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ACS Med. Chem. Lett., **Just Accepted Manuscript** • Publication Date (Web): 27 Apr 2020

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Development of selective steroid inhibitors for the glucose-6-phosphate dehydrogenase from *Trypanosoma cruzi*

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KEYWORDS: Chagas disease, *Trypanosoma cruzi*, G6PDH, selective inhibitors, steroids

ABSTRACT: Chagas disease is a parasitic infection affecting millions of people across Latin America, imposing a dramatic socio-economic burden. Despite the availability of drugs, nifurtimox and benznidazole, lack of efficacy and incidence of side-effects prompt the identification of novel, efficient and affordable drug candidates. To address this issue, one strategy could be probing the susceptibility of *Trypanosoma* parasites toward NADP-dependent enzyme inhibitors. Recently, steroids of the androstane group have been described as highly potent but non-selective inhibitors of parasitic glucose-6-phosphate dehydrogenase (G6PDH). In order to promote selectivity, we have synthesized and evaluated 26 steroid derivatives of epiandrosterone in enzymatic assays, whereby 17 compounds were shown to display moderate to high selectivity for *T. cruzi* over the human G6PDH. In addition, three compounds were effective in killing intracellular *T. cruzi* forms infecting rat cardiomyocytes. Altogether, this study provides new SAR data around G6PDH, and further supports this target for treating Chagas disease.

Discovered in the early 20th century by the Brazilian physician Carlos Chagas, Chagas disease is attributed to the infection by the flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). According to the World Health Organization, it is estimated that about 6 to 7 million people worldwide are at risk of infection by *T. cruzi*.¹ Currently there are two licensed drugs for the treatment of Chagas disease: nifurtimox (NFX) and benznidazole (BNZ). Both have serious side effects such as neurological and digestive disorders, hypersensitivity in the form of rashes, in addition to having low efficacy and efficiency during the chronic stage.^{2,3} Given this current scenario, it is necessary to develop more effective and efficient drugs for the treatment of all stages of infection. The enzyme glucose-6-phosphate dehydrogenase (G6PDH) is the first of the oxidative branch of the pentose-phosphate pathway (PPP). This pathway is key to cellular metabolism because it is a major producer of dihydronicotinamide adenine dinucleotide phosphate (NADPH), a molecule necessary for biosynthetic reductions and important in the maintenance of cellular redox homeostasis, by counteracting the deleterious effects of oxygen radicals.⁴ The PPP is especially important for parasites, since mammalian hosts use oxidative burst as the first line of defense against infection via the formation of reactive oxygen species, which are counterbalanced in the parasite by NADPH-dependent reactions. This assertion is corroborated by in vitro experiments performed in *T. cruzi* by Maugeri and co-workers, which points out the increased flow through the PPP in response to oxidative stress.⁵ G6PDH is of central importance for PPP, since it often presents a high control coefficient, being considered a potential target for the development of drugs.⁶ In addition, the genetic validation of this enzyme as a target was demonstrated in *T. brucei* by Cordeiro and co-workers through the partial depletion of this enzyme using tetracycline inducible RNA interference.⁷

Steroids such as dehydroepiandrosterone (DHEA, **1**) and epiandrosterone (EA, **2**) have long been known for inhibiting human glucose-6-phosphate dehydrogenase (*HsG6PDH*).⁸ Recently, *HsG6PDH* inhibition by DHEA in erythrocytes was shown to promote resistance towards infection by *Plasmodium falciparum*, the causative agent of malaria.⁹ DHEA is further believed to act as an immunomodulating agent by increasing the host's immunity toward infections by *T. cruzi*.^{10–12} Epiandrosterone (EA) analogues have been studied in the context of their potential anticancer activity in phenotypic assays, with modifications occurring mainly at the C3 or the C16/17 of the androstane core (Figure 1).^{13,14} Hamilton and co-workers, in particular, have developed a series of EA-derivatized inhibitors of *HsG6PDH*, allowing for the identification of key features that promote or disfavor binding to this enzyme.¹⁵ 16 α -Bromoepiandrosterone (BrEA, **3**), which is a potent inhibitor of *HsG6PDH*, was also shown to strongly inhibit *T. cruzi* G6PDH (*TcG6PDH*),¹⁶ and its parasitocidal effects have been reported for *Taenia* and *Entamoeba*.¹⁷ In particular, the safety of BrEA (also registered as HE2000) has been confirmed in clinical trials in HIV and malaria patients while its immune-boosting properties were assessed.^{18–20} Although the mode of inhibition (uncompetitive mechanism) has long been elucidated,²¹ the exact mode of binding of steroid inhibitors to the enzyme remains unknown, despite a combination of computational and biochemical efforts suggesting it lies near the binding site of its substrate, G6P, and co-factor, NADP.^{22,23} So far, attempts by Mercaldi and co-workers have yielded the crystal structure of *TcG6PDH* obtained in a ternary complex with the substrate G6P and reduced cofactor NADPH, however the desired quaternary complex with a steroid inhibitor, for instance, has not yet been achieved.²⁴ Despite BrEA displaying high affinity for the parasitic enzyme *TcG6PDH*, it is only twice less active against

