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Article

1,4,9-Triazaspiro[5.5]undecan-2-one Derivatives as Potent and Selective METTL3 Inhibitors

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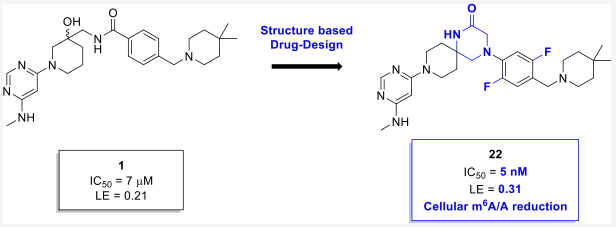
ABSTRACT: N^6-methyladenosine (m^6A) is the most frequent of the 160 RNA modifications reported so far. Accumulating evidence suggests that the METTL3/METTL14 protein complex, part of the m^6A regulation machinery, is a key player in a variety of diseases including several types of cancer, type 2 diabetes, and viral infections. Here we report on a protein crystallography-based medicinal chemistry optimization of a METTL3 hit compound that has resulted in a 1400-fold potency improvement (IC_{50} of 5 nM for the lead compound **22** (**UZH2**) in a time-resolved Förster resonance energy transfer (TR-FRET) assay). The series has favorable ADME properties as physicochemical characteristics were taken into account during hit optimization. **UZH2** shows target engagement in cells and is able to reduce the m^6A/A level of polyadenylated RNA in MOLM-13 (acute myeloid leukemia) and PC-3 (prostate cancer) cell lines.



INTRODUCTION

mRNA is at the heart of multiple chemical modifications, grouped into the epitranscriptome,[1] that are involved in a wide array of biological processes.[2] Nowadays, over 160 distinct RNA modifications have been reported,[3] among which N^6-methyladenosine (m^6A) is the most prevalent one, representing around 50% of the total methylated ribonucleotides in global cellular RNAs.[4] m^6A has been found in all kinds of cellular RNA,[5] including mRNA, tRNA, and rRNA, and its roles in gene expression regulation are various, ranging from splicing to translation, stability, and degradation.[6] Therefore, it is not surprising that abnormal levels of m^6A have been correlated with certain cancers, such as acute myeloid leukemia (AML) and lymphomas, among others.[5,7] Furthermore, other biological mechanisms may be influenced by m^6A dysfunctional levels like the circadian clock,[8] stem cell differentiation, or even viral gene expression.[9]

The dynamic and reversible nature of m^6A is regulated by three types of proteins called "writers", "readers", and "erasers". While the last two recognize or remove m^6A modifications, the former are the starting point by installing the methyl mark.[10] The most important m^6A writer system comprises the heterodimer complex formed by the two methyltransferase-like proteins, METTL3 and METTL14. Structural insights by X-ray crystallography as well as biological function analyses have been reported in the last years, revealing METTL3 as the catalytic unit, whereas METTL14 facilitates RNA substrate binding and stabilizes the complex.[11] Another relevant m^6A methyltransferase is METTL16, which has a homodimer structure and a charged groove comprising unique features.[12] Due to these structural differences, METTL16 binds prominently to pre-mRNA, in particular in introns, while METTL3/METTL14 has a broader panel of RNA targets.[13] The biological role of METTL3 and METTL14 is wide but remains elusive; for instance, METTL3 promotes translation and regulates cell proliferation, cell migration, and inflammatory response, among others.[14] METTL3 and METTL14 seem to be key players in several cancer types; for instance, they were found in the top 10% of the essential genes for AML cell survival, and their knockdown raised the growth, self-renewal, and tumorigenesis of glioblastoma stem cells.[15] In addition to cancer, METTL3/METTL14 plays roles in type 2 diabetes, heart failure, and viral infections.[16] Thus, the METTL3/METTL14 complex is an attractive target for small molecules, but to date, only a high-nanomolar inhibitor (called **UZH1a**) and the very recent disclosure of **STM2457** have been described in the literature.[17] Here we report a medicinal chemistry campaign toward chemical probes with single-digit nanomolar potency against METTL3, selectivity against METTL16 and against non-m^6A RNA methyltransferases, favorable ADME properties, and submicromolar m^6A/A reduction in a leukemia cell line.

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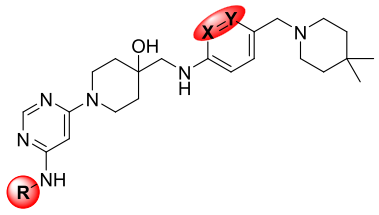
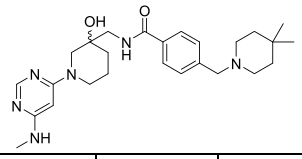
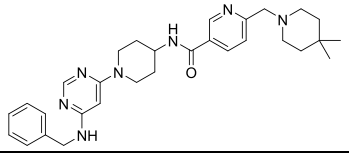
Table 1. Early Modifications of the Original Scaffold



Compound no.	R	X	Y	IC_{50}[a]	MW[b]	LE[c]	LLE[d]	Kinetic solubility[e]	$P_{app\ A\text{-}B}$ (Efflux)[f]	RLM[g]
1				7	466.63	0.21	2.5	-	-	-
2	Me	CH	CH	5.0	438.62	0.23	2.5	-	-	-
3	Me	N	CH	4.6	439.61	0.23	3.2	-	-	-
4	Me	CH	N	5.8	439.61	0.22	3.4	-	-	-
5	Bn	CH	N	0.79	515.70	0.22	2.8	78	8 (5)	5
6				3.6	513.69	0.20	1.7	-	-	-

[a]Time-resolved Förster resonance energy transfer (TR-FRET) assay (μM). Means of at least three measurements. [b]Molecular weight (g/mol). [c]Ligand efficiency (kcal·mol^{-1}·heavy atom count^{-1}). [d]Lipophilic ligand efficiency (pIC_{50} − logP). [e]μM. [f]10^{-6} cm·s^{-1} (efflux ratio), apparent permeability, Caco-2 experiment. [g]Rat liver microsomes, $t_{1/2}$ (min).

RESULTS AND DISCUSSION

Medicinal Chemistry Campaign. Our design started at the roots of one of our early inhibitors (**1**, Table 1), which arose from an adenine-based library screening,[18] followed by a short protein structure-based potency optimization against METTL3. The X-ray structure of **1** with METTL3 revealed several interesting interactions: the pyrimidine moiety was engaged in two hydrogen bonds with a NH backbone from Asn549 and Ile378 while being involved in an edge-to-face π-stacking with Phe534 and π interactions with the Asn549 side chain (Figure 1A). In the meantime, the methylamine group interacted with the Asp377 side chain. On the other end of the binding pocket, the gem dimethyl group filled a lipophilic pocket formed by Lys513, Pro514, Trp457, and Trp431 residues, whereas the charged piperidine formed a salt-bridge with Asp395. The central part of **1** did not show clear interactions, except for the tertiary alcohol, which formed a hydrogen bond with the side chain of Asn549. Thus, the central part was selected to simplify the structure and reduce the molecular weight (Figure 1B). For this purpose, changing the methylene position from 1,3 to 1,4 on the piperidine ring removed the chiral center. In addition, according to the X-ray structure of **1** with METTL3, the amide C═O group deletion would allow the original vector and distance between the piperidine and the aryl group to be kept (Figure 1B).These two modifications led to **2** and its two pyridine containing derivatives **3** and **4**, which exhibited not only similar potency compared to the parent molecule (IC_{50} = 5.0, 4.6, and 5.8 μM respectively; Table 1) but also no chirality, a reduced heavy atom count, and hence a higher ligand efficiency (LE = 0.23, 0.23, and 0.22, respectively). Because **4** had better lipophilic ligand efficiency (LLE = 3.4, calculated with DataWarrior),[19] its pyridine core was conserved in the next optimization stage. According to the crystal structure of the complex of METTL3 with inhibitor **1**, methylamine to benzylamine replacement on

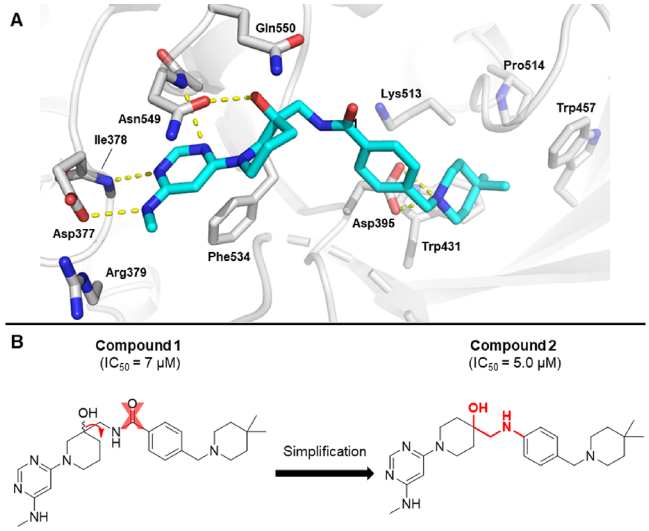


Figure 1. Initial optimization. (A) Crystal structure of compound **1** (carbon atoms in cyan) in the METTL3 binding site with relevant residues (gray). The main interactions are shown (yellow dashes; PDB code: 7NHI). (B) Design of METTL3 inhibitor **2** from hit compound **1**. The bonds formed are depicted in red. The IC_{50} values refer to the biochemical assay based on time-resolved Förster resonance energy transfer (TR-FRET).

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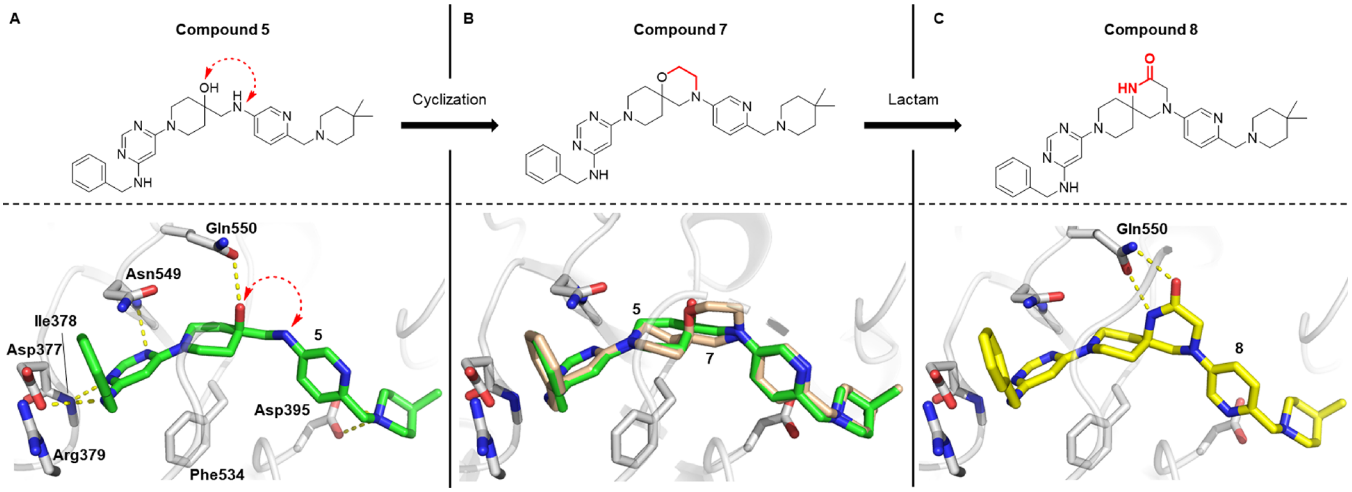
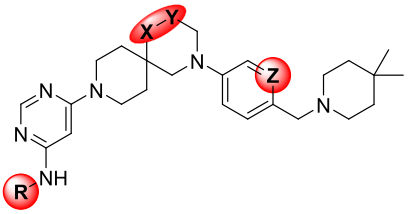
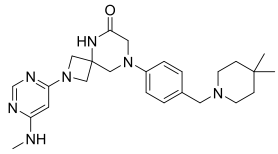
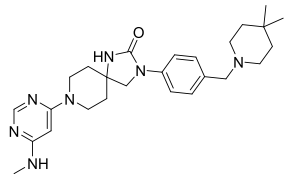


Figure 2. Design of compound **8** starting from **5**. (A) Crystal structure of inhibitor **5** (carbon atoms in green) in the METTL3 binding site with relevant residues (gray). The main intermolecular interactions are displayed (yellow dashed lines, PDB code: 7O08). (B) Superimposition of inhibitors **5** (green) and **7** (beige, PDB code: 7O09). The Gln550 side chain in the structure of the complex with compound **7** is not visible due to poor electron density. (C) Crystal structure of inhibitor **8** (yellow) in the METTL3 binding site and interactions of the lactam with the side chain amide of Gln550 (PDB code: 7O0L).

Table 2. Derivatization from the Spirocycle Scaffold



Compound no.	R	X	Y	Z	IC_{50}[a]	MW[b]	LE[c]	LLE[d]	Kinetic solubility[e]	$P_{app\ A\text{-}B}$ (Efflux)[f]	RLM[g]
7	Bn	O	CH_2	N	0.28	541.74	0.22	2.8	61	15 (0.6)	12
8	Bn	NH	C=O	N	0.037	554.74	0.25	4.4	44	2 (17)	8
9	Bn	NH	C=O	CH	0.026	553.75	0.25	3.6	69	9 (3)	9
10	Me	NH	C=O	CH	0.089	477.65	0.28	4.5	108	2 (9)	107
11					0.44	449.60	0.26	4.5	86	-	70
12					1.8	463.63	0.23	2.6	-	-	-
13	Me	NMe	C=O	CH	1.5	491.68	0.22	3.0	-	-	-

[a]TR-FRET assay (μM). Means of at least three measurements. [b]Molecular weight (g/mol). [c]Ligand efficiency (kcal·mol^{-1}·heavy atom count^{-1}). [d]Lipophilic ligand efficiency (pIC_{50} − logP). [e]μM. [f]10^{-6} cm·s^{-1} (efflux ratio), apparent permeability, Caco-2 experiment. [g]Rat liver microsomes, $t_{1/2}$ (min).

the pyrimidine ring seemed beneficial to inhibition thanks to a potential cation−π interaction with Arg379. This proved to be true as the corresponding derivative **5** showed a 6-fold increase in potency compared to compound **2** (IC_{50} = 0.79 and 5.0 μM, respectively).

One striking feature of this inhibitor series is its linear shape coupled with sp^3 acyclic atom linkers that make it highly flexible. Rigidifying the structure is a viable way to freeze a ligand in its preferred conformation, which in turn can enhance the binding energy by reducing entropic penalties. Thus, we envisioned two different strategies to achieve this goal: either an amide connection could be made between the piperidine and the pyridine ring, or according to compound **5**'s conformation, a spirocycle could be formed by connecting

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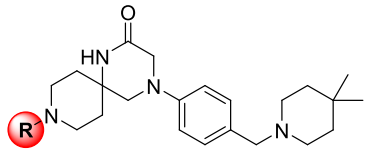
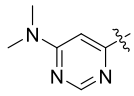
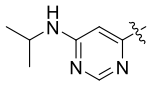
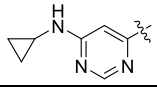
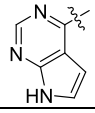
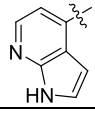
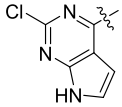
Table 3. Optimization of the Aminopyrimidine Ring



Compound no.	R	IC_{50}[a]	MW[b]	LE[c]	LLE[d]	Kinetic solubility[e]	$P_{app\ A\text{-}B}$ (Efflux)[f]	RLM[g]
14		0.97	491.68	0.23	3.2	-	-	-
15		0.33	505.71	0.24	3.2	-	-	-
16		0.084	503.69	0.26	4.0	111	4 (7)	118
17		0.061	487.65	0.27	4.3	73	2 (14)	93
18		4.5	486.66	0.20	2.3	-	-	-
19		0.024	522.09	0.28	3.9	45	-	32

[a]TR-FRET assay (μM). Means of at least three measurements. [b]Molecular weight (g/mol). [c]Ligand efficiency (kcal·mol^{-1}·heavy atom count^{-1}). [d]Lipophilic ligand efficiency (pIC_{50} − logP). [e]μM. [f]10^{-6} cm·s^{-1} (efflux ratio), apparent permeability, Caco-2 experiment. [g]Rat liver microsomes, $t_{1/2}$ (min).

the tertiary alcohol with the aniline (Figure 2A). The two methods brought opposite results; the amide derivative **6** lost the previous potency boost (IC_{50} = 3.6 μM, Table 1), while spirocycle **7** was promising in terms of inhibition (IC_{50} = 0.28 μM, Table 2) and novelty. We managed to soak both **5** and **7** in crystals of holo METTL3/METTL14. Compound **5** recapitulated most of the interactions of **1**, except for the tertiary alcohol that this time formed a hydrogen bond with Gln550 instead of Asn549 (Figure 2A). A strong structural overlap was found between **5** and **7**, the sole difference being, for the latter, the missing aforementioned hydrogen bond with the Gln550 side chain due to the alcohol transformation into an ether (Figure 2B). In fact, the electron density for the Gln550 side chain in the complex of METTL3 with compound **7** was very poor, which suggested no hydrogen bonding. We envisaged that replacing the ether by a lactam could restore this interaction and even make an additional hydrogen bond thanks to the C═O group of the ligand and the NH_2 amide of Gln550. Indeed, we obtained a strong potency boost for the corresponding derivative **8** (IC_{50} = 0.037 μM), and our hypothesis was confirmed by the two hydrogen bond interactions found in the crystal structure (Figure 2C). Furthermore, both LE and LLE improved substantially (0.25 and 4.4, respectively; Table 2).

ADME properties, such as solubility, cell permeability, and metabolic stability, are essential for chemical probes,[20] so they were considered early on in the project. Our newly synthesized inhibitors (**5**, **7**−**8**) displayed mixed results; nonetheless, all of them had mediocre stability toward enzymatic degradation with half-lives lower than 12 min upon incubation with rat liver microsomes (Tables 1 and 2). Therefore, we focused on improving ADME properties while getting better biochemical potency. The initial approach was to substitute the pyridine nitrogen atom by a carbon atom, yielding **9** with moderate permeability (9×10^{-6} cm·s^{-1}) and, surprisingly, slightly increased solubility (Table 2). However, metabolic stability remained unchanged, so the benzylamine was replaced with methylamine (**10**). Indeed, solubility and metabolic stability were significantly improved (108 μM and 107 min, respectively) as well as LE and LLE values (0.28 and 4.5, respectively) but at the expense of limited permeability (2×10^{-6} cm·s^{-1}) and a 3.4-fold potency reduction. From **10**, two other possibilities of decreasing the size of our molecules were pursued: replacing spiropiperidine with spiroazetidine (**11**) and replacing spirolactam with spirourea (**12**). Unfortunately, both displayed a substantial loss in potency (5- and 20-fold, respectively). Yet, the spiroazetidine moiety remains a potential alternative helping to reduce molecular weight and to improve physicochemical properties at a later stage. Next, our strategy was oriented toward permeability improvement. Lactam methylation in compound **13** resulted in a serious decrease in potency (19-fold), demonstrating the crucial role of the lactam hydrogen bond interactions.

After thorough spirocycle scaffold optimization, we turned our attention to the pyrimidine motif. Addition of one more methyl on the aniline (**14**) was highly detrimental to binding

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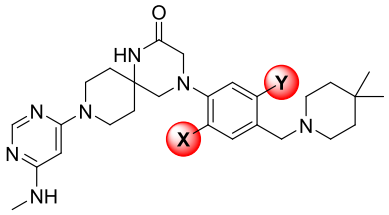
Table 4. Fluorine Scan on the Phenyl Ring



compound no.	X	Y	IC_{50}[a]	MW[b]	LE[c]	LLE[d]	Kinetic solubility[e]	$P_{app\ A\text{-}B}$ (efflux)[f]	RLM[g]
20	F	H	0.038	495.64	0.28	4.8	72	9 (2)	63
21	H	F	0.032	495.64	0.29	4.8	92	3 (8)	46
22 (UZH2)	F	F	0.005	513.64	0.30	5.3	54	12 (2)	24

[a]TR-FRET assay (μM). Means of at least three measurements. [b]Molecular weight (g/mol). [c]Ligand efficiency (kcal·mol^{-1}·heavy atom count^{-1}). [d]Lipophilic ligand efficiency (pIC_{50} − logP). [e]μM. [f]10^{-6} cm·s^{-1} (efflux ratio), apparent permeability, Caco-2 experiment. [g]Rat liver microsomes, $t_{1/2}$ (min).

compared to **10** (0.97 and 0.089 μM, respectively; Table 3), probably due to the loss of the hydrogen bond to the side chain of Asp377, while methyl to isopropyl substitution (**15**) showed a less pronounced reduction as the hydrogen bond was preserved (0.33 μM). These two modifications illustrated the limited space available for branched sp^3 carbons at this position. Surprisingly, substitution with a cyclopropyl group (**16**) was not only well tolerated (0.084 μM), but it also slightly improved the three ADME properties (Table 3) and could become a promising alternative for lead optimization. *S*-Adenosyl methionine (SAM) is the natural ligand of METTL3 that contains an adenosine scaffold overlapping with the pyrimidine group of our inhibitors; thus, we thought to test a few bicyclic heteroaromatic modifications. The pyrrolopyrimidine **17** had a slight increase in potency in comparison to **10** but similarly low permeability and a larger efflux ratio in the Caco-2 assay (Table 3). The interaction geometry between the N^3 pyrimidine atom and Asn549 nitrogen backbone did not seem optimal; thus, we thought to remove this pyrimidine nitrogen atom to improve permeability and possibly to suppress a partial desolvation penalty. The latter proved to be false since pyrrolopyridine **18** exhibited a severe binding loss (74-fold). The incorporation of a chlorine atom between the two pyrimidine nitrogen atoms (**19**) was beneficial for potency (0.024 μM); however, solubility and metabolic stability were critically impaired (45 μM and 32 min, respectively), which prompted us to look for different modifications.

