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BRD4 Structure–Activity Relationships of Dual PLK1 Kinase/BRD4 Bromodomain Inhibitor BI-2536

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KEYWORDS bromodomain; BRD; BET; protein–protein interaction; kinase; BI-2536

ABSTRACT: A focused library of analogues of the dual PLK1 kinase/BRD4 bromodomain inhibitor BI-2536 was prepared and then analyzed for BRD4 and PLK1 inhibitory activities. Particularly, replacement of the cyclopentyl group with a 3-bromobenzyl moiety afforded the most potent BRD4 inhibitor of the series (**39j**) with a K_i = 8.7 nM, which was equipotent against PLK1. The superior affinity of **39j** to BRD4 possibly derives from improved interactions with the WPF shelf. Meanwhile, substitution of the pyrimidine NH with an oxygen atom reversed the PLK1/BRD4 selectivity to convert BI-2536 into a BRD4-selective inhibitor, likely owing to the loss of a critical hydrogen bond in PLK1. Most compounds inhibited growth of the acute myeloid leukemia cell line MV4-11 with sub-micromolar GI_{50} values. We believe further fine-tuning will furnish a BRD4 "magic bullet" or an even more potent PLK1/BRD4 dual inhibitor towards the expansion and improved efficacy of the chemotherapy arsenal.

Cancer remains one of the most challenging pathologies of our time owing to its manifestation through the aberrant regulation of multiple signaling pathways; for example, the upregulation of the anti-apoptotic Bcl-2 proteins and the dysregulation of various kinases.[1] A conventional strategy to block cancer cell growth is to inhibit at least one of the proteins involved in these pathways. However, whilst target-selective inhibitors may function well in vitro, the upregulation of compensatory signaling pathways often compromises their efficacies in cells. Rationally designed polypharmacology, in which a single synthetic agent is fashioned to recognize several key biological targets, carries the promise to deliver drugs that are more efficacious in cells, as well as overcome the drawbacks associated with multi-drug regimens.[2] Imatinib, like most of the other FDA-approved kinase inhibitors, owes much of its success to targeting multiple kinases involved in tumor development and progression.[3] Given the efficacy of imatinib, a notion that is gaining much traction is that the next generation of anti-cancer agents should go one stage further and target multiple protein targets of *different* families that are involved in tumorigenesis.[4]

Polo-like kinase 1 (PLK1) and bromodomain 4 (BRD4) are both drivers in acute myeloid leukemia (AML).[5,6] They are also intricately involved in mitosis.[7,8] Their dual inhibition by a single drug molecule might provide the platform for a new strategy to treat AML as well as various other cancers. Recently, the laboratories of Knapp and Schönbrunn independently discovered that the PLK1 inhibitor BI-2536 (**1**) is also a potent inhibitor of BRD4, which was confirmed through an Alpha Screen (IC_{50} = 25 nM),[9] isothermal titration calorimetry (K_d = 37 nM),[10] and co-crystal structures.[9,10] The BRD proteins recognize ε-*N*-acetylated lysines of histones and have been dubbed epigenetic "readers".[11] The bromo and extra-terminal domain (BET) proteins of the BRD family (BRD2, BRD3, BRD4 and BRDT) have recently emerged as druggable targets for the development of new anti-cancer agents owing to their roles in the transcriptional regulation of genes involved in tumour development (e.g. *c-MYC*) and survival (e.g. *BCL2*).[12-14] Particularly, since the BET proteins regulate the transcription of c-Myc, their inhibition provides an alternative and indirect strategy to counter tumorigenesis, which is urgently needed given the difficulties associated through targeting c-Myc with synthetic agents.[15–21]

Herein, we describe a focused structure–activity relationship (SAR) study of BI-2536 (**1**) against BRD4. Our findings hint at possible strategies to enhance the dual inhibitory activity of BI-2536 as well as to render the ligand more selective for BRD4 over PLK1. The ability to fine tune a ligand in this way may help in the expansion of personalized medicines, and, more generally, may assist in the delineation of biochemical pathways targeted by other drugs with polypharmacological profiles.

