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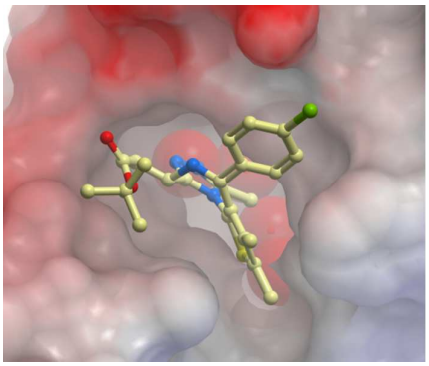

Publicly available bromodomain inhibitors led to discoveries of key functions of BET-proteins in disease and development of new therapeutic strategies.

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ARTICLE

Discovery of BET bromodomain inhibitors and their role in target validation

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Bromodomains (BRDs) are protein interaction modules that selective recognize ε-N-acetylated lysine residues. BRDs are present in diverse proteins that play key functions in chromatin organization and regulation of gene transcription. Aberrant transcription is a hallmark of many diseases in particular cancer and inflammation. The complexity of molecular processes regulating gene transcription identified transcriptional regulators as interesting target for the development of specific chemical tool molecules (chemical probes) that help to understand the molecular mechanisms of transcription and to explore the potential of BRD mediated interactions as sites for pharmaceutical intervention. Recently a number of highly specific inhibitors have been developed against the BET (bromo and extra terminal) family of bromodomains. The availability of selective BRD inhibitors had a significant impact on the validation of bromodomain containing protein as targets for drug development and for our understanding of the biological roles of these proteins. In this review we will summarize the discovery of BET bromodomain inhibitors and their roles in target validation.

Introduction

Acetylation of lysine residues is a frequently observed posttranslational modification[1]. While the functional consequences of acetylation of cytoplasmic and mitochondrial proteins is poorly understood the role of acetyl-lysines in the regulation of chromatin structure and transcription has been well documented. Dense packing of DNA into chromatin requires neutralization of the high negative charge density of DNA by basic histones. Acetylation has a profound effect on the physicochemical properties of the lysine side chain by neutralizing the charge of ε-N amine. In histones, this property favours therefore an open, more loosely packed state of chromatin, leading to increased accessibility of regulatory regions on DNA and as a consequence transcriptional activation[2]. In many diseases, aberrant lysine acetylation leads to changes in gene expression resulting for instance in the inactivation of tumour suppressor genes and the activation of pro-survival and proliferation promoting pathways in cancer. Enzymes that "write" (acetyltransferases, HATs) and "erase" (histone deacetylases, (HDACs) ε-N-acetyl-lysine (Kac) modifications balance the level of acetyl-lysine in histones and participate in creating the so called epigenetic code, a complex language of post- translational modifications that regulate all aspects of chromatin biology. Due to the often observed deregulation of chromatin modifying enzymes in disease, targeting epigenetic mechanisms of transcription control has emerged as an interesting strategy for the development of novel therapies. Current drug discovery programs targeting acetylation homeostasis has been mainly focussed on the development of inhibitors for HDAC[3-5]. However, deregulation of the writers (HATs) and readers of acetylation marks has also been implicated in the development of a large number of diseases suggesting novel potential therapeutic applications of HAT and bromodomain inhibitors[6-10].

ε-N-acetyl-lysine containing sequences in proteins are specifically recognized by the bromodomain family of protein interaction modules. Bromodomains have been named after the Drosophila gene "brahma" for which the core bromodomain sequence motif was first identified[11]. The bromodomain family comprises 61 diverse domains in human that have been described in 41 usually nuclear proteins[12, 13]. Structure based alignments resulted in the identifications of 8 highly diverse bromodomain groups (group I-VIII) that comprise proteins of diverse function including histone acetyl transferases such as PCAF (P300/CBP associated factor), GCN5L2 (General control of amino acid synthesis protein 5-like 2), CREBBP (CREB-binding protein) and EP300 (E1A Binding Protein p300), ATP-dependent chromatin remodelling factors (SMARCA2/4 (SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin) and ATAD2A/B (AAA domain-containing

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protein 2), the methyl transferase ASH1L (Absent, small or homeotic-like), transcriptional modulators such as BRPF (Bromodomain and PHD finger-containing protein), TAF1/TAF1L (Transcription initiation TFIID associated factor), TRIMs (Transcription intermediary factor), BETs (Bromodomain and extra-terminal) and the nuclear body proteins (SP100, SP110 and SP140).

