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Structure-Based Discovery of 4-(6-Methoxy-2-methyl-4-(quinolin-4-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (CD161) as a Potent and Orally Bioavailable BET Bromodomain Inhibitor

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Abstract

We have designed and synthesized 9H-pyrimido[4,5-b]indole-containing compounds to obtain potent and orally bioavailable BET inhibitors. By incorporation of an indole or a quinoline moiety to the 9H-pyrimido[4,5-b]indole core, we identified a series of small molecules showing high binding affinities to BET proteins and low nanomolar potencies in inhibition of cell growth in acute leukemia cell lines. One such compound, 4-(6-methoxy-2-methyl-4-(quinolin-4-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (**31**) has excellent microsomal stability and good oral pharmacokinetics in rats and mice. Orally administered, **31** achieves significant antitumor activity in the MV4;11 leukemia and MDA-MB-231 triple-negative breast cancer xenograft models in mice. Determination of the co-crystal structure of **31** with BRD4 BD2

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Supporting Information

The Supporting Information is available: Crystallography Data Collection and Refinement Statistics (PDF) and Molecular formula strings (CSV).

Accession Code

Atomic coordinates have been deposited in the Protein Data Bank (PDB code 5UOO for **31**). Authors will release the atomic coordinates and experimental data upon article publication.

Conflict-of-Interest

The authors declare the following competing financial interest(s): Multiple patents have been filed by University of Michigan on this class of BET bromodomain inhibitors, which have been licensed by OncoFusion Therapeutics Inc. Shaomeng Wang, Yujun Zhao, Xu Ran, Liu Liu, Longchuan Bai, Chao-Yie Yang, Bo Wen, Ting Zhao, Duxin Sun, Donna McEachern, and Xiaoqin Li, are inventors of the BET Bromodomain inhibitors reported in this manuscript and receive royalties from University of Michigan. Shaomeng Wang also owns stock in and serves as a consultant for OncoFusion Therapeutics Inc. The University of Michigan and Shaomeng Wang have also received a research contract from OncoFusion Therapeutics Inc.

provides a structural basis for its high binding affinity to BET proteins. Testing its binding affinities against other bromodomain-containing proteins shows that **31** is a highly selective inhibitor of BET proteins. Our data show that **31** is a potent, selective and orally active BET inhibitor.

Graphical Abstract

31, A potent, selective, orally bioavailable BET inhibitor

INTRODUCTION

Acetylation of lysine residues at the histone tail plays a central role in the dynamic regulation of chromatin-based gene transcription. Bromodomain-containing proteins (BRDs) function as one of epigenetic “readers” by binding to acetyl lysine residues in the histone tail and regulate gene expression.^{1,2} Bromodomain and extra-terminal (BET) proteins are one sub-family of BRDs which has four members, BRD2, BRD3, BRD4, and testis-specific BRDT. BET proteins have been implicated in a variety of human diseases, including NUT midline carcinoma,³ MLL-fusion leukemia,⁴ inflammatory diseases,⁵ and acute heart failure.⁶

Structurally, BET proteins consist of two tandem bromodomains (BD1 and BD2) both of which bind to acetyl-lysine (Ac-K) residues in the histone tails. Recently, small molecule BET inhibitors, such as JQ-1 (**1**, Figure 1),³ have been discovered that bind to the acetyl-lysine binding pocket in the BET proteins.^{3,5,7–17,18,19} Potent and selective BET inhibitors are powerful tools with which to investigate the role of BET proteins in different biological and pathological processes.^{3–6,18,20–27} In preclinical studies, BET inhibitors have demonstrated their therapeutic potential in the treatment of human cancers, inflammation, HIV infection, acute heart failure, and other disorders. Several BET inhibitors, including OTX-015 (**2**),¹⁸ CPI-0610 (**3**)²⁸ I-BET762 (**4**) (Figure 1) are currently being evaluated in human clinical trials for the treatment of different cancers. Recently obtained clinical data have provided an important proof of the concept that BET small-molecule inhibitors may have a therapeutic potential for the treatment of several forms of human cancer.^{29–32}

Our laboratory previously reported the design of a new class of BET bromodomain inhibitors containing a tricyclic 5*H*-pyrido[4,3-*b*]indole structure, exemplified by RX-37 (**6**).³³ This compound binds to BET proteins with nanomolar affinities and demonstrates high selectivity over other bromodomain-containing proteins.³³ It potently inhibits cell growth in the MV4;11 and MOLM-13 acute leukemia cell lines harboring mixed lineage leukemia 1 (MLL1) fusion protein and has a high specificity for the K562 leukemia cell line harboring BCR-ABL fusion protein.³³ Hence, **6** represents a promising lead compound for further optimization toward our goal of development of a new class of potent, selective, efficacious BET inhibitors for the treatment of human cancers and other medical conditions.

Our previously reported synthetic routes to **6** are not suited to large scale synthesis, and this has limited the exploration of its structure-activity relationships (SAR) and potential *in vivo* evaluation of this class of compounds. We have therefore decided to develop alternative tricyclic core structures, which allowed us to explore the SAR more extensively and comprehensively. In the present study, we report our efforts to obtain potent, efficacious, and orally active BET inhibitors. This has resulted in the discovery of compound **31**, which binds to BET proteins with high affinities and potently inhibits cell growth in acute leukemia cell lines harboring the MLL1 fusion protein and in a panel of human breast cancer cell lines. Significantly, compound **31** has an excellent oral pharmacokinetic profile and orally administered, effectively inhibits tumor growth in mice.

RESULTS AND DISCUSSION

Analysis of the co-crystal structure of **6** in a complex with BRD4 BD2 protein shows that one additional nitrogen atom can be inserted into the 5*H*-pyrido[4,3-*b*]indole structure (Table 1) without causing any unfavorable interactions with the protein. This led to the design of compounds containing the 9*H*-pyrimido[4,5-*b*]indole core structure, which can be more efficiently synthesized than the core structure of **6** as shown in Schemes 1 and 2.

We first synthesized compound **7** to test this new core for the design of potent BET inhibitors. This compound binds to recombinant human BRD4 BD1 and BD2 proteins with $K_i = 387$ nM and 476 nM, respectively (Table 1), in our competitive fluorescence-polarization assays³³. Since this compound still lacks a moiety to interact with the well-defined “WPF” hydrophobic pocket in the BRD4 protein, it therefore represents a reasonable starting point for further optimization.

Our previous SAR data of compound **6** and its analogues demonstrated that targeting the “WPF” hydrophobic pocket in BET proteins is a very effective way of improving the binding affinities of BET inhibitors.³³ Overlay of our modeled structure of **7** in a complex with BRD4 BD2 and the co-crystal structure⁴ of compound **5** (I-BET151) complexed with BRD4 BD2 led to the design of **8** (Table 1), which contains the same hydrophobic group as was used in **5** to target the “WPF” hydrophobic pocket. Compound **8** binds to BRD4 BD1 and BD2 with $K_i = 12.0$ nM and 10.4 nM (Table 1) and is thus >30-times more potent than **7**, further supporting the precept that targeting the WPF binding pocket can indeed significantly improve the binding affinity of BET inhibitors. In comparison, compound **8** is as potent as **1** and **6** in binding to both BDR4 BD1 and BD2 proteins.

In our previous study, we found that substituted 5-membered aromatic rings such as that in compound **6** can be used to effectively target the “WPF” pocket in BET proteins.^{7,10–17,28,33} Accordingly, we next further explored the SAR resulting from targeting the “WPF” pocket using different 5-membered aromatic rings (Table 2). Among compounds **9–13**, which contain an unsubstituted 5-membered ring, compounds **11** and **12** containing a furan and a pyrrole respectively, have the highest binding affinities and are both >5-times more potent than **7** in binding to both BRD4 BD1 and BD2 proteins.

In the co-crystal structure of compound **6** complexed with BRD4 BD2, the cyclopropyl group is inserted deeply into the WPF hydrophobic pocket,³³ suggesting that additional hydrophobic substituent(s) on the 5-membered aromatic ring may further improve binding affinity to the BRD4 protein. Indeed, **14** and **15** with dimethyl substituents on the 3-isoxazole and 3-(1*H*-pyrazole) groups are both more potent than the unsubstituted compounds **9** and **10**. Compound **14** is 9–10 times more potent than **9**, and **15** is 3–7 times more potent than **10** in binding to both the BD1 and BD2 proteins from BRD4. In particular, **15** has K_i values of 19.7 and 20.2 nM to the BD1 and BD2 proteins, and is therefore a fairly potent BRD4 inhibitor.

Encouraged by the good binding affinities of **15** to BRD4 BD1 and BD2 proteins, we further modified **15** to obtain **16** and **17**. Compound **16** with an additional methyl substituent on the 5-membered 1*H*-pyrazole ring of **15** has similar binding affinities to BRD4 BD1 and BD2 proteins, as compared to **15**, but **17**, which targets the WPF pocket with the same pyrazole group as compound **6**, binds to BRD4 BD1 and BD2 with K_i values of 7.0 nM and 2.7 nM, respectively and is thus more potent than compound **6**. It is surprising to observe such potency gap between **6** and **17** given these two molecules differ by only one atom, i.e. a carbon to a nitrogen atom.

BET inhibitors such as compound **1** and **6** have been shown to be very effective in inhibition of cell growth in MV4;11 and MOLM-13 acute leukemia cell lines harboring MLL1 fusions.^{3,33} Indeed, **17** potently inhibits cell growth in both the MV4;11 and MOLM-13 cell lines with IC₅₀ values of 6 and 36 nM, respectively (Table 3). In direct comparison, **17** is more potent than **1**, **5** and **6** in inhibition of cell growth in these two acute leukemia cell lines, and is a very potent BET inhibitor.

We evaluated compound **17** in a quick pharmacokinetic study with three time points to assess its oral exposure in mice. Compound **17** achieves drug concentrations of 2200 ± 331 , 872 ± 351 and 72 ± 46 ng/ml, respectively, at 1, 3 and 6 h time-points with 25 mg/kg oral administration, indicating that **17** has a reasonable oral exposure in mice.

We next turned our attention to modifications of **12**, which contains a pyrrole ring. Compound **12** has reasonable binding affinities to both BRD4 BD1 and BD2 with K_i values of 30.6 nM and 51.9 nM, respectively and sub-micromolar potencies in cell growth inhibition in the MV4;11 and MOLM-13 cell lines (Table 3). Our computational modeling suggested that a fused [5,6] bicyclic system can effectively target the WPF pocket and we therefore synthesized a series of compounds containing a bicyclic [5,6] ring system and whose activities are summarized in Table 4.

Compound **18**, containing an indole group, binds to BRD4 BD1 and BD2 with K_i values of 4.9 nM and 1.1 nM, respectively and is therefore 10-times more potent than **12** in binding to both the BD1 and BD2 domains of BRD4. Replacement of the indole in **18** with 1*H*-pyrrolo[2,3-*c*]pyridine resulted in **19**, which has a binding affinity to BRD4 BD1 similar to that of **18** but is >5-times less potent than **18** in binding to BRD4 BD2. Installation of a fluorine atom at C4 of the indole in **18** yielded **20**, which binds to BRD4 BD1 and BD2 proteins with very high affinities (K_i = 2.6 and 1 nM, respectively). Addition of a methyl group at C2 of **18** led to compound **21**, which also achieves a very high binding affinity to both BRD4 BD1 and BD2 (K_i < 1 nM). Combination of the fluorine and methyl substitutions in compounds **20** and **21** yielded **22**, which has a high binding affinity to both BRD4 BD1 and BD2 proteins (K_i < 1 nM). Replacing C6 of the indole group in **21** by a nitrogen atom led to **23**, which is 10-times less potent than **21**. The weaker binding affinity of **23** than **21** is consistent with the hydrophobic nature of the WPF binding pocket.

Encouraged with the high binding affinities of these indole-containing compounds, we synthesized a series of compounds containing [5,6] bicyclic, non-indole groups. Compound **24**, with a 2-methylbenzofuran and **25** containing a benzoimidazole bind to BRD4 BD1 and BD2 with high affinities but are less potent than **18**. Compound **26**, containing a pyrazolo[1,5-*a*]pyridine is 4-times less potent than **18** and compound **27**, containing a 1-methyl-1*H*-indazole is >10-times less potent than **18**. Compound **28**, with a 1*H*-benzo[*d*] [1,2,3]triazole fails to show any appreciable binding to BRD4 BD1 and BD2 proteins at 1 μ M. These data thus show that not only the shape but also the electronic properties of the bicyclic aromatic rings at this site play an important role in the binding to BRD4 BD1 and BD2 proteins.

We evaluated these compounds for their ability to inhibit cell growth in the MV4;11 and MOLM-13 cell lines, and the results are summarized in Table 3. Consistent with their very high binding affinities to BRD4 BD1 and BD2 proteins, compounds **21** and **22** display very potent cell growth inhibitory activity in both the MV4;11 and MOLM-13 cell lines. Compound **21** has IC_{50} values of 6.6 nM and 65 nM, respectively, in the MV4;11 and MOLM-13 cell lines, while **22** has IC_{50} values of 5.1 nM and 51 nM, respectively in these two leukemia cell lines. Compounds with weaker binding affinities to BRD4 BD1 and BD2 proteins also display weaker cell growth inhibitory activity in both the MV4;11 and MOLM-13 cell lines.

