The thiol-polyamine metabolism of Trypanosoma cruzi: molecular targets and drug repurposing strategies

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Abstract: Chagas´ disease continues to be a challenging and neglected public health problem in many American countries. The etiologic agent, Trypanosoma cruzi, develops intracellularly in the mammalian host, which hinders treatment efficacy. Progress in the knowledge of parasite biology and host-pathogen interaction has not been paralleled by the development of novel, safe and effective therapeutic options. It is then urgent to seek for novel therapeutic candidates and to implement drug discovery strategies that may accelerate the discovery process. The most appealing targets for pharmacological intervention are those essential for the pathogen and, whenever possible, absent or significantly different from the host homologue. The thiol-polyamine metabolism of T. cruzi offers interesting candidates for a rational design of selective drugs. In this respect, here we critically review the state of the art of the thiol-polyamine metabolism of T. cruzi and the pharmacological potential of its components. On the other hand, drug repurposing emerged as a valid strategy to identify new biological activities for drugs in clinical use, while significantly shortening the long time and high cost associated with de novo drug discovery approaches. Thus, we also discuss the different drug repurposing strategies available with special emphasis in its application to the identification of drug candidates targeting essential components of the thiol-polyamine metabolism of T. cruzi.

Keywords: therapy, trypanothione, spermidine, polyamines, Chagas disease, bioinformatics, screening, approved drugs, drug repositioning, drug repurposing.

1. INTRODUCTION

Trypanosoma cruzi is the etiological agent of Chagas disease, a zoonotic disease endemic in tropical rural regions of Latin America, and extended to other American zones and continents by migration phenomena [1-3]. The parasite has a complex life cycle that alternates between intra and extracellular stages in their mammalian hosts and hematophagous insect vectors [4]. The notable plasticity to sense and adapt to different environments allows the pathogen to infect the vector gut, and the bloodstream, macrophages, muscle cells or cardiomyocytes of mammals [5, 6].

The vectorial transmission occurs by mucosal or wound contact with T. cruzi-contaminated feces of Rhodnius and Triatoma insects, vertically during pregnancy or delivery, horizontally by blood transfusion or organ transplantation and, less commonly, by oral transmission by food and drink contaminated with infected vectors or infected feces [7, 8]. The disease is characterized by an acute bloodstream phase that soon after evolves into a chronic stage, where the parasites colonize different organs and tissues.

It is estimated that there are almost 8 million people infected around the world [2], only 10% of them with the correct diagnose and less than 1% with access to treatment [9]. The available treatment is based in two drugs, benznidazole and nifurtimox, discovered in later 60´s and early 70´s. Both drugs are primarily used for the treatment of acute cases, while they show poor efficacy in the chronic phase and present serious safety issues [10–12]. Although effective in killing proliferating parasites, the clinical failure of both nitroheterocycle drugs against chronic Chagas may be associated to their incapacity to kill dormant parasites, as suggested by experiments performed in mouse [13].

Despite increasing efforts over the past decades, drug development for Chagas disease is still lagging behind. To overcome the lack of efficacy and safety of the current treatments, the novel drug candidates should target molecules that are essential for parasite survival in the host and unique to the pathogen, or at least significantly different –from a biochemical or structural point of view– to their host-counterpart (Figure 1).

The thiol-polyamine metabolism of T. cruzi has long been shown to be a suitable pharmacological target because of its unique configuration and/or dependency on external supply.

Polyamines are a group of low molecular weight basic compounds that are present in all living organisms [14–16]. The most abundant polyamines are putrescine (Put), spermidine (Spd) and spermine (Spm); however there exists other less frequent polyamines as cadaverine that was found in auxotrophic mutants of Escherichia coli

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Figure 1. Properties to be met by drug target candidates. The features above the dashed line are absolutely necessary for a molecule to be considered a suitable drug target candidate, while those below are not exclusive but “recommended” to increase the success of the pharmacological approach.

and in T. cruzi but not in T. brucei [17, 18]. Structurally, polyamines are carbonated polycations able to partner with different macromolecules (DNA, RNA, membranes and proteins) via electrostatic or hydrophobic interactions, and hydrogen bonds [14, 19]. By these interactions, polyamines have a direct molecular effect on nucleic acids and proteins function, biosynthesis and structure conservation and signal transduction as well as in essential cellular process such as proliferation, survival, differentiation, aging, autophagy, stress tolerance, apoptosis, etc [20–24].

In most living organisms, glutathione (GSH) is an important metabolite that contributes to metal and redox homeostasis [25]. In trypanosomatids, however, this function is efficiently superseded by the thiol-polyamine conjugate trypanothione or (N1,N8-bis(glutathionyl)spermidine) [26, 27]. Trypanothione is completely absent in the host whereas in the parasite it plays an essential role in maintaining the intracellular thiol-redox homeostasis. The limited capacity of T. cruzi to deal with oxidative stress was suggested by a pioneer study of Dr. Stoppani [28] and then endorsed by the fact that the apparent selectivity of nifurtimox and benznidazole to kill trypanosomes is related to their limited capacity to detoxify highly electrophilic and toxic byproducts (aldehydes and open chain nitrile) [29] and because they lack redundant redox systems to restore their redox balance [30, 31].

In the next sections, we present an updated review of the thiol-polyamine metabolism of T. cruzi, the components thereof with most prominent profile as drug candidates and novel drug discovery strategies that may pave the way toward the development of urgently needed safe, effective and cheap drugs. It is worth to stress that metabolic

and/or drug target validation data from the related trypanosomatids Trypanosoma brucei and Leishmania spp. are included for comparison purposes.