The crystal structure of BI-2536 bound to the first bromodomain of BRD4, BRD4(1), is shown in Figure 1 (PDB ID: 4OGI[11]). The methylated amide of BI-2536 functions as a

mimetic of ε-*N*-acetylated lysine wherein the carbonyl forms a water-mediated hydrogen bond with the side chain amide of N140 while the methyl group is directed into a hydrophobic sub-pocket formed from F83, M132 and C136. The aniline NH and one of the pyrimidine nitrogen atoms bind the backbone amide of Q85 also through an intermediary water molecule. The ethyl group is projected into a small hydrophobic sub-pocket (V87/L92/L94/Y97), while the cyclopentyl moiety and the *N*-methylpiperidine point to the solvent. Close inspection of the binding site indicates that replacement of the amide methyl group with a slightly larger ethyl or isopropyl might enhance binding affinity to BRD4; large groups, such as benzyl, conversely, are not expected to be accommodated here. Introduction of a kink into the cyclopentyl group might allow it to interact better with the WPF shelf (W81, P82, F83). Finally, elaboration of the 3-methoxy group is predicted to improve binding affinity through interactions with L92 and/or L94. One of the pyrimidine nitrogens and the aniline NH form critical hydrogen bonds with C133 in PLK1 (PDB ID = 2RKU). As suggested by Knapp *et al.*, substitution of this NH with NMe will result in the loss of a hydrogen bond donor and a concomitant reduction in PLK1 binding affinity.[10] At the same time, this is expected to have limited impact on the inhibition of BRD4 since this group points to the solvent. Thus, towards the optimization of the BRD4 inhibitory activity of BI-2536, the methyl, ethyl and cyclopentyl moieties were varied, as was the 3-methoxy group. Substitution of the aniline NH with an oxygen atom should render this fragment incapable of functioning as a hydrogen bond donor, and was, therefore, predicted to furnish BRD4 selectivity. The aminopiperidine moiety was maintained to assist with compound solubility.

