Regular Article

Discovery of 3,5-Dimethylpyridin-4(1H)-one Derivatives as Activators of AMP-Activated Protein Kinase (AMPK)

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Received September 18, 2019; accepted October 15, 2019

Novel 3,5-dimethylpyridin-4(1H)-one scaffold compounds were synthesized and evaluated as AMP-activated protein kinase (AMPK) activators. Unlike direct AMPK activators, this series of compounds showed selective cell growth inhibitory activity against human breast cancer cell lines. By optimizing the lead compound (4a) from our library, 2-[[1\[^{1}\text{H}]\text{-[(4-fluorophenyl)methyl]-2-methyl-1,2,3,6-tetrahydro[3,4-bipyrinid]-6-yl}]]oxy)methyl]-3,5-dimethylpyridin-4(1H)-one (25) was found to have potent AMPK activating activity. Compound 25 also showed good aqueous solubility while maintaining the unique selectivity in cell growth inhibitory activity.

Key words AMP-activated protein kinase activator; precision medicine; aqueous solubility; dihedral angle; hydrophobicity; human breast cancer

Introduction

AMP-activated protein kinase (AMPK) is a master regulator of cellular metabolism in response to sources of metabolic stress, such as nutrient starvation, hypoxia and muscle contraction.\(^1,2\) AMPK is switched on by a rise in the cellular AMP/ATP ratio. Binding of AMP to the allosteric pocket of AMPK appears to lead to activation, with binding to the AMPK\(^\gamma\) subunit in particular serving as an important regulatory feature of the conformational switch.\(^3\) AMPK activation stimulates fatty acid oxidation and glucose uptake, inhibition of cholesterol synthesis, triglyceride synthesis and lipogenesis.\(^4\)

Recent studies have suggested that AMPK activation via several signalling pathways may be a feasible therapeutic strategy for the treatment of various cancers,\(^5,6\) such as 1) enhancement of the stability of p53, a known tumor suppressor protein;\(^7\) 2) inhibition of the expression level of cyclin D1, an established cell cycle regulator that is overexpressed in human cancers;\(^8\) and 3) inhibition of the AKT/mammalian target of rapamycin (mTOR) signalling pathway, which is dysregulated in some human cancer cells.\(^9\)

A number of direct and indirect AMPK activators have been reported to date.\(^3,9,10\) Some of the known direct activators are shown in Fig. 1. 5-Aminoimidazole-4-carboxamide riboside (AICAR) was the first direct AMPK activator to be discovered. This compound is an adenosine analog that is taken up into cells and phosphorylated to generate AICAR monophosphate, which mimics the effects of AMP.\(^11,12\)

Additionally, some of the reported indirect activators are shown in Fig. 2. Among these, metformin, a biguanide-type compound, is used as a first line antidiabetic agent. This compound inhibits mitochondrial complex I, leading to an increase in the AMP/ATP ratio.\(^13,14\)

To acquire a novel series of AMPK activators, we performed whole cell enzyme-linked immunosorbent assay (ELISA) to measure the Ser79 phosphorylation of acetyl-CoA carboxylase (ACC), a substrate of AMPK, using the human breast cancer cell line MDA-MB-453. By screening our compound library, we discovered that compound 4a, a 3,5-dimethylpyridin-4(1H)-one-type compound, was a moderate AMPK activator (Fig. 3).

Next, we evaluated AICAR and compound 4a for cell growth inhibitory activity against human breast cancer cell lines MDA-MB-453 and SK-BR-3. The results are shown in Table 1. We found that, unlike the direct AMPK activator AICAR, which inhibited cell growth in both cell lines, 4a exhibited growth inhibitory activity against only MDA-MB-453 cells. This data suggests that 4a indirectly, rather than directly, activates AMPK via another target molecule.

We hypothesized that this unique selectivity in cell growth

![Fig. 1. AMPK Direct Activators](image-url)
inhibitory activity may lead to the identification of interesting and unique candidates for anti-cancer precision medicine compared to currently available direct AMPK activators. Herein, we present the synthesis and structure activity relationship (SAR) of novel AMPK activators with the unique 3,5-dimethylpyridin-4(1H)-one scaffold. We dramatically improved in vitro AMPK activation activity and cell growth inhibitory activity in MDA-MB-453 cells, and successfully obtained a compound with both potent cellular activity and high aqueous solubility.

Chemistry

Chart 1 illustrates the synthesis of analogs 4a–4c. Alkylation of commercially available hydroxypyridine derivatives

Table 1. \textit{In Vitro} Antiproliferative Activity of AICAR and 4a

| Compound | MDA-MB-453 growth inhibition IC$_{50}$ (µM)$^a$ | SK-BR-3 growth inhibition IC$_{50}$ (µM)$^a$ |
|----------|-----------------------------------------------|-----------------------------------------------|
| AICAR    | 1700                                         | 180                                         |
| 4a       | 0.39                                         | 29% inh. at 1 µM                             |

$a$ The IC$_{50}$ values were determined in triplicate in one experiment.
The synthetic route for 1a–1c with 2-(chloromethyl)-4-methoxy-3,5-dimethylpyridine and silver carbonate yielded 2a–2c, respectively. The O-alkylated compounds were then converted to corresponding biaryl compounds 3a–3c by palladium-catalyzed Suzuki–Miyaura cross coupling with (4-fluorophenyl) boronic acid using sodium carbonate as a base. Finally, analogs 4a–4c were obtained by AlCl₃ demethylation of the corresponding methyl ethers in dichloromethane solvent.

The synthetic route for 4d–4i is described in Chart 2. Suzuki–Miyaura cross coupling of 2c was performed with the requisite commercially available aryl boronic acid derivatives using Pd(OAc)₂ and SPhos in the presence of tripotassium phosphate base to give the intermediates 3d–3i. As with 4a–4c, the corresponding methyl ethers were dealkylated using AlCl₃ or methionine to give analogs 4d–4i.