1.1. Thiol-polyamine Metabolism of Trypanosoma cruzi

1.1.1 Polyamine Synthesis and Uptake

The first step of polyamine biosynthesis occurs, in most of the organisms, by the conversion of ornithine into the diamine putrescine. This reaction is catalyzed by ornithine decarboxylase (ODC; EC 4.1.1.17), an enzyme subjected to multiple regulatory mechanism [32] (Figure 2). Putrescine is converted into spermidine by spermidine synthase (SpdS, EC 2.5.1.16). Each aminopropyl moiety is donated by decarboxylated S-adenosyl-L-methionine (dAdoMet), produced by S-adenosyl-L-methionine decarboxylase (AdoMetDC; EC 4.1.1.50). Putrescine can be synthetized by an alternative route present in plants, some bacteria and certain mammalian cells, where arginine is decarboxylated to agmatine by arginine decarboxylase (ADC; EC 4.1.1.19); consecutively, agmatine can be converted into putrescine by different enzymatic activities depending on the cell type [33–35]. Intracellular polyamines are also regulated by back-conversion throw steps of N–acetyl transference and oxidation [36–38]. Cellular transport of polyamines and N-acetyl derivatives in and out of the cell complete the metabolic landscape of intracellular levels of polyamines [6, 39–41]. The therapeutic potential of polyamine metabolism of trypanosomatids gained interest upon the demonstration that inhibition of ODC by the irreversible inhibitor difluoromethyl ornithine proved curative in a murine model of African trypanosomiasis [18]. In fact, DFMO is so far the only drug in clinical use against human African trypanosomiasis with a known specific molecular target.

At variance with most trypanosomatid species, T. cruzi is naturally auxotrophic for putrescine [42] because it lacks ODC as well as ADC [43, 44]. This condition makes T. cruzi entirely dependent on uptake of exogenous polyamines from the surrounding environment, making the polyamine uptake an attractive target for drug design. A high-affinity putrescine permease, namely TcPAT 12 (also called TcPOT1.1), has been identified as critical for polyamine incorporation in T. cruzi [45, 46]. TcPAT 12 belongs to the Amino Acid/Auxin Permeases family (AAAP family) [47]. Members of this protein family are found in the genome of other protozoan (e.g. Plasmodium) and in plants but are completely absent in mammals [6, 47, 48]. Other metabolic step that could be targeted is SpdS, whose biological relevance for T. cruzi still remains unknown. Nonetheless, based on evidences of essentiality for T. brucei [49] and virulence for Leishmania donovani [50], the protein has been investigated as a potential drug target [51-53].

1.1.2 Glutathione Synthesis

The biosynthetic pathway for glutathione (GSH) is shared between mammals and T. cruzi (Figure 2). It starts with the ligation of L-glutamate to L-cysteine, which produces γ-glutamylcysteine and is catalyzed by γ-glutamylcysteine synthetase (GSHA; EC 6.3.2.2). In the second step, glutathione synthetase (GSHB; EC 6.3.2.3) binds covalently L-glycine to γ-glutamylcysteine yielding GSH. Both reactions are ATP-dependent.

For T. cruzi, kinetic [54] and cellular assays with the irreversible inhibitor L-buthionine-S,R-sulfoximine (BSO) showed that GSHA is the bottleneck of this pathway [55]. GSH may participate in thiol-disulfide exchange and other redox reactions, which in most cases results in its oxidation to glutathione disulfide (GSSG). While most organisms are endowed with a glutathione reductase (GR; EC 1.8.1.7) activity to recover the reduced form of glutathione, genes encoding for this enzyme are absent T. cruzi [56] and all other trypanosomatids. In this respect, and based on their kinetic performance, the parasite oxidoreductases glutaredoxin (Grx; EC 1.20.4.1) and tryparedoxin (TXN; EC 1.6.4.8) have recently been suggested as candidates to take over this function using T(SH)2 as electron donor [57].

The therapeutic potential of targeting GSH (and hence trypanothione) biosynthesis in T. cruzi has been supported by in vitro and in vivo experiments. Parasites exposed to BSO displayed an enhanced sensitivity against the cytotoxic

action of nifurtimox and/or benznidazole [58, 59]. The weight thiols have recently been ascribed to the multitarget inhibition exerted by the drug on GSHA and trypanothione synthetase (TryS) of T. cruzi [55].

1.1.3 Trypanothione Synthesis, Reduction and Usage

Trypanothione biosynthesis consists in the stepwise formation of a C-N linkage between the free amines of spermidine and the glycyl carboxylate group from two GSH molecules. The first reaction yields mono-glutathionyl spermidine (Gsp) and the second one bis-glutathionyl spermidine (T(SH)2). Depending on the trypanosomatid species, a single or two independent enzymatic entities may catalyze these reactions using ATP as additional substrate. Glutathionylspermidine synthetase (GspS; EC 6.3.1.8) has selectivity for Gsp synthesis, while trypanothione synthetase (TryS; EC 6.3.1.9) evolved acquiring the capacity to produce both, Gsp and T(SH)2 (Figure 2). T. cruzi harbors genes for GspS and TryS, although only the latter has been biochemically characterized [60]. Importantly from a pharmacological point of view, the occurrence of GspS and TryS sequences is restricted to several Eubacteria lineages and Kinetoplastids [57, 61]. Trypanothione and TryS have been shown to be indispensable for the survival of T. brucei [62] and L. infantum [63] and a similar scenario can be predicted for T. cruzi on the light of the strict conservation of redox systems among trypanosomatids.

