Structure-Based Design of Orally Bioavailable 1H-Pyrrolo[3,2-c]pyridine Inhibitors of Mitotic Kinase Monopolar Spindle 1 (MPS1)

Sébastien Naud,† Isaac M. Westwood,‡ Amir Faisal,† Peter Sheldrake,† Vassilios Bavetsias,† Butrus Atrash,† Kwai-Ming J. Cheung,† Manjuan Liu,† Angela Hayes,† Jessica Schmitt,† Amy Wood,† Vanessa Choi,† Kathy Boxall,† Grace Mak,† Mark Gurden,§ Melanie Valenti,† Alexis de Haven Brandon,† Alan Henley,† Ross Baker,† Craig McAndrew,† Berry Matijssen,† Rosemary Burke,† Swen Hoelder,† Suzanne A. Eccles,† Florence I. Raynaud,† Spiros Linardopoulos,§,‡ Rob L. M. van Montfort,*†‡ and Julian Blagg*†

†Cancer Research UK Cancer Therapeutics Unit, Division of Cancer Therapeutics, The Institute of Cancer Research, London SM2 5NG, United Kingdom
‡Division of Structural Biology, The Institute of Cancer Research, London SW3 6JB, United Kingdom
§The Breakthrough Breast Cancer Research Centre, Division of Breast Cancer Research, The Institute of Cancer Research, London SW3 6JB, United Kingdom

Supporting Information

ABSTRACT: The protein kinase MPS1 is a crucial component of the spindle assembly checkpoint signal and is aberrantly overexpressed in many human cancers. MPS1 is one of the top 25 genes overexpressed in tumors with chromosomal instability and aneuploidy. PTEN-deficient breast tumor cells are particularly dependent upon MPS1 for their survival, making it a target of significant interest in oncology. We report the discovery and optimization of potent and selective MPS1 inhibitors based on the 1H-pyrrolo[3,2-c]-pyridine scaffold, guided by structure-based design and cellular characterization of MPS1 inhibition, leading to 65 (CCT251455). This potent and selective chemical tool stabilizes an inactive conformation of MPS1 with the activation loop ordered in a manner incompatible with ATP and substrate-peptide binding; it displays a favorable oral pharmacokinetic profile, shows dose-dependent inhibition of MPS1 in an HCT116 human tumor xenograft model, and is an attractive tool compound to elucidate further the therapeutic potential of MPS1 inhibition.

INTRODUCTION

The main role of the cell cycle is to enable error-free DNA replication, chromosome segregation, and cytokinesis. Surveillance mechanisms, the checkpoint pathways, monitor passage through the cell cycle at several stages. During mitosis, the spindle assembly checkpoint (SAC) prevents anaphase onset until the appropriate tension and attachment across kinetochores is achieved.1,2 One of the first components of the SAC signal, identified by a genetic screen in budding yeast, was dubbed MPS1 (monopolar spindle 1, also known as TTK) because of the monopolar spindles produced by MPS1 mutant cells.3 Subsequently, the MPS1 gene was shown to encode an essential dual-specificity kinase conserved from yeast to humans.4,5 MPS1 activity peaks at the G2/M transition, is enhanced upon activation of the SAC with nocodazole,6 and is dependent upon autophosphorylation of a threonine at position 676 in the activation loop.7 MPS1 is required for normal function of the mitotic spindle checkpoint and subsequent cell division; it is aberrantly overexpressed in a wide range of human tumors including bladder, anaplastic thyroid, breast, lung, esophagus, and prostate cancers.8–12 In addition, MPS1 has been identified in the...
signature of the top 25 genes overexpressed in tumors with chromosomal instability\textsuperscript{13} and aneuploidy,\textsuperscript{14} with PTEN-deficient breast tumor cells particularly dependent upon MPS1 for their survival such that RNAi-mediated knockdown or chemical inhibition of MPS1 leads to cell death.\textsuperscript{14} This body of work has engendered significant interest in the discovery of selective small-molecule chemical tools to elucidate further the therapeutic potential of MPS1 inhibition for the treatment of cancer.

First-generation nonselective inhibitors of MPS1 have been described, for example, 1 (SP600125), a JNK (c-Jun amino-terminal kinase) inhibitor that disrupts SAC function in a JNK-independent manner via the inhibition of MPS1,\textsuperscript{16,18} and 2 (reversine), an MPS1, Aurora A, and Aurora B inhibitor.\textsuperscript{19} More recently, several selective small-molecule MPS1 inhibitors have been utilized to explore the cellular function of MPS1.\textsuperscript{20,21} These include 3 (MPI-0479605),\textsuperscript{22} 4 (AZ3146),\textsuperscript{19} 5 (NMS-P715),\textsuperscript{23,24} a series of selective diaminopyridine-based inhibitors exemplified by 6 that demonstrates inhibition of growth of A549 human tumor xenografts, and a set of indazole-based inhibitors represented by 7 (Figure 1).

Here, we describe the discovery of orally bioavailable small-molecule inhibitors of MPS1 based on the 1H-pyrrolo[3,2-c]-pyridine scaffold in a medicinal-chemistry program enabled by structure-based design and cellular characterization of MPS1 inhibition. We show that optimized compounds in this series display potent and selective inhibition of MPS1 in vitro and intranslate well to cellular assays of MPS1 autophosphorylation and antiproliferative activity when compared to other recently reported inhibitors,\textsuperscript{8} which was itself prepared by sequential palladium-mediated displacement of the 6-bromo substituent of intermediates \textsuperscript{11} with the appropriate aniline gave the desired products \textsuperscript{12} (Scheme 1). This general route was adapted depending on the identity of the N-1 and C-2 substituents as described below. We consistently observed that when \( R_1 = H \) the transformation of \textsuperscript{11} to \textsuperscript{12} was low-yielding; therefore, we employed a protecting-group strategy whereby a Boc substituent was installed at the N-1 position prior to introduction of the C-6 amino substituent into the 1H-pyrrolo[3,2-c]pyridine scaffold.

Compounds \textsuperscript{8} and \textsuperscript{21–28} shown in Table 1 were prepared according to the general strategy depicted in Scheme 1 using palladium-mediated substitution of key 6-bromo-pyrrolopyridine intermediate \textsuperscript{17}, which was itself prepared by sequential Sonogashira cross coupling and base-catalyzed ring closure of sulfonamide \textsuperscript{16}; introduction of the sulfonamide was necessary to optimize the efficiency of the domino cyclization reaction, presumably by increasing the acidity of the remaining anilinic proton (Scheme 2). Cyclization precursor \textsuperscript{16} was prepared from corresponding 4-amino-2-bromopyridine \textsuperscript{13}; iodonation of \textsuperscript{13} was unselective, and desired regiosomer \textsuperscript{15} was purified from its partner, \textsuperscript{14}, by chromatographic separation in 38 and 37% yields, respectively; subsequent dimesylation with methanesulfonylchloride and base-mediated removal of one of the two mesyl groups provided intermediate \textsuperscript{16} in 54% yield. The required tert-butyl 4-ethyl-1H-pyrazole-1-carboxylate \textsuperscript{20} was prepared.
in 56% overall yield from 4-iodopyrazole 18 by Boc protection followed by Sonogashira-mediated coupling with trimethylsilylacetylene and subsequent TBAF-mediated deprotection of alkyne 19 (Scheme 2).

Compounds 30–33, 35, and 36 in Table 2 were also prepared according the general procedure (Scheme 1), which necessitated multiple protection/deprotection steps in addition to the need for bespoke synthesis of the appropriate...
ethynylheterocycle (see the Supporting Information). For compounds 29 and 37−44 (Table 2), 48−54 (Table 5), and 61−68 (Table 6) bearing a preferred 1-methylpyrazole substituent at C-2 of the pyrrolopyridine scaffold, we developed a more efficient route. Thus, Sonagashira cross coupling of 4-ethynyl-1-methyl-1H-pyrazole 45 with unprotected 4-amino-2-bromo-5-iodopyridine 15 gave cyclization precursor 46 in 88% yield, which was subjected to smooth base-mediated conversion to the pyrrolopyridine core followed by substitution of the N-1 position with a tert-butylcarbonate group to give key intermediate 47 in 75% yield (Scheme 3). This two-step, base-mediated approach to the formation of the pyrrolopyridine scaffold obviated the need for sulfonamide-mediated activation of the anilinic cyclization precursor. Subsequent palladium-mediated substitution at the C-6 position, as described above, gave N-1-Boc derivatives 48−54 (Table 5), 61, and 63−68 (Table 6). TFA-mediated removal of the N-Boc substituent furnished N-1-unsubstituted compounds 29, 37−44 (Table 2), and 62 (Table 6), as depicted in Scheme 3.

The required alkynes and substituted anilines were prepared according to the methods summarized in Scheme 4 (Supporting Information), whereas the appropriate iodo heterocycles were prepared from commercially available starting materials by iodination (Supporting Information). However, the 5-iodooxazole required for the synthesis of 1H-pyrrolo[3,2-c]pyridin-2-yl)oxazoles 34 and 55 proved elusive, and an alternative synthetic route was developed in which desired oxazole intermediate 60 was constructed from acetal 58 in 70% overall yield by liberation of the aldehyde and reaction with p-toluenesulfonylmethyl isocyanide (TOSMIC) followed by subsequent N-1 Boc protection. Acetal 58 was obtained from intermediate 16 in 45% yield by Sonagashira cross coupling with 3,3-diethoxyprop-1-yne 56 and concomitant ring closure in a procedure similar to that described by Le Brazidec et al. for the preparation of 2-substituted pyrrolopyrimidines followed by base-mediated removal of the N-1 sulfonamide (Scheme 5).

**RESULTS AND DISCUSSION**

Compound 8 (Figure 2) exemplifies a series of 1H-pyrrolo[3,2-c]-pyridines discovered by HTS of an in-house kinase-focused compound library versus MPS1. Although compound 8 demonstrated potent, ligand-efficient binding to MPS1 (IC₅₀ = 0.025 μM, LE = 0.43; Table 1) with evidence for cell-based antiproliferative activity in the HCT116 human colon cancer cell line (MTT assay, GI₅₀ = 0.55 μM), its overall profile suffered from poor selectivity, particularly against CDK2 (IC₅₀ = 0.043 μM), poor in vitro metabolic stability in mouse and human liver
microsomes, and significant efflux in Caco-2 permeability assays (Figure 2). An important aim of our initial hit-improvement strategy was to eradicate activity versus cell cycle kinases (e.g., CDK2) and other kinases known to affect mitotic function (e.g., Aurora kinases A and B) to study the profile of a highly selective MPS1 inhibitor on mitotic function in cellular mechanistic assays and in vivo. In addition, we set out to improve the metabolic stability and membrane permeability of compound 8 to discover a chemical tool suitable for in vivo PK/PD studies.

