Folates in *Trypanosoma brucei*: achievements and opportunities

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Abstract: *Trypanosoma brucei* (*T. brucei*) is the agent of Human African Trypanosomiasis (HAT), a neglected disease that threats the life of 65 million people in sub Saharan Africa every year. Unfortunately, available therapy is unsatisfactory, mainly due to safety issues and developing resistances. Significant efforts over the last decades have been made in the discovery of new potential agents from the World Health Organization and public–private partnership organizations such as the Drugs for Neglected Diseases Initiative (DNDi). Whereas antifolates have been a precious source of drugs against bacterial infections and malaria, no effective molecules towards *T. brucei* have been identified so far. Considering the simple *T. brucei* folate metabolism, and the results obtained up to now in this research field, we believe that further investigation might lead to effective chemotherapeutic agents. We present herein a selected collection of the more promising results obtained so far in this field, underlining the opportunities that could lead to successful therapeutic approaches in the future.

1. Introduction

Trypanosoma brucei (*T. brucei*) is a protozoa of the Trypanosomatidae family which causes the Human African Trypanosomias (HAT). The disease is spread in the sub Saharan Africa where it threats the life of 65 million people although only 2,804 new cases have been reported in 2015, and less than 20,000 were estimated.[1] The parasites enter in the human body through the bites of *Glossina spp* flies (tsetse flies). The first hemolymphatic phase of the disease is characterized by unspecific symptoms like local edema, intermittent fever, and headache. When the parasite penetrates the central nervous system (neuronal phase), more characteristic symptoms such as sleep-rest cycle alterations, mood disturbs, and lethargy, appear. The last stage of the infection leads to the death of the human host without an appropriate pharmacologic intervention. Two forms of the disease are described: the eastern HAT (r-HAT), caused by *T. brucei rhodesiense*, spread in eastern and southern Africa, and the western HAT (g-HAT), caused by the subspecies *T. brucei gambiense*, mainly occurring in western and central Africa. The first form rapidly evolves to the neurological phase and, because of this, is often referred to as the acute and lethal HAT (it indeed represents less than 2% of the total cases).[1a, 2] A third subspecies, *T. b. brucei*, is not normally infective to humans and is often used to perform *in vitro* experiments.

Since a vaccine is not available,[1b] the treatment of the disease is based on diagnosis (with better outcomes for earlier diagnosis) and treatment with the few old drugs approved for this application (fig. 1).[3] Among them, pentamidine and suramine are indicated for the first stage of g-HAT and r-HAT, respectively. To treat second stage HAT, drugs should be able to cross the blood-brain barrier (BBB). Currently, melarsoprol, an organ-arsenic compound, remains the first choice for the first-line treatment of second stage r-HAT, whereas the nifurtimox-eflornitine combination therapy (NECT) stands as the most promising first-line treatment for second-stage *T. b. gambiense* HAT. Compared with eflornithine monotherapy, NECT is preferred for a synergistic effect of the two drugs and for an easier administration regimen.[1b, 4] These agents are far from ideal, presenting many shortcomings, such as high cost, poor selectivity, toxicity, emergence of resistance, and they often require hospitalization for their administration.[5] Moreover, some of these drugs show serious toxicity issues, as happens with melarsoprol, which causes highly lethal (10-70% of the cases) reactive encephalopathy to the 5-18% of treated patients.[1b] Recently, fexinidazole (fig. 2) has successfully completed a phase II/III clinical trial, showing comparable efficacy and safety to the NECT during the treatment of g-HAT. If approved and registered, fexinidazole would represent the first new chemical entity for the disease since the early 1980s and could become the drug of choice for the treatment of the disease, also considering its favorable therapeutic scheme (single daily oral dose).[6]

