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# 4-Acyl Pyrroles: Mimicking Acetylated Lysines in Histone Code Reading**

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Chromatin remodeling is a key epigenetic mechanism of gene expression regulation controlled through the posttranscriptional modification of histones. Several enzymes, including histone deacetylases and lysine methyltransferases, add or remove functional groups at a variety of residues on histone tails.[1] The recognition of this histone code by "reader" proteins, such as bromodomains (BRDs) and tudor domains, has a critical impact in the regulation of gene expression.

The human genome encodes up to 61 different BRDs present in transcriptional co-regulators and chromatin modifying enzymes, including histone acetyl transferases and the bromodomain extra-terminal domain (BET) family. They specifically recognize ε-N-acetylated lysine residues ($K_{ac}$). BRDs fold into an evolutionary conserved four anti-parallel helix motif, linked by diverse loop regions of variable length (ZA and BC loops), which define the $K_{ac}$ binding site.[2] In most BRDs, this site features an asparagine residue mainly responsible for substrate recognition.[3] The biological function of BRDs and their potential as therapeutic targets have been thoroughly reviewed.[4]

The large amount of crystallographic data available for most BRDs has recently shown the druggability of human BRDs, including the BET subfamily, namely BRD2, BRD3, BRD4, and BRDT, which modulate gene expression by recruiting transcriptional regulators to specific genomic locations.[5] BRD2 and BRD4 have crucial roles in cell cycle control of mammalian cells.[6] Along with BRD3, they are functionally linked to pathways important for cellular viability and cancer signaling and are co-regulators in obesity and inflammation.[7] Specifically, BRD4 has been characterized as a key determinant in acute myeloid leukemia, multiple myeloma, Burkitt's lymphoma, NUT midline carcinoma, colon cancer, and inflammatory disease.[7a,8] Because of its continued association with $K_{ac}$ in mitotic chromosomes, BRD4 has been postulated to be important for the maintenance of epigenetic memory.[9]

Small molecules that inhibit BRD4 have potential as anti-inflammatory, antiviral, and anticancer agents.[10] Anticancer activity is mainly due to down-regulation of the key oncogene *c*-MYC.[7a,8b] Recently, cytotoxicity in LAC cells by BRD4 inhibition has been related to suppression of the oncogenic transcription factor FOSL1 and its targets.[11] Currently, two 1,4-diazepine derivatives, namely (+)-JQ1 and I-BET, are in preclinical development in cancer and inflammation, respectively, as potent antagonists of the BET bromodomains BRD2, BRD3, and BRD4.[8e,12] Recent fragment-based screenings and QSAR-based lead optimizations for the discovery of small molecules with BRD4 inhibitory activity have shed a few new relevant chemical scaffolds, including Compound 6a, an isoxazole derivative at an initial developmental stage in the clinics.[13]

During recent years, a large amount of structural knowledge about the binding features of inhibitors of BRD4 has become available by means of X-ray crystallography, providing an invaluable resource for drug discovery.[14] The three key areas of interaction in ligand-BET bromodomain complexes are the acetyl-lysine recognition site, the WPF shelf, and the ZA channel (Figure 1c). On this basis, we performed a high-throughput virtual screening experiment using a library containing more than 7 million small molecules, aiming at the identification of novel inhibitors of the first bromodomain of BRD4 (BRD4(1); Supporting information). We selected 22