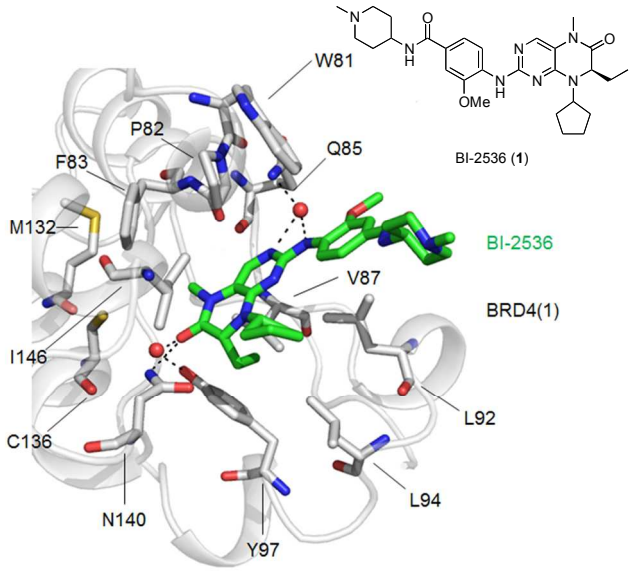
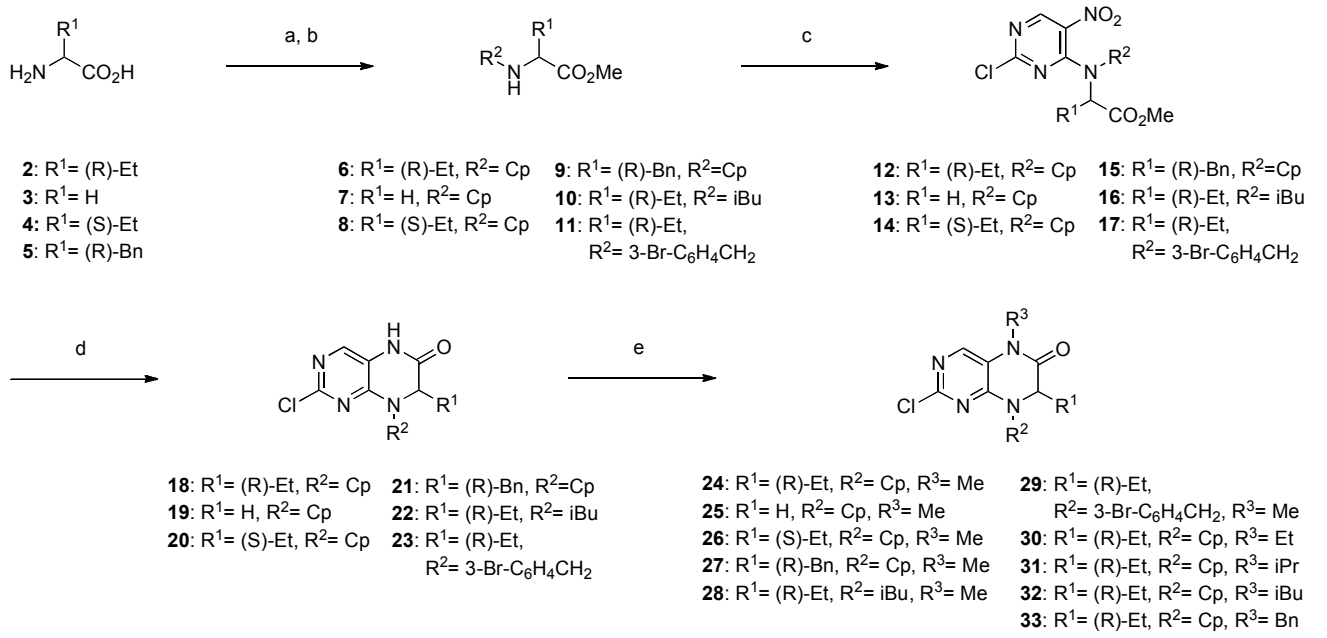


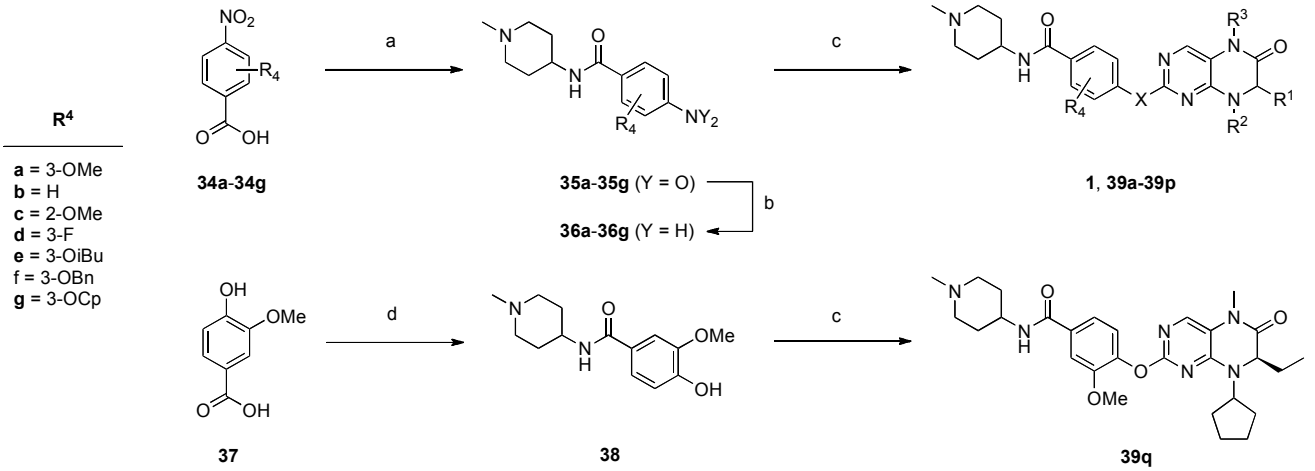
Figure 1. Crystal structure of BI-2536 bound to BRD4(1) (PDB ID = 4OGI).[10] Key side chains of BRD4(1) are shown in stick format and colored by atom type. BI-2536 is shown in stick format and colored by atom type (green = carbon).

