; mp 128–130 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 8.35 (d, *J* = 2.3, 1H), 8.01 (d, *J* = 8.2, 2H), 7.65 (d, *J* = 2.3, 1H), 7.60 (d, *J* = 8.2, 2H), 7.53 (dd, *J* = 2.9, 1.2, 1H), 7.49 (dd, *J* = 2.9, 5, 1H), 7.42 (dd, *J* = 1.2, 5, 1H), 2.92 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.0, 155.1, 145.3, 141.6, 139.3, 135.1, 133.0, 132.0, 129.2, 128.4, 126.4, 125.6, 121.2, 118.4, 28.8; LC–MS (6 min) *m/z* 310 $[M+H]^+$, t_R = 2.39 min, purity >95%; HRMS found $[M+H]^+$ 310.1003, $C_{17}H_{16}N_3OS$ requires 310.1009.

7.10. 4-(2-Amino-5-bromopyridin-3-yl)benzamide (35)

Method A: A mixture of 5-bromo-3-iodopyridin-2-amine **16** (0.16 g, 0.535 mmol), 4-carbamoylphenyl-boronic acid (0.097 g, 0.589 mmol), Na_2CO_3 aq (2 M, 0.68 mL) and $Pd(PPh_3)_4$ (0.031 g, 0.027 mmol) in DME (6.0 mL) was heated at 135 °C in a microwave reactor for 20 min. The reaction mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with CH_2Cl_2/EtOAc 1:1, gave **35** (0.107 g, 0.366 mmol, 68%) as a light yellow powder. mp 233–235 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.04 (d, *J* = 2.4, 1H), 8.02 (br s, 1H), 7.97 (d, *J* = 8.0, 2H), 7.5–7.54 (m, 3H), 7.37 (br s, 1H), 5.90 (s, 2H); ^{13}C (126 MHz, DMSO) δ 168.2, 156.4, 148.4, 140.4, 140.0, 134.3, 129.2, 129.0, 122.6, 106.9; LC–MS (6 min) *m/*

z 292 $[M+H]^+$, t_R = 3.00 min, purity >95%; HRMS found $[M+H]^+$ 292.0086, $C_{12}H_{11}BrN_3O$ requires 292.0080.

Method B: A mixture of 3-bromo-2-aminopyridine **22** (1.14 g, 6.59 mmol), 4-carbamoylphenyl-boronic acid (1.196 g, 7.25 mmol), Na_2CO_3 aq (7.25 mL, 14.5 mmol) and $Pd(PPh_3)_4$ (0.381 g, 0.329 mmol) in DME (8 mL) was heated at 135 °C in a microwave reactor for 40 min. The reaction mixture was partitioned between water (50 mL) and EtOAc (3 × 50 mL). The organic extracts were dried, filtered and concentrated. The residue was washed with hot EtOAc to give 4-(2-aminopyridin-3-yl)benzamide **34** (0.44 g, 2.06 mmol, 31%) as a pale yellow solid; mp 219–221 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.04–7.91 (m, 4H), 7.52 (d, *J* = 8.3, 2H), 7.35–7.37 (m, 2H), 6.67 (dd, *J* = 4.9, 7.3, 1H), 5.60 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 167.5, 156.3, 147.4, 141.1, 137.6, 133.0, 128.2, 128.1, 119.6, 113.1; LC–MS (4 min) *m/z* 214 $[M+H]^+$, t_R = 0.82 min, purity >95%;; HRMS found $[M+H]^+$ 214.0970, $C_{12}H_{12}N_3O$ requires 214.0975. 1,3-Dibromo-5,5-dimethylhydantoin (0.100 g, 0.349 mmol) was added to a solution of **34** (0.149 g, 0.699 mmol) in DMF (3 ml) at 20 °C and the resulting mixture was stirred for 24 h. The mixture was concentrated and purified by MPLC, eluting with CH_2Cl_2/EtOAc 1:1, to give **35** (0.125 g, 0.428 mmol, 61%).