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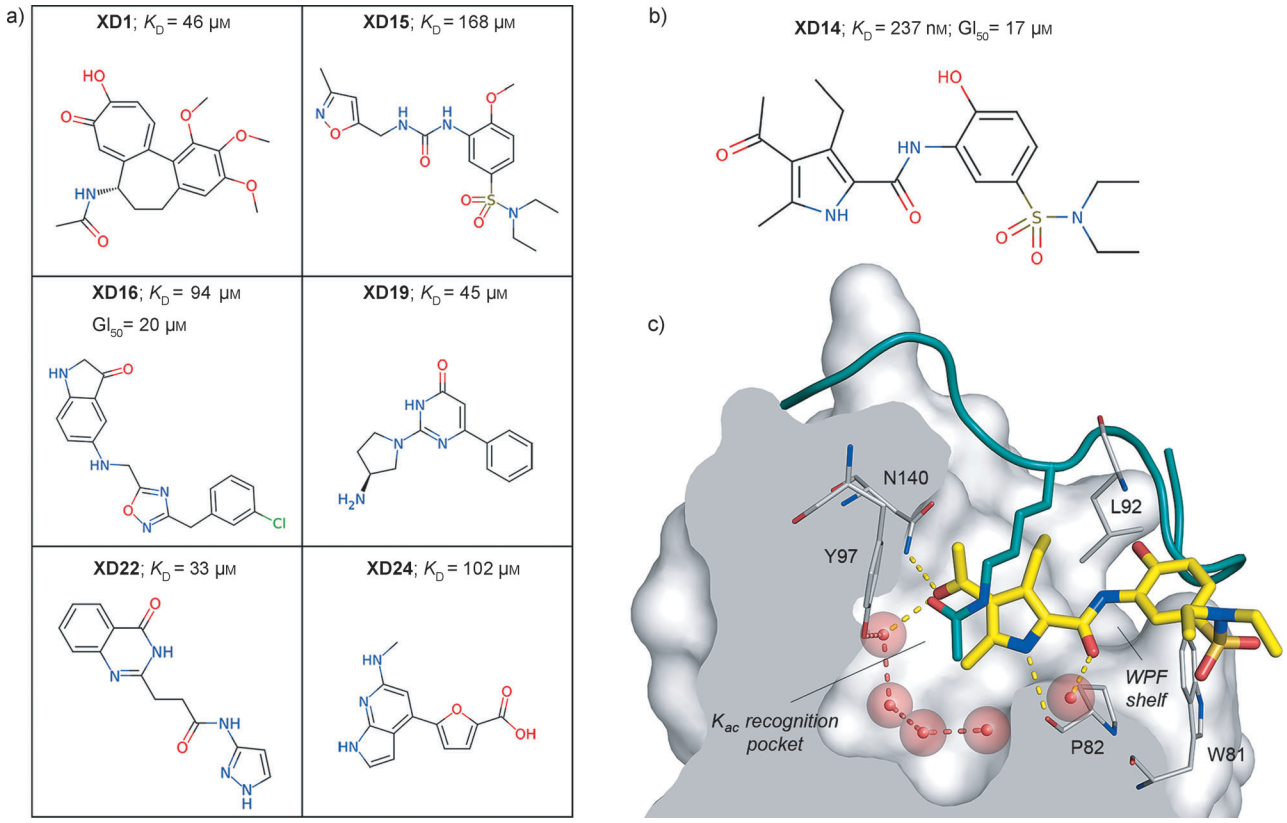



***Figure 1.*** Identified hits binding to BRD4(1). a) Chemical structure and dissociation constant of identified inhibitors. $GI_{50}$ of XD16 is indicated. b) Chemical structure of XD14 with $K_D$ and $GI_{50}$ values. c) X-ray crystal structure of XD14, in yellow sticks, in complex with BRD4(1). The recognition site of the protein is shown along the ZA channel. An acetyllysine histone-4-peptide interacting with BRD4(1) (PDB ID: 3UVW)[2] is shown in green sticks after superimposition with the BRD4(1)-XD14 complex crystal.

candidate compounds (Supporting Information, Table S2) for further experimental validation by ITC measurements, eventually leading to the discovery of 7 compounds with significant binding affinity towards BRD4(1) (Figure 1; Supporting Information, Table S4). The validated hits comprise 6 different previously unknown scaffolds that are potent binders of BRD4(1). Notably, the 4-acyl pyrrole XD14 showed a 700-fold increase in binding affinity as compared to the structurally closely related 5-isoxazole XD15 ($K_D = 0.2$ μM and 168 μM, respectively), rendering XD14 the most potent binder of the entire screening. Furthermore, we also found that the poisonous alkaloid colchiceine (XD1) and its methoxylated analogue colchicine (XD25) have affinity for the protein ($K_D = 46$ μM and 20 μM, respectively). For the subsequent co-crystallization and X-ray structure analysis we chose the most promising candidates, XD14, XD1, and XD25.