Because the spirocycle scaffold and the pyrimidine moiety were already optimized, we considered the phenyl ring as the next target region. Several publications discuss the unique properties of fluorine atoms that can translate into unexpected and promising results in drug design. Indeed, fluorine atoms are able to make unusual interactions, and aromatic fluorine atoms tend to increase permeability.[21,22] A fluorine scan was performed on the phenyl ring, affording two novel derivatives: **20** and **21**. Compared to the inhibitor **10**, both compounds improved binding to a similar extent (0.038 and 0.032 μM, respectively); however, permeability was considerably increased only for **20** (Table 4).

X-ray structures in complex with METTL3 were solved for each molecule and revealed that the fluorine in **21** displayed hydrophobic contacts (Figure 3B), whereas the fluorine atom of **20** was engaged in an unusual interaction with the nitrogen π system of Pro397 (Figure 3A).[22] Inhibitor **20** was preferable because of its strong improvement in permeability and small

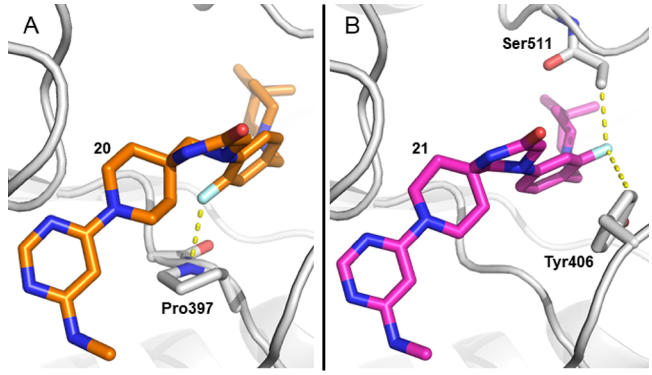


Figure 3. (A) Unusual interaction of the fluorine atom of compound **20** (carbon atoms in orange) with the Pro397 amide π-system, PDB code: 7O29. (B) Van der Waals contacts between the fluorine atom of inhibitor **21** (purple) and the side chains of Ser511 and Tyr406, PDB code: 7O2E. The side chain hydroxyl group of Ser511 in the complex with compound **21** is not visible due to poor electron density.

efflux ratio (9×10^{-6} cm·s^{-1} and 2, respectively), but the combination of both fluorine atoms quickly emerged as the key solution to achieve excellent potency and to keep adequate ADME properties. Indeed, compound **22** (**UZH2**) exhibited single-digit nanomolar IC_{50} (0.005 μM) in the TR-FRET assay (Table 4 and Figure 4A), high cell permeability (12×10^{-6} cm·s^{-1}), and favorable values of LE and LLE (0.3 and 5.3, respectively), as well as acceptable metabolic stability ($t_{1/2}$ = 24 min).

To investigate the selectivity of **UZH2** toward other m^{6}A writer proteins and RNA methyltransferases, we tested our inhibitor in a thermal shift assay against METTL16 and METTL1. The latter is a writer of the 7-methylguanosine mark on tRNA, mRNA, and miRNAs and served as a representative of non-m^{6}A methyltransferase proteins.[23] We employed as positive control *S*-adenosyl-L-homocysteine (SAH), a by-product of RNA methyltransferase catalytic activity and a natural binder, which showed ΔTm of 3.7, 0.8, and 6.3 °C at 1000 μM for METTL3/METTL14, METTL16, and METTL1, respectively (Figures S1, S2, and S3). **UZH2** at 100 μM was able to shift the melting temperature of METTL3/METTL14 by 4.7 °C compared to the DMSO control. On the contrary, no shift was observed for METTL16 and METTL1 with **UZH2** up to 100 μM, which indicates the lack of binding to the off-targets (Figures S2 and S3).

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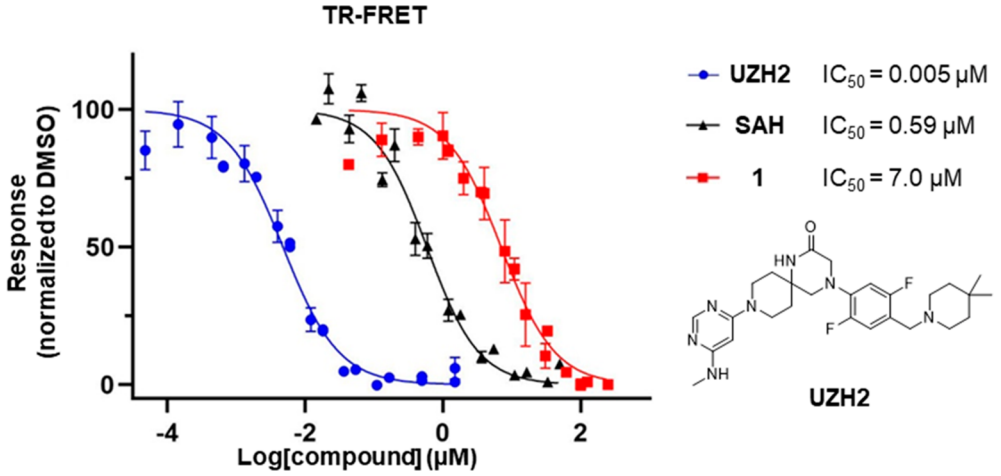

Figure 4. Inhibitory activity of **UZH2**. TR-FRET dose−response curves of METTL3 inhibition (mean ± SEM, $n \geq 3$ technical replicates) measured for **UZH2**, compound **1**, and **SAH**.

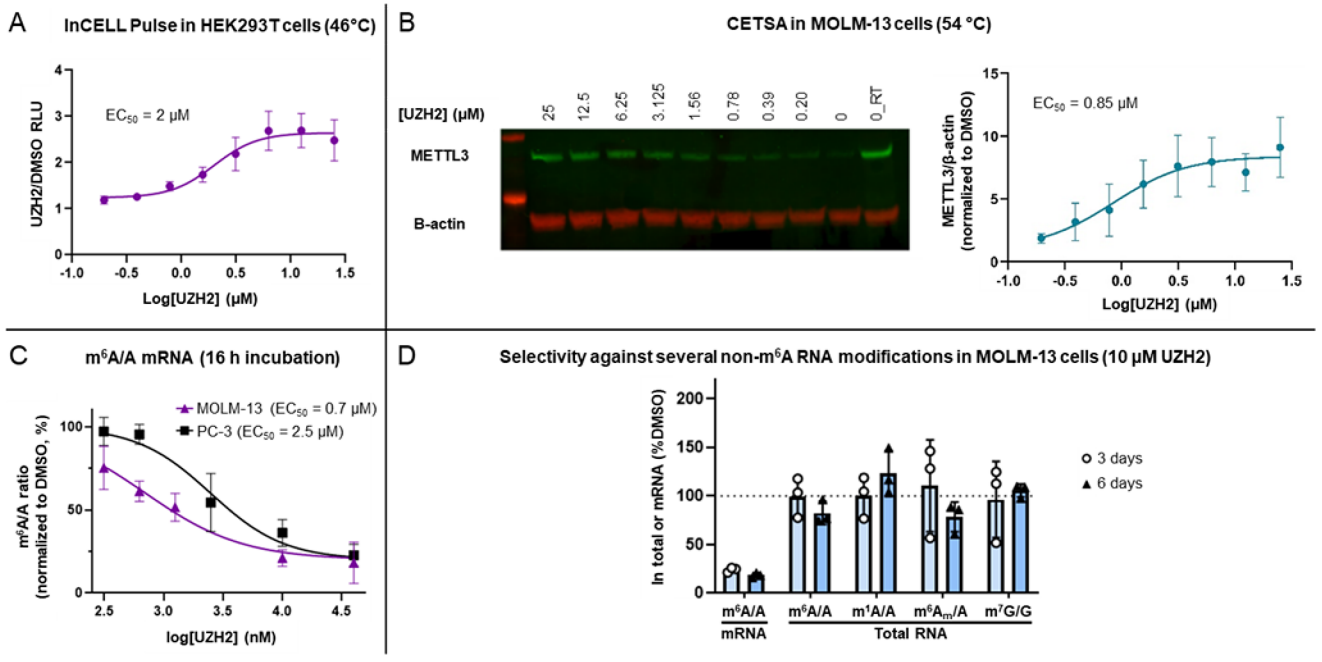

Figure 5. Activity and selectivity of **UZH2** in cellular assays. (A) InCELL Pulse dose−response curve of **UZH2** (mean ± SD, $n = 3$ biological replicates) in HEK293T cells at 46 °C; DMSO-treated cells served as the normalization control. (B) Representative Western blot image for CETSA at 54 °C in MOLM-13 cells and dose−response curve of **UZH2** (mean ± SEM, $n = 3$ biological replicates), normalized to DMSO-treated samples. (C) Dose−response curves of m^{6}A/A reduction upon **UZH2** incubation in the polyadenylated RNA fraction in MOLM-13 (mean ± SD, $n = 5$ biological replicates) and PC-3 (mean ± SD, $n = 3$ biological replicates) cell lines measured by UPLC−MS/MS, normalized to DMSO-treated samples for each cell line. (D) Results of UPLC−MS/MS assay of the modified nucleoside level in the mRNA fraction and in total RNA from MOLM-13 cells upon 10 μM **UZH2** treatment for 3 and 6 days (mean ± SD, $n = 3$ biological replicates, normalized to DMSO-treated samples).

The enhanced thermal stabilization of METTL3 by **UZH2** allowed us to study its cellular target engagement in two orthogonal assays based on protein thermal denaturation. The binding of **UZH2** was evaluated in an InCELL Pulse assay where the enhanced ProLabel (ePL) enzyme fragment fused to the *N*-terminus of the truncated METTL3 (residues 354−580) was expressed in HEK293T cells. After the incubation of these cells with **UZH2** for 1 h at 37 °C, cells were heated at 46 °C for 3 min, and the non-aggregated METTL3-ePL protein was quantified using a luminescence-based assay (Figure 5A). **UZH2** stabilized the METTL3-ePL fusion protein with an EC_{50} of 2 μM in HEK293T cells. Encouraged by these results, we also conducted a CETSA assay in MOLM-13 cells on an endogenously expressed full-length METTL3. Similarly to the InCELL Pulse assay, CETSA in MOLM-13 cells demonstrated that **UZH2** significantly stabilized METTL3 in a dose-dependent manner at 54 °C with an EC_{50} of 0.85 μM as determined by Western blotting (Figure 5B). Altogether, both experiments brought clear evidence of cell permeability and cellular target engagement. The discrepancy between the IC_{50} values measured in the biochemical and cellular assays is due, at least in part, to the high concentration of SAM in the cells.

To highlight the biological potential of **UZH2** as an inhibitor of METTL3 enzymatic activity in cells, we measured the m^{6}A/A ratio in polyadenylated RNA in two distinct cancer cell lines, MOLM-13 (AML) and PC-3 (prostate cancer) cells,

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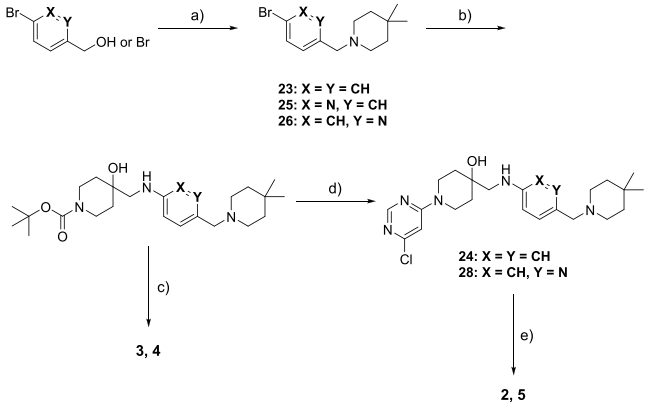
after 16 h of compound treatment. We found that **UZH2** was able to reduce this ratio down to 10−20% of DMSO-treated control samples with a certain degree of selectivity between the two cell lines (EC_{50} = 0.7 and 2.5 μM for MOLM-13 and PC-3, respectively; Figure 5C). These results translated into impaired cell growth for MOLM-13 (GI_{50} = 12 ± 3 μM, Figure S4) after 3 days of compound treatment but not for PC-3 (GI_{50} = 70 ± 10 μM), probably owing to the differing reliance of cell lines on METTL3 for survival. Importantly, METTL3 expression in MOLM-13 was not altered by **UZH2** treatment as shown by Western blotting (Figure S5). Selectivity over other RNA methyltransferases was demonstrated *in vitro*, but evidence in cells would also be advantageous for further biological experiments. Thus, we performed analyses of m^6A level and several RNA modifications (m^6Am, m^1A, and m^7G) in total RNA by an LC−MS/MS assay. Because tRNAs and rRNAs, the major components of cellular total RNA, have longer half-lives than mRNA,[24] measurements were conducted at 3 and 6 days of treatment for unambiguous results. **UZH2** showed specificity for m^6A in mRNA since it did not induce changes to m^1A and m^7G levels even after 6 days of incubation at 10 μM, and m^6A and m^6Am in total RNA were marginally affected after 6 days of treatment (Figure 5D).

Synthesis. The target compounds **2**−**5** were synthesized following a general strategy starting from the alkylation of 4,4-dimethylpiperidine (Scheme 1). Either commercial 4-bromo-

Scheme 1. General Synthesis Route for Compounds 2−5[a]

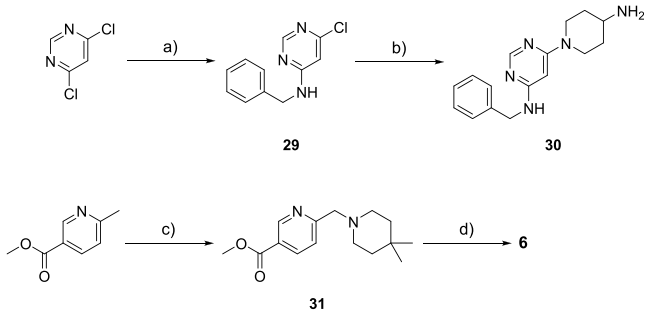


[a]Reagents and conditions: (a) For **23**: 4-bromobenzyl bromide, 4,4-dimethylpiperidine hydrochloride, K_2CO_3, DMF, 25 °C, 17 h, 99%. For **25** and **26**: (i) (6-chloropyridin-3-yl)methanol (**25**) or (5-chloropyridin-2-yl)methanol (**26**), $SOCl_2$, DMF, DCM, 25 °C, 2−3 h; (ii) 4,4-dimethylpiperidine hydrochloride, K_2CO_3, DMF, 70 °C, 3 h, 67% over two steps (**25**)/86% over two steps (**26**); (b) *tert*-butyl 4-(aminomethyl)-4-hydroxypiperidine-1-carboxylate, Pd Ruphos G4, Ruphos, Cs_2CO_3, dioxane, N_2, 150 °C, 17 h; (c) (i) HCl (37% aq.), MeOH, 25 °C, 4 h; (ii) 6-chloro-*N*-methylpyrimidin-4-amine, Et_3N, *i*PrOH, 140 °C, 5 h, μw, 1.6% over three steps (**3**)/23% over three steps (**4**); (d) (i) HCl (37% aq.), MeOH, 25 °C, 4 h; (ii) 4,6-dichloro-pyrimidine, Et_3N, *i*PrOH, 80 °C, 3 h, μw, 36% over three steps (**24**)/80% over three steps (**28**); (e) for **2**: $MeNH_2$, EtOH, 130 °C, 3 h, μw, 33%; for **5**: $BnNH_2$, 140 °C, 8 h, μw, 22%.

benzyl bromide or the adequate alkyl chlorides prepared from the corresponding alcohols were used. Next, the aryl halides **23**, **25**, and **26** were subjected to a Buchwald−Hartwig coupling followed by Boc group deprotection to afford the corresponding secondary amines. The latter underwent either S_NAr with 6-chloro-*N*-methylpyrimidin-4-amine to afford the desired compounds **3** and **4** or S_NAr with 4,6-dichloro-pyrimidine followed by a second S_NAr with methylamine or benzylamine to furnish compounds **2** and **5**, respectively.

For compound **6**, a different approach with a final amide coupling was used (Scheme 2). First, amine **30** was synthesized

Scheme 2. Synthesis Route for Compound 6[a]



[a]Reagents and conditions: (a) $BnNH_2$, Et_3N, *i*PrOH, 25 °C, 3 days, 98%; (b) (i) *tert*-butyl piperidin-4-ylcarbamate, Et_3N, *i*PrOH, 150 °C, 8 h, μw, 32%; (ii) HCl (37% aq.), MeOH, 25 °C, 4 h, 75%; (c) (i) NBS, Bz_2O_2, CCl_4, N_2, 75 °C, 21 h; (ii) 4,4-dimethylpiperidine hydrochloride, K_2CO_3, DMF, 25 °C, 17 h; (d) (i) LiOH, MeOH/H_2O, 25 °C, 17 h; (ii) **30**, HATU, DIPEA, DMF, 25 °C, 17 h, 29%.

in 24% overall yield from 4,6-dichloro-pyrimidine. After benzylic bromination and amine alkylation, ester **31** was hydrolyzed under basic conditions to the corresponding carboxylic acid, which reacted with amine **30** in the presence of HATU and DIPEA to yield compound **6**.

According to the general strategy used for compounds **2**−**5**, the synthesis of most of the next target molecules required the preparation of spirocycle intermediate **33** (Scheme 3). The latter was prepared by modification of a known route[25] where amine **32** was obtained in three steps from commercial 1-Boc-4-piperidone. Subsequent alkylation with ethyl 2-bromoacetate followed by reduction of the Cbz group and concomitant intramolecular cyclisation afforded intermediate **33** in 18% overall yield.

The synthesis route of compound **7** started with the Buchwald−Hartwig coupling between bromo aryl **26** and the corresponding commercial spirocycle intermediate (Scheme 4). After Boc removal, an S_NAr was performed to obtain the desired product **7**. A similar strategy was pursued for compounds **8**−**10** and **14**−**16** with **33** being the spirocycle for cross-coupling reactions, and after Boc deprotection, two S_NArs, first with 4,6-dichloro-pyrimidine and then with the appropriate amines, were carried out. For **13**, an intermediate alkylation step to yield *N*-methyl amide **45** was done prior to Boc deprotection, while for **17** and **18**, the final step consisted of a Buchwald−Hartwig coupling, and only one S_NAr using an activated 7*H*-pyrrolo[2,3-d]pyrimidine derivative was required for **19**.

In the case of compound **11**, the preparation of spirocycle intermediate **40** was envisaged (Scheme 5). Contrary to **33**, the first step afforded the Henry product (β-nitro alcohol) despite the use of strictly the same conditions as **33**. Therefore, a three-step procedure was followed, including a DAST mediated dehydration of the Henry product to obtain intermediate **38** in quantitative yield. The next steps for compound **11** (Scheme 6) were then performed similarly to spirocycle **33** and compound **10**.

