Chiral Analogs of PFI-1 as BET Inhibitors and Their Functional Role in Myeloid Malignancies

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ABSTRACT: Structural analogs of PFI-1 varying at the sulfur core were prepared and their activity as BET inhibitors in myeloid cell lines and primary cells from patients with acute myeloid leukemia (AML) was studied. Docking calculations followed by molecular dynamics (MD) simulations revealed the binding mode of the newly prepared inhibitors suggesting explanations for the observed high enantiospecificity of the inhibitory activity.

The bromodomain and extra-terminal (BET) motif protein BRD4 was first linked to cancer after the discovery of the BRD4-NUT (nuclear protein in testis) gene translocation resulting in NUT midline carcinoma, which is a very aggressive squamous cell carcinoma occurring primarily in adolescents.[1] Importantly, clinical responses were seen in selected patients, using BET inhibitors that targeted BRD4.[2,3]

BET inhibitors are structurally diverse.[4-8] For example, **1** [(+)-JQ1][9] and **2** (OTX-015, MK8628, or Birabresib)[10] are triazolodiazepines showing potencies in the nanomolar range with pronounced selectivities in the BET family. Following work by Conway and co-workers[11] and Prinjha and co-workers,[12] Pfizer introduced 3,4-dihydro-3-methyl-2(1*H*)-quinazolinone **3** (PFI-1) as BRD4 selective BET inhibitor.[13] The latter compound caught our attention because we hypothesized that its sulfonamidoyl group could be modified by (formal) atom exchange reactions leading to unprecedented sulfoximines **4** and sulfondiimides **5** (Chart 1).[14-16] In other systems, such sulfur core modifications had led to products with improved properties, including, for example, increased water-solubility.[17,18] Furthermore, contrasting **3**, compounds **4** and **5** are chiral, allowing studies of individual enantiomers.[19] Here, we report on the preparation of these compounds and their characterization as new BET inhibitors in myeloid cell lines and primary cells from patients with acute myeloid leukemia (AML).

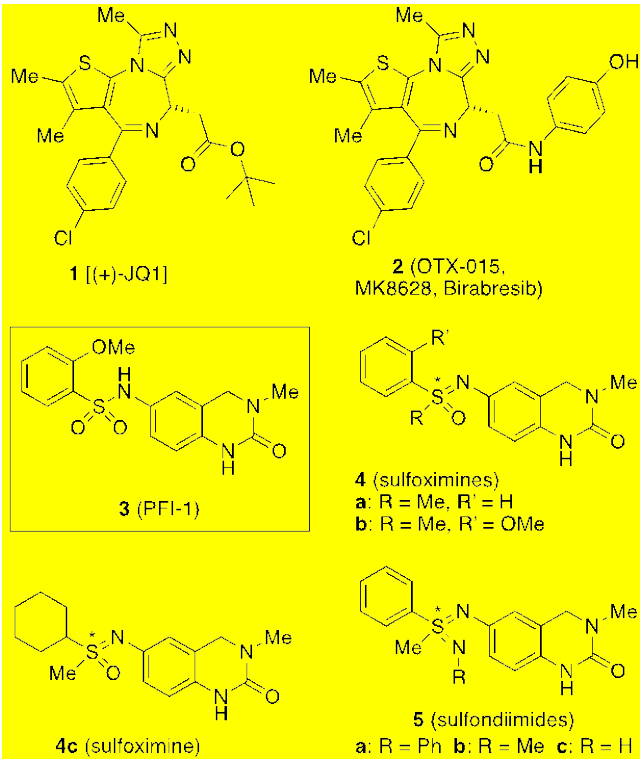
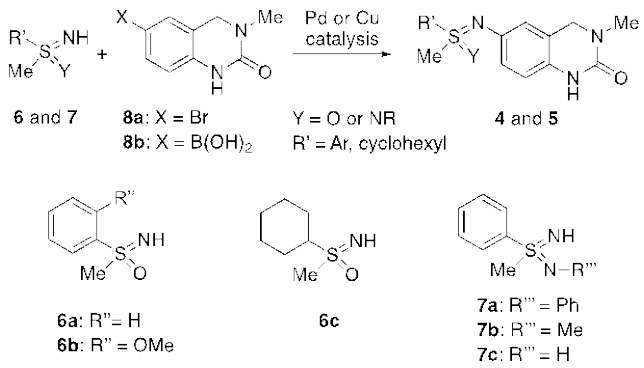


Chart 1. Known BET inhibitors **1**–**3** and newly prepared compounds **4** and **5**.