Based upon its high binding affinities to BRD4 BD1 and BD2 proteins and potent cell growth inhibitory activity, we evaluated **21** for its oral bioavailability in rats. The pharmacokinetic (PK) data show that **21**, orally administered to rats at 25 mg/kg can achieve a reasonable plasma exposure and achieves a C_{max} of 1470 ng/ml, or approximately 3 μ M (Table 5).

A shortcoming we discovered in **21** is that although it has a good microsomal stability in rat microsomes ($T_{1/2}$ > 60 min), it has a poor microsomal stability in mouse and human microsomes with $T_{1/2}$ of 4 and 8 minutes, respectively (Table 6). Our investigation of **21** in human liver microsomes showed that oxidation of the indole group is the major metabolic

weak spot and consequently, we next focused our modifications on the indole group in **21** with the objective of improving its metabolic stability and oral pharmacokinetics.

First, we introduced a methyl group onto the tricyclic core structure in **21**, generating compound **29** (Table 7) which binds to BRD4 BD1 and BD2 proteins with very high affinities ($K_i < 1$ nM). Consistent with its high binding affinity, **29** potently inhibits cell growth in the MV4;11 and MOML-13 cell lines achieving IC_{50} values of 2 and 20 nM, respectively (Table 3), and is therefore 3-times more potent than **21**. Compound **29** has an excellent microsomal stability in mouse and rat microsomes ($T_{1/2} > 60$ min) but has a poor microsomal stability in human microsomes ($T_{1/2} = 9$ min) (Table 6).

We next explored if replacement of the [5,6] bicyclic indole ring system with [6,6] bicyclic ring systems could lead to potent BET inhibitors with improved metabolic stability. Another issue we have found for compound **21** was its poor aqueous solubility. We have synthesized a series of compounds containing a quinoline ring system, which has a basic nitrogen in the bicyclic aromatic system and should have an improved solubility over compound **21** containing a less basic indole aromatic group. The test data from these compounds are summarized in Table 7.

Compound **31** (CD161) containing a quinolin-4-yl ring, binds to BRD4 BD1 and BD2 with K_i values of 8.2 nM and 1.4 nM, respectively (Table 7) and potently inhibits cell growth in the MV4;11 and MOLM-13 cell lines with IC_{50} values of 26 and 53 nM, respectively (Table 3). Significantly, **31** has excellent microsomal stability in mouse, rat and human microsomes (Table 6). These data show that replacing the [5,6] bicyclic indole ring system with a [6,6] bicyclic quinoline ring system can maintain high binding affinities to BRD4 BD1 and BD2, and at the same time achieve potent cell growth inhibitory activity and excellent microsomal stability.

Based upon these encouraging results for **31**, we synthesized a series of compounds containing quinoline ring systems in which the nitrogen in the quinoline ring is located in different positions. Compound **34** containing a quinolin-3-yl system is slightly less potent than **31** in both binding affinities to BRD4 BD1 and BD2 and also in inhibition of cell growth in the MV4;11 and MOLM-13 cell lines. Similarly, **32** with a quinolin-5-yl system is slightly less potent in its binding to BRD4 BD1 and BD2 proteins than **31** and is 2-times less potent than **31** in inhibition of cell growth in MV4;11 and MOLM-13 cell lines (Table 3). Compound **35**, **36** and **37** containing a nitrogen in the 5, 6 and 7-positions respectively, are much less potent than **31** in both their binding affinities to BRD4 BD1 and BD2 proteins, as well as in inhibition of cell growth. Hence, among these compounds containing a quinolinyl ring system, **31** has the best binding affinities and cellular potencies.

We evaluated **31** for its pharmacokinetics in rats with the data provided in Table 5. At 25 mg/kg, orally administered, compound **31** achieves a C_{max} of 7333 ng/mL, 6-times higher than that for **21** and an oral bioavailability of 93%. Compound **31** was also found to have much better solubility than **21**; while **31** was readily soluble in the dosing vehicle (5% DMSO+10% Cremophor+85% saline), **21** was difficult to dissolve in the vehicle. One possible reason for the superior oral pharmacokinetics of **31** over **21** may be due to the

improved solubility for **31**. We further determined the pharmacokinetics of **31** in mice (Table 5). Compound **31** achieves a C_{max} of 983.1 ng/mL with 25 mg/kg, oral administration and an oral bioavailability of 50% (Table 5). Hence, **31** has excellent oral pharmacokinetics in rats and moderate oral pharmacokinetics in mice.

To gain an understanding of its target selectivity, we evaluated compound **31** for its binding affinities to BET family proteins (BRD2–4 and BRDT) and other sub-families of bromodomain-containing proteins in the BROMOScan platform by DiscoverX³⁴ and the data are summarized in Table 8.

Compound **31** has K_d values of 4.5, 0.9, 2, 0.69, 8.4, 2, 15 and 5.5 nM, to BRD2 BD1, BRD2 BD2, BRD3 BD1, BRD3 BD2, BRD4 BD1, BRD4 BD2, BRDT BD1 and BRDT BD2 proteins, respectively. It binds to CREBBP, EP300 and CECR2 with K_d values of 520, 550 and 1600 nM, respectively, and has K_d values > 4,000 nM against other 21 bromodomain-containing proteins tested. Hence, **31** is a highly potent BET inhibitor and demonstrates a high selectivity over the other bromodomain-containing proteins.

Compound **31** was further tested for its potential inhibitory activity against 372 human kinases in the KINOMEScan platform by DiscoverX. The kinase screening data showed that **31** only has a modest inhibitory activity against PLK4 with an IC₅₀ value of 3.9 μM and has IC₅₀ values >50 μM against all other 371 kinases.

We next tested compound **31** for its *in vivo* efficacy in the MV4;11 xenograft model, with compound **5** used as a control (Figure 2). The efficacy data showed that both **5** and **31** have similar strong antitumor activity with a tumor growth inhibition of >80%, and both compounds did not cause weight loss or other signs of toxicity in mice (Figure 2).

In addition to acute leukemia cell lines, we evaluated the cell growth inhibitory activity of **31** in a panel of 19 human breast cancer cell lines. The resulting data, provided in Table 9 show that **31** potently inhibits cell growth in 9 breast cancer cell lines with IC₅₀ values < 1 μM, displays IC₅₀ values between 1–2 μM in 6 other cell lines and has IC₅₀ values > 2 μM in the remaining 4 cell lines. Therefore, while **31** is less potent against breast cancer cell lines than against acute leukemia MV4;11 and MOLM-13 cell lines carrying MLL1 fusion, it nevertheless potently inhibits cell growth against nearly 50% of 19 breast cancer cell lines tested with IC₅₀ values of 1 μM or better.

To further evaluate its therapeutic potential, we evaluated **31** for its antitumor activity in the MDA-MB-231 xenograft model in mice (Figure 3). The data show that **31** is very effective in inhibition of tumor growth in the MDA-MB-231 xenograft model. During the treatment period, **31** at 40 mg/kg dosing daily achieved essentially complete tumor growth inhibition. Mice treated with **31** did not experience weight loss or other signs of toxicity throughout the entire experiment.

Previous studies have shown that BET inhibitors can effectively down-regulate c-Myc and upregulate p21 in tumor cells.⁴ Accordingly, we examined the effect of **31** in the MV4;11 cells on c-Myc and p21 levels (Figure 4). The resulting data showed that **31** is very effective in inducing rapid down-regulation of c-Myc at as early as the 1 h time-point and in a dose-

dependent manner (Figure 4A). Furthermore, compound **31** is effective in inducing upregulation of the cell cycle regulator p21 and cleavage of PARP, a biochemical marker of apoptosis, in a dose-dependent manner (Figure 4B). Hence, our western blotting data further showed that compound **31** functions as a potent BET inhibitor in cells.

To understand the structural basis for the high binding affinity of **31** to BRD4, we successfully determined the co-crystal structure of **31** complexed with BRD4 BD2 (Figure 5). Overall, the co-crystal structure shows **31** binding to BRD4 BD2 with a binding mode similar to that assumed by compound **6**. The dimethylisoxazole group of **31** penetrates deeply into the binding pocket and forms an extensive hydrogen bonding network with Asn433 and two conserved water molecules. One of the methyl groups in the dimethylisoxazole group binds to a hydrophobic pocket formed by residues Tyr332, Val380, Leu387 and Tyr390, and the other methyl group has hydrophobic contacts with Pro375, Phe376 and Val439. The tricyclic ring binds to the lysine channel. As envisaged in our design, the quinolinyl group inserts into the “WPF” pocket with the nitrogen atom exposed to solvent and the fused phenyl group in the hydrophobic pocket formed by Trp374, Pro375, Val439, Met442 and the hydrophobic portion of Glu438. The quinolinyl group of **31** has more extensive interactions than the cyclopropyl group of **6**, consistent with the higher binding potency of **31** to BRD4. This co-crystal structure provides a solid structural basis for the high binding affinity of **31** to BRD4 BD2 and for further structure-based optimization.

CHEMISTRY

The synthesis of 9*H*-pyrimido[4,5-*b*]indole-containing intermediate **7** is shown in Scheme 1.

Reaction conditions: a. Pd(PPh₃)₄, DME-H₂O, reflux overnight, >80% yield; b. NaH, anhydrous DMF, overnight, 53% yield; c. Zn, HOAc, 85 °C, 3 h, 47% yield; d. Ammonium formate 2.0 equiv, formamide, 175 °C, 16 h, 55% yield; e. POCl₃, 90 °C, 5 h, 66% yield

Suzuki coupling of a previously reported substituted bromobenzene (**S1**) and its regioisomer 1-bromo-2-fluoro-4-methoxy-5-nitrobenzene (ratio 2:1)³⁵ and **S2** furnished **S3** as a mixture of two isomers in >80% yield. S_NAr substitution of the fluorine of **S3** by the cyanoacetate carbanion afforded **S5**, which was obtained as a single isomer after flash column chromatography purification. **S5** was reduced, giving **S6** by treatment with Zn-HOAc at 85 °C.³⁶ Condensation of a mixture of **S6** and ammonium formate in formamide at 175 °C yielded **S7** in 55% yield. Finally, chlorination of **S7** provided the final key intermediate (**7**) in 66% yield.

The key 9*H*-pyrimido[4,5-*b*]indole-containing intermediate **S10** was synthesized from **S6** following the synthetic route depicted in Scheme 2.³⁶

Reaction conditions: a. **S6** in dry MeCN, bubble HCl gas for 30 min, then reflux 2.5 h; b. **S9** in EtOH, 10% NaOH aq., reflux 6 h, 78% over two steps; c. POCl₃, 90 °C, 5 h, 75% yield

To a dry solution of **S6** in MeCN, excess hydrogen chloride gas was bubbled and the mixture was heated at reflux for 2.5 h. Removal of the volatile components furnished the crude

intermediate **S8**, which cyclized into **S9** upon reflux in basic aqueous EtOH. Chlorination of **S9** provided the key intermediate **S10** in 75% yield.

Compound **8** in Table 1 was synthesized following the synthetic route depicted in Scheme 3.

Reaction conditions: a. 1-methylpyrrolidin-2-one (NMP), 140 °C, overnight.

An anhydrous solution in NMP of **7** and the corresponding amine was heated at 140 °C in a sealed tube overnight. The final products (**8**) were purified by reverse phase HPLC and obtained in the form of the trifluoroacetic acid salt. Compounds **13** and **25** were synthesized in a similar manner.

Compounds **9–12**, **14–24**, **26**, **27**, **29–40** in Tables 2–4 and 7 were synthesized from key intermediates **7** or **S10** as shown in Scheme 4.

Reaction conditions: a. Pd(PPh₃)₄, DME-H₂O, K₂CO₃ 5.0 equiv., reflux overnight; or Pd(dppf)₂Cl₂-CH₂Cl₂, DME-Na₂CO₃ 2 M, reflux overnight.

Most boronic acids or pinacolboronic esters are commercially available. Others were synthesized according to known methods reported previously.^{37–39} Suzuki coupling^{37,40,41} of the corresponding boronic acids or pinacolboronic esters and **7** or **S10** followed by reverse phase HPLC purification furnished pure final products as salts of trifluoroacetic acid.

Compound **28** was synthesized from **7** and 1H-benzo[d][1,2,3]triazole following a literature reported method with modifications.⁴²

CONCLUSIONS

In this study, we have designed and synthesized a class of 9*H*-pyrimido[4,5-*b*]indole-containing compounds with the goal of obtaining potent and orally bioavailable BET bromodomain inhibitors. These efforts have led to the discovery of 4-(6-Methoxy-2-methyl-4-(quinolin-4-yl)-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (**31**), which binds to BET family proteins with low nanomolar affinities and demonstrates high selectivity over non-BET bromodomain-containing proteins. Compound **31** potently inhibits cell growth in acute leukemia cell lines and breast cancer cell lines. Significantly, it achieves excellent oral bioavailability and is metabolically stable. It also displays potent antitumor activity in MV4;11 acute leukemia and MDA-MB-231 breast cancer xenograft models in mice at well-tolerated dose-schedules. Compound **31** is a potent, selective, orally bioavailable and efficacious BET inhibitor.