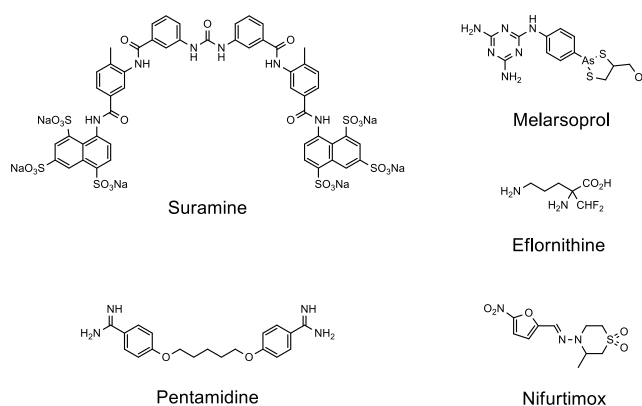
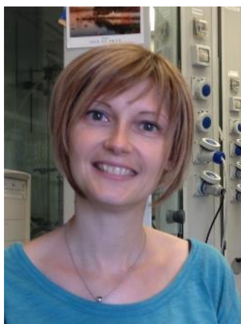


Figure 1. Drugs approved for the treatment of I stage (*i.e.*, suramin, pentamidine) and II stage (*i.e.*, melarsoprol, eflornithine, nifurtimox) of HAT.

Gregorio Cullia is currently a postdoctoral fellow at the IBMM in Montpellier (France). He obtained his PhD in Chemistry in 2017 from the University of Milan. In 2015, he joined the group of Prof. M. Zanda at the IMS, University of Aberdeen (Scotland). His main interest is the design of new potential antimicrobials to cover unmet medical needs exploiting new mechanisms of action.



Lucia Tamborini graduated in 2004 in Medicinal Chemistry at the University of Milan, and in 2007 received her PhD in Medicinal Chemistry from the same University. During the PhD course, she developed part of the research project at the Innovative Technology Centre (Cambridge University, UK), under the supervision of Prof Steven V. Ley and Dr Ian R. Baxendale. She is currently Assistant Professor at the Department of Pharmaceutical Sciences of the University of Milan. Her research activities are mainly focused on the development of innovative synthetic methodologies for sustainable multistep preparation of biologically active compounds.

Paola Conti is an Associate Professor of Medicinal Chemistry at the University of Milan (Italy). After obtaining her PhD in Medicinal Chemistry at the University of Milan in 1999, she joined the group of Alan P. Kozikowski at Georgetown University Medical Center (Washington D.C., U.S.A) as a post-doctoral fellow. Her research activity is focused on the design and synthesis of biologically active molecules, with particular regard to covalent or non-covalent enzymatic inhibitors as chemotherapeutic agents for the treatment of infectious and cancer diseases.

Carlo De Micheli graduated in Chemistry and Pharmacy at the University of Pavia. He has been Full Professor of Medicinal Chemistry at the University of Milan for more than 20 years. His research goal is the study of the structure-activity relationship of ligands acting at muscarinic, adrenergic and glutamatergic receptors. Recently, his interest was devoted to inhibitors of enzymes essential to the survival and replication of parasites that cause a variety of tropical diseases. Carlo De Micheli directed for five years the "European School of Medicinal Chemistry", the only accredited school of the European Federation for Medicinal Chemistry (EFMC). Actually, he serves the journal ChemMedChem as a member of the International Advisory Board.

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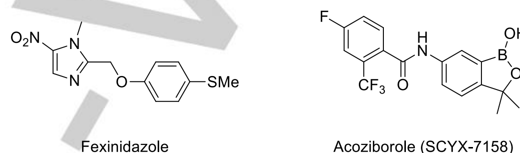


Figure 2. Agents under clinical evaluation for HAT. Fexinidazole successfully completed phase II/III studies, whereas acoziborole is still under phase II/III clinical trial.

Another molecule, acoziborole (or SCYX-7158, fig. 2), is currently under phase II/III clinical trial for the treatment of second stage g-HAT.[7] Although current cases are relatively few, attention to the disease should be keep high, since resurgence of the disease has been already occurred in the past.[2a] For this reason, it is important to continue the research for novel chemotherapeutic agents. Folate pathway has been a precious source of pharmaceutical targets for the treatment of cancer and microbial infections (examples of antifolate drugs are reported in fig. 3).[8] Despite this, no effective antifolate compounds have been described for the treatment of HAT, and this route has just been initially explored in *T. brucei* from a pharmacological point of view. We present herein a selected collection of the more promising results obtained so far in this research field, underlining the opportunities that, in our opinion, could lead to successful therapeutic approaches in the future.