The syntheses of compounds **4** and **5** are summarized in Scheme 1. In general, they involved metal-catalyzed Buchwald/Hartwig-[20] or Chan/Lam-type cross coupling reactions[21] of 3,4-dihydro-3-methyl-2(1*H*)-quinazolinones **8a** and **8b** with respective *N*H-sulfur derivatives (for details, see Supporting Information). The latter were prepared by standard synthetic

protocols leading to *N*-unfunctionalized sulfoximines **6**,[22,23] and sulfondiimides **7**.[24] Enantiopure compounds **4** and **5a** were obtained by preparative CSP-HPLC (for **4c** and **7a**),



Scheme 1. Cross coupling partners.

resolution of diastereomeric salts (for **6a**), and asymmetric synthesis (for **6b**).

Since the initial description of the BRD4-NUT fusion, BRD4 has been examined in a variety of cancer types and found to be a potential therapeutic target in AML and myeloproliferative neoplasms (MPN).[25,26] AML is a heterogeneous disease, characterized by a differentiation block and uncontrolled proliferation of hematopoietic stem and progenitor cells.[27,28] Approximately 97% of patients harbor clonal somatic abnormalities, including mutations in the FMS-like tyrosine kinase 3 (FLT3) in up to 38%.[29] Two main variations of mutations are described: the more frequent internal-tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations.[27] Recently, the FLT3 inhibitor midostaurin has been approved as targeted therapy in combination with chemotherapy for the treatment of FLT3-mutant AML, based upon a phase 3 clinical.[30] However, additional mutations increase the risk of therapy failure in FLT3-mutant and other subtypes of AML.

The BET motif family consists of four members in mammals: BRD2, BRD3, BRD4, and the testis-specific BRDT.[4-8] They share a conserved structure of two bromodomains (BRDs), an extra-terminal recruitment domain (ET) and some other motifs, like A, B or SEED motifs. Furthermore, BRD4 and BRDT have a C-terminal motif (CTM).[31] While the ET domain is taking part in protein-protein interactions, the C-terminal domain interacts with the positive transcription elongation factor b (p-TEFb).[32,33] Bromodomains contain around 110 amino acids and structurally form two loops (ZA, BC) and four α-helices (α_Z, α_A, α_B, α_C).[34,35] The four α-helices build a hydrophobic pocket, which recognizes acetylated lysines (K_{ac}) on proteins, such as histones.[36,37] K_{ac} is a posttranslational modification, which is involved in a variety of cellular processes through the interaction of protein complexes.[38]

BRD4 and the other BET-BRDs can bind to acetylated lysine residues of N-terminal histone tails through its bromodomains and recruits the mediator and p-TEFb complex. Hence, BET-BRD proteins are termed epigenetic readers.[38] Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) inhibits the p-TEFb complex through binding to 7SK small nuclear RNA. BRD4 displaces HEXIM1 from p-TEFb, which then becomes activated and subsequently phosphorylates the carboxy-terminal domain of RNA polymerase II to start transcription of BRD4-dependent genes.[38,39] Together, induction of HEXIM1 and reduction of MYC expression proved to be a potential biomarker in a clinical trial of BET.[40]

The activity of BRD4 to regulate and accelerate cell cycle progression through the regulation of gene expression in myeloid malignancies makes it a promising pharmaceutical target. Although BET inhibition is a matter of intensive ongoing research, recent clinical trials have encountered unexpected dose-limiting toxicity of BET inhibitors, including vomiting, headaches, and back pain,[40] as well as thrombocytopenia.[41] Thus, novel BET inhibitors that show optimized BET family member specificity are of interest to improve tolerability in order to include long-term improvements of prognosis and quality of life in patients with AML and other myeloid malignancies. Optimization of dosing regiments and providing treatment options in case of resistance emergence are conceivable.