the human homologous enzyme. Likewise, already known 16 α -halogenated analogues (**4**, **6** and **7**) have also proven strong inhibitors of *HsG6PDH*, which could potentially hamper their use for the treatment of this parasitic disease, on the basis of undesired drug-host interactions.

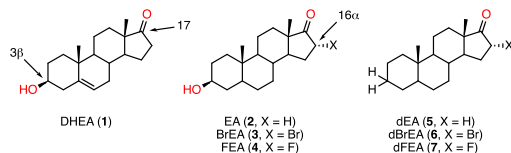


Figure 1. Endogenous steroid hormone DHEA (**1**) and synthetic analogues (**2-7**).

According to Hamilton and co-workers, the presence of an H-bond donor at C3 β substituents is essential for human G6PDH inhibition,¹⁵ but this has not yet been reported for *TcG6PDH*. Thus, the derivatization of the C3 β -OH group would allow for the removal of the key H-bonding feature, while introducing chemical diversity to the androstane core, potentially yielding *TcG6PDH* selective inhibitors. As such, we herein describe the synthesis and biochemical evaluation of a series of C3 β -O-modified analogues of EA and BrEA, composed of carboxylic and sulfonic esters, as well as ethers, where aliphatic and heterocyclic groups are introduced. To the best of our knowledge, these compounds have not been described in the literature in the context of G6PDH inhibition and would thus provide the scientific community with novel data in this field.

The first set of compounds (**8-24**) was composed of carboxylic and sulfonic esters of EA and BrEA²⁵ containing either lipophilic or heterocyclic moieties. Compounds **8-15** represented esters of increasing size, as well as the simple methyl sulfonic esters, and were prepared by reacting steroids **2** and **3** with the desired acyl or sulfonyl chlorides (Scheme 1A). Intermediates **16-18** were synthesized using the same conditions with the appropriate reagent, and further treated with either morpholine or *N*-methylpiperazine to yield heterocyclic steroid analogues **19-24**. Although the 16 α -brominated equivalents of compounds **16-18** (Scheme 1A, X = Br) were successfully synthesized (data not shown), their reaction with the two heterocyclic amines led to partial elimination of the bromine atom, and attempts to brominate compounds **20** and **24** with copper(II) bromide in methanol at reflux led to the elimination of the carboxylic or sulfone ester moieties at C3.

Because of the potential lability of esters in vivo, we sought to produce C3-ether derivatives, thus demanding the use of harsher conditions to perform *O*-alkylations at C3. As a consequence, the protection of the C17-ketone as a ketal (**25**) was required and was performed under conditions described by Hitchin and co-workers (Scheme 1B).²⁶ We chose the simple methyl ethers of EA and BrEA, as well as the equivalent of compounds **10** and **14**, that is, the isobutyl ethers. Ketal (**25**) was thus treated with sodium hydride in methanol under reflux, followed by the addition of methyl iodide to form the desired methyl ether (**26**).²⁷ Deprotection of the ketal group under acidic condition delivered the target methyl ether of EA (**27**), and further treatment with copper(II) bromide generated the 16 α -brominated analogue **28**. In a similar fashion, isobutyl tosylate (**29**) was reacted with ketal **25** and sodium hydride in the presence of sodium iodide to yield the desired isobutyl ether (**30**). After deprotection of the ketal, ketone **31** was treated with copper(II) bromide, thus producing its 16 α -brominated

analogue **32**. In order to introduce polar groups similar to the heterocycle-containing compounds **19-24**, we focused on analogues of derivatives **22** and **24**, where the carbonyl/sulfonyl group and the methylene adjacent to the nitrogen atom would be inverted, producing amide **33** and sulfonamide **34**, respectively. From a synthetic point of view, these new compounds would be formed by an SN2 or a Michael addition-type *O*-alkylation of the corresponding α -bromoacetamide (**35**) and vinyl sulfone (**36**) derivatives, which were prepared by known procedures.^{28,29} Treating ketal **25** with *n*-butyllithium, compound **35** and tetrabutylammonium iodide (TBAI) gave the desired ether (**37**) in modest yield,³⁰ and further deprotection of the ketal delivered the target compound (**33**). For the alkylation of **25** with vinyl sulfone **36**, sodium hydroxide and tetrabutylammonium bromide (TBAB) were used, giving the desired ether (**38**) in 66% yield.³¹ Likewise, removal of the ketal group yielded the proposed compound (**34**). Pleasingly, we were able to introduce the 16 α -bromine atom under the usual conditions, delivering target compounds **39** and **40**.