The synthesis of 4j–4m was accomplished following slight modifications to the synthetic route shown in Chart 2. As outlined in Chart 3, key intermediate 5 was synthesized by metal-halogen exchange of 2c and addition to triisopropyl borate. Subsequent Suzuki–Miyaura cross coupling reaction of 5 with the requisite commercially available heteroaryl halides using Pd(PPh₃)₄ in the presence of aqueous sodium carbonate base in 1,4-dioxane followed by deprotection of the methyl ether group yielded 4j–4m.

Analogs 10 and 12 were generated using the synthetic route outlined in Chart 4. Lithiation of 2c with n-BuLi, followed by trapping with DMF gave aldehyde 6. Treatment of commercially available 7 with triethyl phosphate at 150°C gave phosphonate 8. With intermediates 6 and 8 in hand, aldehyde 6 was subjected to the Horner–Wadsworth–Emmons reaction with phosphonate 8 using NaH as a base to give compound 9. Deprotection of the methyl group in compound 9 using methionine in MsOH as a solvent gave compound 10. For 12, palladium-catalyzed hydrogenation of 9 was performed to obtain 11, and a subsequent deprotection reaction yielded 12.

The synthesis of analogs 19a–19c and 21 required an alternative synthetic strategy, as our initial attempt to generate these analogs by hydrogenation of the corresponding 3,6-dihydropyridine-1(2H)-carboxylate derivative after introduction of the 4-methoxy-3,5-dimethylpyridine moiety was unsuccessful. As shown in Chart 5, our synthesis began with benzylolation of commercially available 1c using silver carbonate to give...
Suzuki–Miyaura cross coupling reaction of 13 with commercially available 3,6-dihydropyridine-1(2H)-carboxylate boronate yielded intermediate 14. Subsequent hydrogenation reaction using Pearlman’s catalyst under high hydrogen pressure gave compound 15. With intermediate 15 in hand, the hydroxypyridine was subjected to alkylation with commercially available benzyl chloride using silver carbonate to provide the key intermediate 17 after TFA deprotection of the Boc group. For the synthesis of 19a–19c, the amine was alkylated with various aldehydes by reductive amination reaction using sodium triacetoxyborohydride followed by deprotection of the methyl ether group. For 21, 1-bromo-4-fluorobenzene was subjected to Buchwald–Hartwig amination with intermediate 17 to give 20. As with the analogs described above, the corresponding methyl ether was dealkylated using methionine to give 21.

Analog 25 was synthesized as shown in Chart 6. Suzuki–Miyaura cross coupling reaction of intermediate 2c with commercially available 3,6-dihydropyridine-1(2H)-carboxylate boronate gave compound 22, which upon deprotection with TFA in dichloromethane followed by reductive amination reaction with 4-fluorobenzaldehyde gave 24. Finally, removal of the methyl group of intermediate 24 with AlCl₃ in dichloromethane as shown in Charts 1 and 2 yielded the desired compound 25.

**Results and Discussion**

The AMPK activation activity of the synthesized compounds was measured by phospho-ACC whole cell ELISA using the MDA-MB-453 human breast cancer cell line. Selected compounds were also evaluated for aqueous solubility in the Japanese Pharmacopoeia 2nd fluid for disintegration test.
The initial SAR of derivatives of lead compound 4a is illustrated in Fig. 4 (data not shown). No AMPK activation effect was observed for the 4-methoxypyridine intermediate. Additionally, shifting the central pyridine nitrogen led to reduced cellular potency. These early modifications demonstrated that the 3,5-dimethylpyridin-4(1H)-one nucleus and position of the nitrogen atom in the central heteroaromatic ring were key elements of the pharmacophore. These elements were readily incorporated using efficient chemistry methodologies, allowing us to optimize the position of the methyl group of the central aromatic ring and substitution of the terminal aromatic moiety for cellular potency.

First, we explored the importance of the position of a substituted methyl group on the central aromatic ring via preparation of compounds 4b and 4c. The SAR of analogs 4b and 4c (Table 2) revealed that substitution of a methyl group at the 6-position was key to improving AMPK activation activity. Compound 4b showed 3-fold more potent cellular activity than compound 4a. Furthermore, 6-methyl analog 4c displayed 10-fold more potent AMPK activation activity than 3-methyl analog 4a.

In the comparison of the 3-methyl derivative (4a) with the 4-methyl (4b) or the 6-methyl (4c) derivative, we focused on the dihedral angle in the biphenyl moiety of these derivatives. To simplify the calculation of the global minimum conformation, we generated the virtual compounds 4a.uni2032–4c.uni2032, which do not possess common 3,5-dimethylpyridin-4(1H)-one moieties in 4a–4c. Figure 5 shows the dihedral angles of the 2-methoxypyridine analogs 4a.uni2032, 4b.uni2032 and 4c.uni2032 calculated using Molecular Operating Environment software (MOE). This calculation indicated that 4b.uni2032 and 4c.uni2032 had larger dihedral angles than 4a.uni2032 (Fig. 5; 4a.uni2032: 52.6°, 4b.uni2032: 81.4°, 4c.uni2032: 81.2°). Based on this result, we concluded that a perpendicular conformation was important for potent cellular activity. On the other hand, 6-methyl derivative 4c showed 4-fold more potent AMPK activation and a similar dihedral angle to that of 4-methyl derivative 4b. As mentioned above, these compounds likely do not activate AMPK directly. However, because the molecule to which these compounds directly bind is unclear, we reasoned the difference between the potency of 4b and 4c based on their cellular activity and chemical structure. There are some potential reasons that explain why we observed different ac-

Table 2. SARs of Methyl Group-substituted Derivatives

| Compound | R  | AMPK activation EC_{50} (µM) |
|----------|----|-----------------------------|
| 4a       | 3-Me | 0.89                        |
| 4b       | 4-Me | 0.28                        |
| 4c       | 6-Me | 0.074                       |

a) The EC_{50} values were examined by phospho-ACC whole cell ELISA using the MDA-MB-453 cell line, and determined in triplicate in one experiment.
activities among these compounds, as one of them, we speculate that the 6-methyl group may occupy a hydrophobic pocket in its target molecule.