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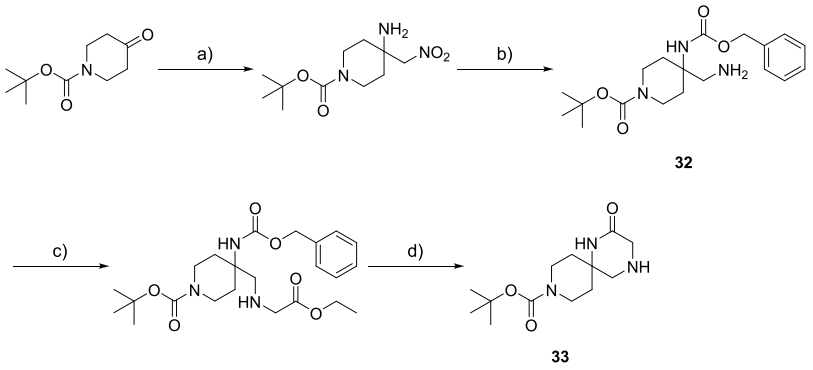
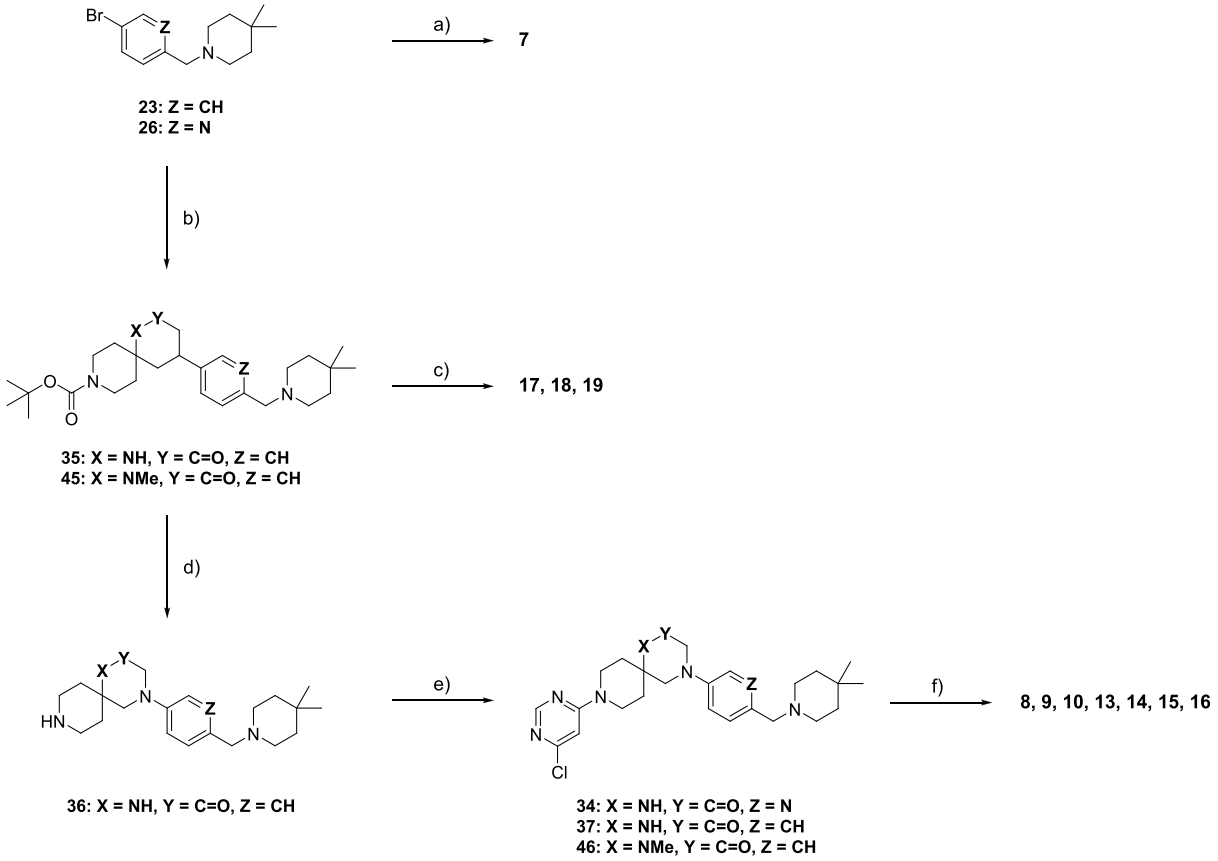
Scheme 3. Synthesis Route toward Intermediate 33[a]



[a]Reagents and conditions: (a) $MeNO_2$, NH_3, MeOH, 25 °C, 17 h; (b) (i) CbzCl, $NaHCO_3$, DCM/H_2O, 0−25 °C, 17 h; (ii) $NiCl_2{\cdot}6H_2O$, $NaBH_4$, MeOH, N_2, 0−25 °C, 1 h, 32% over three steps; (c) ethyl 2-bromoacetate, Et_3N, DCM, 25 °C, 2 h; (d) Pd/C, ammonium formate, *i*PrOH, 80 °C, 4 h, 55% over two steps.

Scheme 4. Synthesis Route for Compounds 7−10 and 13-19[a]



[a]Reagents and conditions: (a) **26**, *tert*-butyl 1-oxa-4,9-diazaspiro[5.5]undecane-9-carboxylate, Pd Ruphos G4, Ruphos, Cs_2CO_3, dioxane, N_2, 150 °C, 17 h; (ii) HCl (37% aq.), MeOH, 25 °C, 4 h; (iii) **29**, Et_3N, *i*PrOH, 150 °C, 8 h, *μ*w, 6% over three steps. (b) **23** or **26**, **33**, Pd Ruphos G4, Ruphos, Cs_2CO_3, dioxane, N_2, 150 °C, 17 h, 93% (**35**); (ii) only for **45**: MeI, KOtBu, THF, 0−25 °C, N_2, 2 h, 54%; (c) (i) HCl (37% aq.), MeOH, 25 °C, 4 h; (ii) for **17** and **18**: 4-chloro-7*H*-pyrrolo[2,3-d]pyrimidine (**17**) or 4-chloro-1*H*-pyrrolo[2,3-b]pyridine (**18**), Pd Ruphos G4, Ruphos, LiHMDS, THF, N_2, 65 °C, 4 h, 36% (**17**)/70% (**18**). For **19**: 2,4-dichloro-7*H*-pyrrolo[2,3-d]pyrimidine, Et_3N, *i*PrOH, 100−130 °C, 6 h, 42%; (d) HCl (37% aq.), MeOH, 25 °C, 4 h, 36% over two steps from **23** (**36**); (e) 4,6-dichloro-pyrimidine, Et_3N, *i*PrOH, 80 °C, 3 h, *μ*w, 27% over three steps from **26** (**34**)/63% from **36** (**37**)/57% over two steps from **45** (**46**); (f) for **8** and **9**: $BnNH_2$, 140 °C, 8 h, *μ*w, 25% (**8**)/5% over two steps from **36** (**9**). For **10** and **13**: $MeNH_2$, EtOH, 130 °C, 3 h, *μ*w, 3% over two steps from **36** (**10**)/85% from **46** (**13**). For **14**: Me_2NH, EtOH, 130 °C, 3 h, *μ*w, 80% from **37**. For **15**: *i*PrNH$_2$, EtOH, 130 °C, 8 h, *μ*w, 52% from **37**. For **16**: cyclopropylamine, *i*PrOH, 130 °C, 6 h, *μ*w, 25% from **37**.

The route toward compound **12** began with the synthesis of aniline **41** in two steps (Scheme 7). In the meantime, 4-amino-1-(*tert*-butoxycarbonyl)piperidine-4-carboxylic acid was reduced to the *β*-amino alcohol thanks to the $I_2/NaBH_4$ system, and the amine was protected with the Troc group to yield intermediate **42**. After oxidation of alcohol **42** to the aldehyde and reductive amination with **41**, the Troc group could be cleanly cleaved in 74% yield with Zn in THF/AcOH. The

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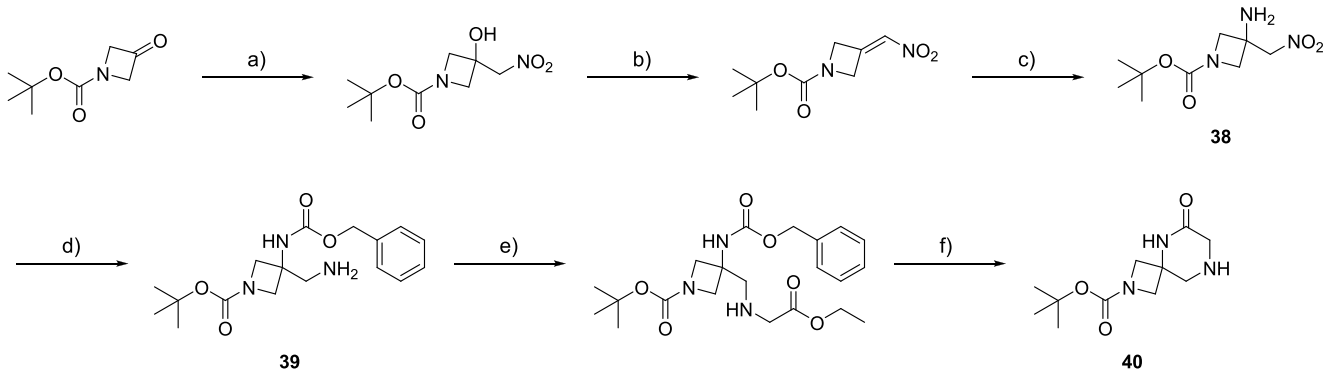
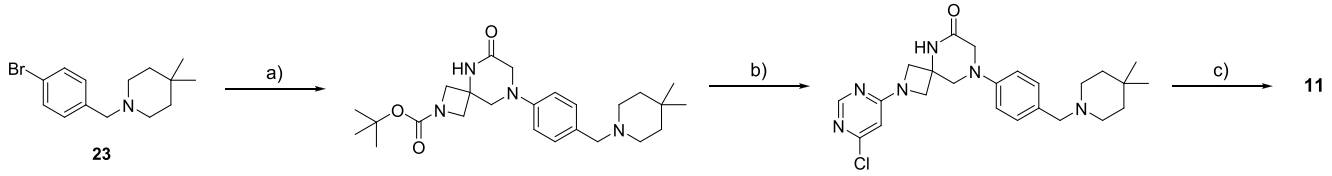
Scheme 5. Synthesis Route to Intermediate 40[a]



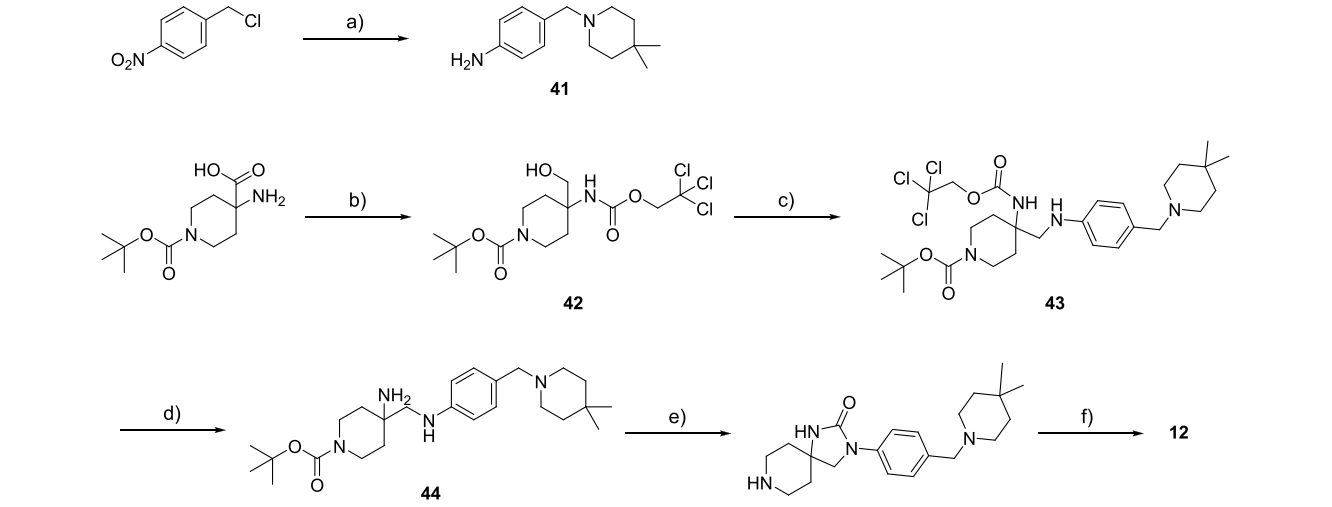
[a]Reagents and conditions: (a) $MeNO_2$, K_2CO_3, EtOH, 25 °C, 17 h; (b) DAST, DCM, N_2, −78 °C, 3 h; (c) NH_3, MeOH, 25 °C, 2 h, quantitative over three steps; (d) (i) CbzCl, $NaHCO_3$, DCM/H_2O, 0−25 °C, 17 h; (ii) $NiCl_2{\cdot}6H_2O$, $NaBH_4$, MeOH, N_2, 0−25 °C, 1 h, 54% over two steps; (e) ethyl 2-bromoacetate, Et_3N, DCM, 25 °C, 2 h; (f) Pd/C, ammonium formate, *i*PrOH, 80 °C, 4 h, 32% over two steps.

Scheme 6. Synthesis Route toward Compound 11[a]



[a]Reagents and conditions: (a) **40**, Pd Ruphos G4, Ruphos, Cs_2CO_3, dioxane, N_2, 150 °C, 17 h; (b) (i) HCl (37% aq.), MeOH, 25 °C, 4 h; (ii) 4,6-dichloro-pyrimidine, Et_3N, *i*PrOH, 80 °C, 7 h, *μ*w; (c) $MeNH_2$, EtOH, 130 °C, 3 h, *μ*w, 19% over four steps.

Scheme 7. Synthesis Route of Compound 12[a]



[a]Reagents and conditions: (a) (i) dimethylpiperidine hydrochloride, K_2CO_3, DMF, 90 °C, 17 h; (ii) Fe, AcOH, EtOH, 80 °C, 4 h, 56% over two steps; (b) (i) $NaBH_4$, I_2, THF, N_2, 0−75 °C, 17 h; (ii) TrocCl, $NaHCO_3$, DCM/H_2O, 0−25 °C, 3 h, 77% over two steps; (c) (i) Dess−Martin periodinane, DCM, 25 °C, 1.5 h, 92%; (ii) **41**, AcOH, $NaBH(OAc)_3$, DCE, 25 °C, 17 h, 71%; (d) Zn, THF/AcOH, 25 °C, 3 h, 74%; (e) (i) CDI, DCM, 25 °C, 17 h; (ii) HCl (37% aq.), MeOH, 25 °C, 4 h; (f) 4,6-dichloro-pyrimidine, Et_3N, *i*PrOH, 80 °C, 3 h, *μ*w; (ii) $MeNH_2$, EtOH, 130 °C, 3 h, *μ*w, 28% over four steps.

resulting amine **44** was cyclized using CDI. Then, Boc group deprotection and final S_NAr gave access to compound **12**. The synthesis of the last target compounds (**20**−**22**, Scheme 8) used the same general route as that of compound **10**. Either the commercial aryl bromides or the alkyl chloride obtained in two steps from the corresponding carboxylic acid reacted with 4,4-dimethylpiperidine to yield bromo aryl **47**, **49**, and **52**. After Buchwald−Hartwig couplings and Boc group cleavage, the corresponding secondary amines were subjected to two S_NArs to afford the desired final molecules.

CONCLUSIONS

We successfully improved the potency (by a factor of 1400), efficiency parameters, and ADME properties of a series of METTL3 inhibitors by protein crystallography-guided medicinal chemistry. The key features were rigidification thanks to the design of spirocycle scaffolds and the use of fluorine atoms

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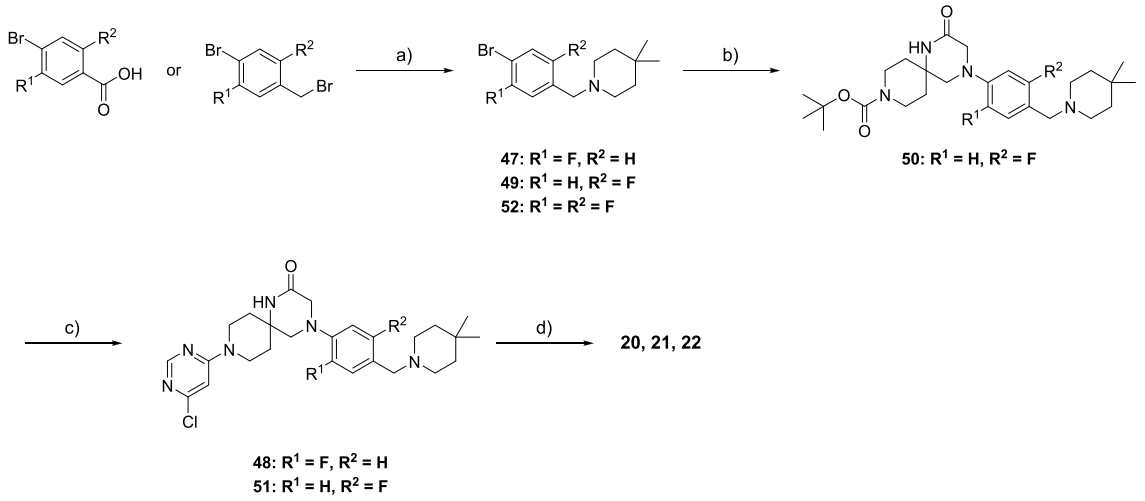
Scheme 8. General Synthesis Route for Compounds 20-22[a]



[a]Reagents and conditions: (a) for **47** and **49**: 1-bromo-4-(bromomethyl)-2-fluorobenzene (**47**) or 4-bromo-1-(bromomethyl)-2-fluorobenzene (**49**), 4,4-dimethylpiperidine hydrochloride, K_2CO_3, DMF, 25 °C, 17 h, 98% (**47**)/99% (**49**). For **52**: (i) 4-bromo-2,5-difluorobenzoic acid, BH_3SMe_2, THF, N_2, 25 °C, 17 h, 83% °C; (ii) $SOCl_2$, DMF, DCM, 25 °C, 3 h; (iii) 4,4-dimethylpiperidine hydrochloride, K_2CO_3, DMF, 25 °C, 17 h, 92%; (b) **33**, Pd Ruphos G4, Ruphos, Cs_2CO_3, dioxane, N_2, 150 °C, 17 h, 83% (**50**); (c) (i) HCl (37% aq.), MeOH, 25 °C, 4 h; (ii) 4,6-dichloropyrimidine, Et_3N, *i*PrOH, 80 °C, 3 h, μw, 15% over three steps from **47** (**48**)/58% over two steps from **50** (**51**); (d) $MeNH_2$, EtOH, 130 °C, 3 h, μw, 47% (**20**)/69% (**21**)/7% over four steps from **52** (**22**).

at specific positions. The most potent inhibitor (**UZH2**) shows an IC_{50} of 5 nM in a TR-FRET assay. Evidence of selectivity against METTL16 and METTL1 was provided by the thermal shift assay up to **UZH2** concentrations of 100 μM. Cellular target engagement of **UZH2** was demonstrated using two different assays. Furthermore, EC_{50} values of 0.7 and 2.5 μM were measured for the reduction of m^6A/A in polyadenylated RNA in MOLM-13 (leukemia) and PC-3 (prostate cancer) cell lines, respectively. In addition, selectivity in cells over other RNA methyltransferase marks was established. Thus, **UZH2** is a small molecule inhibitor with a potential for studying the functional role of METTL3/METTL14 and its involvement in cancer.

EXPERIMENTAL SECTION

Chemistry. All reagents were used as received unless otherwise noted. The solvents were dried over activated molecular sieves of appropriate size. All reactions were monitored by TLC or NMR. Chromatography was carried out over silica gel by hand or automated flash chromatography (Büchi C-850 flash prep). ^1H and ^{13}C NMR spectra were recorded on an AV2 400 MHz Bruker spectrometer. Chemical shifts are given in ppm. The spectra are calibrated to the residual ^1H and ^{13}C signals of the solvents. Multiplicities are abbreviated as follows: singlet (s), doublet (d), triplet (t), doublet−doublet (dd), doublet−doublet−doublet (ddd), triplet−doublet (td), multiplet (m), and broad (br). Melting points were determined on a Büchi M-560 melting point instrument. High-resolution electrospray ionization mass spectrometry was performed on an Agilent 1290 Infinity LC system coupled to an Agilent 6540 quadrupole time-of-flight mass spectrometer. The jet stream electrospray source was operated in positive mode with following parameter settings: nebulizer pressure 35 psig, nozzle voltage 0 V, sheath gas flow 11 L/min, sheath gas temperature 375 °C, drying gas flow 8 L/min, drying gas temperature 250 °C, capillary voltage 3000 V, and fragmentor voltage of 175 V. Accurate mass spectra were acquired in profile mode over an *m/z* range of 100−1000 by 1 spectrum per second. The Q-TOF instrument was operated in high-resolution mode with 1700 *m/z* instrument mass range at a resolving power of 33,000 (measured at *m/z* 322). The purity was acquired by HPLC on an Agilent LC device using a NUCLEOSHELL RP18 column (50 × 4.6 mm, 2.7 μm). The eluent at a flow rate of 1.5 mL/min consisted of MeCN and 0.01 M $(NH_4)_2HPO_4$ pH = 6.6 as solvents. All compounds showed ≥95% purity. Marvin (version 19.1, 2019) was used for drawing, displaying, and characterizing chemical structures and protonation states as well as for generating conformers.

General Procedures. *General Procedure for Dimethylpiperidine Alkylation.* To a stirred solution of the corresponding haloalkane (Cl or Br) or haloalkane hydrochloride salt (1 equiv) in DMF (0.3 mM), 4,4-dimethylpiperidine hydrochloride (1 equiv) and K_2CO_3 (2−3.2 equiv) were added. The reaction mixture was stirred at 25 °C for 17 h and concentrated under reduced pressure. The obtained residue was purified by flash column chromatography.