The structure of the BRD4(1)-XD14 complex at 1.95 Å resolution (Supporting Information, Figure S6) reveals the already well-characterized bromodomain fold with a bundle of four α-helices, termed αZ, αA, αB, and αC from N- to C-terminus.[2] These helices are interconnected by three loops with differing length, ZA, AB, and BC. The termini of the helix bundle are flanked by elongated loops, which pack tightly around the protein core, producing a compact and rather rigid structure. The binding mode of XD14 in the recognition site of BRD4(1) is shown in Figure 1c. XD14 is bound in a pocket located at the end of the longitudinal axis running through the helix bundle that points towards the N-terminus. Consequently, it occupies the same pocket as the native $K_{ac}$ substrate.[15] Moreover, the compound mimics the $K_{ac}$ interaction with BRD4(1) by positioning the 4-acyl substitution in the pyrrole ring towards the highly conserved Asn140, thus engaging in hydrogen bond interactions with Asn140 and the equally conserved water molecule that bridges to the conserved Tyr97 (Figure 1c). The pyrrole ring is located deep inside the recognition pocket, and complements the hydrophobic pocket defined by the four conserved waters with a 5-methyl substitution. The surface complementarity between the ligand and the recognition pocket is further achieved by the 3-ethyl substitution in the pyrrole ring. The presence of the heteroatom in the core of XD14 allows for a key hydrogen-bond donor interaction with the backbone of proline 82. To the best of our knowledge, such an interaction has not been described previously and may play an important role in fixing the compound in the recognition site.

BET bromodomain inhibitors of the isoxazole and quinazoline series identified so far featuring a sulfonamide group mainly block the WPF shelf of the pocket, as previously observed in the *p*-chlorophenyl substitution of the 1,4-diazepines (+)-JQ1 and I-BET.[8e,12,13,16] In the case of XD14, the phenyl sulfonamide moiety locates along the ZA channel, so that a T-shaped CH–π interaction with Trp81 is

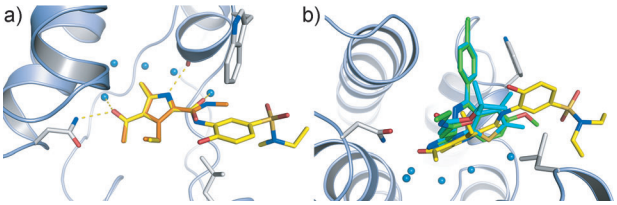
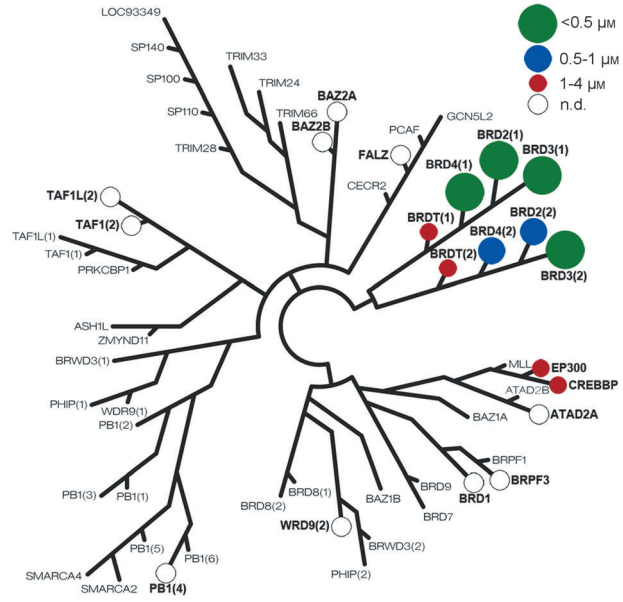



***Figure 2.*** Positioning of novel 4-acyl pyrrole based inhibitors in the recognition pocket of BRD4(1). a) XD14 exploits both the selectivity pocket with Asn140 and an additional π-system provided by Trp81 with Leu92 as lid, the WL trap. The core structure XD46 (orange) only binds to the selectivity pocket. b) Structural overlay of XD14 (yellow) with (+)-JQ1 (cyan) and I-BET (green). Only XD14 intercalates in the WL trap.

