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Design, synthesis, and target identification of new hypoxia-inducible factor 1 (HIF-1) inhibitors containing 1-alkyl-1*H*-pyrazole-3-carboxamide moiety

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ABSTRACT

Hypoxia-inducible factor 1 (HIF-1) is a promising drug target for cancer chemotherapy. In our screening program aimed at identifying new HIF-1 inhibitors by using a hypoxia-responsive luciferase reporter gene assay, **KUSC-5001** containing the 1-alkyl-1*H*-pyrazole-3-carboxamide moiety was found as a potential hit molecule. During an extensive structure–activity relationship (SAR) study, we developed a more effective HIF-1 inhibitor **KUSC-5037** (IC_{50} = 1.2 μM). Under hypoxic conditions, **KUSC-5037** suppressed the HIF-1α (a regulatory subunit of HIF-1) mRNA, causing decreases in the gene expression of HIF-1 target genes such as carbonic anhydrase 9 (*CA9*) and vascular endothelial growth factor (*VEGF*) genes. Furthermore, by applying our fluorescent and bifunctional probes, ATP5B, a catalytic β subunit of mitochondrial F_oF_1-ATP synthase, was identified as a target protein of **KUSC-5037**. These results indicate that the derivatives of **KUSC-5037** containing the 1-alkyl-1*H*-pyrazole-3-carboxamide moiety are promising lead molecules for the inhibition of HIF-1 signaling via F_oF_1-ATP synthase suppression.

1. Introduction

Hypoxia-inducible factor 1 (HIF-1) plays a pivotal role in tumor survival and malignancy.[1] The transcriptional activity of HIF-1, a heterodimer comprising HIF-1α and HIF-1β, depends on HIF-1α, which degrades in an O_2 environment. HIF-1 binds to hypoxia-responsive elements (HRE) to regulate the expression of >800 genes, including carbonic anhydrase 9 (*CA9*) and vascular endothelial growth factor (*VEGF*) genes, leading to diverse phenotypes for adaptation to the tumor microenvironment; metabolic modulation, angiogenesis, invasion, and metastasis.[1] Therefore, HIF-1α is one of the most promising targets for cancer chemotherapy. Inhibitors affecting the HIF-1 signaling pathway have been identified; they include the synthetic molecules YC-1, IACS-01759, and CAY10585[2,3] and the natural products echinomycin and oligomycin.[4,5] For example, YC-1, the most widely used HIF-1 inhibitor, suppresses HIF-1-mediated transcription under hypoxic conditions, suppresses tumor metastasis and prevents the incidence of post-irradiation tumor recurrence.[6] IACS-01759 and oligomycin inhibit HIF-1 activity by targeting mitochondrial respiratory complex I and complex V, respectively.

Thus far, we have identified microbial metabolite verucopeptin[7] and synthetic 1-ethylpyrazole-3-carboxamides[8] as promising lead molecules for the inhibition of HIF-1 activity.[9] During the ongoing screening program aimed at identifying new HIF-1 inhibitors by using a hypoxia-responsive luciferase reporter gene assay, **KUSC-5001** containing the 1-alkyl-1*H*-pyrazole-3-carboxamide moiety was found as an appropriate

Abbreviations: HIF-1, hypoxia-inducible factor 1; Boc, *t*-butoxycarbonyl; CA, carbonic anhydrase; DIPEA, *N*,*N*-diisopropylethylamine; SAR, structure-activity relationship; HRE, hypoxia-response element; VEGF, vascular endothelial growth factor; TBAI, tetrabutylammonium iodide; HATU, 1-(Bis(dimethylamino)methylene)-1*H*-1,2,3-triazolo(4,5-*b*)pyridinium 3-oxide hexafluorophosphate.

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hit molecule from our in-house chemical library. Herein, we report our finding on the structure–activity relationship (SAR) of **KUSC-5001** and its derivatives and the HIF-1 inhibitory activity (e.g. potency, target protein, and mode of action) of a more effective HIF-1 inhibitor **KUSC-5037**.

2. Results and discussion

2.1. Design and synthesis of KUSC-5001 and its derivatives

KUSC-5001 with the 1-alkyl-1*H*-pyrazole-3-carboxamide moiety was identified from our in-house chemical library found as a hit molecule for the inhibition of HIF-1 activity. An hypoxia-responsive element (HRE)-driven luciferase assay using HeLa/HRE/Luc cells revealed that **KUSC-5001** inhibited HIF-1 activity with an IC_{50} of 18 μM.

We synthesized **KUSC-5001** and its derivatives by conjugating the substituted aniline (left segment), 1-(bromomethyl)-1*H*-pyrazole-3-carboxylate (middle segment) and 4-nitro-1*H*-pyrazole (right segment). Substitutions of *p*-bromoaniline (region 1) and 4-nitro-1*H*-pyrazole (region 2) were conducted and the 1*H*-pyrazole ring in the middle segment was replaced by other aromatic rings (region 3).

The synthesis route of the **KUSC-5001** analogues is shown in Scheme 1. First, 1-(bromomethyl)-1*H*-pyrazole-3-carboxylate was synthesized as reported.[10] Commercially available 4-nitro-1*H*-pyrazole was reacted with an excess of 1-(bromomethyl)-3-carboxylate in the presence of potassium carbonate in DMF to produce the conjugated ester **S1** in 96% yield. The ester was hydrolyzed with NaOH to generate the carboxylic acid **S2** in 61% yield, which was condensed with various aniline analogues to yield the corresponding amides **KUSC-5001** ~ **KUSC-5008**, **KUSC-5010** ~ **KUSC-5013**, **KUSC-5027**, and **KUSC-5028**. The synthesis route of **KUSC-5015** and **KUSC-5021** is shown in Scheme S1.

KUSC-5014, **KUSC-5019**, **KUSC-5020**, and **KUSC-5029** were synthesized following the procedure for **KUSC-5001**, but 4-nitro-1*H*-pyrazole was replaced with 1*H*-imidazole, 1*H*-pyrrole, 4-nitro-1*H*-imidazole and 3-nitro-1*H*-pyrrole, respectively (Scheme 2A). The synthesis route of **KUSC-5017** and **KUSC-5025** is shown in Scheme 2B. The nitro group of ethyl 1-((4-nitro-1*H*-pyrazol-1-yl)methyl)-1*H*-pyrazole-3-carboxylate **S1** was reduced by catalytic hydrogenation using 10% Pd/C to produce amine, which was simultaneously protected by $(Boc)_2O$ to yield the *N*-Boc protected carbamide **S10** in 87% yield. The obtained carbamide was hydrolyzed with NaOH to generate carboxylic acid **S11**, which was condensed with *p*-bromoaniline to yield the amide **S12** in 87% yield over two steps. The *N*-Boc-protected amide **S12** was deprotected with 4 M HCl in dioxane to generate the amine hydrochloride **KUSC-5017** in 73% yield, which was acetylated by acetyl chloride to yield the corresponding analogue **KUSC-5025** in 75% yield.

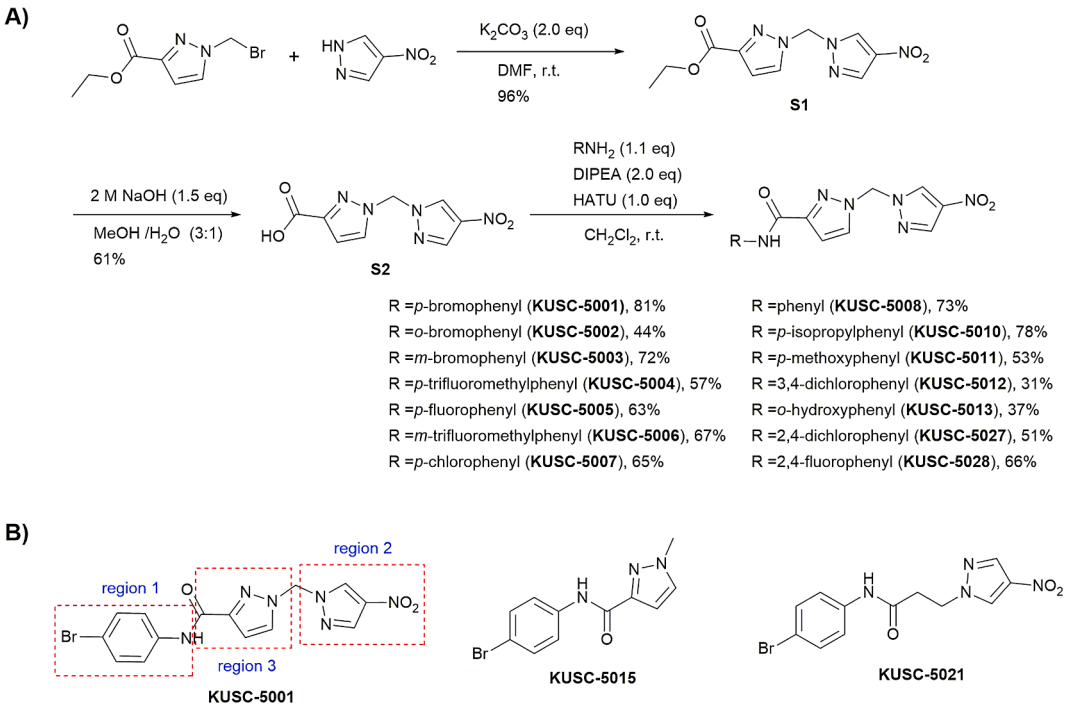
The synthesis route of **KUSC-5035** ~ **KUSC-5040** is shown in Scheme 3A. **KUSC-5035** ~ **KUSC-5040** were synthesized following the procedure for **KUSC-5017** and **KUSC-5025**, but *p*-trifluoromethylaniline was used instead of *p*-bromoaniline. In addition, **KUSC-5035** was acylated by various acyl chlorides in the final step to yield the corresponding analogues **KUSC-5036** ~ **KUSC-5040**. The synthesis schemes of **KUSC-5046**, **KUSC-5049**, and **KUSC-5056** are shown in Scheme S2.

Finally, BODIPY-FL NHS ester was activated with DIPEA and condensed with **KUSC-5035** to yield the fluorescent probe **KUSC-5054**. Moreover, **KUSC-5035** was condensed with 3-(3-(pent-4-yn-1-yl)–3*H*-diazirin-3-yl)propanoic acid **S15** to produce the bifunctional probe **KUSC-5051** (Schemes 3C and 3D).

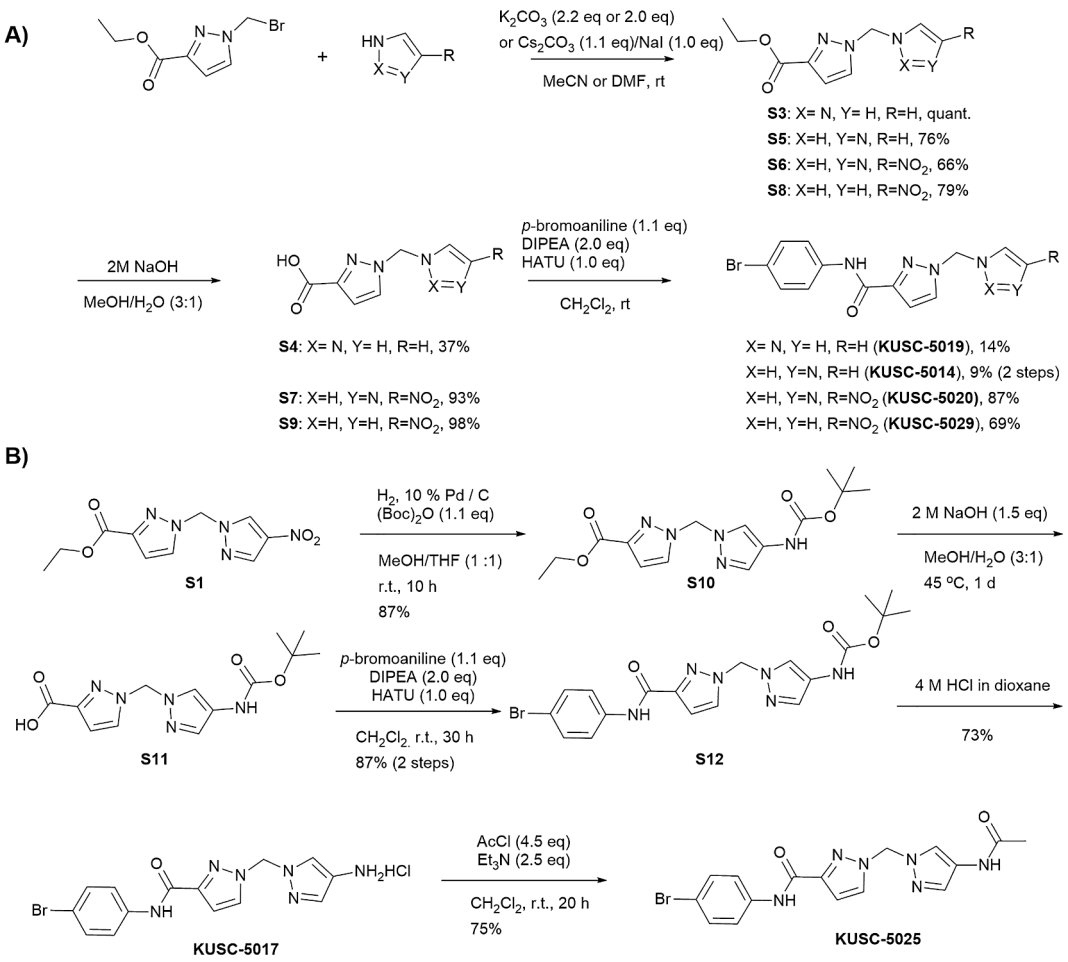
2.2. Structure-activity relationship of KUSC-5001 and its derivatives

The HIF-1 inhibitory activity of the synthetic derivatives was examined using HeLa cells stably-expressing x5HRE-luciferase reporter.[11] Camptothecin, a well-known topoisomerase I inhibitor, was used as a positive control. As mentioned above, **KUSC-5001** showed HIF-1 inhibitory activity with an IC_{50} of 18 μM. In contrast, **KUSC-5015** and **KUSC-5021** (Scheme 1B) lacking region 2 and region 3, respectively, did not show any HIF-1 inhibitory activity. Therefore, the three aromatic groups of regions 1–3 significantly affect the inhibitory activity of the synthesis derivatives.

