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Aim for the readers! Bromodomains as new targets against Chagas' disease

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Abstract: Bromodomains recognize and bind acetyl-lysine residues present in histone and non-histone proteins in a specific manner. In the last decade they have raised as attractive target for drug discovery because the miss-regulation of human bromodomains was discovered to be involved in the development of a large spectrum of diseases..However, targeting eukaryotic pathogens bromodomains continues to be almost unexplored. We and others have reported the essentiality of diverse bromodomain-containing proteins in protozoa, offering a new opportunity for the development of antiparasitic drugs, especially for *Trypansoma cruzi*, the causative agent of Chagas' disease. Mammalian bromodomains were classified in eight groups based on sequence similarity but parasitic bromodomains are very divergent proteins and is hard to assign them to any of these groups, suggesting that selective inhibitors can be obtained. In this review we describe the importance of lysine acetylation and bromodomains in *T. cruzi* as well as the current knowledge on mammalian bromodomains. Also, we summarize the myriad of small-molecules under study to treat different pathologies and which of them have been tested in trypanosomatids and other protozoa. All the information available led us to propose that *T. cruzi* bromodomains should be considered as important potential targets and the search for small-molecules to inhibit them should be empowered.

Keywords: Bromodomains, *Trypanosoma cruzi*, Acetylation, Bromodomain inhibitors, Chagas diseases, Drug discovery.

1. INTRODUCTION

Trypanosoma cruzi has many unique features that distinguish it from its mammalian hosts. One of the most striking characteristics of kinetoplastid parasites, *T. cruzi* included, is the way their protein-coding genes are arranged in the genome and how nuclear gene expression is controlled. Trypanosomatid's genes are tandemly arranged in polycistronic transcription units (PTUs), clusters of genes consecutively organized in a head-to-tail manner. Each chromosome can harbor several PTUs, which are separated by "strand switch regions" (SSRs) that can be either convergent or divergent (i.e., with genes oriented either towards or away from the SSR, respectively) [1–4]. Protein-coding genes are transcribed polycistronically without canonical promoters or typical gene transcription initiation control mechanisms. Indeed, gene expression in trypanosomatids is accepted to be trancriptionally regulated through the control of *trans*-splicing, polyadenylation, mRNA stability, translation, post-translational modifications or protein stability [5]. Even though no precise sequences specifying transcription initialization or termination have been determined, this atypical gene organization suggests that transcription should start at divergent SSRs and finish at convergent ones. In fact, experiments on *Leishmania major* Friedlin chromosomes 1 and 3 showed that transcription initiation and termination take place at SSRs [6, 7], and this appears to be true for most SSRs in kinetoplastids, although other minor transcription starting sites have been also described [8, 9].

It is worth noting that besides typical histone variants there are kinetoplastid-specific histone variants [10, 11]. Both canonical and variant trypanosomatid histones are subjected to different PTMs, suggesting that their histone code can be as sophisticated as other eukaryotes. Several trypanosomatid histone PTMs have been described showing their importance for different nuclear functions [12–15]. But fewer modifications were reported in the N-terminal tails of trypanosomatid histones compared to other organisms. In contrast, the C-terminal region of H2A appears to be hyper-acetylated. Interestingly, mono-methylation at the N-terminal alanines of H2A, H2B, and H4, a modification that has not been described previously for histones was found in *Trypanosoma brucei* [12, 16]. More recently, Picchi and coworkers identified a total of 13 distinct modification types, some of them similar to those found in many organisms and others rather novel or unusual such as alternative lysine acylations, serine/threonine acetylation, and N-terminal methylation [17].

The finding that acetylated and methylated histones are enriched at divergent SSRs but absent from convergent SSRs and intergenic regions within clusters in *T. cruzi* supports the idea that transcription initiates at the SSRs [18, 19]. Similarly, in *Leishmania,* acetylated H3 histone is enriched at divergent SSR and also present at chromosome ends and within polycistronic gene clusters. Interestingly, TATA-binding protein (TBP) and Small Nuclear Activating Protein complex (SNAP50), two basal transcription initiation factors, appeared to be associated with acetylated H3-rich sites, evidence that they represent transcription initiation sites [20]. Furthermore, in *T. brucei* H2AZ and H2BV

histone variants, associated with less stable nucleosomes, as well as acetylated histone H4 and a bromodomain factor were found to be enriched up to 300-fold at probable RNA polymerase II (RNAPII) transcription initiation sites. Additionally, histone variants H3V and H4V were enriched at probable RNAPII TTS, suggesting that histone modifications and histone variants play crucial roles both in transcription initiation and termination in trypanosomes [11]. Other epigenetic marks such as histone variants and the modified DNA base J have been localized to SSR regions in *T. brucei, T. cruzi,* and/or *L. major* [21]. Thus, even though the final protein-levels are determined by post-transcriptional events, emerging evidence suggests that epigenetic mechanisms play important roles in gene expression control and also in the biology and pathogenesis of trypanosomatid parasites.

Lysine (K) acetylation is a reversible PTM found in all domains of life from bacteria to humans. However, the exact biological role of this modification has been elusive. In the last ten years, several authors have identified thousands of acetylated proteins in different organisms using high-resolution mass spectrometry. The proteins identified are involved in diverse cellular functions including transcription, DNA repair, chromatin remodeling, cell cycle, splicing, metabolism, cytoskeletal dynamics, apoptosis, nuclear import, protein folding, and cellular signaling among others [22]. The acetylomes sizes range from around 300 proteins in prokaryotes to more than 2,000 in mammals and acetylation is distributed in almost all cellular compartments and organelles. Proteome-wide surveys in protozoan parasites have uncovered hundreds of acetylated proteins in *Toxoplasma gondii* and *Plasmodium falciparum* in every subcellular compartment [23, 24]. More recently, it has been reported that protein acetylation in Trypanosomes is involved in the differential regulation of multiple cellular processes. Moretti and coworkers identified a number of acetylated proteins and acetylated sites in *T. cruzi* and T. *brucei* that is similar to that identified in some bacteria and other protozoan parasites, but smaller than the number reported in yeast and metazoa. They also identified N-terminal and ε-lysine acetylated proteins in *T. cruzi* and *T. brucei* and showed that acetylation is variable among species and cellular stages. Acetylated proteins were found in almost every cellular compartment in both parasites, although the majority of the acetylated proteins were predicted to be cytosolic. In *T. brucei* bloodstream (BS) form, most acetylated proteins were nuclear followed by those found in the flagellum, being the paraflagellar rod protein 1 (10 acetyl-K sites), alpha- tubulin (7 acetyl-K sites), beta-tubulin (5 acetyl-K sites) and histone H2A (7 acetyl-K sites) the more extensively acetylated proteins [25]. Trypanosomatids are characterized by the presence of a particular cytoskeleton responsible for the modulation of cell shape between the different life cycle stages and for motility and attachment to the host cell surface [26]. Acetylated α-tubulin is found in the subpellicular microtubules and in the flagella of *T. cruzi* and *T. brucei* [27, 28]. This PTM is also present in the ephemeral microtubules of the mitotic spindle of *T. brucei* [28].

