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Comparative mapping of on-targets and off-targets for the discovery of anti-trypanosomatid folate pathway inhibitors

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ABSTRACT

Background: Multi-target approaches are necessary to properly analyze or modify the function of a biochemical pathway or a protein family. An example of such a problem is the repurposing of the known human anti-cancer drugs, antifolates, as selective anti-parasitic agents. This requires considering a set of experimentally validated protein targets in the folate pathway of major pathogenic trypanosomatid parasites and humans: (*i*) the primary parasite on-targets: pteridine reductase 1 (PTR1) (absent in humans) and bifunctional dihydrofolate reductase-thymidylate synthase (DHFR–TS), (*ii*) the primary off-targets: human DHFR and TS, and (*iii*) the secondary on-target: human folate receptor β, a folate/antifolate transporter.
Methods: We computationally compared the structural, dynamic and physico-chemical properties of the targets. We based our analysis on available inhibitory activity and crystallographic data, including a crystal structure of the bifunctional *T. cruzi* DHFR–TS with tetrahydrofolate bound determined in this work. Due to the low sequence and structural similarity of the targets analyzed, we employed a mapping of binding pockets based on the known common ligands, folate and methotrexate.
Results: Our analysis provides a set of practical strategies for the design of selective trypanosomatid folate pathway inhibitors, which are supported by enzyme inhibition measurements and crystallographic structures.
Conclusions: The ligand-based comparative computational mapping of protein binding pockets provides a basis for repurposing of anti-folates and the design of new anti-trypanosmatid agents.
General significance: Apart from the target-based discovery of selective compounds, our approach may be also applied for protein engineering or analyzing evolutionary relationships in protein families.

1. Introduction

Most biochemical pathways are complex networks that are composed of multiple nodes (e.g., enzymes) connected by different relationships. Thus, characterizing or modifying the functions performed by such a network often requires consideration of the whole pathway(s), including the key enzymes, substrates and other participating molecules. In particular, it is known that small molecules, such

Abbreviations: PTR1, Pteridine reductase 1; DHFR, Dihydrofolate reductase; dUMP, Deoxyuridine monophosphate; dTMP, Deoxythymidine monophosphate; hDHFR, Human dihydrofolate reductase; TS, Thymidylate synthase; hTS, Human thymidylate synthase; FOLRβ, Human folate receptor β; MTX, Methotrexate; FOL, Folate; Lm, *Leishmania major*; Ld, *Leishmania donovani*; Li, *Leishmania infantum*; Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; parDHFR, Parasitic dihydrofolate reductase; THF, (6S)-5,6,7,8-tetrahydrofolate; PABA, Para-amino benzoyl (group); MIF, Molecular Interaction Field

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as substrates and drugs, generally bind many macromolecular targets [1]. Their effects should be considered in the global context of their influence on the biochemical networks involving the targets. This means that structure-based design of low molecular weight compounds typically requires simultaneous consideration of several on-targets and off-targets. Here, we apply a systematic ligand-based mapping of protein binding sites to inform the discovery of anti-parasitic agents that target the folate pathway. This mapping is designed to facilitate the discovery of new multi-target inhibitors and the repurposing of anti-cancer antifolate drugs as anti-trypanosomatid agents.

The Trypanosomatidae family of protozoans comprises parasitic species that cause human diseases such as leishmaniasis, Chagas disease and sleeping sickness [2]. Currently, the available treatments are hindered by problems such as drug toxicity, parasite resistance and ineffective drug delivery. Therefore, there is a need to improve the existing anti-parasitic drugs and to discover new compounds [3]. Here, we consider the targeting of multiple proteins in multiple parasite species, and we address the issue of side effects and toxicity due to off-target binding. We focus on trypanosomatid species causing the most prevalent infections: (*i*) *Trypanosoma brucei* (Tb; sleeping sickness), (*ii*) *Trypanosoma cruzi* (Tc; Chagas disease), (*iii*) *Leishmania major* (Lm; cutaneous leishmaniasis); (*iv*) *Leishmania donovani* (Ld) and *L. infantum* (Li; visceral leishmaniasis).

One approach to tackling these parasites is to target the folate pathway in parasites [5]. Drug discovery targeted to the folate pathway has been successful against bacterial infections and cancer [6]. As shown in Fig. 1a, trypanosomatid parasites activate folate (FOL) using dihydrofolate reductase (DHFR). However, in a salvage pathway, they can also use pteridine reductase 1 (PTR1), which is absent in humans and predominantly involved in the biopterin reduction pathway (both enzymes use the nicotinamide adenine dinucleotide phosphate [NADP] cofactor). The reduced FOL is further used for deoxythymidine monophosphate (dTMP) synthesis catalyzed by thymidylate synthase (TS, with deoxyuridine monophosphate [dUMP] as one of the substrates) [7–9]. Whereas for blocking the folate pathway in humans, only DHFR needs to be inhibited, in trypanosomatid parasites efficient inhibition of this pathway also requires inactivation of PTR1 [8]. Inhibitors developed against human DHFR and TS, such as methotrexate (MTX) or nolatrexed, also show activity against parasitic variants of these enzymes [10–13]. They therefore can serve as good starting points for optimization towards the bifunctional parasitic DHFR–TS. However, these compounds are less active against PTR1 (see activity data for MTX in Table S1A, Supplementary material and refs. [4,7,10,12-16]). Thus, we here focus on PTR1 as the primary trypanosomatid-specific folate pathway target.