established (Figure 1c and Figure 2). Thus, a perfect orthogonal orientation of both aromatic systems is created, with Trp81 directly pointing towards the center of the phenyl moiety of XD14. Such interactions have already been reported in other drug–protein complexes.[17] Furthermore, Leu92 serves as a lid from the opposite side, and together with Trp81 it forms what we termed the WL trap. To verify this hypothesis, we have solved the X-ray crystal structure of BRD4(1) in complex with a fragment of XD14, XD46 (Figure 2a; $K_D = 16\ \mu M$; Supporting information). XD46, lacking the phenyl sulfonamide extension, indeed perfectly fits into the recognition site. Yet it fails to establish the interaction with the WL trap. In conclusion, the WL trap significantly adds up to the specificity of binding. Notably, both Trp81 and Leu92 are conserved within the BET family.[14a] Descriptions of the high-resolution X-ray crystal structures of BRD4(1) in complex with XD1 (Colchicein) and XD25 (Colchicin) are included in the Supplementary information, Figures S7, S8). They show that XD1 and XD25 occupy the same ε-acetyl lysine recognition pocket as XD14 underlining the inhibitory potential of all of the ligands. All crystal structures presented here confirmed the predicted binding modes (Supporting Information, Figure S3).

We assessed the target selectivity of XD14 within the human BRD family by means of BROMO*scan* (DiscoveRx Corp., Fremont, CA). The measurements of this large-scale test were in agreement with ITC data ($K_{D,BROMOscan} = 160\ nM$, $K_{D,ITC} = 237\ nM$, Figure 3; Supporting Information, Figure S9). XD14 bound specifically to the BET bromodomain family. It exhibited $K_D$ values in the nM range for most of the members, namely BRD2, BRD3, and BRD4, and low μM affinity to BRDT. So far, no molecules exhibiting binding selectivity within the BET family have been reported, as expected from the similarity of their binding pockets both in terms of three-dimensional structure and amino acid sequence.[18]

A recent classification of the BRD family pointed out that the BRDs in CREBBP and EP300 had binding site amino acid signatures in common with the BET bromodomains.[5a] Indeed, XD14 showed potent binding affinity to these two BRDs ($K_D = 1.6$ and $2.6\ \mu M$, respectively). This result is remarkable, as only a few small molecules displaying significant binding to the BRD of CREBBP have been described so far. The most potent compound reported is ischemin ($K_D =$ 19 μM), which was found to inhibit the association with p53, further blocking apoptosis in cardiomyocytes.[19] Moreover, no compounds have been reported to inhibit the BRD of EP300.




| | BRD2(1) | BRD2(2) | BRD3(1) | BRD3(2) | CREBBP |
|---|---|---|---|---|---|
| $K_D$ (nM) | 170 | 830 | 380 | 490 | 1600 |

| | BRD4(1) | BRD4(2) | BRDT(1) | BRDT(2) | EP300 |
|---|---|---|---|---|---|
| $K_D$ (nM) | 160 | 850 | 1200 | 3700 | 2600 |

***Figure 3.*** Phylogenetic tree showing the selectivity profile of XD14 within the human BRD family. Sphere size and color indicate the binding affinity of XD14 to the specific BRDs, as observed in the BROMO*scan* assay. Additionally, binding to PB1(4) was assessed by ITC.

BRD4 is a validated therapeutic target in NUT midline carcinoma and in acute myeloid and MLL-fusion leukemia.[8a,12,21] We assessed the proliferation inhibition potential of the virtual screening hits in HL60 and HeLa cell lines using an MTS assay, and obtained growth inhibition (GI) curves for the promising candidates XD14 and XD16 (Supporting Information, Table S10). These two compounds exhibited $GI_{50}$ values around 20 μM in HL60 cells and no antiproliferative activity against HeLa at a high concentration of 50 μM.