A crystal structure of the kinase domain of MPS1 with compound 8 (Figure 3) indicated binding of 8, albeit with relatively weak electron density. Nevertheless, the ligand could be modeled with partial occupancy along with a molecule of poly(ethylene glycol) wrapped around the active-site Lys553 side chain, a consequence of the presence of a high concentration of PEG300 in the crystallization conditions. The structure revealed the 6-amino-pyrrolopyridine motif interacting with the hinge region of the ATP-binding site by virtue of an H-bond-donor interaction between the backbone amide group of Gly605 and the pyridine nitrogen hydrogen-bond acceptor of the pyrrolopyridine scaffold. In addition, the anilinic NH of compound 8 formed a hydrogen bond with the carbonyl group of hinge residue Gly605, thereby positioning the anilinic moiety at the entrance of the MPS1 ATP-binding site, stacked above the post-hinge region (residues 606−611) and pointing toward the solvent. Furthermore, it revealed an H-bond between the C-2 pyrazole and Lys553 as well as a van der Waals interaction between lipophilic C-3 to C-4 atoms and the gatekeeper residue, Met602 (Figure 3).

A striking difference between the binding of compound 8 and published compound 6 is their respective hydrogen-bond interactions with the hinge. Whereas the backbone functionalities of hinge residue Cys604 were not involved in interactions with compound 8, a peptide flip of Cys604 in the structure of MPS1 complexed with compound 6 (PDB code 3VQU) allowed an H-bond interaction between the backbone carbonyl of Cys604 and the anilinic NH of compound 6.26

We initially focused our attention on modification of the pyrrolopyridine 6-anilino substituent to replace the electron-rich 3,4-dimethoxy aniline, which we regarded as a metabolic liability. 4-Methoxy analogue 21 proved equipotent and replacement with a range of 4-substituents maintained activity (compounds 22−24), consistent with the crystal structure of 8 bound to MPS1, which showed that this vector projects out of the entrance of the MPS1 ATP-binding site, stacked above the post-hinge region (residues 606−611) and pointing toward the solvent. Furthermore, it revealed an H-bond between the C-2 pyrazole and Lys553 as well as a van der Waals interaction between lipophilic C-3 to C-4 atoms and the gatekeeper residue, Met602 (Figure 3).

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potency while also significantly improving selectivity versus CDK2 and enhancing selectivity versus Aurora A, Aurora B, and GSK3β. This SAR is consistent with previous reports on the use of 2-substituted anilines in other chemical series to enhance selectivity versus MPS1 through the exploitation of a small lipophilic pocket adjacent to Cys604 in the hinge region (see below).25

Compounds 25–28, although selective for MPS1, remained metabolically unstable, and our attention turned to exploration of the pyrrolopyridine C-2 substituent. This was prompted by our observation that solutions of compound 8 underwent slow air oxidation across the double bond between C-2 and C-3 (Supporting Information Figure S1). We hypothesized that the unsubstituted C-2 pyrazole rendered the pyrrole moiety of the pyrrolopyridine scaffold susceptible to electrophilic attack. Gratifyingly, N-methylation of the C-2 pyrazole was tolerated with only a 5-fold reduction in potency (compound 29, MPS1 IC_{50} = 0.12 μM, versus compound 25, MPS1 IC_{50} = 0.025 μM; Table 2), resulting in an air-stable compound and a slight improvement in metabolic stability (MLM = 72% for 29 versus 99% for 25) (Table 2). Similarly, electron-withdrawing trifluoromethyl-substituted pyrazole analogue 30 maintained potency (MPS1 IC_{50} = 0.079 μM) and also improved metabolic stability (53% turnover in MLM); however, corresponding difluoromethyl analogue 31 proved surprisingly weak, with a 6-fold loss of activity with respect to the trifluoroethyl analogue (IC_{50} = 0.46 μM for 31 versus 0.079 μM for 30). 1,3- and 1,5-Disubstituted pyrazole analogues (32 and 33) and the 3,5-disubstituted isoxazole 35 also lost potency despite an improvement in metabolic stability; we rationalized this loss of potency in terms of the sterically encumbered pocket into which the C-2-pyrazole substituent projects. Imidazole analogue 36 also lost potency in comparison with pyrazole 25 despite the presence of a potentially isosteric H-bond-donor or -acceptor interaction with Lys553, depending on the protonation state of the imidazole. Taken together, these results suggested tight SAR along the C-2 vector from the pyrrolopyridine scaffold and were consistent with the crystal structure of MPS1 complexed with compound 8 that showed that the interaction with Lys553 is important (Figure 3). Unsubstituted oxazole 34 proved to be the only potential C-2 pyrazole replacement that maintained potency with enhanced metabolic stability. The crystal structure of MPS1 in complex with oxazole 34 showed unambiguous electron density for the ligand, supporting the initial binding mode for compound 8 and consistent with the SAR observed for the aniline and C-2 heterocycle modifications made to the pyrrolopyridine scaffold (Figure 4). The hinge-binding motif was the same as that observed with compound 8, and the aniline C-2-methoxy substituent was positioned, as expected, in a small hydrophobic pocket lined by Lys529, Ile531, Gln541, and the gatekeeper +2 residue, Cys604. This pocket is not accessible in many other kinases, including CDK2, GSK3β, Aurora A, and Aurora B, which have bulkier residues at the corresponding gatekeeper +2 position (Phe in CDK2 and Tyr in GSK3β and Aurora A and B). This is consistent with improved selectivity for MPS1 versus related kinases observed for compounds with an aniline 2-substituent (Tables 1 and 2).25 Like the pyrazole of compound 8, the oxazole of 34 was oriented toward the catalytic Lys553 residue, and an H-bond was observed between the pyrrolopyridine N-1 atom and a water molecule. However, despite the presence of a preferred aniline C-2-substituent, the weak CDK2 activity of 34 coupled with the relative complexity of the synthetic route to C-2 oxazoles (Scheme 5) disfavored this approach. We selected 1-methylpyrazole in preference to the 1-trifluoroethyl pyrazole as the optimal pyrrolopyridine C-2 substituent because of its lower lipophilicity and improved ligand efficiency.

Figure 3. Crystal structure of MPS1 with compound 8 bound. Compound 8 is shown with orange carbon atoms and is modeled with partial occupancy along with a PEG molecule, shown with orange and cyan carbon atoms for the two alternate conformers. Selected amino acids that contact the ligand are shown with green carbon atoms. The electron density shown in green is from an F_{o} − F_{c} omit map and is contoured at 3σ. Key H-bond interactions are shown as black dotted lines. The interaction between the C-2 pyrazole and Lys553 has been omitted for clarity. All structural figures were produced with CCP4MG.31

Figure 4. Crystal structure of MPS1 with compound 34 bound. Compound 34 is shown with orange carbon atoms. Selected amino acids that contact the ligand are shown with dark green carbon atoms. H-bond interactions are shown as black dotted lines. The electron density shown in green is from an F_{o} − F_{c} omit map and is contoured at 3σ.
Table 2. Effect of Pyrrolopyridine C-2 and Aniline C-4 Substituents on Metabolism

| Compd | R<sup>1</sup> | R<sup>2</sup> | MPS<sub>1</sub> (μM) | CDK2 (μM) | Aurora A (μM) | Aurora B (μM) | MLM<sup>o</sup> |
|-------|--------------|--------------|----------------|----------|---------------|---------------|--------------|
| 25    | HN<sub>N</sub>N | C<sub>H</sub> | 0.025±0.011 | 0.97±0.08 | 28.00±21.00 | > 100         | 99%          |
| 29    | N<sub>N</sub>N | C<sub>H</sub> | 0.12±0.08  | 53.00±38.00| > 100        | > 100         | 72%          |
| 30    | N<sub>F</sub>C<sub>N</sub>N | C<sub>H</sub> | 0.079±0.029| > 100    | > 100        | > 100         | 53%          |
| 31    | F<sub>F</sub>N<sub>F</sub>N | C<sub>H</sub> | 0.46±0.03  | > 100    | > 40         | > 100         | 61%          |
| 32    | N<sub>F</sub>C<sub>F</sub> | C<sub>H</sub> | 0.99±0.41  | > 100    | > 100        | > 100         | 30%          |
| 33    | N<sub>N</sub>N | C<sub>H</sub> | 1.60±0.27  | > 100    | > 100        | > 100         | 97%          |
| 34    | O<sub>O</sub> | C<sub>H</sub> | 0.026±0.010| 2.90±0.57| > 40         | > 100         | 11%          |
| 35    | O<sub>O</sub> | C<sub>H</sub> | 4.90±2.10  | > 100    | > 100        |              | 38%          |
| 36    | N<sub>N</sub>N | C<sub>H</sub> | 0.64±0.08  | 7.80±0.62| 72.00±29.00 | 47.00<sup>o</sup>| 87%          |
| 37    | N<sub>N</sub>N | C<sub>F</sub><sub>C</sub> | 4.40±0.34  | > 100    | > 100        | > 100         | 56%          |
| 38    | N<sub>N</sub>N | C<sub>H</sub> | 0.074±0.005| 46.00±7.40| > 100        | > 100         | 41%          |
| 39    | N<sub>N</sub>N | C<sub>H</sub> | 0.015±0.004| 7.80±1.60| > 40         | > 100         | 25%          |
We then investigated a range of aniline substitutions with the aim of further improving metabolic stability by reduction of both lipophilicity and electron density in the aniline moiety. 2-Methoxy-5-trifluoromethyl analogue \( \text{37 (IC}_{50} = 4.4 \mu M; \text{Table 2)} \) illustrates poor tolerance of a 2,5-disubstitution pattern on the aniline ring. Analysis of the compound \( \text{34-bound MPS1 structure} \) suggested that the addition of a CF\(_3\) substituent to the 5-position of the aniline ring would induce a steric clash with Asp608 (Figure 4). This observation is consistent with the SAR described for a series of Leucine Rich Repeat Kinase 2 (LRRK2) inhibitors in which a 2,5-disubstituted aniline was employed to drive selectivity for LRRK2 over MPS1.\(^{32}\) Exploitation of the aniline C-4 vector, which extends into the solvent channel (Figure 3), was more successful and led to the synthesis of compounds \( \text{39-44}, \) all of which displayed good potency compared to their unsubstituted parent \( \text{38, improved selectivity, and in vitro metabolic stability (Table 2). However, the measured aqueous thermodynamic solubility was low (e.g., 0.01 mg/mL for compound 42).} \)

2-Chloro-4-dimethylcarboxamido-substituted aniline \( \text{39} \) was selected for pharmacokinetic evaluation on the basis of its excellent potency, in vitro selectivity, and improved metabolic stability in mouse and human liver microsomes (25 and 20% turnover after a 30 min incubation, respectively). This compound displayed an improved efflux ratio in Caco-2 (10) compared to original hit compound \( \text{8} \) and demonstrated good in vivo pharmacokinetics in mouse with a low unbound clearance and moderate oral bioavailability, consistent with our strategy of targeting improved in vitro metabolic stability versus compound \( \text{8} \) (Table 2).