General Procedure for Buchwald−Hartwig Coupling. To a stirred solution of the corresponding halide (1 equiv) in dioxane (0.3 M), under a nitrogen atmosphere, the corresponding amine (1 equiv) was added. Nitrogen gas was bubbled through the reaction for 2 min, and Cs_2CO_3 (1.2 equiv), Ruphos Pd G4 (10 mol %), and Ruphos (10 mol %) were added. The reaction mixture was stirred at 150 °C for 17 h and concentrated under reduced pressure, and the obtained residue was purified by flash column chromatography.

General Procedure for Boc Group Deprotection. To a stirred solution of the corresponding Boc protected amine in MeOH (0.3 M), HCl (0.9 M, 37% aq.) was added. The reaction mixture was stirred at 25 °C for 4 h, and the reaction mixture was concentrated under reduced pressure. The obtained residue was directly engaged in the next step without further purification.

General Procedure for S_NAr with 4,6-Dichloropyrimidine. To a stirred solution of the corresponding amine (1 equiv) or amine hydrochloride salt (1 equiv) in *i*PrOH (0.3 M), 4,6-dichloropyrimidine (1.2 equiv) and Et_3N (1−4 equiv) were added. The reaction mixture was stirred at 80 °C for 3 h in the microwave and concentrated under reduced pressure. The crude residue was dissolved in *n*BuOH, washed three times with water and once with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude residue was coevaporated with toluene several times to remove the residual *n*BuOH and then purified by flash column chromatography.

General Procedure for S_NAr with Chloropyrimidine Derivatives. The corresponding chloropyrimidine (1 equiv) was dissolved in methylamine (0.1 M, 8 M in EtOH) or benzylamine (0.3 M) or the corresponding amine in EtOH (33 % wt.) and the reaction mixture was stirred at 130 °C for 3 h or 140 °C for 8 h ($BnNH_2$) in the

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microwave. The crude residue was concentrated under reduced pressure and purified by flash column chromatography. For reactions with benzylamine, the crude residue was coevaporated with water and then toluene several times to remove the benzylamine before performing the purification.

1-(4-Bromobenzyl)-4,4-dimethylpiperidine (23). Intermediate **23** was obtained following the general procedure for dimethylpiperidine alkylation (chromatography: EtOAc/heptane = 0:10 to 3:7). Yellow oil, 99% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46−7.40 (m, 2H), 7.24−7.17 (m, 2H), 3.45 (s, 2H), 2.37 (dd, J = 6.6, 4.3 Hz, 4H), 1.39 (t, J = 5.6 Hz, 4H), 0.92 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 137.9, 131.2 (2C), 130.8 (2C), 120.6, 62.7, 50.0 (2C), 38.7 (2C), 28.4 (2C), 28.2 (br); LRMS (ESI) m/z calcd for $[C_{14}H_{21}BrN]^+$: 282.1, found: 282.1.

1-(6-Chloropyrimidin-4-yl)-4-(((4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)amino)methyl)piperidin-4-ol (24). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling but heating at 130 °C for 8 h in the microwave (chromatography: EtOAc/heptane = 8:2 to 10:0). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. After evaporation, the crude residue was poured into a saturated aqueous Na_2CO_3 solution, and the aqueous layer was extracted three times with nBuOH. The combined organic layers were washed once with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude residue was coevaporated with toluene several times to remove the residual nBuOH, and the impure desired product was engaged in the next step without further purification.

Intermediate **24** was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine, but DCM was used for extraction (column chromatography: DCM/MeOH = 100:5 to 100:8 to 100:10 to 100:15). Orange solid, 36% yield over three steps. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.37 (s, 1H), 7.43−7.33 (m, 2H), 6.73−6.64 (m, 2H), 6.53 (s, 1H), 4.71 (br s, 1H), 4.19 (br s, 2H), 3.99 (br s, 2H), 3.41 (td, J = 12.4, 2.0 Hz, 2H), 3.26 (br s, 2H), 3.16 (d, J = 5.8 Hz, 2H), 2.75−2.65 (m, 2H), 2.17 (br s, 2H), 1.86 (d, J = 13.2 Hz, 2H), 1.62 (td, J = 12.9, 4.6 Hz, 2H), 1.46 (br d, J = 12.4 Hz, 2H), 1.06 (s, 3H), 0.98 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 162.0, 159.8, 158.1, 150.2, 132.4 (2C), 116.1, 112.8 (2C), 101.3, 69.8, 60.6, 54.2, 48.3, 40.2, 35.1, 34.6, 27.9 (2C). One carbon is missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{24}H_{35}ClN_5O]^+$: 444.3, found: 444.3.

4-(((4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)amino)methyl)-1-(6-(methylamino)pyrimidin-4-yl)piperidin-4-ol (2). Compound **2** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:5 to 100:10 to 100:15 to 100:20). The obtained impure product was triturated in diethyl ether plus a few drops of DCM, filtered, and washed with ether to afford the desired product as an off-white solid (33% yield). Mp: 242−246 °C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.99 (s, 1H), 7.25−7.18 (m, 2H), 6.78−6.71 (m, 2H), 5.64 (s, 1H), 4.14 (s, 2H), 4.09 (br d, J = 13.9 Hz, 2H), 3.15 (s, 2H), 3.07 (br s, 2H), 2.85 (s, 3H), 1.73−1.66 (m, 4H), 1.66−1.59 (m, 4H), 1.08−1.02 (m, 6H). Two protons are missing due to overlapping with the solvent residual peak; ^{13}C NMR (101 MHz, MeOD-d_4): δ 164.7, 163.4, 157.7, 152.4, 133.5 (2C), 117.3, 113.8 (2C), 71.6, 61.7, 54.9, 41.7 (2C), 36.8 (2C), 35.4 (2C), 29.0, 28.5. Two carbons are missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{25}H_{39}N_6O]^+$: 439.3185, found: 439.3180.

2-Chloro-5-((4,4-dimethylpiperidin-1-yl)methyl)pyridine (25). To a stirred solution of (6-chloropyridin-3-yl)methanol (1 g, 7 mmol) in DCM (16 mL), $SOCl_2$ (1.5 equiv, 10 mmol, 758 μL) and DMF (1 drop) were added. The reaction mixture was stirred at 25 °C for 3 h and concentrated under reduced pressure to afford the chloroalkane, which was engaged in the next step without further purification.

Intermediate **25** was obtained following the general procedure for dimethylpiperidine alkylation, but the reaction mixture was stirred at 70 °C for 3 h (column chromatography: EtOAc/heptane = 1:9 to 3:7). Yellow solid, 67% yield over two steps. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (d, J = 2.3 Hz, 1H), 7.65 (dd, J = 8.1, 2.3 Hz, 1H), 7.28 (d, J = 7.4 Hz, 1H), 3.48 (s, 2H), 2.39−2.35 (m, 4H), 1.40−1.37 (m, 4H), 0.92 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 150.01, 149.95, 139.5, 133.3, 123.9, 59.6, 49.9 (2C), 38.6 (2C), 28.3 (2C), 28.2 (br); LRMS (ESI) m/z calcd for $[C_{13}H_{20}ClN_2]^+$: 239.1, found: 239.2.

4-(((5-((4,4-Dimethylpiperidin-1-yl)methyl)pyridin-2-yl)amino)methyl)-1-(6-(methylamino)pyrimidin-4-yl)piperidin-4-ol (3). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:2 to 100:5 to 100:8 to 100:10). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. After evaporation, the crude residue was triturated in acetone, and the resulting precipitate was filtered, washed with acetone, and dried. The obtained impure desired product was engaged in the next step without further purification.

To a stirred solution of the corresponding impure amine (1 equiv) in iPrOH (0.3 M), 6-chloro-N-methylpyrimidin-4-amine (1.2 equiv) and Et_3N (4 equiv) were added. The reaction mixture was stirred at 140 °C for 5 h in the microwave and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:5 to 100:10 to 100:15 to 100:20 to 100:25 to 100:30), and the obtained impure product was triturated in acetonitrile, filtered, and dried to afford the desired product as a white solid (1.6% yield over three steps). Mp: decomposition; ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.95 (s, 1H), 7.92 (s, 1H), 7.48 (dd, J = 8.6, 2.0 Hz, 1H), 6.62 (d, J = 8.6 Hz, 1H), 5.60 (s, 1H), 3.95 (d, J = 13.7 Hz, 2H), 3.71 (br s, 2H), 3.39 (s, 4H), 2.82 (s, 3H), 2.81−2.66 (m, 4H), 1.70−1.61 (m, 4H), 1.55−1.48 (m, 4H), 0.99 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 165.1, 163.5, 161.5, 158.1, 150.8, 141.2, 115.6, 110.4, 81.8, 71.8, 59.2, 52.7, 50.0 (2C), 41.8 (2C), 37.2, 35.5 (2C), 29.1 (2C), 28.5; HRMS (ESI): m/z: calcd for $[C_{24}H_{38}N_7O]^+$: 440.3138, found: 440.3132.

5-Chloro-2-((4,4-dimethylpiperidin-1-yl)methyl)pyridine (26). To a stirred solution of (5-chloropyridin-2-yl)methanol (2.43 g, 17 mmol) in DCM (40 mL), $SOCl_2$ (1.5 equiv, 25.5 mmol, 1.85 mL) and DMF (1 drop) were added. The reaction mixture was stirred at 25 °C for 2 h and concentrated under reduced pressure to afford the chloroalkane, which was engaged in the next step without further purification.

Intermediate **26** was obtained following the general procedure for dimethylpiperidine alkylation, but the reaction mixture was stirred at 70 °C for 3 h (column chromatography: EtOAc/heptane = 1:9 to 3:7). Yellow solid, 86% yield over two steps. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.51 (d, J = 2.0 Hz, 1H), 7.62 (dd, J = 8.4, 2.5 Hz, 1H), 7.39 (d, J = 8.4 Hz, 1H), 3.63 (s, 2H), 2.50−2.37 (m, 4H), 1.45−1.38 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 157.6, 147.9, 136.0, 130.1, 123.8, 64.2, 50.2 (2C), 38.6 (2C), 28.3 (2C), 28.2 (br s); LRMS (ESI) m/z calcd for $[C_{13}H_{20}ClN_2]^+$: 239.1, found: 239.2.

4-(((6-((4,4-Dimethylpiperidin-1-yl)methyl)pyridin-3-yl)amino)methyl)piperidin-4-ol hydrochloride (27). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:1 to 100:3 to 100:5 to 100:10 to 100:20). The impure desired product was engaged in the next step without further purification.

Intermediate **27** was obtained following the general procedure for Boc group deprotection. After evaporation, the crude residue was triturated in acetone, and the resulting precipitate was filtered, washed with acetone, and dried to afford the desired product as a brown solid (40% yield over four steps). ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.27 (d, J = 2.8 Hz, 1H), 7.84 (d, J = 9.1 Hz, 1H), 7.74 (dd, J = 8.9, 2.8 Hz, 1H), 4.53 (s, 2H), 3.38−3.32 (m, 5H), 3.30−3.26 (m, 3H), 1.98−1.82 (m, 4H), 1.73 (br s, 4H), 1.08 (s, 6H). Two protons are missing due to overlapping with the solvent residual peak; ^{13}C NMR (101 MHz, MeOD-d_4): δ 150.7, 131.8, 129.2, 128.2, 127.7, 69.4, 56.4, 53.9,

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50.9 (2C), 41.2 (2C), 36.7 (2C), 32.3 (2C), 28.7 (2C). One carbon is missing due to overlapping or broadening; LRMS (ESI) *m/z* calcd for $[C_{19}H_{33}N_4O]^+$: 333.3, found: 333.5.

4-(((6-((4,4-Dimethylpiperidin-1-yl)methyl)pyridin-3-yl)-amino)methyl)-1-(6-(methylamino)pyrimidin-4-yl)piperidin-4-ol (4). To a stirred solution of **27** (200 mg, 0.45 mmol) in *i*PrOH (2.4 mL), 6-chloro-*N*-methylpyrimidin-4-amine (1.5 equiv, 0.68 mmol, 106 mg) and Et_3N (4 equiv, 1.81 mmol, 251 μL) were added. The reaction mixture was stirred at 140 °C for 5 h in the microwave and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:5 to 100:10 to 100:15 to 100:20), and the obtained impure product was triturated in Et_2O, filtered, and dried to afford the desired product as a pale brown solid (45 mg, 23% yield). Mp: decomposition; ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.11 (d, *J* = 2.2 Hz, 1H), 7.96 (s, 1H), 7.26 (d, *J* = 8.6 Hz, 1H), 7.11 (dd, *J* = 9.0, 2.2 Hz, 1H), 5.61 (s, 1H), 4.15 (s, 2H), 4.04 (d, *J* = 13.4 Hz, 2H), 3.23−3.08 (m, 6H), 2.83 (s, 3H), 1.76−1.57 (m, 8H), 1.05 (s, 6H). Two protons are missing due to overlapping with the solvent residual peak; ^{13}C NMR (101 MHz, MeOD-d_4): δ 165.2, 163.6, 158.2, 147.6, 136.7, 126.9, 119.9, 81.8 (br s), 71.6, 62.3, 54.6, 50.5 (2C), 41.6 (2C), 37.3, 35.3 (2C), 29.0 (2C), 28.5. One carbon is missing due to overlapping or broadening; HRMS (ESI): *m/z*: calcd for $[C_{24}H_{38}N_7O]^+$: 440.3138, found: 440.3132.

1-(6-Chloropyrimidin-4-yl)-4-(((6-((4,4-dimethylpiperidin-1-yl)methyl)pyridin-3-yl)amino)methyl)piperidin-4-ol (28). Intermediate **28** was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. The aqueous workup was omitted, and the crude residue was directly purified by flash column chromatography (DCM/MeOH = 10:1). Beige solid, 80% yield. ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.25 (d, *J* = 0.8 Hz, 1H), 8.06 (d, *J* = 2.8 Hz, 1H), 7.25 (d, *J* = 8.6 Hz, 1H), 7.11 (dd, *J* = 8.5, 2.9 Hz, 1H), 6.84 (s, 1H), 4.24 (br s, 2H), 4.00 (s, 2H), 3.43−3.36 (m, 2H), 3.17 (s, 2H), 2.98 (br s, 4H), 1.78−1.61 (m, 4H), 1.61−1.53 (m, 4H), 1.02 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 171.7, 162.1, 159.0, 157.5, 146.0, 135.1, 125.4, 118.4, 101.3, 69.9, 60.2, 53.0, 49.0 (2C), 40.0 (2C), 35.8 (2C), 33.8 (2C), 27.5 (2C). One carbon is missing due to overlapping or broadening; LRMS (ESI) *m/z* calcd for $[C_{23}H_{34}ClN_6O]^+$: 445.3, found: 445.4.

1-(6-(Benzylamino)pyrimidin-4-yl)-4-(((6-((4,4-dimethylpiperidin-1-yl)methyl)pyridin-3-yl)amino)methyl)piperidin-4-ol (5). Compound **5** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:10 in 15 min, 100:10 for 10 min, 100:10 to 100:15 in 10 min, and 100:15 for 5 min). Beige solid (42% yield). Mp: < 50 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (s, 1H), 7.99 (d, *J* = 2.8 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 1H), 7.38−7.32 (m, 4H), 7.31−7.28 (m, 1H), 6.98 (dd, *J* = 8.6, 2.8 Hz, 1H), 5.44 (s, 1H), 5.37 (br s, 1H), 4.91−4.84 (m, 1H), 4.46 (d, *J* = 5.8 Hz, 2H), 4.10 (s, 2H), 4.05 (d, *J* = 14.4 Hz, 2H), 3.31 (t, *J* = 11.4 Hz, 2H), 3.13 (d, *J* = 5.8 Hz, 2H), 3.06 (br s, 4H), 1.83−1.78 (m, 2H), 1.80 (br s, 4H), 1.65−1.57 (m, 2H), 1.02 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.1, 162.5, 157.9, 143.5, 138.2, 135.6, 128.7 (2C), 128.6, 127.5, 127.3 (2C), 124.3, 119.7, 81.0, 70.2, 63.9, 54.4, 49.9 (2C), 45.8, 40.1 (2C), 38.1 (2C), 34.7 (2C), 28.3 (2C). One carbon is missing due to overlapping or broadening; HRMS (ESI): *m/z*: calcd for $[C_{30}H_{42}N_7O]^+$; 516.3451, found: 516.3445.

***N*-Benzyl-6-chloropyrimidin-4-amine (29).** To a stirred solution of 4,6-dichloro-pyrimidine (5 g, 33.6 mmol) in *i*PrOH (100 mL), benzylamine (1.2 equiv, 40.3 mmol, 4.4 mL) and Et_3N (1.2 equiv, 40.3 mmol, 5.59 mL) were added. The reaction mixture was stirred at 25 °C for 3 days and concentrated under reduced pressure. The crude residue was triturated in water, filtered, and dried to afford the desired product as a beige solid (7.21 g, 98% yield). ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.24 (s, 1H), 7.37−7.29 (m, 4H), 7.29−7.22 (m, 1H), 6.53 (br s, 1H), 4.59 (br s, 2H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 165.1, 159.5, 158.8, 140.0, 129.8 (2C), 128.7, 128.5 (2C), 105.4, 45.6; LRMS (ESI) *m/z* calcd for $[C_{11}H_{11}ClN_3]^+$: 220.1, found: 220.1.

6-(4-Aminopiperidin-1-yl)-*N*-benzylpyrimidin-4-amine (30). To a stirred solution of *tert*-butyl piperidin-4-ylcarbamate (2 g, 10 mmol) in *i*PrOH (10 mL), **29** (1.2 equiv, 12 mmol, 2.64 g) and Et_3N (1 equiv, 10 mmol, 1.39 mL) were added. The reaction mixture was stirred at 150 °C for 8 h in the microwave and concentrated under reduced pressure. The obtained residue was triturated in a mixture of ethyl acetate and acetone, the resulting precipitate was filtered and washed with acetone, and the filtrate was concentrated under reduced pressure. The crude residue was purified by flash column chromatography (EtOAc/heptane = 5:5 to 7:3 to 10:0) to afford the Boc protected amine as a white solid (1.22 g, 32% yield).

To a stirred solution of the corresponding Boc protected amine (1.22 g, 3.2 mmol) in MeOH (10 mL), HCl (37% aq., 1.5 mL) was added. The reaction mixture was stirred at 25 °C for 18 h and concentrated under reduced pressure. The obtained residue was poured in Na_2CO_3 aq. sat. solution, and the resulting precipitate was filtered, washed with water, and dried to afford the desired product as a white solid (678 mg, 75% yield). ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.98 (s, 1H), 7.34−7.29 (m, 4H), 7.26−7.22 (m, 1H), 5.63 (s, 1H), 4.47 (s, 2H), 4.26−4.16 (m, 2H), 2.97−2.91 (m, 1H), 2.90−2.82 (m, 2H), 1.90−1.82 (m, 2H), 1.36−1.22 (m, 2H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 164.7, 163.6, 158.5, 140.6, 129.7 (2C), 128.4 (2C), 128.3, 83.0, 50.0, 46.0, 44.5 (2C), 34.3 (2C); LRMS (ESI) *m/z* calcd for $[C_{16}H_{22}N_5]^+$: 284.2, found: 284.2.

***N*-(1-(6-(Benzylamino)pyrimidin-4-yl)piperidin-4-yl)-6-((4,4-dimethylpiperidin-1-yl)methyl)nicotinamide (6).** To a stirred solution of methyl 6-methylnicotinate (1 g, 6.6 mmol) in CCl_4 (4 mL), under a nitrogen atmosphere, NBS (1.15 equiv, 7.6 mmol, 1.35 g) and Bz_2O_2 (0.2 equiv, 1.32 mmol, 426 mg, 75% pure) were added. The reaction mixture was stirred at 75 °C for 4 h, AIBN (0.1 equiv, 0.66 mmol, 108 mg) was added, and the reaction was stirred an additional 17 h at 75 °C. The solution was cooled down to 0 °C, the resulting precipitate was filtered and washed with cold CCl_4, and the filtrate was concentrated under reduced pressure to afford the impure bromoalkane, which was engaged in the next step without further purification.

Intermediate **31** was obtained following the general procedure for dimethylpiperidine alkylation (column chromatography: EtOAc/heptane = 10:90 to 20:80 to 30:70), and the obtained impure product was engaged in the next step without further purification.

To a stirred solution of the corresponding ester (1 equiv) in a mixture of methanol (0.4 M) and water (0.8 M), $LiOH·H_2O$ (3 equiv) was added. The reaction mixture was stirred at 25 °C for 17 h and concentrated under reduced pressure. The pH was adjusted to 6 with aq. HCl (2 M), and the solution was concentrated under reduced pressure. The obtained residue was triturated in acetone, and the resulting precipitate was filtered, washed with acetone, and dried to afford the desire impure acid, which was engaged in the next step without further purification.

