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Discovery of a Biologically Active Bromodomain Inhibitor by Target-Directed Dynamic Combinatorial Chemistry

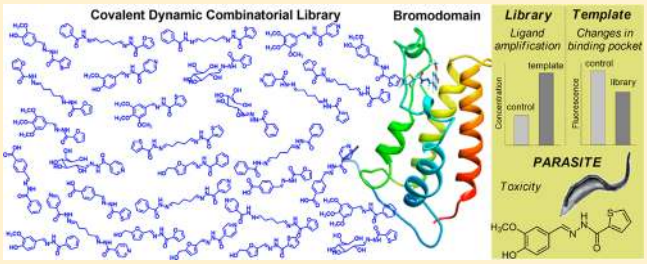
Paula García,[†,§] Victoria L. Alonso,[†,‡,§] Esteban Serra,[†,‡] Andrea M. Escalante,[†] and Ricardo L. E. Furlan*[,†]

[†]Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET, Suipacha 531, S2002LRK Rosario, Argentina

[‡]Instituto de Biología Molecular y Celular de Rosario (IBR), Universidad Nacional de Rosario, CONICET, Ocampo y Esmeralda, 2000 Rosario, Argentina

Supporting Information

ABSTRACT: Target-directed dynamic combinatorial chemistry (DCC) has emerged as a strategy for the identification of inhibitors of relevant therapeutic targets. In this contribution, we use this strategy for the identification of a high-affinity binder of a parasite target, the *Trypanosoma cruzi* bromodomain-containing protein *Tc*BDF3. This protein is essential for viability of *T. cruzi*, the protozoan parasite that causes Chagas disease. A small dynamic library of acylhydrazones was prepared from aldehydes and acylhydrazides at neutral pH in the presence of aniline. The most amplified library member shows (a) high affinity for the template, (b) interesting antiparasitic activity against different parasite forms, and (c) low toxicity against Vero cells. In addition, parasites are rescued from the compound toxicity by *Tc*BDF3 overexpression, suggesting that the toxicity of this compound is due to the *Tc*BDF3 inhibition, i.e., the binding event that initially drives the molecular amplification is reproduced in the parasite, leading to selective toxicity.



KEYWORDS: *Dynamic combinatorial libraries, target-directed chemistry, Trypanosoma cruzi, bromodomain inhibitor*

The identification of small-molecule modulators of protein function is a key activity in modern drug discovery and chemical biology. For targets with limited biostructural information available, random hit-identification strategies are frequently used, wherein target affinity is measured to select the best binder out of large compound libraries.[1] Target binding can also be exploited for the molecular recognition of reactive small molecule building blocks and for both the chemical ligand assembly and the identification of high-affinity building block combinations, integrating in this way chemical synthesis and library screening.[2] When this ligand assembly is achieved through dynamic covalent chemistry, reversibility allows proofreading to increase the yield of good binders.[3,4]

Dynamic combinatorial chemistry (DCC) allows the combination of building blocks through reversible reactions. Since the product distribution of these dynamic combinatorial libraries (DCLs) is dictated by the stability of the library members, it can adapt to environmental changes that affect such stability. Therefore, appropriate analysis of a DCL response to a given stimulus can give useful information about some properties of library members.[5] Addition of a template molecule is a popular strategy to induce composition changes in DCLs. It has been used to discover synthetic receptors for guest templates as well as ligands for biomacromolecular templates,[3] including proteins such as enzymes,[6−10] lectins,[11] and transporters.[12,13] Although some of these proteins are potential therapeutic targets, examples of biologically active molecules resultant of template-induced molecular amplification from dynamic combinatorial libraries are very rare.[6]

One limitation of covalent DCC as a tool for the identification of drug discovery relevant ligands is the limited number of reversible reactions that can be carried out under biomacromolecule-compatible conditions and form bonds that are stable under physiological conditions.[14] The exploitation of hydrazones in biomacromolecule-compatible DCC was precluded for more than a decade because of the required acid conditions for their exchange.[12,13,15] The introduction of aniline[16,17] and related amines as catalysts has paved the way toward the few examples of biomacromolecule-induced adaptation of hydrazone based DCLs.[6,9,11,16,18] Another potential limitation for the exploitation of DCC in drug discovery is the unproductive ligand amplification, i.e., a concentration increase driven by noncovalent interactions with the template that do not affect the biomolecule functionality. This type of interaction cannot be detected by DCL composition analysis in the presence and in the absence of

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the template. It requires a parallel analysis of the effect of the DCL on the template.