We then evaluated the effect of aromatic substitution in region 1 (Table 1) on the HIF-1 inhibitory activity of the synthetic derivatives. **KUSC-5002**, **KUSC-5003**, and **KUSC-5008** exhibited lower HIF-1 inhibitory activity than **KUSC-5001**, indicating that the *p*-substitution



Scheme 1. A) Synthesis of KUSC-5001 ~ KUSC-5008, KUSC-5010 ~ KUSC-5013, KUSC-5027, and KUSC-5028. B) Structures of KUSC-5001, KUSC-5015, and KUSC-5021.



Scheme 2. A) Synthesis of KUSC-5014, KUSC-5019, KUSC-5020, and KUSC-5029. B) Synthesis of KUSC-5017 and KUSC-5025.

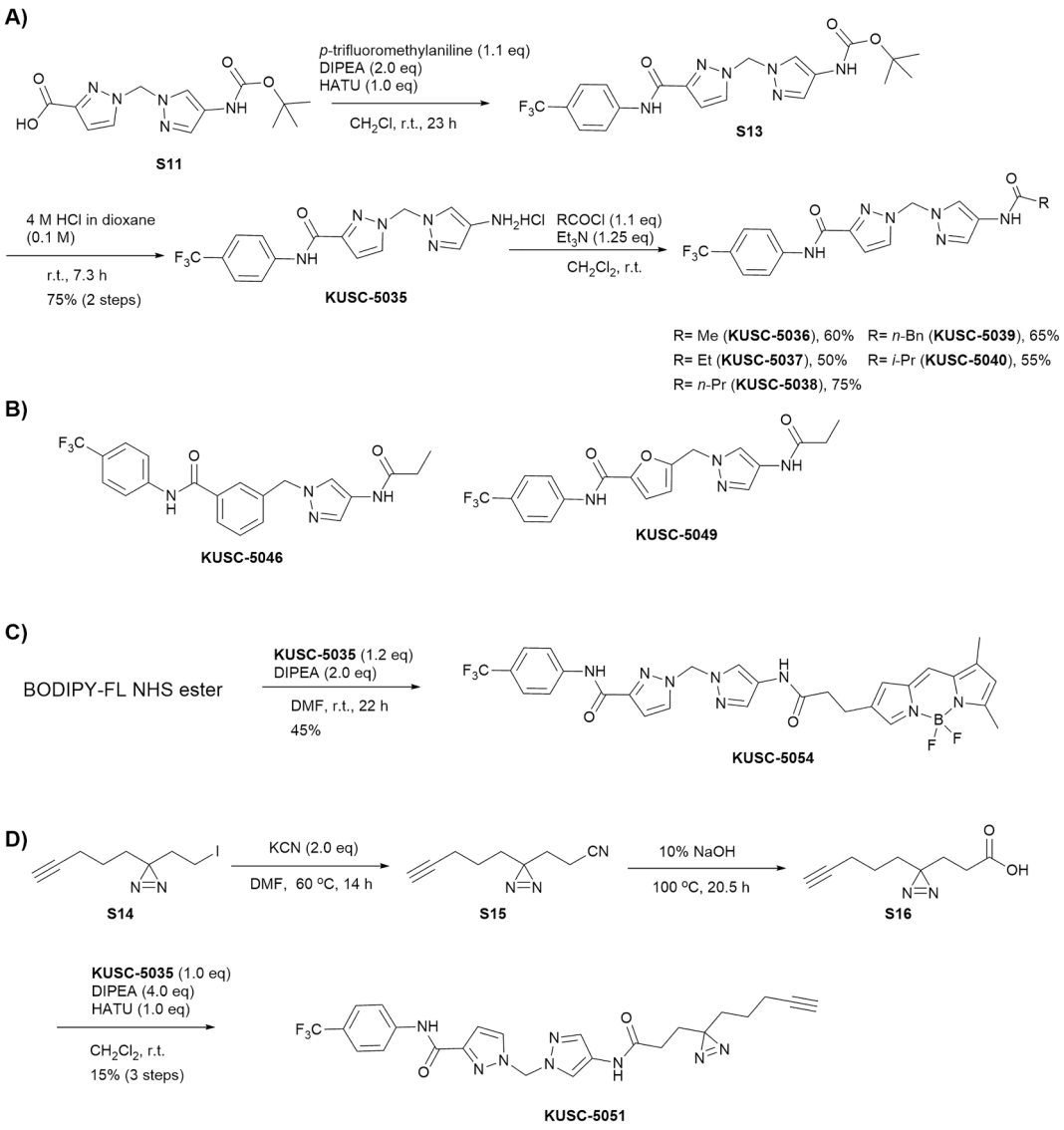
on the phenyl group was the most effective at improving inhibitory activity. Further derivatives were synthesized and evaluated in accordance with the Topliss Tree.[12] The inhibitory activities of **KUSC-5004** containing *p*-CF_3 and **KUSC-5001** were almost the same, whereas that of **KUSC-5006** containing *m*-CF_3 was lower. Therefore, *p*-substitution using electron-withdrawing groups (**KUSC-5001**, **KUSC-5004**, **KUSC-5005**, and **KUSC-5007**), not electron-donating groups (**KUSC-5010** and **KUSC-5011**), improved the HIF-1 inhibitory activity of the synthetic derivatives.

We next attempted to optimize the 4-nitro-1*H*-pyrazole moiety in the region 2 (Table 2). The inhibitory activity of **KUSC-5019** containing an unsubstituted pyrazole ring was *ca.* 3.5 times less than that of **KUSC-5001**. A comparison of the effects of 1*H*-pyrazole, 1*H*-imidazole, and 1*H*-pyrrole (**KUSC-5001**, **KUSC-5020**, and **KUSC-5029**) revealed that the pyrazole ring (**KUSC-5001**) was the most effective at enhancing inhibitory activity. In addition, the nitro group on the pyrazole ring was replaced by amine (**KUSC-5017**) and the *N*-acyl group (**KUSC-5025**). Compared with **KUSC-5017**, the inhibitory activity of **KUSC-5025** was enhanced, which was attributed to the effect of the hydrophobic *N*-acyl group on membrane permeability.

Based on the optimization results of region 1, as shown in Table 1, we proceeded to evaluate **KUSC-5036** containing *p*-CF_3 on the phenyl group in region 1 and 4-*N*-acetyl pyrazole in region 2, respectively, which was three times more potent than **KUSC-5001** in terms of HIF-1 inhibitory activity. We replaced the acetyl group with a set of acyl groups, namely propionyl, butyryl, benzyl, and *i*-propionyl groups (denoted as **KUSC-5037**, **KUSC-5038**, **KUSC-5039**, and **KUSC-5040**, respectively) (Table 3). These four molecules showed almost the same HIF-1 inhibitory activity, indicating the structural tolerance for these substituents. Among them, **KUSC-5037** exhibited the most effective HIF-1 inhibitory activity with an IC_{50} of 1.2 μM (Fig. S1), which was comparable to that of **KUSC-5056** containing alkylamine moiety. Encouraged by these results, we designed and synthesized a fluorescent probe **KUSC-5054** containing the BODIPY-FL functional group and a photoaffinity probe **KUSC-5051** containing the diazirine and alkyne functional groups, which were attached at the C-4 position on the pyrazole ring (Schemes 3C and 3D).[13–15] Finally, we replaced the pyrazole ring in region 3 of **KUSC-5037** with phenyl (**KUSC-5046**) and furan ring (**KUSC-5049**), both of which reduced the inhibitory activity of **KUSC-5037**, indicating the significance of the pyrazole ring in region 3.

2.3. Biological activity of an effective HIF-1 inhibitor KUSC-5037

We evaluated the effectiveness of **KUSC-5037** at inhibiting HIF-1 transcriptional activity by measuring the endogenous mRNA levels of HIF-1 target genes, specifically the carbonic anhydrase 9 (*CA9*) and vascular endothelial growth factor (*VEGF*) genes. Camptothecin was also used as a positive control. As shown in Fig. 1A, quantitative RT-PCR analysis revealed that **KUSC-5037** suppressed *CA9* and *VEGF* expression with comparable to its HRE-Luc inhibition. Moreover, HIF-1 α transcription was also suppressed by **KUSC-5037** under hypoxic conditions, which in turn significantly suppressed hypoxia-induced *CA9* and *VEGF* expression. We then examined the effect of **KUSC-5037** on the HIF-1α protein level. When the cells were treated with **KUSC-5037** under hypoxic conditions, the HIF-1α protein level decreased in a dose-dependent manner, whereas the α-tubulin protein level was not affected



Scheme 3. A) Synthesis of KUSC-5035 ~ KUSC-5040. B) Structures of KUSC-5046 and KUSC-5049. C) Synthesis of a fluorescence probe KUSC-5054. D) Synthesis of a bifunctional probe KUSC-5051 for pull-down of binding protein (s) of KUSC-5037.

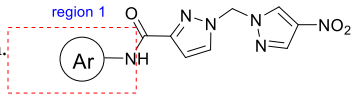
(Fig. 1B).

To determine its mode of action, we investigated the subcellular localization of **KUSC-5037**. Our SAR study indicated that a few *N*-acyl chains, as substituents in region 2, did not significantly affect the HIF-1 inhibitory activity of the synthetic derivatives. Therefore, we designed and synthesized a fluorescent probe **KUSC-5054** containing the BODIPY-FL functional group. After treating the HeLa cells with **KUSC-5054**, mitochondria and nuclei were stained by MitoTracker Red CMXRos and Hoechst 33342, respectively. As shown in Fig. 1C, **KUSC-5054** fluorescence merged with the MitoTracker Red CMXRos stains, indicating that **KUSC-5054** was localized in mitochondria.

Next, we attempted to identify the binding protein(s) of **KUSC-5037** by using a bifunctional fishing probe **KUSC-5051** containing the diazirine and alkyne functional groups. The diazirine and alkyne functional groups were introduced to facilitate a photoaffinity labeling and pull-down by click-chemistry, respectively. HeLa cell lysates were fractionated into mitochondria, cytoplasm, and debris. The molecular probe **KUSC-5051** was irradiated at 365 nm in the absence or presence of **KUSC-5037** and then incubated with each fractionated lysate (mitochondria, cytoplasm, or debris). Results of the pull-down experiments suggested that the *ca.* 60 kDa protein in the mitochondria fraction was a binding protein of **KUSC-5051** (Fig. 2A), which was strongly supported by the competition with an excess of **KUSC-5037**. LC-MS/MS analysis indicated that the 60 kDa protein was the ATP5B protein (a F_oF_1-ATP synthase subunit beta) (Table S1).[16] To confirm this, western blotting was performed using an anti-ATP5B antibody and the 60 kDa protein was determined as the ATP5B protein, as shown in Fig. 2B. Because F_oF_1-ATP synthase is localized in the mitochondria membrane, it is plausible that **KUSC-5037** targets ATP5B in the cells. Furthermore, we tested the effect of **KUSC-5037** on the enzymatic activity of F_oF_1-ATP synthase using the Complex V activity assay kit. As shown in Fig. 2C, **KUSC-5037** significantly inhibited the F_oF_1-ATP synthase as oligomycin did, presumably by binding to ATP5B.