To a stirred solution of the corresponding acid (1 equiv) in dimethylformamide (0.3 M), HATU (1 equiv) and DIPEA (3 equiv) were added. The reaction mixture was stirred at 25 °C for 5 min, **30** (1 equiv) was added, and stirring was continued for an additional 17 h. The reaction was diluted with water, and the aqueous layer was extracted three times with *n*BuOH. The combined organic layers were washed five times with water and once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:2 to 100:4 to 100:6 to 100:8) to afford the desired product as a white solid (22 mg, 29%). Mp: 187−188 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.88 (s, 1H), 8.20 (s, 1H), 8.05 (dd, *J* = 8.0, 2.2 Hz, 1H), 7.57 (d, *J* = 7.9 Hz, 1H), 7.39−7.32 (m, 4H), 7.32−7.29 (m, 1H), 5.95 (d, *J* = 7.1 Hz, 1H), 5.47 (s, 1H), 5.13 (br s, 1H), 4.48 (d, *J* = 5.6 Hz, 2H), 4.33−4.20 (m, 3H), 3.77 (s, 2H), 3.02 (t, *J* = 11.7 Hz, 2H), 2.52 (br s, 4H), 2.16−2.07 (m, 2H), 1.55−1.41 (m, 6H), 0.94 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 165.1, 163.0, 162.4, 161.6, 157.5, 147.4, 138.1, 135.4, 128.7 (2C), 128.6, 127.4, 127.2 (2C), 123.0, 80.9, 64.2, 50.2 (2C), 47.6, 45.7, 43.2 (2C), 38.2 (2C), 31.5 (2C), 28.2 (2C), 28.0 (br s); HRMS (ESI): *m/z*: calcd for $[C_{30}H_{40}N_7O]^+$: 514.3294, found: 514.3289.

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***tert*-Butyl 4-(Aminomethyl)-4-(((benzyloxy)carbonyl)-amino)piperidine-1-carboxylate (32).** To a stirred solution of $MeNO_2$ (1.3 equiv 130 mmol, 7 mL) in NH_3 (53 mL, 7 N in MeOH), *tert*-butyl 4-oxopiperidine-1-carboxylate (20 g, 100 mmol) was added portionwise. The reaction mixture was stirred at 25 °C for 17 h and concentrated under reduced pressure. The crude residue was diluted with DCM and water. The two phases were separated, and the aqueous layer was extracted two times with DCM. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding amine (100 mmol) in dichloromethane (130 mL), a solution of K_2CO_3 (2 equiv, 200 mmol, 27.6 g) in water (130 mL) was added. The reaction mixture was cooled to 0 °C, and CbzCl (1.1 equiv, 110 mmol, 15.6 mL) was added dropwise. The reaction mixture was stirred at 25 °C for 17 h, and the two phases were separated. The aqueous layer was extracted two times with DCM. The combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding nitroalkane (100 mmol) in dry MeOH (450 mL), under a nitrogen atmosphere, at 0 °C, $NiCl_2{\cdot}6H_2O$ (1 equiv, 100 mmol, 27.3 g) was added followed by $NaBH_4$ (5 equiv, 500 mmol, 18.9 g) portionwise to avoid strong H_2 evolution. Caution should be taken when adding $NaBH_4$ because the reaction is highly exothermic and produces hydrogen gas. The reaction mixture was stirred at 25 °C for 1 h and quenched by adding a saturated aqueous $NaHCO_3$ solution. The mixture was filtered through a pad of Celite, the filtrate was concentrated under reduced pressure, and the obtained residue was diluted with water. The aqueous layer was extracted three times with DCM, and the combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH/NH_4OH = 100:0:0 to 100:3:0 to 100:3:1 to 100:5:1 to 100:10:1 to 100:15:1) to afford the desired product as a white solid (11.7 g, 32% yield over three steps). ^1H NMR (400 MHz, $CDCl_3$): δ 7.41−7.31 (m, 5H), 5.07 (s, 2H), 4.66 (s, 1H), 3.80 (br s, 2H), 3.06 (t, J = 11.4 Hz, 2H), 2.90 (s, 2H), 2.03 (d, J = 13.2 Hz, 2H), 1.53−1.47 (m, 2H), 1.46 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.8, 154.7, 136.4, 128.6 (2C), 128.2, 128.0 (2C), 79.6, 66.4, 54.7 (2C), 48.3, 39.2 (br s), 32.1 (2C), 28.4 (3C); LRMS (ESI) m/z calcd for $[C_{19}H_{30}N_3O_4]^+$: 364.2, found: 364.3.

***tert*-Butyl 2-Oxo-1,4,9-triazaspiro[5.5]undecane-9-carboxylate (33).** To a stirred solution of **32** (14.5 g, 40 mmol) in DCM (133 mL) at 0 °C, Et_3N (0.8 equiv, 32 mmol, 4.4 mL) and ethyl 2-bromoacetate (0.7 equiv, 28 mmol, 3.1 mL) were added. The reaction mixture was stirred at 25 °C for 2 h and diluted with a saturated aqueous $NaHCO_3$ solution. The aqueous layer was extracted three times with EtOAc, and the combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the impure desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding Cbz protected amine (40 mmol) in *i*PrOH (400 mL), Pd/C (5 mol %, 2 mmol, 2.1 g, 10% wt) and ammonium formate (6 equiv, 240 mmol, 15 g) were added portionwise. The reaction mixture was stirred at 80 °C for 4 h, cooled to 25 °C, filtered through a pad of Celite, and concentrated under reduced pressure. The obtained residue was dissolved in DCM, and the organic layer was washed once with water and once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:5 to 100:8 to 100:10 to 100:15) to afford the desired product as a white solid (4.14 g, 55% yield over two steps). ^1H NMR (400 MHz, $CDCl_3$): δ 6.00 (br s, 1H), 3.51 (s, 2H), 3.49−3.43 (m, 4H), 2.91 (s, 2H), 1.74−1.63 (m, 4H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.0, 154.6, 80.0, 52.9, 51.6, 49.7, 39.4 (br s), 36.0, 28.4 (3C); LRMS (ESI) m/z calcd for $[C_{26}H_{47}N_6O_6]^+$ = [2 M + H]$^+$: 539.4, found: 539.4.

***N*-Benzyl-6-(4-(6-((4,4-dimethylpiperidin-1-yl)methyl)-pyridin-3-yl)-1-oxa-4,9-diazaspiro[5.5]undecan-9-yl)-pyrimidin-4-amine (7).** The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: EtOAc/heptane = 7:3 to 9:1 to EtOAc/MeOH = 100:0 to 100:1 to 100:5). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. After evaporation, the crude residue was triturated in acetone, and the resulting precipitate was filtered, washed with acetone, and dried to afford the impure desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding amine (1 equiv) in *i*PrOH (0.3 M), **29** (1.5 equiv) and Et_3N (4 equiv) were added. The reaction mixture was stirred at 150 °C for 8 h in the microwave and concentrated under reduced pressure. The reaction was diluted with water, and the aqueous layer was extracted three times with DCM. The combined organic layers were washed five times with water and once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:5 to 100:8 to 100:10 to 100:13 to 100:20) to afford the desired product as a brown solid (6% yield over three steps). Mp: 61−62 °C; ^1H NMR (400 MHz, MeOD-d_4): δ 8.21 (d, J = 2.0 Hz, 1H), 7.99 (d, J = 0.8 Hz, 1H), 7.41−7.36 (m, 2H), 7.35−7.29 (m, 4H), 7.26−7.20 (m, 1H), 5.63 (s, 1H), 4.47 (s, 2H), 3.96−3.87 (m, 4H), 3.84 (br s, 2H), 3.27−3.18 (m, 4H), 3.09 (s, 2H), 2.72 (br s, 4H), 2.07−1.98 (m, 2H), 1.64−1.55 (m, 2H), 1.53−1.46 (m, 4H), 0.98 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.0, 162.6, 157.7, 146.5, 138.2, 137.7, 128.7 (2C), 127.4, 127.3 (2C), 125.3, 123.1, 120.0, 80.9, 70.4, 62.7, 60.0, 57.7, 49.5 (2C), 48.4, 45.8 (2C), 39.7 (2C), 36.9 (br s, 1C), 32.0 (2C), 28.1 (2C). One carbon is missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{32}H_{44}N_7O]^+$: 542.3607, found: 542.3602.

9-(6-Chloropyrimidin-4-yl)-4-(6-((4,4-dimethylpiperidin-1-yl)methyl)pyridin-3-yl)-1,4,9-triazaspiro[5.5]undecan-2-one (34). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:2 to 100:5 to 100:8 to 100:12 to 100:15 to 100:20). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. After evaporation, the crude residue was triturated in acetone, and the resulting precipitate was filtered, washed with acetone, and dried to afford the impure desired product, which was engaged in the next step without further purification.

Intermediate **34** was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine (column chromatography: DCM/MeOH = 100:2 to 100:5 to 100.8 to 100:10 to 100:15 to 100:20) to afford the desired product as a yellow solid (123 mg, 27% yield over three steps). ^1H NMR (400 MHz, MeOD-d_4): δ 8.30 (d, J = 0.5 Hz, 1H), 8.26 (d, J = 2.0 Hz, 1H), 7.46−7.38 (m, 2H), 6.91 (d, J = 0.8 Hz, 1H), 4.00−3.89 (m, 4H), 3.88−3.73 (m, 4H), 3.57 (s, 2H), 2.83−2.63 (m, 4H), 1.99−1.89 (m, 2H), 1.89−1.79 (m, 2H), 1.54−1.46 (m, 4H), 0.98 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.2, 163.9, 160.8, 159.1, 146.4, 145.4 (br s, 1C), 137.1, 126.3, 123.6, 103.1, 63.0, 54.2 (2C), 53.7, 51.6, 50.9 (2C), 41.4 (2C), 38.2, 35.7 (2C), 29.1 (2C), 28.2 (br s); LRMS (ESI) m/z calcd for $[C_{25}H_{35}ClN_7O]^+$: 484.3, found: 484.3.

9-(6-(Benzylamino)pyrimidin-4-yl)-4-(6-((4,4-dimethylpiperidin-1-yl)methyl)pyridin-3-yl)-1,4,9-triazaspiro[5.5]undecan-2-one (8). Compound **8** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:5 to 100:10 to 100:15 to 100:20 to 100:30). The obtained impure product was triturated in diethyl ether; filtered; and washed twice with ether, once with water, and once with ether to afford the desired product as a white solid (14 mg, 25% yield). Mp: 208−209 °C; ^1H NMR (400 MHz, MeOD-d_4): δ 8.16 (s,

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1H), 8.01 (s, 1H), 7.41 (s, 2H), 7.37−7.27 (m, 4H), 7.26−7.20 (m, 1H), 5.67 (s, 1H), 4.48 (s, 2H), 3.88 (s, 2H), 3.83−3.74 (m, 2H), 3.61−3.50 (m, 6H), 2.46 (br s, 4H), 1.92−1.83 (m, 2H), 1.83−1.72 (m, 2H), 1.47−1.36 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.3, 164.8, 163.7, 158.5, 148.6 (br s), 146.1, 140.6, 136.4, 129.7 (2C), 128.4 (2C), 128.3, 126.0, 124.1, 83.2, 64.4, 54.4, 53.9, 51.8, 51.1, 46.0, 41.6, 39.3, 35.6, 29.3, 28.8 (br s); HRMS (ESI): m/z: calcd for $[C_{32}H_{43}N_8O]^+$: 555.3560, found: 555.3554.

***tert*-Butyl 4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-2-oxo-1,4,9-triazaspiro[5.5]undecane-9-carboxylate (35).** Intermediate **35** was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:5 to 100:8 to 100:12 to 100:20). Beige solid, 93% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (br s, 2H), 6.84−6.76 (m, 2H), 6.54 (s, 1H), 3.85 (s, 2H), 3.57−3.50 (m, 4H), 3.50−3.42 (m, 2H), 3.34 (s, 2H), 2.39 (br s, 4H), 1.87−1.79 (m, 2H), 1.78−1.71 (m, 2H), 1.48 (s, 9H), 1.41 (br s, 4H), 0.92 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.8, 154.5, 148.0, 130.6 (2C), 114.7 (2C), 80.0, 62.6, 55.0, 52.8 (2C), 51.9, 49.8 (2C), 39.5 (br s), 38.4, 35.4, 28.4 (2C), 28.4 (3C). Two carbons are missing due to overlapping or broadening.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-1,4,9-triazaspiro[5.5]undecan-2-one hydrochloride (36). Intermediate **36** was obtained following the general procedure for Boc group deprotection. After evaporation, the crude residue was triturated in acetone, and the resulting precipitate was filtered, washed with acetone, and dried to afford the desired product as a beige solid (50% yield over two steps from intermediate 33). ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.49−7.37 (m, 2H), 7.11−7.04 (m, 2H), 4.23 (s, 2H), 3.90 (s, 2H), 3.55 (s, 2H), 3.39−3.34 (m, 4H), 3.17−3.07 (m, 2H), 2.14−1.98 (m, 4H), 1.69−1.59 (m, 4H), 1.07 (br s, 6H). Two protons are missing due to overlapping with the solvent residual peak; LRMS (ESI) m/z calcd for $[C_{22}H_{35}N_4O]^+$: 371.3, found: 371.3.

9-(6-Chloropyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)-1,4,9-triazaspiro[5.5]undecan-2-one (37). Intermediate **37** was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. Instead of chromatography, after evaporation of the crude mixture, the residue was triturated in water. The obtained precipitate was filtered, washed with water, and dried to afford the desired product as a brown solid (63% yield). ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.30 (s, 1H), 7.28−7.21 (m, 2H), 6.98−6.92 (m, 2H), 6.90 (s, 1H), 4.01 (br s, 2H), 3.80 (s, 2H), 3.77−3.67 (m, 2H), 3.48 (s, 2H), 3.47 (s, 2H), 2.43 (br s, 4H), 2.02−1.92 (m, 2H), 1.87−1.78 (m, 2H), 1.47−1.35 (m, 4H), 0.93 (s, 6H). ^{13}C NMR (101 MHz, MeOD-d_4): δ 171.1, 163.9, 160.8, 159.1, 150.3, 132.4 (2C), 129.1, 116.2 (2C), 103.0, 63.6, 55.0, 54.4, 52.9, 50.8, 41.6, 39.2, 35.8, 29.4. One carbon is missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{26}H_{36}ClN_6O]^+$: 483.3, found: 483.3.

9-(6-(Benzylamino)pyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)-1,4,9-triazaspiro[5.5]undecan-2-one (9). The corresponding chloropyrimidine was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. Instead of chromatography, after evaporation, the crude residue was triturated in water, filtered, and washed once with water. The obtained sticky solid was dissolved in MeOH and concentrated under reduced pressure to afford the desired product, which was engaged in the next step without further purification.

Compound **9** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:8 in 20 min, 100:8 for 10 min, and 100:8 to 100:10 in 10 min). The obtained impure product was triturated in water, filtered, and washed once with water to afford the desired product as a pale yellow solid (5% yield over two steps). Mp: 228−231 °C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.01 (s, 1H), 7.35−7.29 (m, 4H), 7.27−7.20 (m, 3H), 6.95−6.90 (m, 2H), 5.67 (s, 1H), 4.48 (s, 2H), 3.87−3.76 (m, 4H), 3.54−3.47 (m, 4H), 3.45 (s, 2H), 2.52−2.38 (m, 4H), 1.92−1.84 (m, 2H), 1.81−1.73 (m, 2H), 1.46−1.37 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, DMSO-d_6): δ 167.0, 163.2, 161.6, 157.4, 148.2, 140.2, 129.8, 128.2 (2C), 127.1 (2C), 126.6, 114.2, 81.5, 61.7, 52.6, 52.4, 51.3, 49.1, 43.6, 38.2 (br s), 34.3, 28.2 (2C). Three carbons are missing due to overlapping; HRMS (ESI): m/z: calcd for $[C_{33}H_{44}N_7O]^+$: 554.3607, found: 554.3602.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-9-(6-(methylamino)pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]undecan-2-one (10). The corresponding chloropyrimidine batch was the same as the one used for compound **9**.

Compound **10** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:12 in 20 min, 100:12 for 10 min, 100:12 to 100:15 in 10 min, and 100:15 for 10 min). White solid (3% yield over two steps). Mp: 235−236 °C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.99 (s, 1H), 7.37−7.27 (m, 2H), 7.02−6.94 (m, 2H), 5.65 (s, 1H), 3.90−3.84 (m, 4H), 3.61−3.53 (m, 2H), 3.52 (s, 2H), 2.93−2.69 (m, 7H), 1.97−1.88 (m, 2H), 1.86−1.77 (m, 2H), 1.57−1.47 (m, 4H), 0.99 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.8, 165.4, 163.7, 158.3, 151.3, 133.3 (2C), 115.9 (2C), 82.2 (br s), 62.0, 54.4, 54.2, 52.2, 50.2, 41.7, 37.5, 35.7, 30.9 (br s), 29.1, 28.5. One carbon is missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{27}H_{40}N_7O]^+$: 478.3294, found: 478.3289.

***tert*-Butyl 4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-1-methyl-2-oxo-1,4,9-triazaspiro[5.5]undecane-9-carboxylate (45).** To a stirred solution of **35** (400 mg, 0.85 mmol) in dry THF (4.2 mL), at 0 °C, under a nitrogen atmosphere, KO*t*Bu (1.5 equiv, 0.13 mmol, 130 μL, 1 M in THF) was added. The reaction mixture was stirred at 25 °C for 5 min, MeI (1.2 equiv, 1.02 mmol, 63 μL) was added, and the solution was stirred for 2 h. The reaction was concentrated under reduced pressure, and the crude residue was purified by flash column chromatography (chromatography: DCM/MeOH = 100:3 to 100:5 to 100:8) to afford the desired product as a brown solid (222 mg, 54% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66−7.55 (m, 2H), 6.88−6.82 (m, 2H), 4.18 (br s, 2H), 4.08 (br s, 2H), 3.92 (s, 2H), 3.58 (br s, 2H), 3.34 (br s, 2H), 2.98 (s, 3H), 2.94 (br s, 2H), 2.79 (br s, 2H), 2.30 (br s, 2H), 2.10−1.98 (m, 2H), 1.74 (d, J = 13.7 Hz, 2H), 1.59−1.54 (m, 2H), 1.50 (s, 9H), 1.09−0.96 (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.6, 154.5, 149.7, 133.1 (2C), 114.2 (2C), 80.3, 60.1, 57.5, 51.8, 51.2, 48.4, 40.5 (br s), 34.9, 30.8, 28.4 (3C), 27.8, 27.0. Two carbons are missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{28}H_{45}N_4O_3]^+$: 485.3, found: 485.4.

9-(6-Chloropyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)-1-methyl-1,4,9-triazaspiro[5.5]undecan-2-one (46). The corresponding amine was obtained following the general procedure for Boc group deprotection. The impure desired product was engaged in the next step without further purification.

The corresponding chloropyrimidine was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. Instead of chromatography, after evaporation of the crude mixture, the residue was triturated in Et_2O. The obtained precipitate was filtered, washed with Et_2O, and dried to afford the desired product as a brown solid (57% yield over two steps). ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.31 (s, 1H), 7.33−7.23 (m, 2H), 7.02−6.94 (m, 2H), 6.91 (s, 1H), 4.48 (br s, 2H), 3.87 (s, 2H), 3.67 (s, 2H), 3.55 (s, 2H), 2.94 (s, 3H), 2.51 (br s, 4H), 2.23−2.10 (m, 2H), 1.94 (d, J = 12.9 Hz, 2H), 1.49−1.37 (m, 4H), 0.94 (s, 6H).^{13}C NMR (101 MHz, MeOD-d_4): δ 169.8, 163.9, 160.8, 159.1, 150.6, 133.0 (2C), 116.0 (2C), 103.1, 62.7, 59.6, 53.4, 53.3, 50.5, 42.0, 38.3, 31.5, 29.2, 27.9, 25.4. One carbon is missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{27}H_{38}ClN_6O]^+$: 497.3, found: 497.3.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-1-methyl-9-(6-(methylamino)pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]-undecan-2-one (13). Compound **13** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:8 in 15 min and 100:8 for 10 min). Yellow solid (85% yield). Mp: 110−113 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.19 (s, 1H), 7.39 (br s, 2H), 6.88−6.81 (m, 2H), 5.46 (s, 1H), 4.82−4.73 (m, 1H), 4.36 (d, J = 13.2 Hz, 2H), 3.91 (s, 2H), 3.67 (br s, 2H), 3.59 (s, 2H), 3.16−3.03 (m, 2H), 2.96 (s, 3H), 2.91 (d, J = 5.3 Hz, 3H), 2.59 (br s, 4H), 2.13 (td, J = 13.1, 5.2 Hz, 2H), 1.88 (d, J = 13.4 Hz, 2H), 1.55 (br s, 4H), 0.96; ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.0, 164.0, 162.4, 157.6, 148.5, 131.4 (2C), 125.9 (br s), 114.4 (2C), 80.1, 61.6, 57.6, 52.5, 52.4, 49.2, 40.9,

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37.1, 30.4, 28.4, 28.1, 27.7 (br s), 27.0; HRMS (ESI): *m/z*: calcd for $[C_{28}H_{42}N_7O]^+$: 492.3451, found: 492.3445.