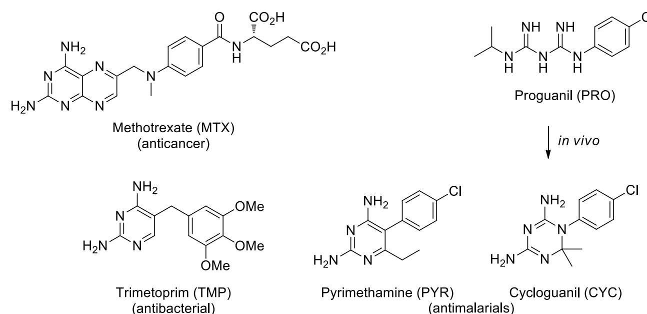


Figure 3. Antifolate drugs currently used in therapy.

1.1. The *Trypanosoma brucei* folate pathway

Folate metabolism in human has been exhaustively described.[9] It consists of several, often redundant, interconnected paths that lead to different modifications of folate and its derivatives. Tetrahydrofolate (THF), reduced form of folate, represents its functional state. All the other modifications have, in ultimate, the same scope: link a monocarbon unit to the nitrogen 5 and/or 10 of THF. These “charged” folates serve as donors of C_1 units in the synthesis of purines, pyrimidines and amino acids.

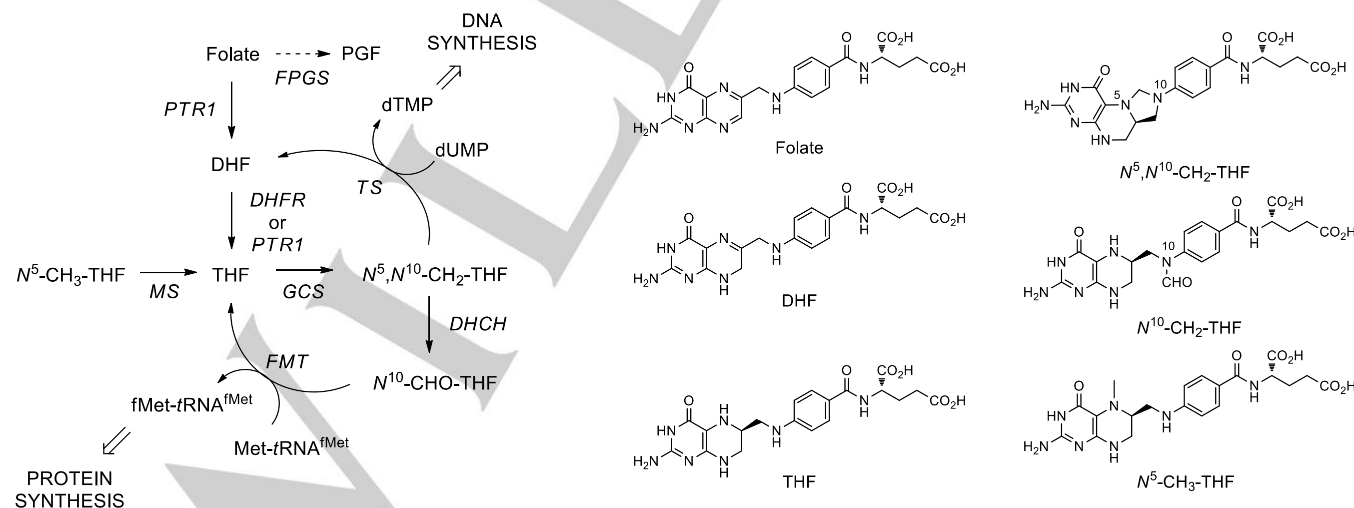
The biochemical routes that stand behind the folates reduction and functionalization in *T. brucei* (scheme 1) are far simpler compared to the human ones. *T. brucei* is folate auxotroph, so it has to salvage it from the environment. Putative folate transporters have been recently identified, together with a putative folylpolyglutamyl synthetase (FPGS).[10] The parasite, differently from humans, expresses a fused dihydrofolate reductase-thymidylate synthase (DHFR-TS) and an enzyme, the pteridine reductase-1 (PTR1, absent in humans), that are able to reduce folates. Basing on the up-to-date knowledge, THF can be converted in only two active forms, N^5,N^{10}-methylenetetrahydrofolate (N^5,N^{10}-CH_2-THF) and N^{10}-formyltetrahydrofolate (N^{10}-CHO-THF), cofactors for the synthesis of deoxythimidylate monophosphate (dTMP) and of formylmethionyl-*t*RNA^{Met} (fMet-*t*RNA^{Met}), respectively. *T. brucei* can also uptake N^5-methyltetrahydrofolate (N^5-CH_3-THF, the major circulating form of THF in the human body) that can be used as a cofactor for the synthesis of methionine (Met) from homocysteine (hCys), catalyzed by methionine synthase (MS).[11] Considering the simplicity of the pathway and the historical impact of antifolates as chemotherapeutics, a deep characterization of the enzymes involved in the folate pathway could be useful to accelerate the drug discovery process towards novel, more selective and affordable inhibitors. In the following paragraphs, we collected some significant results presented in literature targeting the *T. brucei* folate metabolism.