Consequently, we decided to characterize the functional role of the aforementioned PFI-1 analogs in myeloid cell lines and primary cells from patients with AML. For analyzing, whether **4** and **5** had activity in cellular assays, MTT viability assays with HEL, Molm-14 and K562 cells, which are erythroleukemia, AML and CML (terminal blast phase) cell lines positive for JAK2V617F-, FLT3-ITD-, and BCR-ABL, respectively, were performed. Initially, only racemates of the potential inhibitors were applied. This study showed that several compounds were active, and among them, sulfoximine **4b** and sulfondiimide **5a** were most efficient in reducing the relative metabolic activity of HEL and Molm-14 cells at a concentration of 10 μM (Supporting Information, Figure S1). Sulfondiimides **5b** and **5c** showed lower efficacies. None of the compounds significantly affected the growth of the BET-inhibitor insensitive cell line K562.

With the intention to take advantage of the stereochemistry at sulfur, the inhibitory activity of racemic **4a** on HEL, Molm-14 and K562 was compared to effects induced by the individual enantiomers of **4a**. PFI-1 (**3**) was applied as control. As envisaged, (*S*)-**4a** and (*R*)-**4a** behaved very different. While treatment with 1 μM of (*S*)-**4a** led to a significant decrease in relative metabolic activity of the HEL cells in comparison to 1 μM of PFI-1 (37 % ±6 vs. 73 % ±13) (Figure 1A), no effect was observed with (*R*)-**4a** (1 μM), where the result was comparable to the DMSO control (Figure 1A, 1B). Thus, the stereogenic center at sulfur significantly affected the efficacy of **4a** with its (*S*)-enantiomer being superior to both the racemic mixture of **4a** and standard compound PFI-1 (**3**). In the Molm14 cell line similar effects were observed (55 % ±1 vs. 80 % ±1 at 1 μM of (*S*)-**4a** and PFI-1, respectively) (Figure 1C). The IC_{50} values calculated in HEL and Molm-14 cells showed that (*S*)-**4a** was the most efficient compound with concentrations of 0.5 μM (PFI-1: 2.0 μM) and 1.0 μM (PFI-1: 2.7 μM), respectively (Figure 1B, 1D). In contrast to HEL and Molm-14 cells, K562 cells remained mostly unaffected and never reached an IC_{50} (Figure 1E, 1F).

Applying single enantiomers of **4b** and **5a** in MTT assays revealed that also for **4b**, the (*S*)-enantiomer was superior over

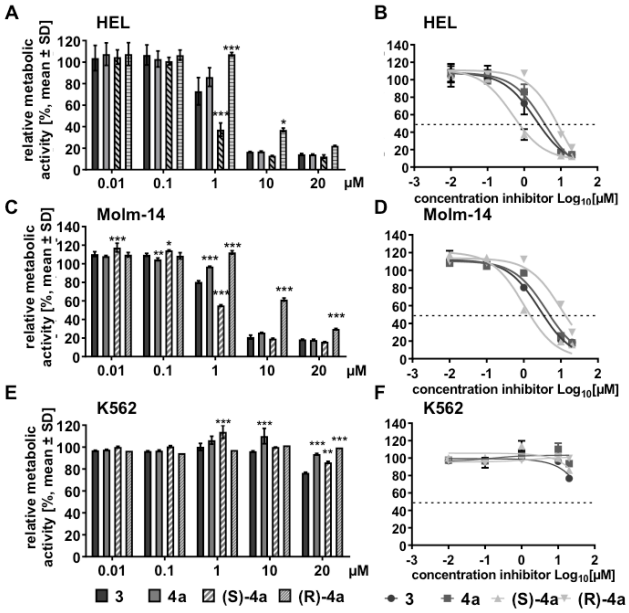


Figure 1. Marked differences in the efficacy of enantiomeric compounds. A) HEL, (C) Molm-14 and (E) K562 cells were treated with PFI-1 (**3**), *rac*-**4a**, (*S*)-**4a**, and (*R*)-**4a** in concentrations of 10, 100 nM, 1, 10 and 20 μM for 72 h. IC_{50} values calculated from MTT assays with (B) HEL, (D) Molm-14 and (E) K562 cells after compound treatment for 72 h. All data are normalized to DMSO control and shown as mean ± SD. Graphs show a combination of three independent experiments. Statistics refer to PFI-1 (**3**). **p<0.01, ***p<0.001.