A crystal structure of compound \( \text{39} \) bound to MPS1 was obtained by soaking MPS1 crystals for 24 h in a solution containing 1.25 mM of the inhibitor. The crystal structure showed that the overall binding mode of \( \text{39} \) was very similar to those of HTS hit compound \( \text{8} \) and oxazole \( \text{34}. \) However, the crystallographic data revealed significant electron density along a vector aligned from the pyrrolopyridine N-1 position (Figure 5A), which could not be explained by the interacting water molecule observed in the compound \( \text{8-bound and compound 34-bound structures. Analysis of the compound sample used for the soaking experiment revealed a 2% impurity of the N-Boc synthetic precursor (48; Table 5), which fitted well with the additional electron density observed in the crystal structure. We reasoned that to preferentially occupy the ATP-binding site in a soaking experiment involving a 50-fold excess of compound \( \text{39}, \) compound \( \text{48} \) must be significantly more potent than compound \( \text{39}. \) Subsequent elucidation of the crystal structure of MPS1 bound to the N-Boc-containing precursor, compound \( \text{48}, \) confirmed this hypothesis because the overall binding mode of \( \text{48} \) was entirely consistent with the other crystal structures and the Boc group was clearly present in the same location as the additional electron density in the compound \( \text{39-bound structure (Figure 5B). The aniline C-2-chloro substituent of compound 48 was located in the same lipophilic pocket as the aniline C-2-methoxy group in compound 34, consistent with the improved selectivity afforded by small lipophilic substituents in the aniline C-2 position (Tables 1 and 2).} \)

Compounds 48 displayed potency at the low end of the dynamic range of our in vitro MPS1 assay (\( \text{IC}_{50} = 0.006 \mu M; \text{Table 5; MPS1 enzyme concentration = 3–12.5 nM, see the Supporting Information). We therefore set up a high-throughput cell-based assay that measures the inhibition of ectopic MPS1 autophosphorylation at Thr33 and Ser37 using...
The electron density shown in green in each panel is from an atoms. Selected amino acids that contact the ligands are shown with dark green carbon atoms. H-bond interactions are shown as black dotted lines.

Figure 5. Crystal structure of MPS1 with compounds 39 (panel A) and 48 (panel B) bound. Compounds 39 and 48 are shown with orange carbon atoms. Selected amino acids that contact the ligands are shown with dark green carbon atoms. H-bond interactions are shown as black dotted lines. The electron density shown in green in each panel is from an Fo – Fc omit map and is contoured at 3σ.

Table 3. In Vivo Mouse Plasma Pharmacokinetic Profile of 39 after Oral and iv Dosing (10 mg/kg)

| t1/2 (h) | Cl (mL/min/kg) | PPB (%) | Clu (mL/min/kg) | Vd (L/kg) | F (%) |
|----------|----------------|---------|----------------|-----------|-------|
| 1.05     | 4.74           | 94.1    | 80             | 0.32      | 48    |

Table 4. In Vivo Mouse Plasma Pharmacokinetic Profile of 48 after Oral and iv Dosing (5 mg/kg)

| t1/2 (h) | Cl (mL/min/kg) | PPB (%) | Clu (mL/min/kg) | Vd (L/kg) | F (%) |
|----------|----------------|---------|----------------|-----------|-------|
| 3.26     | 12.44          | 99.5    | 2589           | 1.99      | 78    |

an MSD electrochemiluminescent readout (see the Supporting Information) to discriminate more potent compounds. Comparison of compound 39 and its N-Boc precursor 48 in this assay revealed a 7-fold increase in cellular potency because of the N-Boc substituent (P-MPS1 IC50 = 0.60 μM for compound 48 versus 4.10 μM for compound 39 and HCT116 GL50 = 2.20 μM for compound 48 versus 9.80 μM for compound 39; Table 5). We hypothesized that a combination of increased in vitro potency and lipophilicity-driven cell penetration was responsible for the increase in cellular potency. Gratifyingly, compound 48 not only retained metabolic stability in mouse and human liver microsomes (48 and 33% turnover after a 30 min incubation, respectively; Table 5) despite increased lipophilicity (48 AlogP = 5.1, 39 AlogP = 3.0) but also displayed reasonable chemical stability in aqueous acid and base (38% cleavage of the Boc group was observed after a 75 min incubation of 48 in a simulated gastric acid fluid at 37 °C, and no cleavage was observed after a 5 h incubation of 48 in a simulated duodenum solution at 37 °C), indicating that the N-1-Boc substituent in further analogues. We next turned our attention to improvement of the cell-based GI50 in HCT116 cells, which remained relatively weak for compound 48 (GI50 = 2.20 μM; Table 5).

As expected, further exploration of the aniline C-4 vector in the N-Boc-substituted pyrrolopyridine series revealed broad tolerance for a variety of substituents, with optimal translation to cell-based potency observed for azetidine amide 51, piperidine amides (52 and 53), and thiomorpholine 1,1-dioxide 54. Consistent with previous SAR, we were pleased to note that C-2-oxazole 55 was also tolerated in this series (Table 5), and the crystal structure of 55 bound to MPS1 confirmed that the oxazole maintains an interaction with Lys553 (Figure 6), consistent with the structure of MPS1 with compound 48 (GI50 = 2.20 μM; Table 5). Finally, we were concerned that increased lipophilicity imparted by our serendipitous discovery of the influential N-Boc substituent might erode the in vitro selectivity profile; however, selectivity versus CDK2 and Aurora A was maintained (Table 5), and we also observed complete selectivity over other mitotic kinases, for example, NIMA-related kinase 2 (NEK2) and Polo-Like Kinase 1 (PLK1) (IC50 > 100 μM). Thus, compound 48 resolved many of our issues with original hit compound 8, and we elected to maintain the N-1-Boc substituent in further analogues. We now turned our attention to improvement of the cell-based GI50 in HCT116 cells, which remained relatively weak for compound 48 (GI50 = 2.20 μM; Table 5).

Table 3. In Vivo Mouse Plasma Pharmacokinetic Profile of 39 after Oral and iv Dosing (10 mg/kg)

| t1/2 (h) | Cl (mL/min/kg) | PPB (%) | Clu (mL/min/kg) | Vd (L/kg) | F (%) |
|----------|----------------|---------|----------------|-----------|-------|
| 1.05     | 4.74           | 94.1    | 80             | 0.32      | 48    |

Table 4. In Vivo Mouse Plasma Pharmacokinetic Profile of 48 after Oral and iv Dosing (5 mg/kg)

| t1/2 (h) | Cl (mL/min/kg) | PPB (%) | Clu (mL/min/kg) | Vd (L/kg) | F (%) |
|----------|----------------|---------|----------------|-----------|-------|
| 3.26     | 12.44          | 99.5    | 2589           | 1.99      | 78    |

an MSD electrochemiluminescent readout (see the Supporting Information) to discriminate more potent compounds. Comparison of compound 39 and its N-Boc precursor 48 in this assay revealed a 7-fold increase in cellular potency because of the N-Boc substituent (P-MPS1 IC50 = 0.60 μM for compound 48 versus 4.10 μM for compound 39 and HCT116 GL50 = 2.20 μM for compound 48 versus 9.80 μM for compound 39; Table 5). We hypothesized that a combination of increased in vitro potency and lipophilicity-driven cell penetration was responsible for the increase in cellular potency. Gratifyingly, compound 48 not only retained metabolic stability in mouse and human liver microsomes (48 and 33% turnover after a 30 min incubation, respectively; Table 5) despite increased lipophilicity (48 AlogP = 5.1, 39 AlogP = 3.0) but also displayed reasonable chemical stability in aqueous acid and base (38% cleavage of the Boc group was observed after a 75 min incubation of 48 in a simulated gastric acid fluid at 37 °C, and no cleavage was observed after a 5 h incubation of 48 in a simulated duodenum solution at 37 °C), indicating that the N-1-Boc substituent in further analogues. We next turned our attention to improvement of the cell-based GI50 in HCT116 cells, which remained relatively weak for compound 48 (GI50 = 2.20 μM; Table 5).
This suggested that replacement of the aniline 4-amido substituent with an appropriate heterocycle may be tolerated, and we were keen to explore the effect of this modification on cellular potency (Table 6). Gratifyingly, C-4-pyrazolo analogues 
61, 62, and 63 proved to be potent inhibitors of MPS1 in the in vitro biochemical assay, with acceptable metabolic stability in mouse and human liver microsomes. Analogous to our observations with compounds 
39 and 48, we observed a significant (43-fold) increase in inhibition of MPS1 autophosphorylation in cells for N-1-Boc-substituted compound 61 versus its N-1-H analogue 62 (P-MPS1 IC50 = 0.16 μM versus 6.90 μM), and this improvement was also observed in an assay of cell proliferation (61, HCT116 GI50 = 0.50 μM; 62, HCT116 GI50 = 4.60 μM). Although oxazole-substituted analogue 64 and those substituted with six-membered heterocycles, 66–68, all maintained potent inhibition of MPS1 in vitro, translation to cell-based activity was not improved compared to pyrazole 61 (Table 6). However, the 1-methyl-imidazol-5-yl moiety at the 4-position of the aniline (compound 65) conferred improved translation to cell-based potency (P-MPS1 IC50 = 0.04 μM and HCT116 GI50 = 0.16 μM), which is comparable to or better than the cell-based potency of reported MPS1-selective inhibitors tested in our assays (Table 7).