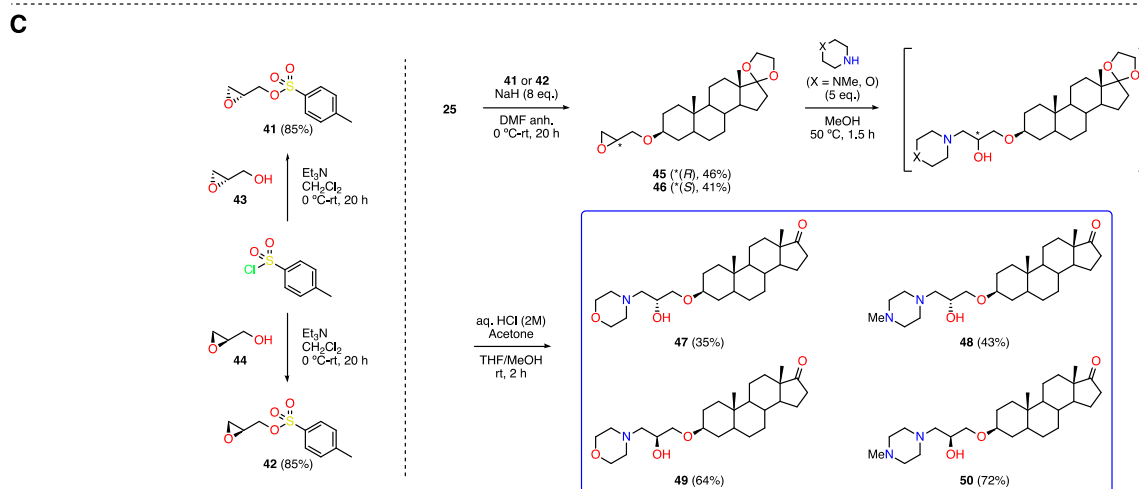
Finally, we designed a series of compounds deprived of hydrolyzable groups, where the attachment to the steroid and the introduction of heterocycles would be facilitated by using the reactivity of an epoxide (Scheme 1C). Seeking to produce both epimers of the resulting secondary alcohols, we used the adequate glycidyl tosylate surrogates (*R*)-**41** and (*S*)-**42**, obtained from the commercially available (*S*)-(+)-glycidol (**43**) and (*R*)-(-)-glycidol (**44**), respectively. Substitution of the tosylate group by the C3-alcohol of **25** occurred best using an excess of sodium hydride at room temperature, despite the modest yields observed for the glycidyl ethers **45** and **46**. Next, the desired target compounds **47-50** were achieved by aminolysis of the epoxides with either morpholine or *N*-methylpiperazine under gentle heating, subsequent treatment of the crude reactions mixtures with aqueous hydrochloric acid to remove the ketal group, and purification by recrystallization.

All 26 target compounds were evaluated in biochemical assays against *Tc* and *HsG6PDH* to calculate the selectivity of the inhibitors and perform a structure-activity relationship (SAR) analysis (Supporting Information, Table S1). Reference compounds DHEA (**1**), EA (**2**), and their C3-deoxygenated and 16 α -halogenated analogues (**3-7**) were also assayed. The potency of the inhibitors towards both enzymes are illustrated in Figure 2, facilitating the SAR analysis.

As mentioned in the introduction, all reference compounds (**1-7**)* are *HsG6PDH*-selective (Figure 2, above the gray dotted line), to the exception of BrEA (**3**), which is, however, only twice more potent for *TcG6PDH*. These compounds all have in common the free C3 β -alcohol (**1-4**) or its absence (**5-7**), further supporting our strategy to produce C3 β -derivatized steroids to favor the inhibition of *TcG6PDH*. Indeed, most of the synthesized EA-analogues are found below the dotted gray line. Special attention should be given to the ester derivatives of BrEA, (**12-14**), in the dotted green triangle, which showed highest potency ($0.15 < \text{IC}_{50} \text{ (}\mu\text{M)} < 0.38$) and selectivity toward *TcG6PDH*, especially compound **14** for which no activity was detected for *HsG6PDH* even at the highest assayed concentration of 80 μM . Further, it appears that increasing the bulkiness of the ester group contributes to increased selectivity, as can be noted by comparing the acetyl (**12**, 160-fold) and the isobutyryl (**14**, > 400-fold) esters of BrEA. This same trend is observed for the non-halogenated analogues (**8-10**), in the dotted orange rectangle, which display moderate selectivity,

due to lower potencies for *Tc*G6PDH (IC₅₀ > 3 μM; pIC₅₀ < 5.5, Figure 2).