Finally, we investigated the effect of **KUSC-5037** on HeLa cells using the XF mito-stress test.[17] As shown in Fig. 2D, **KUSC-5037** inhibited mitochondrial respiratory complex V with similar potency of a F_oF_1-ATP synthase inhibitor oligomycin inhibition.[5,18] The oxygen consumption rate (OCR) decreased after **KUSC-5037** (0.1, 1.0 μM) was injected, and the OCR level immediately recovered after an uncoupled reagent FCCP was subsequently injected. Furthermore, a final injection of rotenone/antimycin A reduced the OCR level, suggesting that **KUSC-5037** functioned as an inhibitor of the mitochondrial respiratory complex V.

Table 1
Effects of KUSC-5001 derivatives (region 1) on HIF-1 transcriptional activity. HeLa/HRE/Luc cells were treated with solvent or the test compounds for 1 h under normoxic conditions (21% O_2) and further incubated under hypoxic conditions (1% O_2) for 24 h. Luciferase activity was measured using an EnVision luminometer ($n = 3$). One μM of camptothecin exhibited the 62.3% inhibitory activity. Representative data from two independent experiments are shown.



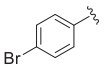
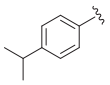
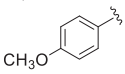
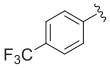
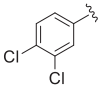
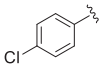
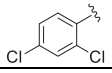
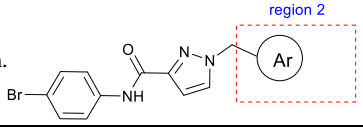
KUSC-	R	IC_{50} (μM)	KUSC-	R	IC_{50} (μM)
5001		18	**5008**		>100
5002		45	**5010**		21
5003		35	**5011**		>100
5004		18	**5012**		13
5005		28	**5013**		>100
5006		33	**5027**		35
5007		20	**5028**		81

Table 2
Effects of KUSC-5001 derivatives (region 2) on HIF-1 transcriptional activity. HeLa/HRE/Luc cells were treated with solvent or the test compounds for 1 h under normoxic conditions (21% O_2) and further incubated under hypoxic condition (1% O_2) for 24 h. Luciferase activity was measured using an EnVision luminometer ($n = 3$). Representative data from two independent experiments are shown.



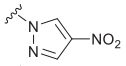
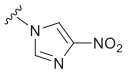
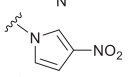
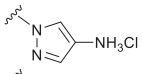
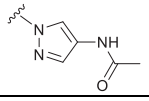
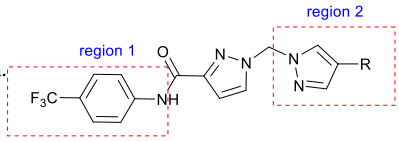
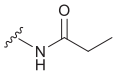
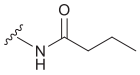
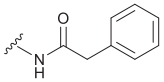
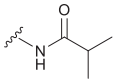
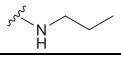
KUSC-	R	IC_{50} (μM)
5001		18
5019		66
5020		58
5014		34
5029		27
5017		20
5025		14

Table 3
Effects of KUSC-5036 derivatives on HIF-1 transcriptional activity. HeLa/HRE/Luc cells were treated with solvent or the test compounds for 1 h under normoxic conditions (21% O_2) and further incubated under hypoxic conditions (1% O_2) for 24 h. Luciferase activity was measured using an EnVision luminometer ($n = 3$). Representative data from two independent experiments are shown.



KUSC-	R	IC_{50} (μM)
5036		5.5
5037		1.2
5038		1.8
5039		1.4
5040		6.9
5056		1.2

Rotenone and antimycin A are inhibitors of mitochondrial respiratory complex I and complex III, respectively.[17,18] In addition, OCR reduction by **KUSC-5037** elevated the intracellular local oxygen concentration, which likely suppressed the HIF-1α level. Furthermore, we investigated the effect of **KUSC-5037** on the OCR level of cells treated with oligomycin as shown in Fig. S2. After the addition of **KUSC-5037**, the OCR level recovered by FCCP was significantly reduced in the presence of oligomycin, suggesting that the mode of action of **KUSC-5037** slightly differs from that of oligomycin. Furthermore, **KUSC-5037** exhibited cell growth inhibitory activity with IC_{50} values of 34.5 μM and 0.6 μM against human cervical cancer HeLa S3 cells and fibrosarcoma HT1080 cells, respectively, as shown Fig. 2E, while those of oligomycin were 5.3 μM and < 0.1 μM (data not shown), respectively.

3. Conclusion

In the present study, we identified **KUSC-5001** containing the 1-alkyl-1*H*-pyrazole-3-carboxamide moiety as a potential HIF-1 inhibitor and developed a more effective derivative **KUSC-5037**. Our extensive SAR study revealed that: i) *p*-substitution of the phenyl group in region 1 using electron-withdrawing groups, not electron-donating groups improved HIF-1 inhibitory activity; ii) the hydrophobic *N*-acyl group at C-4 on the pyrazole ring in the region 3 enhanced inhibitory activity by facilitating membrane permeability; and iii) the pyrazole ring in the region 3 played a key role in HIF-1 inhibitory activity. **KUSC-5037** blocked the transcription of HIF-1 α gene. Based on the SAR study, a fluorescent probe **KUSC-5054** containing the BODIPY-FL functional group and a bifunctional probe **KUSC-5051** containing the diazirine and alkyne functional groups were designed and synthesized, both of which indicated that **KUSC-5037** targeted the ATP5B protein, a catalytic β subunit of F_oF_1-ATP synthase. Oligomycin also inhibits the HIF-1 signaling pathway via the inhibition of a target protein F_oF_1-ATP synthase.[5] F_oF_1-ATP synthase plays a key role in the energy production of cancer cells, which is essential for cell survival.[15,19,20] The scaffold of **KUSC-5037** is different from those of the reported natural and synthetic F_oF_1-ATP synthase inhibitors.[20] Recently, a new synthetic small molecule Gboxin was reported to exhibit antitumor activity especially against

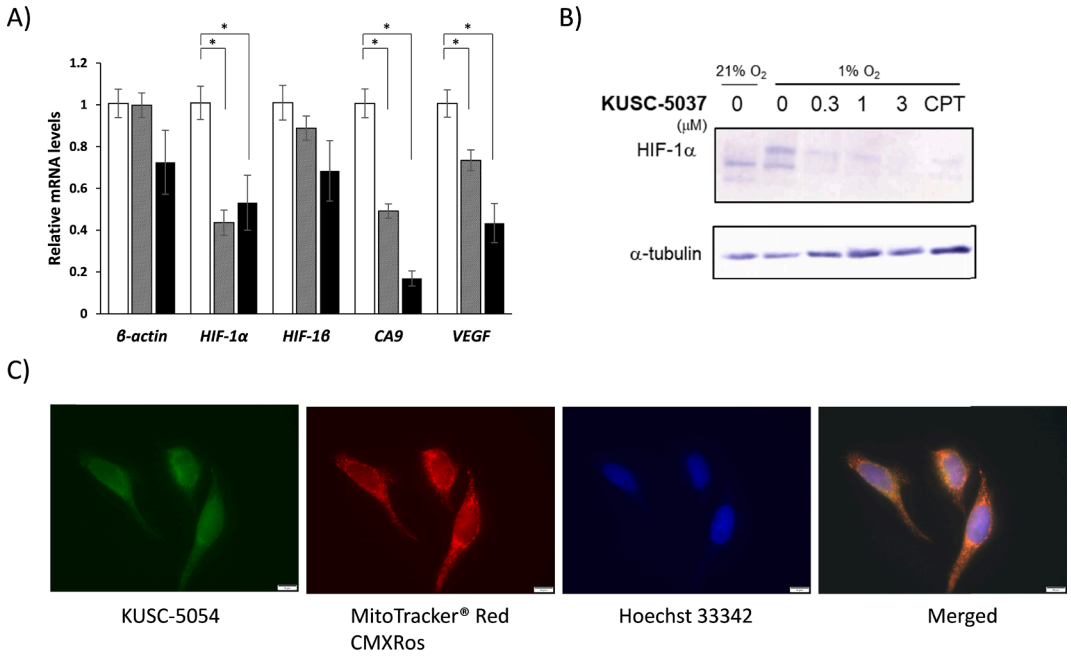


Fig. 1. Effects of **KUSC-5037** on transcriptional activity of HIF-1 and HIF-1α protein level.

A) Cells were treated with solvent (blank column), camptothecin (CPT, 0.5 μM, hatched column), or **KUSC-5037** (3 μM, black column) for 1 h under normoxic conditions (21% O_2), and further incubated under hypoxic conditions (1% O_2) for 4 h. RNA was then collected and subjected to qRT-PCR to quantify the mRNA levels of the indicated genes ($n = 2$). Representative data from three independent experiments are shown. $^*P < 0.05$ (Student's *t*-test).

B) Cells were treated with solvent, camptothecin (CPT, 0.5 μM), or **KUSC-5037** (0.3, 1, 3 μM) for 1 h under normoxic conditions (21% O_2), and further incubated under hypoxic conditions (1% O_2) for 25 h. The cells were lysed and subjected to western blotting using antibodies against HIF-1α and α-tubulin Representative data from three independent experiments are shown.

C) Cells were treated with 1 μM **KUSC-5054** for 3 h. MitoTracker Red CMXRos (100 nM) and Hoechst 33342 (10 μM) were then added, and the cells were fixed and observed using a fluorescence microscope. Scale bar, 10 μm. Representative data from three independent experiments are shown.

glioblastoma by inhibiting of F_oF_1-ATP synthase activity.[21] Our experiments on the HeLa cells using the XF mito-stress test also revealed that **KUSC-5037** inhibited mitochondrial respiratory complex V, although its mode of action was slightly different from that of oligomycin. While the link between F_oF_1-ATP synthase activity and HIF-1 activity remains unclear, **KUSC-5037** can be a promising lead compound for the development of new antitumor drugs.

4. Experimental section

4.1. Chemistry

4.1.1. General

^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA 500 (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR), or a JEOL ECZ600 (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR) spectrometer. Chemical shifts are denoted in δ (ppm) relative to residual solvent peaks as internal standard ($CDCl_3$, ^{1}H δ 7.26, ^{13}C δ 77.16; CD_3OD, ^{1}H δ 3.31, ^{13}C δ 49.00; $(CD_3)_2CO$ ^{1}H δ 2.05, ^{13}C 29.84; DMSO-d_6 ^{1}H δ 2.50, ^{13}C 39.52). ESI-MS and LC-MS experiments were recorded on a Shimadzu LCMS-IT-TOF. High performance liquid chromatography (HPLC) experiments were performed with SHIMADZU HPLC system equipped with LC-20AD intelligent pump. All reactions sensitive to air and/or moisture were conducted under nitrogen atmosphere using dry, freshly distilled solvents, unless otherwise noted. All reagents were used as supplied unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using E. Merck Silica gel 60 F_{254} pre-coated plates. Column chromatography was performed using 40–50 μm Silica Gel 60 N (Kanto Chemical Co., Inc.).

4.1.2. Synthesis of KUSC-5001 ~ KUSC-5008, KUSC-5010 ~ KUSC-5013, KUSC-5027, and KUSC-5028

4.1.2.1. Ethyl 1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxylate (S1). To a stirred solution of 4-nitro-1*H*-pyrazole (100.2 mg, 0.836 mmol) in DMF (0.49 mL) were added ethyl 1-(bromomethyl)-3-carboxylate (227.1 mg, 0.974 mmol) and potassium carbonate (244.9 mg, 1.77 mmol) at room temperature. After being stirred for 5.3 h, the reaction was quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1) to afford the carboxylate **S1** as a colorless solid (225.1 mg, 96%).

^{1}H NMR (500 MHz, DMSO-d_6): δ1.27 (t, $J = 7.0$ Hz, 3H), 4.26 (q, $J = 7.0$ Hz, 2H), 6.59 (s, 2H), 6.81 (d, $J = 3.0$ Hz, 1H), 7.74 (d, $J = 2.0$ Hz, 1H), 8.18 (d, $J = 3.0$ Hz, 1H), 8.35 (s, 1H), 9.21 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 14.17, 60.50, 65.35, 109.16, 131.42, 133.40, 135.61, 136.82, 144.41, 161.29.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{10}H_{11}N_5O_4Na^+$ 288.0703; found, 288.0725.