Accordingly, we prepared a focused library of analogues of BI-2536 by adapting a previously described synthesis of BI-2536.[22] Briefly, the appropriate amino acids **2–5** were first esterified and then *N*-alkylated by reductive amination to deliver compounds **6–11**. Regioselective nucleophilic aromatic substitutions (S_NAr) of 2,6-dichloro-3-nitropyrimidine furnished tertiary anilines **12–17**, wherein the nitro group directed *ortho* attack of amines **6–11** through an intramolecular, hydrogen-bonded, six-membered transition state.[23,24] The dihydropteridinone scaffold was next constructed by reductive heterocyclizations of **12–17** into **18–23**, respectively, with iron powder in hot AcOH. Introduction of the R^3 group was achieved by deprotonation of the anilide NH followed by quenching with various alkyl iodides and bromides to yield the 2-chloropyrimidines **24–33**. Meanwhile, to complete the synthesis of BI-2536 and its analogues, the requisite anilines were prepared as in Scheme 2. Briefly, various 4-nitrobenzoic acids (**34a-g**) were activated by HBTU and then coupled to 4-amino-1-methylpiperidine to deliver amides **35a-g**, which were subsequently reduced with tin (II) chloride ($SnCl_2$) to yield anilines **36a-g**. Phenol **38** was prepared by the condensation of vanillic acid (**37**) with 4-amino-1-methylpiperidine. Finally, HCl-assisted S_NAr of the 2-chloropyrimidines **24–33** with anilines **36a-g** and phenol **38** furnished the lead compound BI-2536 (**1**) and its analogues **39a–q**, whose structures are given in full in Tables 1 and 2.

Table 1 shows the derivatives with variations of substituents on the dihydropteridinone ring along with associated BRD4 and PLK1 inhibitory activities. The data is presented as K_i values, which were obtained with BROMOscan® and KINOMEscan®, DiscoveRx's proprietary ligand binding competition assays that measure interactions between test compounds and bromodomains or kinases, respectively. We obtained similar binding data for the parent compound BI-2536 to that reported previously for BRD4[9,10] and PLK1.[25] It is unsurprising that BI-2536 is >250-fold more selective for PLK1 given the extensive optimization that led to its discovery.[25] Replacement of the CONMe group with a CONH group reduced its activity by almost 150-fold, supporting its functional role as a mimetic of ε-*N*-acetylated lysine moieties. Interestingly, although the binding pocket that accommodates this methyl group appears large enough to allow occupancy by slightly larger groups such as ethyl and isopropyl, all groups bigger than methyl resulted in weaker inhibitors. For example, the K_i values for compounds with R^3 = Me, Et, iPr, Bn were 56, 470, 4500 and >10,000 nM, respectively. Although still potent inhibitors, increasing the size of the R^3 group was not tolerated by PLK1, either.



Scheme 1. (a) $SOCl_2$, MeOH, 0 °C to reflux, 16 h; (b) cyclopentanone, isobutyraldehyde or 3-bromobenzaldehyde, $NaBH(OAc)_3$, NaOAc, CH_2ClCH_2Cl, RT, 16 h; (c) 2,4-dichloro-5-nitropyrimidine, K_2CO_3, acetone, RT, 16 h; (d) Fe, AcOH, 70 °C, 1 h, 100 °C, 4 – 5 h; (e) alkyl iodide or benzyl bromide, NaH, DMF, 0 °C to RT, 3 h.