Scheme 1. Synthesis of EA (2) and BrEA (3) derivatives comprising: A) aliphatic and heterocycle-containing esters; B) aliphatic and heterocycle-containing ethers; C) non-hydrolysable moieties - target compounds are highlighted in blue rectangles.



Methyl sulfones **11** and **15** showed weak (2-fold) and moderate (6-fold) selectivity, respectively.

More polar EA ester analogues containing morpholine and *N*-methylpiperazine moieties (**19-22**) were weak inhibitors ($IC_{50} = 10 \mu M$), while the heterocyclic sulfone esters (**23-24**) performed better ($IC_{50} = \sim 5 \mu M$), only **23** showing moderate selectivity. In the C3 β -ether series, 16 α -brominated ethers **28** and **32** achieved high selectivities (50-fold range), while morpholine-containing analogues **39** and **40** showed moderate selectivity (5 to 10-fold). Non-halogenated methyl ether **27** achieved some selectivity toward *Tc*G6PDH, while unexpectedly, isobutyl ether **31** proved 3-fold *Hs*G6PDH-selective, but also a weak inhibitor. Compounds **47-50** showed almost no activity ($IC_{50} > 40 \mu M$) toward either of the G6PDH homologues, noting that they all contain a hydrogen-bond donating group in their propanolamine moiety. Similarly, compound **33**, which bears a hydrogen-bond acceptor (amide) at the same position, was also one of the weakest compounds of the series, although the addition of the 16 α -bromine somewhat restored potency toward *Tc*G6PDH, as shown with compound **39**. Compound **34** was also a weak inhibitor ($IC_{50} = 20 \mu M$) and non-selective.

Finally, looking at the distribution of the compounds according to their calculated partition coefficient (cLogP), compounds with values higher than 3.5 tend to make the more potent inhibitors, while polar compounds (cLogP < 3.5) are poor inhibitors (Figure 2). Clearly, the most potent inhibitors owe their high affinities to the presence of the 16 α -bromine atom.

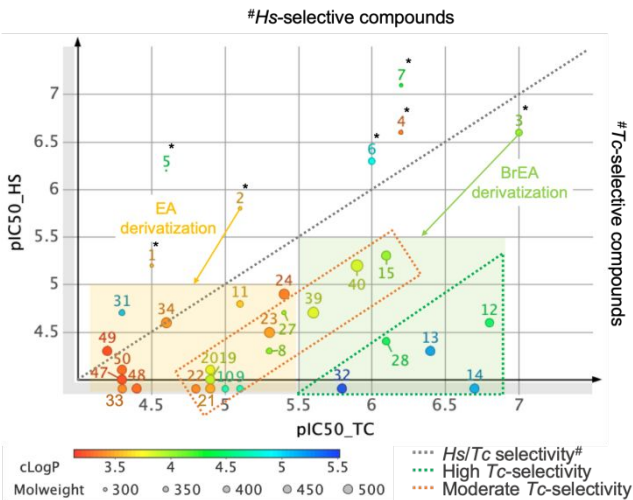


Figure 2. SAR analysis of EA (**2**) and BrEA (**3**) derivatives against the parasitic and human G6PDHs. Affinities are shown as pIC₅₀ values, where IC₅₀ is expressed in μM ($pIC_{50} = -\log(IC_{50}.10^{-6})$). The green dotted triangle represents highly *Tc*-selective compounds, defined by an IC_{50_Hs}/IC_{50_Tc} ratio > 40. Moderate *Tc*-selectivity ($5 < \text{ratio} < 30$) is represented by a dotted orange rectangle. The light green filled rectangle covers BrEA derivatives, whereas the light orange filled rectangle represents EA derivatives. The chart was generated with the DataWarrior software (Osiris).³³

In summary, our strategy to remove a H-bond donating feature at epiandrosterone's C3 β substituents, which is key for *Hs*G6PDH binding, proved successful for producing *Tc*G6PDH selective inhibitors. Despite a recent comparative study

highlighting structural features unique to *Tc*G6PDH,³² the exact mechanism underlying high selectivities for 16 α BrEA-*O*-acetylated inhibitors, for instance, still cannot be rationalized.