4.1.2.2. 1-((4-Nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxylic acid (S2). To a stirred solution of the carboxylate **S1** (53.0 mg, 0.20 mmol) in MeOH (3.0 mL) and water (1.0 mL), was added sodium hydroxide (11.2 mg, 0.28 mmol) at room temperature. After being stirred reflux for 18 h, the mixture was then cooled to room temperature. After the reaction solution was acidified with conc. HCl at pH 2, $CHCl_3$ was added and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layers were dried over Na_2SO_4, and concentrated in vacuo to afford the carboxylic acid **S2** as a colorless

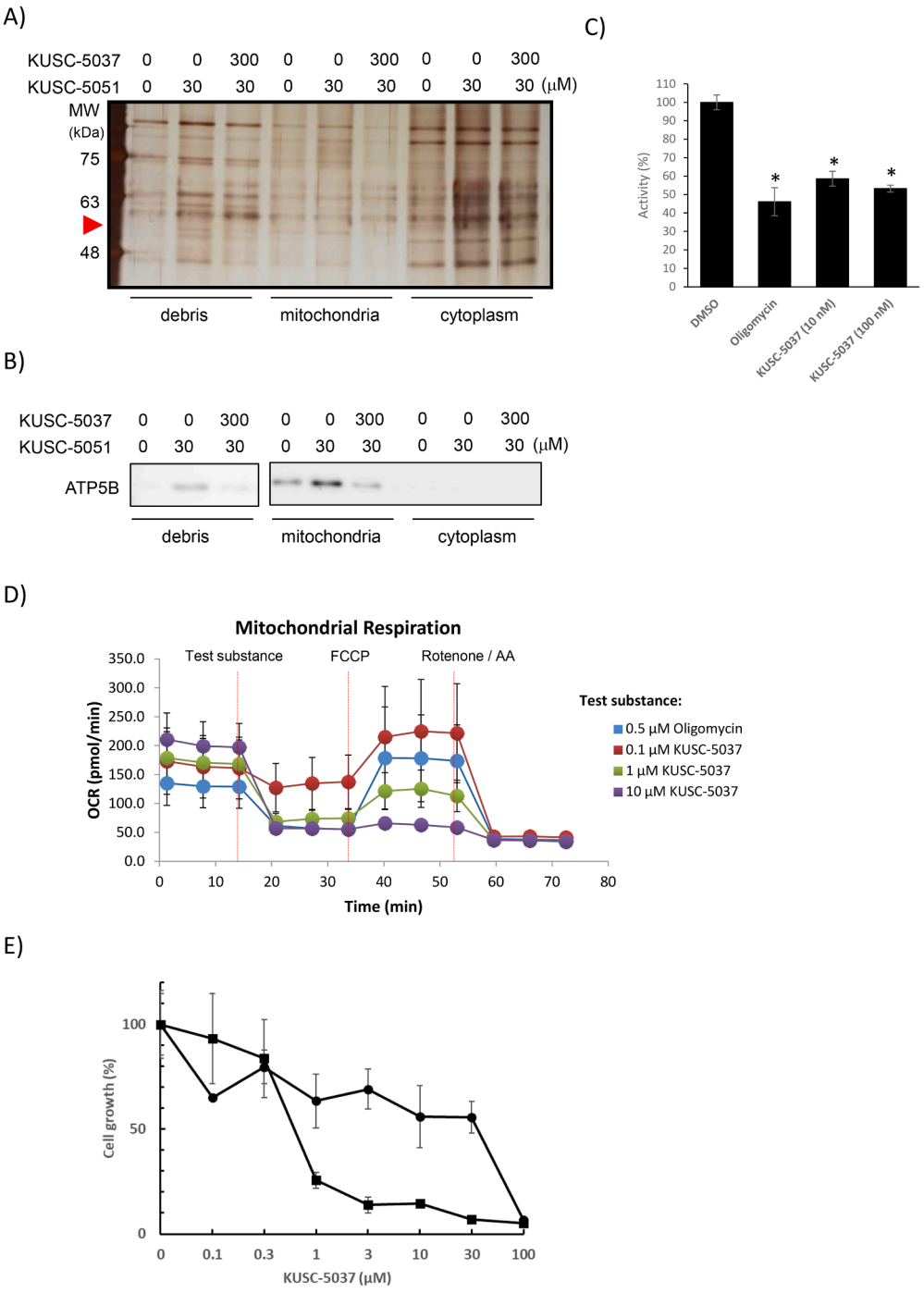


Fig. 2. Target identification of **KUSC-5037** using a bifunctional probe **KUSC-5051** and effects of **KUSC-5037** on mitochondrial oxygen consumption rate.

A) Subcellular fractioned HeLa S3 lysates were incubated with **KUSC-5051** in the presence or absence of a competitor **KUSC-5037**. The mixtures were photo-cross-linked and subjected to click chemistry with biotin-azide followed by NeutrAvidin enrichment. Binding proteins were eluted by boiling in SDS-loading buffer, subjected to SDS-PAGE, and detected with silver stain followed by tryptic digestion and LC-MS/MS analysis. Arrowhead marks an ATP5B protein band, the binding protein of **KUSC-5051**. Representative data from three independent experiments are shown.

B) Binding protein of **KUSC-5051** prepared in Fig. 2A was subjected to western blotting using an antibody against ATP5B. Representative data from three independent experiments are shown.

C) Mitochondrial complex V activity was measured by using a bovine heart mitochondria solution. Absorbance at 340 nm was measured with a spectrophotometer after the solution was incubated for 2 h in the presence of DMSO (1.0%), oligomycin (12.6 μM), and **KUSC-5037** (10, 100 nM) ($n = 3$). Representative data from three independent experiments are shown. *$P < 0.05$ (Student's t-test).

D) Cells were seeded in Seahorse microplates and OCR was measured. Cells were treated with test substance (oligomycin (0.5 μM) or **KUSC-5037** (0.1, 1, 10 μM)). After injections of FCCP (0.5 μM) and rotenone/antimycin A (AA) (0.5 μM each), OCR was measured using a Seahorse XF analyzer (n = 6, mean ± SEM). Representative data from three independent experiments are shown.

E) HeLa S3 cells (circle) and HT1080 cells (square) were treated with solvent or serial dilutions of **KUSC-5037**, respectively, and incubated for 72 h. Cell viability was measured using Cell Counting Kit-8 ($n = 3$). Representative data from two independent experiments are shown.

solid (37.8 mg, 61%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.57 (s, 2H), 6.75 (d, J = 2.0 Hz, 1H), 8.15 (d, J = 2.5 Hz, 1H), 8.35 (s, 1H), 9.19 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 65.36, 109.15, 131.41, 133.19, 135.61, 136.79, 145.36, 162.76.

HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_8H_8N_5O_4^+$ 238.0571; found, 238.0536.

4.1.2.3. N-(4-Bromophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5001). To a stirred solution of the carboxylic acid **S2** (10.0 mg, 0.042 mmol) in dichloromethane (0.57 mL) were added DIPEA (15.0 μL, 0.086 mmol) and HATU (16.0 mg, 0.042 mmol) at room temperature. After being stirred for 30 min, *p*-bromoaniline (8.0 mg, 0.047 mmol) was added, and the mixture was stirred at room temperature for 22 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$ and the combined organic layers were washed with 1 M HCl and sat. $NaHCO_3$ aq., dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford **KUSC-5001** as a colorless solid (13.4 mg, 81%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.38 (s, 2H), 6.96 (m, 1H), 7.47 (dd, J = 8.8 Hz, 2H), 7.59 (d, J = 9.0 Hz, 2H), 7.76 (d, J = 2.5 Hz, 1H), 8.12 (d, J = 2.0 Hz, 1H), 8.43 (s, 1H), 8.58 (br, 1H).

^{13}C NMR (150 MHz, $CDCl_3$): δ 66.44, 109.21, 117.15, 121.45, 129.03, 132.22, 132.71, 136.67, 137.18, 137.26, 149.08, 158.78.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{11}BrN_6O_3Na^+$ 412.9968; found, 412.9983.

4.1.2.4. N-(2-Bromophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5002). A colorless solid.

^{1}H NMR (600 MHz, Acetone-d_6): δ 6.73 (s, 2H), 6.89 (d, J = 2.5 Hz, 1H), 7.09 (td, J = 6.0, 1.5 Hz, 1H), 7.42 (td, J = 6.5, 1.0 Hz, 1H), 7.67 (dd, J = 3.5, 1.0 Hz, 1H), 8.21 (d, J = 2.0 Hz, 1H), 8.22 (s, 1H),8.48 (dd, J = 3.5, 1.0 Hz, 1H), 9.05 (s, 1H), 9.38 (br, 1H).

^{13}C NMR (150 MHz, Acetone-d_6): δ 66.94, 108.23, 114.03, 122.30, 126.12, 129.34, 131.62, 133.39, 134.57, 136.89, 137.37, 137.41, 148.98, 159.62.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{11}BrN_6O_3Na^+$ 412.9968; found, 412.9980.

4.1.2.5. N-(3-Bromophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5003). A colorless solid.

^{1}H NMR (600 MHz, Acetone-d_6): δ 6.66 (s, 2H), 6.87 (d, J = 2.4 Hz, 1H), 7.27–7.31 (m, 2H), 7.73 (dd, J = 1.8, 9.0 Hz, 1H), 8.16–8.17 (m, 2H), 8.21 (s, 1H), 8.94 (s, 1H), 9.58 (br, 1H).

^{13}C NMR (150 MHz, Acetone-d_6): δ 66.95, 108.55, 119.44, 122.71, 123.37, 127.32, 131.28, 131.33, 134.21, 137.20, 141.13, 149.28, 160.30.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{11}BrN_6O_3Na^+$ 412.9968; found, 412.9977.

4.1.2.6. 1-((4-Nitro-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5004). A colorless solid.

^{1}H NMR (500 MHz, Acetone-d_6): δ 6.67 (s, 2H), 6.89 (d, J = 3.0 Hz, 1H), 7.69 (d, J = 9.0 Hz, 2H), 8.04 (d, J = 8.5 Hz, 2H), 8.18 (d, J = 2.5 Hz), 8.22 (s, 1H), 8.95 (s, 1H) 9.76 (s, 1H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 66.97, 108.65, 120.65, 124.36, 125.56, 126.51, 126.82, 126.85, 131.28, 134.29, 137.22, 137.41, 143.09, 149.20, 160.50.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{15}H_{11}F_3N_6O_3Na^+$ 403.0737; found, 403.0757.

4.1.2.7. N-(4-Fluorophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5005). A colorless solid.

^{1}H NMR (500 MHz, Acetone-d_6): δ 6.65 (s, 2H), 6.85 (d, J = 2.0 Hz, 1H), 7.11 (t, J = 8.5 Hz, 2H), 7.81 (t, J = 8.0 Hz, 2H), 8.15 (d, J = 2.5 Hz, 1H), 8.21 (s, 1H), 8.94 (s, 1H), 9.48 (br, 1H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 66.94, 108.44, 115.99, 122.63, 131.24, 134.10, 135.86, 137.18, 149.56, 158.88, 160.07, 160.79.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{11}FN_6O_3Na^+$ 353.0769; found, 353.0774.

4.1.2.8. 1-((4-Nitro-1H-pyrazol-1-yl)methyl)-N-(3-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5006). A colorless solid.

^{1}H NMR (500 MHz, CD_3OD): δ 6.57 (s, 2H), 6.90 (d, J = 1.0 Hz, 1H), 7.42 (d, J = 8.5 Hz, 1H), 7.54 (t, J = 8.0 Hz, 1H), 7.95 (d, J = 8.0 Hz, 1H), 8.04 (s, 1H), 8.16 (s, 1H), 8.20 (s, 1H), 8.92 (br, 1H).

^{13}C NMR (125 MHz, CD_3OD): δ 66.96, 108.90, 118.11, 121.69, 124.45, 124.83, 126.60, 130.74, 131.60, 132.15, 134.30, 137.76, 140.28, 149.42, 162.08.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{15}H_{11}F_3N_6O_3Na^+$ 403.0737; found, 403.0740.

4.1.2.9. N-(4-Chlorophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5007). A colorless solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.33 (s, 2H), 6.97 (s, 1H), 7.33 (d, J = 8.5 Hz, 2H), 7.64 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 2.5 Hz, 1H), 8.12 (s, 1H), 8.43 (s, 1H), 8.58 (br, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 66.45, 109.20, 121.16, 129.03, 129.27,129.55 132.70, 136.17, 137.18, 137.28, 149.10, 158.79.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{11}ClN_6O_3Na^+$ 369.0473; found, 369.0483.