EXPERIMENTAL SECTION

Chemistry

General Methods: All reactions were conducted in a round-bottom flask equipped with a Teflon-coated magnet stirring bar. Experiments involving components sensitive to moisture or air were performed under a nitrogen atmosphere. Commercially available reagents and anhydrous solvents were used without further purification. The crude reaction product was

purified by flash column chromatography packed with silica gel. Further purification was performed on a preparative HPLC (Waters 2545) with a C18 reverse phase column. The mobile phase was a gradient flow of solvent A (water, 0.1% of TFA) and solvent B (CH₃CN, 0.1% of TFA) at a flow rate of 30 mL/min. Proton nuclear magnetic resonance (¹H NMR) and carbon nuclear magnetic resonance (¹³C NMR) spectroscopy were performed in Bruker Advance 300 NMR spectrometers. Low resolution ESI mass spectrum analysis was performed on a Thermo-Scientific LCQ Fleet mass spectrometer. The analytical UPLC model was Waters ACQUITY H class (UV detection at 230 nm and 254 nm wave lengths) and the reverse phase column used was the ACQUITY UPLC® BEH (C18–1.7 μm, 2.1 × 50 mm). All final compounds were purified to ≥ 95% purity as determined by analytical UPLC analysis.

1. Procedures for the synthesis of 9*H*-pyrimido[4,5-*b*]indole-containing cores 7 and S10

4-(4-Fluoro-2-methoxy-5-nitrophenyl)-3,5-dimethylisoxazole (S3)—1-Bromo-4-fluoro-2-methoxy-5-nitrobenzene³⁵ and 1-bromo-2-fluoro-4-methoxy-5-nitrobenzene (3 g, 12 mmol, 2:1 ratio), 3,5-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoxazole (5.35 g, 24 mmol), and K₂CO₃ (5.0 g, 36 mmol) were added to a round-bottom flask. DME (50 mL) and water (30 mL) were added at room temperature. The solution was degassed before addition of Pd(PPh₃)₄ (700 mg, 0.6 mmol) in one portion. The solution was degassed and was then heated at reflux for 16 h. The aqueous layer was extracted with EtOAc and combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. The volatile components were removed on a rotary evaporator and the residue was purified by flash column chromatogram. The desired product (**S3**) and its regioisomer were isolated in > 80% yield (3.38 g). **S3**: ¹H NMR (CDCl₃, 300 MHz): 8.03 (d, *J* = 8.47 Hz, 1H), 6.93 (d, *J* = 12.56 Hz, 1H), 4.00 (s, 3H), 2.40 (s, 3H), 2.24 (s, 3H).

Ethyl 2-cyano-2-(4-(3,5-dimethylisoxazol-4-yl)-5-methoxy-2-nitrophenyl)acetate (S5).—NaH (4.32 g, 60% in mineral oil, 100 mmol, 2.0 equiv) was placed in round-bottom flask and dry DMF (200 mL) was added. Ethyl cyanoacetate (7.35 g, 65 mmol, 1.2 equiv) was added dropwise at 0 °C *via* a syringe. The solution was stirred at room temperature for 30 min. The mixture was cooled to 0 °C, anhydrous DMF solution (30 mL) of **S3** and its regioisomer (14.26 g, 54 mmol, 1.0 equiv) was added *via* a syringe. The reaction mixture was allowed to warm up to room temperature and was then stirred for 16 h. The reaction was quenched by 0.5 N HCl, and the aqueous layer was extracted with EtOAc, the combined organic layers were washed with brine, then dried over anhydrous Na₂SO₄. The volatile components were removed on a rotary evaporator and the residue was purified by flash column chromatogram. Pure **S5** was isolated in 53% yield (6.86 g, 19.1 mmol, based on 66% correct isomer). ¹H NMR (CDCl₃, 300 MHz): 8.10 (s, 1H), 7.27 (s, 1H), 5.78 (s, 1H), 4.35 (q, *J* = 7.12 Hz, 2H), 3.99 (s, 3H), 2.33 (s, 3H), 2.18 (s, 3H), 1.37 (t, *J* = 7.14 Hz, 3H). ¹³C NMR (CDCl₃, 75 MHz): 167.2, 163.6, 161.6, 159.2, 140.0, 129.7, 128.0, 121.6, 114.7, 112.8, 110.7, 64.0, 56.7, 42.0, 14.0, 11.7, 10.8. ESI-MS calculated for C₁₇H₁₈N₃O₆ [M+H]⁺ = 360.12, Observed: 360.58

Ethyl 2-amino-6-(3,5-dimethylisoxazol-4-yl)-5-methoxy-1*H*-indole-3-carboxylate (S6).—To a solution of **S5** (6.86 g, 19.1 mmol, 1.0 equiv) in AcOH (50 mL) at 85 °C, Zn

powder (2.6 g, 40 mmol, 2.0 equiv) were added in small portions. The mixture was stirred at 85 °C for 1 h, a further 2.6 g Zn powder (40 mmol, 2.0 equiv) was added, and the reaction was kept at the same temperature for 2 h. The reaction was cooled, filtered, and washed with AcOH. The AcOH solution was combined and the volatile components were removed on a rotary evaporator. Purification by flash column chromatogram furnished the desired product **S6** (2.62 g, ca. 47% yield based on recovering 13% starting material). ¹H NMR (CDCl₃, 300 MHz): 8.01 (br, s, 1H), 7.44 (s, 1H), 6.78 (s, 1H), 5.73 (br, s, 2H), 4.40 (q, *J* = 7.08 Hz, 2H), 3.82 (s, 3H), 2.29 (s, 3H), 2.15 (s, 3H), 1.45 (t, *J* = 7.08 Hz, 3H). ¹³C NMR (CDCl₃, 75 MHz): 167.2, 165.8, 160.6, 153.7, 153.1, 128.1, 126.7, 114.5, 112.0, 110.6, 101.8, 86.0, 55.6, 53.5, 14.7, 11.5, 10.6. ESI-MS calculated for C₁₇H₂₀N₃O₄ [M+H]⁺ = 330.15, Obtained: 330.25

7-(3,5-Dimethylisoxazol-4-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-4-ol (S7).—**S6** (0.45 g, 1.4 mmol), ammonium formate (1.06 g, 17 mmol), and formamide (16 mL) were heated at 175 °C for 16 h. The reaction mixture was cooled to room temperature and water was added. Filtration of the mixture yielded **S7** as a brown solid (0.24 g, 0.77 mmol, 55% yield). ¹H NMR (DMSO-d₆, 300 MHz): 8.09 (s, 1H), 7.57 (s, 1H), 7.24 (s, 1H), 3.81 (s, 3H), 3.30 (s, 1H), 2.62 (s, 3H), 2.06 (s, 3H), ¹³C NMR (DMSO, 75 MHz): 165.3, 162.9, 159.2, 158.3, 153.9, 152.5, 129.6, 122.7, 115.6, 114.1, 113.6, 102.0, 100.2, 55.5, 11.2, 10.3. ESI-MS calculated for C₁₆H₁₅N₄O₃ [M+H]⁺: 311.11, Obtained: 311.75

4-(4-Chloro-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (7, CD55)—**S7** (0.95 g, 3.05mmol) was dissolved in POCl₃ (15 mL) and the mixture was heated at 90 °C for 5 h. The mixture was cooled to room temperature and the volatile components were removed on a rotary evaporator. The POCl₃ solution was poured into EtOAc (40 mL), followed by saturated NaHCO₃ (60 mL) and water (100 mL). The mixture was quickly filtered and the desired product (**7**) was collected as a brown solid (0.3 g). The aqueous layer was extracted with EtOAc and the combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. The volatile components were removed on a rotary evaporator affording a brown solid (0.36 g). The total yield of **7** is 66% (0.66 g). ¹H NMR (DMSO-d₆, 300 MHz): 8.74 (s, 1H), 7.84 (s, 1H), 7.45 (s, 1H), 3.89 (s, 3H), 3.31 (br, s, 1H), 2.29 (s, 3H), 2.09 (s, 3H) ¹³C NMR (DMSO-d₆, 75MHz): 167.8, 161.2, 155.8, 122.2, 120.3, 117.0, 115.2, 113.1, 105.8, 57.8, 13.4, 12.4. ESI-MS calculated for C₁₆H₁₄³⁵ClN₄O₂ [M+H]⁺ = 329.08, Obtained: 329.67

7-(3,5-Dimethylisoxazol-4-yl)-6-methoxy-2-methyl-9H-pyrimido[4,5-b]indol-4-ol (S9).—To a round-bottom flask, **S6** (0.37 g, 1.1 mmol) and MeCN (20 mL) were added at room temperature. Dry HCl was bubbled through MeCN for 30 min and the reaction mixture was heated at reflux for 2.5 h. The reaction was then cooled to room temperature and the volatile components were removed on a rotary evaporator. To this crude mixture, 10% NaOH aqueous solution (20 mL) and EtOH (50 mL) were added and the solution was heated at reflux for 6 h. The volatile components were then removed on a rotary evaporator and the aqueous residue was acidified with 2N HCl aqueous solution. The product **S9** was allowed to precipitate at 0 °C. Filtration of the mixture furnished **S9** in 0.278 g (78% yield, 2 steps). ¹H NMR (DMSO-d₆, 300 MHz): 7.57 (s, 1H), 7.20 (s, 1H), 3.81 (s, 3H), 2.37 (s, 3H), 2.27

(s, 3H), 2.08 (s, 3H). ^{13}C NMR (DMSO- d_6 , 75 MHz): 165.2, 160.4, 159.4, 157.7, 155.4, 152.2, 130.0, 123.2, 114.5, 114.0, 113.8, 101.9, 97.9, 55.6, 21.9, 11.3, 10.4.

4-(4-Chloro-6-methoxy-2-methyl-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (S10)—S9 (0.278 g, 0.8 mmol) and POCl_3 (8 mL) were added to a round-bottom flask. The mixture was heated at 90 °C for 6 h. The reaction mixture was cooled to room temperature and the volatile components were removed on a rotary evaporator. Water (20 mL) and EtOAc (20 mL) were added and the pH was adjusted to 8 using a large excess of NaHCO_3 saturated aqueous solution. Rapid filtration of the mixture furnished **S10** as a brown solid in 0.208 g (75% yield). ^1H NMR (DMSO- d_6 , 300 MHz): 7.81 (s, 1H), 7.43 (s, 1H), 3.89 (s, 3H), 2.69 (s, 3H), 2.31 (s, 3H), 2.11 (s, 3H). ^{13}C NMR (DMSO- d_6 , 75 MHz): 165.8, 163.6, 159.2, 156.9, 152.5, 151.1, 132.5, 119.5, 118.3, 114.7, 113.2, 108.5, 103.6, 55.8, 25.5, 11.3, 10.4. ESI-MS calculated for $\text{C}_{17}\text{H}_{16}\text{ClN}_4\text{O}_2$ $[\text{M}+\text{H}]^+ = 343.10$, Obtained: 343.58.

2. Synthesis of Final Products

7-(3,5-Dimethylisoxazol-4-yl)-6-methoxy-N-((R)-1-(pyridin-2-yl)ethyl)-9H-pyrimido[4,5-b]indol-4-amine (8).—**7** (80 mg, 0.3 mmol), (*R*)-1-(pyridin-2-yl)ethanamine (122 mg, 1 mmol), and $\text{EtN}(\textit{i}\text{-Pr})_2$ (0.3 mL, 1.5 mmol) were added to a round-bottomed flask. NMP (3 mL) was added at room temperature. The solution was heated at 140 °C for 16 h. The reaction mixture was quenched by water (1 mL). The mixture was purified by reverse phase HPLC. The desired product **8** was isolated as a salt of $\text{CF}_3\text{CO}_2\text{H}$. (16 mg, 20%). ^1H NMR (MeOD- d_4 , 300 MHz): 8.81 (d, $J = 5.58$ Hz, 1H), 8.54 (s, 1H), 8.56–8.47 (m, 1H), 8.23–8.17 (m, 1H), 8.19 (s, 1H), 7.92 (t, $J = 6.39$ Hz, 1H), 7.50 (s, 1H), 6.00 (q, $J = 7.11$ Hz, 1H), 4.02 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H), 2.00 (d, $J = 7.11$ Hz, 3H). ESI-MS calculated for $\text{C}_{23}\text{H}_{23}\text{N}_6\text{O}_2$ $[\text{M}+\text{H}]^+ = 415.19$, Obtained: 415.92

4-(4-(Isoxazol-4-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (9)—Method I using **7** and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoxazole: 13% yield. ^1H NMR (300 MHz, MeOD- d_4): 9.73 (s, 1H), 9.22 (s, 1H), 9.07 (s, 1H), 7.57 (s, 1H), 7.55 (s, 1H), 3.87 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H). ESI-MS calculated for $\text{C}_{19}\text{H}_{16}\text{N}_5\text{O}_3$ $[\text{M}+\text{H}]^+ = 362.13$, Obtained: 362.58.

4-(6-Methoxy-4-(1H-pyrazol-4-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (10),—Method I using **7** and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole: 41% yield. ^1H NMR (300 MHz, MeOD- d_4): 9.09 (s, 1H), 8.61 (s, 2H), 7.79 (s, 1H), 7.60 (s, 1H), 3.87 (s, 3H), 2.33 (s, 3H), 2.15 (s, 3H). ESI-MS calculated for $\text{C}_{19}\text{H}_{17}\text{N}_6\text{O}_2$ $[\text{M}+\text{H}]^+ = 361.14$, Obtained: 361.50.

Method A: Synthesis of **4-(4-(Furan-3-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (11)**, using Suzuki coupling catalyzed by $\text{Pd}(\text{PPh}_3)_4$: **7** (33 mg, 0.1 mmol, 1.0 equiv), 2-(furan-3-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (60 mg, 0.3 mmol, 3.0 equiv), and K_2CO_3 (56 mg, 0.4 mmol, 4.0 equiv) were added to a round-bottom flask. DME (6 mL) and H_2O (4 mL) were added at room temperature. The solution was degassed before $\text{Pd}(\text{PPh}_3)_4$ (10–15 mg, 0.008–0.012 mmol, ca. 0.1 equiv.) was added in one portion.