2. Enzyme involved in reductive metabolism

A fused DHFR-TS enzyme (EC 1.5.1.3-2.1.1.45) is the mayor responsible for the reduction of DHF to the active reduced form THF with the concomitant consumption of NADPH. Currently available DHFR inhibitors did not show a notable antiproliferative activity. This result could be mainly attributed to the presence in *T. brucei* of PTR1, which offers an alternative metabolic route for the generation of THF, in addition to its ability to reduce folate to DHF.

2.1. Dihydrofolate reductase

Sienkiewicz et al. validated *Tb*DHFR as a target on its own.[12] Its activity turned out to be essential for viability and virulence of the parasite, but considerable effects were only observed when the inhibition was complete (as in double knock-out cell lines). The authors also underlined that PTR1 is unable to compensate DHFR deficiency. The crystal structure of the DHFR domain has been solved by Vanichtananankul et al. (PDB: 3RG9): it folds in different secondary structures organized in a C- and N-terminal domains separated by a cleft in which are located the binding sites for substrate and NADPH.[13] The enzyme is quite similar to the isoforms of other parasites (58% and 46% of sequence identity with *Trypanosoma cruzi* and *Leishmania major*, respectively), but it presents important differences with the human one (only 26% of sequence identity).[14]



Scheme 1. Folate pathway in *T. brucei* (left) and structures of folates (right). Folate is collected from the environment. A putative FGPS[10] converts it in polyglutamyl folate (PGF), the cellular pool of folate. DHFR and PTR1 participate to the reduction of folate to THF (only PTR1 can reduce folate to DHF). This last is converted by the glycine cleavage system (GCS) in N^5,N^{10}-CH_2-THF, the cofactor for the synthesis of dTMP. DHCH (N^5,N^{10}-CH_2-THF dehydrogenase/cyclohydrolase) converts N^5,N^{10}-CH_2-THF into N^{10}-CHO-THF, used as cofactor for the synthesis of fMet-*t*RNA^{Met}, catalyzed by the formyl methionyl transferase (FMT). N^5-CH_3-THF is acquired from the human host and converted in THF thanks to the action of the MS, which converts hCys into Met.

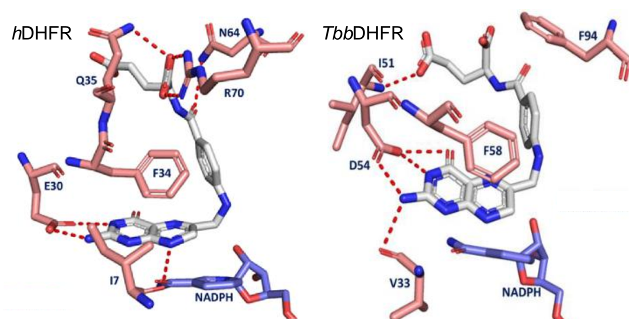


Figure 4. Binding modes of DHF (white) in *h*DHFR and *Tbb*DHFR (rose). NAPDH is represented in light blue. Reproduced from Sharma et al.[15]