7.11. 4-(2-Amino-5-(thiophen-2-yl)pyridin-3-yl)benzamide (36)

A mixture of **35** (0.081 g, 0.277 mmol), thiophen-2-ylboronic acid (0.039 g, 0.305 mmol), Na_2CO_3 aq (2 M, 0.31 mL) and $Pd(PPh_3)_4$ (0.016 g, 0.014 mmol) in DME (4 mL) was heated at 135 °C in a microwave reactor for 20 min. The reaction mixture was partitioned between water (30 mL) and EtOAc (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with hexane/EtOAc 4:1, gave **36** (0.040 g, 0.135 mmol, 49%) as a light yellow powder; mp 222–224 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.29 (d, *J* = 2.2, 1H), 8.02 (s, 1H), 7.99 (d, *J* = 8.3, 2H), 7.58–7.60 (m, 3H), 7.43 (d, *J* = 5.0, 1H), 7.39–7.37 (m, 2H), 7.09 (dd, *J* = 3.5, 5.0, 1H), 5.89 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 167.4, 155.9, 144.3, 140.8, 140.4, 134.7, 133.2, 128.4, 128.2, 128.1, 123.9, 122.2, 119.9, 119.7; LC–MS (4 min) *m/z* 296 $[M+H]^+$, t_R = 2.67 min, purity >95%; HRMS found $[M+H]^+$ 296.0857, $C_{16}H_{14}N_3OS$ requires 296.0852.

7.12. 4-(2-Amino-5-(thiazol-2-yl)pyridin-3-yl)benzamide (37)

$Pd(PPh_3)_4$ (7.91 mg, 0.0685 mmol) was added to a solution of 2-(tributylstannyl)thiazole (24 μl, 0.075 mmol) and **35** (20 mg, 0.068 mmol) in DMF (0.200 mL). The reaction mixture was heated at 130 °C in a microwave reactor for 45 min. The crude mixture was concentrated. MPLC, eluting with CH_2Cl_2/MeOH 95/5–90/10, followed by preparative HPLC gave **37** (3.0 mg, 0.010 mmol, 15%) as a white solid; mp 226–227 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 8.58 (d, *J* = 2.3, 1H), 8.04 (d, *J* = 8.4, 2H), 7.96 (d, *J* = 2.3, 1H), 7.83 (d, *J* = 3.3, 1H), 7.65 (d, *J* = 8.4, 2H), 7.54 (d, *J* = 3.3, 1H); ^{13}C NMR (126 MHz, CD_3OD) δ 171.8, 167.6, 159.0, 146.8, 144.1, 142.1, 137.1, 134.8, 130.0, 129.6, 122.4, 121.5, 119.5; LC–MS (4 min) *m/z* 297 $[M+H]^+$, t_R = 1.47 min, purity >95%; HRMS found $[M+H]^+$ 297.0806, $C_{15}H_{13}N_4OS$ requires 297.0805.

7.13. 4-(2-Amino-5-(2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)pyridin-3-yl)benzamide (53)

A mixture of 5-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-2,3-dihydrothieno-[3,4-*b*][1,4]dioxine (0.151 g, 0.565 mmol), **35** (0.11 g, 0.77 mmol), Na_2CO_3 aq (0.377 mL, 0.753 mmol) and $Pd(PPh_3)_4$ (0.022 g, 0.019 mmol) in DME (3 mL) was heated in a microwave reactor at 135 °C for 40 min. The residue was partitioned between water (30 mL) and ethyl acetate (3 × 30 mL). The organic extracts were dried, filtered and concentrated. MPLC, eluting with CH_2Cl_2/MeOH 9:1, gave **53** (0.077 g, 0.218 mmol, 58%) as a light yellow solid; mp 125–127 °C; ^{1}H NMR (500 MHz, DMSO) δ 8.24 (d, *J* = 2.3, 1H), 7.98 (d, *J* = 8.3, 2H), 7.53–7.55 (m, 3H), 6.51 (s, 1H), 4.27–4.20 (m, 4H); ^{13}C NMR (126 MHz, DMSO) δ 167.3, 155.1, 144.4, 142.1, 140.6, 137.2, 134.6, 133.1, 128.3, 128.1, 119.4, 118.6, 113.4, 96.2, 64.6, 64.1; LC–MS (4 min) *m/z* 354 $[M+H]^+$, t_R = 2.07 min, purity >95%; found $[M+H]^+$ 354.0900, $C_{18}H_{16}N_3O_3S$ requires 354.0907.