The solution was degassed again and then was heated at reflux for 16 h. The aqueous layer was extracted with EtOAc and combined organic layers were washed with brine and dried over anhydrous Na₂SO₄. The volatile components were removed on a rotary evaporator and the residue was treated with TFA (0.5 mL) and then purified by reverse phase HPLC. **11** was isolated as a colorless solid in the form of TFA salt (14 mg, 39% yield). ¹H NMR (MeOD-d₄, 300 MHz): 9.10 (s, 1H), 8.61 (dd, *J* = 1.52, 0.88 Hz, 1H), 8.04 (t, *J* = 1.52 Hz, 1H), 7.78 (s, 1H), 7.59 (s, 1H), 7.28 (dd, *J* = 1.91, 0.88 Hz, 1H), 3.86 (s, 3H), 2.33 (s, 3H), 2.16 (s, 3H). ESI-MS calculated for C₂₀H₁₇N₄O₃ [M+H]⁺ = 361.13, Obtained: 361.33

4-(6-Methoxy-4-(1*H*-pyrrol-3-yl)-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (12).—Method A using *N*-TIPS pyrrole-3-boronic acid pinacol ester: 21% yield. ¹H NMR (MeOD-d₄, 300 MHz): 8.95 (s, 1H), 8.08 (s, 1H), 7.93 (t, *J* = 1.65 Hz, 1H), 7.57 (s, 1H), 7.23 (dd, *J* = 2.91, 1.81 Hz, 1H), 7.03 (dd, *J* = 2.91, 1.62 Hz, 1H), 3.87 (s, 3H), 2.34 (s, 3H), 2.17 (s, 3H). ¹³C NMR (MeOD-d₄, 75MHz): 168.2, 161.2, 157.6, 155.7, 150.4, 148.4, 135.4, 124.6, 123.5, 123.0, 122.0, 117.0, 115.1, 114.7, 110.6, 110.1, 105.4, 56.5, 11.7, 10.8. ESI-MS calculated for C₂₀H₁₈N₅O₂ [M+H]⁺ = 360.15, Obtained: 360.25

4-(4-(1*H*-imidazol-1-yl)-6-methoxy-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (13)—7 (26 mg, 0.08 mmol), imidazole (60 mg, 0.88 mmol), and anhydrous NMP (10 mL) were heated at 170 °C in sealed tube for 24 h. HPLC purification of the reaction mixture provided the title compound in 27% yield (10 mg). ¹H NMR (MeOD-d₄, 300 MHz): 9.73 (s, 1H), 8.95 (s, 1H), 8.46 (s, 1H), 7.93 (s, 1H), 7.53 (s, 1H), 7.47 (s, 1H), 3.85 (s, 3H), 2.33 (s, 3H), 2.15 (s, 3H). ESI-MS calculated for C₁₉H₁₇N₆O₂ [M+H]⁺ = 361.14, Obtained: 361.33

4,4'-(6-Methoxy-9*H*-pyrimido[4,5-*b*]indole-4,7-diyl)bis(3,5-dimethylisoxazole) (14)—Method A using 3,5-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isoxazole ester: 41% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.15 (s, 1H), 7.58 (s, 1H), 7.08 (s, 1H), 3.79 (s, 3H), 2.51 (s, 3H), 2.32 (s, 3H), 2.31 (s, 3H), 2.15 (s, 3H). ESI-MS calculated for C₂₁H₂₀N₅O₃ [M+H]⁺ = 390.16, Obtained: 390.42.

4-(4-(3,5-Dimethyl-1*H*-pyrazol-4-yl)-6-methoxy-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (15).—Method A using 3,5-dimethylpyrazole-4-boronic acid pinacol ester: 67% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.17 (s, 1H), 7.62 (s, 1H), 7.12 (s, 1H), 3.77 (s, 3H), 2.34 (s, 6H), 2.32 (s, 3H), 2.15 (s, 3H). ¹³C NMR (MeOD-d₄, 75MHz): 168.3, 161.1, 157.7, 155.8, 150.3, 148.3, 146.2, 135.7, 123.8, 121.4, 117.2, 114.6, 110.9, 104.9, 56.4, 11.7, 11.6, 10.8. ESI-MS calculated for C₂₁H₂₁N₆O₂ [M+H]⁺ = 389.17, Obtained: 389.83

4-(6-Methoxy-4-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (16).—Method A using 1,3,5-trimethyl-1*H*-pyrazole-4-boronic acid pinacol ester: 42% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.20 (s, 1H), 7.65 (s, 1H), 7.16 (s, 1H), 3.97 (s, 3H), 3.81 (s, 3H), 2.40 (s, 3H), 2.35 (s, 3H), 2.29 (s, 3H), 2.17 (s, 3H). ESI-MS calculated for C₂₂H₂₃N₆O₂ [M+H]⁺ = 403.19, Obtained: 403.50

4-(4-(3-cyclopropyl-5-methyl-1H-pyrazol-4-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (17)—Method A using *tert*-butyl 3-cyclopropyl-5-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole-1-carboxylate: 65% yield; ¹H NMR (MeOD-d₄, 300 MHz): 9.13 (s, 1H), 7.58 (s, 1H), 7.30 (s, 1H), 3.83 (s, 3H), 2.38 (s, 3H), 2.37 (s, 3H), 2.19 (s, 3H), 1.90–1.70 (m, 1H), 1.10–1.00 (m, 1H), 1.00–0.80 (m, 3H).

4-(4-(1H-Indol-3-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (18)—Method A using (1-(*tert*-butoxycarbonyl)-1H-indol-3-yl)boronic acid: 40% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.06 (s, 1H), 8.27 (s, 1H), 7.71 (d, *J* = 8.20 Hz, 1H), 7.57 (s, 1H), 7.50 (d, *J* = 7.96 Hz, 1H), 7.41 (ddd, *J* = 8.24, 7.21, 1.13 Hz, 1H), 7.29 (ddd, *J* = 8.03, 7.08, 0.97 Hz, 1H), 7.15 (s, 1H), 3.42 (s, 1H), 2.32 (s, 1H), 2.13 (s, 1H). ESI-MS calculated for C₂₄H₂₀N₅O₂ [M+H]⁺ = 410.16, Obtained: 410.33

4-(6-Methoxy-4-(1H-pyrrolo[2,3-c]pyridin-3-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (19)—Method A using 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrrolo[2,3-c]pyridine: 37% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.40 (s, 1H), 9.16 (s, 1H), 9.09 (s, 1H), 8.46 (d, *J* = 6.54 Hz, 1H), 8.25 (d, *J* = 6.54 Hz, 1H), 7.58 (s, 1H), 7.19 (s, 1H), 3.58 (s, 3H), 2.32 (s, 3H), 2.14 (s, 3H). ¹³C NMR (MeOD-d₄, 300 MHz): 168.0, 161.2, 158.1, 154.5, 141.4, 137.1, 135.1, 134.0, 130.9, 130.2, 122.3, 120.9, 119.4, 116.3, 114.9, 113.2, 105.6, 56.3, 11.7, 10.8. ESI-MS calculated for C₂₃H₁₉N₆O₂ [M+H]⁺ = 411.16, Obtained: 411.42

4-(4-(6-Fluoro-1H-indol-3-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (20)—Method A using *tert*-butyl 6-fluoro-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indole-1-carboxylate: 44% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.10 (s, 1H), 8.38 (s, 1H), 7.80–7.70 (m, 1H), 7.62 (s, 1H), 7.30–7.10 (m, 3H), 3.52 (s, 3H), 2.35 (s, 3H), 2.16 (s, 3H). ESI-MS calculated for C₂₄H₁₉FN₅O₂ [M+H]⁺ = 428.15, Obtained: 428.25

4-(6-Methoxy-4-(2-methyl-1H-indol-3-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (21)—Method A using *tert*-butyl 2-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indole-1-carboxylate: 26% yield. ¹H NMR (MeOD-d₄, 300 MHz): 9.12 (s, 1H), 7.61–7.58 (m, 1H), 7.58 (s, 1H), 7.37–7.15 (m, 3H), 6.82 (s, 1H), 3.32 (s, 3H), 2.67 (s, 3H), 2.31 (s, 3H), 2.12 (s, 3H). ESI-MS calculated for C₂₅H₂₂N₅O₂ [M+H]⁺ = 424.18, Obtained: 424.42

4-(4-(6-Fluoro-2-methyl-1H-indol-3-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethyl isoxazole (22)—Method A using *tert*-butyl 6-fluoro-2-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indole-1-carboxylate: 52% yield. ¹H NMR (MeOD-d₄, 300 MHz): 12.04 (NH), 9.16 (s, 1H), 7.62 (s, 1H), 7.34 (dd, *J* = 9.33, 2.16 Hz, 1H), 7.27 (dd, *J* = 8.74, 5.06 Hz, 1H), 7.01 (ddd, *J* = 3.42 (s, 3H), 2.67 (s, 3H), 2.33 (s, 3H), 2.14 (s, 3H). ¹³C NMR (MeOD-d₄, 75 MHz): 168.2, 161.7 (d, *J*_{C-F} = 238.7 Hz), 161.1, 157.7, 155.3, 149.3, 148.7, 141.5, 138.0 (d, *J*_{C-F} = 12.8 Hz), 135.5, 123.7, 123.2, 121.8 (d, *J*_{C-F} = 9.8 Hz), 121.5, 116.7, 114.6, 113.4, 110.7 (d, *J*_{C-F} = 24.9 Hz), 106.8, 105.1, 99.6 (d,

J_{C-F} = 26.4 Hz), 55.8, 13.0, 11.7, 10.8. ESI-MS calculated for $C_{25}H_{21}FN_5O_2$ $[M+H]^+$ = 442.17; Obtained: 442.50

4-(6-Methoxy-4-(2-methyl-1H-pyrrolo[2,3-c]pyridin-3-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (23)—Method A using *tert*-butyl 2-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrrolo[2,3-c]pyridine-1-carboxylate: 61% yield. 1H NMR (MeOD- d_4 , 300 MHz): 9.28 (s, 1H), 9.24 (s, 1H), 8.37 (d, J = 6.53 Hz, 1H), 7.87 (d, J = 6.53 Hz, 1H), 7.63 (s, 1H), 6.77 (s, 1H), 3.47 (s, 3H), 2.85 (s, 3H), 2.32 (s, 3H), 2.14 (s, 3H) ESI-MS calculated for $C_{24}H_{21}N_6O_2$ $[M+H]^+$ = 425.17, Obtained: 425.50

4-(6-Methoxy-4-(2-methylbenzofuran-3-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (24)—Method A using 4,4,5,5-tetramethyl-2-(2-methylbenzofuran-3-yl)-1,3,2-dioxaborolane: 31% yield. 1H NMR (MeOD- d_4 , 300 MHz): 9.22 (s, 1H), 7.73 (d, J = 8.32 Hz, 1H), 7.60 (s, 1H), 7.55–7.45 (m, 1H), 7.40–7.30 (m, 2H), 6.85 (s, 1H), 3.37 (s, 3H), 2.68 (s, 3H), 2.30 (s, 3H), 2.12 (s, 3H), ESI-MS calculated for $C_{25}H_{21}N_4O_3$ $[M+H]^+$ = 425.16; Obtained: 425.50

4-(4-(1H-Benzo[d]imidazol-1-yl)-6-methoxy-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (25)—7 (20 mg, 0.06 mmol), benzimidazole (72 mg, 0.6 mmol), and EtN(*i*-Pr) $_2$ (0.1 mL) were dissolved in anhydrous DMSO (4 mL). The solution was heated at 170 °C for 16 h. HPLC purification of the reaction mixture provided **25** in 32% yield. 1H NMR (MeOD- d_4 , 300 MHz): 9.45 (s, 1H), 9.01 (s, 1H), 8.00 (d, J = 8.09 Hz, 1H), 7.67–7.51 (m, 3H), 7.51 (s, 1H), 6.78 (s, 1H), 3.44 (s, 3H), 2.31 (s, 3H), 2.12 (s, 3H). ESI-MS Calculated for $C_{23}H_{19}N_6O_2$ $[M+H]^+$ = 411.16, Found: 411.75

4-(8-Methoxy-1-(pyrazolo[1,5-a]pyridin-3-yl)-5H-pyrido[4,3-b]indol-7-yl)-3,5-dimethylisoxazole (26)—Method A using pyrazolo[1,5-a]pyridin-3-yl-boronic acid pinacol ester: 29% yield. 1H NMR (MeOD- d_4 , 300 MHz): 9.05 (s, 1H), 8.89 (d, J = 7.00 Hz, 1H), 8.76 (s, 1H), 7.76 (d, J = 7.85 Hz, 1H), 7.66–7.58 (m, 1H), 7.56 (s, 1H), 7.25 (dd, J = 6.89, 6.92 Hz, 1H), 7.19 (s, 1H), 3.60 (s, 3H), 2.32 (s, 3H), 2.14 (s, 3H). ESI-MS calculated for $C_{24}H_{20}N_5O_2$ $[M+H]^+$ = 410.16, Obtained: 410.12