Next, we evaluated the efficacy of *Tc*G6PDH inhibitors (with $pIC_{50_TC} > 5$) in killing intracellular *T. cruzi* (Y strain) amastigotes infecting H9C2 rat cardiomyocytes by an image-based cellular assay. Such multiparametric assay enables to assess compound efficacy against parasites and toxicity towards host cells, simultaneously. Initially, compounds were assayed at the single concentration of 20 μM . At this concentration, no compounds were able to reduce infection without showing significant host cell toxicity (Figure 3), as being the case for compounds **3**, **6**, **12**, **13**, **15**, **32**, **39** and **40**, all derived from BrEA. Non-halogenated steroids **2**, **8**, **9**, **10**, **11**, **23**, **24** and **27**, on the other hand, were not cytotoxic towards host cells, but neither were they effective against *T. cruzi*. These observations reinforce the idea that bromine addition at C-16 is important for potent *Tc*G6PDH inhibition but may also be responsible for the pronounced toxicity in cell assays. Concerning fluorine-derivatives **4** and **7**, and BrEA derivative **14**, which were not active in the cell assays despite showing potent enzyme inhibition, one ought to remind that several factors such as solubility and membrane permeability (which needs to occur twice to reach intracellular parasites), as well as metabolism can impact on the general bioavailability of drug candidates. Complex cellular pharmacokinetics have also been suggested by Khare and co-workers for the intracellular *T. cruzi* forms in particular, while assessing proteasome inhibitors of kinetoplastids.³⁴

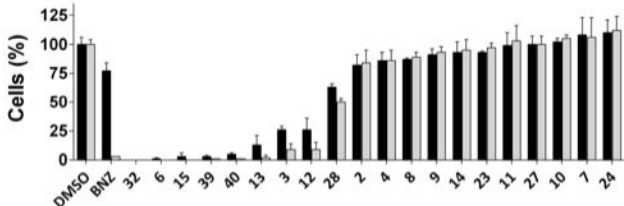


Figure 3. Anti-*T. cruzi* intracellular assay at single concentration of 20 μM . Black and gray bars represent mean and standard deviation of normalized total host and infected cells, respectively. DMSO values were used for data normalization.

Due to the toxicity observed for a set of compounds at 20 μM , these were selected to be retested at lower concentrations in a dose-response assay format for EC₅₀ and selectivity index (S.I.) determination. The results are presented in Table 1.

Table 1: EC₅₀ values (μM) for host cells and intracellular *T. cruzi* forms.

Sample	EC ₅₀ _T. cruzi	EC ₅₀ _host	S.I.
BNZ	2.2 ± 0.4	> 40	> 18
40	2.4 ± 0.4	8.5 ± 1.9	3.5
15	2.4 ± 0.3	7.5 ± 0.6	3.1
39	3.2 ± 1	5.6 ± 1.6	1.8
6	3.5 ± 0.4	8.4 ± 1.8	2.4
32	4.2 ± 0.5	12.3 ± 1.8	2.9
3	6.4 ± 2	13 ± 2	2.0
12	8.5 ± 1.5	14.7 ± 4.2	1.7
13	16.7 ± 4.2	54 ± 3.3	3.2

S.I. = selectivity index calculated as $EC_{50_host}/EC_{50_T. cruzi}$. Mean and standard deviation for 3 independent experiments.

At first glance, the *Tc/HsG6PDH* selectivity and potencies observed in enzymatic assays were not reflected in cell assays. On one hand, highly *Tc*-selective compounds **32**, **12** and **13** showed higher EC₅₀ values for host cells than compounds with no or low *Tc*-selectivity such as **40**, **15**, **39** and **6** (Table 1), which indicates that a certain degree of selectivity in cell assays was achieved, at least in terms of toxicity. However, it is the least *Tc*-selective compounds, which despite having lower potencies for the parasitic enzyme, showed the highest efficacies toward *T. cruzi* in cell assays. On the other hand, BrEA (**3**), which is only twice more potent for *TcG6PDH*, still has a higher EC₅₀_{host} value, and is thus less toxic than its moderately selective congeners. One could propose that possible metabolic pathways could be involved. In fact, BrEA possesses a free C3-OH group which could be prone to sulfatation, as is the case in vivo for DHEA, which renders it inactive toward *HsG6PDH*.³⁵ In a similar fashion, compounds **12** and **13**, which are highly *Tc*-selective but show lower efficacies could also easily be metabolized. Being esters, they could indeed suffer hydrolysis by esterases and further undergo conjugation. As such, these transformations occurring in the host cells' cytosol would lower the concentration of inhibitor entering the parasites, which would account for their higher EC₅₀ values in *T. cruzi*. Conversely, this could explain why non-hydrolyzable ethers **39** and **40** were more active in cell assays. Still, this is merely speculative, since *T. cruzi* pharmacokinetics are reported to be quite complex.³⁴

Nevertheless, we were pleased to observe that compounds **40**, **15** and **32** showed EC₅₀ values for intracellular *T. cruzi* equivalent to BNZ and at least three times higher for host cells (Table 1 and Figure 4).

