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BET Proteins: Investigating BRDT as a Potential Target for Male Contraception

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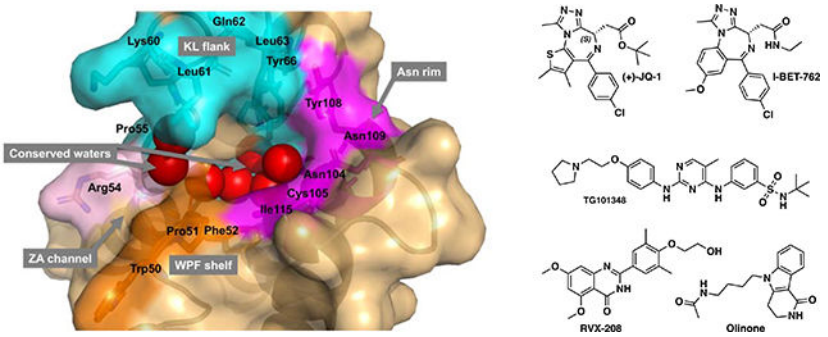
Graphical Abstract

Acetylated lysine binding pocket of BRDT Select BET inhibitors



Importance of and need for male contraceptives

The first oral female contraceptive (oral contraceptive pills, OCP) was introduced in 1960, opening the market to today's plethora of options for women seeking birth control. Hormonal pills and implants, topical patches, vaginal rings, depo injectables, and intrauterine devices are available to provide safe, reliable, effective and reversible contraception, along with other non-contraceptive benefits.[1]

However, many women are unable to use hormonal contraceptives due to preexisting medical or pathological conditions.[2] Additionally, some women may choose to discontinue hormonal contraceptive use due to associated side effects.[3–5] Recently, it was discovered that some women may experience contraceptive failures due to a difference in steroid metabolism that depletes the blood concentration of hormonal contraceptives; even with perfect use of their hormonal contraceptive, these women are at risk of unintended pregnancy.[6] Furthermore, some women can find it difficult to obtain contraceptives due to cost of effective prescription contraception and the associated doctors' visits.[7]

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Contraceptive options for men are limited. Condoms are 98% effective at preventing pregnancy with perfect use, but with average use are only 83–85% effective.[7–9] Vasectomies are another major form of male contraception and while reliable are not easily reversible, making the procedure exclusively a long-term contraceptive method.[8, 9] Withdrawal has an unintended pregnancy rate of 22%.[9] The disproportionate number of contraceptive methods available to men has put the onus of family planning largely on women.

Despite the myriad contraceptive options available, in 2011 the unintended pregnancy rate in the United states was still 45%, down from 51% in 2008, as a percentage of reported pregnancies (Figure 1).[10] Most of these unintended pregnancies can be attributed to lack of contraceptive use, but 43% of unintended pregnancies reported were caused by inconsistent or incorrect use of contraceptives.[7] Globally, nearly half of pregnancies are unplanned.[11]

Contraceptives are only effective at preventing unintended pregnancies with continued and near-perfect use, and the use of multiple methods at one time (i.e., condoms and OCP) is recommended.[9] Clearly, there is a need for a safe, effective, reversible male contraceptive.

Since as early as the 1970s, researchers have been investigating the possibility of a male hormonal contraceptive (MHC), seeking to suppress spermatogenesis by interfering with the normal release of gonadotropin-releasing hormone, and thus the downstream luteinizing hormone and follicle stimulating hormone through negative feedback of exogenous testosterone. Both androgen-only and androgen-progestin combination MHC regimens have been studied and are reviewed elsewhere.[11–16]

The general consensus from MHC studies is both a positive proof of principle and that androgen-progestin combination regimens are more effective than androgen-only conditions. Common side effects include acne, changes in mood, night sweats, a reversible decrease in testicular volume, and changes in cholesterol profile (decreased HDL, LDL, and total cholesterol). The short durations of treatment (typically no longer than one year) have precluded adequate assessment of cardiovascular or thromboembolic events related to use, or other unknown long-term medical problems. Attempts to create a hormonal contraceptive option for men have proven unsuccessful, due to high prevalence of side effects and lack of universal or uniform efficacy, thus no hormonal regimen has progressed to the approval phase.[11, 15]

Another area being explored for potential male contraceptive methods is that of physical occlusion of the vas deferens. Several surgical and non-surgical methods are currently under investigation and are reviewed in greater detail elsewhere.[11, 12, 14] These options generally show good contraceptive properties in clinical and preclinical trials, establishing proof of principle, but studies demonstrating the reversibility of these methods are still required.

Several non-hormonal contraceptive agents have been studied, though none are currently in clinical trials.[17] One of the most well studied potential therapies is gossypol, a naturally occurring phenol originally extracted from the cotton plant. While early studies showed it to be well-tolerated with a strong contraceptive effects, further research indicated inconsistent results as a contraceptive, poor recovery of fertility, and toxicity with prolonged exposure, prohibiting gossypol from use as a modern contraceptive.[12, 14] Another interesting and

validated target for non-hormonal male contraception is the retinoic acid receptor (RAR), which is discussed in more depth elsewhere.[11, 12, 14, 15] Development of RAR antagonists is currently underway, but as yet no RAR antagonists have been shown to inhibit spermatogenesis in humans.[12, 15] Other therapies for non-hormonal male contraception are currently being explored, including adrenergic receptors, phenoxybenzamine, prazosin, tamsulosin, adjudin, H2-gamendazole, and others reviewed elsewhere, though none are currently in clinical testing.[11, 12, 14–16, 18]

Role of bromodomains and BRDT

The concept of epigenetics was first introduced in 1939 and later refined to describe heritable changes in gene expression that are not due to alterations in DNA sequence.[19] Practically, epigenetics is the study of differential gene expression. Expression can be modified many ways, including post-translational modification (PTM) of the chromatin structure. Many expression changes are caused by marks written, read, or erased from histone proteins, with specific classes of proteins responsible for each modification. Writers, such as acetylases, methylases, and phosphorylases, add marks to histone proteins. Readers, such as bromodomains, chromodomains, and PHD fingers, recognize the modifications. Erasers, such as deacetylases, demethylases, and phosphatases, remove the post-translational marks. These marks lead to changes in the chromatin structure, either uncoiling the DNA structure and encouraging higher levels of transcription, or further coiling the DNA around histone proteins, leading to decreased gene expression. Many different PTMs are used, including serine/threonine/tyrosine phosphorylation, lysine/arginine methylation, citrullination, ubiquitination, and others, that make up the histone code. Further exploration of PTMs has been reviewed elsewhere.[20–24]

One of the most frequently occurring PTMs is ε-N-acetylation of lysine residues (Figure 2).[25] Acetylation levels are strictly controlled by two enzyme families. Lysine acetyltransferases (KATs, or HATs for histone specific acetyltransferases) are responsible for appending acetyl groups onto proteins, acting as the “writers” of this PTM. When the *N*-ε-lysine sidechains of histone protein tails are acetylated, an unpacking of the chromatin structure is often seen, due to the decreased affinity of the histones for DNA; however, when the positive charge of the lysine tail is masked by the acetyl group, the histone protein loses affinity for the negatively charged phosphate backbone of DNA as the ionic charge-charge interaction is no longer possible.[26] Histone deacetylases (HDACs) “erase” acetyl groups from modified proteins, which can lead to condensing of the chromatin structure. The class of proteins responsible for recognizing these acetylated lysines (K_{ac}) and recruiting the appropriate binding partners necessary for chromatin-dependent signal transduction are the bromodomains (BRDs).