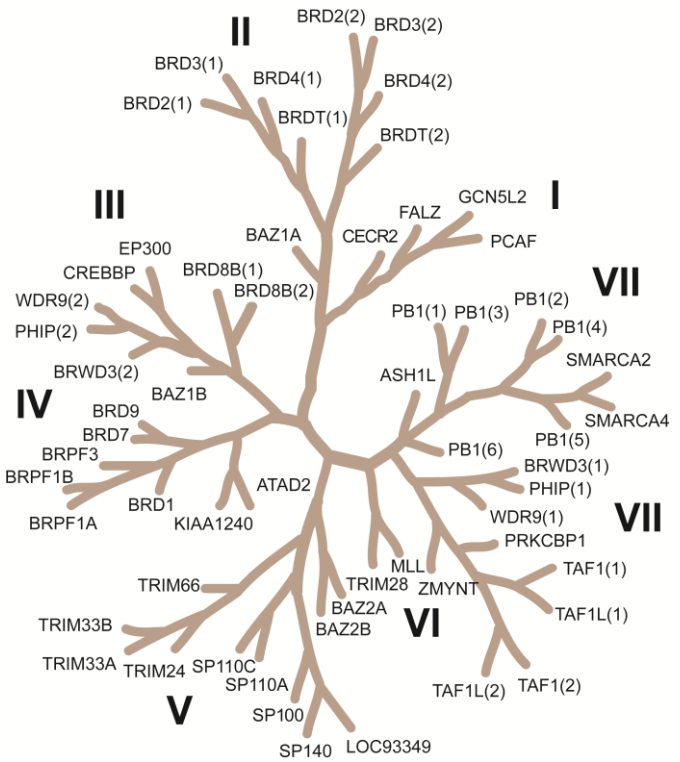

Figure 1: Human bromodomain family

BRD architecture and acetyl-lysine recognition

Bromodomains share a highly conserved fold that comprises a left-handed bundle of four alpha helices (αZ, αA, αB, αC). The four canonical bromodomain helices form a large central cavity. The loop regions (ZA and BC loops) linking the helices ZA and BC constitute the rim of the acetyl-lysine binding

pocket, which determines specificity of the interaction of bromodomains with acetylated peptide sequences. The acetylated lysine of bromodomain interaction sites is anchored by a hydrogen bond to a conserved asparagine residue present in most bromodomains[14]. In addition water-mediated interactions link the acetyl-lysine carbonyl to a conserved tyrosine residue. Some bromodomains recognize several histone marks. The first bromodomain in BET bromodomains (BRD2, BRD3, BRD4 and BRDT) for instance simultaneously bind di-acetylated sequences. Crystal structures of BET peptide complexes showed one of these acetyl-lysines interacting with the conserved asparagine while a second acetyl-lysine forms additional interaction with the BET peptide binding site[15,12].