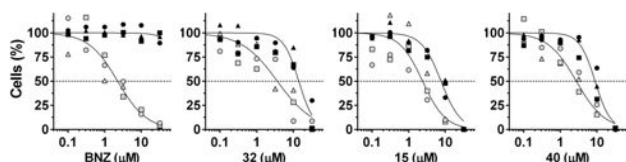


Figure 4. Dose-response curves for normalized host (black) and infected cells (gray) for BNZ, compounds **32**, **15** and **40**. Plots prepared with GraphPad Prism.

Inspection of cell assay images for these compounds acquired at 10 μ M (Figure 5), the concentration closest to the EC₅₀ for host cells, shows a significant reduction in the number of infected cells and infection ratio, equivalent to BNZ. Despite the observed decrease in total host cells, a significant reduction in parasitemia was clearly observed in the remaining cells, supporting the notion that the parasites are more susceptible to the compounds' action than the host cells.

In conclusion, for the first time, selective inhibitors of the parasitic G6PDH enzyme, with respect to the human isoform, have been described. Our data shows that esters of the C3 β -OH group of epiandrosterone (**2**) abolish activity towards the human enzyme, while maintaining high affinity for *TcG6PDH*, especially in the presence of the 16 α -bromine atom, as supported by consistent SAR analyses. On assessing the efficacy of these compounds in cell-based assays, however, most of the halogenated compounds proved cytotoxic at 20 μ M. On performing dose-response experiments, isobutyl ether **32**, sulfone **15** and sulfonamide **40** displayed EC₅₀ values close to BNZ's, but with narrow therapeutic windows. New chemical scaffolds are thus pursued by our group in order to achieve safer

drug candidates.³⁶ Altogether, these are encouraging results showing for the first time that uncompetitive G6PDH inhibitors are able to kill intracellular *T. cruzi* amastigotes, the parasite form associated with the chronic stage of Chagas disease.

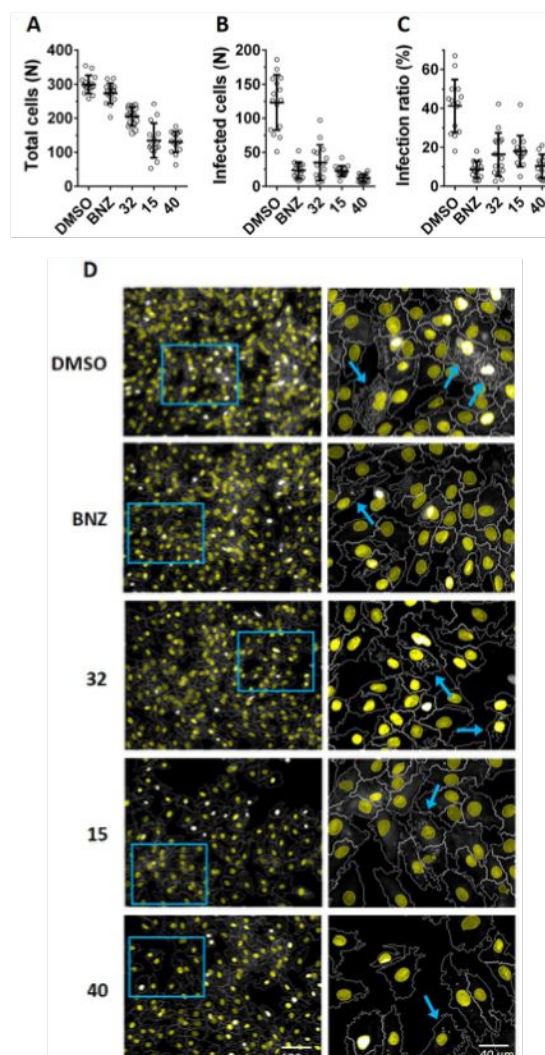


Figure 5. Image analysis of anti-*T. cruzi* intracellular assay of compounds **32**, **15** and **40** (10 μ M). Mean and standard deviation for total host cells (A), infected cells (B) and infection ratio (C) per image (N=15). (D) Representative images (left) and zoom from blue boxes (right) for DMSO, BNZ and compounds **32**, **15** and **40**. Host cell nuclei are colored in yellow and *T. cruzi* amastigotes appear as white spots in the cytoplasm of infected cells (indicated by blue arrows).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures for the synthesis of compounds **3-42** and **45-50**; experimental procedures and data for the biological evaluation of the target compounds - enzymatic and cell-based assays (docx). ¹H and ¹³C NMR spectra as ANNEX (docx).