Bromodomains are protein-interaction modules that bind to acetyl-lysine residues of histone and nonhistone proteins. In recent years, human bromodomains have become attractive drug targets for the treatment of a variety of diseases.[19] The inhibition of eukaryotic pathogen bromodomains is considered a promising strategy to treat infections, even though it continues to be almost unexplored.[20,21] Several bromodomain-containing proteins have been identified in different protozoan parasites. Since some of them are essential for viability, they represent new opportunities for the discovery of antiparasitic drugs.[21−25] We have characterized three bromodomain-containing coding sequences in *Trypanosoma cruzi*, the protozoan parasite that causes Chagas disease.[22−25] *Tc*BDF3 is a rare cytoplasmic bromodomain containing protein that interacts with acetylated α-tubulin and is involved in flagella morphogenesis and differentiation. These distinct features makes it an interesting drug target against *T. cruzi*.[23,24]

Here, we report the preparation and *Tc*BDF3-induced adaptation of a DCL of hydrazones. The most amplified library member binds to the acetyl-lysine recognition pocket of the template and shows high cytotoxicity against different *T. cruzi* parasite forms. On the contrary, it shows low toxicity against Vero cells and *Tc*BDF3 overexpressing parasites.

The library was prepared from a set of six aldehydes (**A1**−**A6**) and four acylhydrazides (**B1**−**B4**) (Figure 1). Their

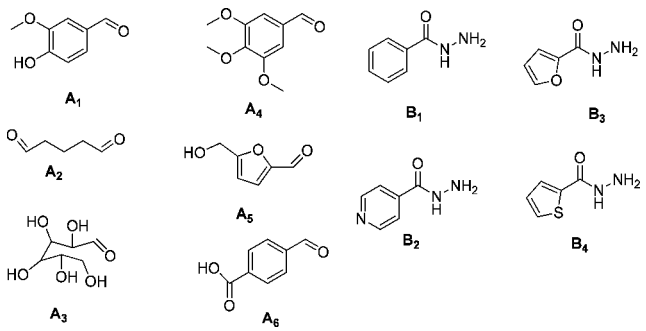

Figure 1. Building blocks used for DCL generation.

combination can produce hydrazones that include, aromatic, aliphatic, polyhydroxylated, and heterocyclic (including N, O, or S) moieties, with phenol, methoxyl, carboxyl, and alcohol substituents as potential recognition groups. One or more of these recognition groups are present in most of the reported compounds that possess affinity for different human bromodomains.[26]

Aldehydes **A1**−**A6** (75 μM each) and acylhydrazides **B1**−**B4** (75 μM each) were dissolved in ammonium acetate buffer (100 mM, pH 6.5) in the presence of aniline (3.75 mM). After 10 h of reaction, the system reached a constant composition. LC−MS analysis showed the presence of 30 hydrazones with unique molecular weights (Table S1). When the dynamic library was exposed to recombinant *Tc*BDF3 (100 μM), a shift in the composition was observed. The template-induced response of the DCL favored mainly the formation of hydrazone **A1B4** that showed a 2.4-fold increase in concentration (Figure 2). Another five library members increased their concentrations to a lesser extent, between 1.5 and 1.8 times, whereas the rest of the library members either increased slightly or decreased their concentrations.