Further optimization efforts focused on the substituent on the terminal aromatic moiety to obtain more potent compounds (Table 3). Replacement of the fluoro group with a cyano group (4d) produced a compound with similar potency (EC_{50} = 0.089 \mu M) to 4c. However, replacement with a methyl group (4e) resulted in a significant improvement in potency (EC_{50} = 0.018 \mu M). Further improvement in cellular activity was observed when the fluoro group was replaced with a CF_{3} group (4f), with the resulting compound showing an EC_{50} of 0.010 \mu M. There was a clear preference for substitution in the 4'-position, as exemplified by the difference in potency between the 3'-CF_{3} compound (4g) and 2'-CF_{3} compound (4h) (EC_{50} = 0.19 and 0.30 \mu M, respectively), compared to 4f.

While compound 4f showed potent cellular activity, its aqueous solubility at pH 6.8 was low (<1 \mu M) because of its high hydrophobicity (log D_{7.4} = 4.6). It is well acknowledged that compounds with poor aqueous solubility in gastric pH are often associated with poor oral absorption.17 To investigate the potential anti-tumor effects of this series of compounds, we speculated that the 6-methyl group may occupy a hydrophobic pocket in its target molecule.

In general, hydrophobicity is the most often encountered cause of poor solubility.19 Therefore, strategies for decreasing hydrophobicity by chemical modification, such as introducing hetero atoms into compounds, is widely used to increase aqueous solubility.19 Accordingly, we initially focused on introducing nitrogen atoms into the terminal phenyl moiety to improve the aqueous solubility of 4f. Table 4 shows the AMPK activation EC_{50} values, aqueous solubility at pH 6.8 and measured log D values at pH 7.4 for compounds 4i–4m.

The hydrophobicity of pyridine analog 4i and 4j (log D_{7.4} = 3.2 and 3.3, respectively) was reduced compared to that of 4f, while their aqueous solubility was slightly improved (7.8 and 9.6 \mu M, respectively). However, their cellular activity was reduced by 3–5 fold (EC_{50} = 0.034 and 0.042 \mu M, respectively) compared to that of 4f. While the measured log D_{7.4} values of the pyrimidine analog (4k) and pyrazine analog (4l) were lower (log D_{7.4} = 3.0 and 2.8, respectively) than those of 4i and 4j, the aqueous solubility was similar (6.3 and 3.8 \mu M, respectively). Additionally, the AMPK activation activity was further reduced (EC_{50} = 0.10 and 0.098 \mu M, respectively). Recently, Walker reported that pyridazine was the most water soluble among 2-nitrogen atom-containing, 6-membered ring heterocycles due to its higher dipole moment, pK_{HBX}, pK_{a}, and lower hydrophobicity compared to corresponding pyrimidines and pyrazines.20 Likewise, our pyridazine analog (4m) had the lowest hydrophobicity (log D_{7.4} = 2.1) of these compounds and its aqueous solubility was markedly improved (22.8 \mu M). Unfortunately, the cellular activity of compound 4m was more than 100-fold lower (EC_{50} = 1.1 \mu M) than that of 4f. Therefore, we concluded that it was difficult to achieve a significant improvement in aqueous solubility through decreasing hydrophobicity while maintaining potency.

A recent study by Brown et al. reported that removal of the biaryl structure led to improved aqueous solubility.21,22 Therefore, we next focused on a strategy for removing the biaryl substituent to improve aqueous solubility. We did this by inserting a linker group between the central pyridine moiety and the terminal aromatic group21 (Table 5) or by aromatic group saturation22 (Table 6).

The SAR of compounds 10 and 12, which contain a carbon...
Table 5. SAR of Ethylene or Alkene Spacer Compounds

| Compound | R | AMPK activation EC₅₀ (µM)ᵃ | JP2 (µM)ᵇ | Measured log D₄₃,₄ |
|----------|---|-----------------------------|-------------|---------------------|
| 4i       |   | 0.034                       | 7.8         | 3.2                 |
| 10       |   | 0.13                        | < 1         | 3.9                 |
| 12       |   | 0.13                        | 2.7         | 3.2                 |

ᵃ The EC₅₀ values were examined by phospho-ACC whole cell ELISA using the MDA-MB-453 cell line, and determined in triplicate in one experiment. ᵇ Aqueous solubility in the Japanese Pharmacopoeia 2nd fluid for disintegration test (JP2; pH = 6.8).

Table 6. SAR of Piperidine or Tetrahydropyridine-Type Compounds

| Compound | R | AMPK activation EC₅₀ (µM)ᵃ | JP2 (µM)ᵇ | Measured log D₄₃,₄ |
|----------|---|-----------------------------|-------------|---------------------|
| 4f       |   | 0.010                       | < 1         | 4.6                 |
| 19a      |   | > 3                         | ≥ 100       | NTᵇ                 |
| 21       |   | 0.074                       | 1.7         | 3.8                 |
| 19b      |   | 0.14                        | ≥ 100       | 2.8                 |
| 19c      |   | 0.095                       | 2.6         | 4.2                 |
| 25       |   | 0.016                       | ≥ 100       | 3.2                 |

ᵃ The EC₅₀ values were examined by phospho-ACC whole cell ELISA using the MDA-MB-453 cell line, and determined in triplicate in one experiment. ᵇ Aqueous solubility in the Japanese Pharmacopoeia 2nd fluid for disintegration test (JP2; pH = 6.8). ᶜ NT: not tested.

The aqueous solubility of ethylene linker analog 10 was undetectable (< 1 µM), probably due to its high lipophilicity (log D₄₃,₄ = 3.9). The hydrophobicity of ethylene linker analog 12 was equal (log D₄₃,₄ = 3.2) to that of biaryl compound 4i, but its aqueous solubility was comparable (2.7 µM) to that of 4i. Therefore, we concluded that it also was difficult to improve the aqueous solubility through insertion of a two-carbon atom linker chain.