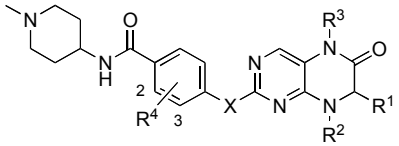
Scheme 2. (a) 4-amino-1-methylpiperidine, HBTU, Et_3N, DMF, RT, 16 h. (b) $SnCl_2 \cdot 2H_2O$, EtOAc/ethanol, 10:3, 50°C, 16 h; (c) **24**–**33**, c. HCl, EtOH/dioxane/H_2O, 1:1:1, 100 °C, 24 – 48 h; (d) 4-amino-1-methylpiperidine, EDCI•HCl, HOBt•H_2O, Et_3N, CH_3CN, RT, 16 h.

In the co-crystal structure of BRD4(1)–BI-2536, the ethyl group at the R[1] position points into the pocket formed by V87, L92, L94 and Y97. Changing the stereochemistry from *R* to *S* did not affect the binding affinity to BRD4 (compare **1** with **39g**) but resulted in a two-fold drop in potency against PLK1. With respect to BRD4 inhibition, the *S*-Et group can point into a pocket on the other side of V87/L92/L94/Y97 formed by P82, F83 and I146, and this accounts for the subtle effect of the chirality change. Deleting the ethyl group afforded a reduction in BRD4 inhibitory activity of 26-fold (**39f**: K_i = 1400 nM), whilst replacing it with a bulky *R*-benzyl group (compound **39h**) completely abolished binding. Together, these data indicate that a small hydrophobic group is optimal here, consistent with the crystal structure. In PLK1 (PDB ID = 2RKU), the *R*-Et group is surrounded by L59, C67, A80, K82 and L130. Similar to BRD4, this pocket is largely tolerant of the chirality change but cannot accommodate a larger benzyl group.

Whilst modifying the R[1] and R[3] groups exhibited only detrimental impacts on BRD4 binding, changing the R[2] group from cyclopentyl to 3-bromobenzyl increased the BRD4 binding affinity by 7-fold (**39j**: K_i = 8.7 nM) although a bulkier isobutyl group (**39i**) was not tolerated. The potent binding affinity of **39j** places it amongst the most active BRD4 inhibitors known,[14] and might be explained by hydrophobic interactions with the WPF shelf.

Notably, compound **39j** bound PLK1 25-fold weaker than BI-2536, such that affinities for BRD4 and PLK1 had become effectively equipotent. Taken together, these data indicate that the R^2 group appears to be a dictator of selectivity, and further optimization here might furnish a selective, sub-nanomolar BRD4 inhibitor.

Table 1. Structure–activity relationships of the R^1, R^2, R^3, R^4 and X groups. Cp = cyclopentyl.