BRDs are ~110 amino acid interaction modules first discovered in 1992 and named for the *brahma* gene in *Drosophila melanogaster* by Tamkun et al.[27] Subsequent research has revealed much about BRDs and to date, 61 human BRDs have been identified in 46 different proteins.[28] BRD-containing proteins are often large, multidomain proteins associated with chromatin remodeling, transcriptional control, or DNA repair, with the BRD motif surrounded by other epigenetic reader domains. These flanking domains may be tightly

linked together to form a single interaction domain, or connected loosely by long, flexible linker regions allowing the motifs to be accommodating of diverse substrates.

A pivotal study of the human BRDs by Filippakopoulos et al. has shown that despite large sequence variations, all BRDs share the same conserved fold of a left-handed bundle of four alpha helices (αZ, αA, αB, and αC) linked by two highly variable loop regions (ZA and BC loops) (see Figure 3).[28] These loops come together to form a long hydrophobic groove (ZA channel) leading to an asparagine residue (Asn109 in the first BRD of BRDT and Asn140 in the first BRD of BRD4) conserved on the BC loop, seen in most BRDs, responsible for K_{ac} binding (Figure 4). The K_{ac} of histone tails are bound by BRDs through a hydrogen bond from the acetyl carbonyl of the K_{ac} to the amide nitrogen of this conserved Asn. A tyrosine residue (Tyr66 in BRDT(1); Tyr97 in BRD4(1)) within the binding site (ZA loop) has also been shown to be essential for K_{ac} binding through a water-mediated hydrogen bond and is found in most BRDs.[28, 29]

By manually aligning and visually inspecting the structures of all 61 human BRDs, Filippakopoulos et al. were able to identify several features conserved throughout the folded protein domain. One of these features is a conserved phenylalanine found in the C terminus of the Z' helix within the ZA loop. This Phe is part of a structure termed the WPF shelf, named for the tryptophan, proline, and phenylalanine residues (Trp81, Pro82, and Phe83) in BRD4(1) (Figure 4). This general motif is present in most BRDs as a hydrophobic ridge along the rim of the K_{ac} binding pocket, though the exact residues involved are variable. The WPF shelf is often involved in van der Waals interactions with BRD binding partners, potentially contributing to ligand selectivity.

Also conserved within the K_{ac} binding site of BRDs are five structural water molecules forming a network along a groove formed by the ZA loop (the ZA channel). These waters contribute to many water-mediated hydrogen bonds that help stabilize the K_{ac} binding pocket and contribute to ligand binding.[29] The importance of these structural waters has been demonstrated by several groups, highlighting the ability of these water molecules to tune selectivity and affinity of BRD-ligands, contributing to the druggability of these proteins.[30–34] This suggests that researchers should investigate the conserved waters as targetable groups, rather than displaceable obstacles in drug design. To date, only one potent BRD ligand is known to displace these conserved waters.[34]

The ZA and BC loops surrounding the K_{ac} binding site are highly variable between BRDs, as are the C and N termini of BRD-containing proteins. This low overall sequence homology between BRD-containing proteins is reflected in the highly diverse surface properties seen, especially in the electrostatic potentials, which range from highly positive to strongly negative in charge, suggesting that BRDs recognize a wide array of binding partners. The variability of the ZA and BC loops at the rim of the K_{ac} binding pocket contributes to ligand specificity.[28, 35] In their study of BRD structure, Filippakopoulos et al. also explored BRD interactions with histone K_{ac} sites and discovered that some BRDs also bind to non-histone K_{ac} sites.[28] They confirmed that BRDs generally have a low affinity for K_{ac} binding, suggesting that additional interactions may be required for higher affinity target-binding. Supporting this is a previous discovery by Morinière et al., showing that diacetylated motifs

were preferentially recognized by murine BRDT over monoacetylated peptides.[36] This tendency to bind double-acetylated histone tails is conserved across a number of BRDs, where both K_{ac}s insert into the BRD pocket, but only the N-terminal modification interacts with the conserved Asn residue, while the second K_{ac} hydrogen binds with residues of the K_{ac}-binding pocket and the N-terminal K_{ac} through a water-mediated hydrogen bond.

To further explore the idea that multiple PTMs might affect BRD-binding, an experiment systematically interrogating combinations of acetylated and trimethylated lysines (K_{me3}) along with phosphoserine (pS) and phospho-threonine (pT) PTMs around each K_{ac} site was developed for a peptide array.[28] The results showed that most BRDs tested were highly sensitive to PTMs surrounding the K_{ac} of histone proteins. For example, the second BRD of BRD4 (BRD4(2)) interacted strongly with a diacetylated H3 ($H3K4_{ac}K9_{ac}$) truncated peptide, while it did not interact with peptides acetylated on K4 alone. Moreover, BRD4(2) also showed no interaction with $H3K4_{ac}K9_{me3}$, indicating that BRDs are sensitive not only to the number of PTMs on a given protein but also to the type of PTM displayed. Preferences for a specific number and identity of PTMs flanking the K_{ac} site contribute to BRD specificity, but more studies are required before the role of each human BRD can be definitively established.

BET family bromodomains

Based on three-dimensional structural alignments, a phylogram was derived clustering human BRDs into eight major families (I-VIII) (Figure 5).[28] The most well-studied BRDs come from family II, the bromodomain and extra-terminal (BET) proteins: bromodomain-containing proteins 2, 3, 4 (BRD2, BRD3, BRD4), and testis-specific bromodomain-containing protein (BRDT). The four BET proteins are distinct from other BRD families due to their unique C-terminal extra terminal domain and N-terminal tandem BRDs. The extra terminal domain is responsible for protein-protein interactions involved in recruiting different effector proteins to the chromatin.[37, 38] The extra terminal domain is highly conserved across the BET family and also exhibits a helical architecture.[39]

Several conserved motifs are also conserved in the modular architecture of the BET family (Figure 6). A SEED motif at the very C-terminus of the extra terminal domain is composed of serine, glutamate, and aspartate residues. The A motif is located between the tandem BRDs of the BET proteins and contains a conserved stretch of 12 amino acids (KGVKRKADTTTP) that is responsible for the nuclear localization of the BETs; deletion of this signature results in mislocalization of the mutant proteins.[40] The BET proteins also contain a conserved ‘motif B’, thought to be responsible for mediating BET protein dimerization, as has been shown in BRD2.[41] BRD4 and BRDT also contain a C-terminal domain motif.