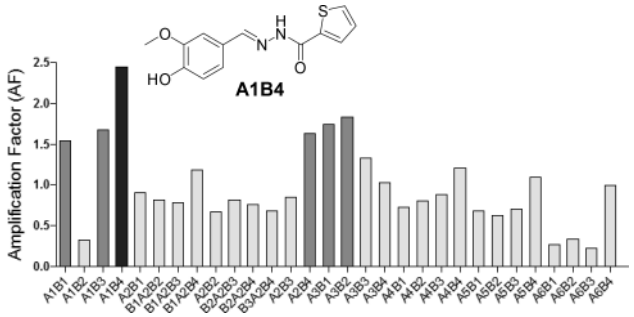

Figure 2. Amplification factors (AFs) observed for library members in the presence of the template *Tc*BDF3. AFs were calculated by dividing the LC−MS peak area of each compound in the templated and untemplated libraries. Black: AF higher than 2. Dark gray: AF between 1.5 and 2. Light gray: AF lower than one.

A template-induced molecular amplification in DCC is ideally driven by specific noncovalent interactions between the amplified ligand and the template. However, different factors can disrupt the correlation between amplification and affinity of the amplified compound to the template.[27,28] In particular, when a biomacromolecule is used as a template to drive amplification of relatively small ligands, interactions between library members with different regions of the template are possible, and some of those interactions may not affect the biological function of the template biomolecule. To gain insight into the type of interaction between the library members and *Tc*BDF3, the effect of the DCL on some of the properties of the template was analyzed.

*Tc*BDF3 possesses a tryptophan residue (W117) placed in a hydrophobic pocket that is the acetyl-lysine binding site. The intrinsic fluorescence of this aromatic residue can be affected by compounds that enter the *Tc*BDF3 hydrophobic pocket.[20,24] In the presence of the DCL, a significant decrease in the *Tc*BDF3 maximum intrinsic fluorescence was observed (Figure 3A), suggesting that one or more library members could enter

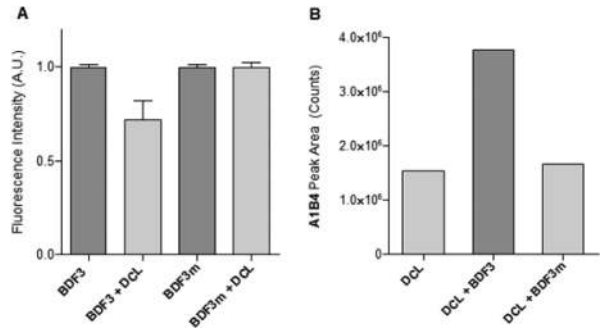

Figure 3. (A) Normalized intrinsic fluorescence of recombinant *Tc*BDF3 or *Tc*BDF3m (10 μM) in ammonium acetate buffer (100 mM, pH 6.5) in the absence and in the presence of DCL. (B) LC−MS peak area for **A1B4** in the untemplated DCL and in the DCLs in the presence of *Tc*BDF3 or *Tc*BDF3m (100 μM).

the hydrophobic pocket. In order to support this observation, the effect of the library was also measured with a mutated version of *Tc*BDF3 (*Tc*BDF3m) that includes two point mutations (Y123A and L130A). *Tc*BDF3m was designed to retain its secondary structure while losing its ability to bind to its acetylated ligand *in vitro*.[24] Neither the maximum intrinsic fluorescence of *Tc*BDF3m was affected by the library (Figure

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3A) nor the **A1B4** concentration was affected by the presence of *Tc*BDF3m (Figure 3B), suggesting a specific binding of **A1B4** to the binding pocket of the wild type protein.[29]

Hydrazone **A1B4** was then prepared individually and its interaction, as well as the interaction of each of its component building blocks **A1** and **B4**, with the template *Tc*BDF3 were evaluated by fluorescence quenching and by differential scanning fluorimetry (DSF) or thermal shift. Individually, building blocks **A1** and **B4** (100 μM) did not produce any detectable change in fluorescence. However, the maximum intrinsic fluorescence of *Tc*BDF3 decreased regularly in the presence of increasing concentrations of **A1B4** (Figure 4). The