Some relevant variations are located in the folate binding site, and, in particular, are: a) a small tunnel is present under the pteridine ring in *Tb*DHFR; b) residues Gly20-Asp21 of *h*DHFR, overall negatively charged, occupy the channel between the substrate and the NADPH binding pocket, while in *T. brucei* the neutral residues Gly45-Thr46 are found in the same positions; c) while in *h*DHFR Phe31 points towards the benzamide moiety of the substrate, in *Tb*DHR we find Met55, resulting in a larger binding pocket in the parasitic enzyme; d) the positions occupied by Gln35 and Asn69 in the human enzyme, are occupied by Arg39 and Phe94, respectively, in *Tb*DHFR. The two binding modes are shown in figure 4. Phe94 is also oriented towards Met55, preventing the interaction between the glutamate tail of the ligand and Arg95.[14-15] The presence of Thr86 in the active site makes *Tb*DHFR less sensitive towards rigid classical antifolates, as PYR (K_i 24 nM) and CYC (K_i 256 nM), rather than more flexible compounds, as WR99210 (K_i 1.1 nM, fig. 5).

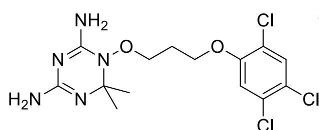


Figure 5. Structure of WR99210.

Diaminopyrimidines and diaminoquinazolines

The first groups of inhibitors have been developed starting from a known 5-benzyl-2,4-diaminopyrimidine inhibitor (**1**) of *Lm*DHFR, which presented interesting inhibitory activity and remarkable selectivity.[16] Starting from the model compound **1**, Chowdhury et al. developed a series of analogues in which they introduced modification at 3', 4' and 6 positions.[17] The diaminopyrimidine ring of these compounds binds into the active site establishing a salt bridge with Asp54, while the group in the 3' position is important for the selectivity, interacting with Phe94 of *Tb*DHFR, while in the human isoform the same position is occupied by Asn69. The peak of activity and selectivity (table 1) has been obtained with derivatives characterized by a linear alkoxy chain in position 3' (**2**, **3** and **4**), with a chain length up to 6 carbon units, and alkoxy chain in 4' (**6**). Compounds with branched alkoxy chains in 3' also possess interesting activity (**5**), while longer chains decrease the selectivity of the inhibitors.

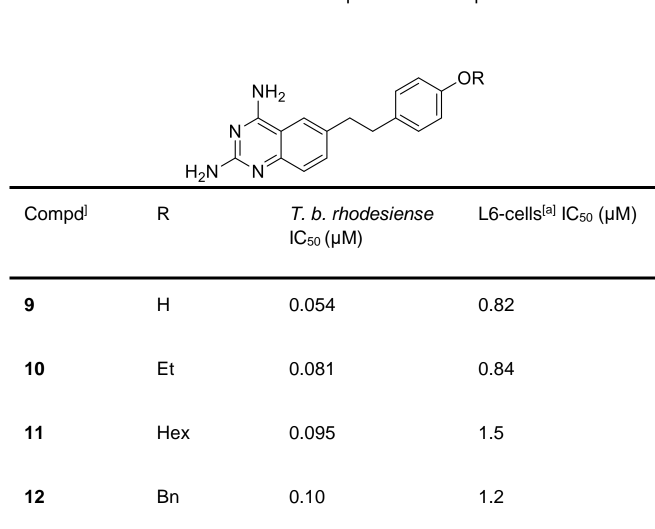
Table 1. Enzyme inhibition and *in vitro* activities for the diaminopyrimidine compounds.

Compd	R^1	R^2	*Tb*DHFR K_i (nM)[a]	*T. brucei rhodesiense* IC_{50} (μM)	L6-cells[b] MIC or IC_{50} (μM)
1	OOct	H	24 (100)	2	<34 (MIC)
2	OEt	H	8.6 (184)	21	>410 (MIC)
3	OBu	H	3.6 (257)	5	121 (MIC)
4	OPent	H	7.1 (154)	4	115 (MIC)
5	O*i*Pr	H	8.8 (156)	14	>388 (MIC)
6	H	OPr	6.4 (442)	10	388 (MIC)
7	OH	OOct	n.d.	0.73	14.0 (IC_{50})
8	OHex	OHex	n.d.	0.77	5.5 (IC_{50})
TMP	/	/	10 (134)	148	n.d.
PYR	/	/	11 (11)	7	n.d.