Compound 65 displayed in vitro potency versus MPS1 at the low end of the dynamic range of our in vitro assay, which together with an excellent translation to cell-based assays prompted further analysis of the binding mode of 65 by X-ray crystallography (Figure 7A). The structure was determined by co-crystallization of the kinase domain of MPS1 with 65 using PEG3350 as the precipitant instead of PEG300 in an attempt to remove the precipitant instead of PEG300 in an attempt to remove the artifactual PEG molecule bound in the ATP-binding site. This resulted in a more physiologically relevant structure without a PEG molecule wrapped around the active-site Lys553 residue, which allowed for the formation of the conserved Lys553–Glu571 ion-pair. Although the binding mode of compound 65 was entirely consistent with our previous compound-bound crystal structures, MPS1 activation-loop residues Ala668–Thr675 adopt an ordered conformation, and the ordered loop forms an antiparallel β-sheet interaction with the P-loop (Figure 7B). In addition, activation-loop residues Met671–Pro673 form a complementary hydrophobic pocket wrapped around the N-1-Boc substituent of 65, completely enclosing the inhibitor in the ATP-binding site. Intriguingly, this activation-loop conformation has previously been observed in the crystal structure of MPS1 with a pyrimidodiazepine ligand (PDB code 3H9F) and is incompatible with binding of a PEG molecule around Lys553 because of steric hindrance. In this structure, a triply phosphorylated Thr–Thr–Ser motif at residues 675–677 formed magnesium-mediated crystal contacts, which may have influenced the activation-loop conformation. A careful analysis of two recently reported crystal structures of MPS1, one in complex with a diamino-pyridine inhibitor (PDB code 3VQU) and one with an early indazole-based inhibitor (PDB code 3W1F), also showed a similarly ordered activation loop in both structures but with the Thr–Thr–Ser motif disordered. In our compound 65-bound MPS1 structure, Thr676 and Ser677 are also disordered, and we did not observe electron density for a phosphate group on Thr675 or for the mediating magnesium atoms. This suggests that in our 65-bound MPS1 structure these residues are not involved in meaningful crystal contacts that could have an effect on the conformation of the activation loop. Further analysis of the crystal packing showed that the only residue of a symmetry-related molecule that is in the vicinity of the activation loop is Ile738. However, a comparison of several compound-bound structures (Supporting Information Figure S2A) showed that Ile738 is located in a region of the protein with only minor conformational flexibility, whereas the activation loop in the respective structures shows a wide range of conformations and a varying degree of order. The absence of any concerted conformational changes between the activation loop and the symmetry-related Ile738 region in these structures led us to conclude that the ordering of the activation loop is not influenced by crystal contacts. Nevertheless, the ordering of the activation loop is clearly prevented by the binding of a PEG molecule in MPS1 structures resulting from the PEG300-containing crystallization conditions. This is supported by soaking MPS1 crystals grown in PEG300 with compound 65, which resulted in a 65-bound MPS1 structure with a disordered activation loop (data not shown).

The structures of MPS1 in complex with the precursors of the diamino-pyridine and indazole-based inhibitors 
66 and 77 show an ordering of the activation loop through interactions with the P-loop and an ethoxy-group, which in both inhibitors is located in a similar position as the N-1-Boc substituent in 65. Moreover, in the pyrimidodiazepine-bound MPS1 structure, the inhibitor interacts with the ordered activation loop via a cyclopentyl moiety in a similar position as the N-1 Boc in compound 65. Taken together, these findings support the hypothesis that the ordering of the activation loop might have a compound-dependent component for inhibitors with substituents similar to the N-1 Boc in compound 65. It is important to note that the overall structure of the MPS1 kinase domain in the compound 65-bound structure has many features of an active kinase conformation. These include the positioning of the αC-helix and the conserved DFG motif in an “in” conformation as well as the presence of the canonical active Lys553–Glu571 ion pair. Further analysis of the conformation in light of the “spine concept” clearly shows the presence of the catalytic C-spine and shows only minor distortions in the regulatory R-spine (Supporting Figure 2B), also indicating that the conformation of MPS1 in this structure...
is close to an active kinase conformation. However, the compound-induced conformation of the activation loop is incompatible with ATP binding and substrate-peptide binding to the kinase because it blocks the phosphate-binding region and the peptide binding site is not formed, a situation that would certainly render MPS1 inactive (Figure 7D).

Introduction of the methylimidazole into compound 65 resulted in compromised passive permeability (PAMPA = 18 × 10⁻⁶ cm/s at pH 7.4) and increased efflux (Caco-2 A to B = 5 × 10⁻⁶ cm/s, B to A = 12 × 10⁻⁶ cm/s, ER = 2.5) compared to compound 48. Although the thermodynamic aqueous solubility of compound 65 was very low (<0.001 mg/mL), solubility in fasted- and fed-state-simulated intestinal fluid (FaSSIF and FeSSIF) was 0.01 and ∼0.55 mg/mL, respectively, consistent with the presence of a weakly basic center (the methylimidazole ring). All other in vitro properties were maintained, and in view of its favorable in vitro profile, compound 65 was selected for more extensive in vitro profiling versus a panel of 121 kinases (Supporting Information Table S1) and in vivo pharmacokinetic evaluation. Of the 121 kinases in the panel, MPS1 showed the greatest inhibition by 65, and only three other kinases showed inhibition greater than 80%. Mouse and rat blood pharmacokinetics revealed a favorable profile with moderate clearance and good to moderate oral bioavailability (Table 8). This compound was progressed to a human tumor xenograft model to test whether pharmacodynamic biomarker modulation could be achieved in vivo. Oral administration of two doses of compound 65 at 50, 75, and 100 mg/kg b.i.d. to mice bearing HCT116 human colon carcinoma xenografts demonstrated dose-dependent modulation of MPS1-driven phospho-histone H3 levels versus control animals at 2 and 10 h but not at 72 h after the last dose, consistent with engagement of MPS1 in vivo (Figure 8A). The compound was well-tolerated at all doses, and the observed decrease in phospho-histone H3 inhibition over time by compound 65 tracks with a decrease in total plasma and tumor tissue exposure measured in the same experiment (Figure 8B,C).

**CONCLUSIONS**

We describe the structure-based optimization of a potent but nonselective and metabolically unstable 1H-pyrrolo[3,2-c]-pyridine HTS hit 8 to compound 65, a highly potent inhibitor
Table 6. Effect of the Aniline Substituent on Cell-Based Potency

| Compd | R<sup>1</sup> | R<sup>2</sup> | Biochemical IC<sub>50</sub> (μM) | Cellular activity (μM) | MLM<sup>a</sup> | HLM<sup>a</sup> |
|-------|-------------|-------------|-----------------------------|------------------------|----------------|----------------|
|       |             |             | MPS1 CDK2 Aurora A P-MPS1 IC<sub>50</sub> HCT116 GI<sub>50</sub> |                        |                |                |
| 61    | Boc         | N            | 0.015±0.013 | >100 >100 | 0.16±0.12 | 0.50±0.15 | 29% 24% |
| 62    | H           | N            | 0.017±0.001 | >100 >100 | 6.90±5.80 | 4.60±0.67 | 49% 33% |
| 63    | Boc         | N            | 0.020±0.002 | >100 >100 | 2.50±0.42 | 4.40±0.11 | 35% 20% |
| 64    | Boc         | N            | 0.019±0.009 | >40 >100 | 0.26±0.02 | 0.97±0.72 | 23% 20% |
| 65    | Boc         | N            | 0.003±0.002 | >100 >40 | 0.043±0.026 | 0.16±0.09 | 28% 51% |
| 66    | Boc         | N            | 0.028±0.018 | >100 >100 | 0.71±0.64 | 1.30±0.96 | 13% 43% |
| 67    | Boc         | N            | 0.032±0.006 | >100 >100 | 0.62±0.54 | 1.30±0.55 | 26% 25% |
| 68    | Boc         | N            | 0.021±0.017 | >50 >50 | 0.21<sup>b</sup> | 0.53<sup>b</sup> | 12% 16% |

<sup>a</sup>MLM/HLM: percentage of parent compound metabolized after a 30 min incubation in mouse and human liver microsomes. <sup>b</sup>n = 1.

Table 7. Comparison of Compound 65 (CCT251455) with Reported MPS1 Inhibitors

| compd | biochemical IC<sub>50</sub> (μM) | cellular activity (μM) |
|-------|-----------------------------|------------------------|
|       | MPS1 CDK2 Aurora A P-MPS1 IC<sub>50</sub> HCT116 GI<sub>50</sub> |                        |
| 65    | 0.003 ± 0.002 >100 >40 | 0.043 ± 0.026 0.16 ± 0.09 |
| 4     | 0.007 ± 0.009 36.00<sup>a</sup> 21.00 ± 11.00 | 0.72<sup>a</sup> 1.20<sup>a</sup> |
| 5     | 0.007 ± 0.003 >100 >30 | 0.16 ± 0.12 0.18 ± 0.09 |
| 6     | 0.011 ± 0.004 8.80<sup>a</sup> >100 | 0.56 ± 0.24 1.60 ± 0.75 |

<sup>a</sup>n = 1.

ATP-binding site and is incompatible with ATP and substrate binding. Despite the increased lipophilicity imparted by the carbamate moiety, 65 demonstrates a good oral pharmacokinetic profile in mouse and rat as well as inhibition of MPS1 activity in vivo following oral administration; 65 is a suitable chemical probe<sup>35</sup> for cell-based assays and in vivo evaluation of the effect of MPS1 inhibition in human tumor xenograft models.

### EXPERIMENTAL SECTION

**Chemistry.** Commercially available starting materials, reagents and dry solvents were used as supplied. Flash column chromatography was...
performed using Merck silica gel 60 (0.025−0.04 mm). Column chromatography was also performed on a FlashMaster personal unit using isolute Flash silica columns or a Biotage SP1 purification system using Merck or Biotage Flash silica cartridges. Preparative TLC was performed on Analtech or Merck plates. Ion-exchange chromatography was performed using acidic Isolute Flash SCX-II columns, Isolute Si-carbonate columns, or basic Isolute Flash NH2 columns. Preparative HPLC was conducted using a Phenomenex Luna column (5 μm, 250 × 21.2 mm, C18, Phenomenex, Torrance, USA) using a Gilson GX-281 liquid handler system combined with a Gilson 322 HPLC pump (Gilson, Middleton, USA) over a 15 min gradient elution from 10:90 to 100:0 MeOH/water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min or over a 15 min gradient elution from 40:60 to 100:0 MeOH/water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min. UV–vis spectra were acquired at 254 nm on a Gilson 156 UV–vis detector (Gilson, Middleton, USA). Collection was triggered by UV signal and collected using a Gilson GX-281 liquid handler system (Gilson, Middleton, USA). Raw data was processed using Gilson Trilution Software.