4.1.2.10. 1-((4-Nitro-1H-pyrazol-1-yl)methyl)-N-phenyl-1H-pyrazole-3-carboxamide (KUSC-5008). A colorless solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.33 (s, 2H), 6.97 (d, J = 2.5 Hz, 1H), 7.15 (t, J = 7.5 Hz, 1H), 7.37 (t, J = 8.0 Hz, 2H), 7.68 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 2.5 Hz, 1H), 8.12 (s, 1H), 8.44 (s, 1H), 8.57 (br, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 66.42, 109.12, 119.96, 124.61, 129.05, 129.25, 132.60, 137.12, 137.24, 137.58, 149.35, 158.83.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{12}N_6O_3Na^+$, 335.0863; found, 335.0864.

4.1.2.11. N-(4-Isopropylphenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5010). A colorless solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.25 (d, J = 7.0 Hz, 6H), 2.90 (septet, J = 7.0 Hz, 1H), 6.32 (s, 2H), 6.96 (d, J = 3.0 Hz, 1H), 7.22 (d, J = 8.5 Hz, 2H), 7.58 (d, J = 8.5 Hz, 2H), 7.75 (d, J = 2.5 Hz, 1H), 8.12 (s, 1H), 8.44 (s, 1H), 8.54 (br, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 24.16, 33.76, 66.40, 109.08, 120.10, 127.13, 129.08, 132.54, 135.23, 137.10, 137.20, 145.37, 149.41, 158.76.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{17}H_{18}N_6O_3Na^+$ 377.1333; found, 377.1352.

4.1.2.12. N-(4-Methoxyphenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5011). A colorless solid.

^{1}H NMR (500 MHz, Acetone-d_6): δ 3.78 (s, 3H), 6.64 (s, 2H), 6.84 (d, J = 2.0 Hz, 1H), 6.91 (d, J = 9.0 Hz, 2H), 7.69 (d, J = 9.0 Hz, 2H), 8.13 (s, 1H), 8.20 (s, 1H), 8.93 (s, 1H), 9.29 (s, 1H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 55.62, 66.90, 108.29, 114.64, 122.37, 131.22, 132.57, 133.99, 137.16, 137.38, 149.85, 157.08, 159.79.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{15}H_{14}N_6O_4Na^+$ 365.0969; found, 365.0990.

4.1.2.13. N-(3,4-Dichlorophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5012). A colorless solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.60 (s, 2H), 6.89 (d, J = 2.0 Hz, 1H), 7.59 (d, J = 9.0 Hz, 1H), 7.79 (dd, J = 9.0, 2.0 Hz, 1H), 8.15 (d, J =

3.0 Hz, 1H), 8.21 (d, $J = 3.0$ Hz, 1H), 8.39 (s, 1H), 9.17 (s, 1H), 10.42 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 65.42, 108.84, 120.41, 121.58, 125.18, 130.51, 130.79, 131.28, 133.71, 135.70, 136.71, 138.74, 147.67, 159.84.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{10}Cl_2N_6O_3Na^+$ 403.0084; found, 403.0092.

4.1.2.14. N-(2-Hydroxyphenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5013). A colorless solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.63 (s, 2H), 6.81 (t, $J = 7.5$ Hz, 1H), 6.85 (d, $J = 2.5$ Hz, 1H), 6.92 (m, 2H), 8.17 (d, $J = 8.0$ Hz, 1H), 8.22 (d, $J = 2.0$ Hz, 1H), 8.36 (s, 1H), 9.22 (d, $J = 7.0$ Hz, 2H), 10.19 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 65.28, 107.32, 114.78, 119.22, 119.74, 124.06, 126.02, 131.31, 134.11, 135.66, 136.76, 146.41, 147.84, 158.43.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{12}N_6O_4Na^+$ 351.0812; found, 351.0808.

4.1.2.15. N-(2,4-Dichlorophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5027). A colorless solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.63 (2H, s), 6.89 (1H, s), 7.47 (1H, d, $J = 8.5$ Hz), 7.73 (1H, s), 7.96 (1H, d, $J = 8.5$ Hz), 8.23 (1H, s), 8.37 (1H, s), 9.21 (1H, s), 9.65 (1H, s).

^{13}C NMR (125 MHz, DMSO-d_6): δ 65.40, 107.58, 125.89, 127.08, 127.87, 128.90, 129.47, 131.47, 133.55, 134.00, 135.71, 136.76, 147.07, 159.10.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{10}Cl_2N_6O_3Na^+$ 403.0084; found, 403.0106.

4.1.2.16. N-(2,4-Difluorophenyl)-1-((4-nitro-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5028). A colorless solid.

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.61 (s, 2H), 6.86 (d, $J = 2.5$ Hz, 1H), 7.10 (t, $J = 3.5$ Hz, 1H), 7.36 (t, $J = 3.0$ Hz, 1H), 7.66 (q, $J = 8.5$ Hz, 1H), 8.20 (d, $J = 2.5$ Hz, 1H), 8.36 (s, 1H), 9.18 (s, 1H), 9.77 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 65.38, 104.29, 107.57, 111.19, 121.89, 127.64, 131.35, 133.68, 135.71, 136.71, 147.37, 154.59, 156.53, 159.53.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{10}F_2N_6O_3Na^+$ 371.0675; found, 371.0683.

4.1.3. Synthesis of KUSC-5015 and KUSC-5021

4.1.3.1. N-(4-bromophnyl)-1-methyl-1H-pyrazole-3-carboxamide (KUSC-5015). To a stirred solution of 1-methyl-1*H*-pyrazole-3-carboxylic acid (30.0 mg, 0.238 mmol) in dichloromethane (3.2 mL) were added DIPEA (83 μL, 0.477 mmol) and HATU (90.5 mg, 0.238 mmol) at room temperature. After being stirred for 30 min, *p*-bromoaniline (45.0 mg, 0.262 mmol) was added, and the mixture was stirred at room temperature for 21 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$ and the combined organic layers were washed with 1 M HCl, sat. $NaHCO_3$ aq., and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was subjected to ODS HPLC on COSMOSIL 5C18 MS II (φ20 × 250 mm) with H_2O/CH_3CN (40 : 60) to afford **KUSC-5015** as a colorless solid (34.81 mg, 52%).

^{1}H NMR (600 MHz, $CDCl_3$): 3.97 (s, 3H), 6.88 (d, $J = 2.5$ Hz, 1H), 7.41 (d, $J = 2.5$ Hz, 1H), 7.46 (d, $J = 9.5$ Hz, 2H), 7.61 (d, $J = 8.5$ Hz, 2H), 8.68 (br, 1H).

^{13}C NMR (150 MHz, $CDCl_3$): δ 39.56, 107.40, 1116.50, 121.22, 132.06, 132.38, 137.20, 146.51, 159.84.

HRMS (ESI) *m/z*: calcd for $[M + Na]^+$ $C_{11}H_{10}BrN_3ONa^+$ 303.9879; found, 303.9828.

4.1.3.2. N-(4-bromophenyl)-3-(4-nitro-1H-pyrazol-1-yl)propanamide (KUSC-5021). To a stirred solution of 3-(4-nitro-1*H*-pyrazol-1-yl)propanoic acid (15.0 mg, 0.0810 mmol) in dichloromethane (1.1 mL) were added DIPEA (28.0 μL, 0.161 mmol) and HATU (30.8 mg, 0.0810 mmol) at room temperature. After being stirred for 30 min, *p*-bromoaniline (15.3 mg, 0.047 mmol) was added, and the mixture was stirred at room temperature for 18.5 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with AcOEt and the combined organic layers were washed with 1 M HCl, sat. $NaHCO_3$ aq., and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 chromatography ($CHCl_3$/MeOH = 20:1), and the purified fraction was further subjected to ODS HPLC on COSMOSIL 5C18 MS II (φ20 × 250 mm) with H_2O/CH_3CN (40 : 60) to afford **KUSC-5021** as a colorless solid (20.3 mg, 74%).

^{1}H NMR (600 MHz, acetone-d_6): δ 3.08 (t, $J = 6.0$ Hz, 2H), 4.60 (t, $J = 7.2$ Hz, 2H), 7.44 (d, $J = 8.4$ Hz, 2H), 7.58 (d, $J = 8.4$ Hz, 2H), 8.10 (s, 1H), 8.64 (s, 1H), 9.43 (br, 1H).

^{13}C NMR (150 MHz, acetone-d_6): δ 36.72, 49.45, 116.18, 121.88, 131.07, 132.48, 132.65, 136.18, 139.26, 169.03.

HRMS (ESI) *m/z*: calcd for $[M-H]^-$ $C_{12}H_{10}BrN_4O_3$ 336.9942; found, 336.9963.

4.1.4. Synthesis of KUSC-5019, KUSC-5014, KUSC-5020, and KUSC-5029

4.1.4.1. Ethyl 1-((1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxylate (S3). To a stirred solution of ethyl 1-(bromomethyl)-3-carboxylate (100.0 mg, 0.429 mmol) in CH_3CN (5.60 mL) were added 1*H*-pyrazole (66.0 mg, 0.969 mmol) and potassium carbonate (130.5 mg, 0.944 mmol). After being stirred at room temperature for 3.5 days, the reaction was quenched with water and the layers were separated. The aqueous layer was extracted with ethyl acetate and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 column chromatography ($CHCl_3$/MeOH = 20:1) to afford the carboxylate **S3** as a colorless solid (94.8 mg, quant.).

^{1}H NMR (600 MHz, $CDCl_3$): δ 1.37 (t, $J = 7.2$ Hz, 3H), 4.39 (q, $J = 7.2$ Hz, 2H), 6.30 (s, 1H), 6.37 (s, 2H), 6.81 (d, $J = 3.0$ Hz, 1H), 7.56 (s, 1H), 7.67 (d, $J = 1.8$ Hz, 1H), 7.70 (d, $J = 1.8$ Hz, 1H).

^{13}C NMR (150 MHz, $CDCl_3$): δ 14.48, 61.30, 65.85, 107.71, 110.21, 130.20, 131.09, 141.38, 144.93, 162.07.

HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{10}H_{13}N_4O_2$ 221.1034; found, 221.1003.

4.1.4.2. 1-((1H-Pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxylic acid (S4). To a stirred solution of the carboxylate **S3** (30.0 mg, 0.136 mmol) in MeOH (2.1 mL) and water (0.7 mL) was added 2 M NaOH (0.10 mL, 0.200 mmol). After being stirred at 48 °C for 15 h, the reaction solution was cooled to room temperature. After the reaction was quenched with conc. HCl at pH 5, added ethyl acetate and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford the carboxylic acid **S4** as a colorless solid (9.7 mg, 37%).

^{1}H NMR (500 MHz, MeOH-d_4): δ 6.35 (d, $J = 2.0$ Hz, 1H), 6.44 (s, 2H), 6.78 (d, $J = 2.0$ Hz, 1H), 7.56 (d, $J = 2.0$ Hz, 1H), 7.90 (d, $J = 2.5$ Hz, 1H), 7.93 (d, $J = 2.5$ Hz, 1H).

^{13}C NMR (125 MHz, MeOH-d_4): δ 66.16, 107.99, 110.31, 132.21, 133.22, 142.20, 146.29, 164.93.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_8H_8N_4O_2Na^+$ 215.0539; found, 215.0503.

4.1.4.3. 1-((1H-pyrazol-1-yl)methyl)-N-(4-bromophenyl)-1H-pyrazole-3-carboxamide (KUSC-5019). To a stirred solution of the carboxylate **S4** (9.72 mg, 0.0506 mmol) in dichloromethane (0.68 mL) were added

DIPEA (18 μL, 0.103 mmol) and HATU (19.43 mg, 0.0505 mmol). After being stirred at room temperature for 30 min, *p*-bromoaniline (9.60 mg, 0.0558 mmol) was added, and the mixture was stirred at room temperature for 11 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layers were washed with sat. $NaHCO_3$ aq. and brine, respectively, dried over Na_2SO_4, concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 100:3) to afford **KUSC-5019** as a colorless solid (15.9 mg, 14%).

^{1}H NMR (500 MHz, Acetone-d_6): δ 6.31 (t, J = 2.0 Hz, 1H), 6.50 (s, 2H), 6.81 (d, J = 2.5 Hz, 1H), 7.49–51 (m, 3H), 7.82 (d, J = 9.0 Hz, 2H), 7.99 (s, 1H), 8.00 (s, 1H), 9.55 (s, 1H).

^{13}C NMR (125 MHz, Acetone-d_6): δ 66.17, 107.52, 108.20, 116.38, 122.48, 122.56, 131.08, 132.45, 133.31, 139.10, 141.34, 148.58, 160.43.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{12}BrN_5ONa^+$ 368.0117; found, 368.0093.