Worth noting, TryS (and also GspS) harbors an N-terminal domain with amidase activity that is capable to convert Gsp and T(SH)2 back to substrates [57, 60]. As stated above, polyamines can participate in a plethora of cellular functions. Thus, the polyamine-glutathione hydrolytic activity of GspS and TryS may play an important role in the maintenance of intracellular polyamine homeostasis in T. cruzi as indirectly suggested by studies conducted in an ODC-null mutant of L. donovani [64] and T. brucei [65].

The maintenance of steady-state levels of T(SH)2 depends not only in the rate of its biosynthesis but, more significantly, in the existence of an efficient system to recycle its oxidized or disulfide form (TS2) back to dihydrotrypanothione. The flavoenzyme trypanothione reductase (TR; EC 1.8.1.12) is in charge of reducing Gsp disulfide and TS2 at expenses of NADPH+ (Figure 2). Despite sharing sequence identity with human GR, both enzymes do not have a reciprocal substrate specificity [66]. TR has been shown to be essential for the infective stages of T. brucei [67] and Leishmania spp. [68], and a similar condition can be expected for T. cruzi. Trypanothione participates in a manifold of cellular functions, which can be classified in non-redox and redox-dependent

Figure 2. Thiol-polyamine metabolism of Trypanosoma cruzi. T. cruzi lacks arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), and hence is auxotrophic for putrescine, which is taken up from the extracellular medium by transporters. Spermidine synthase (SpdS) synthesizes spermidine by adding an aminopropyl group of S-adenosyl-L-methionine (produced by S-adenosyl-L-methionine decarboxylase, AdoMetDC) to putrescine. Cysteine, a precursor of glutathione, can be incorporated from the extracellular medium or synthesized de novo (de novo CB) from methionine, or from serine via the reverse transulfuration pathway (RTS). The RTS pathway is absent in mammals. γ-glutamylcysteine synthetase (GSHA) ligates glutamate to cysteine, rendering γ-glutamylcysteine, which is then bound to glycine by glutathione synthetase (GSHB) to produce the tripeptide glutathione (γ-glutamylcysteinylglycine, GSH). Monoglutathionyl spermidine synthetase (GspS) and trypanothione synthetase (TryS) catalyze the conjugation of one and/or two molecules of GSH to spermidine to produce mono- (GSpd) or bis-glutathionylspermidine (dihydrotrypanothione, T(SH)2). T(SH)2 is a redox cofactor or substrate of the glyoxalase system or different redoxins (i.e. glutaredoxins: Grx, thioredoxin: Trx, tryparedoxin: TXN). The glyoxalase system converts the toxic methylglyoxal into lactate, which is secreted to the medium. The different redoxins maintain the intracellular thiol-redox balance by reducing intra- or inter-molecular disulfides within or between proteins (Protein-S2) or with GSH (Protein-S-SG) to the corresponding thiol form (Protein-SH). TXN is the major oxidoreductase delivering electrons to different types of peroxidases (Px-S2). Oxidation of dihydrotrypanothione to the disulfide

form (TS2) is reverted by the flavoenzyme trypanothione reductase (TR) at expense of NADPH reducing power. The documented targets for the drugs buthionine sulfoximine (BSO), benznidazole (BZN) and nifurtimox (NFX) is shown. Inset: for comparison, the presence and absence of the different pathways or enzymes is depicted with different color codes for the parasite and host cell.

[for thorough reviews see 69-71]. Among the non-redox functions, trypanothione (and Gsp) may serve as reserve of GSH and Spd, which are replenished to the intracellular medium by the amidase activity of TryS (and GspS) [61, 72]. The dithiol can also act as metal ligand, which has been proposed to provide protection against the toxic action of reactive nitrogen and oxygen species generated by free-radical reactions [73], and to contribute to the biogenesis of iron-sulfur clusters, which are important structural or functional cofactors in proteins [74]. Moreover, several studies have linked T(SH)2 to protection against nifurtimox and benznidazole by the capacity of the dithiol to form conjugates with the drugs or their toxic byproducts [29-31, 54, 75-77]. T(SH)2 serves as thiol-cofactor of the glyoxalase system that detoxify the harmful oxoaldehyde methylglyoxal generated during glycolysis [78-80]. The dithiol is also a powerful scavenger of xenobiotics and radiation-induced radicals [81].

As reducing agent, trypanothione has been shown to be superior to GSH in the delivery of electrons to dehydroascorbate, or the oxidants hydrogen peroxide and peroxynitrite [69-71]. T(SH)2 is also very efficient in reducing protein disulfides [69-71]. Among them, several small oxidoreductases (glutaredoxin: Grx, thioredoxin: Trx and tryparedoxin: TXN) have been shown to be excellent redox partners of T(SH)2 [reviewed in 82]. T. cruzi encodes for a single Grx and Trx that are cytosolic proteins, and for two TXN, one displaying a cytosolic localization (TXN1) and a second isoform (TXN2) anchored to the surface of the mitochondria and endoplasmic reticulum [83, 84]. According to in vitro biochemical assays, Grx may contribute to maintain the pool of reduced glutathione and protein thiols at expenses of T(SH)2 [85]. Grx appears to have stage-specific roles, which involve the protection against oxidative stress in amastigotes and a pro-apoptotic effect in non-infective epimastigotes [86]. Trx is a low abundant protein with ability to reduce protein disulfides at expenses of T(SH)2 or TXN1 [83, 87]. Its biological relevance for T. cruzi is so far unknown but the homologue enzyme proved dispensable for African trypanosomes [88]. Overall, the oxidoreductase activity of the membrane-anchored TXN2 is at least one order of magnitude lower than that observed for the cytosolic TXN1. Both TXN catalyze the reduction of the active site cysteine residues of peroxidases and methionine sulfoxide reductases [83], proteins that play important roles in the detoxification of peroxides and in the repair of polypeptides, respectively. The oxidoreductases also proved more efficient than T(SH)2 in reducing several non-protein substrates such as GSSG, nitroso-glutathione and dehydroascorbate [83]. For a pathogen defective on GR activity and in view of the substrate specificity displayed, it is very likely that Grx and TXN contributes to maintain the pool of reduced glutathione at expenses of T(SH)2 [82]. Highlighting its role as multipurpose oxidoreductase, pulldown assays identified a dozen of partner candidates for TXN1 [89] and TXN2 [84]. In line with the enzymatic studies, both TXN interacted with different proteins from the parasite´s antioxidant system. The oxidoreductases also showed certain selectivity for interacting with proteins participating in different cellular processes such as protein synthesis and degradation (for TXN1) or energy metabolism, cytoskeleton and protein translation (for TXN2). This suggests some non-overlapping functions of TXN in the maintenance of protein redox homeostasis and signaling.