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Author Contributions

F.F.N.* and M.B. performed the synthesis; J.N.F.* produced the purified enzymes and performed the enzymatic assays; A.G.E. performed the cell-assays; A.T.C. designed and supervised the biological assays; M.B. planned the target compounds; A.T.C. and M.B. jointly designed the study and analyzed the results. The manuscript was written through contributions of all authors. *These authors contributed equally.

Funding Sources

This research was funded by the São Paulo Research Foundation [FAPESP grants for M.B. (2013/16534-4), and A.T.C. (2016/14271-4 and 2018/22202-8)] and by the Coordination for the Improvement of Higher Education Personnel (CAPES 7775/2014-98). The Brazilian Ministry of Science, Technology, Innovation and Communication sponsored F.F.N. (PCI-DB 313424/2015-2).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

The authors wish to thank Prof. Ronaldo Pilli and the Chemistry Institute / UNICAMP for their support, as well as the Nuclear Magnetic Resonance Laboratory at LNBio/CNPem.

ABBREVIATIONS

NADP, nicotinamide adenine dinucleotide phosphate; G6PDH, glucose-6-phosphate dehydrogenase; NFX, nifurtimox; BNZ, benznidazole; PPP, pentose-phosphate pathway; NADPH, dihydronicotinamide adenine dinucleotide phosphate; DHEA, dehydroepiandrosterone; EA, epiandrosterone; BrEA, 16 α -bromoepiandrosterone; DMSO, dimethyl sulfoxide; HIV, human immunodeficiency virus.