Finally, we investigated saturation of the terminal aromatic moiety to improve aqueous solubility (Table 6). Based on the calculated hydrophobicity, a simple dearomatization of the terminal aromatic group was predicted to increase hydrophobicity (data not shown). Therefore, we designed and introduced a piperidine linker, a saturated cyclic moiety with an additional polar amino group, into our compound. Replacement of the terminal phenyl moiety with methyl piperidine (19a) dramatically improved the aqueous solubility (≥ 100 µM). Although the potency of compound 19a was undetectable (EC₅₀ ≥ 3 µM), this result encouraged us to synthesize piperidine derivatives for further SAR analysis. Replacement of the methyl group of 19a with a 4-fluorophenyl group enhanced the AMPK activation activity (21; EC₅₀ = 0.074 µM), while the aqueous solubility remained low (1.7 µM). Interestingly, 4-fluorobenzylamine analog 19b showed moderate cellular activity (EC₅₀ = 0.14 µM) and high aqueous solubility (≥ 100 µM). Encouraged by this finding, we sought to further optimize 19b to improve potency. Based on the above SAR studies (Table 3), trifluoromethyl analog 19c was synthesized and evaluated. While the potency of 19c was slightly improved (EC₅₀ = 0.095 µM), the modification reduced aqueous solubility (2.6 µM), probably due to an increase in hydrophobicity (log D₄₃,₄ = 4.2). Therefore, we decided to improve the potency of 19b through subtle structural modifications without increasing hydrophobicity. We replaced the fully saturated piperidine moiety with a partially saturated tetrahydropyridine moiety, which we predicted may mimic the perpendicular conformation of biaryl-type compound 4c. As expected, there was a significant increase in potency following addition of an sp² carbon into the piperidine moiety (25; EC₅₀ = 0.016 µM, which was comparable to that of 4f). Furthermore, the hydrophobicity of tetrahydropyridine analog 25 was lower (log D₄₃,₄ = 3.2) than that of 19c but comparable to that of 19b, indicating that 25 was a potent compound with favorable aqueous solubility (≥ 100 µM).

To better understand the increased potency of compound 25, we compared the global minimum energy conformation of 25 with that of 4e and 19b. For the same purpose as the experiments shown in Fig. 5, we generated virtual compounds 19b' and 25'. Figure 6 shows the superimposition of the global minimum energy conformations of 4c, 19b and 25 calculated using MOE. As expected, tetrahydropyridine analog 25' adopted a conformation closer to that of biaryl analog 4c than that of piperidine analog 19b'. We hypothesize that addition of two sp² carbons into the piperidine moiety allowed the compound to adopt the preferred perpendicular conformation shown in Fig. 5. As described above, hydrophobic substituents were preferred at the 4'-position of the phenyl moiety (Table 3; 4c vs. 4f; EC₅₀ = 0.074 vs. 0.010 µM), indicating the presence of a hydrophobic pocket around the 4'-position of the terminal phenyl moiety. Because the hydrophobic 4-fluorobenzyl group in 25' is oriented in the same direction as the fluoro group in 4c, these groups may occupy the same hydrophobic pocket in the target molecule. We hypothesize that the hydrophobic interaction is another important factor for improving the potency of compound 25.

Given the promising solubility and good potency, we further examined compound 25 in a pharmacological study. Compound 25 was evaluated for its antiproliferative activity...
against MDA-MB-453 and SK-BR-3 human breast cancer cell lines. As shown in Table 7, compound 25 showed 70-fold more potent cell growth inhibitory activity against MDA-MB-453 than compound 4a. Furthermore, the unique cellular selectivity of compound 4a was maintained in 25, which showed quite weak antiproliferative activity against SK-BR-3, similar to 4a. The AMPK activation activity and antiproliferative activity of 25 was relatively potent compared to reported AMPK activators, suggesting that compound 25 may be a novel AMPK activating anti-cancer option in precision medicine.

Conclusion
The present study describes a SAR study of novel 3,5-dimethylpyridin-4(1H)-one compounds and the unique biological profiles of a new class of indirect AMPK activators. Compound 4a from our library showed moderate AMPK activation activity and unique cancer cell selectivity. Our structural optimization of the central pyridine ring and terminal aromatic group led to the discovery of trifluoromethyl substituted compound 4f with potent AMPK activation activity. Due to the poor aqueous solubility of 4f, we further optimized the compound using molecular conformation calculations to acquire a compound with both potent activity and high aqueous solubility. As a result, we discovered tetrahydropyridine-type 25, a potent and highly water-soluble compound. Further studies on these unique AMPK activating compounds will be reported in due course. In this report, we will show the results of the target identification study and discuss the background of the cancer cell growth selectivity.

Experimental
Chemistry  
^1H-NMR spectra were recorded on a Varian VNS-400, Varian 400-MR, JEOL JNM Lambda-300, JEOL JNM Lambda-400 or Bruker AVANCE III-HD500 spectrometer. Chemical shifts were expressed in δ values (ppm) using tetramethylsilane as the internal standard (s = singlet, d = doublet, t = triplet, m = multiplet and br = broad peak). MS were recorded on a JEOL GC Mate II, Waters SQD, Waters ZQ-2000, Thermo Fisher LCQ Advantage or Thermo Fisher Exacta Plus Orbitrap. All reactions were performed using commercially available reagents and solvents without further purification. The following abbreviations are used: BINAP, 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene; BuLi, butyllithium; t-BuOK, potassium tert-butoxide; t-BuONa, sodium tert-butoxide; DME, 1,2-dimethoxyethane; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; EtOAc, ethyl acetate; EtOH, ethanol; IPE, diisopropyl ether; NaBH(OAc)₃, sodium triacetoxorobohydride; PdCl₂(PPh₃)₂, bis(triphenylphosphine)-palladium(II) dichloride; Pd₂(dba)₃, tris(dibenzyldieneacetone) dipalladium(0); Pd(OAc)₂, palladium(II) acetate; Pd(PPh₃)₄, tetrakis(triphenylphosphine)palladium(0); SPhos, 2-dicyclohexylphosphino-2',6'-dimethoxybiphenyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

2-[(5-Bromo-3-methylpyridin-2-yl)oxy]methyl]-4-methoxy-3,5-dimethyldpyridine (2a) To a mixture of 2-(chloromethyl)-4-methoxy-3,5-dimethyldpyridine hydrochloride (600 mg, 2.7 mmol) and 5-bromo-2-hydroxy-3-methylpyridine (1a; 510 mg, 2.7 mmol) in toluene (6.0 mL) was added Ag₂CO₃ (1.9 g, 6.8 mmol). The mixture was stirred at 110°C for 6 h. After cooling to room temperature, the mixture was filtered through a Celite pad. The filtrate was concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give the product (360 mg, 39%). ^1H-NMR (DMSO-d₆) δ: 2.11 (3H, s), 2.21 (3H, s), 2.23 (3H, s), 3.74 (3H, s), 5.36 (2H, s), 7.78–7.83 (1H, m), 8.09–8.13 (1H, m), 8.19 (1H, s); MS m/z: 337 (M + H)^+.