4-(6-Methoxy-4-(1-methyl-1H-indazol-3-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (27)—Optimized Suzuki coupling conditions³⁷ were used to synthesize **27**. **7** (34 mg, 0.1 mmol), 1-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indazole (75 mg, 0.3 mmol), and Na $_2$ CO $_3$ (2M aqueous solution, 50 mg) were mixed in round-bottom flask. To this flask, MeOH (4 mL), PhMe (4 mL), and water (1 mL) were added and the system was degassed and refilled with pure N $_2$. Pd(PPh $_3$) $_4$ (20 mg) was then added. The system was degassed and refilled again with pure N $_2$. The mixture was heated at reflux for 12 h. The aqueous layer was extracted with EtOAc and the combined organic layers were washed with brine and dried over anhydrous Na $_2$ SO $_4$. The volatile components were removed on a rotary evaporator and the residue was purified by reverse phase HPLC. The desired product **27** was isolated as TFA salt in 23% yield. 1H NMR (DMSO- d_6 , 300 MHz): 12.36 (s, 1H), 9.31 (s, 1H), 9.05 (s, 1H), 8.82 (d, J = 8.18 Hz, 1H), 7.85 (d, J = 8.48 Hz, 1H), 7.60–7.50 (m, 1H), 7.39 (s, 1H), 7.40–7.32 (m, 1H), 4.36 (s, 3H), 4.00 (s, 3H), 3.37 (s, NH), 2.32 (s, 3H), 2.13 (s, 3H). ^{13}C NMR (DMSO- d_6 , 75 MHz): 165.7, 159.3, 156.9,

153.9, 152.9, 151.7, 141.0, 132.8, 127.0, 123.8, 123.1, 122.4, 119.9, 119.0, 113.4, 110.4, 109.3, 108.1, 55.5, 36.3, 11.4, 10.5. ESI-MS Calculated for $C_{24}H_{21}N_6O_2$ $[M+H]^+ = 425.17$, Found: 425.83

4-(4-(1*H*-Benzo[d][1,2,3]triazol-1-yl)-6-methoxy-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (28)—A modified reported method was followed.⁴² **7** (34 mg, 0.1 mmol), benzotriazole (14 mg, 0.12 mmol), and K_3PO_4 (42 mg, 0.2 mmol) were added into a round-bottom flask. The round-bottom flask was degassed and refilled with pure N_2 gas. In a second round-bottom flask, $Pd_2(dba)_3$ (9 mg, 0.01 mmol) and ($\pm$)-BINAP (15 mg, 0.024 mmol) were added. The round-bottom flask was degassed and refilled with pure N_2 . To this flask, anhydrous toluene (10 mL) was added and the solution was heated at 120 °C for 3 min to generate the active catalyst. The active catalyst was transferred into the first flask and the reaction mixture was heat at reflux for 12 h. The mixture was then diluted with EtOAc and washed with water and brine. The organic layer was collected, the volatile components were removed on a rotary evaporator, and the residue was purified by reverse phase HPLC. The desired product **28** was isolated as a TFA salt in 29% yield. 1H NMR (DMSO- d_6 , 300 MHz): 12.88 (s, 1H), 9.04 (s, 1H), 8.61 (s, 1H), 8.26–8.18 (m, 2H), 7.68–7.60 (m, 2H), 7.48 (s, 1H), 3.87 (s, 3H), 3.30 (s, 3H), 3.15 (s, NH), 2.31 (s, 3H), 2.11 (s, 3H). ^{13}C NMR (DMSO- d_6 , 75 MHz): 167.6, 160.8, 160.3, 155.3, 153.8, 146.7, 135.3, 130.8, 122.3, 120.5, 119.3, 119.0, 115.9, 114.8, 109.0, 105.8, 57.0, 13.1, 12.1. ESI-MS Calculated for $C_{22}H_{18}N_7O_2$ $[M+H]^+ = 412.15$, Found: 412.42

4-(6-Methoxy-2-methyl-4-(2-methyl-1*H*-indol-3-yl)-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (29)—Method A using **S10** and *tert*-butyl 2-methyl-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indole-1-carboxylate afforded a mixture of **29** and a Boc group protected **29**. Treatment of the crude mixture with TFA in DCM followed by HPLC purification yield **29** in 27% yield. 1H NMR (MeOD- d_4 , 300 MHz): 11.94 (NH), 7.62 (d, $J = 8.13$ Hz, 1H), 7.57 (s, 1H), 7.35 (ddd, $J = 8.17, 6.82, 1.30$, 1H), 7.32–7.18 (m, 2H), 6.80 (s, 1H), 3.00 (s, 3H), 2.70 (s, 3H), 2.34 (s, 3H), 2.15 (s, 3H). ^{13}C NMR (MeOD- d_4 , 75 MHz): 168.1, 161.1, 160.1, 158.2, 155.3, 148.8, 141.0, 137.8, 135.5, 127.2, 124.3, 122.7, 122.5, 121.6, 120.7, 116.6, 114.6, 113.4, 111.7, 107.0, 104.5, 55.8, 22.4, 12.9, 11.7, 10.8. ESI-MS calculated for $C_{26}H_{24}N_5O_2$ $[M+H]^+ = 438.19$, Obtained: 438.42.

Method I: Synthesis of 4-(6-Methoxy-2-methyl-4-(2-methylquinolin-4-yl)-9*H*-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (30), using Suzuki coupling catalyzed by $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ complex: **S10** (728 mg, 2.1 mmol, 1.0 equiv) and 2-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)quinoline (1.674g, 6.22 mmol, 3.0 equiv) were placed in a round-bottom flask. DME (60 mL) and Na_2CO_3 (2 M aq solution, 20 mL) were added subsequently. The system was degassed to remove oxygen. $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ complex (257 mg, 0.31 mmol, > 0.1 equiv) was added in one portion. The system was degassed again and refilled with N_2 . The reaction was heated at reflux for 16 h before quenching with H_2O . The reaction was then diluted with H_2O , the aqueous layer was extracted with EtOAc (100 mL $\times$ 3) and the combined organic layers were dried over anhydrous sodium sulfate. The solvent was removed in vacuum and the residue was purified

by flash column chromatography to yield **30** in 0.64 g (63% yield). Further purification was aided by reverse phase HPLC to yield the corresponding products **30** as a salt of CF₃CO₂H. ¹H NMR (300 MHz, MeOD-d₄): 8.32 (d, *J* = 8.47 Hz, 1H), 8.13 (s, 1H), 8.10 (ddd, *J* = 8.44, 7.03, 1.26 Hz, 1H), 7.93 (d, *J* = 7.86 Hz, 1H), 7.75 (t, *J* = 7.71 Hz, 1H), 7.49 (s, 1H), 6.30 (s, 1H), 3.25 (s, 3H), 3.04 (s, 3H), 2.95 (s, 3H), 2.26 (s, 3H), 2.07 (s, 3H). ESI-MS calculated for C₂₇H₂₄N₅O₂ [M+H]⁺ = 450.19, Obtained: 450.42

4-(6-Methoxy-2-methyl-4-(quinolin-4-yl)-9H-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (31).—Method A using **S10** and quinoline-4-boronic acid: 57% yield; ¹H NMR (MeOD-d₄, 300 MHz): 9.31 (d, *J* = 4.59 Hz, 1H), 8.38 (d, *J* = 8.50 Hz, 1H), 8.08 (d, *J* = 4.63 Hz, 1H), 8.12–8.00 (m, 1H), 7.88 (d, *J* = 7.76 Hz, 1H), 7.78–7.70 (m, 1H), 7.53 (s, 1H), 6.21 (s, 1H), 3.21 (s, 3H), 3.00 (s, 3H), 2.26 (s, 3H), 2.07 (s, 3H). ¹³C NMR (MeOD-d₄, 75 MHz): 168.2, 162.1, 161.0, 158.6, 155.3, 151.0, 150.3, 148.3, 141.3, 136.1, 133.3, 130.8, 129.9, 126.7, 126.2, 123.7, 123.6, 120.4, 117.0, 114.5, 112.9, 105.3, 55.9, 23.6, 11.6, 10.7. ESI-MS Calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Found: 436.50.

4-(6-Methoxy-2-methyl-4-(quinolin-5-yl)-9H-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (32).—Method A using **S10** and quinoline-5-boronic acid: 27%. ¹H NMR (300 MHz, MeOD-d₄): 9.15 (dd, *J* = 4.39, 1.39 Hz, 1H), 8.61–8.53 (m, 1H), 8.41 (d, *J* = 8.60 Hz, 1H), 8.25 (d, *J* = 1.33 Hz, 1H), 8.24 (s, 1H), 7.72 (dd, *J* = 7.74, 4.42 Hz, 1H), 7.57 (s, 1H), 6.30 (s, 1H), 3.28 (s, 3H), 3.03 (3H), 2.29 (s, 3H), 2.10 (s, 3H). ESI-MS calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Obtained: 436.33.

4-(6-Methoxy-2-methyl-4-(6-methylquinolin-5-yl)-9H-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (33).—Method A using **S10** and 3-methylquinoline-4-boronic acid: 36%. ¹H NMR (300 MHz, MeOD-d₄): 9.08 (d, *J* = 4.56 Hz, 1H), 8.46 (*J* = 8.81 Hz, 1H), 8.22 (d, *J* = 8.56 Hz, 1H), 8.17 (d, *J* = 8.86 Hz, 1H), 7.69 (dd, *J* = 8.63, 4.64 Hz, 1H), 7.55 (s, 1H), 6.14 (s, 1H), 3.28 (s, 3H), 3.01 (s, 3H), 2.47 (s, 3H), 2.25 (s, 3H), 2.06 (s, 3H). ESI-MS calculated for C₂₇H₂₄N₅O₂ [M+H]⁺ = 450.19, Obtained: 450.50.

4-(4-(Isoquinolin-4-yl)-6-methoxy-2-methyl-9H-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (34).—Method A using **S10** and isoquinoline-4-boronic acid: 54%. ¹H NMR (300 MHz, MeOD-d₄): 9.78 (s, 1H), 8.98 (s, 1H), 8.53–8.46 (m, 1H), 8.04–7.92 (m, 2H), 7.90–7.82 (m, 1H), 7.55 (s, 1H), 6.23 (s, 1H), 3.20 (s, 3H), 3.01 (s, 3H), 2.26 (s, 3H), 2.07 (s, 3H). ESI-MS calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Obtained: 436.50.

4-(4-(Isoquinolin-5-yl)-6-methoxy-2-methyl-9H-pyrimido[4,5-*b*]indol-7-yl)-3,5-dimethylisoxazole (35).—Method A using **S10** and isoquinoline-5-boronic acid: 29%. ¹H NMR (300 MHz, MeOD-d₄): 9.85 (s, broad, 1H), 8.80 (d, *J* = 8.32 Hz, 1H), 8.75–8.50 (broad, 1H), 8.56 (dd, *J* = 7.24, 1.11 Hz), 8.26 (dd, *J* = 8.29, 7.30 Hz, 1H), 8.04 (d, *J* = 6.08 Hz, 1H), 6.32 (s, 1H), 3.29 (s, 3H), 3.00 (s, 3H), 2.27 (s, 3H), 2.08 (s, 3H). ¹³C NMR (75 MHz, MeOD-d₄): 168.1, 162.2, 161.0, 158.7, 155.2, 153.2, 151.8, 141.8, 136.6, 136.0, 135.6, 134.1, 130.6, 130.1, 123.4, 121.1, 120.7, 112.0, 114.5, 113.1, 105.1, 55.9, 23.6, 11.6, 10.7. ESI-MS calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Obtained: 436.68.

4-(4-(Isoquinolin-8-yl)-6-methoxy-2-methyl-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (36)—Method A using **S10** and isoquinoline-8-boronic acid: 35%. ¹H NMR (300 MHz, MeOD-d₄): 9.54 (s, 1H), 8.74 (s, 1H), 8.61 (dd, *J* = 7.75, 1.22 Hz, 1H), 8.52 (d, *J* = 6.18 Hz, 1H), 8.47–8.35 (m, 2H), 7.54 (s, 1H), 6.42 (s, 1H), 3.32 (s, 3H), 2.99 (s, 3H), 2.26 (s, 3H), 2.08 (s, 3H). ¹³C NMR (75 MHz, MeOD-d₄): 168.1, 162.8, 161.1, 158.8, 155.1, 152.5, 149.7, 140.6, 139.4, 135.9, 134.2, 133.6, 132.8, 132.0, 126.8, 125.0, 123.2, 120.7, 116.9, 114.6, 113.1, 104.9, 56.0, 24.1, 11.6, 10.7. ESI-MS calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Obtained: 436.56.

4-(6-Methoxy-2-methyl-4-(quinolin-8-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (37)—Method A using **S10** and quinoline-8-boronic acid: 18%. ¹H NMR (300 MHz, MeOD-d₄): 8.89 (dd, *J* = 4.28, 1.72 Hz, 1H), 8.65 (dd, *J* = 8.41, 1.68 Hz, 1H), 8.47 (dd, *J* = 8.28, 1.31 Hz, 1H), 8.37 (dd, *J* = 7.18, 1.36 Hz, 1H), 8.02 (dd, *J* = 8.20, 7.26 Hz, 1H), 7.74 (dd, *J* = 8.38, 4.28 Hz, 1H), 7.54 (s, 1H), 6.43 (s, 1H), 3.33 (s, 3H), 2.99 (s, 3H), 2.27 (s, 3H), 2.08 (s, 3H). ESI-MS calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Obtained: 436.83.