In contrast, in both insect stages, there were a larger number of acetylated proteins in the glycosome and the mitochondria. The most abundant non-histone acetylated proteins from *T. cruzi* are cytosolic, mitochondrial and glycosomal and this highlights the regulatory importance of this PTM in a myriad of cellular processes [25]. It is worth mentioning that the glycosome is a peroxisome-like organelle specific from trypanosomatids. It contains the first six or seven glycolytic enzymes together with some auxiliary pathways apparently involved in the re-oxidation of NADH and in the regeneration of the ATP consumed in the activation of glucose molecules [29, 30]. It has been reported in *T. brucei* that all Embden-Meyerhof segment of glycolytic enzymes, except for glucose phosphate isomerase, contain acetylated lysines [25]. Interestingly, as mentioned below, we identified a glycosomal bromodomain-containing protein in *T. cruzi* [31], which led us to suspect some kind of acetylation-dependent regulation in this particular organelle.

Even though in many proteins the significance of acetylation is not completely understood, acetylated K has physicochemical properties radically different from the non-acetylated amino-acid and it can, hence, modify the function of the whole protein. This PTM is controlled by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs and NADH-dependent sirtuins). In addition to these enzymes, which are considered "writers" and "erasers" of acetylation, the acetyl-K residues are recognized or "read" by a structurally conserved domain called bromodomain (BRD).

2. BROMODOMAINS

The bromodomain was identified as a novel structural motif studying the *Drosophila* gene *brahma*, also called *brm* [32]. *Brahma* received its name from a creator god in Hinduism traditionally represented with four faces and four arms, an image that resembles the trithorax developmental defect observed in *brm* lost-of-function mutations. *Brahma* encodes for a transcriptional activator that also has a DNA-dependent ATPase motif and a helicase motif. It is part of a large protein complex and the trithorax phenotype is caused by a decrease in the expression of the ultrabithorax gene. The BRD is a motif of 110 amino-acids, evolutionarily conserved and found in all eukaryotes studied. On December 12th, 2017, the Protein Data Base (https://www.rcsb.org/pdb/[33]) reported 1,312 structural entries related to BRDs, 1,261 of them obtained by X-ray crystallography and 51 by solution NMR. All bromodomains structures share a conserved structural fold of a left-handed four-helix bundle (αZ, αA, αB and αC), first described in the human Histone Acetyltransferase (HAT) co-activator P/CAF (p300/CBP-associated factor) [34]. The sequences between αZ-αA (ZA) and αB-αC (BC) form loops that constitute a hydrophobic pocket that recognize the acetylated K. Despite this structural

conservation, the overall sequence similarity between members of the BRD family is low, and there are significant variations in the amino-acidic sequences of the ZA and BC loops. Some hydrophobic side chain amino-acids located between the ZA and BC loops have been indicated as responsible for the hydrophobicity of the pocket and the recognition of the acetyl-K residue. Among them, V752, Y760, Y802 and Y809 from P/CAF are essential for acetyl-K recognition [34]. Interestingly, the replacement of tyrosine residues Y760 and Y802 for alanines (A) generates a correctly folded domain that is unable to bind to a derived peptide from histone H4 acetylated at K8 [34]. In addition to these hydrophobic amino-acids, a conserved asparagine (N) residue located in the BC loop (N798 in P/CAF and N1168 in human CREB-binding protein, hCBP) is essential for acetyl-K binding through a hydrogen bond between its nitrogen amide and the oxygen of the acetyl carbonyl group [35–37]. However, neither the asparagine nor the tyrosines are completely conserved in the reported BRDs sequences. In nature, Y can be found replaced by other hydrophobic amino-acids keeping the domain ability to bind acetyl-K, as can be observed in BRDs from Trypanosomatids (Figure 1A). The conserved asparagine seems to be quite essential but there are some functional BRDs where this amino-acid has been replaced by other hydrogen bond donors such as threonine or tyrosine [40–42]. As can been seen in the logos alignment in figure 1B, *T. cruzi* bromodomains are quite divergent but the representative amino-acids of this domain (Pfam HMM profile, figure 1C) are maintained.

BRD-binding to acetylated peptides can be influenced by the environment of the modified K, giving some specificity to the interaction. This selectivity can be influenced by the presence of other modified amino-acids in the surroundings of the K in the target protein, and some BRDs from the bromodomain extra-terminal domain (BET) family can recognize two neighbor acetylation marks in a single binding pocket [38–40]. In this scenario, one of the acetyl-K interacts with the conserved N and the second one binds to the edge of the hydrophobic pocket, through hydrogen bonds with the peptide backbone. Even though the BRD selectivity for the recognition of acetylated proteins is weak, some degree of selectivity exists for the sequence surrounding the acetylated peptide. Only in a few cases, the residues responsible for this specificity at the peptide-binding site have been identified [36, 38, 39, 41, 42]. Another characteristic feature of the BRD acetyl-K recognition site is the presence of a network of water molecules that interacts, through hydrogen bonds, with carbonyl groups of the protein backbone at the base of the binding pocket [43]. These water molecules are relatively conserved in the reported structures of almost all BRD families and differences in this network have been associated to the selectivity of the BRDs for the different acetylated ligands [44].

The domain architecture of BRD-containing proteins, usually called BDFs (for BRD-factor), is very diverse. For instance, BRDs can be present as a unique domain or in tandem and a BET domain can accompany double BRDs.