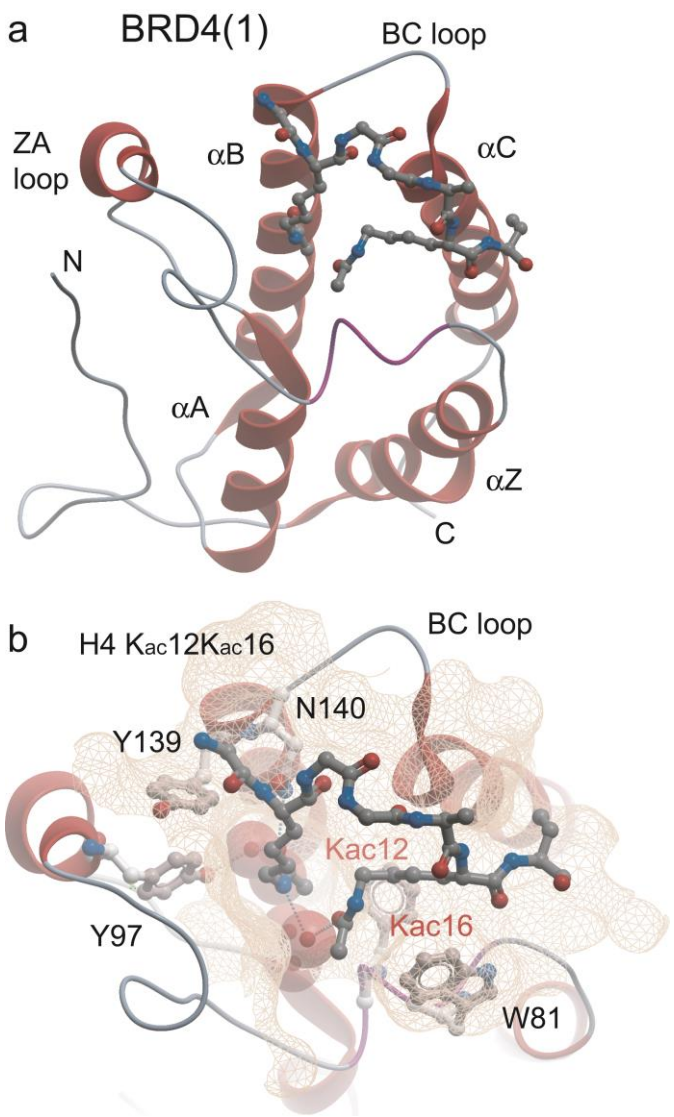

Figure 2: Architecture of bromodomains and details of the acetyl-lysine recognition site. Shown is a ribbon diagram of the first bromodomain of BRD4 (BRD4(1) (A) as well as details of the interaction formed with the di-acetyl-lysine peptide from histone H4 (Kac12Kac16). Conserved water molecules are shown as semitransparent cpk spheres.

Acetylation of the lysine side chains neutralizes the charge of the lysine amine. The consequence of this drastic change in side chain physiochemical property, the bromodomain acetyl-lysine binding pocket is lined by mainly aromatic and hydrophobic residues suggesting that chemical inhibitors with good cell permeability can be developed. In addition, the acetyl-lysine binding pocket is sufficiently large to accommodate chemical inhibitors of 300-500 Da and usually has a good level of enclosure. These properties suggest good "druggability" and the comparable weak interaction of bromodomains with their targeting sequences make the acetyl-lysine binding pocket an attractive site for the development of inhibitors[16].

The BET family of bromodomains

The bromo and extra terminal (BET) family of bromodomain proteins consists of 4 members in human (BRD2, BRD3, BRD4 and BRDT). Each BET family member contains two highly similar N-terminal bromodomains as well as an extra terminal (ET) protein interaction motif. BET bromodomains recognize acetylated lysine residues in histones H3 and H4. A number of additional recognition sequences have been described as well[12,15, 17-19]. BET family members play critical roles in cellular proliferation and cell cycle progression as well as in chromatin compaction[20].

The role of the BET family members BRD4 and the testis specific BRDT in transcription is at least in part mediated through interaction with the "positive transcription elongation factor b" (P-TEFb), a complex of the kinase CDK9 and its activator cyclin T. It has been shown that BRD4 recruits P-TEFb to acetylated chromatin, where it phosphorylates the C-terminal heptad repeat region of RNA polymerase II, a required post-translational modification for efficient transcriptional elongation of mRNA[21,22, 23]. BRD4 is known to interact with the mediator complex present at gene enhancers controlling transcriptional elongation by RNA Polymerase II. Recent work has demonstrated that BRD4 and potentially also other BET family members are not only localized to the core promoter regions of genes. High expression levels of many growth promoting genes is driven by transcriptional enhancers and BRD4 has been shown to be particularly enriched in enhancer regions[24]. Phosphorylation of the FOSL1 enhancer at the histone H3 residue serine 10 leads to recruitment of the histone acetyltransferase MOF and subsequent acetylation of the promoter region stimulating the recruitment of BRD4/PTEFb and FOSL1 transcription[25,26]. Interestingly, recent studies demonstrated that key lineage-specific survival genes are regulated by so-called super-enhancer regions. These transcriptional enhancers are considerably larger than typical gene enhancer regions and are densely populated by transcription factors leading to strong activation of gene transcription. Super-enhancers are present in loci of key oncogenic drivers. BRD4 is particularly enriched in these critical control regions suggesting that specific targeting of the bromodomains of BRD4 will lead to transcriptional repression of key oncogenic drivers[27, 28].