The tandem BRDs (BD1 and BD2) of the BET family are conserved in eukaryotes, while plant BET proteins contain only one BRD, supporting the hypothesis that the tandem BRDs arose from a duplication within the ancestral BET gene. Since that time, the first and second have diverged significantly, creating greater similarity between BD1 or BD2 across the mammalian BET family than there is between BD1 and BD2 for a given BET protein. For

example, BD1 of BRDT (BRDT(1)) is 73% identical to BRD2(1) and BRDT(2) is 76% identical to BRD2(2), while BRDT(1) is only 40% identical to BRDT(2), though the domains still share high homology and most of the sequence differences lie outside the K_{ac} binding pocket.[33, 39] This difference between the tandem BRDs has allowed for probe development, showing proof of principle for selective targeting of BD1 or BD2.[42] Chromatin binding is predominantly controlled by BD1, but genetic and chemical-genetic studies have shown roles for both BRDs in transcriptional activation.

The BET proteins are expressed throughout the human body and are responsible for transcriptional regulation in cellular proliferation, mitosis, cell cycle progression, apoptosis, and other cellular functions. BRD2 helps control neuronal differentiation, cell-cycle progression, and cell-cycle exit in neuroepithelial cells in central nervous system development.[43] BRD3 is involved in regulating the expression of erythroid and megakaryocyte-specific genes.[44] BRD4 is a central regulator of transcriptional elongation by recruiting the positive transcription elongation factor b (P-TEFb) complex to chromatin, leading to the activation of ribonucleic acid polymerase II (RNA Pol II), a protein complex that catalyzes DNA transcription.[44] Additional evidence shows BRD4 interacts with a large portion of the genome, allowing the protein to stimulate elongation of both protein-coding and non-coding enhancer RNA transcripts.[45] BRD4 also binds many M/G1 genes programmed to be expressed at the end of mitosis, and may even play a role in "bookmarking" certain genes for post-mitotic transcriptional reactivation by enhancing transcription kinetics and helping to decompact chromatin in daughter cells.[46, 47] BRDT is required for spermatogenesis, playing an important role in reorganizing chromatin and facilitating histone eviction and replacement by transition proteins during germ cell differentiation, and it also acts as a central regulator of transcriptional elongation through P-TEFb.[38, 48]

With the central role that BET proteins play in many important cellular functions, misregulation of BET activity is associated with many diverse disease states, as is discussed in more detail elsewhere.[44, 49] BRD2 has been shown to play a role in obesity, acute inflammatory response, neuronal development, hematopoiesis, and hematologic malignancies.[44, 49–51] BRD3 has been implicated in many aggressive leukemias.[44, 49] BRD3 and BRD4 have been involved in chromosomal translocations leading to tumor-causing fusions with nuclear protein in testes (NUT), leading to rare and aggressive carcinomas that have no specific tissue or organ of origin, known as NUT midline carcinomas (NMCs).[44] BRD4, in particular, has been implicated in basal-like breast cancer, as a coactivator protein for oncoprotein MYC, and NMCs, as well as many other cancers.[44, 49, 52, 53] While BRDT is exclusively expressed in the testes, it has also been seen in lung cancer.[54] BETs have also been shown to have a role in tethering viral genomes to mitotic chromosomes.

BET inhibitors

Due to the established role of BET proteins in many disease states, many small molecule inhibitors have been developed. The first potent and selective BET inhibitor was discovered through a phenotypic screen for up-regulators of apolipoprotein A1 (ApoA1) to inhibit inflammation.[55, 56] Though the triazolobenzodiazepine **I-BET/I-BET-762** was identified

without a known target protein, structure-activity relationship (SAR) studies revealed that the benzodiazepine core and the 5-position aryl group were required for activity (Figure 7). Additionally, the C3 stereochemistry strongly affects the potency of the molecule, with the (*S*)-enantiomer showing activity and the (*R*)-enantiomer showing no activity. An affinity matrix was used to identify the BET proteins as the ultimate protein target of **I-BET**, with no other BRDs showing affinity for the molecule; this selectivity was confirmed by thermal stability and other biochemical assays.[56] The binding of **I-BET** to tandem BET proteins was demonstrated by in vitro ITC and SPR experiments, with a K_d of 50.5–61.3 nM, and similar affinity found in a competitive FRET assay where **I-BET** displaced a tetra-acetylated histone peptide $H_{41–14}K5_{ac}K8_{ac}K12_{ac}K16_{ac}$ (IC_{50} of 32.5–42.4 nM for tandem BETs).[55, 56]

Crystal structures with select BET proteins have revealed nearly identical binding modes within the K_{ac} binding pocket: N3 of **I-BET** binds the conserved Asn140 of BRD4 and makes an additional hydrogen bond to the amide nitrogen while N2 binds the conserved Tyr97 through a structural water molecule. Shape complementarity between ligand and binding site is very good, with van der Waals interactions between the 4-chlorophenyl moiety and the conserved WPF shelf.

The first clinical trial of **I-BET** was launched in 2012 investigating the effects of the drug on NMC and many other cancers.[57] Since then, four other phase I/II clinical trials have begun, though no results have yet been released.[58–61]

Simultaneously, another triazolodiazepine compound was discovered that selectively inhibited BET proteins, **(+)-JQ1**, also from an anti-inflammatory phenotypic study.[62] Like **I-BET**, only the **(+)-JQ1** enantiomer is active against the BET family, with **(−)-JQ1** enantiomer showing no activity against any of the BRDs tested.[62] With good selectivity for the BET family proteins, **(+)-JQ1** has a K_d of 49 nM (ITC) and an IC_{50} of 77 nM (AlphaScreen) against BRD4(1). **(+)-JQ1** has also been shown to inhibit the interaction of BRD4 with nuclear chromatin in human cells.[62] As with **I-BET**, **(+)-JQ1** shows excellent shape complementarity to the K_{ac} binding site. The methylated-1,2,4-triazole moiety of **(+)-JQ1** forms hydrogen bonds to the conserved Asn and, through a water molecule, to the Tyr within the K_{ac} binding pocket and the 4-chlorophenyl group interacting with the WPF shelf.[62]

A widely used chemical probe, **(+)-JQ1** has been proven an effective inhibitor of many cancers, including NMC, acute myeloid leukemia, multiple myeloma, and triple-negative breast cancer, among others.[62–66] **(+)-JQ1** (licensed by Oncoethix and slightly modified to **OTX-015b**) has been used in five clinical trials treating various cancers and hematologic malignancies.[67–71] Though most of these trials have been phase I, determining appropriate doses of **OTX-015b**, one phase II study treating recurrent glioblastoma multiforme was terminated due to a lack of clinical activity.[68]

Other triazolodiazepine BET inhibitors include **GSK525762**, **MS417**, and **CPI-203.** Additional chemotypes of BET inhibitors include 3,5-dimethylisoxazoles like **I-BET-151**, benzimidazoles like **BIC1**, quinazolinones like **PFI-1**, and many others reviewed elsewhere.

[72, 73] As with other drug targets, not all BET inhibitors are drug candidates but instead find use as probe compounds.