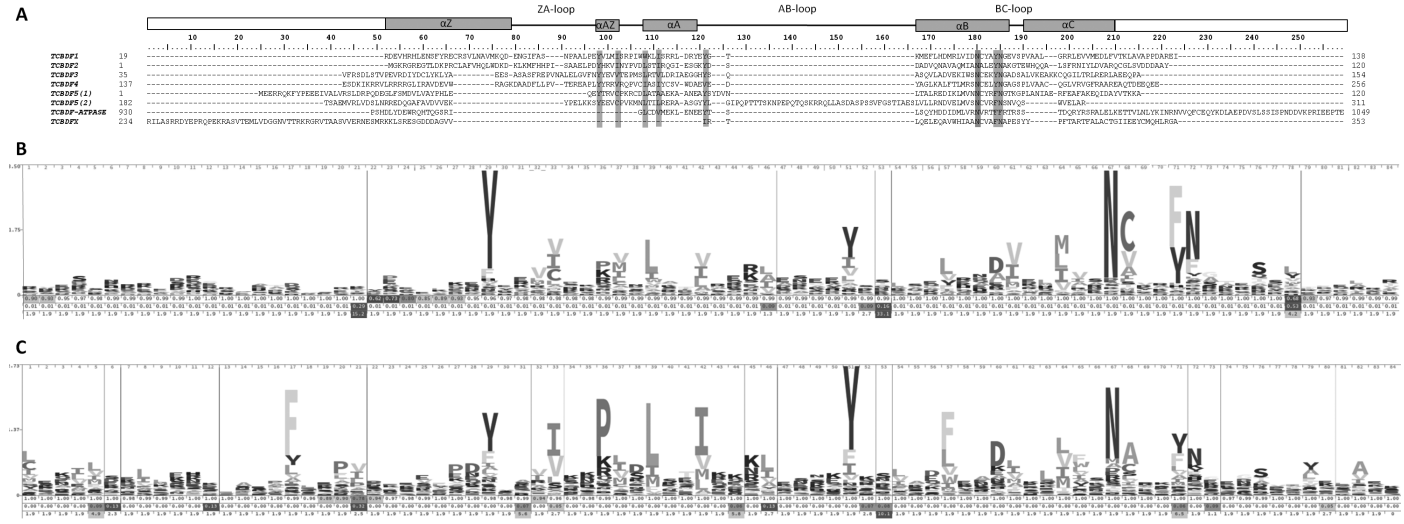

Figure 1: Alignment of the profile HMM for *T. cruzi* bromodomains was constructed using *hmmalign* (default parameters) from HMMER 3.1 on the Pfam seed alignment (A). The conserved asparagine (N) residue that mediate interaction with the acetylated lysine is shaded dark gray and the hydrophobic amino-acids light gray. Logos were built with Skylign (http://skylign.org) based on profile Hidden Markov Model (HMM) from *T. cruzi* bromodomains (B) and Pfam (PF00439) of mammalian bromodomains (C). In the bottom of each logo there is a table which indicates: occupancy (top row) and with dark grey background lower occupancy; insert probability (middle row); and expected insert length (lower row). For both of the latter rows, a black background indicates higher values. The thin vertical bar drawn indicated that the insertion will produce letters between the neighboring positions.

BET domain-containing BRDs define the BET family that includes the transcription regulators BRD2, BRD3, BRD4 and BRDT and are the most studied ones [45]. Furthermore, BRDs can be accompanied by a large diversity of different domains. The Pfam database (release 31.0, posted 8 March 2017) has 17,577 protein sequences from 824 eukaryotic

species that contain BRDs (PF00439). These sequences are organized in 641 unique architectural domains in which BRDs are found in combination with a number of other domains, such us chromatin-related modules like other BRDs, BET and HAT (among the most frequents), PDH (plant homeodomain), PWWP, helicase, ATPase, zinc fingers, SAND (named after Sp100, AIRE-1, NucP41/75, DEAF-1 proteins), FY Rich, SET (named after Su(var)3-9, Enhancer-of-zeste and Trithorax proteins), BAH (bromo adjacent homolog), WD40 repeat and MBD (methyl-CpG binding domain) [46].

Like the acetylome size, the number of BRD-containing proteins increases with the complexity of the species, ranging from seven in yeast (one of them considered a BRD-like domain, *Saccharomyces* Genome Database, SGD; http://www.yeastgenome.org) to 46 in humans [38]. The architectural complexity is also a reflection of the biological complexity. Two yeast BRD proteins belong to the BET family and only these two plus another non-BET BRD have extra domains (BAH and HAT). On the other hand, the 46 human proteins contain 61 individual BRDs, being BAF180 the largest poly-bromo with six bromodomains. Currently, there is a consensus classification for the human BRD family into 8 subgroups [37, 38]. Due to the BRD ability to bind acetyl-K residues in histones, many of them serve as chromatin-targeting modules that "decipher" the histone code [47]. Hence, BRD-containing proteins can modulate transcription by recognizing acetyl-K residues in nucleosomal histones and recruiting proteins of the transcription machinery. When BRDs are associated to a HAT activity, the acetylated histone recognition is usually followed by a massive acetylation of the surrounding nucleosomes. Under this model, transcription modulation through BRD-containing complexes may result in activation, but also in inhibition of transcriptional activity. Nevertheless, transcription modulation is not the only function associated to BRDs. Many protein complexes with chromatin remodeling activity, as well as ubiquitin and SUMO ligase activities and methyltransferases bare this domain [38, 44]. Finally, even though histones are the most common ligands of BRDs, acetylation is also a frequent PTM in many nuclear non-histone proteins that were also described as ligands for BRDs [44].

The study of the BRD mechanisms of action is a very active field of research. Since this domain was accepted as a target for many cell-proliferative associated diseases, a large amount of structural information has been produced, most of it for the human BRD family and for the structure-guided development or optimization of bromodomain inhibitors (especially for the BET family). This phenomenon explains the explosion of BRD structural entries in the PDB in the last years: out of 1,312 structures, 1,272 (95,2 %) belong to the 46 human BRD-containing proteins (most of them to the four members of the BET family). Currently, it is known that small molecules with BRD-inhibiting activity have potential effect against specific pathologies, but the detailed function of many BRD-containing proteins remains unknown. Regardless of this, BRDs are considered as key proteins for epigenetic regulation and their malfunction may directly cause or maintain a disease state. In many cancers, BRD-containing proteins are deregulated and transformed into oncogenic proteins by gene fusions or point mutations [48]. In such cases BRDs may be good candidates for direct drug targeting. Alternatively, BRDs may be the effector of one (or more than one) upstream signaling pathway that ends in transcription regulation. A good example of this is the participation of BRD4 in the c-Fos/AP1-mediated papillomavirus-induced tumorigenesis (originally initiated by E2 viral transcription factor). This signaling cascade involves c-Fos/AP1, a transcription factor which is not druggable and BRD4 is downstream of the cascade and can be targeted to treat this viral disease [49]. In situations like this, when the main drivers of the disease are hardly druggable, BRDs may be good targets to interfere indirectly with the outcome of the disease.

Finally, even though the BRD is considered a nuclear domain, there are some reports of proteins containing BRDs with dual cytoplasmic-nuclear localization. Human BRD2 is a protein that has been detected in many tissues. During proliferation and differentiation of mouse spinal cord and dorsal root ganglia, BRD2 switches its localization from the nucleus in proliferating cells to the cytoplasm in differentiated neurons that are no longer cycling [50]. During ovarian folliculogenesis, BRD2 localization also shows a change from nuclear to cytoplasmic with the advance of oocyte maturation and meiotic competence [51]. BRD7 is considered a negative regulator of cell proliferation and growth and also shows a broad expression distribution in the nucleus of human fetus cells from cerebellum, pancreas, intestines, liver and kidney. However, in cardiomyocytes BRD7 shows high cytoplasmic expression [52]. The BRD-PHD finger-containing protein BRPF1 was found in the cytoplasm of NIH 3T3 cells, from where it is translocated to the nucleus in complex with MOZ (monocytic leukemia zinc finger), ING5 (inhibitor of growth 5) or EAF6 (Esa1-associated factor 6 ortholog) [53]. Conversely, in J558 cells the BRD-PHD-containing protein LYSp100 delocalize from the nucleus to the cytoplasm in the presence of the nuclear B cell regulator of IgH transcription (Bright), a transactivator of the immunoglobulin heavy chain (IgH) intronic enhancer [54]. BRD dual localization was also observed in the Histone Acetyltransferase GCN5 of *Candida albicans* that moves from the nucleus to the cytoplasm during vegetative growth [55]. The fact that the available data about extra nuclear localization of BRD-containing proteins is very limited suggests that this is an atypical situation in nature. However, the increasing interest for developing inhibitors to be used as epigenetic modulators have focused the interest of research to nuclear BRDs, leaving out the study of the possible function of BRDs out of the nucleus.