9-(6-(Dimethylamino)pyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)-1,4,9-triazaspiro[5.5]undecan-2-one (14). Compound **14** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:10 in 15 min and 100:10 for 10 min). Brown solid (80% yield). Mp: 245−246 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.26 (s, 1H), 6.86−6.77 (m, 2H), 6.24 (s, 1H), 5.52 (s, 1H), 3.88 (s, 2H), 3.76−3.63 (m, 4H), 3.48 (br s, 2H), 3.40 (s, 2H), 3.09 (s, 6H), 2.39 (br s, 4H), 1.98−1.89 (m, 2H), 1.89−1.79 (m, 2H), 1.40 (br s, 4H), 0.94 (s, 6H). Two protons are missing due to overlapping with the solvent residual peak; ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.7, 163.3, 162.5, 157.3, 148.3, 131.0 (2C), 114.7 (2C), 80.6, 62.1, 55.0, 53.0, 51.8, 49.5, 40.3, 37.8 (br s), 37.3 (2C), 35.0, 28.3. Two carbons are missing due to overlapping or broadening; HRMS (ESI): *m/z*: calcd for $[C_{28}H_{42}N_7O]^+$: 492.3451, found: 492.3445.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-9-(6-(isopropylamino)pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]-undecan-2-one (15). Compound **15** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:8 in 15 min, 100:8 for 10 min, and 100:8 to 100:10 in 10 min). Beige solid (52% yield). Mp: 175−176 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (s, 1H), 7.44 (br s, 2H), 6.88−6.79 (m, 2H), 6.38 (s, 1H), 5.45 (s, 1H), 4.62 (d, *J* = 7.9 Hz, 1H), 3.89 (s, 2H), 3.87−3.77 (m, 2H), 3.75−3.60 (m, 5H), 3.42 (s, 2H), 2.63 (br s, 4H), 1.95−1.88 (m, 2H), 1.87−1.80 (m, 2H), 1.53 (br s, 4H), 1.25 (d, *J* = 6.3 Hz, 6H), 0.98 (br s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.2, 162.5, 162.4, 157.8, 149.0 (br s), 131.9 (2C), 114.6 (2C), 81.0, 61.2 (br s), 54.6, 53.1, 51.5, 49.0, 42.7, 40.3, 36.4 (br s), 35.0, 28.1, 22.8 (2C). Three carbons are missing due to overlapping; HRMS (ESI): *m/z*: calcd for $[C_{29}H_{44}N_7O]^+$: 506.3607, found: 506.3602.

9-(6-(Cyclopropylamino)pyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)-1,4,9-triazaspiro[5.5]-undecan-2-one (16). Compound **16** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:10 in 15 min, 100:10 for 10 min, and 100:10 to 100:15 in 15 min). Brown solid (25% yield). Mp: 172−174 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (s, 1H), 6.87−6.80 (m, 2H), 6.21 (s, 1H), 5.83 (s, 1H), 5.13 (s, 1H), 3.89 (s, 2H), 3.80−3.64 (m, 5H), 3.42 (br s, 2H), 2.50 (br s, 4H), 1.97−1.90 (m, 2H), 1.90−1.83 (m, 2H), 1.63 (br s, 4H), 0.94 (br s, 6H), 0.85−0.79 (m, 2H), 0.63−0.56 (m, 2H). Two protons are missing due to overlapping with the solvent residual peak, and two protons are missing due to broadening; ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.2, 164.4, 162.6, 157.8, 131.2, 114.8, 81.3, 62.1 (br s), 55.0, 53.1, 51.9, 49.5, 40.3, 35.2, 29.7, 28.3, 23.2, 7.5 (2C). Three carbons are missing due to overlapping; HRMS (ESI): *m/z*: calcd for $[C_{29}H_{42}N_7O]^+$: 504.3451, found: 504.3445.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-9-(7*H*-pyrrolo[2,3-d]pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]undecan-2-one (17). To a stirred solution of **36** (100 mg, 0.21 mmol) in dry THF (700 μL), under a nitrogen atmosphere, 4-chloro-7*H*-pyrrolo-[2,3-d]pyrimidine (1 equiv, 0.21 mmol, 32 mg) was added. Nitrogen gas was bubbled through the reaction for 2 min, and Ruphos Pd G4 (5 mol %, 0.011 mmol, 8.9 mg), Ruphos (5 mol %, 0.011 mmol, 5.1 mg), and LiHMDS (6.6 equiv, 1.39 mmol, 1.39 mL, 1 M THF) were added. The reaction mixture was stirred at 65 °C for 4 h, cooled down to 25 °C, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:3 to 100:5 to 100:8 to 100:10 to 100:15) to afford the desired product as a yellow solid (37 mg, 36% yield). Mp: 250−252 °C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.15 (s, 1H), 7.38−7.31 (m, 2H), 7.14 (d, *J* = 3.5 Hz, 1H), 7.06−6.98 (m, 2H), 6.65 (d, *J* = 3.8 Hz, 1H), 4.31−4.20 (m, 2H), 4.01−3.89 (m, 4H), 3.87 (s, 2H), 3.57 (s, 2H), 2.91 (br s, 4H), 2.07−1.96 (m, 2H), 1.96−1.87 (m, 2H), 1.59−1.50 (m, 4H), 1.01 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.8, 158.3, 152.6, 151.8, 151.4, 133.4, 122.7, 115.9, 104.5, 102.6, 61.9, 54.5, 54.3, 52.2, 50.1, 43.3, 37.4, 36.2, 29.1. Two carbons are missing due to overlapping or broadening; HRMS (ESI): *m/z*: calcd for $[C_{28}H_{38}N_7O]^+$: 488.3138, found: 488.3132.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-9-(1*H*-pyrrolo[2,3-b]pyridin-4-yl)-1,4,9-triazaspiro[5.5]undecan-2-one (18). To a stirred solution of **36** (100 mg, 0.21 mmol) in dry THF (700 μL), under a nitrogen atmosphere, 4-chloro-1*H*-pyrrolo-[2,3-b]pyridine (1 equiv, 0.21 mmol, 32 mg) was added. Nitrogen gas was bubbled through the reaction for 2 min, and Ruphos Pd G4 (5 mol %, 0.011 mmol, 8.9 mg), Ruphos (5 mol %, 0.011 mmol, 5.1 mg), and LiHMDS (6.6 equiv, 1.39 mmol, 1.39 mL, 1 M THF) were added. The reaction mixture was stirred at 65 °C for 4 h, cooled down to 25 °C, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:0 to 100:10 in 15 min, 100:10 for 5 min, 100:10 to 100:15 in 10 min, and 100:15 for 5 min). The obtained impure product was triturated in Et_2O, filtered, washed with Et_2O, and dried to afford the desired product as a beige solid (71 mg, 70% yield). Mp: 177−179 °C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.94 (d, *J* = 5.8 Hz, 1H), 7.42−7.35 (m, 2H), 7.19 (d, *J* = 3.5 Hz, 1H), 7.06−7.00 (m, 2H), 6.58−6.51 (m, 2H), 4.06 (s, 2H), 3.88 (s, 2H), 3.83−3.75 (m, 2H), 3.62−3.52 (m, 4H), 3.04 (br s, 4H), 2.11−1.95 (m, 4H), 1.64−1.54 (m, 4H), 1.03 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.8, 153.3, 151.6, 150.4, 144.3, 133.6, 123.5, 115.8, 112.1, 103.0, 101.3, 61.5, 54.3, 54.1, 52.1, 50.0, 46.7, 37.1, 36.2, 29.0. Two carbons are missing due to overlapping or broadening; HRMS (ESI): *m/z*: calcd for $[C_{29}H_{39}N_7O]^+$: 487.3185, found: 487.3180.

9-(2-Chloro-7*H*-pyrrolo[2,3-d]pyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)phenyl)-1,4,9-triazaspiro[5.5]-undecan-2-one (19). To a stirred solution of **36** (100 mg, 0.21 mmol) in *i*PrOH (1 mL), 2,4-dichloro-7*H*-pyrrolo[2,3-d]pyrimidine (1.2 equiv, 0.25 mmol, 47 mg) and Et_3N (4 equiv, 0.84 mmol, 116 μL) were added. The reaction mixture was stirred at 100 °C for 3 h and an additional 3 h at 130 °C, both in the microwave. The reaction mixture was concentrated under reduced pressure, and the crude residue was triturated in water. The resulting precipitate was filtered, washed with water, and dried to afford the desired impure product, which was further purified by flash column chromatography (DCM/MeOH = 100:0 to 100:10 in 15 min and 100:10 for 10 min) to afford the desired product as a beige solid (46 mg, 42% yield). Mp: 199−201 °C; ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.31−7.24 (m, 2H), 7.09 (d, *J* = 3.5 Hz, 1H), 7.01−6.95 (m, 2H), 6.64 (d, *J* = 3.5 Hz, 1H), 4.32−4.22 (m, 2H), 3.99−3.89 (m, 2H), 3.83 (s, 2H), 3.64 (br s, 2H), 3.53 (s, 2H), 2.59 (br s, 4H), 2.07−1.97 (m, 2H), 1.95−1.86 (m, 2H), 1.51−1.41 (m, 4H), 0.95 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.9, 158.5, 154.12, 154.10, 151.0, 133.0, 125.0, 122.7, 116.0, 102.8, 102.5, 62.5, 54.6, 54.4, 52.4, 50.4, 43.2, 38.0, 36.2, 29.2. One carbon is missing due to overlapping or broadening; HRMS (ESI): *m/z*: calcd for $[C_{28}H_{37}ClN_7O]^+$: 522.2748, found: 522.2731.

***tert*-Butyl 3-Amino-3-(nitromethyl)azetidine-1-carboxylate (38).** To a stirred solution of *tert*-butyl 3-oxoazetidine-1-carboxylate (10.65 g, 62 mmol) in EtOH (31 mL), $MeNO_2$ (13 mL) and K_2CO_3 (1 mol %, 0.62 mmol, 86 mg) were added. The reaction mixture was stirred at 25 °C for 17 h and filtered. The filtrate was concentrated under reduced pressure to afford the desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding alcohol (62 mmol) in dry DCM (250 mL), under a nitrogen atmosphere and cooled to −78 °C, DAST (1.2 equiv, 74.4 mmol, 9.8 mL) was added dropwise. The cooling bath was removed, and the reaction mixture was stirred for 3 h, cooled to 0 °C, and quenched slowly by the addition of a saturated aqueous $NaHCO_3$ solution. The aqueous layer was extracted three times with DCM, washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to obtain the desired product, which was engaged in the next step without further purification.

The corresponding nitromethylene intermediate (62 mmol) was dissolved in ammonia (17.7 mL, 7 N in MeOH), and the reaction mixture was stirred for 2 h at 25 °C. The reaction mixture was concentrated under reduced pressure to afford the desired product as an orange solid (15.67 g, quantitative yield over three steps). ^{1}H

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NMR (400 MHz, $CDCl_3$): δ 4.65 (s, 2H), 4.00 (d, J = 9.6 Hz, 2H), 3.80 (d, J = 9.6 Hz, 2H), 1.45 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.1, 81.7, 80.3, 60.5 (br s), 50.2 (2C), 28.3 (3C); LRMS (ESI) m/z calcd for $[C_5H_{10}N_3O_4]^+$ = [M-*t*Bu + 2H]$^+$: 176.1, found: 176.1.

***tert*-Butyl 3-(Aminomethyl)-3-(((benzyloxy)carbonyl)-amino)azetidine-1-carboxylate (39).** To a stirred solution of **38** (62 mmol) in dichloromethane (100 mL), a solution of $NaHCO_3$ (2 equiv, 124 mmol, 10.42 g) in water (100 mL) was added. The reaction mixture was cooled to 0 °C, and CbzCl (1 equiv, 62 mmol, 8.8 mL) was added dropwise. The reaction mixture was stirred at 25 °C for 17 h, and the two phases were separated. The aqueous layer was extracted two times with DCM. The combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding nitroalkane (62 mmol) in dry MeOH (300 mL), under a nitrogen atmosphere at 0 °C, $NiCl_2 \cdot 6H_2O$ (1 equiv, 62 mmol, 16.9 g) was added followed by $NaBH_4$ (5 equiv, 310 mmol, 11.7 g) portionwise to avoid strong H_2 evolution. Caution should be taken when adding $NaBH_4$ because the reaction is highly exothermic and produces hydrogen gas. The reaction mixture was stirred at 25 °C for 1 h and quenched by adding a saturated aqueous $NaHCO_3$ solution. The mixture was filtered through a pad of Celite, the filtrate was concentrated under reduced pressure, and the obtained residue was diluted with brine and a saturated aqueous Na_2CO_3 solution. The aqueous layer was extracted three times with DCM, and the combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH/NH_4OH = 100:3:0 to 100:3:1 to 100:5:1 to 100:8:1 to 100:12:1 to 100:20:1) to afford the desired product as a white solid (11.3 g, 54% yield over two steps). ^1H NMR (400 MHz, $CDCl_3$): δ 7.40−7.31 (m, 5H), 5.46 (br s, 1H), 5.10 (s, 2H), 4.04 (br s, 2H), 3.79 (d, J = 8.9 Hz, 2H), 3.06 (s, 2H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.4, 154.9, 136.1, 128.6 (2C), 128.3, 128.2 (2C), 79.7, 66.7, 57.4 (br s), 52.5, 47.0, 28.3 (3C); LRMS (ESI) m/z calcd for $[C_{34}H_{51}N_6O_8]^+$ = [2 M + H]$^+$: 671.4, found: 671.4.

***tert*-Butyl 6-Oxo-2,5,8-triazaspiro[3.5]nonane-2-carboxylate (40).** To a stirred solution of **39** (11.3 g, 33.7 mmol) in DCM (110 mL) at 0 °C, Et_3N (1 equiv, 33.7 mmol, 4.7 mL) and ethyl 2-bromoacetate (1 equiv, 33.7 mmol, 3.7 mL) were added. The reaction mixture was stirred at 25 °C for 17 h and diluted with a saturated aqueous $NaHCO_3$ solution. The aqueous layer was extracted three times with DCM and the combined organic layers were washed once with water, once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the impure desired product (12.4 g, 29 mmol), which was engaged in the next step without further purification.

To a stirred solution of the corresponding Cbz protected amine (29 mmol) in *i*PrOH (240 mL), Pd/C (5 mol %, 1.5 mmol, 1.6 g, 10% wt) and ammonium formate (6 equiv, 174 mmol, 11 g) were added portionwise. The reaction mixture was stirred at 80 °C for 4 h, cooled to 25 °C, filtered through a pad of Celite, and concentrated under reduced pressure. The obtained residue was partitioned between DCM and water, the two phases were separated, and the aqueous layer was extracted three times with DCM. The combined organic layers were washed once with water and once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:5 to 100:8 to 100:10 to 100:15 to 100:20) to afford the desired product as a white solid (2.6 g, 32% yield over two steps). ^1H NMR (400 MHz, $CDCl_3$): δ 6.44 (br s, 1H), 3.96 (d, J = 9.9 Hz, 2H), 3.85 (d, J = 9.6 Hz, 2H), 3.51 (s, 2H), 3.16 (s, 2H), 1.45 (s, 9H).^{13}C NMR (101 MHz, $CDCl_3$): δ 169.8, 156.1, 80.2, 60.9 (br s), 51.7, 51.3, 49.5, 28.3 (3C); LRMS (ESI) m/z calcd for $[C_7H_{12}N_3O_3]^+$ = [M-*t*Bu + 2H]$^+$: 186.1, found: 186.2.

8-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-2-(6-(methylamino)pyrimidin-4-yl)-2,5,8-triazaspiro[3.5]nonan-6-one (11). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:5 to 100:8 to 100:11 to 100:15). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. The impure desired product was engaged in the next step without further purification.

The corresponding chloropyrimidine was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. Due to **40** derivative impurities still present, 7 equiv of the pyrimidine and 7 equiv of Et_3N were used, and the reaction was heated for 7 h at 80 °C in the microwave. The impure desired product was engaged in the next step without further purification.

Compound **11** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:10 in 15 min, 100:10 for 5 min, 100:10 to 100:13 in 5 min, and 100:13 for 5 min). Yellow solid, 19% yield over four steps. Mp: decomposition; ^1H NMR (400 MHz, MeOD-d_4): δ 7.97 (s, 1H), 7.38−7.31 (m, 2H), 7.10−7.02 (m, 2H), 5.32 (s, 1H), 4.14 (d, J = 8.9 Hz, 2H), 4.00 (d, J = 8.9 Hz, 2H), 3.91−3.78 (m, 4H), 3.74−3.72 (m, 2H), 2.93−2.67 (m, 7H), 1.57−1.48 (m, 4H), 0.99 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.7, 164.7, 164.6, 158.3, 151.2, 133.3, 116.8, 61.9 (br s), 61.8, 54.8, 53.2, 52.7, 50.3, 37.6, 30.9 (br s, 1C), 29.1, 28.4. One carbon is missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{25}H_{36}N_7O]^+$: 450.2981, found: 450.2976.

4-((4,4-Dimethylpiperidin-1-yl)methyl)aniline (41). To a stirred solution of 1-(chloromethyl)-4-nitrobenzene (4 g, 23 mmol) in DMF (56 mL), 4,4-dimethylpiperidine hydrochloride (1 equiv, 23 mmol, 3.49 g) and K_2CO_3 (2 equiv, 46 mmol, 6.36 g) were added. The reaction mixture was stirred at 90 °C for 17 h, cooled down to 25 °C, and diluted with water. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed three times with water and once with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The obtained residue was engaged in the next step without further purification.

To a stirred solution of the corresponding nitroaryl (23 mmol) in EtOH (74 mL), acetic acid (16 mL) and iron powder (3 equiv, 69 mmol, 3.85 g) were added. The reaction mixture was vigorously stirred at 80 °C for 4 h and cooled down to 25 °C. An aqueous solution of KOH (2 M) was added to adjust the pH to 11, and the aqueous layer was extracted with DCM. The combined organic layers were washed once with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (EtOAc/heptane = 1:1 to 7:3 to 10:0) to afford the desired product as an orange solid (2.8 g, 56% yield over two steps). ^1H NMR (400 MHz, $CDCl_3$): δ 7.13−7.06 (m, 2H), 6.68−6.62 (m, 2H), 3.61 (br s, 2H), 3.41 (s, 2H), 2.42−2.29 (m, 4H), 1.38 (t, J = 5.6 Hz, 4H), 0.91 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 145.2, 130.4 (2C), 128.5, 114.8 (2C), 63.0, 49.8 (2C), 38.6 (2C), 28.5 (2C), 28.2 (br s); LRMS (ESI) m/z calcd for $[C_{14}H_{23}N_2]^+$: 219.2, found: 219.3.

***tert*-Butyl 4-(Hydroxymethyl)-4-(((2,2,2-trichloroethoxy)-carbonyl)amino)piperidine-1-carboxylate (42).** To a stirred solution of $NaBH_4$ (2.4 equiv, 98 mmol, 3.72 g) in THF (256 mL), 4-amino-1-(*tert*-butoxycarbonyl)piperidine-4-carboxylic acid (10 g, 41 mmol) was added. The mixture was cooled down to 0 °C, and a solution of I_2 (1 equiv, 41 mmol, 10.37 g) in THF (73 mL) was added dropwise. When the H_2 evolution stopped, the reaction mixture was heated at 75 °C for 17 h, cooled down to 25 °C, quenched with MeOH, and concentrated under reduced pressure. The obtained residue was poured in an aqueous solution of KOH (20%, 1 L), and the solution was stirred for 4 h at 25 °C. The aqueous layer was extracted three times with DCM, and the combined organic layers were washed once with brine, dried over $MgSO_4$, and concentrated under reduced pressure to afford the desired product, which was engaged in the next step without further purification.

To a stirred solution of the corresponding amino alcohol (41 mmol) in DCM (81 mL), a solution of $NaHCO_3$ (2.4 equiv, 98 mmol, 8.2 g) in water (81 mL) was added. The reaction mixture was cooled to 0 °C, and TrocCl (1.2 equiv, 49 mmol, 6.7 mL) was added

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dropwise. The reaction mixture was stirred at 25 °C for 3 h, and the two phases were separated. The aqueous layer was extracted two times with DCM. The combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (EtOAc/heptane = 3:7 to 1:1 to 7:3 to 10:0 to EtOAc/MeOH = 100:1 to 100:5) to afford the desired product as a white solid (12.79 g, 77% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.94 (br s, 1H), 4.72 (s, 2H), 3.85−3.68 (m, 4H), 3.24−3.12 (m, 2H), 2.58 (br s, 1H), 1.98 (d, J = 14.2 Hz, 2H), 1.70−1.62 (m, 2H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.7, 153.8, 95.5, 79.9, 74.2, 67.4, 55.6, 39.2 (br s), 31.2, 28.4 (3C); LRMS (ESI) m/z calcd for $[C_9H_{16}Cl_3N_2O_3]^+$ = [M-Boc + 2H]$^+$: 305.0, found: 305.1.