1H NMR spectra were recorded on a Bruker Avance-500. Samples were prepared as solutions in a deuterated solvent and referenced to the appropriate internal deuterated solvent peak or tetramethylsilane. Chemical shifts were recorded in ppm (δ) downfield of tetramethylsilane. LC/MS analysis was performed on a Waters Alliance 2795 Separations Module and Waters 2487 dual-wavelength absorbance detector coupled to a Waters/Micromass LCt time-of-flight mass spectrometer with ESI source. Analytical separation was carried out at 30 °C either on a Merck Chromolith SpeedROD column (RP-18e, 50 × 4.6 mm) using a flow rate of 2 mL/min in a 3.5 min gradient elution with detection at 254 nm or on a Merck Purospher STAR column (RP-18e, 30 × 4 mm) using a flow rate of 1.5 mL/min in a 4 min gradient elution with detection at 254 nm. The mobile phase was a mixture of MeOH (solvent A) and water (solvent B), both containing formic acid at 0.1%. Gradient elution was as follows: 1:9 (A/B) to 9:1 (A/B) over 2.25 min, 9:1 (A/B) for 0.75 min, and then reversion back to 1:9 (A/B) over 0.3 min, and finally 1:9 (A/B) for 0.2 min (also referred to as ESI-HRMS method A). LC/MS and HRMS analyses were performed on an Agilent 1200 series HPLC and diode array detector coupled to a 6210 time-of-flight mass spectrometer with dual multmode atmospheric pressure CI/ESI source. Analytical separation was carried out at 30 °C either on a Merck Chromolith SpeedROD column (RP-18e, 50 × 4.6 mm) using a flow rate of 2 mL/min in a 4 min gradient elution with detection at 254 nm or on a Merck Purospher STAR column (RP-18e, 30 × 4 mm) using a flow rate of 1.5 mL/min in a 4 min gradient elution with detection at 254 nm.

Table 8. Mouse and Rat in Vivo PK Profile of Compound 65 Dosed at 5 mg/kg iv/po

| species | t1/2 (h) | Cl (mL/min/kg) | PPB (%) | Vd (L/kg) | F (%) |
|---------|----------|----------------|---------|-----------|-------|
| mouse   | 4.2      | 23.8           | 99.93   | 2.3       | 83    |
| rat     | 3.1      | 7.04           | ND      | 1.75      | 32    |
| ND = not determined. |  |  |  |  |  |

Figure 7. Crystal structure of MPS1 with compound 65 bound. (A) Selected amino acids are shown with dark green carbon atoms. Compound 65 is shown with orange carbon atoms. H-bond interactions are shown as black dotted lines. The electron density shown in green is from an Fo − Fc omit map and is contoured at 3σ. (B) Compound 65 is shown as a surface, and MPS1 is shown in dark green as ribbons. Activation-loop residues Ala668–Thr675 are highlighted with blue carbon atoms and blue ribbon representation. (C) Superposition of the compound 65-bound MPS1 structure in green and the structure of MPS1 complexed with an indazole-based inhibitor (PDB code 3W1F)27 in cyan, with an ethoxy-group in similar position as the N-1 Boc in compound 65. The ordered activation loops interacting with the respective inhibitors are indicated. (D) Comparison of the ATP-bound structure of MPS1 (PDB code 3HMN)34 shown in lilac with the compound 65-bound structure shown in green. The activation loop in the MPS1-65 complex structure is highlighted in blue, and clashes with all three phosphates of ATP in the 3HMN structure.
Figure 8. (A) Representative immunoblots of phospho-histone H3 showing dose-dependent PD modulation in HCT116 human tumor xenografts following two oral doses of 65 (50, 75, and 100 mg/kg) or vehicle. Total histone H3, cleaved poly ADP ribose polymerase (PARP, a measure of apoptosis), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, for protein loading) are also shown. (B, C) Plasma and tumor exposure levels from the same experiment measured 2 (light gray bars) or 10 h (dark gray bars) after the last dose.

The mobile phase was a mixture of MeOH (solvent A) and water (solvent B), both containing formic acid at 0.1%. Gradient elution was as follows: 1:9 (A/B) over 2.5 min, 9:1 (A/B) for 1 min, and then reversion back to 1:9 (A/B) over 0.3 min, and finally 1:9 (A/B) for 0.2 min (default method, also referred to as ESI-HRMS method B). The following references masses were used for HRMS analysis: ca 0.2 min (default method, also referred to as ESI-HRMS method B). The following two oral doses of 622.02896 or reserpine \([\text{M} + \text{H}]^+\) 609.280657. All tested compounds gave >95% purity as determined by either method A or method B.

Preparation of Compounds in Table 1 (Exemplified by the Preparation of Compound 3). 2-Bromo-5-iodopyridin-4-amine (15). 4-Amino-2-bromopyridine 13 (22.8 g, 131.8 mmol) and sodium acetate (20.8 g, 254 mmol) were stirred in AcOH (82 mL), and a solution of iodine monochloride (1 M in AcOH, 134 mL, 134 mmol) was added. The mixture was stirred and heated at 75 °C for 3 h. Most of the AcOH was evaporated, and the residue was partitioned between water and EtOAc. The aqueous fraction was again extracted with EtOAc. The combined extracts were washed twice with 10% sodium thiosulfate solution, water, and brine, dried, and evaporated. This gave 40.3 g of a crude product that was purified by chromatography on a silica column (9 cm internal diameter with 28 cm bed of silica) eluting with 5% EtOAc in CH2Cl2, then 10% EtOAc in CH2Cl2, and then 20% EtOAc in CH2Cl2. The deposited white solid was dried in a vacuum desiccator over sodium hydroxide to give \(N\)-(2-bromo-5-iodopyridin-4-yl)-N-(methylsulfonyl)methanesulfonamide (16).

N-(2-Bromo-5-iodopyridin-4-yl) methanesulfonamide (16). 4-Amino-2-bromopyridine 15 (3.055 g, 10.2 mmol) was stirred in CH2Cl2 (34 mL), and Et3N (6.9 mL, 49.1 mmol) was added. The mixture was cooled in ice. To the cold solution was added dropwise a solution of methanesulfonyl chloride (3.2 mL, 40.6 mmol) in CH2Cl2 (11.5 mL) over a period of 14 min. The cold bath was removed, and the reaction was stirred at rt for 1.5 h. The reaction was diluted with CH2Cl2 and washed twice with water. The solution was dried and evaporated. Trituration with ether gave a solid (5.01 g). The crude product was passed in 5% EtOAc in CH2Cl2 through a 2.5 cm pad of silica in a 10 cm diameter sinter to give \(N\)-(2-bromo-5-iodopyridin-4-yl)-N-(methylsulfonyl)methanesulfonamide (3.01 g, 64%). \(^1\)H NMR (500 MHz, CDCl3): \(\delta\) 3.60 (s, 6H), 7.53 (s, 1H), 8.89 (s, 1H). LC (method A)-MS (ESI, m/z) \(t_f\) 1.91 min, 455 \([\text{M} + \text{H}]^+\), 100%. \(^1\)N-(2-Bromo-5-iodopyridin-4-yl)-N-(methylsulfonyl)methanesulfonamide (228 mg, 0.50 mmol) was stirred with THF (1.3 mL) and at rt for 3 h. The THF was evaporated, and the aqueous phase was neutralized using a 10% citric acid solution. The deposited white solid was filtered off, washed with water, and dried in a vacuum desiccator over sodium hydroxide to give 16 (159 mg, 84%). \(^1\)H NMR (500 MHz, DMSO-d6): \(\delta\) 3.29 (s, 3H), 7.54 (s, 1H), 6.84 (s, 1H). LC (method A)-MS (ESI, m/z) \(t_f\) 1.89 min, 377 \([\text{M} + \text{H}]^+\), 100%.

\textbf{tert-Butyl 4-[[Trimethylsilyl]ethyl]yl]-1H-pyrazole-1-carboxylate (19).} 4-Iodopyrazole 18 (7.85 g, 40.4 mmol) was dissolved in THF (120 mL), and Et3N (8.5 mL, 60.5 mmol) and di-tert-butyl dicarbonate (9.7 g, 44.5 mmol) were added. The reaction was stirred at rt for 3 h. The THF was evaporated, and EtOAc was added. The solution was washed with water and brine, dried, and evaporated to leave an oil (14.2 g). The crude product was purified by chromatography on a pad of silica in a sinter (10 cm diameter, 6 cm thick) eluted with 10% EtOAc in cyclohexane and then 20%
tert-Butyl 4-Ethynyl-1H-pyrazole-1-carboxylate (20). tert-Butyl 4-(trimethylsilyl)ethyl)-1H-pyrazole-1-carboxylate 19 (3.88 g, 14.69 mmol) was dissolved in THF (40 mL) and cooled to −5 °C. A 1 M solution of tetrabutylammonium fluoride in THF (16 mL, 16 mmol) was added, and the reaction was stirred for 20 min. The THF was evaporated, and the residue was taken up in EtOAc and washed with water and brine, dried, and evaporated. The crude product was purified by flash chromatography (silica, eluting with 10% EtOAc in cyclohexane) to give 19 (3.88 g, 92%).

H NMR (500 MHz, CDCl3): δ 1.68 (s, 9H), 2.98 (s, 1H), 7.73 (s, 1H), 8.17 (s, 1H).

tert-Butyl 6-Bromo-2-(1-(tert-butoxycarbonyl)-1H-pyrazol-4-yl)-1H-pyrrole (3.2-cypionate-1-carboxylate (17)). To a mixture of N-(4-bromo-5-nitrophenyl)-acetamide (239 mg, 1.06 mmol) and Xantphos (12.8 mg, 0.0216 mmol) in toluene (2.5 mL) was added 1-Methyl-4-iodo-pyrazole (5.0 g, 24.04 mmol) and potassium carbonate (4.26 g, 30.9 mmol). The reaction was cooled and diluted with EtOAc. The solution was washed with water and brine, dried, and evaporated. The residue was kept at ambient temperature overnight. It was adsorbed on four 2 mm, 20 ash silica, packed onto a dipalladium(0) (10 mg, 0.0108 mmol) was added, and the flask was flushed again with argon. The reaction was heated at 60 °C for 1.25 h. The reaction was cooled and added to water. The product was extracted with ether. The combined extracts were washed with water and brine, dried, and evaporated. The crude product was purified by preparative thin layer chromatography (silica, eluting with 1% EtOAc in cyclohexane) to give 17 (1.2 g, 77%).