3. BROMODOMAINS IN TRYPANOSOMATIDS

3.1. *Trypanosoma cruzi* bromodomains

According to the TritrypDB database (http://tritrypdb.org/tritrypdb/) there are seven coding sequences (CDSs) for BRD-containing proteins in trypanosomatids, named BDF1 to BDF5, BDFx and BDF-ATPase (Table 1).

Table 1: Accession numbers from TriTrypDB for bromodomains coding sequences

	T. cruzi	*T. brucei*	*L. major*
BDF1	TcCLB.506247.80	Tb927.10.8150	LmjF.36.6880
BDF2	TcCLB.507769.30	Tb927.10.7420	LmjF.36.2980
BDF3	TcCLB.509747.110	Tb927.11.10070	LmjF.36.3360
BDF4	TcCLB.508857.150	Tb927.7.4380	LmjF.14.0360
BDF5	TcCLB.510359.130	Tb927.11.13400	LmjF.09.1260
BDFx	TcCLB.510889.330	Tb927.1.3400	LmjF.12.0430
BDF-ATPase	TcCLB.506297.110	Tb927.11.6350	LmjF.11.0910

Even though the orthologous genes can be easily identified by sequences alignment, the Tritryp BRDs share limited identity, even at a structural level (Figure S1 and S2). Among trypanosomatids, the inner core of each BRD is different and the only identical amino-acid is the conserved asparagine previously mentioned. The hydrophobic pocket is constituted by non-identical amino-acids with conserved hydrophobicity. In *T. cruzi*, *Tc*BDF1-3 and *Tc*BDF-ATPase bare a unique domain. *Tc*BDF1-3 BRD represents almost half of the protein and the remaining sequence contains long stretches of identical amino-acids with low complexity. *Tc*BDF5 has two BRDs in tandem. The second BRD is highly divergent and its boundaries are hard to establish. On the contrary, the second BRD from *L. major* ortholog (*Lm*BDF5) is well recognized by the Pfam database search engine (Figure S3). The second domain from *T. cruzi* and *T. brucei* seem to have an insertion at the AB-loop (Figure S1). *Tc*BDF4 and *Tc*BDF-ATPase are the only BRD-containing proteins from Tritryps that bear an additional domain, a CW type zinc finger and an ATPase domain, respectively (Figure S1). The seventh BRD-containing protein, *Tc*BDFx, has a very divergent BRD that seem to be absent in the orthologous gene from *L. major* (Figure S1). Neither Pfam nor SMART motif-search engines detect this putative bromodomain when the search is performed under default parameters, suggesting that it may be an extremely divergent BRD or may not be a real bromodomain at all.

When aligned and analyzed together with the human BRDs, the *T. cruzi* BRDs showed to be very divergent and localized at the root of the phylogenetic tree, with very long branches. Similar results were obtained when the alignment was analyzed by Neighbor-Joining or Maximum likelihood methods [56]. Only *Tc*BDF1 clustered within the human group V, but the statistical consistence (bootstrap parameter) of the group containing the human and *Tc*BDF1 sequences was between 30% and 50% of that obtained when only the human sequences were used (depending on the method used). This suggests that *Tc*BDF1 is clearly more similar to group V but we cannot asseverate that it belongs to this group (data not shown).

In the past years we identified and characterized *Tc*BDF1, *Tc*BDF2 and *Tc*BDF3 from *T. cruzi* [31, 57–59]. *Tc*BDF2 was the first bromodomain to be described in *T. cruzi*. It is expressed throughout the whole life cycle of the parasite showing no differences in the protein content among the different stages. By immunocytolocalization and subcellular fractionation we determined that *Tc*BDF2 locates in discrete regions of the nucleus, but localization and its function in the nucleus are still unknown. *Tc*BDF2 recognizes histones H2 and H4, when assayed by far-western blot (WB) over a histone fraction purified from *T. cruzi* nucleus. Furthermore, co-immunoprecipitation and far-WB assays using peptides derived from the N-terminal domain of histone H4 showed that *Tc*BDF2 binds preferentially to acetylated K10 and K14 of histone H4. This bromodomain accumulated after exposure of the parasites to UV light, suggesting it may be playing key roles in the chromatin remodeling that occurs during the DNA repair process [58]. On the other hand, overexpression of *Tc*BDF2 lacking its C-terminal domain causes a decrease in the replication rate of epimastigotes, suggesting that this mutant protein could be acting as negative dominant interfering with the normal functions of the protein. The overexpression of a wild type version of the protein did not show any effect on the growth rate but it did diminish the parasite sensitivity to UV radiation (unpublished results from our group) supporting the idea of a role in DNA repair.

Most of the BRDs studied in trypanosomatids were found in the nucleus. To our knowledge, we were the first to describe and characterize extra-nuclear BRDs that seem not to have a dual cytoplasmic-nuclear localization. We found that *Tc*BDF1 and *Tc*BDF3 are mainly localized in the cytoplasm of *T. cruzi* cells. *Tc*BDF1 localized out of the nucleus and it is developmentally regulated throughout the *T. cruzi* life cycle. Higher protein levels were found in the infective stage (trypomastigotes) than in the replicative stages (amastigotes and epimastigotes). Confocal microscopy and subcellular fractionation confirmed that *Tc*BDF1 locates outside the nucleus and co-localization assays with a GFP-PTS1 (peroxisome target signal 1) expression plasmid [60], showed that *Tc*BDF1 is found in the glycosome, a unique organelle present in trypanosomes that contains the majority of the enzymes related to the glucose metabolism, as mentioned earlier [61, 62]. Later, a PTS2 sequence was identified at the N-terminus of *Tc*BDF1, and the deletion of the first 28 amino acids of the protein changed dramatically its localization from the glycosome to the cytosol [62]. Even though we were not able to determine the exact biological function, nor the proteins associated with *Tc*BDF1, its importance for the parasite fitness has been well established. Overexpression of the wild type protein affected epimastigotes replication rate causing the parasites

death probably through an apoptotic mechanism [62]. A mutated form of *Tc*BDF1 (where the bromodomain structure is unaltered but the putative ability to bind the acetylated lysine is abolished) had no effect on epimastigotes replication rate but it did affect trypomastigotes infection rates and amastigotes replication. Metacyclogenesis was also affected by the mutated version showing a decrease in the number of trypomastigotes obtained while the wild type version had no effect [62]. These results allowed us to gain insights into the role of *Tc*BDF1 related to the parasite's fitness. Also, as mentioned before, many glycolytic enzymes located in the glycosomes were found to be acetylated in *T. cruzi* [25], and we know from mammalian models that acetylation plays a major role in metabolic regulation [63]. So, it is not unrealistic to think that *Tc*BDF1 could be playing regulatory roles inside the glycosome. In agreement with this idea, Moretti and coworkers suggested a role for acetylation of glycosomal enzymes in the regulation of the metabolic switch during the differentiation from the bloodstream to the procyclic form of *T. brucei* [25].