1.3 Molecular Targets within the Thiol-Polyamine Metabolism: a Critical Revision

Before addressing the discussion on the subject of this section, it is important to recall the requirements that a molecular target should fulfill to be considered as a suitable drug target candidate (Figure 1). Worth noting, while some features are absolutely necessary (i.e. indispensability, druggability) others are not excluding but highly recommended (i.e. no potential for developing resistance, biochemical properties, absence in the host, structural specificity) to warrant a successful outcome. Applying these criteria, a similar number of components from the thiol-polyamine metabolism of trypanosomatids can be regarded and disregarded as targets for specific pharmacological

development or repurposing. Genetic validation is lagging behind for T. cruzi, thus, whenever required, the essentiality of the different components of the parasite redox metabolism is inferred from data obtained for related trypanosomatids.

Regarding the polyamine pathway, the auxotrophy of T. cruzi for polyamine precursors set the transport of these metabolites and SpdS as the only potential candidates for drug repurposing. Focusing on polyamine transporters, the functional uptake and the resultant intracellular polyamine levels have been shown involved in epimastigotes growth rates and survival under stress conditions elicited by hydrogen peroxide, nifurtimox or benznidazole [90], in parasite autophagy and differentiation from epimastigote to metacyclic trypomastigote [91, 92] and in trypomastigote infectivity and infection progression [21, 93]. While recently five new sequences encoding for putative polyamine transporters have been identified in T. cruzi genome, the functionality of those genes has not been yet confirmed [90]. In this sense, a knockout of TcPAT12 was not fully lethal for T. cruzi amastigotes [93]. Considering the key role of polyamine transport in T. cruzi, it is reasonable to suspect that additional permeases might function as secondary low-affinity uptake mechanism/s, contributing to maintain a basal level of polyamines in a small subset of parasites with a lower replication rate. Such hypothetical scenario would result detrimental for targeting polyamine transport in drug discovery campaigns. However, TcPAT12 appears as the sole high-affinity diamine permease of T. cruzi that is key for robust survival inside the host cell [93]. Moreover, TcPAT12 and all the other members of the AAAP protein family present a highly variable region (5% of consensus) at the N-terminal domain -implied in ligand specificity- and a C-terminal domain highly conserved (more than 70% of consensus) [94-96]. This highly conserved structure makes AAAP family attractive for multitarget inhibition, approach that could present increased treatment efficacy, prevent the development of drug resistance, reduce treatment duration, and -eventually- decrease the treatment costs [97]. The essentiality of SpdS for survival of the infective stages of T. cruzi remains to be elucidated. However, the capacity of te parasite to take up Spd from the extracellular medium [98] shed doubts about the potential of SpdS as drug target, except that polyamine transport is simultaneously inhibited. Nonetheless, recent efforts focused on the structural characterization and discovery of inhibitors against T. cruzi SpdS [see next section; 51-53].

Analysis of metabolic control through the thiol-polyamine pathway and downstream components of the electron chain (TR, TXN and Px) of T. cruzi provided clues on the targets amenable for therapeutic intervention [54, 99]. The main bottlenecks of the pathway upstream to T(SH)2 resulted GSHA > TryS >> polyamine transport. For the downstream steps, TXN was recognized as the enzyme exerting major flux control on the pathway, whereas the contribution of TR and the different peroxidases can be neglected. Overall, these studies suggested that simultaneous and moderate inhibition of GSHA and TryS and/or TXN may be sufficient to impair redox homeostasis with lethal consequences for the pathogen. As matter of fact, RNAi-mediated downregulation of the expression of each of these proteins demonstrated their indispensability in African trypanosomes [62, 100, 101].

Although several studies supports the druggability of GSHA or TryS [55, 102, 103], and, to a minor extent, of TXN [54, 99] from T. cruzi, these targets present additional features that allows for a more precise assessment of their potential for effective and selective identification or development of inhibitors. Despite the high degree of sequence similarity between human and trypanosomal GSHA, a study revealed kinetic differences between the T. brucei and mammalian enzyme that may eventually be exploited for the development of specific inhibitors [104]. These findings should be corroborated for the T. cruzi GSHA. Although, TXN is absent in mammals, the trypanosomal protein has a folding and active site that is similar to that of Trx [105], an abundant protein in host cells. In fact, the most potent inhibitors identified against TXN in a large high throughput screening (HTS) were covalent inhibitors that modified irreversible the active site cysteines of the redoxin and also interacted with human Trx [106]. Against the infective form of African trypanosomes, the most active compounds showed selectivity indexes of >10- or even 83-folds. The higher selectivity of the compounds towards trypanosomes might be related to the highly proliferative rate of these parasites compared to mammalian cell, which demand a constant supply of reducing power for DNA replication. It remains to be determined whether these compounds are equally effective against T. cruzi parasites and lack toxicity in long term treatments, as required for chronic Chagas.