Compound	R^1	R^2	R^3	R^4	X	BRD4 K_i (nM)[a]	PLK1 K_i (nM)[a]
BI-2536	(R)-Et	Cp	Me	3-OMe	NH	56 ± 9	0.22 ± 0.01
39a	(R)-Et	Cp	H	3-OMe	NH	7950 ± 1550	ND
39b	(R)-Et	Cp	Et	3-OMe	NH	470 ± 10	0.91 ± 0.13
39c	(R)-Et	Cp	iPr	3-OMe	NH	4500 ± 300	ND
39d	(R)-Et	Cp	iBu	3-OMe	NH	>10000	ND
39e	(R)-Et	Cp	Bn	3-OMe	NH	>10000	ND
39f	H	Cp	Me	3-OMe	NH	1400 ± 400	ND
39g	(S)-Et	Cp	Me	3-OMe	NH	54 ± 4	0.42 ± 0.04
39h	(R)-Bn	Cp	Me	3-OMe	NH	>10000	4.20 ± 0.99
39i	(R)-Et	iBu	Me	3-OMe	NH	970 ± 30	6.95 ± 1.63
39j	(R)-Et	3-Br-$C_6H_4CH_2$	Me	3-OMe	NH	8.7 ± 1.3	5.80 ± 0.99
39k	(R)-Et	Cp	Me	H	NH	60 ± 7	ND
39l	(R)-Et	Cp	Me	2-OMe	NH	37 ± 5	ND
39m	(R)-Et	Cp	Me	3-F	NH	46 ± 2	ND
39n	(R)-Et	Cp	Me	3-OiBu	NH	29 ± 9	ND
39o	(R)-Et	Cp	Me	3-OBn	NH	100 ± 0	ND
39p	(R)-Et	Cp	Me	3-OCp	NH	27 ± 8	0.84 ± 0.01
39q	(R)-Et	Cp	Me	3-OMe	O	99 ± 11	240 ± 14

[a]Data represent the mean and standard deviation of two independent experiments.

Shifting the 3-methoxy group to the 2-position (compound **39l**) did not have a significant impact on the binding to BRD4. Moreover, its deletion or replacement with a fluorine atom also had little effect. Substitution of the 3-methoxy group with bulkier isobutoxy (**39n**) and cyclopentyloxy (**39p**) moieties afforded a 2-fold enhancement in binding affinity to BRD4, which may be due to improved contacts with L92 and/or L94, with a concomitant 4-fold reduction in binding affinity to PLK1, as predicted.[10] Akin to our findings with the R^2 group, careful modification of the R^4 group might provide further improvements to BRD4 inhibitory activity. One of the most striking observations of our SAR work is seen when comparing the in vitro data for BI-2536 with **39q** wherein substitution of the pyrimidine NH with an isosteric O afforded more than a 1000-fold drop in activity against PLK1, yet only resulted in a 2-fold reduction in activity against BRD4. This result is consistent with the NH engaging in a critical hinge interaction with C133 as a hydrogen bond donor in the active site of PLK1, an interaction that is lost upon its replacement with an oxygen atom; this group points to the solvent in BRD4 and so a limited effect on binding affinity was observed. The dramatic impact on binding profiles indicates that the isosteric identity here is a key determinant of ligand specificity, and may be exploited towards achieving BRD4 selectivity. Indeed, the data for **39q** implies that the PLK1 selective inhibitor BI-2536 has been transformed into a BRD4 selective inhibitor.

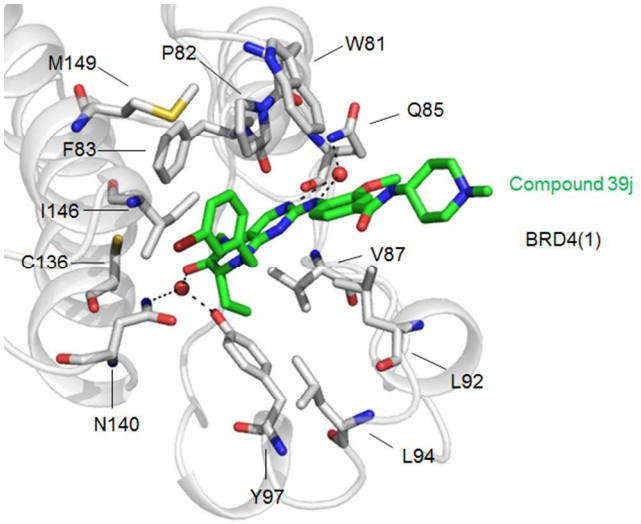


Figure 2. A high scoring, GOLD-predicted docking mode of compound **39j** in BRD4(1). Compound **39j** shown in stick format and colored by atom type (green = carbon).