We further assessed the antiproliferative potential of XD14 by means of the NCI60 human tumor cell line anticancer drug screen.[20] Currently, this assay consists of 56 cell lines representing nine different cancer types. The results are summarized in Figure 4. The compound showed potent and selective activity against the leukemia cell lines represented in the assay. Indeed, the most sensitive cells were HL60(TB) and SR, from the leukemia panel, whereas the most resistant were OVCAR-5 and COLO 205, from ovarian and colon cancer, respectively. The results indicate that XD14 selectively represses growth of leukemia cells and suggest that it is a promising candidate for further development of selective leukemia anticancer agents.

Rigorous analyses of chemical, biochemical, and clinical data have led to the acceptance that intrinsic physicochemical

a)

| | Cell line | GI (%) |
|---|---|---|
| Breast cancer | T-47D | 31.0 |
| | HS 578T | 18.0 |
| | BT-549 | 15.3 |
| | MCF7 | 15.1 |
| | MDA-MB-468 | 7.7 |
| | MDA-MB-231/ATCC | 2.4 |
| Colon cancer | KM12 | 24.3 |
| | HCT-116 | 10.9 |
| | SW-620 | 7.3 |
| | HCT-15 | 4.7 |
| | HT29 | 1.2 |
| | COLO 205 | -4.7 |
| Leukemia | HL-60(TB) | 65.0 |
| | SR | 64.2 |
| | K-562 | 42.0 |
| | MOLT-4 | 34.8 |
| | CCRF-CEM | 23.0 |
| | RPMI-8226 | 21.8 |
| Melanoma | UACC-62 | 23.5 |
| | M14 | 15.2 |
| | SK-MEL-5 | 12.9 |
| | UACC-257 | 10.3 |
| | MDA-MB-435 | 9.6 |
| | SK-MEL-2 | 9.1 |
| | SK-MEL-28 | 7.2 |
| | MALME-3M | 4.7 |
| Prostate cancer | PC-3 | 17.8 |
| | DU-145 | 2.6 |

| | Cell line | GI (%) |
|---|---|---|
| CNS cancer | SNB-75 | 29.4 |
| | SF-295 | 10.0 |
| | U251 | 6.6 |
| | SNB-19 | 1.3 |
| | SF-268 | 0.2 |
| Non-small cell lung cancer | NCI-H522 | 42.4 |
| | NCI-H322M | 25.4 |
| | HOP-92 | 15.8 |
| | NCI-H226 | 9.7 |
| | NCI-H23 | 8.9 |
| | HOP-62 | 5.7 |
| | A549/ATCC | 4.1 |
| | NCI-H460 | 3.2 |
| Ovarian cancer | IGROV1 | 27.3 |
| | OVCAR-4 | 14.8 |
| | OVCAR-3 | 11.2 |
| | OVCAR-8 | 6.7 |
| | SK-OV-3 | 6.4 |
| | NCI/ADR-RES | 3.2 |
| | OVCAR-5 | -6.7 |
| Renal cancer | UO-31 | 31.3 |
| | 786-0 | 15.6 |
| | A498 | 14.5 |
| | SN12C | 9.4 |
| | CAKI-1 | 6.1 |
| | ACHN | 3.6 |
| | TK-10 | -0.7 |
| | RXF 393 | -1.0 |

b)

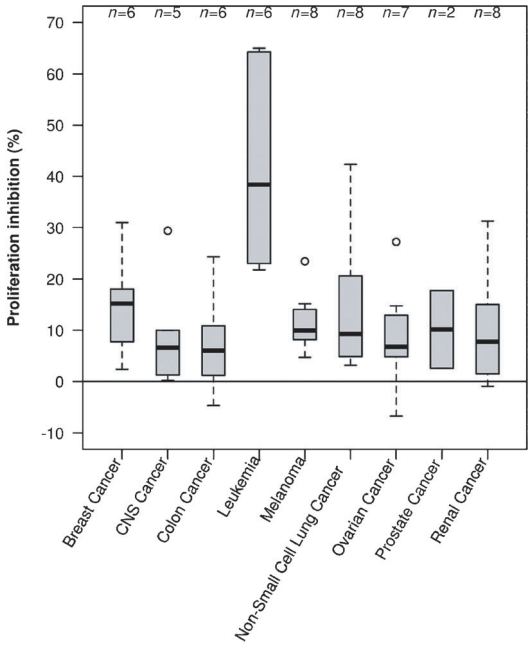



***Figure 4.*** NCI60 human tumor cell line anticancer drug screen for XD14. a) GI (%) observed after incubation of each cell line with XD14 at a concentration of 10 μM for 48 h.[20] GIs are colored, ranging from higher to lower sensitivity, in red and green, respectively. b) Box plot representation of the proliferation inhibition (%) grouped by cancer type.