4-(6-Methoxy-2-methyl-4-(2-(trifluoromethyl)quinolin-4-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (38)—Method I using **S10** and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2-(trifluoromethyl)quinoline: 10% yield. ¹H NMR (300 MHz, MeOD-d₄): 8.44 (d, *J* = 8.61 Hz, 1H), 8.28 (s, 1H), 8.04 (t, *J* = 7.71 Hz, 1H), 7.90 (d, *J* = 8.19 Hz, 1H), 7.76 (t, *J* = 8.03 Hz, 1H), 7.45 (s, 1H), 6.20 (s, 1H), 3.21 (s, 3H), 2.93 (s, 3H), 2.26 (s, 3H), 2.07 (s, 3H). ESI-MS calculated for C₂₇H₂₁F₃N₅O₂ [M+H]⁺ = 504.16, Obtained: 504.58.

4-(6-Methoxy-4-(2-methoxyquinolin-4-yl)-2-methyl-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (39)—Method I using **S10** and 2-methoxy-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)quinoline: 35% yield. ¹H NMR (300 MHz, MeOD-d₄): 8.11 (d, *J* = 8.44 Hz, 1H), 7.83 (ddd, *J* = 8.39, 7.05, 1.30 Hz, 1H), 7.63 (d, *J* = 8.29 Hz, 1H), 7.54 (s, 1H), 7.49 (s, 1H), 7.44 (t, *J* = 7.63 Hz, 1H), 6.27 (s, 1H), 4.20 (s, 3H), 3.21 (s, 3H), 3.00 (s, 3H), 2.26 (s, 3H), 2.07 (s, 3H). ESI-MS calculated for C₂₇H₂₄N₅O₃ [M+H]⁺ = 466.19, Obtained: 466.68.

4-(6-Methoxy-2-methyl-4-(quinolin-3-yl)-9H-pyrimido[4,5-b]indol-7-yl)-3,5-dimethylisoxazole (40)—Method A using **S10** and quinoline-3-boronic acid: 22% yield. ¹H NMR (300 MHz, MeOD-d₄): 9.47 (d, *J* = 2.01 Hz, 1H), 9.21 (d, *J* = 2.03 Hz, 1H), 8.29 (t, *J* = 7.52 Hz, 2H), 8.08 (ddd, *J* = 8.50, 6.99, 1.40 Hz, 1H), 7.88 (ddd, *J* = 8.05, 7.14, 1.00 Hz, 1H), 7.57 (s, 1H), 7.29 (s, 1H), 3.60 (s, 3H), 3.00 (s, 3H), 2.31 (s, 3H), 2.13 (s, 3H). ESI-MS calculated for C₂₆H₂₂N₅O₂ [M+H]⁺ = 436.18, Obtained: 436.83. The procedures for FP-Based Protein Binding Assay, Cell Growth Inhibition Assay, and Western Blotting have been reported in our previous paper and please refer that paper for experimental details.

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Determination of Biochemical Binding Affinities to BET Proteins

Recombinant human proteins corresponding to BRD2 BD1 (residues 72–205), BRD2 BD2 (residues 349–460), BRD3 BD1 (residues 24–144), BRD3 BD2 (residues 306–417), BRD4 BD1 (residues 44–168), BRD4 BD2 (residues 333–460) were used in the biochemical binding assays. Binding affinities of BET inhibitors to BRD2 (BD1 and BD2 proteins), BRD3 (BD1 and BD2 proteins), BRD4 (BD1 and BD2 proteins) were determined using our established fluorescence-polarization binding assays as described previously.³³

Determination of the co-crystal structure of compound **31** in complex with BRD4 BD2 protein

Prior to crystallization, BRD4 BD2 concentrated to 7.1 mg/mL in 25 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM TCEP was incubated with a 2-fold molar excess of compound **31** for 1 hr then incubated with 0.5% β -mercaptoethanol for an additional 2 hr at room temperature. Crystals were grown at 20 °C from drops containing equal volumes of protein complex and well solution (50% polyethylene glycol 400, 10 mM imidazole pH 8.0, and 0.5 mM ATP). For data collection, the crystals were mounted directly from the drop and flash frozen in liquid nitrogen. Data were collected at the Advance Photon Source at Argonne National Lab on the LS-CAT beamlines 21-ID-G. The complex crystallized in space group P2₁2₁2 and contained 1 molecule per asymmetric unit. Data were processed with HKL2000⁴³ and the structure was solved by molecular replacement with Phaser⁴⁴ using an in-house structure as a model. The structure was refined with BUSTER⁴⁵ and electron density maps fit with COOT⁴⁶. Coordinates and restraints for the compounds were developed using Grade with the mogul+qm option⁴⁵. Structures were validated using Molprobit⁴⁷. Ligand statistics were obtained from the wwPDB Validation Server. Data refinement and statistics are given in Supporting Information Table S1. The coordinates were deposited in the PDB with the following ID: 5UOO.

Cell Growth Inhibition and Western Blotting

The SUM-149PT human breast cancer cell line was developed at the University of Michigan Comprehensive Cancer Center.⁴⁸ All the other human cancer cell lines were purchased from the American Type Culture Collection. Cells were used within one month after thaw and were cultured as recommended.

In cell growth assay, cells were seeded in 96-well cell culture plates at 10,000 cells/well for leukemia cells and 3,000 cells/well for breast cancer cells in 75 μ L of culture medium. Compounds were serially diluted in culture medium, and 75 μ L of the diluted compounds was added to the plates. The cells were then cultured for 4 days. Cell growth was evaluated by the dehydrogenases-based WST-8 assay (Dojindo Molecular Technologies). The WST-8 reagent was added to the plate at a final concentration of 10% (v/v), incubated for 1–3h, and read at 450 nm using a Tecan Infinite M1000 multimode microplate reader (Tecan, Morrisville, NC). The readings were normalized to the DMSO-treated cells and the IC₅₀ was calculated by nonlinear regression analysis using GraphPad Prism 6 software.

For Western blot analysis, cells were treated with compounds at indicated conditions. Cells were collected and lysed in RIPA buffer containing protease inhibitors. Whole cell lysates

were separated by 4–20% SDS- PAGE gels and blotted into PVDF membranes. For immunoblotting, rabbit polyclonal antibodies for BRD2, BRD3 and BRD4 were from Bethyl Laboratories (Montgomery, TX, USA), mouse monoclonal antibody for c-Myc was from Cell Signaling Technology (Danvers, MA, USA) and GAPDH was from Santa Cruz Biotechnologies (Dallas, TX, USA).

Microsomal Stability Studies

The metabolic stability was assessed using pooled CD-1 mouse liver microsomes, Pooled SD rat liver microsomes and pooled human liver microsome (purchased from Xeno Tech). Briefly, 1 μ M of each compound (**21**, **29**, **30** and **31**) was incubated with 0.5 mg/mL microsomes and 1.7 mM cofactor β -NADPH in 0.1 M phosphate buffer (pH = 7.4) containing 3.3 mM MgCl_2 at 37 °C. The DMSO concentration was less than 0.1% in the final incubation system. At 5, 10, 15, 30, 45, and 60 min of incubation, the 40 μ L of reaction mixture were taken out, and the reaction is stopped immediately by adding 3-fold excess of cold acetonitrile containing 100 ng/mL of internal standard for quantification. The collected fractions were centrifuged at 15000rpm for 10 min to collect the supernatant for LC–MS/MS analysis, from which the amount of compound remaining was determined. The natural log of the amount of compound remaining was plotted against time to determine the disappearance rate and the half-life of tested compounds.

Pharmacokinetic Studies in Rats and Mice

All animal experiments were approved by the University of Michigan Committee on Use and Care of Animals and Unit for Laboratory Animal Medicine under the approved protocol (PRO00005315, P.I. Shaomeng Wang).

The pharmacokinetics of compound **21** and **31** was determined in Sprague Dawley rats following intravenous (iv) dosing at 5 mg/kg or oral (PO) dosing at 25 mg/kg. The quick pharmacokinetics of compound **17** was determined in SCID Mice following PO dosing at 25 mg/kg.

A tested compound was dissolved in the vehicle containing 20% (v/v) PCP and 70% (v/v) PBS. Blood samples (100 μ L) were collected from rats with a catheter at different time points (0, 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 6hr, 8 hr and 24 hr) after the treatment of drugs. The blood samples were centrifuged at 15 000 rpm for 10 min, then the supernatant plasma was saved at –80 °C until analysis.

Plasma concentrations of the compounds were determined by the LC–MS/MS method developed and validated for this study. The LC–MS/MS method consisted of a Shimadzu HPLC system, and chromatographic separation of tested compound was achieved using a Waters XBridge-C18 column (5 cm $\times$ 2.1 mm, 3.5 μ m). An AB Sciex QTrap 4500 mass spectrometer equipped with an electrospray ionization source (Applied Biosystems, Toronto, Canada) in the positive-ion multiple reaction monitoring (MRM) mode was used for detection. The mobile phases were 0.1% formic acid in purified water (A) and 0.1% formic acid in acetonitrile (B). The gradient (B) was held at 10% (0–0.3 min), increased to 95% at 0.7 min, then stayed at isocratic 95% B for 2.3 min, and then immediately stepped back

down to 10% for 2 min re-equilibration. Flow rate was set at 0.4 mL/min. All pharmacokinetic parameters were calculated by noncompartmental methods using WinNonlin, version 3.2 (Pharsight Corporation, Mountain View, CA, USA).

Efficacy Studies in the MV4;11 and MDA-MB-231 Xenograft Models in Mice

All efficacy experiments were done under the guidelines of the University of Michigan Committee for Use and Care of Animals and using an approved animal protocol (PRO00005315, P.I. Shaomeng Wang).

To develop xenograft tumors, 5×10^6 MV4;11 or MDA-MB-231 cells with 50% Matrigel were injected subcutaneously on the dorsal side of severe combined immunodeficient (SCID) mice, obtained from Charles River, one tumor per mouse. When tumors reached $\sim 100 \text{ mm}^3$, mice were randomly assigned to treatment and vehicle control groups. Animals were monitored daily for any signs of toxicity and weighed 2–3 times per week during the treatment and weighed at least weekly after the treatment was ended. Tumor size was measured 2–3 times per week by electronic calipers during the treatment period and at least weekly after the treatment was ended. Tumor volume was calculated as $V = L \times W^2/2$, where L is the length and W is the width of the tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

DME	1,2-Dimethoxyethane
DMF	Dimethylformamide
NMP	1-Methylpyrrolidin-2-one
C_{max}	The peak plasma concentration of a drug after administration
V_{ss}	The apparent volume in which a drug is distributed at steady state
CL	The volume of plasma cleared of the drug per unit time
PARP	Poly (ADP-ribose) polymerase

CL-PARP

cleaved PAPR

REFERENCES

- (1). Arrowsmith CH; Bountra C; Fish PV; Lee K; Schapira M Epigenetic protein families: a new frontier for drug discovery. *Nat. Rev. Drug Discovery* 2012, 11, 384–400. [PubMed: 22498752]
- (2). Belkina AC; Denis GV BET domain co-regulators in obesity, inflammation and cancer. *Nat. Rev. Cancer* 2012, 12, 465–477. [PubMed: 22722403]
- (3). Filippakopoulos P; Qi J; Picaud S; Shen Y; Smith WB; Fedorov O; Morse EM; Keates T; Hickman TT; Felletar I; Philpott M; Munro S; McKeown MR; Wang Y; Christie AL; West N; Cameron MJ; Schwartz B; Heightman TD; La Thangue N; French CA; Wiest O; Kung AL; Knapp S; Bradner JE Selective inhibition of BET bromodomains. *Nature* 2010, 468, 1067–1073. [PubMed: 20871596]
- (4). Dawson MA; Prinjha RK; Dittmann A; Giotopoulos G; Bantscheff M; Chan WI; Robson SC; Chung CW; Hopf C; Savitski MM; Huthmacher C; Gudgin E; Lugo D; Beinke S; Chapman TD; Roberts EJ; Soden PE; Auger KR; Mirguet O; Doehner K; Delwel R; Burnett AK; Jeffrey P; Drewes G; Lee K; Huntly BJ; Kouzarides T Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* 2011, 478, 529–533. [PubMed: 21964340]
- (5). Nicodeme E; Jeffrey KL; Schaefer U; Beinke S; Dewell S; Chung CW; Chandwani R; Marazzi I; Wilson P; Coste H; White J; Kirilovsky J; Rice CM; Lora JM; Prinjha RK; Lee K; Tarakhovsky A Suppression of inflammation by a synthetic histone mimic. *Nature* 2010, 468, 1119–1123. [PubMed: 21068722]
- (6). Anand P; Brown JD; Lin CY; Qi J; Zhang R; Artero PC; Alaiti MA; Bullard J; Alazem K; Margulies KB; Cappola TP; Lemieux M; Plutzky J; Bradner JE; Haldar SM BET bromodomains mediate transcriptional pause release in heart failure. *Cell* 2013, 154, 569–582. [PubMed: 23911322]
- (7). Gosmini R; Nguyen VL; Toum J; Simon C; Brusq JM; Krysa G; Mirguet O; Riou-Eymard AM; Boursier EV; Trotter L; Bamborough P; Clark H; Chung CW; Cutler L; Demont EH; Kaur R; Lewis AJ; Schilling MB; Soden PE; Taylor S; Walker AL; Walker MD; Prinjha RK; Nicodeme E The discovery of I-BET726 (GSK1324726A), a potent tetrahydroquinoline ApoA1 up-regulator and selective BET bromodomain inhibitor. *J. Med. Chem* 2014, 57, 8111–8131. [PubMed: 25249180]
- (8). Gehling VS; Hewitt MC; Vaswani RG; Leblanc Y; Cote A; Nasveschuk CG; Taylor AM; Harmange JC; Audia JE; Pardo E; Joshi S; Sandy P; Mertz JA; Sims RJ 3rd; Bergeron L; Bryant BM; Bellon S; Poy F; Jayaram H; Sankaranarayanan R; Yellapantula S; Bangalore Srinivasamurthy N; Birudukota S; Albrecht BK Discovery, design, and optimization of isoxazole azepine BET inhibitors. *ACS Med. Chem. Lett* 2013, 4, 835–840. [PubMed: 24900758]
- (9). Wong C; Laddha SV; Tang L; Vosburgh E; Levine AJ; Normant E; Sandy P; Harris CR; Chan CS; Xu EY The bromodomain and extra-terminal inhibitor CPI203 enhances the antiproliferative effects of rapamycin on human neuroendocrine tumors. *Cell Death Dis.* 2014, 5, e1450–e1450. [PubMed: 25299775]
- (10). Fish PV; Filippakopoulos P; Bish G; Brennan PE; Bunnage ME; Cook AS; Fedorov O; Gerstenberger BS; Jones H; Knapp S; Marsden B; Nocka K; Owen DR; Philpott M; Picaud S; Primiano MJ; Ralph MJ; Sciammetta N; Trzupek JD Identification of a chemical probe for bromo and extra C-terminal bromodomain inhibition through optimization of a fragment-derived hit. *J. Med. Chem* 2012, 55, 9831–9837. [PubMed: 23095041]
- (11). Bamborough P; Diallo H; Goodacre JD; Gordon L; Lewis A; Seal JT; Wilson DM; Woodrow MD; Chung CW Fragment-based discovery of bromodomain inhibitors part 2: optimization of phenylisoxazole sulfonamides. *J. Med. Chem* 2012, 55, 587–596. [PubMed: 22136469]
- (12). McKeown MR; Shaw DL; Fu H; Liu S; Xu X; Marineau JJ; Huang Y; Zhang X; Buckley DL; Kadam A; Zhang Z; Blacklow SC; Qi J; Zhang W; Bradner JE Biased multicomponent reactions to develop novel bromodomain inhibitors. *J. Med. Chem* 2014, 57, 9019–9027. [PubMed: 25314271]
- (13). Hewings DS; Fedorov O; Filippakopoulos P; Martin S; Picaud S; Tumber A; Wells C; Olcina MM; Freeman K; Gill A; Ritchie AJ; Sheppard DW; Russell AJ; Hammond EM; Knapp S;

