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Inhibition of bromodomain-containing protein 9 for the prevention of epigenetically-defined drug resistance

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ABSTRACT

Bromodomain-containing protein 9 (BRD9), an epigenetic "reader" of acetylated lysines on post-translationally modified histone proteins, is upregulated in multiple cancer cell lines. To assess the functional role of BRD9 in cancer cell lines, we identified a small-molecule inhibitor of the BRD9 bromodomain. Starting from a pyrrolopyridone lead, we used structure-based drug design to identify a potent and highly selective in vitro tool compound **11**, (**GNE-375**). While this compound showed minimal effects in cell viability or gene expression assays, it showed remarkable potency in preventing the emergence of a drug tolerant population in EGFR mutant PC9 cells treated with EGFR inhibitors. Such tolerance has been linked to an altered epigenetic state, and **11** decreased BRD9 binding to chromatin, and this was associated with decreased expression of ALDH1A1, a gene previously shown to be important in drug tolerance. BRD9 inhibitors may therefore show utility in preventing epigenetically-defined drug resistance.

Bromodomains are a series of 61 different ~110 amino-acid protein modules that recognize acetylated lysine residues on histones as well as on other proteins.[1–7] Significant progress has been made in elucidating the biological function of the bromodomain and extra terminal domain (BET) family consisting of dual bromodomains in bromodomain-containing protein 2 (BRD2), bromodomain-containing protein 3 (BRD3), bromodomain-containing protein 4 (BRD4), and bromodomain testis-specific protein (BRDT).[8,9] However, little is known regarding the biological role of the other bromodomain family members. Because of this, it is important to identify potent and selective bromodomain inhibitors for cellular phenotypic screening in order to elucidate the therapeutic significance of targeting bromodomains for oncology, immunology, and inflammatory diseases. Bromodomain-containing protein 9 (BRD9) is one such non-BET bromodomain, and inhibitors of BRD9 have been disclosed recently.[10–13] Herein we report our discovery of novel inhibitors of BRD9.

While the function of BRD9 is unknown, over-expression has been observed for several disease tissues. The gene encoding BRD9 is located on the 5p arm of chromosome 5 and is over-expressed in cervical cancer as well as non-small cell lung cancer.[14,15] Proteomic analysis has also identified BRD9 as a dedicated member of the mammalian SWI/SNF complex, which has been postulated as being involved in tumor suppression.[16] Finally, Hohmann, et al. have disclosed that BRD9 may play a role in hematopoietic cancers and have used a combination of protein engineering and inhibitor studies to validate the bromodomain as a target.[17]

To further understand the functional role of BRD9, we sought an in vitro BRD9 bromodomain inhibitor tool compound. Our goal was to achieve <100 nM cellular potency against BRD9, while maintaining at least 100-fold selectivity over bromodomain family members in our internal panel of fluorescence resonance energy transfer (FRET) assays. We also sought BRD4 FRET activity > 10 μM to ensure that any phenotypic readout at full BRD9 target coverage would not be obscured by the broad cellular activities observed for

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BET inhibitors. The BRD4 bromodomains served as a surrogate for all BET bromodomains due to the high degree of sequence identity among BET family members.

We recently reported the identification of compound **1**, a fragment-derived BRD9 small-molecule lead (Fig. 1).[18] Importantly, this molecule incorporates a highly ligand-efficient pyrrolopyridone core that forms a two-point hydrogen bonding interaction with the conserved asparagine N100 (PDB Code: 5I7Y, 1.45 Å).[18] The *N*-crotyl substituent extending from the pyridone induces a hydrophobic channel adjacent to the conserved binding-pocket water network and conveys significant selectivity for BRD9 over the majority of the bromodomain family.

Lead-optimization efforts targeted substitution along multiple vectors from compound **1**. Structural analysis of **1** bound to BRD9 indicated significant accessible volume extending from the para position of the benzamide deeper into the ZA channel. Also, we believed there was an opportunity to occupy a small pocket formed by the Gly43-Phe44-Phe45 lipophilic shelf in BRD9 by introduction of small substituents at either the meta or ortho position of the benzamide. While potentially beneficial to BRD9 potency, we believed substitution would also result in an unfavorable interaction with the corresponding WPF shelves found in CECR2, TAF1(2), and BET bromodomains, and thereby improve the selectivity profile. It also appeared possible to introduce functionality at the pyrrolopyridone 2-position, extending toward the BC loop region with a small aliphatic substituent.

As we probed SAR on the 4-phenyl ring, we found that polar substituents at the para position such as 2-propanol **2** or morpholine amide **5** both improved BRD9 potency, while also providing increased selectivity over all bromodomains screened (Table 1). We believe that this may be the result of the ability of the polar functional groups to interact with solvent in the relatively large binding pocket found in BRD9.