its *R*-configured compound (Supporting Information, Figure S2). For **5a**, however, the absolute configuration had only a minor effect on the compound efficacy.

Next, we analyzed the selectivity of (*S*)-**4a** and PFI-1 (**3**) for 76 bromodomain-containing proteins in a thermal shift assay (BromoMELT™ Assay, Reaction Biology Corp., PA, USA). The TSA demonstrated a slightly higher selectivity of (*S*)-**4a** vs. PFI-1 (**3**) for BRD2/4 (ΔTm of 5 vs. 4 for BRD4 and 3.5 vs. 2.5 for BRD2-1) (Figure 2A, 2B and Supporting Information for the full data set). At the same time, the ΔTm is reduced for BRDT-2 and slightly for BRD3 [(*S*)-**4a** vs. **3**]. Therefore, we confirmed the binding of (*S*)-**4a** to BET proteins and demonstrated a better selectivity profile of (*S*)-**4a** for BRD2 and BRD4 in comparison to PFI-1.

Several studies confirmed induction of growth arrest and apoptosis using the BET inhibitors JQ1 (**1**) and OTX015 (**2**).[10,25,42,43] Therefore, we evaluated if (*S*)-**4a** and further compounds led to reduced proliferation and disturbed cell cycle progression. Indeed, proliferation of HEL and Molm-14 cells was significantly reduced by (*S*)-**4a** when compared to PFI-1 (Figure 2C and Supporting Information, Figure S3A-C), while the cell number of K562 cells was only slightly affected by (*S*)-**4a** treatment in line with data on another BET inhibitor.[44]

Analyzing the cell cycle progression confirmed that PFI-1 and (*S*)-**4a** led to an increase of the G0/G1 phase, a decrease of the S-phase and in some cases to an increase of Sub-G1 phase (Supporting Information, Figures S3D and S2E). Again, (*S*)-**4a** was more effective in perturbing G1 to S