The second non-nuclear bromodomain to be described in trypanosomes was *Tc*BDF3. This protein is also expressed throughout the whole life cycle of the parasite, but no differences in the protein content among the stages were observed. Several approaches were performed to demonstrate its localization. First, subcellular fractionation followed by WB assays showed that *Tc*BDF3 was detected in the cytoplasmic and in the insoluble fraction associated with cytoskeletal and flagellar proteins. Next, by transmission electronic microscopy we showed that no signal was detected in the DNA containing structures such as nucleus and kinetoplast, but we did detect labeling in the flagella, especially in the inner part, which is attached to the cell body. A similar pattern was obtained by confocal microscopy where *Tc*BDF3 was dispersed in the cytoplasm and close to the flagellar pocket region both in epimastigotes and amastigotes. However, the flagellar pocket region was more deeply marked and the plasma membrane appears to be also labeled in amastigotes. On the other hand, *Tc*BDF3 in trypomastigotes showed a different subcellular localization pattern when compared to the replicative stages. Surprisingly, in this stage *Tc*BDF3 was localized exclusively in the flagellum [57]. Regarding its biological relevance, overexpression of wild type *Tc*BDF3 showed no effect on the replication rate of epimastigotes. On the contrary, when the bromodomain was mutated (unable to bind acetyl-K), it caused a delay in the growth. Furthermore, the phenotype of these parasites was affected: a detachment of the flagella was evidenced by confocal microscopy suggesting that this BRD is involved in the genesis and maintenance of this cellular structure in epimastigotes. The relevance of *Tc*BDF3 in the metacyclogenesis process was also shown. Overexpression of mutated and wild type *Tc*BDF3 diminishes the differentiation rate from epimastigotes to trypomastigotes, suggesting that *Tc*BDF3 may be involved in this process independently from its ability to bind acetyl-K. Regarding its capacity to infect mammalian cells, *Tc*BDF3 may play also a determinant role since overexpression of *Tc*BDF3 in trypomastigotes decreased their infectivity, whereas mutant *Tc*BDF3 increased it. The replication of intracellular amastigotes was also improved when the mutated version was overexpressed, in correlation with an increased number of trypomastigotes released when the cells bursted. In contrast, overexpression of wild type *Tc*BDF3 did not seem to affect amastigote growth but it appears to be necessary for the development of the trypomastigote flagella during differentiation from amastigotes [59]. Although tubulin acetylation is a widespread modification present in all eukaryotic cells, its precise function in cytoskeleton dynamics has not been completely elucidated yet. Unlike other organisms, *T. cruzi* contains most of its α-tubulin acetylated [27]. *Tc*BDF3 was the first bromodomain found to interact with this tubulin PTM, and acetylated tubulin from *T. cruzi* is the first and only cytoplasmic ligand identified from any BRD so far. Supporting this, we observed by far-WB assays using recombinant *Tc*BDF2 and *Tc*BDF3 with acetylated and not-acetylated peptides derived from histone H4 and α-tubulin that the interactions *Tc*BDF2/acetylated-H4 and *Tc*BDF3/acetylated-α-tubulin are specific and do not cross react [57].

*Tc*BDF4 and *Tc*BDF5 have not been characterized yet but *in silico* predictions suggest that they could be nuclear proteins since both of them have a bipartite nuclear localization signal. As already mentioned, *Tc*BDF4, like its homologues in *T. brucei* and *Leishmania*, contains a zinc finger motif within its coding sequence. *Tc*BDF-ATPase as well as their orthologues from *T. brucei* and *Leishmania*, have a predicted ATPase motif, which is a common feature found in several BRD-containing proteins that takes part in chromatin remodeling complexes [64].

Further studies must be performed to elucidate the exact function of *T. cruzi* BRDs, and how the direct interaction with their acetylated ligands can contribute to the regulation of key roles in the biology of this parasite.

3.2. *Trypanosoma brucei* bromodomains

Four BRDs have been characterized in *T. brucei* and all of them have been localized to the nucleus (*Tb*BDF1, *Tb*BDF2, *Tb*BDF3 and *Tb*BDF5) [11]. It appears that *Tb*BDF2 has a nuclear localization that is more restrained to the nucleolus region, implying a role in the genic regulation of this parasite. This BRD binds to histone variant H2AZ/H2BV dimers and it was demonstrated that it specifically recognizes the hyperacetylated N-terminus of H2AZ [65]. Depletion of *Tb*BDF2 resulted in a normal growth for up to 48 hours, after which a growth defect emerged, showing a cell cycle perturbation [66].

Endogenous tagging helped to demonstrate that *Tb*BDF3 also binds to modified histones, especially H4K10ac (a PTM that is enriched in probable RNAPII transcription initiation site). *Tb*BDF3 essentiality was evidenced through RNA interference (RNAi) where the protein content was lowered. In these parasites, the reduction in the growth rate was clear

after 48hs where most of the cells died. Even though *Tb*BDF3 colocalized with H4K10ac, the specific binding to this PTM is yet to be validated [11]. RNA-seq assays showed that many insect-stage specific genes were up-regulated when *Tb*BDF3 was depleted, such as the variable surface glycoprotein (VSG) genes, which are located at silent BS expression sites (BESs) [66].

Through a genome-wide RNA interference viability screen, Alsford and coworkers selected five essential candidates as chromatin associated factors and among them it was *Tb*BDF5. This BRD was also found in the nucleus and it is proposed to be a key factor in regulating VSG gene expression sites. Inducible knockdown showed a decrease in the replication rates of both BS and insect stages but further studies must be done to elucidate its specific role in chromatin biology [67]. Even though the characterization of BRD-containing proteins from *T. cruzi* and *T. brucei* is still partial, it is clear that orthologous from these species identified by sequence similarity are not necessarily functional homologs.

4. BROMODOMAIN INHIBITORS

The idea of using small molecules that disrupt molecular interactions as therapeutic drugs has been accepted and developed in the last years as an alternative to classical approaches of enzymatic inhibition. In 2007, two groups described for the first time two compounds that inhibited the interaction between components of a protein complex, setting the bases for the "druggability" of this kind of complexes. The microbial natural products FR901464 and pladienolide B originally discovered as anticancer agents, were found to inhibit the spliceosome activity by disrupting its quaternary structure [68, 69]. Three years later, the first inhibitors of the interaction between bromodomains and their acetylated partners were described, demonstrating that this protein interaction is "druggable" [70–72]. Since then, the number of BRD inhibitors described has increased exponentially. In parallel, many new screening methods, most of them borrowed from structural biology, have been developed. Here, we summarize the different experimental approaches used to discover BRD inhibitors (for a detailed discussion of this topic the reader can refer to a review by Chung and Witherington [73]). We also compile the different chemical structures that have been associated to BRD-inhibiting activities.