Brennan PE; Conway SJ Optimization of 3,5-dimethylisoxazole derivatives as potent bromodomain ligands. *J. Med. Chem* 2013, 56, 3217–3227. [PubMed: 23517011]

- (14). Seal J; Lamotte Y; Donche F; Bouillot A; Mirguet O; Gellibert F; Nicodeme E; Krysa G; Kirilovsky J; Beinke S; McCleary S; Rioja I; Bamborough P; Chung CW; Gordon L; Lewis T; Walker AL; Cutler L; Lugo D; Wilson DM; Witherington J; Lee K; Prinjha RK Identification of a novel series of BET family bromodomain inhibitors: binding mode and profile of I-BET151 (GSK1210151A). *Bioorg. Med. Chem. Lett* 2012, 22, 2968–2972. [PubMed: 22437115]
- (15). Mirguet O; Lamotte Y; Donche F; Tourn J; Gellibert F; Bouillot A; Gosmini R; Nguyen VL; Delannee D; Seal J; Blandel F; Boullay AB; Boursier E; Martin S; Brusq JM; Krysa G; Riou A; Tellier R; Costaz A; Huet P; Dudit Y; Trotter L; Kirilovsky J; Nicodeme E From ApoA1 upregulation to BET family bromodomain inhibition: discovery of I-BET151. *Bioorg. Med. Chem. Lett* 2012, 22, 2963–2967. [PubMed: 22386529]
- (16). Zhao L; Cao D; Chen T; Wang Y; Miao Z; Xu Y; Chen W; Wang X; Li Y; Du Z; Xiong B; Li J; Xu C; Zhang N; He J; Shen J Fragment-based drug discovery of 2-thiazolidinones as inhibitors of the histone reader BRD4 bromodomain. *J. Med. Chem* 2013, 56, 3833–3851. [PubMed: 23530754]
- (17). Hewings DS; Rooney TPC; Jennings LE; Hay DA; Schofield CJ; Brennan PE; Knapp S; Conway SJ Progress in the development and application of small molecule inhibitors of bromodomain–acetyl-lysine interactions. *J. Med. Chem* 2012, 55, 9393–9413. [PubMed: 22924434]
- (18). Chapuy B; McKeown MR; Lin CY; Monti S; Roemer MG; Qi J; Rahl PB; Sun HH; Yeda KT; Doench JG; Reichert E; Kung AL; Rodig SJ; Young RA; Shipp MA; Bradner JE Discovery and characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma. *Cancer Cell* 2013, 24, 777–790. [PubMed: 24332044]
- (19). Wang S; Sun W; Zhao Y; McEachern D; Meaux I; Barriere C; Stuckey JA; Meagher JL; Bai L; Liu L; Hoffman-Luca CG; Lu J; Shangary S; Yu S; Bernard D; Aguilar A; Dos-Santos O; Besret L; Guerif S; Pannier P; Gorge-Bernat D; Debussche L SAR405838: An optimized inhibitor of MDM2-p53 interaction that induces complete and durable tumor regression. *Cancer Res.* 2014, 74, 5855–5865. [PubMed: 25145672]
- (20). Banerjee C; Archin N; Michaels D; Belkina AC; Denis GV; Bradner J; Sebastiani P; Margolis DM; Montano M BET bromodomain inhibition as a novel strategy for reactivation of HIV-1. *J. Leukocyte Biol* 2012, 92, 1147–1154. [PubMed: 22802445]
- (21). Matzuk MM; McKeown MR; Filippakopoulos P; Li Q; Ma L; Agno JE; Lemieux ME; Picaud S; Yu RN; Qi J; Knapp S; Bradner JE Small-molecule inhibition of BRDT for male contraception. *Cell* 2012, 150, 673–684. [PubMed: 22901802]
- (22). Mertz JA; Conery AR; Bryant BM; Sandy P; Balasubramanian S; Mele DA; Bergeron L; Sims RJ 3rd Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc. Natl. Acad. Sci. U. S. A* 2011, 108, 16669–16674. [PubMed: 21949397]
- (23). Asangani IA; Dommeti VL; Wang X; Malik R; Cieslik M; Yang R; Escara-Wilke J; Wilder-Romans K; Dhanireddy S; Engelke C; Iyer MK; Jing X; Wu YM; Cao X; Qin ZS; Wang S; Feng FY; Chinnaiyan AM Therapeutic targeting of BET bromodomain proteins in castration-resistant prostate cancer. *Nature* 2014, 510, 278–282. [PubMed: 24759320]
- (24). Romero FA; Taylor AM; Crawford TD; Tsui V; Côté A; Magnuson S Disrupting acetyl-lysine recognition: progress in the development of bromodomain inhibitors. *J. Med. Chem* 2016, 59, 1271–1298. [PubMed: 26572217]
- (25). Waring MJ; Chen HW; Rabow AA; Walker G; Bobby R; Boiko S; Bradbury RH; Callis R; Clark E; Dale I; Daniels DL; Dulak A; Flavell L; Holdgate G; Jowitt TA; Kikhney A; McAlister M; Mendez J; Ogg D; Patel J; Petteruti P; Robb GR; Robers MB; Saif S; Stratton N; Svergun DI; Wang WX; Whittaker D; Wilson DM; Yao Y Potent and selective bivalent inhibitors of BET bromodomains. *Nat. Chem. Biol* 2016, 12, 1097–1104. [PubMed: 27775716]
- (26). Bradbury RH; Callis R; Carr GR; Chen HW; Clark E; Feron L; Glossop S; Graham MA; Hattersley M; Jones C; Lamont SG; Ouvre G; Patel A; Patel J; Rabow AA; Roberts CA; Stokes S; Stratton N; Walker GE; Ward L; Whalley D; Whittaker D; Wrigley G; Waring MJ Optimization of a series of bivalent triazolopyridazine based bromodomain and extraterminal inhibitors: the discovery of (3R)-4-[2-[4-[1-(3-methoxy-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-4-

piperidyl]phenoxyethyl]-1,3-dimethyl-piperazin-2-one (AZD5153). *J. Med. Chem* 2016, 59, 7801–7817. [PubMed: 27528113]

- (27). Tanaka M; Roberts JM; Seo HS; Souza A; Paulk J; Scott TG; DeAngelo SL; Dhe-Paganon S; Bradner JE Design and characterization of bivalent BET inhibitors. *Nat. Chem. Biol* 2016, 12, 1089–1096. [PubMed: 27775715]
- (28). Albrecht BK; Gehling VS; Hewitt MC; Vaswani RG; Côté A; Leblanc Y; Nasveschuk CG; Bellon S; Bergeron L; Campbell R; Cantone N; Cooper MR; Cummings RT; Jayaram H; Joshi S; Mertz JA; Neiss A; Normant E; O'Meara M; Pardo E; Poy F; Sandy P; Supko J; Sims RJ; Harmange J-C; Taylor AM; Audia JE Identification of a benzoisoxazoloazepine inhibitor (CPI-0610) of the bromodomain and extra-terminal (BET) family as a candidate for human clinical trials. *J. Med. Chem* 2016, 59, 1330–1339. [PubMed: 26815195]
- (29). Dombret H; Preudhomme C; Berthon C; Raffoux E; Thomas X; Vey N; Gomez-Roca C; Ethell M; Yee K; Bourdel F; Herait P; Michallet M; Recher C; Roumier C; Quesnel B A phase 1 study of the BET-bromodomain inhibitor OTX015 in patients with advanced acute leukemia. *Blood* 2014, 124, 117–117.
- (30). Stathis A; Quesnel B; Amorim S; Thieblemont C; Zucca E; Raffoux E; Dombret H; Peng Y; Palumbo A; Vey N; Thomas X; Michallet M; Gomez-Roca C; Recher C; Karlin L; Yee K; Rezai K; Preudhomme C; Facon T; Herait R Results of a first-in-man phase I trial assessing OTX015, an orally available BET-bromodomain (BRD) inhibitor, in advanced hematologic malignancies. *Eur. J. Cancer* 2014, 50, 196–196.
- (31). Vazquez R; Astorgues-Xerri L; Riveiro ME; Di Marino M; Beltrame L; Bekradda M; Cvitkovic E; Erba E; Frapolli R; D'Incalci M Evaluation of the pan-BET-bromodomain inhibitor OTX015 as a single agent and in combination with everolimus (RAD001) in triple-negative breast cancer. *Eur. J. Cancer* 2014, 50, 187–187.
- (32). Herait PE; Berthon C; Thieblemont C; Raffoux E; Magarotto V; Stathis A; Thomas X; Leleu X; Gomez-Roca C; Odore E; Roumier C; Bourdel F; Quesnel B; Zucca E; Michallet M; Recher C; Cvitkovic E; Rezai K; Preudhomme C; Facon T; Palumbo A; Dombret H BET-bromodomain inhibitor OTX015 shows clinically meaningful activity at nontoxic doses: interim results of an ongoing phase I trial in hematologic malignancies. *Cancer Res.* 2014, 74, DOI: 10.1158/1538-7445.AM2014-CT231.
- (33). Ran X; Zhao Y; Liu L; Bai L; Yang C-Y; Zhou B; Meagher JL; Chinnaswamy K; Stuckey JA; Wang S Structure-based design of γ -carboline analogues as potent and specific BET bromodomain inhibitors. *J. Med. Chem* 2015, 58, 4927–4939. [PubMed: 26080064]
- (34). <https://www.discoverx.com/services/drug-discovery-development-services/epigenetic-profiling/bromoscan> (Access date: April 12, 2017).
- (35). Breslin HJ; Lane BM; Ott GR; Ghose AK; Angeles TS; Albom MS; Cheng M; Wan W; Haltiwanger RC; Wells-Knecht KJ; Dorsey BD Design, synthesis, and anaplastic lymphoma kinase (ALK) inhibitory activity for a novel series of 2,4,8,22-tetraazatetracyclo[14.3.1.1^{3,7}.1^{9,13}]docosa-1(20),3(22),4,6,9(21),10,12,16,18-nonaene macrocycles. *J. Med. Chem* 2011, 55, 449–464. [PubMed: 22172029]
- (36). Showalter HD; Bridges AJ; Zhou H; Sercel AD; McMichael A; Fry DW Tyrosine kinase inhibitors. 16. 6,5,6-tricyclic benzothieno[3, 2-d]pyrimidines and pyrimido[5,4-b-] and -[4,5-b]indoles as potent inhibitors of the epidermal growth factor receptor tyrosine kinase. *J. Med. Chem* 1999, 42, 5464–5474. [PubMed: 10639288]
- (37). Jimenez JM; Boyall D; Brenchley G; Collier PN; Davis CJ; Fraysse D; Keily SB; Henderson J; Miller A; Pierard F; Settimo L; Twin HC; Bolton CM; Curnock AP; Chiu P; Tanner AJ; Young S Design and optimization of selective protein kinase C theta (PKCtheta) inhibitors for the treatment of autoimmune diseases. *J. Med. Chem* 2013, 56, 1799–1810. [PubMed: 23398373]
- (38). Li W; Nelson DP; Jensen MS; Hoernner RS; Cai D; Larsen RD; Reider PJ An improved protocol for the preparation of 3-pyridyl- and some arylboronic acids. *J. Org. Chem* 2002, 67, 5394–5397. [PubMed: 12126436]
- (39). Lefoix M; Dailliant JP; Routier S; Merour JY; Gillaizeau I; Coudert G Versatile and convenient methods for the synthesis of C-2 and C-3 functionalised 5-azaindoles. *Synthesis* 2005, 3581–3588.