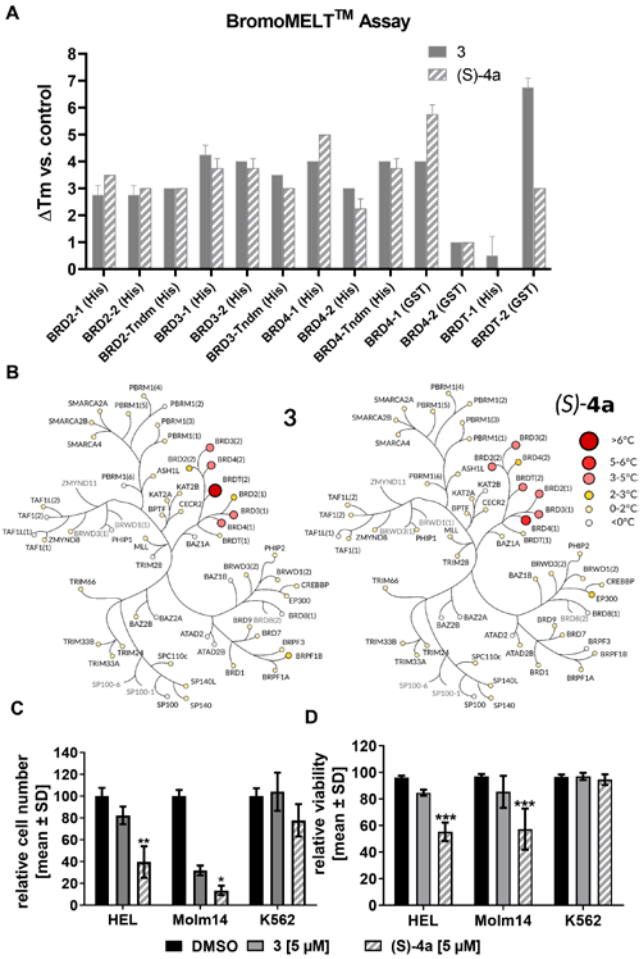


Figure 2. Higher selectivity and potency of (*S*)-**4a**. (A) Thermal shift assay (TSA, BromoMELT™ Assay) of compound **3** and (*S*)-**4a** in duplicate at 10 μM. Illustrated are the ΔTm vs. control of the BET domain protein family. Full analyses see Supporting Information and (B) TSA data of PFI-1 (**3**) and (*S*)-**4a**. Temperature shifts are indicated by reddish circles with increasing radii for higher Tm values as indicated.[45] (C) HEL, Molm-14 and K562 cells were treated with either DMSO or 5 μM of compounds PFI-1 (**3**) or (*S*)-**4a**. Comparison of cell proliferation after 72 h of treatment. Three independent experiments combined. All data displayed as mean ± SD. Statistics: 5 μM of PFI-1 compared to 5 μM of (*S*)-**4a**. (D) Bar graph illustrating significant viability loss by 5 μM (*S*)-**4a** treatment of HEL and Molm-14 cells after 72 h of treatment. All data are shown as mean ± SD. Statistics: **3** compared to (*S*)-**4a**. *p<0.05, **p<0.01, ***p<0.001.

phase transition than PFI-1 and an increase of the subG1 peak was observed.

Next, direct effects of the novel compounds on viability and apoptosis were determined. In HEL as well as Molm-14 cells, 5 μM of PFI-1 reduced the cell viability to 85 % (± 2) or 85 % (± 12), while 5 μM of (*S*)-**4a** diminished it to 55 % (± 7) or 57 % (± 16) after 72 hours of treatment, respectively (Figure 2D and Supporting Information, S4A-C). PARP1 cleavage, as a marker of apoptosis, was confirmed in (*S*)-**4a** treated HEL cells and more pronounced in Molm-14 cells (Supporting Information, Figure S4D). In the DMSO control, PARP1

cleavage was partly detected, mostly due to massive proliferation of cells without BRD4 inhibitor and therefore, a lack of nutrients (Supporting Information, Figure S4D),[46] also supported by the higher amount of uncleaved PARP1 protein (upper band) in DMSO and (*R*)-**4a** treated cells (Supporting Information, Figure S4D; prominent in Molm14 cells). As expected, no reduction of viability and PARP1 cleavage was observed in K562 cells (Supporting Information, Figure S4C, S4D).

Taken together, (*S*)-**4a** was more efficient in reducing cell viability of Molm-14 and HEL cells than PFI-1 (**3**). The major effect of BRD4 inhibition was the block of proliferation and cell cycle progression.

BET protein inhibition correlates with decreased expression of Aurora kinase B (AURKB) and increased expression of HEXIM1, and HEXIM1 participates in the subsequent growth arrest of AML cell lines.[47,48] Therefore, we performed Western Blot analysis and observed that HEXIM1 protein was increased in HEL and Molm-14 cells after 24 hours of treatment with 5 μM of PFI-1 and even stronger after treatment with (*S*)-**4a** (Figures 3A, 3B). In contrast, (*R*)-**4a** treatment did not increase the HEXIM1 protein level. Surprisingly, (*R*)-**4a** reduced AURKB in Molm-14 cells and K562 cells after 48 hours, but, at least, the result in K562 cells was DMSO dependent. Nevertheless, (*S*)-**4a** most efficiently reduced AURKB protein levels in HEL and Molm-14 cells (Figure 3).