[a] When determined, the selectivity index (*h*DHFR K_i/*Tb*DHFR K_i) is reported in brackets. [b] Rat skeletal myoblast cell line. N.d.: not determined.

Some compounds also showed *in vitro* activity on *T. b. rhodesiense* cultures, generally in the micromolar range, with a selective toxicity towards the parasite. However, especially for the 3'-substituted analogues, there is not a clear correlation between the range of enzyme inhibition and *in vitro* growth inhibition. Since the most active compounds possess longer alkyl chain, this effect might be due to an improved rate of cell penetration. Some compounds, such as **1**, also prolonged the life span of mice infected by *T. brucei*. Additional 4'-alkyloxy- and 3',4'-dialkyloxy-substituted analogues were described by the same group.[18] The inhibitory activity of these compounds was determined against *Lm*DHFR and *Tc*DHFR. *In vitro* activities were generally comparable with the ones of the first series of diaminopyrimidines but these compounds showed a modest selectivity. Actually, it is difficult to extrapolate a clear trend in activity for this set of compounds due to the low range in the *in vitro* activity, which, in addition, is likely affected by their different physicochemical properties. The 2,4-diaminoquinazoline scaffold appeared to be promising for the design of parasitic DHFR inhibitors.[19] Khabnadideh et al. linked, with a two-carbon atom chain, this heterocycle to a substituted phenyl ring, essential to generate selectivity.[20] These compounds (table 2) showed good inhibitory activity towards *Lm*DHFR and interesting trypanocidal activity (from 0.67 to 0.054 μM), but only modest selectivity between parasitic and mammalian cells (4-16 folds).

Table 2. *In vitro* activities of the diaminoquinazoline compounds.



Compd	R	*T. b. rhodesiense* IC_{50} (μM)	L6-cells[a] IC_{50} (μM)
9	H	0.054	0.82
10	Et	0.081	0.84
11	Hex	0.095	1.5
12	Bn	0.10	1.2

[a] Rat skeletal myoblast cell line.

The alkylation of the phenolic OH always showed a detrimental effect on the IC_{50} profile, but to a lesser extent in the case of ethyl, hexyl and benzyl substituents. Compounds with a flexible linker (ethyl-) are generally more potent than the ones with a rigid linker (ethenyl-/ethynyl-), in accordance with the findings of Vanichtananankul et al.[13]

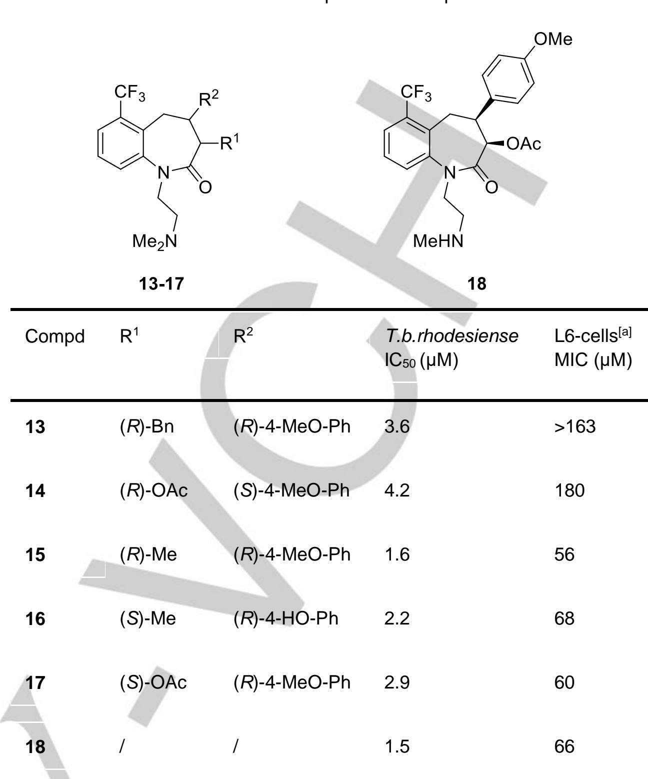
Benzoazepinones and benzodiazepines

Zuccotto and co-workers[21] identified *Tc*DHFR inhibitors characterized by a benzoazepin-2-one (**13**) or a benzo-1,4-diazepine structure (**19**). A series of derivatives with these two scaffolds were synthesized and assayed for their growth inhibitory activity on *T. brucei* (table 3 and 4) showing good potencies and a selectivity in the range from modest to good.[21]