The discovery and design of numerous BET inhibitors is ongoing to refine specificity within the BET family. Part of the difficulty of this goal is the nature of the K_{ac} binding site. The binding of a BRD to an acetylated histone tail is a protein-protein interaction. These interfaces are notoriously difficult to inhibit with small molecules due to the broad, shallow, and featureless surfaces involved, making intra-BET family selectivity challenging. While most current BET inhibitors are active against all of the BET proteins, a few have been developed to selectively inhibit the first or second of the BET family's tandem BDs. Hexahydropyridoindolone **Olinone** (Figure 7) is a BET-specific inhibitor that prefers BD1 over BD2.[74] This selectivity is achieved through a hydrogen bond interaction of the indanone carbonyl with Asp144 of BRD4(1) (and corresponding Asp residues of BRD2(1), BRD3(1), and BRDT(1)). The corresponding residue in BRD4(2) is His435 (and corresponding His residues of BRD2(1), BRD3(1), and BRDT(1)), which crowds out the indolone from the K_{ac} binding pocket.[74]

RVX-208 and **RVX-297** are related quinazolinone BET inhibitors that preferentially bind BD2.[42, 75] While both compounds form hydrogen bonds with Asn140 and a water-mediated hydrogen bond to Tyr97 of BRD4(1), the remaining interactions with the K_{ac} binding site are largely hydrophobic and non-specific. **RVX-208** and **RVX-297** bind to the canonical Asn437 of BRD4(1) but are also able to occupy the entire ZA channel, which is narrowed in BRD2 by the presence of His438 (as compared to Asp144 in BRD4(1)). This tighter pocket of BD2 shows much better shape complementarity with **RVX-208** and **RVX-297** than BD1, providing some selectivity.[42, 75]

While intra-BET selectivity continues to prove challenging, the examples of **Olinone**, **RVX-208** and **RVX-209** show proof of principle for further selectivity within the family. Continued exploration and exploitation of the protein sequence differences between the BET proteins should allow for tuned specificity.

A newly emerging class of pharmaceuticals is the proteolysis-targeting chimeras (PROTACs), compounds that induce protein knockdown by recruiting the cell's natural protein degradation processes. PROTACs are chimeric molecules composed of three parts: a target protein ligand, a degradation machinery recruiting unit, and a chemical linker to join the functionalities together. When both ligand moieties have bound their respective partners, the proximity of the proteins leads to the polyubiquitination of the target protein by the E3 ubiquitin ligase complex, and the target protein is subsequently degraded by the proteasome. While first described as large, polar, peptide-based molecules by Sakamoto et al. in 2001, PROTACs have since also been developed into cell-permeable, small-molecule-like entities.[76] First generation small-molecule PROTACs matched the peptide-based entities in potency, but exploration of degradation machinery recruiting units (often an E3 ligand component) and linker moieties have greatly increased both potency and selectivity profiles of PROTACs, allowing for some tunability of their effects.[77, 78] Additionally, PROTACs have been shown to have catalytic, superstoichiometric target degradation.[78] PROTAC development and applications have been reviewed extensively elsewhere.[78–81]

The BET proteins have been targeted by several PROTAC efforts, most often utilizing **(+)-JQ1** or **OTX-015** as the BET-targeting ligand. PROTAC **ARV-825** combines the BET ligand **OTX-015** with pomalidomide, a ligand of the (Cul4-Rbx1-DDB1-)cereblon E3 ubiquitin ligase complex, leading to BET protein degradation (Figure 8). In Burkitt's lymphoma cell lines, **ARV-825** creates a prolonged degradation of BRD4 and a suppression of c-MYC levels and other downstream signalling.[82] **ARV-825** was found to have similar degradation effects on BRD2 and BRD3 but appears not to have been tested in BRDT. **ARV-771** also uses **OTX-015** as a BET ligand but contains a Von Hippel-Lindau (VHL)-binding ligand as its E3 recruiting element (**Figure 1.2.8.**). Like **ARV-825**, this PROTAC degrades BRD2, BRD3, and BRD4, but it has not been tested against BRDT. **ARV-771** dosing leads to BET degradation and depletion of the c-MYC transcript and protein in cells.[83] Additionally, while **ARV-771** has a similar K_d value to that of **(+)-JQ1**, the PROTAC demonstrates a more than 10-fold higher efficacy in reducing c-MYC levels. These favorable preclinical assessments of **ARV-825** and **ARV-771** have created high expectations for clinical testing.

dBET1 is another PROTAC developed using **OTX-015** and pomalidomide, differing from **ARV-825** in the linker used to combine these moieties (Figure 8). **dBET1** induces a BET-selective cereblon-dependent protein degradation in vitro and in vivo, and additionally was able to delay leukemia progression in mice.[84] Quantitative expression proteomics analysis showed a near equal knockdown of BRD2, BRD3, and BRD4 in leukemia cells. However, unlike **ARV-825** and **ARV-771**, **dBET1** was also tested against BRDT in a BROMOscan assay (DiscoveRx) and showed equal potency against this testis-specific BET (K_d values ranging from 6 to 19 nM for the BET proteins).[84]

PROTAC **MZ1** is similar in structure to **ARV-771**, differing in linker length and a slight modification to the VHL-ligand (Figure 8). Interestingly, **MZ1** is somewhat selective for BRD4 degradation over BRD2 and BRD3 but was not tested against BRDT.[85, 86] Further investigation of this BRD4 selectivity lead to crystallization of the ternary BRD4(2)-MZ1-VHL complex.[86] The crystal structure solution revealed that **MZI** forms specific intermolecular interactions with both BRD4(2) and VHL, but intermolecular interactions are also seen between BRD4(2) and VHL. These extensive, newly identified protein-protein interactions drive the BRD4 selectivity and also lead to increased stabilization of the ternary complex. The ternary complex crystal structure allowed for structure-based drug design efforts resulting in **AT1**, a PROTAC compound with even greater BRD4 selectivity over BRD2 and BRD3 (again, BRDT was not tested).[86] Further exploration of such de novo protein-protein interactions may allow for the selective degradation of other BET proteins, creating intra-family specificity.

BRDT(1) target validation

As mentioned above, BRDT is required for spermatogenesis, encouraging chromosome compaction for the first meiotic division in sperm production. This chromatin reorganization protects the genetic information, and the location and modification state of the remaining histones prepare the genome for reactivation after fertilization. BRDT(1) may act to facilitate post-meiotic chromatin compaction by binding to acetylated histone tails, facilitating the replacement of hyperacetylated histones with sperm-specific, highly basic

proteins known as protamines.[38, 87] Knock-out experiments have shown that BRDT(1) is essential for spermatogenesis, with male mice maturing to healthy but sterile adults.[39, 62, 88, 89]

During mouse studies of **(+)-JQ1**, the researchers noted that the testes of the test animals were unusually small. As a pan-BET inhibitor, **(+)-JQ1** also inhibits BRDT, and the small size of the **(+)-JQ1**-treated testes was due to a lack of mature spermatids.[88] As a BET inhibitor, **(+)-JQ1** competitively binds BRDT(1), preventing the chromatin remodeling required for spermatogenesis, as shown in Figure 9.A, adapted from the in vitro studies performed by Matzuk and coworkers using both murine (circles) and human (squares) BRDT-(1).[88] Breeding studies (Figure 9.B) demonstrated the reversibility of JQ1-induced infertility, where adult males were pretreated with vehicle control or **(+)-JQ1** for 6 weeks and then caged continuously with two adult females with continued **(+)-JQ1** dosing. Throughout this period, no litters were birthed. The **(+)-JQ1** treatment was discontinued and male fertility was restored, with litters born by month 4 of the experiment (one month after treatment cessation). Not only was full fertility restored within one month of treatment cessation, but litter sizes and pups born following **(+)-JQ1** treatment proved normal.[87]

This demonstrates the reversibility of BRDT(1) inhibition by **(+)-JQ1**, and acts as a proof of principle for BRDT(1)-targeted male contraception. As BRDT is expressed only in mid- to later-term spermatocytes and not in mitotically dividing spermatogonia, a BRDT-specific inhibitor would not affect the spermatogonial stem cell population, making any small molecule-BRDT inhibition reversible and leading to no ill effects on subsequent mature spermatids.[39, 48] Altogether, this makes BRDT(1) a promising target for a reversible, non-hormonal male contraceptive.