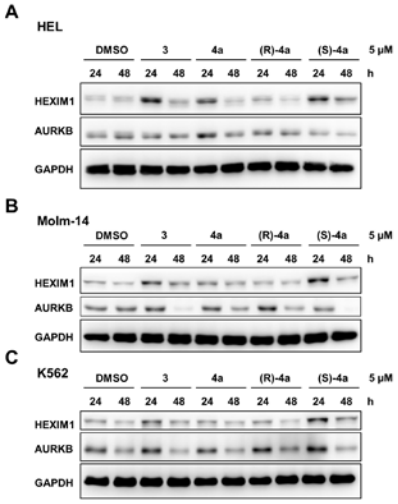


Figure 3. Western Blot analysis of HEXIM1 protein in compound treated HEL, Molm14 and K562 cells. (A) HEL (B) Molm14 and (C) K562 cells were incubated with DMSO or 5 μM of the indicated compounds for 24 and 48 h. Specific antibodies detecting HEXIM1, Aurora kinase B (AURKB) and GAPDH were used. GAPDH served as loading control.

Although, HEXIM1 protein was upregulated in K562 cells treated with PFI-1 and (*S*)-**4a** (Figure 3C), cell viability was not reduced by BRD4 inhibition (Figure 1-2). Hence, HEXIM1 alone cannot ablate CML cell line proliferation and viability. Interestingly, HEXIM1 protein decreased already 48 h after treatment, demonstrating a relatively short half-life of the used BRD4 inhibitors and probably explaining relatively low induction of apoptosis. On the other hand, AURKB protein behaved in the opposite way, most likely demonstrating the protein half-life before its proteasomal degradation. Preincubation of **3** and (*S*)-**4a** in medium and subsequent treatment of HEL, K562 and Molm-14 cells demonstrated compound stability and robust induction of HEXIM1 especially by (*S*)-**4a** (Figure S5).

To support our cell line data, AML patient PBMCs (peripheral blood mononuclear cells), carrying FLT3-ITD or tyrosine kinase domain (FLT3-TKD) mutations were isolated and applied into colony formation (CFU) assays. Healthy donor peripheral blood mononuclear cells (PBMC) or CD34+ cells were used to analyze general cytotoxicity.

Clonogenic growth of healthy donor cells (PBMCs and CD34+) was reduced in both 1 μM and 5 μM compound treatments (Figure 4A), as demonstrated already for PFI-1 (**3**) and OTX015 (**2**).[44, 47,49] Importantly, 1 and 5 μM of (*S*)-**4a** reduced the colony number of bone marrow mononuclear cells (BMMC) isolated from AML patients more efficiently than the standard compound PFI-1 (Figure 4B). More HD and AML samples need to be analyzed in the future.

With the goal to gain a more fundamental understanding of the binding mode of the newly prepared inhibitors and to develop a comprehension for the enantiospecific inhibitory activity, *in silico* models of the structural determinants of BRD4-ligand complexes were obtained by performing docking calculations followed by molecular dynamics (MD) simulations. For this purpose, the X-ray structure of the first bromodomain of human BRD4 in complex with the inhibitor PFI-1 (PDB_ID:4E96) was used. The best-performing ligand according to the Glide XP scoring function (see Supporting Information for details) is (*S*)-**4a**, while (*R*)-**4a** is the worst (see Supporting Information, Table S8).

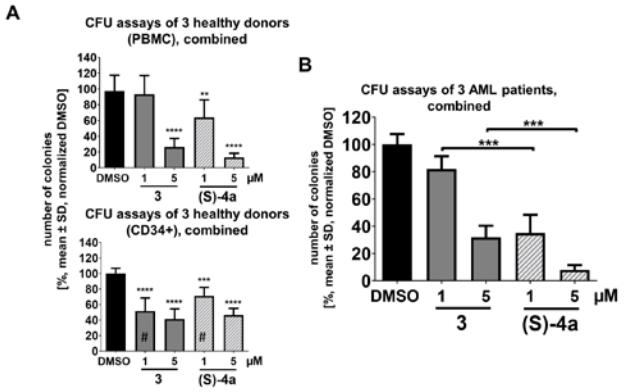


Figure 4. (*S*)-**4a** reduces clonogenic growth of primary AML patient cells more efficiently than PFI-1. (A) CFU assays with healthy donor peripheral blood mononuclear cells (PBMC, upper graph) or CD34+ cells (lower graph, # only two treatments with 1 μM). (B) CFU assays with bone marrow monocuclear cells (BMMC) of three FLT3 ITD or TKD mutated AML patient samples combined and normalized to the DMSO control. Experiments were performed in triplicates. All data are shown as mean ± SD. **p<0.01, ***p<0.001.