***tert*-Butyl 4-(((4-((4,4-Dimethylpiperidin-1-yl)methyl)-phenyl)amino)methyl)-4-(((2,2,2-trichloroethoxy)carbonyl)-amino)piperidine-1-carboxylate (43).** To a stirred solution of **42** (5 g, 12.3 mmol) in dry DCM (41 mL), under a nitrogen atmosphere, Dess−Martin periodinane (1.1 equiv, 13.5 mmol, 5.74 g) was added. The reaction mixture was stirred for 1.5 h at 25 °C and diluted with DCM. The organic layer was washed once with an aqueous $Na_2S_2O_3$ solution (20%), once with a saturated aqueous $NaHCO_3$ solution, and once with brine; dried over $MgSO_4$; filtered; and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (EtOAc/heptane = 1:9 to 3:7 to 1:1 to 7:3) to afford the desired product as a white solid (4.6 g, 92%).

To a stirred solution of the corresponding aldehyde (4.6 g, 11.4 mmol) in dichloroethane (40 mL), **41** (1 equiv, 11.4 mmol, 2.5 g) and AcOH (3 equiv, 34.2 mmol, 1.95 mL) were added. The reaction mixture was stirred at 25 ° for 3 h, $NaBH(OAc)_3$ (1.5 equiv, 17.1 mmol, 3.62 g) was added, and the mixture was stirred a further 17 h. The reaction was quenched by the addition of a saturated aqueous K_2CO_3 solution, and the two phases were separated. The aqueous layer was extracted three times with DCM, and the combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH = 100:5 to 100:10 to 100:15 to 100:20) to afford the desired product as an orange solid (4.91 g, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.13 (d, J = 7.4 Hz, 2H), 6.57 (d, J = 8.4 Hz, 2H), 4.88 (br s, 1H), 4.71 (s, 2H), 3.91 (br s, 2H), 3.46 (br s, 2H), 3.42 (d, J = 5.6 Hz, 2H), 3.05 (t, J = 11.8 Hz, 2H), 2.42 (br s, 4H), 2.13 (d, J = 12.7 Hz, 2H), 1.70−1.62 (m, 2H), 1.51−1.38 (m, 13H), 0.92 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.6, 153.1, 147.5, 130.8 (2C), 112.5 (2C), 95.6, 79.9, 74.1, 62.6, 55.2, 50.9, 49.6, 38.1, 32.5, 28.4 (3C). Four carbons are missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{28}H_{44}ClN_4O_4]^+$: 605.2, found: 605.3.

***tert*-Butyl 4-Amino-4-(((4-((4,4-dimethylpiperidin-1-yl)-methyl)phenyl)amino)methyl)piperidine-1-carboxylate (44).** To a stirred solution of **43** (4.85 g, 8.01 mmol) in a mixture of THF (16 mL) and AcOH (10 mL), zinc powder (10 equiv, 80.1 mmol, 5.2 g) was added. The reaction mixture was stirred at 25 ° for 3 h and filtered over a pad of Celite, and the filter cake was washed with MeOH. The filtrate was concentrated under reduced pressure, and the pH was adjusted to 11 with a saturated aqueous Na_2CO_3 solution. The aqueous layer was extracted three times with DCM, and the combined organic layers were washed once with brine, dried over $MgSO_4$, filtered, and concentrated reduced pressure. The crude residue was purified by flash column (chromatography (DCM/MeOH/NH_4OH) = 100:2:0 to 100:4:0 to 100:4:1 to 100:6:1 to 100:8:1 to 100:10:1 to 100:12:1 to 100:20:1) to afford the desired product as a yellow solid (2.54 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.14−7.09 (m, 2H), 6.63−6.58 (m, 2H), 4.06 (t, J = 5.6 Hz, 1H), 3.76−3.63 (m, 2H), 3.41 (s, 2H), 3.27 (ddd, J = 13.6, 10.2, 3.3 Hz, 2H), 3.02 (d, J = 5.8 Hz, 2H), 2.37 (br s, 4H), 1.68−1.57 (m, 2H), 1.50−1.45 (m, 11H), 1.39 (br t, J = 5.1 Hz, 4H), 0.91 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 154.8, 147.8, 130.5 (2C), 127.1, 112.6 (2C), 79.5, 63.0, 54.7, 50.3, 49.7 (2C), 39.6 (br s), 38.5, 36.1 (2C), 28.5 (2C), 28.4 (3C). One carbon is missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{25}H_{43}N_4O_2]^+$: 431.3, found: 431.4.

3-(4-((4,4-Dimethylpiperidin-1-yl)methyl)phenyl)-8-(6-(methylamino)pyrimidin-4-yl)-1,3,8-triazaspiro[4.5]decan-2-one (12). To a stirred solution of **44** (300 mg, 0.70 mmol) in DCM (3 mL), CDI (2 equiv, 1.4 mmol, 226 mg) was added. The reaction mixture was stirred at 25 °C for 17 h and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (chromatography: DCM/MeOH = 100:3 to 100:5 to 100:8 to 100:10). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. The impure desired product was engaged in the next step without further purification.

The corresponding chloropyrimidine was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. Due to the CDI derivative impurities still present, 4 equiv of the pyrimidine and 6 equiv of Et_3N were used (chromatography: DCM/MeOH = 100:1 to 100:3 to 100:5 to 100:8 to 100:10 to 100:15). The impure desired product was engaged in the next step without further purification.

Compound **12** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:7 in 20 min and 100:7 for 5 min). Yellow solid (28% yield over four steps). Mp: 204−207 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (s, 1H), 7.64−7.51 (m, 4H), 5.71 (br s, 1H), 5.47 (s, 1H), 4.98 (br s, 1H), 3.99 (s, 2H), 3.79−3.63 (m, 6H), 3.05−2.75 (m, 7H), 1.88 (t, J = 5.4 Hz, 4H), 1.76 (br s, 4H), 1.00 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.8, 162.5, 157.5, 157.4, 140.7, 131.6 (2C), 117.7 (2C), 80.1 (br s), 60.8 (br s), 55.8 (s), 52.6 (s), 48.8 (s), 41.0 (s), 36.8 (s), 35.8 (br s), 28.5 (s), 28.0 (s). Two carbons are missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{26}H_{38}N_7O]^+$: 464.3138, found: 464.3132.

1-(4-Bromo-3-fluorobenzyl)-4,4-dimethylpiperidine (47). Intermediate **47** was obtained following the general procedure for dimethylpiperidine alkylation (column chromatography: EtOAc/heptane = 1:9). Colorless oil, 98% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (t, J = 7.6 Hz, 1H), 7.15 (dd, J = 9.6, 1.8 Hz, 1H), 6.99 (dd, J = 8.4, 1.3 Hz, 1H), 3.45 (s, 2H), 2.37 (t, J = 5.1 Hz, 4H), 1.39 (t, J = 5.8 Hz, 4H), 0.92 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.0 (d, J = 246.2 Hz, 1C), 141.2 (d, J = 6.9 Hz, 1C), 132.9, 125.6 (d, J = 3.5 Hz, 1C), 116.7 (d, J = 22.5 Hz, 1C), 106.8 (d, J = 20.8 Hz, 1C), 62.3, 50.0 (2C), 38.6 (2C), 28.3 (2C), 28.2 (br s); LRMS (ESI) m/z calcd for $[C_{14}H_{20}BrFN]^+$: 300.1, found: 300.1.

9-(6-Chloropyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)-2-fluorophenyl)-1,4,9-triazaspiro[5.5]undecan-2-one (48). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:3 to 100:5 to 100:8). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. The impure desired product was engaged in the next step without further purification.

Intermediate **48** was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine (column chromatography: DCM/MeOH = 100:3 to 100:5 to 100.8 to 100:10) to afford the desired product as a white solid (15% yield over three steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.40 (s, 1H), 7.15−6.99 (m, 2H), 6.87 (t, J = 8.5 Hz, 1H), 6.56 (s, 1H), 6.48 (s, 1H), 3.91−3.80 (m, 2H), 3.77 (s, 2H), 3.74−3.63 (m, 2H), 3.45 (br s, 2H), 3.29 (s, 2H), 2.46−2.27 (m, 4H), 2.09−1.99 (m, 2H), 1.91−1.81 (m, 2H), 1.47−1.34 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.1, 162.0, 160.1, 158.1, 155.0 (d, J = 245.4 Hz, 1C), 136.3 (d, J = 9.5 Hz, 1C), 133.8, 125.3 (br s), 118.2 (d, J = 2.6 Hz, 1C), 117.1 (d, J = 20.8 Hz, 1C), 101.4, 62.0, 57.1 (d, J = 3.5 Hz, 1C), 53.1 (2C), 53.1, 49.8 (2C), 40.0, 38.3 (2C), 35.2 (2C), 28.3 (2C), 28.1 (br s); LRMS (ESI) m/z calcd for $[C_{26}H_{35}ClFN_6O]^+$: 501.3, found: 501.3.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)-2-fluorophenyl)-9-(6-(methylamino)pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]-undecan-2-one (20). Compound **20** was obtained following the

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general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:8 in 20 min and 100:8 for 15 min). White solid (47% yield). Mp: 202−204 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.17 (s, 1H), 7.18−7.04 (m, 2H), 6.88 (t, J = 8.6 Hz, 1H), 6.07 (s, 1H), 5.45 (s, 1H), 4.82−4.73 (m, 1H), 3.78 (s, 2H), 3.77−3.71 (m, 2H), 3.66−3.59 (m, 2H), 3.52 (br s, 2H), 3.30 (s, 2H), 2.90 (d, J = 5.1 Hz, 3H), 2.58−2.31 (m, 4H), 2.06−1.98 (m, 2H), 1.87−1.79 (m, 2H), 1.52−1.39 (m, 4H), 0.94 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.3, 164.0, 162.5, 157.6, 155.2 (d, J = 245.4 Hz, 1C), 136.6 (d, J = 9.5 Hz, 1C), 125.4, 118.4 (d, J = 2.6 Hz, 1C), 117.3 (d, J = 20.8 Hz, 1C), 80.0, 62.1, 56.8 (d, J = 4.3 Hz, 1C), 53.5, 53.3, 49.9, 40.3, 38.2, 35.4, 28.5, 28.4. Two carbons are missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{27}H_{39}FN_7O]^+$: 496.3200, found: 496.3195.

1-(4-Bromo-2-fluorobenzyl)-4,4-dimethylpiperidine (49). Intermediate **49** was obtained following the general procedure for dimethylpiperidine alkylation. Colorless liquid, 99% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32−7.27 (m, 1H), 7.27−7.25 (m, 1H), 7.25−7.19 (m, 1H), 3.53 (d, J = 1.3 Hz, 2H), 2.48−2.34 (m, 4H), 1.44−1.35 (m, 4H), 0.91 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 161.0 (d, J = 249.7 Hz), 132.5 (d, J = 5.2 Hz), 127.1 (d, J = 3.5 Hz), 124.6 (d, J = 14.7 Hz), 120.6 (d, J = 9.5), 118.7 (d, J = 25.1 Hz), 55.0 (d, J = 1.7 Hz), 49.7 (2C), 38.6 (2C), 28.3 (2C), 28.1 (br s); LRMS (ESI) m/z calcd for $[C_{14}H_{20}BrFN]^+$: 300.1, found: 300.1.

tert-Butyl 4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)-3-fluorophenyl)-2-oxo-1,4,9-triazaspiro[5.5]undecane-9-carboxylate (50). Intermediate **50** was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:5 to 100:8 to 100:12 to 100:16). Brown solid (83% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40−7.28 (m, 1H), 6.59 (d, J = 7.9 Hz, 1H), 6.49 (dd, J = 12.5, 2.4 Hz, 1H), 6.44 (s, 1H), 3.86 (s, 2H), 3.63−3.53 (m, 3H), 3.53−3.44 (m, 3H), 3.36 (s, 2H), 2.46 (br s, 4H), 1.84−1.71 (m, 4H), 1.48 (s, 9H), 1.47 (br s, 4H), 0.93 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.1, 162.3 (d, J = 245.4 Hz), 154.5, 149.8 (d, J = 11.3 Hz), 133.1 (d, J = 4.3 Hz), 109.9 (d, J = 2.6 Hz), 101.3 (br d, J = 26.9 Hz), 80.1, 54.7, 54.3, 52.7, 51.3, 49.3, 39.4 (br s, 1C), 38.0, 35.3, 28.4 (3C), 28.3 (2C). Two carbons are missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{27}H_{42}FN_4O_3]^+$: 489.3, found: 489.4.

9-(6-Chloropyrimidin-4-yl)-4-(4-((4,4-dimethylpiperidin-1-yl)methyl)-3-fluorophenyl)-1,4,9-triazaspiro[5.5]undecan-2-one (51). The corresponding amine was obtained following the general procedure for Boc group deprotection. The impure desired product was engaged in the next step without further purification.

Intermediate **51** was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. Instead of chromatography, after evaporation of the crude mixture, the residue was triturated in water. The obtained precipitate was filtered, washed with water, and dried to afford the desired product as a brown solid (58% yield over two steps). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.37 (s, 1H), 8.34 (s, 1H), 7.15 (t, J = 8.6 Hz, 1H), 7.01 (s, 1H), 6.80−6.69 (m, 2H), 3.86 (br s, 2H), 3.81−3.65 (m, 4H), 3.44 (s, 2H), 3.37 (s, 2H), 2.38−2.25 (m, 4H), 1.81−1.70 (m, 2H), 1.70−1.57 (m, 2H), 1.34−1.24 (m, 4H), 0.86 (s, 6H).^{13}C NMR (101 MHz, DMSO-d_6): δ 166.8, 161.9, 161.8 (d, J = 242.8 Hz), 159.2, 158.0, 149.8 (d, J = 10.4 Hz), 132.1 (d, J = 5.2 Hz), 113.8 (br s), 109.4, 101.7, 100.6 (d, J = 27.7 Hz), 54.5, 52.2, 52.0, 50.7, 49.0, 38.2, 34.2, 28.1 (2C). Two carbons are missing due to overlapping or broadening; LRMS (ESI) m/z calcd for $[C_{26}H_{35}ClFN_6O]^+$: 501.3, found: 501.3.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)-3-fluorophenyl)-9-(6-(methylamino)pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]-undecan-2-one (21). Compound **21** was obtained following the general procedure for S_NAr with chloropyrimidine derivatives (chromatography: DCM/MeOH = 100:0 to 100:8 in 20 min, 100:8 for 5 min, 100:8 to 100:12 in 10 min, and 100:12 for 5 min). Yellow solid, 69% yield. Mp: 210−211 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (s, 1H), 7.40−7.29 (m, 1H), 6.65−6.56 (m, 1H), 6.50 (dd, J = 12.8, 2.4 Hz, 1H), 6.33 (br s, 1H), 5.45 (s, 1H), 4.81−4.71 (m, 1H), 3.88 (s, 2H), 3.80−3.72 (m, 2H), 3.69−3.47 (m, 4H), 3.40 (s, 2H), 2.91 (d, J = 5.3 Hz, 3H), 2.48 (br s, 4H), 1.95−1.76 (m, 4H), 1.53−1.33 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.4, 164.1, 162.5, 162.2 (d, J = 245.4 Hz), 157.6, 149.4 (d, J = 9.5 Hz), 132.7 (d, J = 6.1 Hz), 115.7 (d, J = 15.6 Hz), 109.9, 101.5 (d, J = 26.9 Hz), 80.0, 55.1, 54.6, 53.0, 51.5, 49.6, 40.1, 38.5, 35.0, 28.4, 28.3, 28.1 (br s). HRMS (ESI): m/z: calcd for $[C_{27}H_{39}FN_7O]^+$: 496.3200, found: 496.3195.

1-(4-Bromo-2,5-difluorobenzyl)-4,4-dimethylpiperidine (52). To a stirred solution of 4-bromo-2,5-difluorobenzoic acid (1 g, 4.2 mmol) in dry THF (10 mL), under a nitrogen atmosphere, $BH_3{\cdot}SMe_2$ (2 equiv, 8.4 mmol, 4.2 mL, 2 M THF) was added. The reaction mixture was stirred for 17 h at 25 °C, cooled down to 0 °C, and quenched by the addition of a saturated aqueous Na_2CO_3 solution. The aqueous layer was extracted three times with EtOAc, and the combined organic layers were washed once with brine, dried over $MgSO_4$, and concentrated under reduced pressure to afford the desired product as a brown solid (789 mg, 83% yield).

To a stirred solution of the corresponding alcohol (789 mg, 3.54 mmol) in DCM (10 mL), $SOCl_2$ (1.5 equiv, 5.3 mmol, 385 μL) and DMF (1 drop) were added. The reaction mixture was stirred at 25 °C for 3 h and concentrated under reduced pressure to afford the desired chloroalkane, which was engaged in the next step without further purification.

To a stirred solution of the corresponding chloroalkane (425 mg, 1.76 mmol) in dimethylformamide (5 mL), 4,4-dimethylpiperidine hydrochloride (1 equiv, 1.76 mmol, 263 mg) and K_2CO_3 (2 equiv, 3.52 mmol, 486 mg) were added. The reaction mixture was stirred at 25 °C for 3 days and concentrated under reduced pressure. The obtained residue was purified by flash column chromatography (EtOAc/heptane = 3:100 to 10:100) to afford the desired product as a colorless liquid (514 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.27−7.21 (m, 2H), 3.50 (s, 2H), 2.46−2.38 (m, 4H), 1.45−1.37 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.7 (d, J = 245.4 Hz), 155.5 (d, J = 242.8 Hz), 127.2 (dd, J = 17.3, 6.1 Hz), 119.8 (d, J = 27.7 Hz), 117.8 (dd, J = 24.7, 5.6 Hz), 106.9 (dd, J = 23.4, 10.4 Hz), 54.9, 49.9 (2C), 38.7 (2C), 28.3 (2C), 28.2 (br s); LRMS (ESI) m/z calcd for $[C_{14}H_{19}BrF_2N]^+$: 318.1, found: 318.1.

4-(4-((4,4-Dimethylpiperidin-1-yl)methyl)-2,5-difluorophenyl)-9-(6-(methylamino)pyrimidin-4-yl)-1,4,9-triazaspiro[5.5]-undecan-2-one (22/UZH2). The corresponding Boc protected amine was obtained following the general procedure for Buchwald−Hartwig coupling (chromatography: DCM/MeOH = 100:3 to 100:5 to 100:8). The impure desired product was engaged in the next step without further purification.

The corresponding amine was obtained following the general procedure for Boc group deprotection. The impure desired product was engaged in the next step without further purification.

The corresponding chloropyrimidine was obtained following the general procedure for S_NAr with 4,6-dichloropyrimidine. The impure desired product was engaged in the next step without further purification.

UZH2 was obtained following the general procedure for S_NAr with chloropyrimidine derivatives chromatography: DCM/MeOH = 100:0 to 100:10 in 15 min, 100:10 for 10 min, and 100:10 to 100:12 in 10 min. White solid, 7% yield over four steps. Mp: 214−216; ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.98 (s, 1H), 7.17 (dd, J = 13.2, 6.6 Hz, 1H), 6.87 (dd, J = 11.3, 7.2 Hz, 1H), 5.64 (s, 1H), 4.00−3.89 (m, 2H), 3.72 (s, 2H), 3.54 (s, 2H), 3.46−3.39 (m, 2H), 3.39 (s, 2H), 2.84 (s, 3H), 2.47 (br s, 4H), 2.05−1.95 (m, 2H), 1.87−1.77 (m, 2H), 1.48−1.37 (m, 4H), 0.93 (s, 6H); ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.5, 165.4, 163.6, 158.3, 159.2 (d, J = 241.9 Hz), 152.5 (d, J = 241.0 Hz), 140.3−139.7 (m), 120.0 (dd, J = 23.0, 5.6 Hz), 118.7 (dd, J = 18.2, 6.9 Hz), 107.4 (d, J = 28.6 Hz), 82.1, 56.3 (d, J = 3.5 Hz), 55.5, 54.7, 53.9, 50.7, 41.9, 39.3, 36.0, 29.3, 28.5. One carbon is missing due to overlapping or broadening; HRMS (ESI): m/z: calcd for $[C_{27}H_{38}F_2N_7O]^+$: 514.3106, found: 514.3100.

Materials. MOLM-13 and PC-3 cells were obtained from DSMZ−German Collection of Microorganisms and Cell Cultures. HEK293T cells were obtained from ATCC. Dulbecco's modified essential medium (DMEM) supplemented with 4.5 g/L glucose and GlutaMAX, RPMI 1640 medium, Opti-MEM, Gibco Fetal Bovine

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Serum, Gibco Penicillin−Streptomycin, 0.05% Trypsin−EDTA, nuclease-free water, and Lipofectamine RNAiMAX were purchased from Thermo Fisher Scientific (Waltham, MA). Cell culture treated dishes, flasks, and multiwell plates were obtained from Corning Inc. (Corning, NY). The InCELL Pulse assay kit was obtained from DiscoverX Corp. (Fremont, CA). The GENEzol reagent was purchased from Geneaid Biotech (New Taipei, Taiwan). Sera-Mag Oligo(dT)-coated magnetic particles were obtained from GE Healthcare (Chicago, IL). NEBuffer 2 and nucleoside digestion mix were obtained from NEB (Ipswich, MA). Adenosine was purchased from Sigma-Aldrich (St. Louis, MO) and N6-methyladenosine (m^6A) was purchased from Chemie Brunschwig (Basel, Switzerland) as UPLC−MS/MS standards.