4.1.4.4. Ethyl 1-((1H-imidazol-1-yl)methyl)-1H-pyrazole-3-carboxylate (S5). To a stirred solution of ethyl 1-(bromomethyl)-3-carboxylate (50.0 mg, 0.215 mmol) in CH_3CN (2.8 mL) were added potassium carbonate (65.2 mg, 0.472 mmol) and 1*H*-imidazole (33.0 mg, 0.485 mmol). After being stirred at room temperature for 3 days, the reaction was quenched with water and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1) to afford the carboxylate **S5** as a colorless solid (36.1 mg, 76%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.38 (t, J = 7.0 Hz, 3H), 4.39 (q, J = 7.0 Hz, 2H), 6.21 (s, 2H), 6.83 (d, J = 3.0 Hz, 1H), 7.07 (s, 1H), 7.10 (s, 1H), 7.51 (d, J = 2.5 Hz, 1H), 7.73 (s, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 14.44, 61.43, 110.58, 118.36, 118.74, 130.25, 131.01, 137.14, 145.38, 161.85.

HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{10}H_{13}N_4O_2^+$, 221.1033; found, 221.1012.

4.1.4.5. 1-((1H-imidazol-1-yl)methyl)-N-(4-bromophenyl)-1H-pyrazole-3-carboxamide (KUSC-5014). To a stirred solution of the carboxylate **S5** (36.1 mg, 0.164 mmol) in MeOH (2.4 mL) and water (0.8 mL) was added 2 M NaOH (0.12 mL). After being stirred at 45 °C for 20 h, the reaction mixture was cooled to room temperature. After the reaction solution was acidified with 1 M HCl at pH 5, ethyl acetate was added, and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were concentrated in vacuo. The residue was used without further purification.

To a stirred solution of the residue (5.6 mg, 0.0291 mmol) in dichloromethane (0.39 mL) were added DIPEA (10.2 μL, 0.0586 mmol) and HATU (11.1 mg, 0.0292 mmol). After being stirred at room temperature for 30 min, *p*-bromoaniline (5.0 mg, 0.0291 mmol) was added, and the mixture was stirred at room temperature for 22 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with ethyl acetate and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford **KUSC-5014** as a colorless solid (5.0 mg, 9% in 2 steps).

^{1}H NMR (600 MHz, DMSO-d_6): δ 6.42 (s, 2H), 6.84 (d, J = 1.8 Hz, 1H), 6.94 (s, 1H) 7.40 (s, 1H), 7.52 (d, J = 9.0 Hz, 2H), 7.77 (d, J = 9.0 Hz, 2H), 7.94 (s, 1H), 8.16 (d, J = 1.8 Hz, 1H), 10.23(1H, br).

^{13}C NMR (150 MHz in DMSO-d_6): δ 60.05, 107.60, 115.43, 119.30, 122.32, 129.23, 131.46, 132.83, 137.55, 138.04, 147.57, 159.83.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{12}BrN_5ONa^+$ 368.0118; found, 368.0154.

4.1.4.6. Ethyl 1-((4-nitro-1H-imidazol-1-yl)methyl)-1H-pyrazole-3-carboxylate (S6). To a stirred solution of ethyl 1-(bromomethyl)-pyrazole-3-carboxylate (100.0 mg, 0.429 mmol) in DMF (4.3 mL) were added Cs_2CO_3 (153.8 mg, 0.472 mmol), NaI (64.3 mg, 0.429 mmol), and 4-nitro-1*H*-imidazole (72.8 mg, 0.644 mmol). After being stirred at 80 °C for 19 h, the reaction was quenched with water and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1) to afford the carboxylate **S6** as a colorless solid (74.8 mg, 66%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.27 (t, J = 6.0 Hz, 3H), 4.27 (q, J = 6.0 Hz, 2H), 6.54 (s, 2H), 6.83 (d, J = 1.5 Hz, 1H), 8.15 (s, 1H), 8.23 (d, J = 2.5 Hz, 1H), 8.59 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 14.17, 60.53, 60.90, 109.36, 120.98, 133.09, 137.42, 144.59, 147.39, 161.24.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{10}H_{11}N_5O_4Na^+$ 288.0703; found, 288.0726.

4.1.4.7. 1-((4-Nitro-1H-imidazol-1-yl)methyl)-1H-pyrazole-3-carboxylic acid (S7). To a stirred solution of the carboxylate **S6** (30.0 mg, 0.113 mmol) in MeOH (1.8 mL) and water (0.6 mL) was added 2 M NaOH (0.085 mL). After being stirred at 48 °C for 10 h, the reaction solution was cooled to room temperature. After the reaction was quenched with conc. HCl at pH 5, ethyl acetate was added, and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were dried over Na_2SO_4, and concentrated in vacuo to afford the carboxylic acid **S7** as a colorless solid (24.9 mg, 93%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.51 (s, 2H), 6.77 (d, J = 2.0 Hz, 1H), 8.13 (d, J = 2.0 Hz, 1H), 8.19 (d, J = 2.5 Hz, 1H), 8.56 (d, J = 1.5 Hz, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 60.86, 109.23, 120.95, 132.84, 137.37, 145.54, 147.35, 162.68.

HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_8H_8N_5O_4$ 238.0571; found, 238.0548.

4.1.4.8. N-(4-bromophenyl)-1-((4-nitro-1H-imidazol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5020). To a stirred solution of the carboxylic acid **S7** (18.2 mg, 0.0765 mmol) in CH_2Cl_2 (1.0 mL) were added DIPEA (27.0 μL, 0.155 mmol) and HATU (29.3 mg, 0.0770 mmol). After stirred for 30 min, *p*-bromoaniline (9.60 mg, 0.0558 mmol) was added, and the mixture was stirred for 12 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 flash chromatography ($CHCl_3$/MeOH = 100:3) to afford **KUSC-5020** as a colorless solid (26.1 mg, 87%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.54 (s, 2H), 6.88 (d, J = 2.5 Hz, 1H), 7.53 (d, J = 8.5 Hz, 2H), 7.75 (d, J = 8.5 Hz, 2H), 8.16 (d, J = 1.5 Hz, 1H), 8.22 (d, J = 2.5 Hz, 1H), 8.59 (s, 1H), 10.20 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 60.89, 107.74, 115.52, 120.98, 122.35, 131.46, 133.36, 137.41, 137.89, 147.36, 148.15, 159.59

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{14}H_{11}BrN_6O_3Na^+$ 412.9968; found, 412.9965.

4.1.4.9. Ethyl 1-((3-nitro-1H-pyrrol-1-yl)methyl)-1H-pyrazole-3-carboxylate (S8). To a stirred solution of 3-nitro-1*H*-pyrrole (30.0 mg, 0.268 mmol) in DMF (1.5 mL) were added Ethyl 1-(bromomethyl)-pyrazole-3-carboxylate (68.6 mg, 0.294 mmol) and potassium carbonate (74.0 mg, 0.535 mmol). After being stirred at room temperature for 23 h, the reaction was quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$ and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo.

The residue was purified by SiO_2 flash chromatography ($CHCl_3$/MeOH = 100:3) to afford the carboxylate **S8** as a colorless solid (55.7 mg, 79%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.38 (t, J = 7.0 Hz, 3H), 4.39 (q, J = 7.0 Hz, 2H), 6.18 (s, 2H), 6.71 (dd, J = 2.0 Hz, 1H), 6.82 (t, J = 2.5 Hz, 1H), 6.85 (d, J = 2.0 Hz, 1H), 7.64 (d, J = 2.5 Hz, 1H), 7.74 (s, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 14.25, 61.34, 64.33, 106.81, 110.55, 121.19, 121.30, 130.69, 138.13, 145.64, 161.63.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{11}H_{12}N_4O_4Na^+$, 287.0751; found, 287.0774.

4.1.4.10. 1-((3-Nitro-1H-pyrrol-1-yl)methyl)-1H-pyrazole-3-carboxylic acid (S9). To a stirred solution of the carboxylate **S8** (20.0 mg, 0.0757 mmol) in MeOH (1.20 mL) and water (0.40 mL) was added 2 M NaOH (57.0 μL, 0.114 mmol). After being stirred at 45 °C for 13 h, the reaction solution was cooled to room temperature. After the reaction was quenched with conc. HCl at pH 3–4, was added ethyl acetate, and the layers were separated. The aqueous layer was extracted with ethyl acetate, dried over Na_2SO_4, and concentrated in vacuo to afford the carboxylic acid **S9** as a colorless solid (17.58 mg, 98%).

^{1}H NMR (600 MHz, DMSO-d_6): δ 6.39 (s, 2H), 6.71 (d, J = 3.0 Hz, 1H), 6.75 (d, J = 2.0 Hz, 1H), 7.15 (t, J = 2.5 Hz, 1H), 8.17 (d, J = 2.0 Hz, 1H), 8.20 (t, J = 2.0 Hz, 1H).

^{13}C NMR (150 MHz, DMSO-d_6): δ 63.19, 105.48, 109.25, 122.77, 123.01, 132.63, 136.66, 145.26, 162.83.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_9H_8N_4O_4Na^+$ 259.0438; found, 259.0437.

4.1.4.11. N-(4-bromophenyl)-1-((3-nitro-1H-pyrrol-1-yl)methyl)-1H-pyrazole-3-carboxamide (KUSC-5029). To a stirred solution of the carboxylic acid **S9** (17.58 mg, 0.0744 mmol) in dichloromethane (1.0 mL) were added DIPEA (26.0 μL, 0.149 mmol) and HATU (28.3 mg, 0.0744 mmol). After being stirred at room temperature for 30 min, *p*-bromoaniline (14.1 mg, 0.0820 mmol) was added and the mixture was stirred at room temperature for 14 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified with SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1). The purified fraction was further subjected to ODS HPLC on COSMOSIL $_5C_{18}$ MSII (φ20 × 250 mm) with H_2O/MeCN (40:60) to afford **KUSC-5029** as a colorless solid (20.2 mg, 69%).

^{1}HNMR (500 MHz, Acetone-d_6): δ 6.49 (s, 2H), 6.70 (s, 1H), 6.86 (d, J = 2.5 Hz, 1H), 7.15 (s, 1H), 7.50 (d, J = 9.0 Hz, 2H), 7.78 (d, J = 8.5 Hz, 2H), 8.06 (s, 1H), 8.15 (s, 1H), 9.57 (s, 1H).

^{13}C NMR (125 MHz in Acetone-d_6): δ 64.71, 106.43, 108.51, 116.52, 122.63, 122.98, 123.06, 132.47, 133.61, 138.46, 138.91, 149.27, 160.23.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{15}H_{12}BrN_5O_3Na^+$ 412.0016; found, 412.0037.

4.1.5. Synthesis of KUSC-5017 and KUSC-5025

4.1.5.1. Ethyl 1-((4-((tert-butoxycarbonyl)amino)-1H-pyrazol-1-yl)methyl)-1H-pyrazole-3-carboxylate (S10). To a stirred solution of the carboxylate **S1** (150.0 mg, 0.566 mmol) in MeOH (0.72 mL) and THF (0.72 mL) were added $(Boc)_2O$ (0.13 mL, 0.566 mmol) and 10% Pd/C (39.5 mg). After being stirred at room temperature for 10 h under H_2 atmosphere, the reaction solution was filtered with celite, and the solution was concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1) to afford the carboxylate **S10** as a colorless solid (164.9 mg, 87%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.38 (t, J = 7.0 Hz, 3H), 1.47 (s, 9H), 4.39 (q, J = 7.0 Hz, 2H), 6.27 (s, 2H), 6.38 (s, 1H), 6.79 (d, J = 2.0 Hz, 1H), 7.48 (s, 1H), 7.61 (d, J = 1.5 Hz, 1H), 7.84 (s, 1H).

^{13}C NMR (125 MHz, $CDCl_3$): δ 14.47, 28.39, 61.31, 66.41, 80.81, 110.26, 120.14, 123.41, 130.85, 132.77, 144.87, 152.92, 162.12.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{15}H_{21}N_5O_4Na^+$ 358.1486; found, 358.1487.

4.1.5.2. tert-Butyl (1-((3-((4-bromophenyl)carbamoyl)-1H-pyrazol-1-yl)methyl)-1H-pyrazol-4-yl)carbamate (S12). To a stirred solution of the carboxylate **S10** (50.0 mg, 0.149 mmol) in MeOH (3.0 mL) and water (1.0 mL) was added 2 M NaOH (0.45 mL). After being stirred at 45 °C for 1 day, the reaction solution was cooled to room temperature. After the reaction was quenched with conc. HCl at pH 2, added $CHCl_3$ and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layers were dried over Na_2SO_4, and concentrated in vacuo to afford 1-((4-((*tert*-butoxycarbonyl)amino)-1*H*-pyrazol-1-yl)methyl)-1*H*-pyrazole-3-carboxylic acid (**S11**) as a colorless solid (51.1 mg).