MTX, with a scaffold similar to FOL (see Fig. 1a), is an example of a potent hDHFR inhibitor that also targets other folate pathway enzymes. It displays pico- to nanomolar inhibition of human and parasite DHFR and submicromolar inhibition of other folate pathway targets, including PTR1 (see Table S1A). Due to its multi-target inhibition capability, MTX provides a good starting scaffold for the design of dual- or multiple-target anti-trypanosomatid antifolates, but has the risk of major off-target effects on hDHFR. On the other hand, MTX is not a strong binder to hTS (with a nearly millimolar K_d value [16]).

Attempts to design potent MTX derivatives optimized for PTR1 have been reported [10,17]. The synthesized compounds appear promising but still require optimization of their selectivity against the enzymes and potency against the parasites. Ideally, inhibitors should be active against PTR1 enzymes of multiple species, in order to have a single inhibitor for multiple pathogenic trypanosomatids. However, while MTX inhibits PTR1 from different species with similar inhibition constants (K_i in the range 39–180 nM, see Table S1A in the Supplementary material and refs. [7,10,15]), other PTR1 inhibitors, including MTX derivatives as well as entirely different scaffolds such as flavonols, often show distinctly differing inhibitory activities against PTR1 variants [10,18,19]. Many anti-PTR1 drug design efforts have focused on discovering inhibitors specifically targeting a single parasitic PTR1. For example, in a virtual screening strategy, thiadiazoles and benzothiazoles were identified as *Leishmania major* PTR1 (LmPTR1) inhibitors [20], and aminobenzimidazoles were iteratively optimized to target species-specific subpockets of *Trypanosoma brucei* PTR1 (TbPTR1, Mpamhanga et al. [21], Spinks et al. [22]). Thus, we here systematically describe structural differences that may result in differential binding of inhibitors to PTR1 variants, and investigate if similarities between these protein targets could be exploited to design multi-species inhibitors.

The efficacy of inhibitors is dependent on their ability to reach the target site of action. In infections caused by *T. cruzi* and *Leishmania*, the parasite amastigote form proliferates inside human cells (in particular in fibroblasts and macrophages, respectively [23–25]). Thus, to reach their targets, PTR1 and DHFR, antifolates need to cross the membranes of the human cell, the parasitophorous vacuole and the parasite (see Fig. 1b). From this perspective, improving the capacity to bind transporters might improve inhibitor efficacy. In human cells, the *β* isoform of the folate receptor (FOLR*β*) is involved in the transport mechanism exploited by folate and pteridine-based antifolates, e.g., MTX [4,26,27]. This suggests that receptors such as FOLR*β* may serve as transporters of antifolates into the human macrophage cell. Moreover, crystal structures of FOLR*β* are available [4]. Thus, while targeting the trypanosomatid folate pathway, simultaneous optimization towards FOLR*β* should be considered.

In the present work, as illustrated in Fig. 1c, we compare sequence and structural properties of the proteins discussed above for the purpose of structure-based antifolate design against trypanosomatids. We analyze the properties of PTR1 and the following folate pathway enzymes (Fig. 1a,b): parasite DHFR–TS bifunctional enzyme (further on-target), human TS (hTS) and DHFR (hDHFR) enzymes (off-targets), and FOLR*β* as a potential transporter and secondary on-target. Such comparisons are not straightforward, since the proteins, despite binding the same substrates, have dissimilar folds. Therefore, for our analyses, we use FOL (which is, depending on the oxidation state, a substrate, a semi-product or a product of the enzymatic reaction) and MTX (an inhibitor) as reference compounds known to bind to all the targets considered. Based on the available crystallographic data and the determination of the crystal structure of the TcDHFR–TS complex with a reduced form of FOL we identify similar and dissimilar regions of the protein binding pockets and discuss the consequences of these observations for the design of antifolates. We, in particular, compare PTR1 and human/parasitic DHFR variants to aid the design of dual inhibitors against PTR1 and parasitic DHFR, particularly those of *T. cruzi*, whose PTR1 has not been the focus of previous inhibitor design studies. Our findings allow us to construct a guide for the design and derivatization of PTR1-targeting compounds considering multiple-target inhibition and selectivity against off-targets.

2. Materials and methods

2.1. Analyzed parasite species and protein sequences

Representative disease-causing trypanosomatid species were chosen for this study. Protein sequences were retrieved from the UniProt database [28], see Table S2 (Supplementary material). For *Leishmania* PTR1, we mostly focused on the structural data for *L. major*. When comparing PTR1 pockets, the TbPTR1 sequence numbering was used, since TbPTR1 was taken as the reference for defining the subpockets of the PTR1 binding site. In further comparisons of PTR1 and DHFR enzymes, unless otherwise noted, we mostly used the *T. cruzi* amino acid sequence numbering, since only for this species was a complete set of PTR1 and DHFR crystal structures with the reference ligands FOL (in part in reduced form) and MTX available. Sequence alignments were performed with the Clustal Omega tool [29] available at the UniProt webpage, and were visualized with the TeXshade LaTeX package [30].