In their best binding poses (see Methods in the Supporting Information for details), the anchoring hydrogen bond interactions with ASN140 and the water-mediated interaction with TYR97 of BRD4, observed in the X-ray complex of PFI-1 (**3**) (Figure 5E), are preserved for both (*S*)-**4a** and (*R*)-**4a** (Figures

5A, B and Figure 5C, D). However, (*S*)-**4a** orients the anchoring 3,4-dihydro-3-methyl-2(1*H*)-quinazolinone core towards the BC-loop and the sulfoximidoyl group points towards the hydrophobic patch consisting of ILE146, PRO82, and TRP81 (Figures 5A, B).[50] Differently, in (*R*)-**4a** the latter substituent points toward the opposite direction, suggesting the loss of the hydrophobic-patch interactions and the exposure of the apolar phenyl moiety toward the solvent (Figures 5C, D). Therefore, we expect (*R*)-**4a** to be a weaker binder with respect both (*S*)-**4a** and PFI-1.

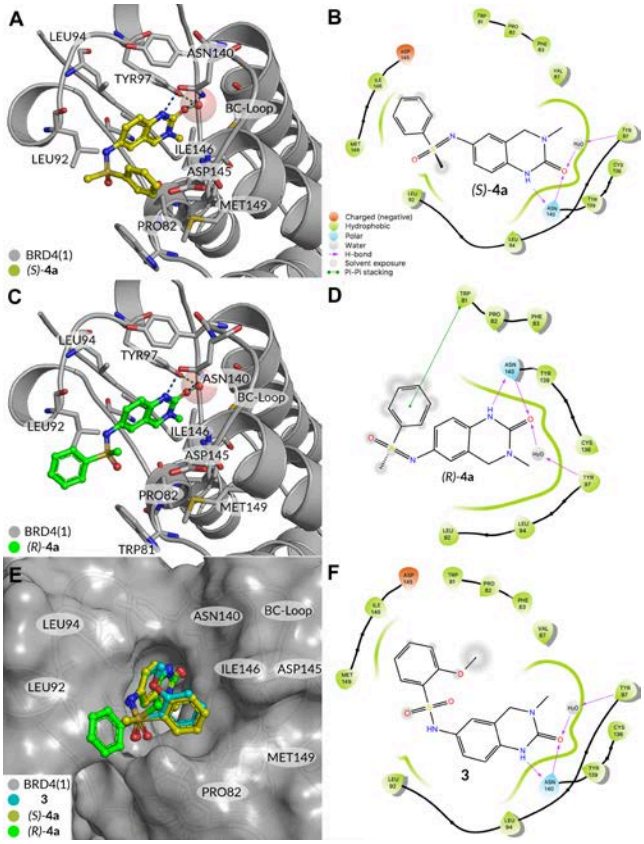


Figure 5. (A-D) Best binding poses of (*S*)-**4a** (A) and (*R*)-**4a** (C) in 3D and corresponding 2D representations (B and D**,** respectively) of non-bonded interactions. In A and C, hydrogen bonds between ASN140 and the anchoring 3,4-dihydro-3-methyl-2(1*H*)-quinazolinone core are shown in blue. TYR97 is forming a mediated hydrogen bond with the ligand and is denoted as a green dashed line. Water position 380 is shown as a red sphere. The phenyl moiety of (*S*)-**4a** shows a distinct kink towards ILE146 (dihedral angle φ(-CNSC) = 56.6). The (*R*)-enantiomer does not show the kink in comparison to the (*S*)-enantiomer (dihedral angle φ(-CNSC) = 175.5 vs. 56.6). In B and D**,** hydrogen bonding is denoted by the arrows colored in magenta. The hydrophobic surface area is shown as a green line and the color coding of the pocket residues is explained in the legend of panel B. (E) Direct comparison of crystal pose orientation of PFI-1 (**3**) and the best binding pose of (*S*)-**4a** and (*R*)-**4a** with van der Waals surface of BRD4, shown as a gray surface. (F) 2D representation of PFI-1 (**3**) binding conformation in crystal structure (PDB_ID: 4E96). Same color code used in B and D was implemented.