To a stirred solution of the carboxylic acid **S11** (51.1 mg, 0.149 mmol) in dichloromethane (2.0 mL) were added DIPEA (52.0 μL, 0.299 mmol) and HATU (56.7 mg, 0.164 mmol). After being stirred at room temperature for 30 min, *p*-bromoaniline (28.2 mg, 0.164 mmol) was added, and the mixture was stirred at room temperature for 30 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1) to afford the carbamate **S12** as a colorless solid (60.0 mg, 87% in 2 steps).

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.43 (s, 9H), 6.42 (s, 2H), 6.82 (d, J = 2.0 Hz, 1H), 7.38 (s, 1H), 7.50 (d, J = 9.0 Hz, 2H), 7.78 (d, J = 8.5 Hz, 2H), 7.97 (s, 1H), 8.08 (d, J = 2.5 Hz, 1H), 9.27 (s, 1H), 10.28 (s, 1H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 28.10, 65.09, 78.90, 107.53, 115.33, 119.44, 122.32, 123.27, 131.37, 132.81, 138.10, 147.32, 147.43, 152.69, 159.83.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{19}H_{21}BrN_6O_3Na^+$ 485.0751; found, 485.0727.

4.1.5.3. 1-((4-amino-1H-pyrazol-1-yl)methyl)-N-(4-bromophenyl)-1H-pyrazole-3-carboxamide hydrochloride (KUSC-5017). A solution of the carbamate **S12** (24.1 mg, 0.0522 mmol) in 4 M HCl in 1,4-dioxane (0.52 mL) was stirred at room temperature for 2.7 h and concentrated in vacuo to afford **KUSC-5017** as a colorless solid (15.06 mg, 73%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 6.55 (s, 2H), 6.87 (d, J = 2.5 Hz, 1H), 7.50 (d, J = 8.5 Hz, 2H), 7.65 (s, 1H), 7.78 (d, J = 8.5 Hz, 2H), 8.17 (d, J = 2.5 Hz, 1H), 8.29 (s, 1H), 10.29 (br, 2H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 65.05, 107.60, 114.41, 115.38, 122.32, 126.01, 131.38, 133.18, 135.32, 138.08, 147.65, 159.74.

HRMS (ESI) *m/z*: $[M-HCl + Na]^+$ calcd for $C_{14}H_{13}BrN_6ONa^+$ 383.0226; found, 383.0175.

4.1.5.4. 1-((4-acetamido-1H-pyrazol-1-yl)methyl)-N-(4-bromophenyl)-1H-pyrazole-3-carboxamide (KUSC-5025). To a stirred solution of **KUSC-5017** (15.1 mg, 0.0379 mmol) in dichloromethane (0.38 mL) was added Et_3N (13.2 μL, 0.0950 mmol). After being stirred at room temperature for 30 min, acetyl chloride (12 μL, 0.169 mmol) was added and the mixture was stirred at room temperature for 20 h. The reaction was then quenched with MeOH, added water and AcOEt, and the layers were separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified with preparative TLC ($CHCl_3$/MeOH = 20:1) to afford **KUSC-5025** as a colorless solid (11.5 mg, 75%).

^{1}H NMR (600 MHz, MeOH-d_4): δ 2.07 (s, 3H), 6.42 (s, 2H), 6.83 (d, J = 1.5 Hz, 2H), 7.48 (d, J = 4.0 Hz, 2H), 7.55 (s, 1H), 7.68 (d, J = 4.0 Hz, 2H), 7.93 (d, J = 2.0 Hz, 1H), 8.24 (s, 1H).

^{13}C NMR (150 MHz, MeOH-d_4): δ 22.73, 66.49, 108.57, 117.79, 122.78, 123.34, 124.06, 132.82, 133.48, 133.70, 138.77, 148.86,

162.10, 170.46.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{16}H_{15}BrN_6O_2Na^+$ 425.0332; found, 425.0314.

4.1.6. Synthesis of KUSC-5035 ~ KUSC-5040

4.1.6.1. tert-Butyl (1-((3-((4-fluorophenyl)carbamoyl)-1H-pyrazol-1-yl) methyl)-1H-pyrazol-4-yl)carbamate (S13). To a stirred solution of the carboxylic acid **S11** (24.1 mg, 0.0785 mmol) in dichloromethane (1.1 mL) at room temperature were added DIPEA (27.0 μL, 0.155 mmol) and HATU (29.9 mg, 0.0786 mmol). After being stirred at room temperature for 30 min, *p*-trifluoromethyl aniline (11.0 μL, 0.0876 mmol) was added, and the mixture was stirred for 23 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$ and the combined organic layers were washed with sat. $NaHCO_3$ aq., brine, dried over Na_2SO_4, and concentrated in vacuo to afford the crude solid **S13**. This crude **S13** was subjected to the next reaction without further purification.

4.1.6.2. 1-((4-amino-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl) phenyl)-1H-pyrazole-3-carboxamide hydrochloride (KUSC-5035). A solution of the carbamate **S13** (42.2 mg) was dissolved in 4 M HCl in 1,4-dioxane (0.94 mL) and stirred at room temperature for 7.3 h. After the reaction mixture was concentrated in vacuo, the residue was subjected to ODS HPLC on COSMOSIL $_5C_{18}$ MSII (φ20 × 250 mm) with 0.01% TFA in H_2O/CH_3CN (70:30) to afford **KUSC-5035** as a colorless solid (22.75 mg, 75% in 2 steps).

^{1}H NMR (500 MHz, MeOH-d_4): δ 6.53 (s, 2H), 6.89 (d, J = 2.5 Hz, 1H), 7.66 (d, J = 8.5 Hz, 2H), 7.68 (s, 2H), 9.34 (d, J = 8.5 Hz, 2H), 7.94 (d, J = 8.5 Hz, 2H), 7.99 (d, J = 2.0 Hz, 1H), 8.19 (s, 1H).

^{13}C NMR (125 MHz, MeOH-d_4): δ 66.69, 108.77, 116.56, 121.27, 124.64, 126.78 (q), 127.06, 127.09, 133.92, 136.48, 142.98, 149.07, 162.09.

HRMS (ESI) *m/z*: $[M-HCl + Na]^+$ calcd for $C_{15}H_{13}F_3N_6ONa^+$ 373.0995; found, 373.1019.

4.1.6.3. 1-((4-acetamido-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl) phenyl)-1H-pyrazole-3-carboxamide (KUSC-5036). A colorless solid.

^{1}H NMR (500 MHz, MeOH-d_4): δ 2.07 (s, 3H), 6.44 (s, 2H), 6.86 (d, J = 2.0 Hz, 1H), 7.56 (s, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.94–96 (m, 3H), 8.25 (s, 1H)

^{13}CNMR (125 MHz, MeOH-d_4): δ 22.72, 66.52, 108.69, 121.29, 122.81, 124.08 (d), 126.83 (t), 127.03 (d), 133.57, 133.74, 143.09, 148.75, 162.29, 170.46.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{17}H_{15}F_3N_6O_2Na^+$ 415.1101; found, 415.1071.

4.1.6.4. 1-((4-propionamido-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5037). To a stirred solution of **KUSC-5035** (16.8 mg, 0.0435 mmol) in dichloromethane (0.44 mL) at room temperature was added Et_3N (7.6 μL, 0.0545 mmol). After being stirred at room temperature for 30 min, propionyl chloride (4.2 μL, 0.0481 mmol) was added, and the mixture was stirred for 15 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with ethyl acetate and the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) and the purified fraction was further subjected to ODS HPLC on COSMOSIL $_5C_{18}$ MSII (φ20 × 250 mm) H_2O/CH_3CN (50:50) to afford **KUSC-5037** as a colorless solid (8.8 mg, 50%).

^{1}H NMR (500 MHz, MeOH-d_4): δ 1.18 (t, J = 7.5 Hz, 3H), 2.34 (q, J = 7.5 Hz, 2H), 6.44 (s, 2H), 6.86 (d, J = 3.0 Hz, 1H), 7.57 (s, 1H), 7.65 (d, J = 3.5 Hz, 1H), 7.94–96 (m, 3H), 8.26 (s, 1H).

^{13}C NMR (125 MHz, MeOH-d_4): δ 10.25, 30.18, 66.51, 108.69, 121.27, 122.75, 124.11, 124.84, 126, 0.76 (q), 127.01 (d), 133.55, 133.79, 143.06, 148.73, 126.27, 174.24.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{18}H_{17}F_3N_6O_2Na^+$ 429.1257; found, 429.1263.

4.1.6.5. 1-((4-butyramido-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5038). A colorless solid.

^{1}H NMR (500 MHz, MeOH-d_4): δ 0.96 (t, J = 8.0 Hz, 3H), 1.68 (sextet, J = 7.5 Hz, 2H), 2.29 (t, J = 7.5 Hz, 2H), 6.43 (s, 2H), 6.86 (d, J = 3.0 Hz, 1H), 7.57 (s, 1H), 7.64 (d, J = 9.0 Hz, 2H), 7.93 (d, J = 8.5 Hz, 2H), 7.94 (d, J = 2.5 Hz, 1H), 8.26 (s, 1H).

^{13}C NMR (125 MHz, MeOH-d_4): δ 13.97, 20.28, 39.01, 66.52, 108.70, 121.28, 122.78, 124.05, 124.66, 126.73 (q), 127.00 (d), 133.55, 133.81, 143.05, 148.74, 162.26, 173.39.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{19}H_{19}F_3N_6O_2Na^+$ 443.1414; found, 443.1433.

4.1.6.6. 1-((4-(2-phenylacetamido)-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5039). A colorless solid.

^{1}H NMR (600 MHz, DMSO-d_6): δ 3.56 (s, 2H), 6.46 (s, 2H), 6.85 (d, J = 2.4 Hz, 1H), 7.22–24 (m, 1H), 7.28–7.31 (m, 4H), 7.52 (s, 1H), 7.69 (d, J = 8.4 Hz, 2H), 8.03 (d, J = 7.8 Hz, 2H), 8.09 (d, J = 2.4 Hz, 1H), 8.23 (s, 1H), 10.30 (br, 1H), 10.51 (br, 1H).

^{13}C NMR (150 MHz, DMSO-d_6): δ 42.49, 65.10, 107.73, 120.26, 120.77, 122.58, 123.51 (q), 125.34, 125.89, 126.58, 128.36, 129.04, 131.81, 132.98, 135.95, 142.38, 147.18, 160.18, 167.73.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{23}H_{19}F_3N_6O_2Na^+$ 491.1414; found, 491.1423.

4.1.6.7. 1-((4-isobutyramido-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5040). A colorless solid.

^{1}H NMR (500 MHz, MeOH-d_4): δ 1.17 (d, J = 7.0 Hz, 6H), 2.56 (septet, J = 7.0 Hz, 1H), 6.44 (s, 2H), 6.86 (d, J = 2.0 Hz, 1H), 7.59 (s, 1H), 7.65 (d, J = 8.5 Hz, 2H), 7.94–96 (m, 3H), 8.25 (s, 1H).

^{13}C NMR (125 MHz, MeOH-d_4): δ 19.85, 36.39, 66.53, 108.70, 121.29, 122.78, 124.15, 124.68, 126.74 (q), 127.02 (d), 133.55, 133.87, 143.07, 148.74, 162.28, 177.43.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{19}H_{19}F_3N_6O_2Na^+$ 443.1414; found, 443.1434.

4.1.7. Synthesis of KUSC-5054 and KUSC-5051

4.1.7.1. 1-((4-(3-(5,5-difluoro-7,9-dimethyl-5H-4λ^4,5λ^4-dipyrrolo[1,2-c:2′,1′-f][1,3,2]diazaborinin-2-yl)propanamido)-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5054). To a stirred solution of **KUSC-5035** (6.0 mg, 0.0155 mmol) in DMF (0.13 mL) were added DIPEA (4.5 μL, 0.0258 mmol) and BODIPY FL NHS ester (5.0 mg, 0.0128 mmol) in DMF (0.13 mL). After being stirred at room temperature for 22 h, the reaction solution was concentrated in vacuo. The residue was subjected to ODS HPLC on COSMOSIL $_5C_{18}$ MSII (φ20 × 250 mm) with H_2O/CH_3CN (35:65) to afford **KUSC-5054** as a brown solid (3.63 mg, 45%).

^{1}H NMR (500 MHz, MeOH-d_4): δ 2.27 (s, 3H), 2.50 (s, 3H), 2.75 (t, J = 7.5 Hz, 2H), 3.28 (t, J = 7.5 Hz, 2H), 4.60 (br, 1H), 5.49 (br, 1H), 6.20 (s, 1H), 6.31 (d, J = 4.0 Hz, 1H), 6.44 (s, 2H), 6.86 (d, J = 7.0 Hz, 1H), 6.97 (d, J = 4.0 Hz, 1H), 7.40 (s, 1H), 7.58 (s, 1H), 7.64 (d, J = 8.5 Hz, 2H), 7.94–95 (m, 3H), 8.28 (s, 1H).