TR remains the molecular target for which more drug discovery campaigns have been conducted [reviewed in 107]. Unfortunately, most of the inhibitors failed to impair parasite survival in vitro or in vivo at pharmacologically relevant doses and/or to display an on-target effect. This is probably due to its low metabolic control of the trypanothione pathway that requires a high and sustained inhibition (>95%) of the reductase to induce a defective phenotype in trypanosomes [54, 107]. Although the crystal structure of TR from T. cruzi shows significant differences at the substrate-binding site with the distantly related human GR [108-111], the large volume of this pocket makes difficult the design of small and high affinity ligands.

TryS is, so far, the most attractive drug target within the thiol-polyamine metabolism of trypanosomatids because it is: i) exclusive of the pathogen, ii) a relatively low abundant cytosolic protein, iii) indispensable for parasite survival, iv) kinetically characterized, v) druggable and with a known overall crystal structure [62, 63, 102, 103, 107, 112-114]. Early developments of TryS inhibitors were based on substrate or reaction intermediate analogues [135-137]. However, most of these compounds displayed a poor correlation of TryS vs. parasite inhibition, in part due to the capability of the amidase domain of the enzyme to cleave off C-N bonds of the inhibitors. This and the recently demonstrated species-specific behavior of novel TryS inhibitors [102, 103] should be taken into account in future drug discovery approaches against this enzyme.

For several reasons, the remaining components of the parasite redox systems are not at all or at least less attractive as therapeutic candidates.

Trx and Grx are not essential for the infective form of African trypanosomes, which together with their high structural similarity with the human homologues make them not attractive drug target candidates. Because a species-specific indispensability cannot be disregarded for these proteins, final decision on their suitability as drug-targets await biological validation in T. cruzi.

Different classes of peroxidases play important roles as virulence factors and in protection against oxidative damage generated by endogenous metabolic processes or by the host immune response [115, 116]. However, the identification and/or development of selective inhibitors appears challenging giving the high structural similarity with the human homologues and their low druggability [106].

Metabolic modeling of the trypanothione-dependent detoxification of methylglyoxal by glyoxalases suggests that this pathway is highly efficient and a detrimental phenotype would only be achieved by simultaneous inhibition of several enzymatic activities [80]. This renders the glyoxalase system not a priority for drug development.

1.3 Strategies for Drug Repurposing against Chagas Disease

Drug repurposing involves finding new medical uses for approved, withdrawn, abandoned and investigational drugs [117]. The interest in such strategy has dramatically risen in the last years. Branching the development of a drug to a new therapeutic area can shorten the drug development timeframe, since the new indication is built on previously pharmacokinetic, safety and manufacturing data [118, 119], also resulting in substantial savings.

The first successful repurposing stories emerged from serendipitous/empirical observations (a posteriori knowledge). For example, sildenafil was first investigated as an antihypertensive drug and as treatment for coronary artery disease; while undergoing clinical trials, however, its unexpected effects on penile erection were detected and then exploited [120]. This and many other examples of successful drug repurposing have prompted the drug discovery community to explore systematic (prospective) approaches to identify repurposing opportunities [122-124]. It has been estimated that drug repurposing accounts for roughly 30% of the newly FDA-approved drugs in recent years [122].

The strategy has drawn attention in the fields of neglected and rare conditions [125-129], where resources are limited due to low investment returns. As a matter of fact, a considerable fraction of the (scarce) lately approved

drugs and the drug candidates undergoing clinical trials for the treatment of trypanosomatid-caused diseases are repurposed drugs [127]. For instance, eflornithine (which was originally pursued as an anticancer drug) and nifurtimox, have been successfully introduced for treating African trypanosomiasis, and the long forgotten antimicrobial agent Fexinidazole is currently undergoing clinical trials for African tyrpanosomiasis, Chagas disease and Leishmaniasis. The antifungal Ambisome® and mitefosine (originally pursued as anti-cancer agent) are currently used to treat Leishmaniasis.

Commercial and intellectual property issues that limit repurposing perspectives from an industrial viewpoint [130-132], are often not that relevant in the field of neglected diseases, where most of the investment comes from public and non-for-profit initiatives and the driving force of research is rather humanitarian than purely economic [133].

Systematic approximations to drug repurposing will be briefly overviewed in the next sub-sections and are schematized in Figure 3. Examples of applications in the search for therapeutic solutions for Chagas disease will also be provided, when available.

1.3.1. Systematic Screening and Drug Repurposing

It is possible to implement exhaustive (wet) screening of libraries of approved and withdrawn drugs to uncover repurposing opportunities, sing either phenotypic- or target-based screens, in a low- or high-throughput manner [134-136]. Applying this approach, a set of 100 approved drugs was tested against trypanosomatids, leading to the identification of several hits such as azole antifungals, auranofin, rifamycin, tipranavir and clofazimine [137].

Planer et al. screened the Spectrum Collection of 2,000 biologically active compounds, including about 700 FDA-approved drugs, against T. cruzi amastigotes [138]. 17 hits with EC50 in the high nanomolar or low micromolar range and acceptable pharmacological profile plus 7 trypanocidal compounds retrieved from literature were tested in vitro in all two-way combinations. Compounds displaying synergism were then tested in an acute mouse model of Chagas. The most potent combination was the calcium channel blocker amlodipine plus posaconazole, resulting in a nearly complete suppression of parasitemia.