- (40). de Koning PD; McAndrew D; Moore R; Moses IB; Boyles DC; Kissick K; Stanchina CL; Cuthbertson T; Kamatani A; Rahman L; Rodriguez R; Urbina A; Sandoval A; Rose PR Fit-for-purpose development of the enabling route to crizotinib (PF-02341066). *Org. Process Res. Dev* 2011, 15, 1018–1026.
- (41). Kruger AW; Rozema MJ; Chu-Kung A; Gandarilla J; Haight AR; Kotecki BJ; Richter SM; Schwartz AM; Wang Z The discovery and development of a safe, practical synthesis of ABT-869. *Org. Process Res. Dev* 2009, 13, 1419–1425.
- (42). Ueda S; Ali S; Fors BP; Buchwald SL Me₃(OMe)tBuXPhos: A surrogate ligand for Me₄tBuXPhos in palladium-catalyzed C–N and C–O bond-forming reactions. *J. Org. Chem* 2012, 77, 2543–2547. [PubMed: 22313416]
- (43). Otwinowski Z; Minor W Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* 1997, 276, 307–326.
- (44). McCoy AJ; Grosse-Kunstleve RW; Adams PD; Winn MD; Storoni LC; Read RJ Phaser crystallographic software. *J. Appl. Crystallogr* 2007, 40, 658–674. [PubMed: 19461840]
- (45). Bricogne G B E; Brandl M; Flensburg C; Keller P; Paciorek W; Roversi P; Sharff A; Smart OS; Vonrhein C; Womack TO Grade, BUSTER (version 2.10.0.), Global Phasing Ltd; Cambridge, United Kingdom 2011.
- (46). Emsley P; Lohkamp B; Scott WG; Cowtan K Features and development of Coot. *Acta Crystallogr., Sect. D: Biol. Crystallogr* 2010, 66, 486–501. [PubMed: 20383002]
- (47). Chen VB; Arendall WB; Headd JJ; Keedy DA; Immormino RM; Kapral GJ; Murray LW; Richardson JS; Richardson DC MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr., Sect. D: Biol. Crystallogr* 2010, 66, 12–21. [PubMed: 20057044]
- (48). Ethier SP; Mahacek ML; Gullick WJ; Frank TS; Weber BL Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. *Cancer Res* 1993, 53, 627–635. [PubMed: 8425198]

Figure 1:
Representative, Previously Reported Potent BET Bromodomain Inhibitors

Figure 2.

Antitumor activity of **31** in the MV4;11 acute leukemia xenograft model, with compound **5** included as a control.

Figure 3.
Antitumor activity of **31** in the MDA-MB-231 breast cancer xenograft in mice

Figure 4.

Western blot analysis of the effect of compound **31** on c-Myc, PARP and p21 in the MV4;11 leukemia cells. (A) MV4;11 cells were treated with different concentrations of **31** for 1 h and c-Myc protein was probed by a specific antibody. (B) MV4;11 cells were treated with compound **31** for 24 h, and PARP, cleaved PARP (CL-PARP) and p21 proteins were probed by specific antibodies. β-Actin was used as the loading control.

Figure 5.

Crystal structure of BRD4 BD2 bound to **31** determined at 2.5 Å resolution. (A) BRD4 BD2 is shown as a grey cartoon with its sidechains interacting with **31** shown as sticks (carbon atoms in grey, nitrogens in blue, oxygens in red and sulfurs in gold). **31** is shown as sticks in the same coloring as the protein except carbon atoms are in yellow. (B). The omit Fo-Fc electron density map for compound **31** shown as a green grid contoured at 3σ. (C) Previously determined crystal structure of BRD4 BD2 complexed with **6** overlaid onto the co-crystal structure of BRD4 BD2 complexed with **31**. Panel C is rotating 90° about the x-axis with respect to panel A. Compound **6** is shown as sticks with carbon atoms in cyan.

Scheme 1:
Synthesis of key intermediate **7**

Scheme 2:
Synthesis of common intermediate, **S10**

Scheme 4:
General Suzuki coupling reaction conditions

Table 1.Design of BET inhibitors containing a 9*H*-pyrimido[4,5-*b*]indole tricyclic core structure

	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p>7 (CD55)</p> </div> <div style="text-align: center;"> <p>8 (CD57)</p> </div> </div>				
Compound	BRD4 BD1		BRD4 BD2		
	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)	
1	46.7±4.4	14.9±2.6	42.2±9.1	12.0±3.5	
4	145±22	46.4±6.4	77.5±15.0	23.0±4.2	
5	31.7±7.7	9.0±2.9	226±44	74.8±8.6	
6	75.5±6.2	24.7±1.0	36.5±8.9	12.2±1.6	
7	1125±92	387±46	1760±396	476±86	
8	39.2±11.6	12.0±3.5	26.4±5.3	10.4±1.1	

Table 2.

Design of BET inhibitors targeting the WPF binding pocket using five-membered aromatic rings

	9 (CF231) 10 (CF230) 11 (CD84) 12 (CD85) 13 (CD83)

	14 (CD58) 15 (CD64) 16(CD63) 17 (CD133)			
Compound	BRD4 BD1		BRD4 BD2	
	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)
9	2060±420	655±269	1882±406	641±200
10	393±127	131±50	199±81	57.0±17.3
11	155±47	44.3±13.9	180±37	56.4±11.7
12	165±50	51.9±18.6	108±37	30.6±9.0
13	1456±441	456±150	1139±198	335±25
14	102±40	41.2±16.9	164±44	68.7±21.4
15	51.7±15.0	19.7±8.2	63.5±16.4	20.2±9.2
16	40.9±14.2	18.5±4.0	89.7±18.5	31.2±11.8
17	24.1±5.5	7.0±3.3	11.7±2.0	2.7±0.5

Table 3

Inhibition of cell growth by BET inhibitors in MV4;11 and MOLM-13 cell lines.

Compound	IC ₅₀ (nM)		Compound	IC ₅₀ (nM)	
	MV4;11	MOLM-13		MV4;11	MOLM-13
1	24±19	56±24	24	29	380
4	93±45	241±58	25	15	262
5	162±112	228±52	26	13±8	139±24
6	20±9	66±14	27	>10000	>10000
11	2863	>10000	28	>10000	>10000
12	253±123	612±97	29	2±1	20±4.6
13	1062	9271	30	17±2	69±34
14	182±91	709±116	31	26±5	53±11
15	99±36	233±13	32	28±13	76±45
16	95±30	193±21	33	99±43	220±101
17	6±3	36±2	34	47±11	124±78
18	57	205	35	295±48	656±240
19	190±89	489±9	36	>1000	1140
20	76±31	374±52	37	334	NT
21	6.6±1.6	65±11	38	77	971
22	5.1 ±2.8	51±6	39	69±13	243±23
23	13±6	127±21	40	423	716

Table 4:

BET inhibitors containing [5,6] fused aromatic rings targeting the WPF binding pocket.

	18 (CD101) 19 (CD66) 20 (CD77) 21 (CD80) 22 (CD126) 23 (CD130) 24 (CD138) 25 (CD142) 26 (CD106) 27 (CD151) 28 (CD154)			
Compd	BRD4 BD2		BRD4 BD1	
	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)
12	165±50	51.9±18.6	108±37	30.6±9.0
18	20.3±4.9	4.9±2.2	5.5±2.4	1.1±0.7
19	11.6±3.6	7.0±2.4	22.7±7.3	7.1±2.2
20	12.2±4.2	2.6±0.8	2.2±1.0	< 0.5
21	5.0±1.7	0.7±0.1	1.1±0.5	< 0.5
22	5.6±1.9	0.9±0.3	2.3±1.1	< 0.5
23	10.8±3.0	4.2±1.1	25.9±11.8	9.1±4.3
24	32.4±10.2	9.3±3.8	12.2±4.4	2.8±1.1
25	29.4±.6	9.9±3.3	9.4±2.7	2.8±1.1
26	48.7±17.3	28.0±3.2	26.5±6.1	6.5±0.6
27	147±85	85.1±12.5	74.9±20.7	22.5±6.3
28	> 1000	> 1000	> 1000	> 1000

Table 5.Pharmacokinetics of compounds **21** and **31** in rats and mice

Compound	Species	Route (Dose)	t _{1/2} (h)	C _{max} (ng/mL)	AUC _{0-t} (ngh/mL)	V _{ss} (L/kg)	CL (L/h/kg)	F (%)
21	Rat	PO (25 mg/kg)	1.7	1396.7±277.4	13444.7±933.4	-	1.9	
31	Rat	IV (5 mg/kg)	2.4	-	6343	2.0	0.79	-
	Rat	PO (25 mg/kg)	2.9	7333	29547	-	0.88	93%
	Mouse	IV (5 mg/kg)	0.5	-	3688.8	2.75	1.36	-
	Mouse	PO (25 mg/kg)	1.60	983.1	9135	-	-	50%

Table 6.

Microsomal stability of selected BET inhibitors.

Compound	Microsomal Stability $T_{1/2}$ (min)		
	Mouse	Rat	Human
21	4	>60	8
29	>60	>60	9
30	>60	>60	>60
31	>60	>60	>60

Table 7.

BET Inhibitors containing indole or quinoline to target the WPF pocket.

R =	29 (CD134)	30 (CD179)	31 (CD161)	32 (CD178)	33 (CD180)	34 (CD186)
		35 (CD195)	36 (CD196)	37 (CD202)	38 (CD204)	39 (CD218)	40 (CD173)
Compound	BRD4 BD2		BRD4 BD1				
	IC ₅₀ (nM)	K _i (nM)	IC ₅₀ (nM)	K _i (nM)			
29	1.9±0.7	< 0.5	6.1±1.4	0.8±0.1			
30	5.2±1.4	1.6±0.5	19.7±7.5	5.7±3.6			
31	7.2±1.9	1.4±0.4	28.2±4.4	8.2±1.2			
32	15.5±4.7	3.1±1.0	30.9±7.6	10.1±4.0			
33	36.3±6.9	10.5±1.9	93.8±15.8	38.4±10.5			
34	12.7±5.9	3.8±0.9	35.6±8.8	9.5±2.4			
35	436±219	146±56	451±128	139±32			
36	167±55	52.3±17.1	316±86	78.6±12.6			
37	193±53	58.2±12.6	412±126	129±24			
38	92.7±36.7	26.9±12.6	393±115	122±34			
39	14.6±6.3	4.2±1.4	73.1±12.9	31.8±5.9			
40	128±27	42.6±6.9	397±47	136±12			

Table 8.

Binding affinities of **31** to different BET proteins and selectivity over other bromodomain-containing proteins, as determined using the DiscoverX BROMO Scan platform

Protein	K_d (nM)	Protein	K_d (nM)	Protein	K_d (nM)
BRD2 (BD1)	4.5	CREBBP	520	BRPF1	>4000
BRD2 (BD2)	0.9	CECR2	1600	BRPF3	>4000
BRD2 (BD1-BD2)	1.1	EP300	550	FALZ	>4000
BRD3 (BD1)	2.0	ATAD2A	>4000	GCN5L2	>4000
BRD3 (BD2)	0.69	ATAD2B	>4000	PBRM1(2)	>4000
BRD3 (BD1-BD2)	0.73	BAZ2A	>4000	PBRM1(5)	>4000
BRD4 (BD1)	8.4	BAZ2B	>4000	PCAF	>4000
BRD4 (BD2)	2.0	BRD1	>4000	SMARCA2	>4000
BRD4 (BD1-BD2)	1.8	BRD7	>4000	SMARCA4	>4000
BRDT (BD1)	15	BRD8 (BD1)	>4000	TAF1 (BD2)	>4000
BRDT (BD2)	5.5	BRD8 (BD2)	>4000	TRIM24	>4000
BRDT (BD1-BD2)	9.7	BRD9	>4000	WDR9 (BD2)	>4000

Table 9.Inhibition of cell growth by compound **31** in a panel of breast cancer cell lines

Cell Line	Subtype	IC ₅₀ (nM)	Cell Line	Subtype	IC ₅₀ (nM)
MDA-MB-157	Basal B	39	HCC1954	Basal A	1604
MDA-MB-453	Luminal	188	HCC70	Basal A	1623
MDA-MB-361	Luminal	189	MDA-MB-436	Basal B	1698
MDA-MB-231	Basal B	244	MDA-MB-468	Basal A	1018
HCC38	Basal B	285	BT-20	Basal A	1713
HCC1428	Luminal	319	SUM-159PT	Basal B	>2000
BT-474	Luminal	434	HBL100	Basal-B	>2000
BT-549	Basal B	410	HCC1143	Basal A	>2000
SUM-149PT	Basal B	601	HCC1937	Basal A	>2000
HCC1395	Basal B	1233			