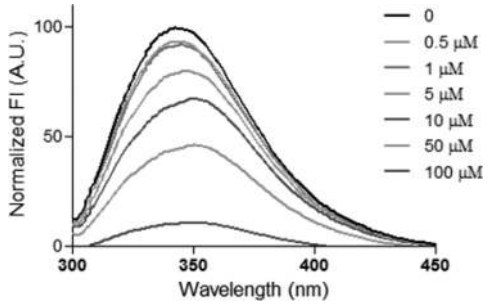

Figure 4. Fluorescence spectra of *Tc*BDF3 (10 μM) alone and with increasing amounts of **A1B4**. λ_{ex} = 295 nm. FI, fluorescence intensity; A.U., arbitrary units.

fluorescence data were analyzed by Stern−Volmer, modified Stern−Volmer, and double logarithmic plots. The Stern−Volmer plot showed a negative deviation (toward the *x*-axis) as previously reported for *Tc*BDF3 with bromodomain inhibitors.[20,24] From the double logarithmic plot a dissociation constant value of 1.7 μM was obtained. In addition, binding to **A1B4** significantly increased the thermal stability of *Tc*BDF3 with ΔT_m observed of 3.43 °C, whereas for *Tc*BDF3m ΔT_m was 0.4 °C, which further supports the binding of **A1B4** to *Tc*BDF3 (Figure S8).

To support these results, the modeled 3D structure of *Tc*BDF3 was used to perform docking predictions using the Swissdock server.[30] The best prediction located the hydrazone **A1B4** inside the hydrophobic pocket of *Tc*BDF3 (Figure S9).

In view of the promising biophysical data and considering the novelty of *Tc*BDF3 as a therapeutic target for Chagas disease, the effect of **A1B4** on the different life cycle stages of *T. cruzi* was evaluated *in vitro*. Amastigotes are present in the mammalian host cells, whereas epimastigotes and the infective metacyclic trypomastigotes are present in the gut of the insect vector. The cytotoxicity was also studied in the Vero cell line to evaluate selectivity. **A1B4** shows a trypanocidal effect on the three life cycle stages of *T. cruzi* with IC_{50} values between 13 and 23 μM (Table 1). Interestingly, its toxicity against Vero Cells was low, with an IC_{50} value higher than 200 μM, giving a selectivity index ≥9 depending on the parasite form.

To gain insight into the specificity of **A1B4** for *Tc*BDF3 *in vivo*, the sensitivity of bromodomain-overexpressing lines was evaluated. *T. cruzi* epimastigotes overexpressing *Tc*BDF3 through a tetracycline-induced plasmid were treated with **A1B4**, at a concentration around its IC_{50} value, in the absence and in the presence of tetracycline (Figure 5). Overexpression

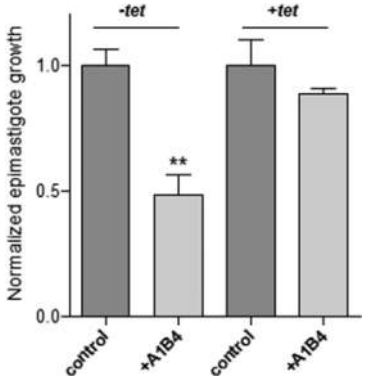

Figure 5. Relative growth of *T. cruzi* Dm28c epimastigotes transfected with p*Tc*INDEXGW-BDF3HA, uninduced (-tet) and induced (+tet) with tetracycline (tet), untreated (control), and treated with **A1B4** (25 μM). The experiment was performed in triplicate, and cell growth was determined after 72 h of culture by counting viable forms. The values obtained were normalized to the condition without compound (control). The bar graph represents the mean SD; $^{**}P < 0.05$ (unpaired, two-tailed Student's *t* test). In the experiment with tetracycline, the observed difference in relative growth in the absence (*+tet* control) and in the presence of inhibitor (*+tet* + **A1B4**) is not significant.

of *Tc*BDF3 completely rescued epimastigotes from the growth inhibition produced by **A1B4**, suggesting that the toxicity of this compound is due to the *Tc*BDF3 inhibition.