H NMR (500 MHz, CDCl3): δ 1.68 (s, 9H), 3.90 (s, 3H), 3.91 (s, 3H), 6.45 (d, J = 1.0 Hz, 1H), 6.89 (m, 2H), 6.96 (m, 1H), 7.45 (m, 1H), 7.81 (d, J = 0.6 Hz, 1H), 8.20 (d, J = 1.0 Hz, 1H), 8.42 (d, J = 1.0 Hz, 1H).

ESI-HRMS calc'd for C22H17N2O2 [M + H]+, 364.1257. Found, 364.1255. Preparation of Compounds in Tables 2 and 5 (Exemplified by the Preparation of Compounds 39, 40, and 48). 4-Ethyl-1-methyl-1H-pyrrole (45). 4-Iodopyrazole 18 (5.0 g, 25.75 mmol) was dissolved in DMF (50 mL) and potassium carbonate (4.26 g, 30.9 mmol) was added and stirred (2 min) before iodomethane (1.76 mL, 22.2 mmol) was added. The reaction was cooled and diluted with EtOAc. The solution was washed with water and brine, dried, and evaporated. The residue was triturated with ether to give a gum after evaporation (56 mg). This was triturated with ether to give a solid (49 mg, 89%).

H NMR (500 MHz, DMSO-d6): δ 3.74 (s, 3H), 3.74 (s, 3H), 6.50 (s, 1H), 6.72 (s, 1H), 6.84 (d, J = 8.5 Hz, 1H), 7.06 (d, J = 8.5, 2.2 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 8.01 (s, 1H), 8.25 (s, 1H), 8.33 (s, 1H), 11.1 (br s, 1H).

ESI-MS calcd for C7H7N2O2 [M + H]+, 137.0714. Found, 137.0712.

The reaction was evaporated to a small volume. EtOAc was added, and the solution was washed with water and brine, dried, and evaporated. The residue was kept at ambient temperature overnight. It was adsorbed on four 2 mm, 20 ash silica, packed onto a dipalladium(0) (10 mg, 0.0108 mmol) was added, and the flask was flushed again with argon. The reaction was heated at 60 °C for 2 h. The reaction was cooled and added to MeOH. The product was recovered using 2 M ammonia in MeOH to give a gum after evaporation (180 µL, 0.18 mmol) was added. The solution was added to a 2 g SCX-2 column, and the column washed with more MeOH. The product was recovered using 2 M ammonia in MeOH to give a gum after evaporation (56 mg). This was triturated with ether to give a solid (49 mg, 89%).

H NMR (500 MHz, DMSO-d6): δ 3.74 (s, 3H), 3.74 (s, 3H), 6.50 (s, 1H), 6.72 (s, 1H), 6.84 (d, J = 8.5 Hz, 1H), 7.06 (d, J = 8.5, 2.2 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 8.01 (s, 1H), 8.25 (s, 1H), 8.33 (s, 1H), 11.1 (br s, 1H).

ESI-MS calcd for C7H7N2O2 [M + H]+, 137.0714. Found, 137.0712.

The glassy material was taken up in MeOH, and 1 M sodium hydroxide solution (180 µL, 0.18 mmol) was added. The solution was applied to a 2 g SCX-2 column, and the column washed with more MeOH. The product was recovered using 2 M ammonia in MeOH to give a gum after evaporation (56 mg). This was triturated with ether to give a solid (49 mg, 89%).

H NMR (500 MHz, DMSO-d6): δ 3.74 (s, 3H), 3.74 (s, 3H), 6.50 (s, 1H), 6.72 (s, 1H), 6.84 (d, J = 8.5 Hz, 1H), 7.06 (d, J = 8.5, 2.2 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 8.01 (s, 1H), 8.25 (s, 1H), 8.33 (s, 1H), 11.1 (br s, 1H).
backwashed with a single 40 mL portion of EtOAc. The EtOAc solution was dried and evaporated, and the residue was chromatographed (silica) and eluted with EtOAc/cyclohexane (1:3) and EtOAc/cyclohexane (1:1) to give 45 (3.18 g, 77%). 1H NMR (500 MHz, CDCl3): δ 3.00 (s, 1H), 3.88 (s, 3H), 7.52 (s, 1H), 7.59 (s, 1H). LC (method A)-MS (ESI, m/z) tR 1.34 min, no ion recorded.

2-Bromo-5-(1-(methyl-1H-pyrazol-4-yl)-ethynyl)pyridine-4-amine (46). 4-Amino-2-bromo-5-iopropidine 15 (2.58 g, 8.63 mmol), copper(1) iodide (164 mg, 0.86 mmol), and bis(triphenylphosphine)-palladium dichloride (216 mg, 0.432 mmol) were dissolved in a 100 mL flask, and DMF (25 mL) with Et3N (22 mL) was added. The mixture was stirred at rt for 15 min under nitrogen. 4-Ethynyl-1-methyl-1H-pyrazole 45 (945 mg, 8.91 mmol) in DMF (10 mL) and Et3N (5 mL) were added to the flask. The reaction was stirred at rt for 1.75 h. The reaction was diluted with EtOAc, and the solution was washed with water and dried, and evaporated. The residue was purified by flash chromatography (silica/EtOAc) to give 46 (2.11 g, 88%). 1H NMR (500 MHz, CDCl3): δ 3.94 (s, 3H), 4.80 (br s, 2H), 6.79 (s, 1H), 7.59 (s, 1H), 7.66 (s, 1H), 8.15 (s, 1H). LC (method A)-MS (ESI, m/z) tR 1.83 min, 277 [(M+H)+, 100%].

tert-Butyl-6-bromo-2-(1-methyl-1H-pyrazol-4-yl)-1H-pyrrolo[3,2-c]pyridine-1-carboxylate (47). Potassium tert-butoxide (315 mg, 2.81 mmol) was dissolved in NMP (3 mL), and 2-bromo-5-(1-methyl-1H-pyrazol-4-yl)ethyl)pyridin-4-amine 45 (375 mg, 1.35 mmol) was added to the stirred solution. The reaction was placed under nitrogen and warmed at 50 °C for 3 h. The reaction was cooled, and 10% ammonium chloride (3 mL) added. Water (21 mL) was heated to about 60 °C, and the product solution in NMP/water was added to the water; a solid immediately crashes out. The suspension was allowed to cool to rt and filtered, and the solid was washed with water. Drying in a vacuum desiccator over KOH for 3 days gave product (347 mg) that was aeotroped with toluene and toluene to give 6-bromo-2-(1-methyl-1H-pyrazol-4-yl)-1H-pyrrolo[3,2-c]pyridine (315 mg, 84%). 1H NMR (500 MHz, DMSO-d6); δ 3.90 (s, 3H), 6.69 (d, J = 1.0 Hz, 1H), 7.47 (t, J = 1.0 Hz, 1H), 7.94 (d, J = 0.6 Hz, 1H), 8.18 (s, 1H), 8.50 (d, J = 1.0 Hz, 1H), 11.92 (br s, 1H). LC (method A)-MS (ESI, m/z) tR 1.07 min, 391 [(M+H)+, 100%]. 6-Bromo-2-(1-methyl-1H-pyrazol-4-yl)-1H-pyrrolo[3,2-c]pyridine (325 mg, 1.35 mmol) was dissolved in EtOAc (93 mL) and Et3N (5.3 mL, 37.8 mmol). To the suspension were added DMAP (622 mg, 5.1 mmol) and diisopropylethylamine (2 M in THF, 0.60 mL, 1.196 mmol), and dimethylamine (2 M in THF, 0.65 mL, 1.196 mmol) was stirred for 30 min at rt. It was then concentrated and filtered on an Isolute Flash NH4 column. The residue was purified via Biotage silica gel column chromatography, eluting with CH2Cl2/EtOH (99:1 to 90:10) to afford tert-butyl-6-(4-(dimethylamino)-2-methoxyphenylamino)-2-(1-methyl-1H-pyrazol-4-yl)-1H-pyrrolo[3,2-c]pyridine (34) as a white solid (15 mg, 0.027 mmol) in TFA (265 μL) was stirred for 30 min at rt. It was then concentrated and filtered on an Isolute Flash NH4 column. The residue was purified via Biotage silica gel column chromatography, eluting with 10% MeOH/aq NH4 H2O (10:1) in CH2Cl2 to afford 40 as a white solid (7 mg, 68%). 1H NMR (500 MHz, CDCl3): δ 3.11 (s, 3H), 3.96 (s, 3H), 6.59 (d, J = 0.8 Hz, 1H), 7.01 (dd, J = 8.2, 1.8 Hz, 1H), 7.06–7.09 (m, 2H), 7.65 (d, J = 8.2 Hz, 1H), 7.85 (s, 1H), 7.95 (s, 1H), 8.40 (d, J = 0.8 Hz, 1H). ESI-HRMS calc for C24H26N4O2 [M+H]+, 391.1877; found, 391.1873.

4-Amino-3-methoxy-N,N-dimethylbenzamide. HATU (0.296 g, 0.778 mmol) was added to a solution of 4-amino-3-methoxybenzoic acid (0.1 g, 0.598 mmol), DIPEA (0.15 mL, 0.897 mmol), and dimethylamine (2 M in THF, 0.65 mL, 1.196 mmol) in THF (1.6 mL) under argon. The reaction mixture was stirred overnight. It was then partitioned between EtOAc and water. The separated organic phase was washed with water, dried over Na2SO4, and evaporated in vacuum. The crude was purified via Biotage silica gel column chromatography, eluting with CH2Cl2/EtOAc (60:40 to 40:60) and then filtered on a SCX-2 column to afford the title compound as a colorless oil (69 mg, 59%). 1H NMR (500 MHz, CDCl3): δ 3.06 (s, 6H), 3.87 (s, 1H), 3.99 (br s, 2H), 6.65 (d, J = 7.9 Hz, 1H), 6.89 (d, J = 7.9, 1.7 Hz, 1H), 6.96 (d, J = 1.7 Hz). LC (method B)-MS (ESI, m/z) tR 1.23 min, 195 [(M+H)+, 100%].