2-[(5-Bromo-4-methylpyridin-2-yl)oxy]methyl]-4-methoxy-3,5-dimethyldpyridine (2b) Compound 2b was prepared from 5-bromo-2-hydroxy-4-methylpyridine (1b) in...
94% yield using a similar approach to that described for 2a. 

1H-NMR (DMSO-d$_6$): δ: 2.22 (6H, s), 2.31 (3H, s), 3.74 (3H, s), 5.33 (2H, s), 6.92 (1H, s), 8.19 (1H, s), 8.26 (1H, s); MS m/z: 337 (M + H$^+$).

2-[[[5-Bromo-6-methylpyridin-2-yl]oxy][methyl]-4-methoxy-3,5-dimethylpyridine (2c) Compound 2c was prepared from 3-bromo-6-hydroxy-2-methylpyridine (1e) in 92% yield using a similar approach to that described for 2a. 

1H-NMR (DMSO-d$_6$): δ: 2.22 (3H, s), 2.24 (3H, s), 2.49 (3H, s), 3.74 (GH, s), 5.33 (2H, s), 6.67 (1H, d, J = 8.8 Hz), 7.86 (1H, d, J = 8.8 Hz), 8.20 (1H, s); MS m/z: 337 (M + H$^+$).

2-[[[4-(Fluorophenyl)-3-methylpyridin-2-yl]oxy][methyl]-4-methoxy-3,5-dimethylpyridine (2a) A mixture of 2-[[[5-bromo-3-methylpyridin-2-yl]oxy][methyl]-4-methoxy-3,5-dimethylpyridine (2a; 100 mg, 0.30 mmol) and 1,4-dioxane (3.0 mL) were added (4-fluorophenyl)boronic acid (83 mg, 0.62 mmol) and PdCl$_2$(PPh$_3$)$_2$ (10 mg, 0.015 mmol). The mixture was stirred at 100°C for 15 h. After cooling to room temperature, the mixture was diluted with H$_2$O and extracted with EtOAc. The organic layer was washed with saturated NaHCO$_3$ aqueous solution and brine, dried over MgSO$_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give the product (100 mg, 100%). 

1H-NMR (CDCl$_3$): δ: 2.20 (3H, s), 2.23 (3H, s), 2.26 (3H, s), 3.76 (3H, s), 3.43 (2H, s), 7.24–7.28 (2H, m), 7.76–7.77 (2H, m), 7.86–7.92 (1H, m), 8.21 (1H, s), 8.26–8.33 (1H, m); MS m/z: 353 (M + H$^+$).

2-[[[4-(Fluorophenyl)-4-methylpyridin-2-yl]oxy][methyl]-4-methoxy-3,5-dimethylpyridine (3b) Compound 3b was prepared from 2b in 100% yield using a similar approach to that described for 3a. 

1H-NMR (CDCl$_3$): δ: 2.20 (3H, s), 2.23 (3H, s), 2.24 (3H, s), 2.34 (3H, s), 3.79 (3H, s), 5.47 (2H, s), 6.73 (1H, s), 7.07–7.16 (2H, m), 7.21–7.30 (2H, m), 7.97 (1H, s), 8.28 (1H, s); MS m/z: 353 (M + H$^+$).

2-[[[4-(Fluorophenyl)-6-methylpyridin-2-yl]oxy][methyl]-4-methoxy-3,5-dimethylpyridine (3c) Compound 3c was prepared from 2c in 100% yield using a similar approach to that described for 3a. 

1H-NMR (DMSO-d$_6$): δ: 2.23 (3H, s), 2.28 (3H, s), 2.36 (3H, s), 3.75 (3H, s), 5.37 (2H, s), 6.75 (1H, d, J = 8.3 Hz), 7.22–7.33 (2H, m), 7.36–7.47 (2H, m), 7.55 (1H, d, J = 8.3 Hz), 8.22 (1H, s); MS m/z: 353 (M + H$^+$).

2-[[[4-(Fluorophenyl)-3-methylpyridin-2-yl]oxy][methyl]-3,5-dimethylpyridin-4(1H)-one (4a) A solution of 2-[[[5-(4-fluorophenyl)-3-methylpyridin-2-yl]oxy][methyl]-4-methoxy-3,5-dimethylpyridine (3a; 100 mg, 0.30 mmol) in CH$_2$Cl$_2$ (3.1 mL) was added AlCl$_3$ (120 mg, 0.89 mmol). The mixture was stirred at 100°C for 15 h. After cooling to room temperature, the mixture was diluted with H$_2$O and extracted with EtOAc. The organic layer was washed with saturated NaHCO$_3$ aqueous solution and brine, dried over MgSO$_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give the product (100 mg, 100%). 

1H-NMR (CDCl$_3$): δ: 2.22 (3H, s), 2.28 (3H, s), 2.38 (3H, s), 3.75 (3H, s), 5.39 (2H, s), 6.75–6.83 (1H, m), 7.57–7.65 (3H, m), 7.88–7.96 (2H, m), 8.21 (1H, s); MS m/z: 360 (M + H$^+$).

4-Methoxy-3,5-dimethyl-2-[[[6-methyl-5-[[4-(4-fluorophenyl)phenyl]pyridin-2-yl]oxy]methyl]pyridine (3e) Compound 3e was prepared from 2e and (4-methylphenyl)boronic acid in 92% yield using a similar approach to that described for 3d.