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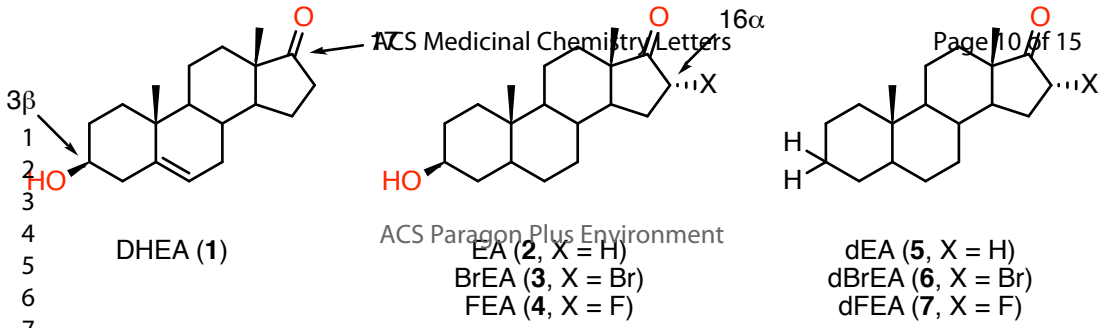
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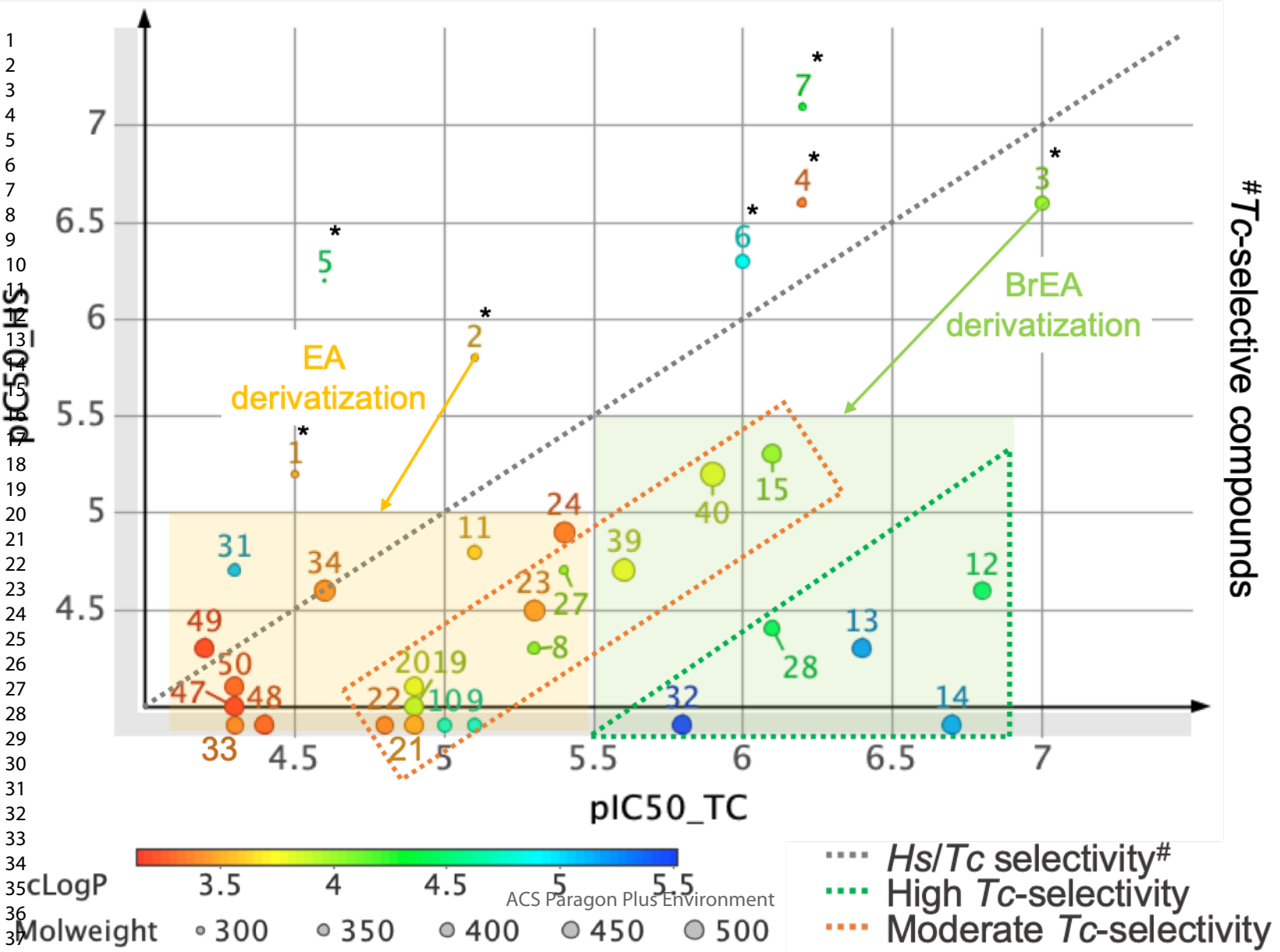
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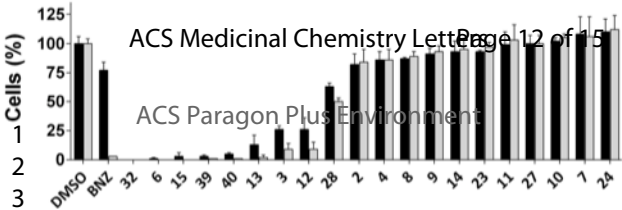
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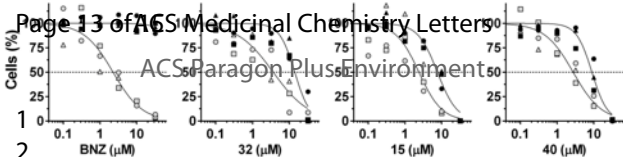


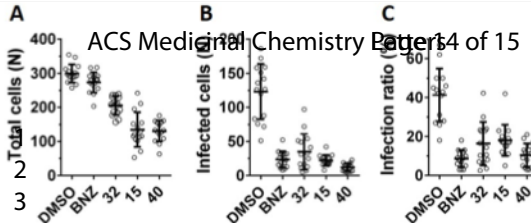
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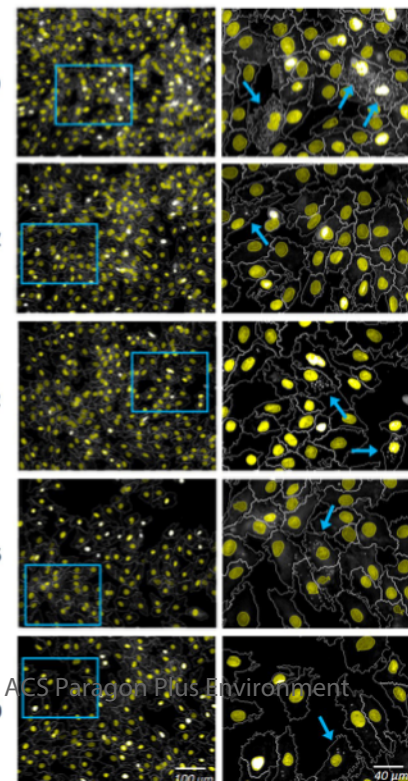
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