parameters of putative pharmacological entities play a crucial role in their pharmacokinetic properties and determine their ultimate success as marketable drugs.[22] We have computed three descriptors to determine whether the identified inhibitors have desirable drug-like properties. Quantitative estimate of drug-likeness (QED) is a recently developed measure of drug-likeness based on the concept of desirability of physicochemical descriptors present in drugs.[23] Ligand efficiency indexes provide a measure of how efficiently a ligand binds to a biomolecule with respect to the magnitude of a physical property of the compound. Thus, we have computed the ligand efficiency in terms of number of heavy atoms (LE) and ligand-lipophilicity efficiency per unit of potency (LLE).[24]

Table 1 summarizes the drug-likeness analysis for XD14, XD46, and the reference compounds, (+)-JQ1 and I-BET. QED scores indicate that the four molecules are drug-like,

***Table 1:*** Summary of drug-likeness and ligand efficiency index parameters computed for compounds XD14, XD46, (+)-JQ1, and I-BET.[a]

| Compound | $pK_D$ | QED | LE | LLE |
|---|---|---|---|---|
| XD14 | 6.6 | 0.41 | 0.31 | 3.0 |
| XD46 | 4.8 | 0.73 | 0.44 | 3.8 |
| (+)-JQ1 | 7.6 | 0.44 | 0.34 | 3.4 |
| I-BET | 7.2 | 0.65 | 0.33 | 3.9 |

[a] QED = quantitative estimate of drug-likeness; LE = ligand efficiency (calculated as $1.37(pK_D/\text{number of heavy atoms})$, kcal mol$^{-1}$·heavy atom$^{-1}$); LLE = ligand–lipophilicity efficiency (calculated as $pK_D - c\log P_{o/w}$).[23,24]

with XD14 and (+)-JQ1 having the lowest values in the range of 0.4. LE has proven to be useful in the estimation of the potency of a compound to disrupt a protein–protein complex.[25] It could be shown that potent inhibitors had LE values above 0.24. Interestingly, XD14 has a LE of 0.31, similar to that of (+)-JQ1 and I-BET. XD46, as a representative of the new 4-acyl pyrrole BET family inhibitors, displayed the highest ligand efficiency indexes among the studied compounds, with an LE value of 0.44 and LLE of 3.8, indicating that it is a reasonable starting point for a fragment-based drug design approach.[26] This technique has been recently applied to the optimization of phenylisoxazole sulfonamides, finally leading to a significant increase of binding affinity to the BET bromodomain family.[13a] In summary, the analysis of drug-likeness and physicochemical properties of XD14 and XD46 indicate that the 4-acyl pyrrole moiety is an interesting scaffold for the development of molecules binding to the BET bromodomains.

We have computed the same descriptors for all identified hits (Supporting Information, Table S11). Their QED value indicates that they all possess drug-like physicochemical properties. Notably, the relatively small and polar compounds XD19, XD22, and XD24 displayed LE and LLE values close to those observed for XD14 and the reference compounds (LEs ranging from 0.29 to 0.31 and LLEs from 3.2 to 4.6). Further optimization is tempting.

The results reported herein stress the validity of high-throughput virtual screening in the identification of novel active molecules in the epigenetics field, and indicate that the newly discovered XD14 is a representative of a new class of BET bromodomain inhibitors featuring a 4-acyl pyrrole moiety. Future studies will be directed at assessing the therapeutic potential of XD14, and performing a knowledge-driven lead optimization of the identified hits. The core compound XD46 will be subjected to fragment-based drug design. The procedure presented herein can be further used

for the identification of small molecules interacting with other druggable epigenetic targets.

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