7.14. Ethyl 2-amino-5-(2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)nicotinate (59)

A mixture of ethyl 2-amino-5-bromonicotinate **57** (0.276 g, 1.13 mmol), 5-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)-2,3-dihydrothieno-[3,4-*b*][1,4]dioxine (0.362 g, 1.35 mmol), $Pd(PPh_3)_4$ (0.065 g, 0.056 mmol) and 2 M Na_2CO_3 aq (0.845 mL, 1.69 mmol) in DME (4 mL) was heated at 135 °C in a microwave reactor for 40 min. Solvent was removed by evaporation and the residue was purified by MPLC, eluting with hexane/EtOAc 3:1, to give **59** (0.202 g, 0.659 mmol, 59%) as a pale yellow solid; mp 165–167 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.59 (d, *J* = 2.4, 1H), 8.39 (d, *J* = 2.4, 1H), 6.57 (br s, 2H), 6.28 (s, 1H), 4.39 (q, *J* = 7.1, 2H), 4.33–4.29 (m, 2H), 4.28–4.23 (m, 2H), 1.42 (t, *J* = 7.1, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.9, 157.8, 151.2, 142.2, 137.6, 137.3, 119.1, 113.8, 106.2, 96.7, 64.8, 64.5, 61.0, 14.3; LC–MS (4 min) *m/z* 307 $[M+H]^+$, t_R = 2.59 min, purity >95%; HRMS found $[M+H]^+$ 307.0744, $C_{14}H_{15}N_2O_4S$ requires 307.0747.

7.15. 2-Amino-5-(2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)-*N*-(2-(dimethylamino)ethyl)nicotinamide (63)

A mixture of **59** (0.067 g, 0.22 mmol), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (0.012 g, 0.087 mmol) and *N,N*-dimethylethylenediamine (0.096 mL, 0.875 mmol) in THF (4 mL) was heated at 100 °C in a microwave reactor for 60 min. Solvent was evaporated and the residue was purified by MPLC, eluting with hexane/EtOAc 1:1, to give **63** (0.044 g, 0.126 mmol, 58% yield) as a colourless oil; ^{1}H NMR (500 MHz, CDCl3) δ 8.44 (d, *J* = 2.3, 1H), 7.94 (d, *J* = 2.3, 1H), 7.02 (br s, 1H), 6.44 (br s, 2H), 6.24 (s, 1H), 4.28–4.23 (m, 4H), 3.52–3.49 (m, 2H), 2.56 (t, *J* = 6.0, 2H), 2.30 (s, 6H); ^{13}C NMR (126 MHz, CDCl3) δ 167.8, 157.1, 149.1, 142.2, 137.5, 133.3, 118.8, 113.9, 110.5, 96.6, 64.8, 64.5, 57.7, 45.1, 37.0; LC–MS (4 min) *m/z* 349 $[M+H]^+$, t_R = 1.18 min, purity >95%; HRMS found $[M+H]^+$ 349.1321, $C_{16}H_{21}N_4O_3S$ requires 349.1329.

Acknowledgments

We thank Dr. A. Mirza and Mr. M. Richards for assistance in the characterisation of test compounds. This work was supported by Cancer Research UK [CUK] Grant Numbers C309/A2187, C309/A2874, C309/A8365 and C302/A8265 and Infrastructure Support Grant C302/A7803, and by the Institute of Cancer Research (studentship to CEA). We acknowledge NHS funding to the NIHR Biomedical Research Centre.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.058.

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