1H-NMR (DMSO-d$_6$): δ: 2.23 (3H, s), 2.28 (3H, s), 2.36 (3H, s), 3.75 (3H, s), 5.37 (2H, s), 6.73 (1H, d, J = 8.44 Hz), 7.21–7.27 (4H, m), 7.52 (1H, d, J = 8.4 Hz), 8.21 (1H, s); MS m/z: 349 (M + H$^+$).

4-Methoxy-3,5-dimethyl-2-[[[6-methyl-5-[[4-( trifluoromethyl)phenyl]pyridin-2-yl]oxy]methyl]pyridine (3f) Compound 3f was prepared from 2f and [4-(trifluoromethyl)phenyl]boronic acid in 77% yield using a similar approach to that described for 3d. 

1H-NMR (DMSO-d$_6$): δ: 2.23 (3H, s), 2.28 (3H, s), 2.38 (3H, s), 3.75 (3H, s), 5.39 (2H, s), 6.80 (1H, d, J = 8.41 Hz), 7.59–7.65 (3H, m), 7.81 (2H, d, J = 8.2 Hz), 8.22 (1H, s); MS m/z: 403 (M + H$^+$).

4-Methoxy-3,5-dimethyl-2-[[[6-methyl-5-[[3- (trifluoromethyl)phenyl]pyridin-2-yl]oxy]methyl]pyridine (3g) Compound 3g was prepared from 2g and [3-(trifluoromethyl)phenyl]boronic acid in 82% yield using a similar approach to that described for 3d. 

1H-NMR (DMSO-d$_6$): δ: 2.23 (3H, s), 2.28 (3H, s), 2.37 (3H, s), 3.75 (3H, s), 5.39 (2H, s), 6.78 (1H, d, J = 8.41 Hz), 7.63 (1H, d, J = 8.4 Hz), 7.66–7.78 (4H, m), 8.22 (1H, s); MS m/z: 403 (M + H$^+$).

4-Methoxy-3,5-dimethyl-2-[[[6-methyl-5-[[2- (trifluoromethyl)phenyl]pyridin-2-yl]oxy]methyl]pyridine (3h) Compound 3h was prepared from 2h and [2-(trifluoromethyl)phenyl]boronic acid in quantitative yield using a similar approach to that described for 3d. 

1H-NMR (DMSO-d$_6$): δ: 2.10 (3H, s), 2.23 (3H, s), 2.29 (3H, s), 3.75
(3H, s), 5.27–5.48 (2H, m), 6.70–6.77 (1H, m), 7.40 (1H, d, J = 7.5 Hz), 7.45 (1H, d, J = 8.6 Hz), 7.60–7.68 (1H, m), 7.71–7.78 (1H, m), 7.86 (1H, d, J = 7.5 Hz), 8.23 (1H, s); MS m/z: 403 (M + H)+.

6-[(4-Methoxy-3,5-dimethylpyridin-2-yl)methoxy]-2-methyl-6-(trifluoromethyl)-3,3′-bipyridine (3i) Compound 3i was prepared from 2c and [6-(trifluoromethyl)pyridin-3-yl]boronic acid in 88% yield using a similar approach to that described for 3d. 1H-NMR (DMSO-d6) δ: 8.23 (1H, s), 2.28 (3H, s), 2.40 (3H, s), 3.75 (3H, s), 5.41 (2H, s), 6.84 (1H, d, J = 8.4 Hz), 7.71 (1H, d, J = 8.4 Hz), 7.98 (1H, d, J = 8.2 Hz), 8.11–8.17 (1H, m), 8.22 (1H, s), 8.78–8.86 (1H, m); MS m/z: 390 (M + H)+ (Calcd for C21H16F3N2: 388.1200).

3,5-Dimethyl-2-[(2-methyl-6-(trifluoromethyl)3,3′-bipyridin-6-yl(methoxy)pyridin-4(1H)-one (4i) Compound 4i was prepared from 3i in 18% yield using a similar approach to that described for 4a. 1H-NMR (DMSO-d6) δ: 1.86 (3H, s), 1.98 (3H, s), 2.41 (3H, s), 5.31 (2H, s), 6.89 (1H, d, J = 8.4 Hz), 7.52 (1H, s), 7.74 (1H, d, J = 8.4 Hz), 7.99 (1H, d, J = 8.2 Hz), 8.11–8.19 (1H, m), 8.78–8.83 (1H, m), 11.27 (1H, brs); MS m/z: 389 (M + H)+; ESI-MS m/z: 390.1491 (M + H)+ (Calcd for C21H16F3N2: 389.1491).
(trifluoromethyl)pyridazine and Pd(PPh3)4 in 72% yield using a similar approach to that described for 3a. 1H-NMR (CDCl3) δ: 2.28 (3H, s), 2.37 (3H, s), 2.60 (3H, s), 3.80 (3H, s), 5.53 (2H, s), 6.83 (1H, d, J = 8.6 Hz), 7.73–7.80 (2H, m), 7.87 (1H, d, J = 8.8 Hz), 8.28 (1H, s); MS m/z: 405 (M + H)+.

3,5-Dimethyl-2-[[2-(methylthio)-5-(trifluoromethyl)2,3'-bipyridin-6-yl]oxy]methyl]pyridin-4(1H)-one (4j) To a solution of i-ButOK (360 mg, 3.2 mmol) in DMF (3.5 mL) was added the solution of 6-[[4-methoxy-3,5-dimethylpyridin-2-yl]methoxy]-2',3'-bipyridine (3j; 260 mg, 0.65 mmol) in DMF (2.5 mL). The mixture was stirred at 60°C for 2 h. After cooling to room temperature, the mixture was concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/CHCl3/MeOH) to give the product (278 mg, 82%).

Diethyl 4-[(trifluoromethyl)pyridin-3-yl]methyloxymethyl)]pyridine (8) A mixture of 5-(chloromethyl)-2-(trifluoromethyl)-pyridine (7; 480 mg, 2.5 mmol) in triethyl phosphite (3.0 mL, 17 mmol) was stirred at 150°C for 4 h. After cooling to room temperature, the mixture was concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/CHCl3/MeOH) to give the product (670 mg, 92%).