Expression and Purification of Recombinant Proteins. Recombinant METTL3/METTL14 complex constructs for crystallization and for the use in enzymatic activity assay were expressed using the baculovirus/Sf9 insect cell expression system as described previously.[11a]

The plasmid expressing the N-terminally hexahistidine-tagged METTL1 protein (residues 32−265) was a gift from Cheryl Arrowsmith (Addgene ID: 25264). The protein was overexpressed for 12 h at 20 °C in *E. coli* BL21 (DE3) cells upon induction with 0.2 mM IPTG. The cells were harvested and resuspended in the lysis buffer containing 100 mM Tris−HCl at pH 8.0 and 500 mM NaCl. The cells were lysed by sonication, and the cell lysate was clarified by centrifugation at 50,000*g* for 2 h and loaded onto a Ni-NTA affinity column (5 mL HisTrap FF from GE Healthcare). After extensive washing with the wash buffer containing 100 mM Tris−HCl at pH 8.0, 500 mM NaCl, and 50 mM imidazole, the target protein was eluted with an elution buffer containing 100 mM Tris−HCl at pH 8.0, 500 mM NaCl, and 250 mM imidazole. The N-terminal hexahistidine-tag was removed by cleavage with tobacco etch virus (TEV) protease at a 1:50 ratio. The excess imidazole was removed by overnight dialysis, and the sample was subjected to a secondary subtractive Ni-NTA affinity chromatography step to remove the protease and uncleaved protein. Finally, the protein was subjected to a gel filtration step using a Superdex 75 16/60 column in a buffer containing 10 mM Tris−HCl at pH 8 and 150 mM NaCl.

The plasmid expressing 6xHis-Strep-SUMO-TEV fused with the core methyltransferase domain of human METTL16 (residues 1−291) was received from Pillai lab.[26] The protein was overexpressed for 12 h at 18 °C in *E. coli* BL21 (DE3) cells upon induction with 0.2 mM IPTG. The cells were harvested and resuspended in the lysis buffer containing 100 mM Tris−HCl at pH 7.4 and 500 mM NaCl. The cells were lysed with a French press three times at 15,000 psi, and the cell lysate was clarified by centrifugation at 50,000*g* for 2 h and loaded onto a Ni-NTA affinity column (5 mL HisTrap FF from GE Healthcare). After extensive washing with the wash buffer containing 100 mM Tris−HCl at pH 7.4, 500 mM NaCl, and 50 mM imidazole, the target protein was eluted with an elution buffer containing 100 mM Tris−HCl at pH 7.4, 500 mM NaCl, and 250 mM imidazole. The N-terminal tags were removed by cleavage with tobacco etch virus (TEV) protease at a 1:50 ratio. The excess imidazole was removed by overnight dialysis, and the sample was subjected to a secondary subtractive Ni-NTA affinity chromatography step to remove the protease and uncleaved protein. Finally, the target protein was subjected to a gel filtration step using a Superdex 75 16/60 column in a buffer containing 25 mM HEPES at pH 7.2 and 150 mM NaCl.

Reader-Based HTRF Assay. Compound potencies were evaluated by using a previously reported METTL3 inhibition assay.[27] Briefly, the level of m^6A in the oligoribonucleotide substrate after the reaction catalyzed by METTL3-METTL14 was quantified by measuring the specific binding of modified oligoribonucleotide to the m^6A reader $YTHDC1_{345-509}$ by homogeneous time-resolved fluorescence (HTRF). Tested compounds that inhibit METTL3 decrease the m^6A level and thus reduce the HTRF signal. Response curves were plotted in GraphPad Prism 8.4 and fitted with nonlinear regression "log(inhibitor) vs. normalized response−variable slope", from which IC_{50} values were determined. The IC_{50} values are given as an average of at least three independent measurements for each compound.

Crystallization. The protein crystals of $METTL3_{354-580}$−$METTL14_{106-396}$ were obtained as previously described.[11a] Briefly, the purified $METTL3_{354-580}$−$METTL14_{106-396}$ complex was diluted to 5 mg/mL in 10 mM Tris−Cl at pH 8.0 and 200 mM KCl. Crystals were obtained using the hanging drop vapor diffusion method by mixing 1 mL of the complex solution with 1 mL of the reservoir solution containing 20% PEG 3350 and 400 mM magnesium acetate. The soaking experiment was carried out by transferring crystals to a 1 μL drop containing 200 mM compound directly dissolved in the buffer containing 100 mM Tris at pH 8.0, 30% PEG 3350, and 400 mM magnesium acetate. After 16 h of incubation at 22 °C, the crystals were harvested and flash-frozen in liquid nitrogen.

Data Collection and Structure Solution. Diffraction data were collected at the PXIII beamline at the Swiss Light Source (SLS) of the Paul Scherrer Institute (PSI, Villigen, Switzerland) and processed using XDS.[28] The crystal structures were solved by molecular replacement techniques using the 5L6D structure as the search model with the Phaser program[29] from the Phenix package. In the crystals not subjected to soaking, the clear electron density for product cofactor *S*-adenosyl-homocysteine (SAH) is visible. Therefore, in this soaking experiment setup, test compounds competed with SAH for the *S*-adenosyl methionine (SAM) binding site. In the crystal structures with test compounds that were able to replace SAH in the binding site, the electron density due to the homocysteine part of SAH was no longer visible. All of the crystallographic models were constructed through iterative cycles of the manual model building with COOT[30] and refinement with phenix.refine. Default XYZ (reciprocal-space), XYZ (real-space), individual B-factors, and occupancy refinement parameters appropriate for the resolution range were utilized. During the first run of the refinement update, water was used in phenix.refine[31] followed by the addition of the missing water molecules manually.

Kinetic Solubility Assay. The kinetic solubility has been evaluated by ChemPartner (Shanghai, China) following standard procedures. Briefly, the compounds (10 mM stock solution in DMSO) are added to a 100 mM phosphate buffer solution (pH = 7.4), and the samples (compound concentration = 100 μM, 0.1% DMSO) are shaken (1000 rpm) for 1 h at room temperature and centrifuged. The supernatants are then analyzed by LCMS/MS to determine the compounds' solubility.

Caco-2 Cell Permeability Assay. The intestinal permeability has been evaluated in a Caco-2 cell permeability assay by ChemPartner (Shanghai, China) following standard procedures. Briefly, Caco-2 monolayer cells were cultured in Millicell-24 cell culture plates (Merk Millipore; surface area of the membrane = 0.7 cm^2, V_A = 0.8 mL (A-to-B) or 0.4 mL (B-to-A)). The cell permeability of the compounds along with the reference compounds (i.e., erythromycin, metoprolol, and atenolol) was tested at 10 μM in an HBSS buffer containing 0.4% DMSO (v/v) final concentration. The compounds were placed in either apical or basolateral chamber, and their concentrations were evaluated in both compartments after the 90 min incubation at 37 °C using the API 4000 LC/MS/MS system (Applied Biosystems, Waltham, MA). The Caco-2 monolayer's leakiness after the treatment was evaluated by measuring concentrations of the fluorescent dye Lucifer Yellow with low cell permeability (5 μM initial concentration) using a fluorometer (at Ex/Em of 485/535 nm). Lucifer Yellow Papp values were lower than 1×10^{-6} cm/s, and transepithelial electrical resistance (TEER) values were higher than 400 Ω·cm^2, verifying that the Caco-2 monolayers were intact. Mass recovery of the compounds indicated low nonspecific adsorption to the assay chambers. Compound permeability was evaluated in duplicates. Compound permeability was calculated according to the following equation, where V_A is the volume in the acceptor well, area is the surface area of the membrane, and time is the total transport time in seconds:

$$P_{app} = \frac{V_A \times [\text{compound}]_{acceptor} \times \text{dilution factor}}{\text{area} \times \text{time} \times [\text{compound}]_{donor,initial}}$$

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Lucifer Yellow permeability was calculated according to the following equation:

$$P_{app} = \frac{V_A \times ([RFU]_{acceptor} - [RFU]_{blank}) \times \text{dilution factor}}{\text{area} \times \text{time} \times ([RFU]_{donor,initial} - [RFU]_{blank})}$$

Compound recovery was calculated according to the following equation:

$$\text{Recovery}(\%) = 100 \times \frac{\text{total compound}_{donor,90\ min} + \text{total compound}_{acceptor,90\ min}}{\text{total compound}_{donor,initial}}$$

Transepithelial electrical resistance (TEER) was calculated according to the following equation:

$$\text{TEER}(\Omega \cdot \text{cm}^2) = (\text{resistance}_{sample} - \text{resistance}_{blank}) \times \text{area}$$

Rat Liver Microsome Assay. The metabolic stability has been evaluated in a rat liver microsome assay by ChemPartner (Shanghai, China) following standard procedures. Briefly, the compounds (10 mM stock solution in DMSO) were incubated at 37 °C with rat liver microsomes in a phosphate buffer solution (pH = 7.4). At several time points (0, 5, 15, 30, and 45 min), the internal standard in acetonitrile was added to the corresponding well to stop the reaction. The samples were shaken for 10 min (600 rpm) and then centrifuged for 15 min (5594*g*), and the supernatants were analyzed by LC−MS.

Thermal Shift Assay. Thermal shift measurements were carried out as previously described[32] in a final volume of 20 μL; protein concentrations of 2 μM; compound **UZH2** concentrations of 10, 50, and 100 μM; and SAH concentrations of 100, 500, and 1000 μM. The reported values (ΔTm) are calculated as the difference between the transition midpoints of an individual sample and the average of the reference wells (containing the protein and the DMSO only) in the same plate. The DMSO concentration was kept at 1% (v/v).

Cell Culture. MOLM-13 cells were cultured in RPMI 1640 medium containing 10% Gibco FBS and 1% penicillin/streptomycin (complete medium) in 5% CO_2 at 37 °C in a humidified incubator, with maintained cell densities at 0.6−2·10^6 cells/mL. PC-3 cells were maintained in RPMI 1640 medium containing 10% Gibco FBS and 1% penicillin/streptomycin (complete medium) in 5% CO_2 at 37 °C in a humidified incubator, with regular passaging twice a week using a 1:5 split ratio. HEK293T cells were maintained in DMEM supplemented with 4.5 g/L glucose and GlutaMAX containing 10% Gibco FBS and 1% penicillin/streptomycin (complete medium) in 5% CO_2 at 37 °C in a humidified incubator, with regular passaging twice a week using a 1:10 split ratio. All cell lines were tested negative for mycoplasma contamination (PCR-based assay by Microsynth, Switzerland).

CETSA. CETSA experiments were performed as previously described.[33] In brief, MOLM-13 cells were concentrated to 2 × 10^7 cells/mL in Hank's balanced salt solution (HBSS) containing 2× Protease Inhibitor Cocktail (Sigma) and added to a compound dilution series in a 1:1 ratio with a final DMSO concentration of 0.5% (v/v). The solutions were incubated for 1 h at 37 °C. Samples were then heated up to 54 °C for 3 min followed by cooling to room temperature. Before the centrifugation at 4 °C, cells were lysed by three freeze−thaw cycles. The amount of METTL3 and β-actin in the supernatants was quantified by Western blotting as previously described.[17a]

Cellular Target Engagement Assay InCELL Pulse. Cellular target engagement of **UZH2** was evaluated using an InCELL Pulse assay according to the manufacturer's instructions. The METTL3 gene encoding the METTL3 catalytic domain (residues 356−580) was amplified using primers listed below.

pICP-N-M3-F: ATATGAATTCACACAGAGTGTCGGAGGT-GATTCC.

pICP-N-M3-R: ATGCTCTAGATTATAAATTCTTAGGTTTA-GAGATGATACCATCTGGG.

The amplified gene was ligated into the pICP-ePL-N vector between *Eco*RI and *Xba*I restriction sites.

HEK293T cells were seeded at 1 × 10^6 cells/well in 2 mL of complete medium in a six-well plate and transfected with 1.5 μg of the plasmid encoding the enhanced ProLabel (ePL) enzyme fragment fused to the N-terminus of the truncated METTL3 (residues 354−580) using Lipofectamine RNAiMAX for 24 h. After the transfection, cells were washed once with the Opti-MEM medium, detached from the plate by gentle washing, and counted. Forty microliters of the Opti-MEM medium containing 5000 transfected cells was mixed with 10 μL of the **22** solution in Opti-MEM or 2.5% (v/v) of DMSO (5×) as a negative control and incubated for 1 h at 37 °C. After the incubation, cells were heated at 46 °C for 3 min followed by incubation at 22 °C for 3 min, and the non-aggregated METTL3-ePL protein was quantified using a luminescence-based assay according to the InCELL Pulse kit's instructions.

Quantification of the m^6A/A Ratio in polyadenylated RNA and m^6A/A, m^6A_m/A, m^1A/A, and m^7G/G in Total RNA by UPLC−MS/MS Analysis. MOLM-13 cells were seeded into six-well plates at a density of 1 × 10^6 cells/mL in 2 mL of the complete medium. After 24 h, cells were treated with increasing concentrations of **UZH2** in DMSO or DMSO alone as a negative control (final concentration of DMSO 0.5% (v/v)) for indicated time points. For the long-term incubation studies and analysis of m^6A_m/A, m^1A/A, and m^7G/G ratios in total RNA, MOLM-13 cells were seeded at 1 × 10^6 cells/well in a six-well plate and treated with 10 μM of **UZH2** in DMSO or DMSO alone as a negative control (final concentration of DMSO 0.5% (v/v)) for 3 days or, after reseeding at 1 × 10^6 cells/well and adding fresh compound, 6 days in total. Following incubation in the cell culture incubator, cells were washed once with PBS, and total RNA was extracted using 0.5 mL of GENEzol reagent according to manufacturer's instructions. The final volume of 50 μL of total RNA eluate was subjected to two rounds of purification using 25 μL of Sera-Mag magnetic oligo(dT) particles per sample. The polyadenylated RNA was eluted with nuclease-free water in a final volume of 25 μL, and its concentration was determined using NanoDrop. One hundred nanograms of mRNA was digested to nucleosides and dephosphorylated in a one-pot reaction using 0.05 μL of the nucleoside digestion mix in 25 μL of the total reaction volume for 16 h at 37 °C for m^6A/A analysis. For the analysis of m^6A_m/A, m^1A/A, and m^7G/G ratios in mRNA or total RNA, 100 ng of RNA was first decapped using RNA 5′ pyrophosphohydrolase for 1 h according to manufacturer's instructions and then digested to nucleosides and dephosphorylated in a one-pot reaction using 0.05 μL of the nucleoside digestion mix in 25 μL of the total reaction volume for 16 h at 37 °C. The samples were used for UPLC−MS/MS analysis without further purification steps.

Nucleoside UPLC−MS/MS was performed at the Functional Genomics Centre Zurich following previously described procedures with slight modifications.[17a] Briefly, the samples were diluted 10- or 100-fold in solvent A (0.1% (v/v) formic acid in water), separated using a Waters Aquity M class (Waters) UPLC, and detected with a TSQ Quantiva (Thermo Scientific) mass spectrometer by using a nano electrospray ionization (ESI) source in SRM mode. Analytes were separated on an HSS T3 column (particle size 1.8 μm, dimensions 0.15 × 60 mm). The mobile phases used for elution were A 0.1% (v/v) formic acid in water and B 0.1% (v/v) formic acid in acetonitrile at a flow rate of 2 μL/min, column temperature was ambient, sample temperature was 8 °C, injection volume was set to 1 μL, and sample loop volume was 1 μL. The analytes were separated using isocratic elution for 6 min at 12.5% B. Adenosine, m^6A, m^1A, and m^6A_m were eluted at 3.30, 3.44, 2.89, and 3.56 min, respectively. Guanosine and m^7G were eluted at 3.34 and 3.27 min, respectively. The mass spectrometer was operated using the following parameters: ESI positive ion mode; SRM acquisition mode; capillary voltage 3 kV; source temperature 300 °C; and collision energy of 15 V for adenosine and guanosine, 20 V for m^7G, and 25 V for m^6A, m^1A, and m^6A_m. Adenosine, m^6A, m^1A, and m^6A_m were detected by monitoring precursor to product transitions of m/z = 268.1 → 136.1, m/z = 282.1 → 150.1, m/z = 282.1 → 150.1, and m/z = 296.1 → 152.1,

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respectively. Guanosine and m^7G were detected by monitoring precursor to product transitions of m/z = 284.1 → 136.1 and m/z = 298.1 → 166.1, respectively. Absolute concentrations of adenosine, guanosine, m^6A, m^1A, m^6A_m, and m^7G in the samples were determined based on an external calibration curve generated using adenosine (Sigma-Aldrich, 01890-5G), guanosine (Sigma-Aldrich, G6264-1G), m^6A (Chemie Brunschwig, CBLQB-1055-1 g), m^1A (MedChem Express, MCE-HY-113081), m^6A_m (Toronto Research Chemicals, D447415), and m^7G (Sigma-Aldrich, M0627-100MG) nucleoside standards. The modified-to-nonmodified nucleoside ratio of compound-treated samples was normalized to the corresponding value of DMSO-treated negative control. All measurements were performed in technical duplicates and repeated in at least three biological replicates on different days. Inhibition curves were plotted in GraphPad Prism 8.4 and fitted with nonlinear regression "log(inhibitor) vs. response−variable slope" with a maximum inhibition of 80%, from which IC_{50} values were determined.

METTL3 Immunoblotting. For immunodetection of the METTL3 expression level after the compound exposure, MOLM-13 cells were seeded in 2 mL of complete RPMI media at 1 × 10^6 cells/mL in a six-well plate. On the same day, cells were treated with 10 μM of **UZH2** or 0.5% (v/v) DMSO as a control. After 16 h of treatment, cells were collected by centrifugation, washed twice with PBS, and resuspended in 40 μL of HBSS containing 1× Protease Inhibitor Cocktail (Sigma). Cells were lysed by three freeze−thaw cycles. After incubating for 15 min on ice, cell lysates were centrifuged for 20 min at 16,000*g* at 4 °C to remove cell debris, and the supernatant containing proteins was collected. The protein concentration was quantified with a Pierce Coomassie (Bradford) protein assay kit, and 25 μg of the protein was loaded per well on a SurePAGE 12% Bis−Tris polyacrylamide gel. METTL3 and β-actin were detected as previously described.[17a]

Cell Viability Assay. MOLM-13 cells were seeded in white clear-bottom 96-well plates at a density of 2 × 10^4 cells/well in 100 μL of the complete RPMI medium. PC-3 cells were seeded in white clear-bottom 96-well plates at a density of 5 × 10^3 cells/well in 100 μL of the complete RPMI medium. After 24 h, cells were treated with 11 μL of increasing concentrations of the indicated compounds dissolved in DMSO (final concentration of compounds 2.5−160 μM and DMSO 0.5% (v/v)) or DMSO only as a negative control and incubated for 72 h at 37 °C with 5% CO_2. The assay was carried out with two technical replicates for each concentration and repeated three to four times on different days. Cell viability was determined using a CellTiter-Glo luminescent cell viability assay based on the detection of ATP according to manufacturer's instructions. Briefly, 100 μL of the CellTiter-Glo reagent was added to each well and incubated for 10 min at room temperature on an orbital shaker. The bottom of the plate was covered with a custom-made white plastic sheet, and luminescence was recorded using a Tecan Infinite 3046 M1000 microplate reader from the top. Background luminescence value was obtained from wells containing the CellTiter-Glo reagent and medium without cells. The cell viability was calculated according to the following formula, where $I_{compound}$ refers to the luminescence intensity of cells treated with different doses of the compounds, $I_{DMSO\ only}$ refers to the luminescence intensity of DMSO-treated cells, and $I_{background}$ refers to the luminescence intensity of medium:

$$\text{Cell viability}(\%) = \frac{I_{compound} - I_{background}}{I_{DMSO\ only} - I_{background}} \times 100\%$$

Cell viability curves were plotted in GraphPad Prism 8.4 and fitted with nonlinear regression "log(inhibitor) vs. normalized response−variable slope", from which IC_{50} values were determined.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00773.

Supplementary figures, crystallographic, biophysical and biological data, as well as ^1H and ^{13}C NMR of the target molecules and their HPLC purity traces (PDF)

Accession Codes

The PDB codes of METTL3 in complex with the following compounds are listed herein: **1**, 7NHI; **5**, 7O08; **7**, 7O09; **8**, 7O0L; **20**, 7O29; **21**, 7O2E; and **22** (**UZH2**), 7O2F. The authors will release the atomic coordinates and experimental data upon article publication.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CETSA, cellular thermal shift assay; CDI, 1,1′-carbonyldiimidazole; DAST, diethylaminosulfur trifluoride; DIPEA, *N*,*N*-diisopropylethylamine; HATU, 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; S_NAr, nucleophilic aromatic substitution; TR-FRET, time-resolved Förster resonance energy transfer; Troc, 2,2,2-trichloroethoxycarbonyl chloride; μw, microwave

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