Preparation of Compounds in Scheme 5 (Exemplified by Compounds 34 and 55). 6-Bromo-2-(diethoxymethyl)-1-(methylsulfonyl)-1H-pyrrolo[3,2-c]pyridine (57). To DMF (3.4 mL) containing Et3N (0.64 mL, 4.5 mmol) were added propargylaldehyde diethyl acetal 56 (183 μL, 1.27 mmol) and N-(2-bromo-5-iopropidine-4-yl)ethanesulfonamide 16 (400 mg, 1.06 mmol) followed by copper(1) iodide (7.1 mg, 0.037 mmol). The reaction was placed under nitrogen. Bis(triphenylphosphine)palladium dichloride (26.1 mg, 0.037 mmol) was added, and the reaction was flushed again with nitrogen and then heated at 80 °C for 2 h. The reaction was cooled and added to water containing NaHCO3 solution. The reaction was extracted with EtOAc. The combined organic layers were washed with water containing NaHCO3 solution and then evaporated in vacuum. The residue was purified using silica gel column chromatography, eluting with 100% CH2Cl2 to give 57 (207 mg, 51%). 1H NMR (500 MHz, CDCl3): δ 1.32 (d, J = 6.9 Hz, 6H), 3.38 (s, 3H), 3.75 (m, 2H), 3.84 (m, 2H), 5.86 (d, J = 1.0 Hz, 1H), 6.94 (t, J = 1.0 Hz, 1H), 8.14 (t, J = 1.0 Hz, 1H),
**Journal of Medicinal Chemistry**

8.67 (d, J = 1.0 Hz, 1H). LC (method A)-MS (ESI, m/z) t θ 2.44 min, 377 ([M + H]+, 100%).

6-Bromo-2-(diethoxymethyl)-1H-pyrrolo[3,2-c]pyridine (58). 6-Bromo-2-(diethoxymethyl)-1H-pyrrolo[3,2-c]-pyridine 57 (202 mg, 0.54 mmol) was stirred in MeOH (2.4 mL) and 1 M sodium hydroxide in water (0.62 mL, 0.62 mmol) was added. The reaction was stirred at 25 °C for 6 h. The MeOH was evaporated, and the residue taken up in EtOAc. The solution was washed with water and brine, and the organic layer was concentrated in vacuo to afford 58 (142 mg, 89%). \[1^H NMR (500 MHz, CDCl3): \delta 1.27 (t, J = 6.9 Hz, 6H), 3.58–3.72 (m, 4H), 5.73 (m, 1H), 6.59 (m, 1H), 7.48 (m, 1H), 8.65 (s, 1H), 8.84 (br s, 1H). LC (method A)-MS (ESI, m/z) t = 2.08 min, 525 ([M + H+]-EtOH, 100%).

5-(6-Bromo-1H-pyrrolo[3,2-c]pyridin-2-yl)-oxazole (59). To a solution of 6-bromo-2-(diethoxymethyl)-1H-pyrrolo[3,2-c]-pyridine 58 (142 mg, 0.47 mmol) in THF (1.4 mL), water (0.28 mL) was added to give a toluene solution (134 mg, 0.705 mmol), and the reaction was stirred at 25 °C for 55 min. The reaction was partitioned between EtOAc and NaHCO₃. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with NaHCO₃ and brine and concentrated in vacuo to afford 59 (142 mg, 89%). \[1^H NMR (500 MHz, CDCl3): \delta 1.0 (m, 1H), 7.62 (s, J = 1.0 Hz, 1H), 8.88 (d, J = 1.0 Hz, 1H), 9.32 (br s, 1H), 9.93 (s, 1H). LC (method A)-MS (ESI, m/z) t = 1.5 min, 225 ([M + H]+, 100%); 6-Bromo-1H-pyrrolo[3,2-c]pyridine-2-carbaldehyde (60): The reaction mixture was heated at 135 °C for 1 h under microwave irradiation. The residue was partitioned between EtOAc and water. The layers were separated, and the organic solution was washed with water and brine and concentrated in vacuo. The residue was purified using silica gel column chromatography, eluting with EtOAc to afford 59 (561 mg, 79%). \[1^H NMR (500 MHz, CDCl3): \delta 7.37 (s, 1H), 7.56 (s, J = 1.0 Hz, 1H), 7.60 (t, J = 1.0 Hz, 2H), 6.81 (s, 1H), 8.30 (s, 1H), 8.67 (d, J = 1.0 Hz, 1H). LC (method A)-MS (ESI, m/z) t = 1.83 min, 264 ([M + H]+, 100%).

Preparation of Compound 65. tert-Butyl 6-(2-methoxyphenylamino)-2-(1-methyl-1H-pyrazol-4-yl)-1H-pyrrolo[3,2-c]pyridine-1-carboxylate (65). tert-Butyl 6-(2-methoxyphenylamino)-2-(1-methyl-1H-pyrazol-4-yl)-1H-pyrrolo[3,2-c]pyridine-1-carboxylate (65) was prepared via silica gel column chromatography, eluting with CH₂Cl₂/EtOH (99:1 to 95:5) to afford 65 as a white solid (45 mg, 60%). \[1^H NMR (500 MHz, CDCl3): \delta 1.60 (s, 9H), 3.10 (s, 6H), 6.84 (d, J = 0.6 Hz, 1H), 7.17 (br s, 1H), 7.37 (dd, J = 8.5, 1.9 Hz, 1H), 7.56 (d, J = 1.9 Hz, 1H), 7.77 (s, 1H), 8.01 (s, 1H), 8.21 (d, J = 8.5 Hz, 1H), 8.61 (d, J = 1.0 Hz, 1H). ESI-HRMS calcd for C₂₃H₂₂ClN₅O₄ [M+H]+, 482.1590; found, 482.1586.

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**10065**
Protein Production for Full-Length MPS1. The coding sequence for full-length human MPS1 was amplified by PCR using a plasmid containing MPS1 as a template (kindly provided by the laboratory of Prof. Dr. Eric Nigg, University of Basel, Basel, Switzerland). The PCR product was inserted into a modified version of pFastBac1 that encodes an N-terminal 6× His-tag followed by a G468R tag and then in HRV 3C protease cleavable version, was generated according to Bac-to-Bac protocols (Invitrogen, Paisley, UK). For protein production, Sf9 insect cells were grown in sf-900 II media to a cell density of around 2 × 10^6 cells per milliliter and infected with 30–100 μL of virus per 10^7 cells. Insect cell cultures were harvested 3 days postinfection. Cell pellets were resuspended in 3 volumes of lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, and 10% (v/v) glycerol) containing 1× complete EDTA-free protease inhibitors, 20 mM β-mercaptoethanol, 10 mM NaF, 2 mM NaVO₄, and 25 μM benzamidine nuclease, and the resuspended cells were lysed by sonication. Following centrifugation, the supernatant was purified over 10 mL of Talon resin using a batch/gravity protocol, eluting with 5 column volumes (CV) of 50 mM HEPES, pH 7.0, 300 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, and 1× complete EDTA-free protease inhibitors. The Talon eluate was subsequently purified over a 5 mL GSTrap FF column equilibrated in GSH buffer A (10 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 0.0001%Tween 20) and eluted with 4 CV of GSH Buffer B (GSH buffer A + 10 mM glutathione). N-terminal His and GST tags were removed by overnight incubation at 4°C with HRV 3C protease. Cleaved protein was concentrated to 0.5 mL and applied to a Superdex 200 HR 10/30 column in series with a 5 mL GSTrap FF column that was equilibrated with 10 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 0.0001% Tween 20. Selected fractions were pooled, concentrated to 1 to 2 mg/mL and frozen.

MPS1 Kinase Assay. The enzyme reaction (10 μL total volume) was carried out in black 384-well low-volume plates containing full-length MPS1 (LifeTechnologies or in-house, in a range from 3 to 12.5 nM to obtain 10% total conversion during the assay), fluorescent-labeled peptide [H236, sequence: SFAM-DHTGFLXYTR- CONH₂, Pepceuticals Ltd., Enderry, UK] (5 μM), ATP (10 μM), either 1% (v/v) DMSO or the test compound (in the range from 0.25 mM to 100 μM in 1% (v/v) DMSO), and assay buffer (50 mM HEPES, pH 7.0, 0.02% (w/v) NaN₃, 0.01% (w/v) BSA, 0.1 mM orthovanadate, 10 μM MgCl₂, 1 μM DTT, and Roche protease inhibitor). The reaction was carried out for either 60 or 90 min at room temperature and stopped by the addition of buffer (10 μL) containing 20 mM EDTA and 0.05% (v/v) Brij-35, in 0.1 M HEPES-buffered saline (Free acid, Sigma, UK). The plate was read on a Caliper EZ reader II (PerkinElmer). The reader provides a Software package (‘Reviewer’) that converts the peak heights into percent conversion by measuring both the product and substrate peaks. The percent inhibition was calculated relative to blank wells (containing no enzyme and 1% (v/v) DMSO). IC₅₀ values were determined by testing the compounds at a range of concentrations from 0.25 mM to 100 μM. The percent inhibition at each concentration was then fitted to a four-parameter logistic fit using the Studies package (Dotmatics, Bisphorpoolstedford, UK): y = c a ( (b a) 1)/(1 (c x )), where a = asym min, b = asym max, c = IC₅₀, and d = Hill coefficient.

CDK2 Kinase Assay. The enzyme reaction (10 μL total volume) was carried out in black 384-well low-volume plates containing full-length CDK2/CyclinA complex (2 mM, LifeTechnologies), fluorescent-labeled peptide [FL-PEptide18, PerkinElmer, sequence: 5FM-QSPKKG-CONH₂ (1.5 μM), ATP (25 μM), either 1% (v/v) DMSO or the test compound (in the range from 0.25 mM to 100 μM in 1% (v/v) DMSO), and assay buffer (50 mM HEPES, pH 7.0, 0.02% (w/v) NaN₃, 0.01% (w/v) BSA, 0.1 mM orthovanadate, 10 μM MgCl₂, and 1 μM DTT). The reaction was carried out for 60 min at room temperature and stopped by the addition of buffer (10 μL) containing 20 mM EDTA and 0.05% (v/v) Brij-35 in 0.1 M HEPES-buffered saline (Free acid, Sigma, UK). The plate was read on a Caliper EZ reader II (PerkinElmer). The reader provides a Software package (‘Reviewer’) that converts the peak heights into percent conversion by measuring both the product and substrate peaks. The percent inhibition was calculated relative to blank wells (containing no enzyme and 1% (v/v) DMSO). IC₅₀ values were determined by testing the compounds at a range of concentrations from 0.25 mM to 100 μM. The percent inhibition at each concentration was then fitted to a four-parameter logistic fit using the Studies package (Dotmatics, Bisphorpoolstedford, UK): y = a ( (b a) 1)/(1 (c x )), where a = asym min, b = asym max, c = IC₅₀, and d = Hill coefficient.

GSK3β Kinase Assay. All GSK3β percentage inhibitions at 1 μM were performed in duplicates by Invitrogen in a Z′LYTE activity assay using their SelectScreen biochemical kinase profiling service.