In addition, translocations of the BET locus give rise to oncogenes that lead to the development of highly aggressive cancer types: Genetic rearrangement of the BRD4 and BRD3 loci have been detected in an aggressive form of squamous carcinoma[29-33]. The identified oncogenic rearrangements comprise the tandem N-terminal bromodomains of BRD4 or BRD3 fused in frame with the protein NUT (nuclear protein in testis). The BRD4/BRD3-NUT oncogene results in the development of NUT midline carcinoma (NMC), an incurable, uniformly fatal subtype of squamous carcinoma which is usually only driven by this oncogene. The high dependency on the BRD-NUT oncogene including the bromodomains makes these cancer types particularly sensitive to BET bromodomain inhibitors[34].

Discovery of BET inhibitors

Using cellular phenotypic assays together with a chemoproteomic approach scientists at GSK discovered the highly potent and selective BET inhibitor iBET. iBET belongs to the benzo-triazolo-1,4-diazepine class (BZDs) and demonstrated strong anti-inflammatory properties[35, 36]. Also Mitsubishi Pharmaceuticals disclosed a series of structurally related thieno-triazolo-1,4-diazepines with strong growth inhibitory activity on an array of cancer cell lines[37, 38]. The disclosure of this patent prompted the development of the novel thieno-triazolo-1,4-diazepine JQ1 and a comprehensive characterization of this inhibitor *in vitro* and *in vivo*[34]. The (S) enantiomer, (+)-JQ1 is highly selective for BET bromodomains binding to the different BET bromodomains with low nM potency while the (R) enantiomer, (-)-JQ1 is inactive. Co-crystallization of (+)-JQ1 and iBET demonstrated that the methyl-triazolo group mimics interactions formed by the acetyl-lysine head group forming a hydrogen bond with the conserved asparagine (N140 in BRD4(1)). The co-crystal structure of (+)-JQ1 revealed also the structural reasons of the inactivity of the (R) isomer which is sterically excluded from the binding site. A robust synthetic route was developed for stereospecific synthesis of (+)-JQ1 and its inactive (-)-JQ1 isomer.

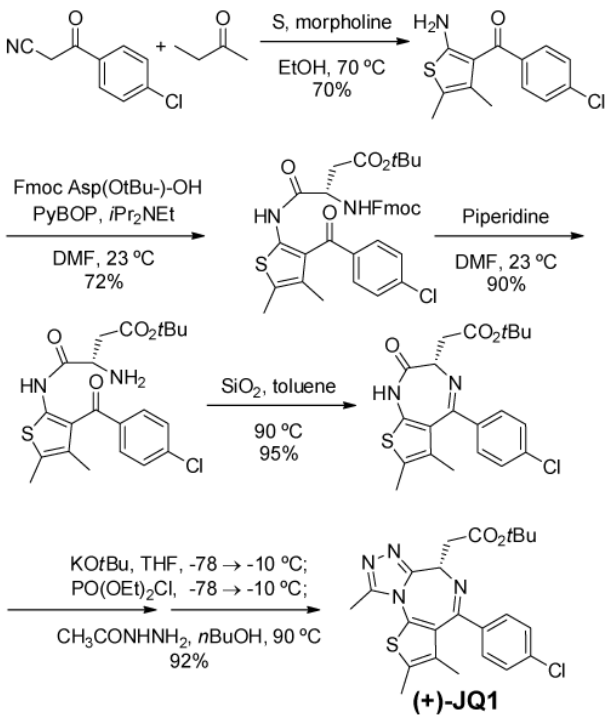

Figure 3: Synthetic route developed for (+)-JQ1[34].