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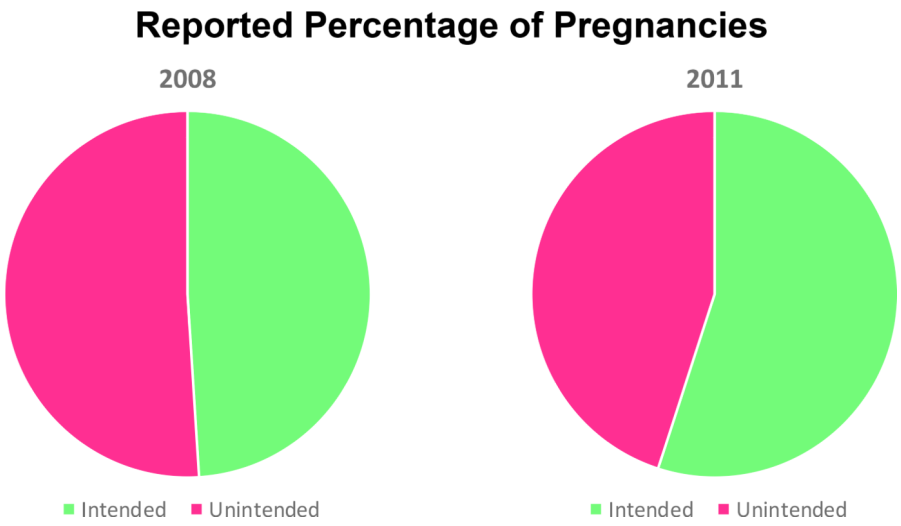

Figure 1.
Reported percentage of pregnancies in the United States in all women.[10]

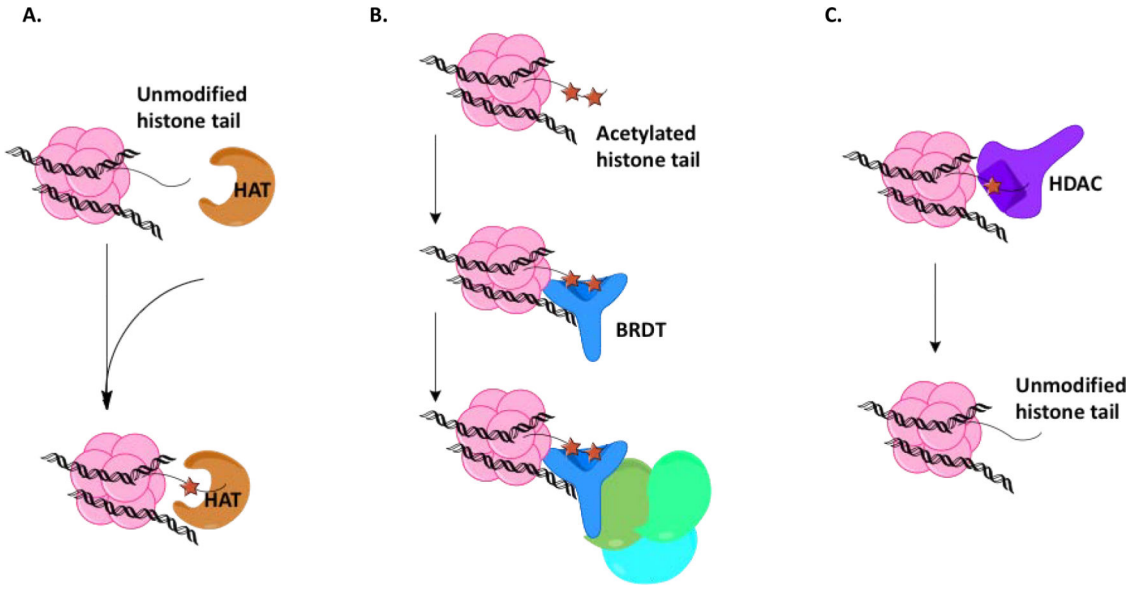

Figure 2.

Lysine acetylation post-translational modification. A. Histone acetyl transferase (HAT; orange enzyme) adds acetyl groups (orange stars) to lysine residue of histone tail at two points (H4K5 and H4K8). B. Testis-specific bromodomain BRDT (blue protein) recognizes acetylated lysine residues and recruits binding partners. C. Histone deacetylase (HDAC; purple enzyme) removes acetyl group from lysine residue, leaving an unmodified histone tail.