Cell Viability Assay. Cell proliferation assays were carried out by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). Briefly, cells were plated in 96-well plates at 1500 cells per well in 100 μL of culture medium in triplicate. On the next day, 2-fold dilutions of the compounds to be tested were made in culture medium so that when diluted 5× the final concentration in the wells ranged from 0 to 20 μM. Twenty-five microliters of compounds dilutions in the medium was added to 100 μL of cells and incubated at 37°C and 5% CO₂ for 3 more days (72 h). Cells were then incubated with 40 μL of 5 mg/mL solution of MTT reagent at 37°C for 3 h. Media was carefully removed, and crystals were dissolved in 100 μL of DMSO. The absorbance was measured at 570 nm with the Wallac VICTOR 1420 multilabel counter (PerkinElmer), and analysis was performed to calculate the GI₀ using GraphPad PRISM.

MPS Assay for MPS1 Autophosphorylation. Cellular IC₅₀ values of MPS1 autophosphorylation inhibition were measured by an in-house electrophoriminescence (Meso Scale Discovery, MSD) assay that measured phosphorylation of ectopic MPS1 at the Thr33 and Ser37 sites. On day 1, 3 × 10⁵ HCT116 cells per well in a 96-well plate were reverse-transfected with 100 ng of wild-type Myc-MPS1 using Lipofectamine LTX (Invitrogen). On the next day, cells were treated with 50 ng/mL of nocodazole. On the following day, cells were treated with 2-fold dilutions of test compounds ranging from 0 to 10 μM for 2 h in the presence of 10 μM MG132. After treatment, cells were washed with PBS and lysed with 60 μL per well of complete lysis buffer (50 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X100, 10 mM NaF, protease inhibitor tablet, and phosphatase inhibitor cocktails) on ice for 30 min with shaking. Cell lysates were mixed thoroughly by pipetting up and down, and 25 μL of lysate was loaded onto MSD plates that were precoated with total MPS1 antibody (mouse monoclonal, Invitrogen, cat. no. 35-9100, 2 μg/mL) and blocked with 3% (w/v) BSA. After a 1 h incubation at room temperature on a shaker, plates were washed three times with MSD wash buffer, and 25 μL of pThr33/pSer37 antibody (Invitrogen, cat. no. 44-1325G) diluted in 1% (w/v) BSA was added followed by incubation for a further 1 h at room temperature. Plates...
were washed again three times with MSD wash buffer and incubated with 25 μL of anti-rabbit sulfo-TAG antibody (Meso Scale Discovery, cat. no. R32AB) diluted in 1% (v/v) BSA) for 1 h. After the final incubation, plates were washed three times with MSD wash buffer and read in the presence of 1× MSD read buffer. IC_{50} values were determined using GraphPad PRISM.

**Solubility Assays.** Aqueous, fed-state-simulated intestinal fluid (FeSSIF) and fasted-state-simulated intestinal fluid (FaSSIF) solubilities were determined at Pharmorphix.

**Crystal Structure Determination of MPS1 with Ligands.** The kinase domain (residues 519–808) of MPS1 was produced in E. coli and purified as described previously. Purified MPS1 was crystallized in the apo form at 18 °C using the sitting-drop vapor-diffusion method. The crystallization drops were composed of 2 μL of protein (8.9 mg/mL) and 2 μL of reservoir solution placed over 200 μL of reservoir solution of 30–45% (v/v) aqueous PEG300 in 48-well plates. Apo crystals typically grew in 72 h. For compounds 8, 34, 39, 48, and 55, apo-protein crystals were soaked for 24 h at 18 °C in 4 μL drops composed of 1–10 mM compound in 35% (v/v) PEG300, 0.1 M HEPES, pH 7.5, and 5% (v/v) DMDSO. Soaked protein crystals were briefly transferred to cryoprotectant solution containing 40% (v/v) PEG300, 0.1 M HEPES, pH 7.5, and 20% (v/v) ethylene glycol prior to cryocooling in liquid nitrogen. MPS1 was cocryocristallized with compound 65 by mixing 2 μL of protein solution, premixed with 1 mM 65 for 30 min on ice, with 2 μL of reservoir solution placed over 200 μL of reservoir solution consisting of 14% (v/v) PEG8000, 0.05 M magnesium acetate, and 0.1 M sodium acetate. Cocrystals of MPS1 with 65 formed within 72 h at 18 °C and were briefly transferred to a cryoprotectant solution containing 10% (v/v) PEG8000, 0.05 M magnesium acetate, 0.1 M sodium acetate, and 25% (v/v) glycerol prior to cryocooling in liquid nitrogen.

X-ray data were collected at Diamond Light Source, Oxfordshire, UK, at beamlines 104 and 104-1. Crystals belonged to the space group I222 and diffracted to a resolution between 2.36 and 2.80 Å. Data were integrated with MOSFLM and XDS and scaled and merged with AIMLESS. The structures were solved by molecular replacement using Phaser and a publicly available MPS1 structure (PDB code 4B11) with ligand and water molecules removed was used as the molecular replacement model. The protein–ligand structures were manually rebuilt in COOT and refined with BUSTER in iterative cycles. Ligand restraints were generated with grade and Mogo. The quality of the structures was assessed with MOLPROBITY. The data collection and refinement statistics are presented in Supporting Information Table S2.

**Mouse Liver Microsomal Stability.** Compounds (10 μM) were incubated with male CD1 mouse liver microsomes (1 mg/mL) protein in the presence of 1 mM NADPH, 2.5 mM UDP-glucuronic acid (UDPGA), and 3 mM MgCl₂ in 10 mM PBS at 37 °C. Incubations were conducted for 0 and 30 min. Control incubations were generated by the omission of NADPH and UDPGA from the incubation reaction. The percentage of compound remaining was determined after analysis by LC/MS.

**Human Liver Microsomal Stability.** Compounds (10 μM) were incubated with mixed-gender pooled human liver microsomes (1 mg/mL) protein in the presence of 1 mM NADPH, 2.5 mM UDPGA, and 3 mM MgCl₂ in 10 mM PBS at 37 °C. Incubations were conducted for 0 and 30 min. Control incubations were generated by the omission of NADPH and UDPGA from the incubation reaction. The percentage of compound remaining was determined after analysis by LC/MS.

**In Vivo Mouse PK.** All procedures involving animals were carried out within The ICR’s Animal Ethics Committee and national guidelines. Mice (female Balb/C) were dosed po or iv (5 or 10 mg/kg) in 10% (v/v) DMDSO and 5% (v/v) Tween 20 in saline. After administration, mice were culled at 5, 15, and 30 min and 1, 2, 4, 6, and 24 h. Blood was removed by cardiac puncture and centrifuged to obtain plasma samples. Plasma samples (100 μL) were added to the analytical internal standard (olomoucine; IS) followed by protein precipitation with 300 μL of methanol. Following centrifugation (1200g, 30 min, 4 °C), the resulting supernatants were analyzed for compound levels by LC/MS. For blood pharmacokinetics, 20 μL was spotted on a Whatman B card and allowed to dry for at least 12 h at room temperature and 6 mm diameter disks were punched and extracted with 200 μL of methanol containing 500 nM olomoucine used as internal standard. Sample extracts were analyzed by LC/MS/MS against calibration curves (six levels) and six quality controls (three levels in duplicate). Separation was carried out by an Acquity UPLC binary system (Waters) on a reverse-phase Kinetex C18 (Phenomenex 50 × 2.1 mm, 1.7 μm particles) analytical column. Elution was achieved with a 4.5 min gradient of 0.1% formic acid/methanol (95:5 formic acid to 0%) following a 0.5 min isocratic period. Detection was performed in positive ion mode ESI multi-reaction monitoring (MRM) on a QTRAP 4000 (AB-SCIEX).

**In Vivo Mouse PK/PhD Study.** Human HCT116 colon carcinoma cells (3 × 10⁶) were sc injected bilaterally in the flanks of female C57BL/6J-FoxI(−/−) athymic mice. Once tumors reached a mean diameter of ~8 mm (day 15), mice were dosed twice at a 12 h intervals with 50, 75, or 100 mg/kg of compound 65 in 10% (v/v) DMDSO and 5% (v/v) Tween 20 in saline. Mice were culled (n = 3 per group) at 2, 10, and 72 h after the second dose. Tumors were snap-frozen and stored at −80 °C until analysis. Tumor samples were homogenized in PBS (3 vol/tumor weight).

**ASSOCIATED CONTENT**

Supporting Information

Experimental procedures for compounds 4–6, 21–33, 35–38, 41–44, 49–54, 61–64, and 66–68. Scheme depicting slow air oxidation of compound 8. Summary of kinase selectivity profiling of compound 65. Crystallographic analysis of compounds 8, 34, 39, 48, 55, and 65. This material is available free of charge via the Internet at http://pubs.acs.org.

**Accession Codes**

Atomic coordinates and structure factors for the crystal structures of MPS1 with compounds 8, 34, 39, 48, 55, and 65 can be accessed using PDB codes 4c4e, 4c4f, 4c4g, 4c4h, 4c4i, and 4c4j, respectively. In the deposited structure of MPS1 soaked with compound 39 (4c4g), the pyrrolo N1-Boc substituted analogue 48 has been modeled.

**AUTHOR INFORMATION**

**Corresponding Authors**

*Phone: +44(0)2087224364; E-mail: rob.vanmontfort@icr.ac.uk (R.L.M.v.M.).
*Phone: +44(0)2087224051; E-mail: julian.blagg@icr.ac.uk (J.B.).

**Notes**

The authors declare the following competing financial interest(s): The authors are current or former employees of The Institute of Cancer Research, which has a commercial interest in the development of kinase inhibitors.

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10063
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**ABBREVIATIONS USED**

Cl, clearance; Clu, unbound clearance; DIPEA, N,N-diisopropylethylamine; GSK3β, glycogen synthase kinase 3β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEPE, 4-[(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HLM, human liver microsome; JNK, c-Jun amino terminal kinase; LRRK2, leucine rich repeat kinase 2; MPM, mouse liver microsomes; MPS1, monopolar spindle 1 kinase; MSD, Meso Scale Discovery; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEK2, NIMA-related kinase 2; PARP, poly ADP ribose polymerase; PLC1, polo-like kinase 1; PTEN, phosphatase and tensin homolog; SAC, spindle assembly checkpoint; TOSMIC, p-toluenesulfonylmethyl isocyanide; UDPGA, UDP-glucuronic acid; Vp, volume of distribution

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