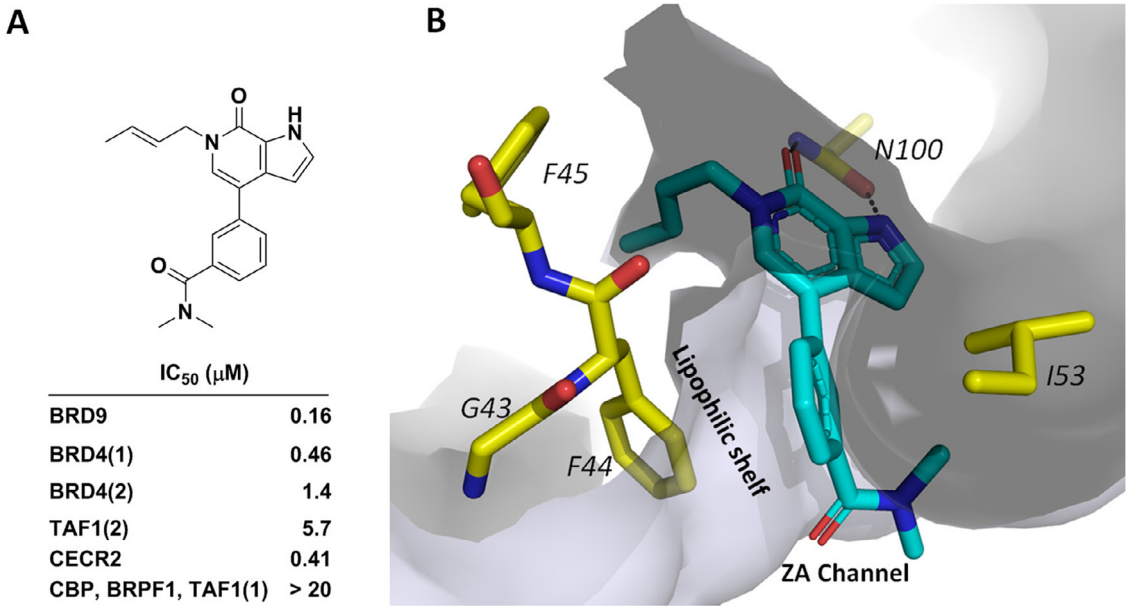
We next incorporated a methoxy group at the ortho position of compound **2**, as this would rest in a low energy conformation planar to the phenyl ring and effectively fill the available volume adjacent to the BRD9 lipophilic shelf region. While the terminal methyl may be beneficial for potency, desolvation of the ether oxygen in this lipophilic region may account for the overall BRD9 potency remaining unchanged. We also hypothesized that the methoxy group would create a steric clash with bromodomains containing the more rigid WPF shelf (TAF1, BRD4, CECR2) and therefore increase the selectivity for BRD9. Indeed, we found the selectivity window for **3** increased at least 5 to 7-fold over most of our internal bromodomain panel. BRD4(1) was an exception, showing only a modest selectivity gain. We believe the retained potency for BRD4(1) may be due to the methoxy group being directed away from the BRD4(1) WPF shelf and into the ZA channel, therefore avoiding the steric clash and subsequent potency loss.

The level of cellular engagement by these early lead compounds was assessed by visualization of the displacement of an inducible ZsGreen fusion protein localized to chromatin that forms aggregation "dots" when displaced.[19] These dots can be quantified in a dose-dependent manner to enable EC_{50} determination (Table 1). We found that both the 2-propanol (**3**) and morpholine amide (**5**) substituents have sub-micromolar cellular affinity, making them attractive candidates for initial phenotypic screening.

We profiled **3** and **5** in a 3-day viability assay across a panel of 652 cancer cell lines representing 32 distinct indications. Activity was largely restricted to a subset of lines derived from blood and lymph node (Fig. 2A,B, Supporting Information Tables S1 and S2). Analysis of **3** in a subset of the full panel (125 lines) in a longer 8-day assay did not reveal any additional sensitivities (Fig. 2C, Supporting information Table S3).

To extend assay conditions beyond conventional 2-dimensional growth, we applied **3** to cells plated at low density and allowed to grow for 9 days into single colonies (clonogenic assays). We analyzed 18 cell lines from skin, lung, breast and pancreatic cancers, and noted that two cell lines (PC9 and HCC1954) showed modest sensitivity to **3**, albeit only at high concentrations (Fig. 3A). This is in contrast to sensitivity to the Brd4 inhibitor JQ1,[20] which is extremely potent in this assay (Fig. 3B).

To further extend phenotypic assays to those with known dependence on epigenetic alterations, we tested the ability of BRD9 inhibitors to affect the drug tolerant state of cells treated with growth inhibitory compounds. This drug tolerant population (DTP) is dynamic, transient, and mediated by differences in chromatin state relative to the untreated parental population.[21]



IC_{50} (μM)	
BRD9	0.16
BRD4(1)	0.46
BRD4(2)	1.4
TAF1(2)	5.7
CECR2	0.41
CBP, BRPF1, TAF1(1)	> 20

Fig. 1. A) Profile of **1** in biochemical bromodomain binding assays (TR-FRET). IC_{50} values are the average from at least 2 independent experiments B) Compound **1** (cyan) bound to BRD9 (residues in yellow). PDB Code: 5I7Y, 1.45 Å.