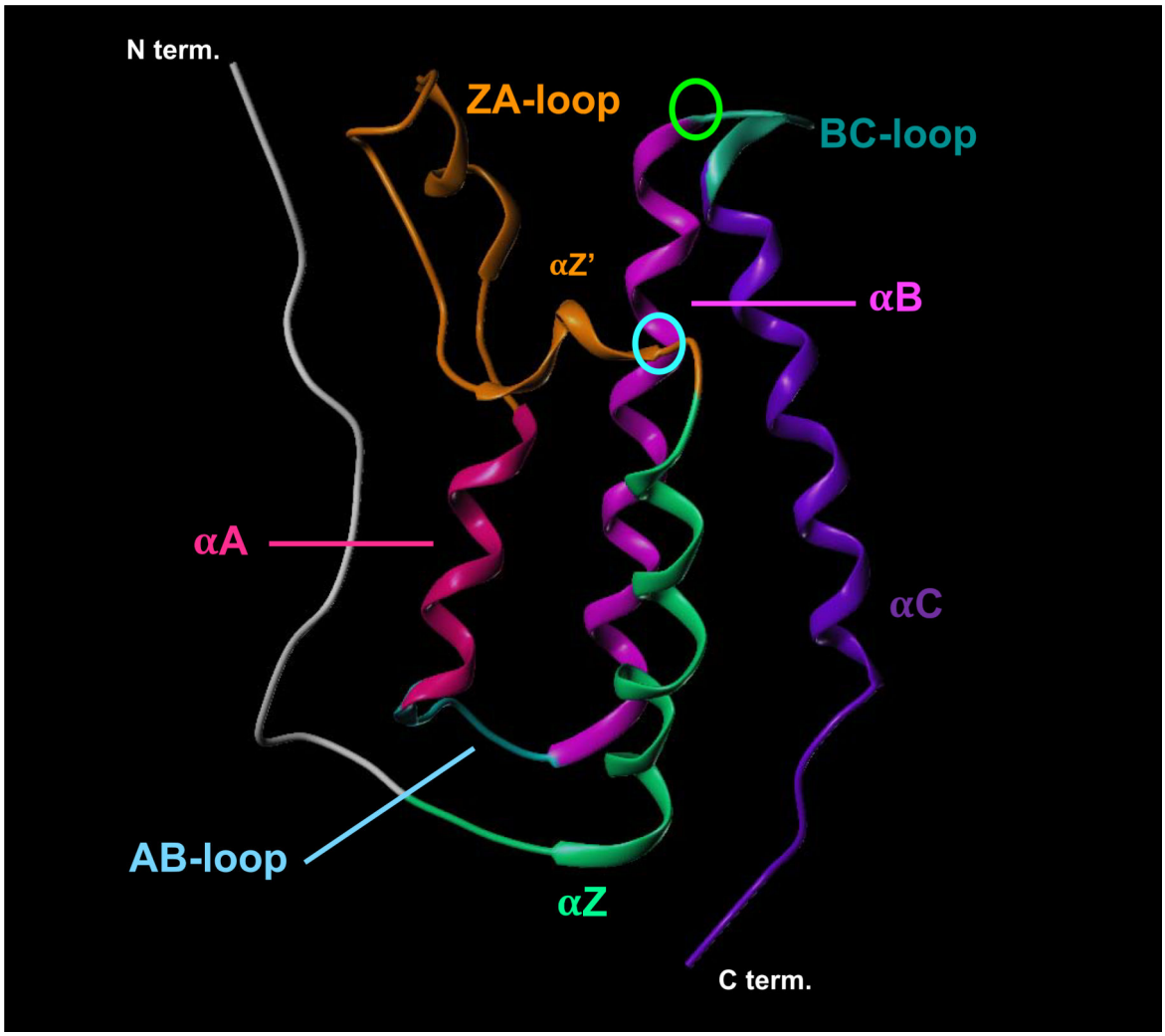

Figure 3.

Ribbon structure of bromodomain containing protein BRD4(1) showing canonical structure of bromodomains. N and C termini are labeled as are canonical secondary structures. Critical K_{ac}-binding Asn residue (Asn109 of BRDT(1)) circled in green, critical Tyr residue (Tyr66 of BRDT(1)) circled in cyan.

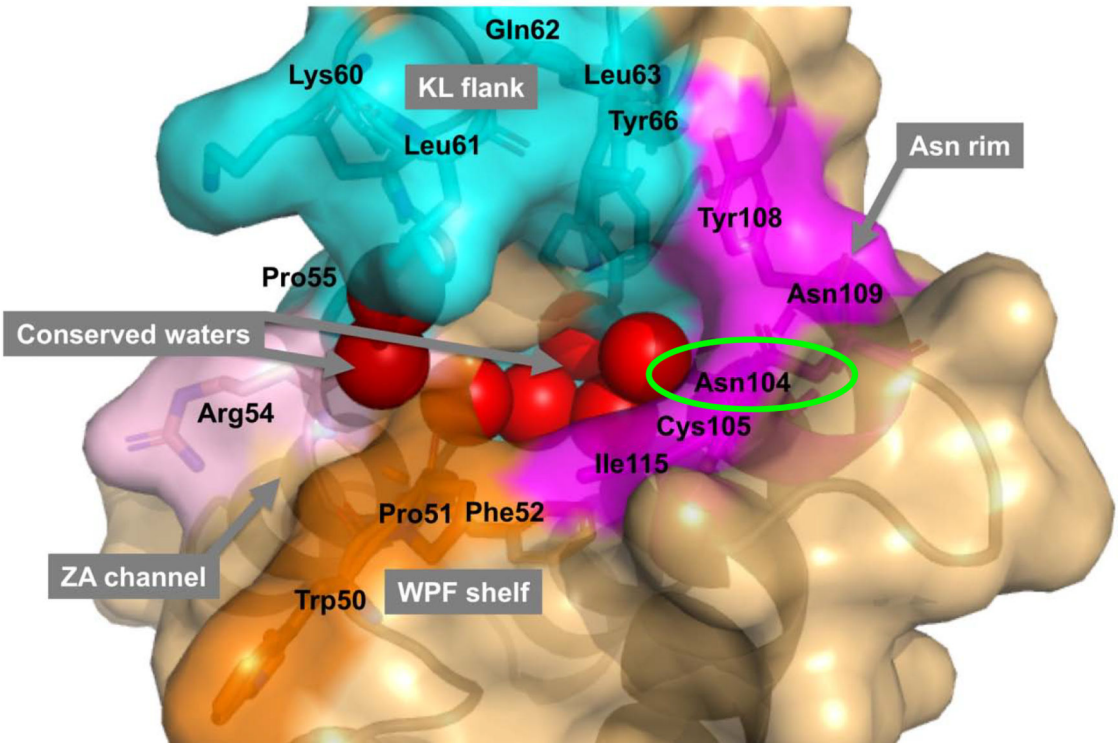

Figure 4.

The K_{ac} binding pocket architecture of BRDT(1) shown as semi-transparent surface with residues labeled and depicted in sticks. Landmarks of K_{ac} binding site highlighted in color: KL flank (teal), WPF shelf (orange), Asn rim (magenta), ZA channel and conserved water molecules (red). Critical Asn109 residue circled in green.