^{13}C NMR (125 MHz, MeOH-d_4): δ 11.16, 14.85, 25.41, 36.06, 66.53, 108.70, 117.51, 121.30, 122.92, 123.99, 124.68, 125.78, 126.78 (q), 127.02 (d), 129.55, 133.59, 133.88, 124.87, 136.56, 143.07, 145.90, 148.74, 158.23, 161.45, 162.29, 171.88.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{29}H_{26}BF_5N_8O_2Na^+$,

647.2084; found, 647.2071.

4.1.7.2. 2-(3-(Pent-4-yn-1-yl)-3H-diazirin-3-yl)ethan-1-cyanide (S15). To a stirred solution of the iodide **S14** (17.38 mg, 0.0663 mmol) in DMF (0.83 mL) was added KCN (8.7 mg, 0.132 mmol). After being stirred at 70 °C for 22 h, the reaction solution was cooled to room temperature. The reaction solution was diluted with water, extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, and then concentrated in vacuo. The crude cyanide **S15** was subjected to the next reaction.

4.1.7.3. 3-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)propanoic acid (S16). To a solution of the crude cyanide **S15** (9.77 mg) was added 10 %NaOH (1.1 mL). After stirred reflux for 15 h, the reaction solution was cooled to room temperature, acidified with 1 M HCl at pH 2, extracted with ethyl acetate, dried over Na_2SO_4, and concentrated in vacuo. The crude carboxylic acid **S16** was used without purification.

4.1.7.4. 1-((4-(3-(3-(pent-4-yn-1-yl)-3H-diazirin-3-yl)propanamido)-1H-pyrazol-1-yl)methyl)-N-(4-(trifluoromethyl)phenyl)-1H-pyrazole-3-carboxamide (KUSC-5051). To a stirred solution of the crude carboxylic acid **S16** (10.6 mg, 0.0588 mmol) in dichloromethane (0.79 mL) were added DIPEA (41.0 μL, 0.235 mmol) and HATU (22.4 mg, 0.0589 mmol). After being stirred at room temperature for 30 min, **KUSC-5035** (22.75 mg, 0.0598 mmol) was added, and the mixture was stirred at room temperature for 13 h. The reaction was then quenched with water and the layers were separated. The aqueous layer was extracted with $CHCl_3$, and the combined organic layers were washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 flash column chromatography ($CHCl_3$/MeOH = 20:1), and the purified fraction was further subjected to ODS HPLC on COSMOSIL $_5C_{18}$ MSII (φ20 × 250 mm) with H_2O/CH_3CN (45:55) to afford the **KUSC-5051** as a brown solid (4.54 mg, 15%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 1.32 (pentet, J = 8.0 Hz, 2H), 1.55 (t, J = 8.5 Hz, 2H), 1.88 (t, J = 7.5 Hz, 2H), 1.93 (t, J = 2.5 Hz, 1H), 2.08 (t, J = 7.5 Hz, 2H), 2.15 (td, J = 7.0, 2.0 Hz, 2H), 6.25 (s, 2H), 6.92 (d, J = 2.5 Hz, 1H), 7.19 (s, 1H), 7.46 (s, 1H), 7.62 (d, J = 9.0 Hz, 2H), 7.68 (d, J = 2.0 Hz, 1H), 7.83 (d, J = 8.5 Hz, 2H), 8.27 (s, 1H), 8.82 (s, 1H).

^{13}C NMR (125 MHz in $CDCl_3$): δ 17.82, 22.56, 27.87, 28.28, 30.42, 31.65, 66.10, 69.12, 83.23, 108.55, 119.33, 122.14, 122.99, 125.47, 126.29, 131.89, 132.28, 140.75, 147.60, 159.35, 168.48.

HRMS (ESI) *m/z*: $[M + Na]^+$ calcd for $C_{24}H_{23}F_3N_8O_2Na^+$, 535.1788; found, 535.1737.

4.2. Biology

4.2.1. Reagents and antibodies

MitoTracker Red CMXRos and Hoechst 33342 were purchased from Cell Signaling Technology and Nacalai Tesque, respectively. The antibodies used in this study are as follows: anti-human HIF-1α mouse monoclonal antibody (54/HIF-1α, BD Transduction Laboratories), anti-α-tubulin mouse monoclonal antibody (B-5–1-2, Sigma), anti-human ATP5B rabbit polyclonal antibody (Proteintech), alkaline phosphatase or horseradish peroxidase conjugated-secondary antibodies (Promega).

4.2.2. Cell-based HRE-driven luciferase reporter gene assay

HeLa/HRE/Luc cells, stably transfected with a luciferase gene driven by the hypoxia responsive element, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.[8,11] For the reporter assay, the cells (6.0×10^3) were seeded in 96-well plates (100 μL) and cultured for 48 h. After 48 h incubation, the cells were treated with test compounds and incubated for 1 h under normoxic conditions prior to the exposure to hypoxic conditions (1% O_2). After further 24 h-incubation under hypoxic conditions, the cells were washed with PBS(-) and lysed in 30 μL of lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100). Twenty μL of the cell lysate was employed for the luciferin-luciferase assay (50 mM Tris pH 8.0, 5 mM $MgSO_4$, 0.125 mM D-luciferin, 0.5 mM ATP, and 0.5 mM coenzyme A). Epiluminescence was measured using an EnVision luminometer (Perkin Elmer). Luciferase activity was determined in triplicate and showed as a relative activity normalized with protein concentration.

4.2.3. RNA extraction and quantitative real-time PCR

Total RNA was isolated from HeLa S3 cells by using Total RNA Extraction kit (Viogene). The RNA (1.5 μg) was reverse transcribed by random hexamer (ReverTra Ace with gDNA remover, TOYOBO). Quantitative real-time PCR analysis was carried out StepOne PCR Real Time System (Thermo Fisher Scientific) employing with SYBR Select. Master Mix. Primers used for PCR were as follows: human β *-actin*, 5′-CTCCTCCCTGGAGAAGAGCTAC-3′ (sense) and 5′-TGCGGATGTCCACGTCACA-3′ (antisense); human *HIF-1* α, 5′-TACTAGCTTTGCAGAATGCTC-3′(sense) and 5′-GCCTTGTATAGGAGCATTAAC-3′ (antisense); human *HIF-1* β, 5′-GGGGGCAGCTTACCTCTAAC-3′ (sense) and 5′-GGGTAGAGGCACTCGAACTG-3′ (antisense); human *CA9*, 5′-ACCCTCTCTGACACCCTGTG-3′ (sense) and 5′-GGCTGGCTTCTCACATTCTC-3′ (antisense); human *VEGF*, 5′-AATGACGAGGGCCTGGAGTGTG-3′ (sense) and 5′-ATGTGCTGGCCTTGGTGAGGT-3′ (antisense); human 18S rRNA, 5′-GCAATTATTCCCCATGAACG-3′ (sense) and 5′-GGGACTTAATCAACGCAAGC-3′ (antisense). Relative expression level was calculated by the ΔΔCT method, and normalized with 18S rRNA level.

4.2.4. Subcellular localization analysis of KUSC-5054

HeLa S3 cells grown on cover glass were treated with **KUSC-5054** (1 μM), MitoTracker Red CMXRos (100 nM) and Hoechst 33342 (10 μM). Epifluorescence was observed under IX81 fluorescence microscope (Olympus).

4.2.5. Western blot analysis

MDA-MB-231 cells were treated with **KUSC-5037** (0.3, 1, 3 μM) or camptothecin (0.5 μM) under the hypoxia (1% O_2, 25 h) and the cells were lysed in PBS(-) containing 0.5% Triton X-100. Whole cell lysate (20 μg) was subjected to SDS-PAGE followed by blotting onto a PVDF membrane (Pall Life Sciences). The membrane was briefly washed with PBST buffer [PBS(-) containing 0.1% Tween-20] and blocked with blocking buffer (PBST buffer containing 5% dry non-fat milk or 3% BSA). The membrane was incubated with the primary antibody in blocking buffer overnight. After three times wash with PBST, the membrane was incubated with the secondary antibody for 1 h. Bands were visualized with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt colorimetric detection system (Nacalai Tesque).

4.2.6. Measurement of oxygen consumption rate (OCR)

OCR was measured by Seahorse XFe96 Flux Analyzer (Agilent Technologies), according to the manufacturer's protocol. Briefly, cells (80–90% confluency) were equilibrated for 1.5 h in a 37 °C incubator lacking CO_2. Oxygen concentration in media was measured at basal conditions and after sequential addition of compounds (KUSC-5037 (0.1, 1, and 10 μM), oligomycin (0.5 μM), FCCP (0.5 μM) and a mixture of rotenone (0.5 μM) and antimycin A (0.5 μM)). OCR was determined from at least 6 samples.

4.2.7. In vitro labeling and identification of KUSC-5037/KUSC-5051 binding proteins

HeLa S3 cells were lysed and fractionated by using Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific) according to the manufacturer's protocol. Debris fraction (1 mg/mL), mitochondrial fraction (0.8 mg/mL) and cytosol fraction (0.6 mg/mL) were incubated with DMSO or 300 μM **KUSC-5037** in the absence or presence of 30 μM **KUSC-5051** for 1 h at 37 °C with agitation, followed by 365 nm UV irradiation for 30 min. A biotin moiety was conjugated to the covalent

protein-compound complex by click chemistry at RT for 1 h [200 μM biotin azide, 1 mM Tris(2-carboxyethyl)phosphine (TCEP, Cayman), 100 μM Tris[(1-*benzyl*-1*H*-1,2,3-*triazol*-4-*yl*)*methyl*]amine (TBTA, Tokyo Chemical Industry) and 1 mM $CuSO_4$]. After the removal of excess labeling reagents by $CHCl_3$-MeOH precipitation,[14] the complexes were enriched on High Capacity NeutrAvidin agarose (Thermo Fisher Scientific) by overnight incubation in PBS(-) containing 0.1% SDS at 4 °C. After a series of extensive wash of the beads at 4 °C with [PBS(-) + 0.02% SDS/50 mM NH_4HCO_3/PBS(-)], the assembled proteins were eluted into Laemmli SDS sample buffer and subjected to SDS-PAGE. The gel was used in either western blot analysis employing an anti-human ATP5B antibody or silver stain by using Pierce Silver Stain Kit (Thermo Fisher Scientific) for the further mass analysis.

4.2.8. Analysis of KUSC-5037/KUSC-5051 binding proteins by LC-MS/MS

The SDS-PAGE separated band was excised and de-stained. The 60 Da protein band shown in Fig. 2A was digested with trypsin (TPCK-treated, Worthington Biochemical) for 12 h at 37 °C. The digested mixture was analyzed by nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) coupled with Easy-nLC 1200 (Thermo Fisher Scientific). The peptides were separated on a nano ESI spray column (75 μm (inner diameter) × 150 mm (length), NTCC analytical column C18, 3 μm (particle diameter), Nikkyo Technos), with a gradient of 0–40% buffer B (80% aqueous acetonitrile with 0.1% (v/v) formic acid) in buffer A (MilliQ water with 0.1% (v/v) formic acid) at a flow rate of 300 nL min^{-1} over 10 min. The mass spectrometer was operated in the positive-ion mode for mass spectrometry and tandem mass spectrometry, and the tandem mass spectrometry spectra were acquired using the data-dependent TOP 10 method. The acquired data were processed using Mascot (version 2.7.0, Matrix Science). The MSMS data were used to search the NCBI-nr protein database (20160711, Taxonomy: H. sapiens 326,427 sequences), using the following parameters: enzyme = trypsin; maximum missed cleavages = 3; variable modifications = Acetyl (Protein *N*-term), Gln-> pyro-Glu (*N*-term Q), Oxidation (M), Propionamide (C); product mass tolerance = ± 15 ppm; product mass tolerance = ± 30 milli mass unit; instrument type = ESI-TRAP. The quantitative data of **KUSC-5037/-5051**-binding protein form LC-MS/MS analysis are shown in Table S1.

4.2.9. ATP hydrolysis activity assay

ATP hydrolysis activity of bovine F_oF_1-ATP synthase was measured by using MitoCheck Complex V Activity Assay Kit (Cayman Chemical). Briefly, 10 and 100 nM of **KUSC-5037** was incubated with ATP synthase. The activity during the 2 h-incubation at 25 °C was monitored by measuring the absorbance at 340 nm which attributed to the consumption of the supplemented NADH. Oligomycin was used as a positive control.

4.2.10. Cell proliferation assay

HeLa S3 cells and HT1080 cells were plated in 96-well plates at a seeding density of 1.5×10^3 cells/well. After 24 h incubation, the cells were treated with test compounds, cell viability at 72 h was determined by Cell Counting Kit-8 (Dojindo).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116375.

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