4.1. Assays used to discover bromodomain inhibitors

Novel BRD inhibitors have been discovered through several strategies, such as high throughput screening (HTS) techniques, fragment-based drug discovery (FBDD), computational techniques, rational design or combinations of them. Also, there are several cell-based assays described to test small molecules against BRDs. The *in vitro* assays can be divided into two groups: label-free and displacement assays. On the first group we have Nuclear Magnetic Resonance (NMR), Surface Plasmon Resonance (SPR), Differential scanning fluorescence (DSF), Isothermal Titration Calorimetry (ITC) and X-ray crystallography. NMR gives information about the mode of action simultaneously to screening but it cannot be used to perform HTS. ^{1}H-^{15}N-NMR has been widely used for fragment-based and structure-based focus screening and can yield validated starting points for chemistry [74, 75]. SPR is very popular for fragment studies and uses far less protein than NMR. It is amenable for HTS and generates also affinity and kinetic data. This method immobilizes one component of the interaction onto the surface of a gold chip, over which solutions of putative binders are sequentially introduced [76]. The other biophysical methods mentioned before have mainly been used as part of the hit confirmation strategy. DSF or Thermal Shift monitor changes in protein thermal stability upon binding of the ligand by using a fluorescent dye that binds more avidly to denatured protein [77]. This strategy has been proven useful for evaluating compound selectivity over multiple BRDs [78]. Also, it needs lower amounts of protein than NMR, it is plate-based and easily scalable and requires less expert knowledge to run and interpret. The downside is that it detects all interactions increasing the protein thermal stability, some of which may be nonspecific with no functional relevance [77]. ITC is considered the gold-standard to determine direct binding modes despite its low throughput and high protein consumption, because it gives direct measurement of the thermodynamics of binding [70, 79]. Finally, X-ray crystallography has been used to characterize specific modes of action and confirmation of hits [80].

The second group of *in vitro* assays is based on the displacement of a known ligand from the BRD. To measure this displacement several techniques have been described, like ELISA [38] and the highly sensitive AlphaScreen. AlphaScreen is a proximity-based reporting system with an acceptor and a donor bead. When these beads are in close proximity, a chemiluminescent signal is produced. This assay is useful for weak interactions and also uses small amounts of proteins [81]. Time-resolved Förster resonance energy transfer (TR-FRET) assays have also been reported. They require a suitable peptide-binding partner for the BRD and have been used to validate hits against the BET BRDs [82]. Also using fluorescence detection, a sensitive fluorescence anisotropy (FA) assay was developed, in which the displacement of a fluorescently labeled probe molecule is detected [83, 84]. A fluorescence polarization competitive assay was also reported to efficiently screen and evaluate inhibitors of the interaction between PCAF bromodomain and a Tat-AcK50 peptide [85]. We have described a microplate assay to measure the changes in the intrinsic fluorescence of *Tc*BDF3 upon exposure to small-molecules. Such fluorescence is produced mainly by a tryptophan (W) residue located in the hydrophobic pocket of the BRD that is quenched when a compound binds to the acetyl-K binding pocket. This method has been successfully used to determine

that human BET-bromodomain inhibitor iBET-151 interacts with *Tc*BDF3 and scaled up by our group [86]. Using this method combined with Thermal Shift assays and a bioguided fractionation of chemically modified plant extracts we identified an oxime hit that interacts with *Tc*BDF3 with affinity in the submicromolar range and that shows interesting antiparasitic properties against the different life cycle stages of *T. cruzi* [87]. With the same strategy but using a small library of acylhydrazones obtained through dynamic combinatorial chemistry we discovered another hit for *Tc*BDF3 active against *T. cruzi* [88].

Finally, an important area of epigenetic drug discovery is the use of cell-based assays. Several of these strategies will be described later, but it is worth mentioning that one of the first BRD inhibitors described was found using a phenotypic screening approach to discover up-regulators of Apolipoprotein A1 (ApoA1) [89]. Also, an in-cell FRET assay for histone acetylation was developed and allowed the identification of a small-molecule inhibitor of the BRD2-histone H4K12ac interaction [90]. Finally, Fluorescence Recovery After Photobleaching (FRAP) experiments have been established for 11 representative human BRDs. These results demonstrate that FRAP offers a potentially pan-bromodomain method for generating cell-based assays [91]. Although these approaches are powerful for identifying cell-permeable small molecules that generate the desired phenotype, the cellular target or targets must be subsequently identified by other methods.

4.2. The chemical diversity of BRD inhibitors

In this section we discuss different chemotypes that have emerged in the last seven years as high-affinity small molecule BRD inhibitors. BRD-containing proteins have come to rival epigenetic writers and readers as promising chemotherapeutic targets in a wide range of diseases, including cancer, neurological diseases, antiviral therapy, inflammatory and autoimmune diseases. A quick search into the ChEpiMod database, which allows the search for ligands (modulators) of epigenetic reader domains [92], finds a total of 2,348 compounds that target human bromodomains. This search was based on literature, patents and HTS campaigns (www.chepimod.org, accessed December 22, 2017) accounting for the amount of research in this area.

Many of the BRD inhibitors act as acetyl-K mimetic, forming hydrogen bonds in the BRD hydrophobic pocket in a similar manner to the binding of the acetyl-K. In the last years BRD inhibitors have been intensely reviewed [93–95], so, here we will concentrate on small molecules that have been tested in *Trypanosoma* and other protozoa.

4.2.1. Triazolodiazepines

Among the first BRD inhibitors described, there are members that share a triazolodiazepine scaffold (Figure 2) [70, 96]. They were described to inhibit BET family BRDs, and are so called iBETs. As mentioned before, BET BRDs contain two N-terminal BRDs and so far the iBETs reported have poor or no selectivity towards specific domains with one exception (RVX-208). Representative members of these type of compounds are: JQ1 [70], iBET-762 [97], OTX015 [98] and CPI-203 [99], which have been proven effective against septic shock, different types of cancer and activation of latent HIV.

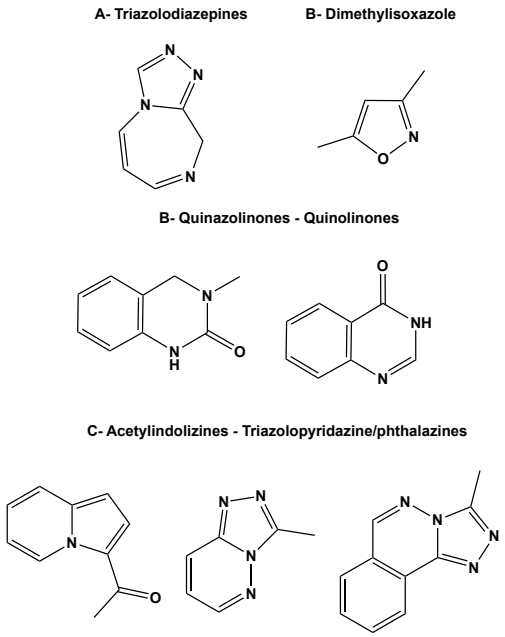

Figure 2: Bromodomain inhibitors' chemical structure of representative scaffolds.