Interestingly, (*S*)-**4a** performed better than PFI-1 in our *in silico* experiments. A significant difference between (*S*)-**4a** and PFI-1 is the hybridization state of the nitrogen atom on passing from sulfonamide to sulfoximine, sp^3 to sp^2, respectively. Increasing the s-character of the nitrogen lone pair decreases the hydrogen-bond acceptor strength ($sp^3 > sp^2 > sp$).[51] The sp^3 hybridized nitrogen in PFI-1 (**3**) is therefore a stronger hydrogen-bond acceptor than the sp^2 nitrogen in (*S*)-**4a**, suggesting a slightly better solvation and higher hydrophilicity. Therefore, the slightly more hydrophobic (*S*)-**4a** would be better stabilized than PFI-1 in the hydrophobic binding pocket of BRD4. Also, the sp^2 hybridized nitrogen in the sulfoximine moiety reduces (*S*)-**4a** flexibility, since the free rotation around the N–S bond (dihedral angle φ(-CNSC)) is lost. As a result, (*S*)-**4a** is restricted in its orientation. The restriction of a small molecule's motion on binding to a protein usually causes a loss of configurational entropy, and thus a penalty in binding affinity.[52,53] The single orientation of the N–S bond in (*S*)-**4a** cause a lower loss of entropy with respect to PFI-1 (**3**) upon binding. These observations together point to (*S*)-**4a** having a higher binding affinity towards BRD4 compared to PFI-1 (**3**).

In summary, we found a new BET inhibitor with activity in myeloid cell lines and primary cells from patients with acute myeloid leukemia (AML). The most effective compound, **4a**, has a stereogenic center at sulfur, and its absolute configuration dictates is efficacy. The data show that (*S*)-**4a** is superior to well-studied PFI-1. The (*R*)-enantiomer of **4a** is essentially inactive.

Although the BET inhibitors, including PFI-1 and our newly prepared (*S*)-**4a**, showed some degree of activity on healthy control cells, the effects on AML cells were more pronounced. Therapy with BET protein bromodomain antagonists is of high interest and may be beneficial for particular patients. In addition, combination therapy of BRD4 inhibitors with specific tyrosine kinase inhibitors should be further evaluated. Thus, subsequent research is needed to investigate, if (*S*)-**4a** can be used as a single therapeutic agent or in combination therapy for AML.

ASSOCIATED CONTENT

Supporting Information

Chemical syntheses, NMR spectra, HPLC traces, biological testings, computational studies (PDF) and BromoMELT™ Assay. The Supporting Information is available free of charge on the ACS Publications website.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally (shared first authorship). § Shared last authorship.

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Notes

Part of this work was generated within the medical thesis work of BA. A patent application was submitted.

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ABBREVIATIONS

AML, acute myeloid leukemia; BET, bromodomain and extra-terminal; BMMC, bone marrow mononuclear cells; CML, chronic myeloid leukemia; CSP, chiral stationary phase; CTM, C-terminal motif; FLT3, FMS-like tyrosine kinase 3; GAPDH, gylceraldehyde-3-phosphatedehydrogenase; HEL, human erythroleukemia cell line; HEXIM1, hexamethylene bisacetamide-inducible protein 1; HPLC, high-performance liquid chromatography; MCAP, mitotic chromosome associated protein; MPN, myeloproliferative neoplasms; NUT, nuclear protein in testis; PMF, primary myelofibrosis; PBMC, peripheral blood mononuclear cells; PV, polycythaemia vera; p-TEFb, positive transcription elongation factor b; SD, Standard Deviation; TKD, tyrosine kinase domain.

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