The success of iBET and (+)-JQ1 in a number of cancer models and in inflammation gave rise to the development of several highly related benzodiazepine and thienodiazepine molecules. These molecules include the benzotriazepines (BzT), in which the asymmetric carbon atom has been replaced by a

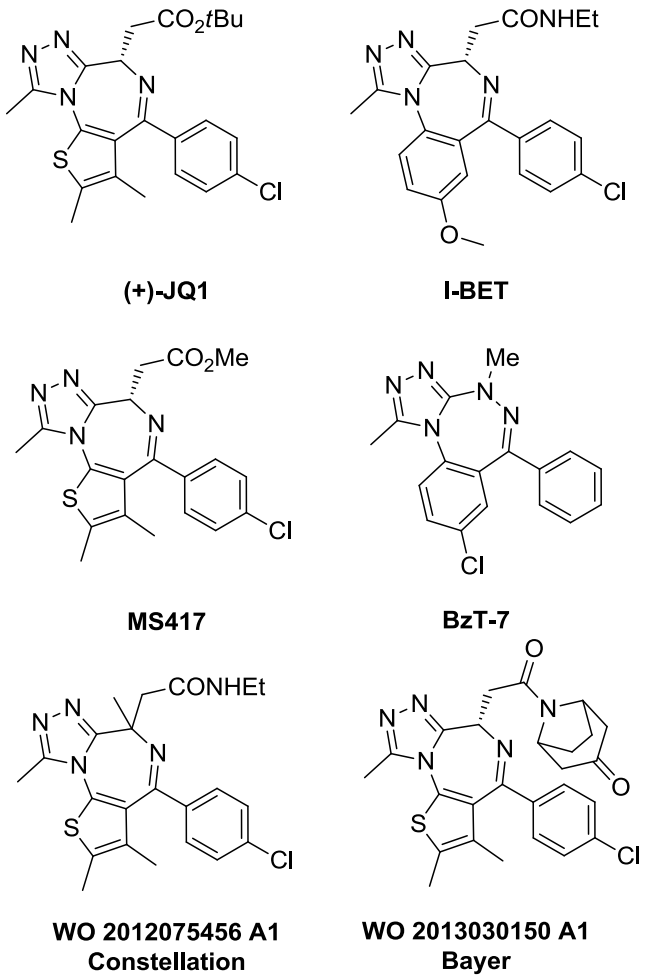

Figure 4: Inhibitors based on benzo-triazolo-1,4-diazepine and thieno-triazolo-1,4-diazepine scaffolds

nitrogen atom[39], the JQ1 methylester MS417[40] as well as a number of inhibitors that were disclosed in patent applications[41-45] (**Figure 4**).

In the search for novel acetyl-lysine mimetic bromodomain inhibitors a number of fragments have been co-crystallized with BRD4(1) (**Figure 5**). AlphaScreen assays revealed that acetyl-lysine (**1**) has an IC50 value of about 7 mM for BRD4(1)[46]. A number of solvent molecules often detected in crystal structures showed similar binding potency. These molecules include typical acetyl-lysine mimetic groups such as dimethylsulfoxide (**2**) (280 mM) and N-methylpyrrolidin-2-one (**3**) (6 mM)[46]. Other fragments include methyl-triazolo (**4**), which is also the acetyl-lysine mimetic group in published benzo and thieno-diazepines. Furthermore, 4-benzimidazole (**5**), N-acetyl-2-methyltetrahydroquinoline (**6**), 1-(1-(Pyridin-2-yl)indolizin-3-yl)ethanone (**7**), acetaminophen (paracetamol) (**8**), 3-methyl-3,4-dihydroquinazolin-2(1H)-one (**9**) and 4-phenyl 3,5-dimethyl isoxazole (**10**) as well as thiazolidinones (**11**) were identified as potential starting points for inhibitor development [47, 48, 49]. A recent *in silico* study revealed a number of additional fragments demonstrating the excellent druggability of BRD4 and other bromodomains[50]. Interestingly, also several kinase inhibitors have been identified inhibiting bromodomains including the clinical CDK inhibitor dinaciclib **(13)** as well as typical ATP mimetic fragments and others[51] **(14-16)**.