JQ1 and iBET-762 (or GSK525762) were both discovered in separated anti-inflammatory phenotypic screens that monitored ApoA1 levels by Mitsubishi Pharmaceuticals and GSK (GlaxoSmithKline) respectively. JQ1 targets all BET BRDs with different specificities and also inhibits the BRD4-NUT fusion protein that causes NUT midline carcinoma. The triazole moiety in JQ1 forms a hydrogen bond with the conserved N and water array, mimicking the acetyl-K. A very useful compound is the enantiomer of JQ1, named (-)-JQ1 that does not bind to BRD4 and serves as a negative control for validating biological effects [70]. On the other hand, iBET-762 has a similar binding affinity and interaction pattern to JQ1/BRD4 and, additionally, it down-regulates several inflammatory genes both *in vitro* and *in vivo* [96]. Currently, iBET-762 has entered phase I clinical trial for treatment of NUT midline carcinoma [100] (Table 2). OTX015 derives from JQ1 and it also targets BET BRDs. It was initially described to decrease c-MYC expression in acute leukemia cells and it has been proven useful against prostate cancer and glioblastoma models [101, 102]. Nowadays it has entered phase I clinical trial for treatment of malignant tumors [103] (Table 2). CPI-203 is an amide analogue of JQ1, it has anti-lymphoma activity and a much higher bioavailability than JQ1 [104, 105].

We have assayed JQ1 and its enantiomer in *T. cruzi* epimastigotes and only JQ1 displayed inhibitory effects. We have also demonstrated that the recombinant *Tc*BDF3 is able to bind this inhibitor *in vitro* in a concentration-dependent manner (*Kd* = 38 μM) and overexpression of the protein *in vivo* decreased parasite sensitivity to the compound [86]. The affinity constants observed for JQ1 were two orders of magnitude higher that the ones described for BET BRDs.

4.2.2. Dimethylisoxazoles

Diazepine-based compounds confer remarkable shape complementarity and selectivity for the BET BRD binding pocket because of their rigidity and hydrophobicity, however, their bioavailability and space for chemical modification is limited. 3,5-dimethylisoxazole emerges as a scaffold more suitable for optimization and diversification. There are a number of BRD inhibitors with this scaffold like CBP30 developed by the SGC (Structural Genome Consortium) and the well-known iBET-151 developed by GSK [89, 106].

CBP30 targets the non-BET bromodomains of CREBBP (cyclic-AMP response element binding protein binding protein) and p300 (E1A binding protein) and its on-target cellular efficacy was evaluated using a FRAP assay in HeLa cells [91]. Recently, it has been reported that CBP30 reduced immune cell production of IL-17A and other pro-inflammatory cytokines with much more selectivity than JQ1 and thus, could be useful for the treatment of inflammatory diseases [107]. iBET-151 is another BET family inhibitor, which disrupts the expression of inflammatory genes that respond to bacterial lipopolysaccharide (LPS) [108]. iBET-151 showed the same potency as the diazepine iBET-762 but with improved pharmacokinetics and terminal half-life in an animal model [89]. Finally iBET-151 has shown promise for the treatment of different types of cancer where the c-MYC oncogen is overexpressed [72, 109].

Both inhibitors have been tested in *T. cruzi* epimastigotes showing promising effects (IC_{50} ≈ 25 μM for CBP30 and 6 μM for iBET-151) and like JQ1, iBET-151 is able to bind recombinant *Tc*BDF3 with a dissociation constant of around 20 μM. Again, in epimastigotes, overexpression of *Tc*BDF3 confers a decreased sensitivity to this compound similar to JQ1 [86]. CBP30 has shown an anti-trypanocidal effect against *T. brucei* and it also blocks the binding of *Tb*BDF5 first BRD to acetylated H4K10 as reported in a recent patent [110]. In *T. brucei,* RNA-seq experiments of parasites treated with iBET-151 revealed changes in the transcriptome similar to those seen in cells differentiating from the BS to the insect stage. Also, BRD inhibition with iBET-151 disrupts two major BS-specific immune evasion mechanisms that trypanosomes harness to evade mammalian host antibody responses: monoallelic expression of VSG and internalization of antibodies bound to VSG [111]. Mice infected with *T. brucei* treated for two days with iBET-151 survived significantly longer than control mice but while repurposing known BRD inhibitors is a promising strategy, *in vivo* treatment with iBET-151 itself is suboptimal because of its low affinity for trypanosome BRD proteins and its high affinity for mammalian BET proteins that affect the immune system as mentioned before [111]. Finally, *Tb*BDF2 crystal structure has been solved in complex with iBET-151. The overall structure adopts the canonical BRD fold comprising four α-helices linked by variable loop regions that form the binding site but is flipped close to 180° with respect to the human BRD4-iBET-151 complex and cannot adopt its classical binding mode consistent with the reduced affinity of iBET-151 with *Tb*BDF2 [111] and consistent with our affinity results with *Tc*BDF3 [86]. There is also evidence of the anti-proliferative effect of iBET-151 in the apicomplexan parasite *Toxoplasma gondii* tachyzoite stage (IC_{50} of ~10 μM) [112].

4.2.3. Quinazolinones and quinolinones

There are several inhibitors with quinazolinone and quinolinone scaffolds that mainly inhibit BET BRD. Resverlogix Pharmaceuticals developed RVX-208, which has entered several clinical trials for cardiovascular diseases (Table 2). RVX-208 is a quinolinone derivative of resveratrol and was identified initially in a phenotypic screening to find modulators of ApoA1 like JQ1 and iBET-762 [113]. It was one of the first iBET described to have selectivity for the second bromodomain (BD2) of BRD4 over the first (BD1) [114]. Another example is the dihydroquinazolinone PFI-1 that was discovered by Pfizer on a fragment-based screening followed by optimization and it is very potent against BET BRDs [115, 116]. PFI-1 was active against leukemic cell lines with mixed lineage leukemia (MLL) rearrangements but was less effective than JQ1 with unfavorable pharmacokinetic properties in *in vivo* studies. Both of these BRD inhibitors were tested in *T. cruzi* epimastigotes showing no anti-proliferative effect [86].

4.2.4. Acetylindolizines and Triazolopyridazine/phthalazines

GSK2801 is an acetylindolizine that was designed to target human BAZ2A/BAZ2B. BAZ2A and BAZ2B constitute the central scaffolding protein of the nucleolar-remodeling complex (NoRC) that regulates the expression of non-coding RNAs. GSK2801 binds to BAZ2A and BAZ2B with dissociation constants (*Kd*) of 136 and 257 nM respectively. Crystal structures demonstrated a canonical acetyl-K competitive binding mode. Cellular activity was demonstrated using FRAP, monitoring the displacement of GFP-BAZ2A from acetylated chromatin. A pharmacokinetic study in mice showed that GSK2801 had a reasonable *in vivo* exposure after oral dosing, with modest clearance and plasma stability [117]. A recent publication using a structure-based virtual screening combined with ITC experiments showed that GSK2081 has a high affinity for *Tb*BDF2. This compound and the hyperacetylated N-terminus of H2AZ have similar binding sites on *Tb*BDF2 and it was demonstrated that it competitively inhibits the hyperacetylated N-terminus of H2AZ binding to *Tb*BDF2.