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4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[(4-

4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[(4-

4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[(4-

4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[(4-
in vacuo. The residue was purified by column chromatography on silica gel (hexane/EtOAc) to give the product (79 mg, 83%). 1H-NMR (CDCl3): δ: 2.26 (3H, s), 2.34 (3H, s), 2.39 (3H, s), 2.82–2.97 (4H, m), 3.18 (3H, s), 5.41 (2H, s), 6.59 (1H, d, J = 8.4 Hz), 7.21 (1H, d, J = 8.4 Hz), 7.55–7.60 (2H, m), 8.26 (1H, s), 8.45 (1H, s); MS m/z: 432 (M + H)+.

3,5-Dimethyl-2-[[6-methyl-5-[2-[[trifluoromethyl]pyridin-3-yl][ethyl]pyridin-2-yl]oxo]methyl]pyridine (19) 1H-NMR (CDCl3): δ: 1.58–1.82 (4H, m), 1.99–2.15 (2H, m), 2.26 (3H, s), 2.33 (6H, s), 2.47 (3H, s), 2.53–2.70 (1H, m), 2.91–3.05 (2H, m), 3.77 (3H, s), 5.40 (2H, s), 6.63 (1H, d, J = 8.6 Hz), 7.41 (1H, d, J = 8.6 Hz), 8.25 (1H, s); MS m/z: 356 (M + H)+.

4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[1-(1-[4-(trifluoromethyl)phenyl][ethyl]pyridin-4-yl)pyridin-2-yl]oxo]methyl]pyridine (18a) To a solution of 4-methoxy-3,5-dimethyl-2-[[6-methyl-5-[[5-[[4-fluorophenyl]methyl]pyridin-4-yl]pyridin-2-yl]oxo][methyl]pyridine (17, 200 mg, 0.59 mmol) in THF (3.0 mL)/EtOH (3.0 mL) was added 1H-benzotriazol-1-ethanol (150 mg, 0.97 mmol). After stirring at room temperature for 10 min, to the mixture was added NaBH(OAc)3 (290 mg, 1.4 mmol). After stirring at room temperature for 18h, to the mixture was added saturated NaHCO3 aqueous solution. The mixture was extracted with CHCl3. The organic layer was dried over Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography on silica gel (CHCl3/MeOH) to give the product (150 mg, 73%). 1H-NMR (CDCl3): δ: 1.58–1.82 (4H, m), 1.99–2.15 (2H, m), 2.26 (3H, s), 2.33 (6H, s), 2.47 (3H, s), 2.53–2.70 (1H, m), 2.91–3.05 (2H, m), 3.77 (3H, s), 5.40 (2H, s), 6.63 (1H, d, J = 8.6 Hz), 7.41 (1H, d, J = 8.6 Hz), 8.25 (1H, s); MS m/z: 356 (M + H)+.

4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[[1-(1-[4-(trifluoromethyl)phenyl][ethyl]pyridin-4-yl)pyridin-2-yl]oxo]methyl]pyridine (18b) To a solution of 4-methoxy-3,5-dimethyl-2-[[6-methyl-5-[[5-[[4-fluorophenyl]methyl]pyridin-4-yl]pyridin-2-yl]oxo][methyl]pyridine (17, 200 mg, 0.59 mmol) in CH2Cl2 (6.0 mL) were added 4-fluorobenzaldehyde (77 µL, 0.73 mmol) and acetic acid (67 µL, 1.2 mmol). After stirring at room temperature for 1h, to the mixture was added NaBH(OAc)3 (250 mg, 1.2 mmol). After stirring at room temperature for 18h, to the mixture was added saturated NaHCO3 aqueous solution. The mixture was extracted with CHCl3. The organic layer was washed with brine, dried over Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography on silica gel (CHCl3/MeOH) to give the product (220 mg, 84%). 1H-NMR (CDCl3): δ: 1.44–1.58 (2H, m), 2.20 (3H, s), 2.54–2.92 (3H, m), 3.91–4.14 (2H, m), 6.11 (1H, d, J = 9.3 Hz), 7.33 (1H, d, J = 9.3 Hz), 11.39 (1H, brs); MS m/z: 293 (M + H)+.

4-Butyl-6-(benzoxalo)-2-methyl-3,6-dihydro[3,4-bipyridine-1(2H)]-carboxylate (15) To a solution of 4-butyl-6-(benzoxalo)-2-methyl-3,6-dihydro[3,4-bipyridine-1(2H)]-carboxylate (14; 2.2 g, 5.75 mmol) in THF (20 mL)/MeOH (20 mL) was added 20% Pd(OH)2/C (wetted with approx. 50% water, 0.73 mmol). After stirring at room temperature for 10 min, to the mixture was added NaBH(OAc)3 (290 mg, 1.4 mmol). After stirring at room temperature for 18h, to the mixture was added saturated NaHCO3 aqueous solution. The mixture was extracted with CHCl3. The organic layer was washed with brine, dried over Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography on silica gel (CHCl3/MeOH) to give the product (150 mg, 73%). 1H-NMR (CDCl3): δ: 1.58–1.82 (4H, m), 1.99–2.15 (2H, m), 2.26 (3H, s), 2.33 (6H, s), 2.47 (3H, s), 2.53–2.70 (1H, m), 2.91–3.05 (2H, m), 3.77 (3H, s), 5.40 (2H, s), 6.62 (1H, d, J = 9.3 Hz), 8.25 (1H, s); MS m/z: 356 (M + H)+.

4-Methoxy-3,5-dimethyl-2-[[6-methyl-5-[[1-(1-[4-(trifluoromethyl)phenyl][ethyl]pyridin-4-yl)pyridin-2-yl]oxo]methyl]pyridine (18c) Compound 18c was prepared from 17 and 4-(trifluoromethyl)benzaldehyde in 69% yield using a similar approach to that described for 18b. 1H-NMR (CDCl3): δ: 1.53–1.71 (4H, m), 2.05–2.18 (2H, m), 2.21 (3H, s), 2.24 (3H, s), 2.31 (3H, s), 2.30–2.42 (2H, m), 2.50 (2H, s), 3.77 (3H, s), 5.28 (2H, s), 6.62 (1H, d, J = 8.4 Hz), 7.49–7.61 (3H, m), 7.69 (1H, d, J = 9.7 Hz), 8.19 (1H, s); MS m/z: 500 (M + H)+.
4(1H)-one (19b) Compound 19b was prepared from 18b in 39% yield using a similar approach to that described for 4f.