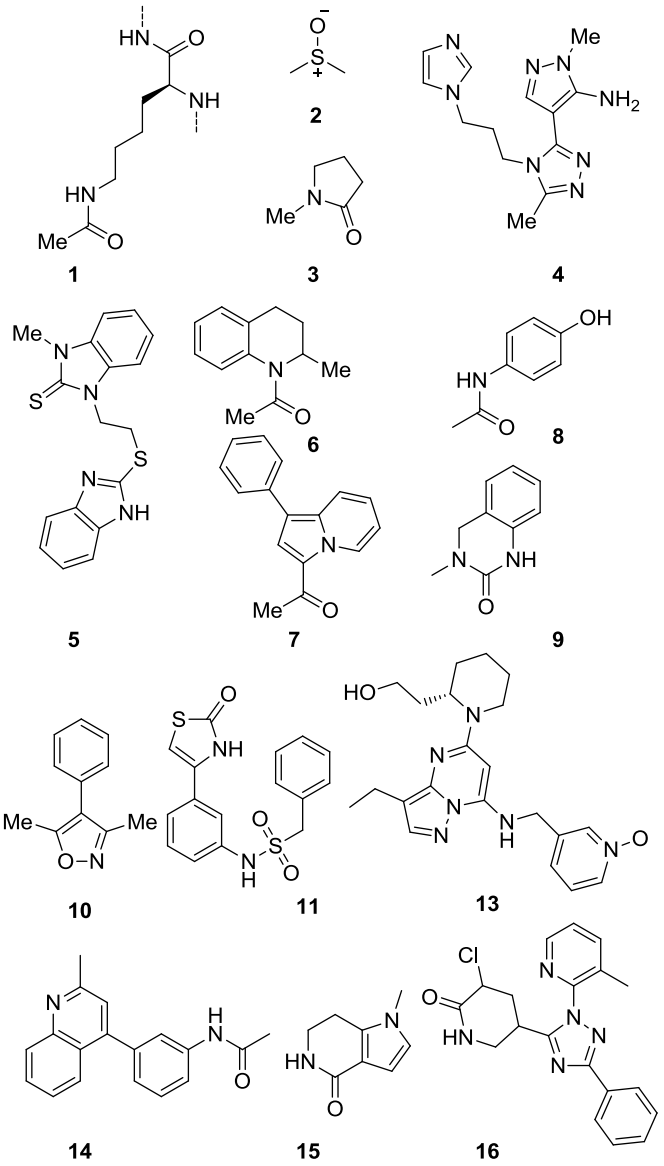

Figure 5: Acetyl-lysine mimetic fragments

Other BET inhibitors

Isosteric ring systems replacing the methyltriazolo acetyl-lysine mimetic moiety in benzo or thieno-triazolo-1,4-diazepines led to the development of 3,5-dimethylisoxazoles as BRD inhibitors[52]. Decoration of isoxazole acetyl-lysine bioisostere at the 4 position with aromatic ring systems resulted in selective BET inhibitors with good ligand efficiency[53] as well as potent and selective BET inhibitors of the 5-and 6-isoxazolyl-benzimidazole class[54]. Importantly, the methyltriazolo ring system was the basis for the development of 7-

isoxazoloquinolines GSK1210151A (I-BET151), a potent and highly selective BET inhibitor with excellent pharmacokinetic properties (**Figure 6**)[55-58]. In addition, a quinazolinone fragment hit (**Figure 5**) prompted a hit expansion series based on sulphonamides and reverse sulphonamide linkers yielding the BET specific inhibitor PFI-1[48]. Co-crystal structures confirmed the acetyl-lysine mimetic binding mode of the quinazolinone head group of PFI-1 which forms two hydrogen bonds with the conserved Asn140 in BRD4(1) as well as a water-mediated hydrogen bond to the conserved tyrosine Tyr97[59]. The excellent druggability of BET family members and the interest in this target class makes it likely that many more potent and diverse BET inhibitors will be developed in the near future.

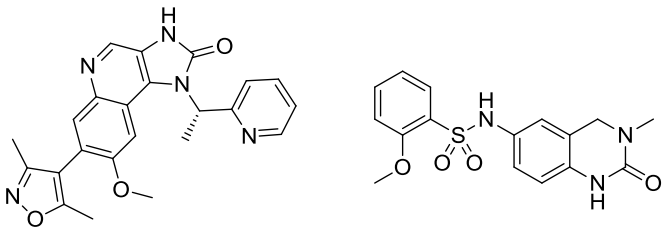

Figure 6:

Chemical structures of the BET inhibitors I-BET151 and PFI-1.