Table 2: Bromodomain inhibitors in clinical trials

Inhibitor	Sponsor/ Collaborators	Phase	Condition	Start Date	Status	ClinicalTrials.gov identifier
iBET-762	GSK	I	Drug interactions	May 2016	Completed	NCT02706535
	GSK	I	Solid tumors	May 2017	Recruiting	NCT03150056
	GSK	I	NUT midline carcinoma	March 2012	Recruiting	NCT01587703
	GSK	II	Breast cancer	February 2017	Recruiting	NCT02964507
	GSK	I	Cancer	May 2014	Recruiting	NCT01943851
OTX015	Oncoethix	I and II	Glioblastoma Multiforme	October 2014	Terminated	NCT02296476
	Oncoethix	I	Acute Myeloid Leukemia, Diffuse Large B-cell Lymphoma, Acute Lymphoblastic Leukemia, Multiple Myeloma	December 2012	Completed	NCT01713582
	Oncoethix	I and II	Acute Myeloid Leukemia	January 2015	Withrawn	NCT02303782
	Oncoethix	I	Solid tumors	October 2014	Completed	NCT02259114
	Merck	I	Solid tumors	May 2016	Terminated	NCT02698176
	Merck	I	AML, DLBCL	May 2016	Active	NCT02698189
iBET-151	GSK	I	Advanced or Recurrent Solid Tumors	April 2016	Recruiting	NCT02630251
RVX-208	Resverlogix	II	Diabetes	November 2012	Completed	NCT01728467
	Resverlogix	II	Dyslipidemia, Coronary Artery Disease	May 2013	Terminated	NCT01863225
	Resverlogix	II	Atherosclerosis, Coronary Artery Disease	December 2009	Completed	NCT01058018
	Resverlogix	I and II	Fabry Disease	April 2018	Not yet recruiting	NCT03228940
	Resverlogix	I and II	Dyslipidemia, Atherosclerosis, Acute Coronary Syndrome, Cardiovascular Disease	September 2008	Completed	NCT00768274
	Resverlogix	I and II	Chronic Kidney Failure	December 2017	Not yet recruiting	NCT03160430
	Resverlogix	III	Diabetes Mellitus Type 2, Coronary Artery Disease, Cardiovascular Diseases	October 2015	Recruiting	NCT02586155
	Resverlogix	II	Coronary Artery Disease, Dyslipidemia	August 2011	Completed	NCT01423188
	Resverlogix	II	Coronary Artery Disease	September 2011	Completed	NCT01067820
CPI-0611	Constellation Pharmaceuticals	I	Lymphoma	September 2013	Recruiting	NCT01949883
	Constellation Pharmaceuticals	I	Multiple Myeloma	July 2014	Recruiting	NCT02157636
	Constellation Pharmaceuticals	I	Acute Myelocytic Leukemia, Myelodysplastic Syndrome (MDS), Myelodysplastic/Myeloproliferative Neoplasm, Myelofibrosis	June 2014	Recruiting	NCT02158858
	University of Texas Southwestern Medical Center	II	Peripheral Nerve Tumors	May 2017	Recruiting	NCT02986919
ABV-075	AbbVie	I	Cancer	April 2015	Recruiting	NCT02391480
INCB057643	Incyte Corporation	I and II	Solid Tumors and Hematologic Malignancy	May 2015	Recruiting	NCT02711137
INCB054329	Incyte Corporation	I and II	Solid Tumors and Hematologic Malignancy	May 2015	Active	NCT02431260

After treatment with GSK2801, *T. brucei* cell growth was inhibited and localization of *Tb*BDF2 was modified [65].

The other chemotypes described here have the ability to inhibit the activity of many different bromodomains (also known as pan-BRDs). Because of the complex multidomain/subunit architecture of BRD proteins, it is complicated to make predictions of the consequences of their pharmacological targeting. In this sense, a promiscuous inhibitor that broadly targets BRDs (including BETs) is a tool for the identification of cellular processes and diseases where BRDs have a regulatory function and interrogate BRD biology. With this aim Bromosporine was designed recently [118] to target mainly BET BRDs and also [1,2,4]triazolo [4,3-1]phthalazines that target non-BET BRDs [119]. Bromosporine was described to potently reactivate HIV-1 replication in different latency models and might be a candidate for future HIV-1 eradication strategies [120]. This pan-inhibitor was assayed against *T. cruzi* epimastigotes with no anti-proliferative effect [86]. The crystal structure of *Leishmania donovani* BDF2 (doi: 10.2210/pdb5c4q/pdb) and the second bromodomain of *Ld*BDF5 (doi: 10.2210/pdb5c4q/pdb) in complex with bromosporine has been deposited in the Protein Data Bank but no information regarding the effect of this inhibitor on the parasite has been published. Also, the crystal structure of *T. brucei* BDF2 in complex with bromosporine is available but again with no biological information (10.2210/pdb5czg/pdb).

4.2.5. Other BRD inhibitors currently in clinical trials

CPI-0610 is a potent and selective benzoisoxazoloazepine BET BRD inhibitor that attenuates BET-dependent gene expression *in vivo*. It has been shown to have antitumor efficacy in a MV-4-11 mouse xenograft model and it is currently undergoing human clinical trials for hematological malignancies [121, 122] (Table 2). Recently a pyrrolopyridone-based inhibitor (ABBV-075) was described with extremely potent BET bromodomain inhibitor activity, excellent pharmacokinetic properties and is currently undergoing phase I clinical trials [123] (Table 2). Other BET inhibitors are currently on phase I and II clinical trials for Solid Tumors and Hematologic Malignancy, like

INCB057643 and INCB054329 [124] (Table 2). None of these inhibitors have been tested in trypanosomatids or other protozoa.

5. CONCLUSIONS

Recent progress in epigenetic research has led to the development of new targets for drug discovery against several pathologies. Epigenetics can influence the level of transcription by downregulating or upregulating genes, and the misregulation of these events can be pathological. The discovery of small molecules that interfere on epigenetic mechanisms has led to the development of pharmacoepigenomics, a fast growing discipline that promises to provide new drugs to treat a broad range of diseases. Among the epigenetic targets, BRDs have demonstrated to have an enormous potential, as evidenced by the continuously growing number of BRD inhibitors.

Following this rationale, many BRD inhibitors have been assayed against parasites, some of them with promising results. Even if the essentiality of parasitic BRD-containing proteins has been probed only in in a few cases, they are emerging as key targets. Parasitic BRDs (including those form Tritryps) are very divergent proteins hard to be assigned to any of the already characterized BRD groups from mammals, suggesting that selective inhibitors can be obtained. In the particular case of *T. cruzi*, the existence of two BRD-containing proteins with cytoplasmic localization and atypical specific acetylated ligands reinforces this assumption, and enlarges the field of potential influence of BRD inhibitors beyond epigenetics. The functional study of BRDs certainly will generate information that can be leveraged for targeting acetylation signaling to treat Chagas' disease. As already mentioned, identifying new molecules that inhibit the BRD-acetylated ligand interaction are currently of great interest for many diseases. We propose here that parasitic BRDs should also be considered as potential targets and empower the search for small-molecules to inhibit them.

6. CONFLICT OF INTEREST

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7. AKNOWLEGMENTS

All authors contributed equally to conceiving and writing this manuscript.

8. SUPPLEMENTARY MATERIAL

Supplementary material includes the alignments of bromodomain coding sequences for *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania mayor* and the identity percentages of the full protein sequence and for only the bromodomain.

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