Engel et al. reported an image-based HTS protocol targeting T. cruzi amastigotes [140]. A pilot screening on 909 clinical compounds resulted in 55 confirmed hits with EC50 ≤50 μM, including known antidepressants, antihistamines and antipsychotics, among others.

De Rickery et al. screened the NIH Clinical Collection and the SelleckChem FDA-approved drug library for potential new drugs against T. cruzi amastigotes [141]. The most promising hits identified were clemastine, azelastine, ifenprodil, ziprasidone and clofibrate. However, the authors argued that these drugs were not suitable for repurposing due to the low levels of the drugs achieved in clinical settings. There were several CNS-targeting amines among them, which were disregarded because of two often overlooked points: their low therapeutic dosage and potential side-effects. Some calcium channel blockers were also identified, in line with previous reports describing the trypanocidal effects of channel blockers and suggesting Ca2+ homeostasis as a good target to develop T. cruzi chemotherapy [140, 142-144].

1.3.2. Cheminformatics-based Drug Repurposing

Computer-aided approximations are among the best examples of rational drug repurposing strategies.

Virtual screening (VS) represents the most frequent cheminformatic approach to drug repurposing for neglected diseases. It involves submitting digital chemical libraries of candidate compounds to computational models or algorithms; the score provided by such models is used to rank the compounds of the library and decide which deserve experimental testing.

In an application of what could today be regarded as target repurposing, Soares et al. searched for T. cruzi sirtuin 2 inhibitors in a small library of compounds containing the nicotamide core, which was retrieved from ZINC database through similarity searches [149]. Nicotamide itself, a known sirtuin inhibitor, as the best candidate that, when tested, proved trypanocidal against the different parasite life stages.

The first VS application specifically focused on drug repurposing to identify novel inhibitors against cruzipain, was performed by Bellera et al [150]. From the selected hits , the antiparkinsonian bromocriptine and the antiarrhythmic amiodarone displayed a weak, concentration-dependent inhibitory effect on cruzipain and more potent antiproliferative effects against epimastigotes. Optimization of the in silico search and validation strategy led this group to other cruzipain inhibitors: L-thyroxine (used to treat thyroid hormone deficiency) [151], the antibiotic clofazimine, the antihypertensive benidipine and the antiviral saquinavir [142]. For almost all hits studied the IC50 against the enzyme was higher than their EC50 against the parasite, suggesting other molecular targets were involved in the trypanocidal effect. In fact, it had already been shown that the trypanocidal effects of amiodarone and its analogue dronedarone could be related to disruption of the parasites' Ca2+ homeostasis and ergosterol biosynthesis inhibition [152, 153], whereas clofazimine has later demonstrated inhibition of putrescine uptake [154].

In vivo testing of the hits from the first two screening campaigns was abandoned because either the effective plasma concentrations reported for the original therapeutic indications were much lower than the effective concentrations against parasites, or because the initial therapeutic activity may become an undesirable side effect in patients with normal physiology. In contrast, the potent activity displayed by benidipine and clofazimine justified therapeutic efficacy tests in mouse models of Chagas. Both proved effective in controlling acute infection [142] and in reducing parasite load in skeletal and heart muscle during the chronic stage of the disease [155].

Figure 3. Drug repositioning approaches. The scheme summarizes the different methodologies that can be applied for drug repurposing. Examples of drug candidates identified against trypanosomatids are provided.

Applying a structure-based approach with cruzipain as molecular target, Palos and co-workers identified several hits from a library of FDA-approved drugs [156]. Four hits were submitted to in vitro testing confirmed their activity against trypomastigotes. In vivo evaluation of the compounds showed that etofillyn clofibrate and piperacillin reduced parasitemia in infected mice.

The same FDA-library was used to screen for inhibitors of trans-sialidase, an enzyme secreted by T. cruzi that plays key roles in host response evasion, cell invasion and pathogenesis [157]. Four hits (including antihistaminic, antibiotic, antihelmintic and antihypertensive drugs) showed better trypanocidal effects than the reference drugs nifurtimox and benznidazole towards trypomastigotes. One of them, the anti-inflammatory sulfasalazine, confirmed moderate inhibition of trans-sialidase; it also showed the best trypanocidal effect in short-term in vivo experiments. The authors emphasized that the combined trypanocidal and anti-inflammatory properties of sulfasalazine could be beneficial to control infection and ameliorate the inflammatory processes associated to Chagas disease.

A different cheminformatic approach to drug repurposing has been reported by Wu et al. [158], in line with previous work by Keiser and collaborators [159, 160]. Their general idea is that different targets or therapeutic indications may be related if each of them includes sets of chemically similar drugs. These studies suggest a pattern of cross-repositioning opportunities between pairs of therapeutic classes. Interestingly, some therapeutic categories, such as antidepressants [140, 161-163], antihypertensives or antiarrhythmics with Ca2+ channel blocking properties [138-

140, 153] and antihistamine drugs [138, 140, 157], seem to display trypanocidal effects in a rather systematic manner (see next section for more examples and references).

1.3.3. Bioinformatics-based Drug Repurposing

Health conditions connected to similar drug targets may be treated with the same drugs. Bioinformatic tools can help revealing protein-protein similarities among different pathogens. Close similarities provide immediate, easily noticeable on-target repurposing opportunities. A good example is the repurposing of azole antifungals to inhibit CYP51 orthologues in other microorganisms, such as trypanosomatids [161].

In contrast, remote similarities between proteins without evolutionary relationship or even without a similar fold or function are more challenging and provide less apparent repurposing possibilities. Much attention has recently been given to the identification of binding site similarity as a basis for spotting repurposing prospects [162-165]: similar binding sites can be found in proteins with low global similarity and numerous case studies reveal that the binding of similar ligands cannot be deduced from fold but from local similarities [162, 166]. It should be recalled that while similar binding sites frequently bind the same ligands, the converse does not apply: a single ligand can bind to very different binding sites [166]. Therefore, binding site comparisons can only cover a fraction of the possible drug repositioning prospects and should be complemented with other bionformatic or cheinformatic approaches.