BET inhibitors as tools for target validation

Interest in BET proteins grew about 10 years ago based on discoveries that linked them to central roles in transcriptional regulation[21, 23, 60] and replication of viruses[61, 62]. In addition, identification of BRD4-NUT rearrangements in an aggressive sub-type of squamous cell carcinoma triggered interest of the oncology field in this protein family. Between 2000 and 2010 there was a steadily growing interest in the biological role of BET family members (**Figure 7**). It is likely that this trend would have continued in a linear fashion until today if BET specific bromodomain inhibitors would not have been discovered. The publication and availability of the BET specific inhibitors JQ1 and iBET at the end of 2010 spawned a tremendous interest of the chemical biology and medical communities. Since then research on BET proteins accelerated dramatically leading to fundamental discoveries for the use of these inhibitors in medicine, some of which are outlined below. However, the important aspect of that impact was the immediate public availability of the developed inhibitors that are now widely distributed by the developing laboratories as well as chemical vendors. In addition, a number of benzodiazepine and thienodiazepine inhibitors such as I-BET762 entered now phase I/II clinical trial for nuclear protein in testis (NUT) midline carcinoma and other cancer forms[63].

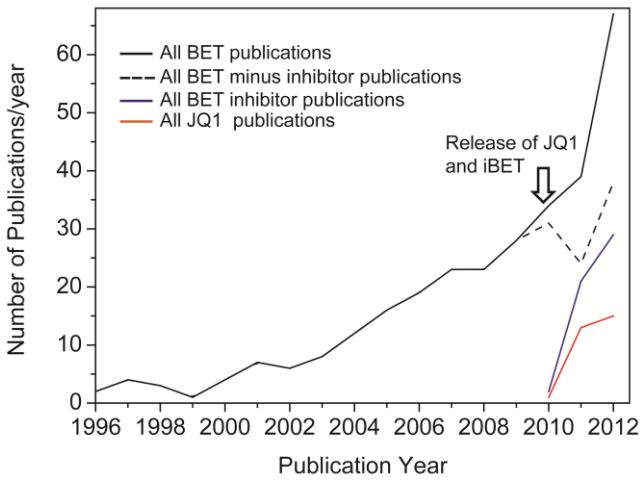

Figure 7: Impact of the availability of BET inhibitors on the chemical biology and biomedical research activity.

BET inhibitors in Oncology

Chromosomal rearrangements between BRD4 and BRD3 with the protein NUT (Nuclear protein in testis) have been detected in an aggressive and poorly differentiated squamous cell carcinoma[33]. BRD-NUT dependent carcinomas arise mainly in the midline of the body affecting the head, neck or mediastinum coining the name NUT midline carcinoma (NMC). Analysis of NMC showed that this tumour type, the BRD-NUT chromosomal translocation, is often the only genetic aberration providing a strong rational targeting BET bromodomains in this incurable cancer that predominantly occurs in children and young adults [29, 31, 64-68]. The BRD-NUT oncogene exclusively localizes to the nucleus while the wild type NUT protein has been detected in both the cytoplasm as well as the nucleus suggesting that the BET bromodomains are responsible for tethering the BRD-NUT oncogene to chromatin. Genetic knock down of the oncogene in patient derived cell lines lead to terminal differentiation and G1 growth arrest which provided further evidence for targeting BRD-NUT in this rare cancer[30, 69]. First evidence of the efficacy of BET inhibitors in NMC was published using the pan-BET inhibitor JQ1[34] which phenocopied the phenotype observed in genetic knock downs and showed compelling efficacy in mouse xenograft models of NMC.