To gain a better appreciation of how our most potent compound **39j** likely binds BRD4(1), we employed molecular modeling with GOLD. Figure 2 shows a GOLD high scoring docking pose of the most potent BRD4 inhibitor compound **39j** bound to BRD4. Comparing to Figure 1, compound **39j** and BI-2536 bind to BRD4 in a very similar pattern, and form largely identical interactions with BRD4. Structurally, the only difference between these two molecules is the replacement of the cyclopentyl group in BI-2536 with a 3-bromobenzyl moiety. In BI-2536, the cyclopentyl group does not appear to be involved in any notable interactions with BRD4 as it is directed out into the solvent. In contrast, the installation of the benzyl CH_2 in **39j** provides the extra flexibility to reach out to the WPF shell (W81, P82, and F83), possibly accounting for the 7-fold improvement in activity. Furthermore, the bromine atom potentially forms a hydrophobic interaction with I146, which might also explain the greater binding affinity for **39j**. Therefore, future investigation into the R^2 position with flexible and hydrophobic arylmethyl groups may lead to more potent BRD4 inhibitors.

Compounds were also analyzed in a CellTiter-Blue® cell viability assay with the MV4-11 cell line, which has an acute myeloid leukemia cell line that expresses high levels of BRD4 and PLK1. As positive controls, the BRD4-selective inhibitor (+)-JQ-1 and the PLK1-selective inhibitor GSK-461364 were used. The growth inhibition (GI_{50}) data is shown in Table 2, which represents the concentration of small-molecule that elicits 50% growth inhibition of the MV4-11 cells. The overall trend of *in vitro* data is mirrored by the cell data, supporting the conclusion that cell death is through inhibition of BRD4 and PLK1. There are a few anomalous results that require discussion. The cell data for **39j** and **39p** are less potent, while **39i** is more potent than would be anticipated given their *in vitro* data. Considering its polypharmacological profile, it is possible that BI-2536, and its analogues, bind other targets in addition to BRD4 and PLK1, such as PLK2 and PLK3,[25] which may account for the imperfect correlation between the *in vitro* and cell data.

Table 3. MV4-11 cell viability exposed to BI-2536 analogues.

Compound	MV4-11, GI50 (μM)[a]	Compound	MV4-11, GI50 (μM)[a]
BI-2536	0.0152	**39h**	1.87
(+)-JQ-1	0.08	**39i**	0.04
GSK-461364	0.679	**39j**	0.675
39a	0.219	**39k**	0.003
39b	0.392	**39l**	0.015
39c	2.67	**39m**	0.004
39d	2.53	**39n**	0.154
39e	7.28	**39o**	0.356
39f	2.19	**39p**	0.240
39g	0.019	**39q**	1.34

[a]Compound concentration that elicits 50% growth inhibition of MV4-11 cells.

In summary, building on the initial discovery by Knapp and Schönbrunn, an SAR campaign of the PLK1 inhibitor BI-2536 has revealed opportunities for its continued optimization as a dual inhibitor of PLK1 and BRD4, as well as a selective inhibitor of the latter. The impacts of structural modifications were largely in agreement with predictions made based on the PDB of the BRD4(1)–BI-2536 co-crystal structure. We believe that combining further modifications to the R^2 and R^4 groups, as well as switching the pyrimidine NH to O, could provide selective, subnanomolar BRD4 inhibitors. At the same time, we anticipate it will be feasible to enhance BRD4 inhibitory activity yet maintain dual PLK1/BRD4 inhibitory activity through careful selection of the R^4 side chain whilst retaining, most critically, the pyrimidine NH group, as well as the cyclopentyl group and the *R*-stereochemistry of the ethyl group. More generally, it is considered that drugs with polypharmacological profiles may likewise be optimized for inhibitory activities against specific deconvoluted targets, which may assist in a better understanding of the biochemical pathways targeted within cells.

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures and assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

BRD4, bromodomain 4; BET, bromodomain and extra-terminal domain; PLK1, polo-like kinase 1.

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