As an example of bioinformatics-based drug repurposing, Rodrigues et al. explored the Kinetoplastid Genomic Resource (TriTrypDB) to enumerate 65 protein sequences related to cellular transport; each of them was subsequently used as query to find homolog proteins with known ligands in DrugBank and the Therapeutic Target Database, finding three hit targets [167].

1.3.4. Other approaches

Other computer-aided approximations to assist systematic drug repurposing include literature mining, and network- and gene signature-based approaches; so far, though, they have been scarcely applied to the discovery of novel medicines against Chagas disease.

Literature mining applies automated text mining tools to screen large volumes of scientific literature and find implicit associations between two concepts or elements (e.g. a drug and a disease) [168].

Networks-based approaches integrate huge volumes (and frequently, different types) of data by generating topological architectures in which entities (e.g. drugs, gene products, diseases) are represented as nodes and relationships between nodes, as edges [169]. Pair-wise relationships between network elements might emerge from computational predictions and metrics (e.g. molecular similarity; sequence similarity) or from observed data (e.g. affinity constants, co-expression patterns, etc.). The information derived from this analysis can turn valuable to identify novel molecular targets and uncover drug-target associations [170-172]. Recently, for instance, Berenstein et al. used data from extensively studied organisms (Homo sapiens, Escherichia coli, yeast and others) to produce a multilayer weighted network comprising proteins and bioactive compounds [173]. The network edges reflected molecular similarities between 170,000 compounds and functional relationships among 167,000 proteins. The authors applied the network to prioritize targets in kinetoplastids; most of the identified candidate proteins were kinases with homologs in humans.

The signature-based drug repurposing relies on the quantitative comparison of the transcriptome of a cell before and after exposure to a chemical [174]. The drug-induced gene expression profile can be compared with a disease-associated signature. It is hypothesized that opposite signatures between drug- and disease-associated signatures will reveal a positive effect of the drug against the targeted disease. The approach has still to be applied in the

context of trypanosomatid-caused diseases, where it could possibly provide new therapeutics beyond the traditionally pursued trypanocidal drugs (e.g. drugs that prevent decline of cardiac function). For that purpose, availability of gene-signatures of patients with Chagas would be invaluable.

Transcriptomics can also be helpful to disclose resistance mechanisms of infectious agents, and to propose drug targets [179]. For example, a combination of transcriptomics with functional genomics recently demonstrated the role of adenine phosphoribosyltransferase in T. cruzi's resistance to benznidazole [180].

1.4. Drug repurposing against enzymes from the thiol-polyamine metabolism of Trypanosoma cruzi

Whereas drug repurposing initiatives with a focus on T. cruzi redox metabolism are still scarce, some examples related to thiol-polyamines metabolism can be found in the literature.

Many of the repurposed candidate drugs in this area have previously been used in the field of psychiatry, were most drugs are known to be multi-target ligands displaying a certain degree of promiscuity [181, 182]. In 1984, Hammond et al. identified several tricyclic antidepressants (such as clomipramine, trimipramine and desipramine) and antipsychotics from the phenothiazine family (e.g. promethazine, perphenazine and related compounds such as thiethylperazine) as anti-T. cruzi agents [183]. Soon later, the same authors focused on the screening of amphiphilic cationic drugs because compounds bearing such characteristic had shown to be effective against T. cruzi and other protozoa [184]. Further studies revealed that one of the molecular targets of phenothiazine derivatives and tricyclic antidepressants is TR [185-189]. From about 30 structurally-related compounds, clomipramine resulted as the most potent TR inhibitor (Ki in the low μM range) that competed with trypanothione binding to the enzyme [185]. Based on the phenothiazine scaffold, Chan et al. designed several derivatives with a conserved inhibition mechanism (i.e. competitive with trypanothione and noncompetitive with NADPH) [190]. This study also pinpointed positions and substituents of the promazine nucleus that may increase ligand affinity. In an attempt to repurpose methylene blue, a phenothiazine derivative and antimalarial agent (inhibitor of P. falciparum glutathione reductase), as drug candidate against trypanosomatid-caused diseases, their potential molecular targets and mode of action were investigated. As expected from its chemical nature, methylene blue behaved as a redox cycler drug affecting the trypanothione-based antioxidative network of trypanosomes by generating ROS and acting as subversive substrate of T. cruzi TR and lipoamide dehydrogenase [187].

Consistently with their promiscuous nature, trypanocidal antipsychotics and antidepressants seem to exert their cidality through additional mechanisms [190]: clomipramine by an anticalmodulin action; trifluoperazine and thioridazine through disruption of mitochondria membrane potential and; prometazine through serious cell membrane disorganization.

Under experimental conditions, clomipramine proved curative in a mouse model of acute Chagas [191] and effective in reducing pathogenesis in a chronic murine model of Chagas [192]. In contrast, methylene blue failed to cure mice infected with T. b. brucei, a behaviour ascribed to differences in drug metabolism and pharmacokinetics between mice and humans [193]. On the other hand, the fact that clomipramine and related compounds are used in low doses and achieve low free drug plasma concentrations when administered for their original indication [138], along with a possible incompatibility between their psychotropic activity and their use as an anti-trypanosomal therapeutics [194] may difficult their effective repurposing. This explains the past and present interest in the design of novel trypanocidal agents based on tricyclic antidepressants and antipsychotics [194-197].