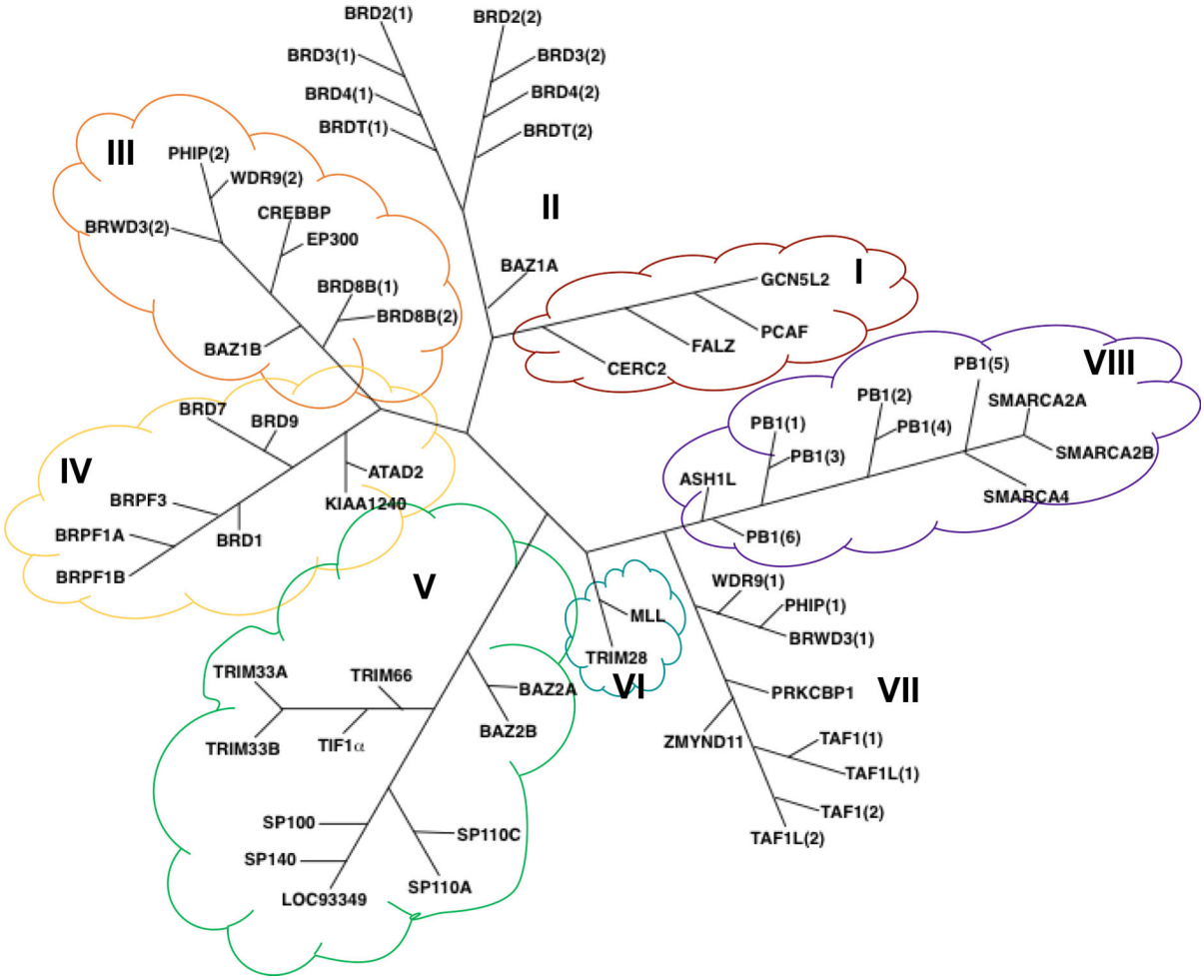

Figure 5.

Phylogenetic tree of bromodomain containing proteins. The different families are labeled in Roman numerals.

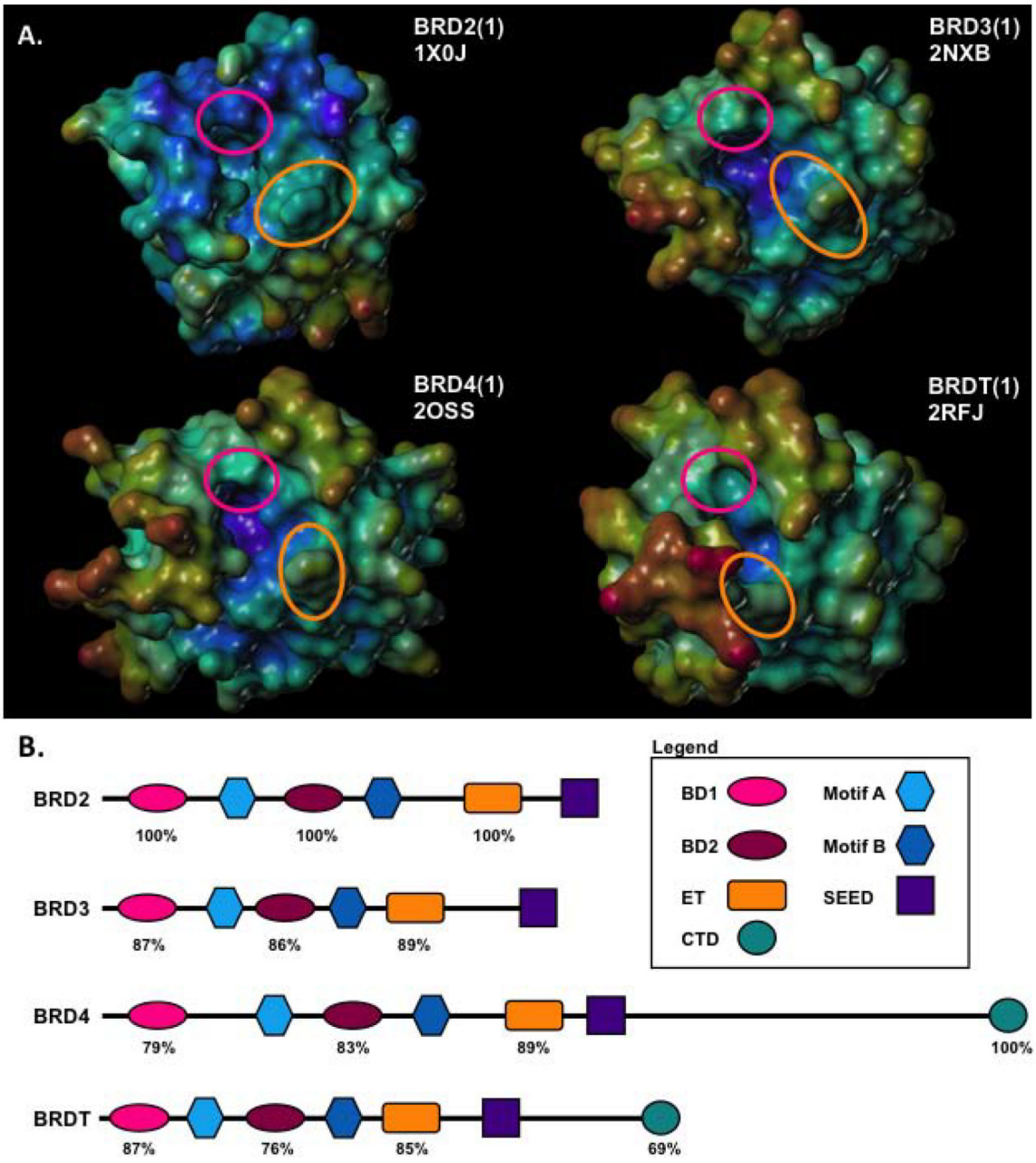

Figure 6.

BET bromodomain proteins. **A.** Electrostatic surface potentials for the first bromodomain of the BET family proteins (blue is negative, red is positive); BRD names and PDB accession codes are shown in the figure. Pink circle highlights critical Asn residue, orange circle shows WPF shelf. **B.** Structural comparison of the BET family members in mice. The percentages under each bromodomain and the ET domain indicate the percentage of identical amino acid residues in the domain as compared to the corresponding domain in murine BRD2, the founding member of the mouse BET family. The percentages under the CTD are compared to murine BRD4.