First indications for the use of BET inhibitors beyond the specific and rare case of NMC were provided in studies on multiple myeloma. Gene expression analysis of treated multiple myeloma cells revealed that only the transcription of a small subset of genes is affected upon exposure of myeloma cells with JQ1[70]. In particular transcription of the general growth promoting oncogene c-myc was dramatically repressed. Following this study, strong down-regulation of c-MYC has been described in a large diversity of cancer types using also other BET specific inhibitors. These cancers include acute myeloid and mixed linage leukemia (AML)[55, 71, 72], lymphoma[73, 74], acute lympho-blastic leukemia (ALL)[75, 76], and

glioblastoma[77]. In addition, also repression of the N-MYC isoform was reported in neuroblastoma[78]. Also other key tumour drivers and anti-apoptotic genes have been shown to be repressed by BET inhibitors including Aurora kinases[59] and the FOSL transcription factor in lung adenocarcinoma cancer[79] as well as BCL2 and CDK6[55]. The unanticipated finding that BET inhibition preferentially supresses transcription of growth promoting and anti-apoptotic genes has been linked to the presence of BRD4 in gene enhancer regions which lead to strong up-regulation of transcription of often linage specific genes[24]. The large number of studies published in the past three years on the utility of BET inhibitors in oncology has provided convincing body of target validation data in the oncology field. However, more studies are needed to understand the function of BET bromodomains in different tissue types and the consequences of BET inhibition in a clinical setting.

Inflammation and viral infection

BRD4 has been reported to associate with and regulate the transcription factor NF-κB. Association with NF-κB is acetyl-lysine dependent suggesting that the BRD4 bromodomains play an important function in this process. NF-κB is a key regulator of inflammatory response[80] and association with BRD4 has been shown to stimulate transcription of NF-κB target genes[81]. In agreement with these findings that pan-BET inhibitor iBET showed strong anti-inflammatory response by efficiently suppressing transcription of pro-inflammatory cytokines in lipopolysaccharide (LPS) stimulated macrophages. In particular, the suppression of secondary response genes led to protection against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis in mice[36]. This effect may not only be due transcriptional modulation of NF-κB. Recent studies suggest that BRD4 also regulates ubiquitination and degradation of NF-κB significantly affecting protein half-life[82]. Also the BET family member BRD2 has been linked to the regulation of inflammation. A recent genome wide association study revealed strong correlation of BRD2 polymorphism and the development of rheumatoid arthritis[83]. Reduction of BRD2 levels in BRD2 hypomorphic mice results in server obesity and reduced inflammatory response of adipose tissue[84, 85]. In agreement with these data, the pan BET inhibitors iBET and JQ1 strongly supress pro-inflammatory cytokine production in endotoxemic mice that have been rescued from LPS-induced death[86].

The key role of BET bromodomains in viral replication has been recognized for a long time. Both BRD2 and BRD4 interact with viral proteins of herpesviruses, human papillomaviruses (HPV) and Merkel cell polyomavirus (MCV). These BET proteins are required for viral transcription and replication as treatment of infected cells with JQ1 lead to enhanced viral DNA replication[87]. Interestingly, recent data showed that inhibition of BET family members by JQ1 efficiently reverses HIV latency. These data suggest that BET inhibition may be a useful strategy for the eradication of the virus from latent reservoirs and may lead to the development of curative treatment in HIV[88, 89].

Other applications

BET family members have been recently identified as a potential targets for the development of new therapies of heart failure. Inhibition of BET bromodomains by pan-BET inhibitors including JQ1 effectively suppresses cardiomyocyte hypertrophy and pathologic cardiac remodelling in mouse models of heart failure by supressing the expression of genes that promote pathogenesis in failing heart[90, 91].

The testis-specific BET family member BRDT is essential for chromatin remodelling during spermatogenesis and male germ cell differentiation. Deletion of the first bromodomain as well as the whole protein has been shown to cause sterility in mice[92, 93]. Also inhibition of this BET isoform in testis by JQ1 blocked sperm cell differentiation suggestion potential applications of BET inhibitors as male contraceptive agents[93, 94].

Conclusions

Since the development of potent and selective BET inhibitors a large number of potential applications for these inhibitors have been discovered. Availability of the developed chemical probes initiated a large number of studies in very diverse disease areas significantly accelerating target validation efforts. It is likely that in the near future further potential application will be reported in the literature. The excellent efficacy of BET inhibitors in NUT midline carcinoma and in acute leukaemia led to the initiation of phase I clinical studies. The quick path from the discovery of BET inhibitors to clinical studies reflects the excitement of oncologist in this novel treatment strategy. However, more studies are necessary to understand the tissue specific roles of BET family members and potentially associated side effects of BET inhibition.

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