3,5-Dimethyl-2-[[[6-methyl-5-(1-[4-(trifluoromethyl)phenyl]methyl)pyridin-4-yl]pyridin-2-yl]oxy]pyridin-4(1H)-one (19c) Compound 19c was prepared from 18c in 46% yield using a similar approach to that described for 4f.

To a mixture of 4-methoxy-3,5-dimethyl-2-[[[6-methyl-5-(piperydin-4-yl)pyridin-2-yl]oxy]methyl]pyridine (17; 200 mg, 0.59 mmol) and tolue (6.0 mL) were added N-bromo-4-flurobenzene (0.13 mL, 1.2 mmol), r-BuONa (110 mg, 2.2 mmol), BINAP (22 mg, 0.03 mmol) and Pd(dba)2 (12 mg, 0.01 mmol) under an argon atmosphere. The mixture was refluxed for 18 h. After cooling to room temperature, the mixture was diluted with EtOAc and filtered through a Celite pad. The filtrate was washed with H2O and brine, dried over Na2SO4 and tert-Butyl6-[4-Methoxy-3,5-dimethylpyridin-2-yl]methyl]-2-methyl-3,6-dihydro[3,4-bipyrindin-1(2H)-carboxylate (22) Compound 22 was prepared from 2c, tert-butyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,6-dihydropyridine-1(2H)-carboxylate and Pd[PPh3]2 in 76% yield using a similar approach to that described for 3a.

4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3,6-dihydropyridine-1(2H)-carboxylate was washed with H2O and brine, dried over Na2SO4 and the residue was purified by column chromatography on silica gel (CHCl3/MeOH). The residue was purified by column chromatography on amino functionalized silica gel (hexane/CHCl3) to give the product (120 mg, 47%).

Cell Lines The breast cancer cell lines MDA-MB-453 and SK-BR-3 were purchased from American Type Culture Collection (Manassas, VA, U.S.A.). MDA-MB-453 was cultured in RPMI 1640 medium (Sigma-Aldrich Co. LLC., St. Louis, MO, U.S.A.) at 37°C in 5% CO2. All media were supplemented with 10% fetal bovine serum (Sigma-Aldrich Co. LLC.) and 1% penicillin/streptomycin (Cat. No. 15070-063; Life Technologies).

Whole Cell ELISA MDA-MB-453 cells were seeded onto 96-well clear flat plates at 1.5 × 10^4 cells/well. The following day, the cells were treated with the test compound at final concentrations of 0 (DMSO only), 0.1, 0.3, 1, 3, 10, 50, 100, 300, and 1000 nmol/L. For probing, the supernatant was discarded and the cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 10 min at room temperature. For probing, the supernatant was discarded and the cells were blocked with ODSYSS Blocking solution (Li-Co Bio-  sciences, Lincoln, NE, U.S.A.) for 1 h at room temperature. After blocking the supernatant, a primary phospho-Acetyl CoA Carboxylase (Ser79) Antibody (1:500 in ODSYSS Blocking solution; Cat. No. 3661, Cell Signaling Technologies, Danvers, MA, U.S.A.) was added. After incubating overnight at 4°C, the plates were washed 3 times with Tris-buffered saline containing 0.05% Tween20 (TBS/Tween). Goat anti-rabbit IRDye 800CW (Li-Co Biosciences; 1:1000 in ODSYSS Blocking solution) was added, and the plates were incubated
for 1 h at room temperature. After the incubation, the plates were washed 3 times with TBS/Tween and dried for at least 3 h at room temperature. Fluorescence signals were quantified using the Aerius automated infrared imaging system (Li-Cor Biosciences). The assays were performed in triplicate, and the data were analyzed using Prism5 software (GraphPad Software Inc., San Diego, CA, U.S.A.). The EC_{50} value of each test compound was calculated using Sigmoid-Emax model nonlinear regression analysis. The average signal was normalized by regarding the signal in the DMSO-treated group as 0% in each experiment.

**Cell Growth Inhibition Assay** Each breast cancer cells above were seeded onto non-adherent 384-well white plates (Thermo Scientific Nunc, Copenhagen, Denmark) at 500 cells/well. The following day, the test compound was added to each well at final concentrations of 0 (DMSO only), 0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nmol/L. The final concentration of DMSO in each well was 0.1% (v/v). Four days after addition of the test compound, cell viability was determined using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, U.S.A.). CellTiter-Glo® Reagent was added to each well, and luminescence was measured using ARVO SX® (PerkinElmer, Inc., Waltham, MA, U.S.A.). The assay was performed in triplicate. Cell viability according to the luminescence intensity was normalized by regarding the average luminescence intensity following treatment with DMSO only as 100% and no luminescence intensity as 0%. The IC_{50} value of each test compound was calculated by Sigmoid-Emax nonlinear regression analysis using Prism5 software (GraphPad Software Inc.).

**Aqueous Solubility** The test compounds in 10 mM DMSO solution (13 µL) were diluted to 130 µM by adding the Japanese Pharmacopoeia 2nd fluid solution (pH 7.4). After incubation at 25°C for 20 h, precipitates were separated by filtration. The filtrate and a standard solution comprising a 10 mM DMSO stock solution of the compound were examined by regarding the signal in the DMSO-treated group as 0% and no luminescence intensity as 100% in each experiment. The ratio of the peak area of the standard solution was determined using liquid chromatography. The ratio of the peak area of the test compound was calculated using Sigmoid-Emax model nonlinear regression analysis. The average signal was normalized by regarding the signal in the DMSO-treated group as 0% in each experiment.

**Conflict of Interest** All authors were employees of Astellas Pharma Inc. when the study was conducted and have no further conflicts of interest to declare.

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