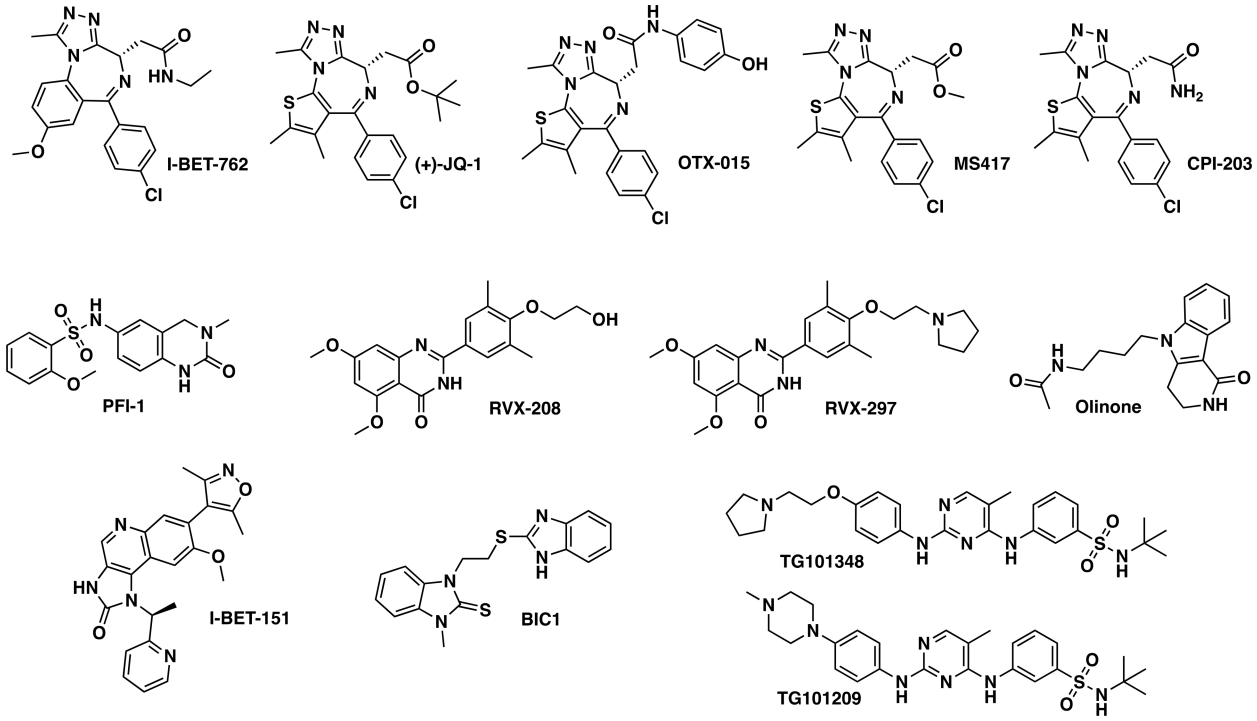

Figure 7.
Structures of select BET inhibitors.

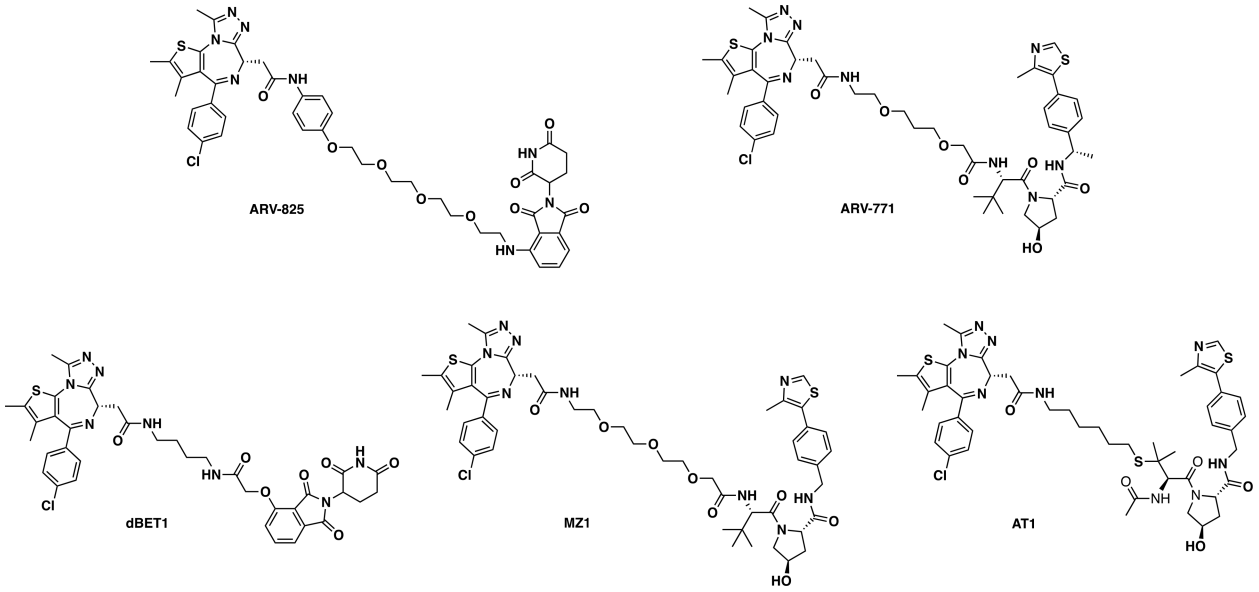

Figure 8.
Structures of select BET-targeting PROTACs.

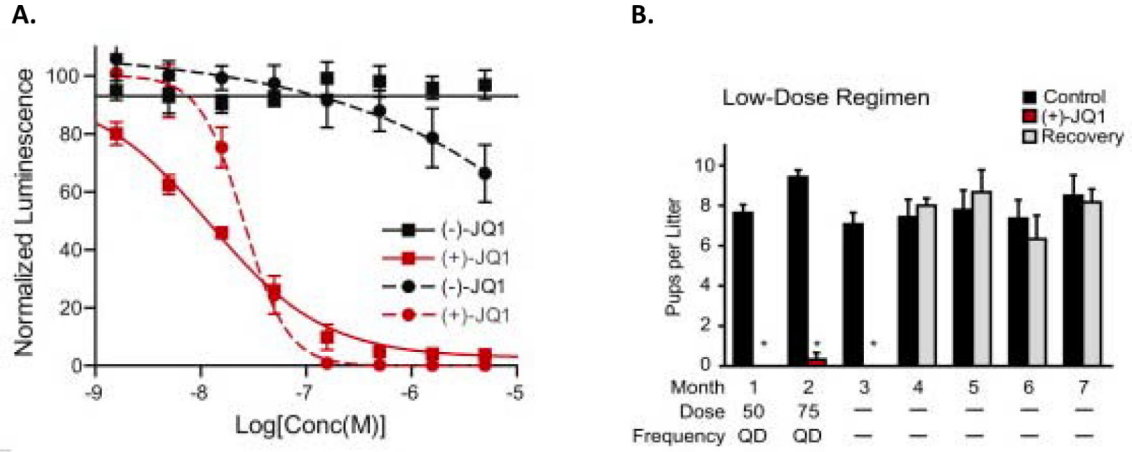

Figure 9.

Inhibition of BRDT(1) by **(+)-JQ1**. Modified from Matzuk et al.[88] **A.** Competitive inhibition of human (squares) and mouse (circles) BRDT(1) binding to synthetic biotinylated H4Kac4 by **JQ1** using proximity detection assays (hBRDT(1) IC_{50} = 11 nM; mBRDT(1) IC_{50} = 10 nM). **B.** Adult males were pretreated for 6 weeks with vehicle control (n = 7) or **(+)-JQ1** (50 mg/kg QD; n = 3) and then caged continuously with two females each while continuing 50 mg/kg QD for month 1 and escalating to 75 mg/kg QD for month 2. **(+)-JQ1** treatment was stopped at the end of month 2 of mating. Graphical representation of pups born in each month to the females reveals a contraceptive effect evident in months 1–3 (data represent mean ± SEM; *$p < 0.001$) and durable restoration of fertility at month 4.