In order to evaluate if amplification of **A1B4** is somehow related to the properties shown by this compound, a poorly amplified compound was prepared and analyzed (**A4B4**). Compared to **A1B4**, **A4B4** was amplified in a lesser extent (AF = 1.21, Figure 2), produced a smaller change in the intrinsic fluorescence and thermal shift of *Tc*BDF3 *in vitro*, and showed lower cytotoxicity against the parasite *in vivo* (Figure S12).

In summary, a hydrazone based DCL was prepared under reaction conditions that are compatible with the biologically relevant template *Tc*BDF3. When mixed, DCL and template affected each other: the template induced changes in the

Table 1. Cytotoxicity of A1B4 on the Different Life Cycle Stages of *T. cruzi* and Vero Cells

	IC_{50} (μM)[a,b] (SI[c])			
compound	epimastigotes	trypomastigotes	amastigotes	Vero cells
A1B4	23 ± 3.8 (>9)	17.8 ± 2.29 (>11)	13.1 ± 1.28(>15)	>200 ± 6
BZN[d]	18.16 ± 5.13 (2)	27.07 ± 4.23 (1)	3.92 ± 1.24 (8)	30.17 ± 12
NFX[e]	8 ± 3 (14)	7 ± 2 (16)	3 ± 1.5 (38)	115 ± 12

[a]The results are averages of three separate determinations. [b]IC_{50} is the concentration required to give 50% inhibition, calculated by nonlinear regression analysis from beta-galactosidase activity at the used concentrations (0−200 μM). [c]Selectivity index (SI) is the ratio between IC_{50} on Vero cell toxicity and IC_{50} activity of extracellular or intracellular forms of the parasite. [d]MTT assay on Vero cells incubated 72 h with BZN (0−250 μM). [e]Values obtained from the literature.[31] BZN, benznidazol; NFX, nifurtimox (reference drugs for the treatment of Chagas disease).

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relative concentration of the library members, and the library produced changes in the intrinsic fluorescence of a tryptophan residue placed in the acetyl-lysine recognition pocket of the bromodomain. Blockage of the entrance to this pocket by specific mutations deleted both effects. Binding of the most amplified library member, **A1B4**, to the template *Tc*BDF3 was studied by fluorescence quenching and thermal shift observing a K_d of 1.7 μM, slightly lower than the value for the best *Tc*BDF3 inhibitor reported.[20] The binding process observed *in vitro* also seems to be possible *in cell* since the compound **A1B4** shows interesting toxicity activity against different parasite forms, but it is not toxic for other eukaryotic cells, such as Vero cells, or for parasites wherein *Tc*BDF3 has been overexpressed. Hydrazone **A1B4** showed very interesting antiparasitic activity and selectivity index.

These results illustrate how biologically active small-molecules can be identified through a DCC casting approach by using mild reaction conditions, biologically relevant templates, and appropriate analysis of DCL adaptation and DCL effect on the template. In addition, the biophysical and biological properties observed for compound **A1B4** give relevance to bromodomain-containing proteins as attractive targets for the discovery of selective antiparasitic molecules.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00247.

*Tc*BDF3 and *Tc*BDF3m purification, library preparation, library analysis, synthesis and characterization of **A1B4** and **A4B4**, binding experiments, and biological activities and docking prediction (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rfurlan@fbioyf.unr.edu.ar.

ORCID

Andrea M. Escalante: 0000-0001-8279-5174
Ricardo L. E. Furlan: 0000-0001-6136-0980

Author Contributions

§These authors contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DCC, dynamic combinatorial chemistry; DCL, dynamic combinatorial library; *Tc*BDF3, *Trypanosoma cruzi* bromodomain

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