Recent efforts have focused on putrescine uptake as a potential target for new repurposed drug candidates against Chagas disease. From a dataset of polyamine analogues, Alberca et al. screened DrugBank and identified five potential hits [198]. Three of them (triclabendazole, sertaconazole and paroxetine) displayed trypanocidal effects and inhibited putrescine uptake in epimastigote parasites. Whereas triclabendazole and sertaconazole also showed inhibitory effects on the uptake of lysine, arginine and uridine, the effect of paroxetine on the putrescine transporter

TcPAT12 seemed to be more selective. None of the hits displayed inhibitory effects against TryS suggesting non-effective competition with the polyamine binding site of this enzyme. Further testing of paroxetine in an acute mice model of Chagas (50 mg/kg/day dose) failed to reduce parasitemia (unpublished data); in contrast, triclabendazole 100 mg/kg reduced parasitemia to some extent. The lack of correlation between in vitro and in vivo activity could be related to the low fraction of free drug in plasma (≥95% of paroxetine is bound to plasma proteins) [199].

Reigada et al. screened a library of 3,000 approved drugs using a combined ligand- and structure-based strategy [200]. Retinol was used as ligand query and a comparative model of TcPAT12 as molecular target. From seven hits, the anti-acne drug isotretinoin showed dose-dependent inhibition of putrescine uptake albeit other amino acid transporters from the same protein family were inhibited. The drug showed a strong inhibition of T. cruzi trypomastigote burst from infected cells (EC50 ~ 130 nM). Further studies revealed that isotretinoin induced autophagic and apoptotic processes, which may be related to nutrient starvation caused by transporter inhibition. Unfortunately, the high distribution volume of isotretinoin [201] together with the relatively low maximum dose (2 mg/kg/day) used for the original indication and the very high extent of plasma protein binding (>99.9%) might conspire against progressing to the preclinical development stage.

In a recent publication, Dietrich et al. used a hybrid ligand- and structure-based VS approach (parallel similarity-based and QSAR-based screen, serially combined with docking) for the discovery of new inhibitors of TcPAT12 [202]. The identified hits cisapride and [2-(cyclopentyloxy)phenyl]methanamine displayed anti-proliferative effect against T. cruzi epimastigotes. Transport assays confirmed that cisapride interferes with putrescine uptake in a specific mode. In a second combined in silico screening campaign using an improved ensemble of QSAR classifiers [154], cinnarizine and clofazimine were identified as novel inhibitors of putrescine uptake in T. cruzi. Interestingly, neither cinnarizine nor clofazimine modified arginine uptake by putative amino acid transporters.

Maccari et al. developed a fast VS protocol for TR that combines reciprocal structure information from the protein and active ligands [203]. This methodology selects for site-specific compounds that shares a similar structural scaffold and/or electrostatic properties, and hence reduces significantly the ratio of false positive hits. The strategy proved successful in picking new low μM inhibitors of TR and holds potential for its application to drug repurposing libraries.

Beig et al. employed a combined and iterative approach of in vitro and 2D in silico screening against TR, that increased significantly the rate of hit identification that each of these screening methods yield separately. Applying this strategy to drug repurposing libraries may prove valuable [204].

A library of almost 4.8 million compounds bearing drug-like properties has been recently screened against T. cruzi SpdS [51]. The hits identified can be rated as moderate inhibitors of SpdS as their IC50 values were one or two orders of magnitude higher than that of the control inhibitor (IC50 = 1.9 μM). Nonetheless, the new inhibitor scaffolds may serve as template structures for cheminformatics-based drug repurposing-based approaches.

CONCLUSION

Trypanosomatids are unique in that the major products of the low molecular weight thiol and polyamine metabolism are juxtaposed, resulting in the formation of trypanothione, a redox substrate and cofactor around which the parasites developed a unique redox system. This makes several components of this metabolism excellent to reasonable drug-target candidates. Targeting a metabolism at the uppermost step of the pathway will almost abolish all downstream processes depending on the metabolic products. Bearing this concept in mind along with the demonstrated auxotrophy of T. cruzi for polyamine precursors and the dependency and uniqueness of trypanothione, it seems reasonable to propose that drug discovery campaigns should focus on polyamine transport and trypanothione biosynthesis and reduction. This is further supported by the specific structural and biochemical features of the corresponding proteins that guarantee a selective impairment of parasite viability with low to null off-target effects if ligands are rationaly designed or identified.

Drug repurposing represents a cost-efficient strategy to develop therapeutic solutions; it is a highly appealing strategy to find innovative solutions for neglected and rare diseases, which are characterized by low invest returns. In silico drug repositioning can be regarded as a particularly efficient approximation because candidates are prioritized using computational techniques, which contributes to a more rational and optimized used of more expensive experimental procedures. Furthermore, in the omics era, computational approaches are well suited to organize the increasing volume of experimental data to generate knowledge from already existing information.

Among the variety of in silico techniques that may be used to guide drug repurposing, virtual screening is so far the more extensively applied. However, other promising approaches have begun to be considered by ongoing investigations, as highlighted by the many reports cited in this review. The in silico selection of candidates drugs for clinical repurposing, should, however, be complemented with pharmacological criteria, including dose compatibility across the expanded therapeutic indications, recommended administration routes, contraindications of the drug for the targeted population, possible additional benefits, etc.

It should be noted that, due to the availability of free-access computational resources, and the advent of cloud and low cost parallel computing, in silico techniques are probably, nowadays, the research field within drug discovery with the smallest technological gap. This allows local players from endemic countries and regions to get actively involved in drug discovery for neglected conditions.

CONFLICT OF INTEREST

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