2.2. Pteridine reductase 1 (PTR1)

PTR1 (EC 1.5.1.33) is a member of the short-chain dehydrogenase/reductase family (SDR) able to catalyze the reduction of both folate, biopterine, and their dihydro forms, *i.e.*, DHF and dihydrobiopterine (DHB), with consumption of NADPH. *Tb*PTR1 has been genetically validated as a target: the lack of the enzyme has cytocidal effects together with phenotypic defects, and reduced *in vivo* virulence. The reduction of the tetrahydrobiopterine (THB) pool seems to be the explanation of these results but the still lacking knowledge of pterines functions in *T. brucei* prevents any clear conclusion.[22] Each monomer of the active asymmetric tetramer presents two α-/β-domains in which seven β-sheets are between two sets of α-helices. The active site is an L-shaped depression mainly formed by a single subunit, but one of its end is created by the C-terminus of a partner subunit. The cofactor and Phe97 contribute to the formation of the catalytic center. The pterine ring of substrates/products is indeed sandwiched between NADPH nicotinamide and Phe97. Other key interactions are established between the polar groups of the ligand, NADPH, and active site residues (fig. 6b). *Tb*PTR1 possesses a closer binding site, in comparison to *Lm*PTR1, caused by a less flexible β-6/α-6 loop.

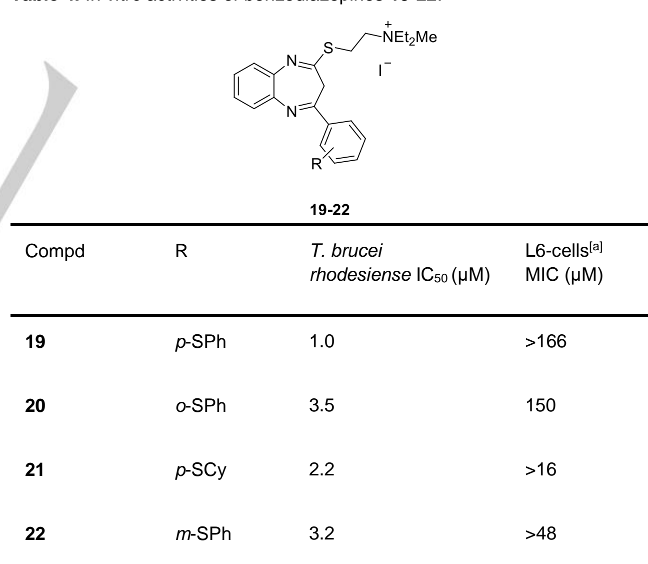
Table 3. *In vitro* activities of benzoazepin-2-one compounds.



Compd	R^1	R^2	*T.b.rhodesiense* IC_{50} (μM)	L6-cells[a] MIC (μM)
13	(*R*)-Bn	(*R*)-4-MeO-Ph	3.6	>163
14	(*R*)-OAc	(*S*)-4-MeO-Ph	4.2	180
15	(*R*)-Me	(*R*)-4-MeO-Ph	1.6	56
16	(*S*)-Me	(*R*)-4-HO-Ph	2.2	68
17	(*S*)-OAc	(*R*)-4-MeO-Ph	2.9	60
18	/	/	1.5	66

[a] Rat skeletal myoblast cell line.

Table 4. *In vitro* activities of benzodiazepines **19**-**22**.



Compd	R	*T. brucei rhodesiense* IC_{50} (μM)	L6-cells[a] MIC (μM)
19	*p*-SPh	1.0	>166
20	*o*-SPh	3.5	150
21	*p*-SCy	2.2	>16
22	*m*-SPh	3.2	>48

[a] Rat skeletal myoblast cell line.

In the case of folates, the *p*ABA and the glutamate residue are directed out of the active site. Vice versa, MTX binds PTR1 with the pterine ring rotated by 180° compared to the orientation of folate (fig. 6a): in this way, the N8 interacts with Arg14 and with the pyrophosphate through a water bridge while the 4-NH_2 interacts through an hydrogen bond with Tyr 174.[23]

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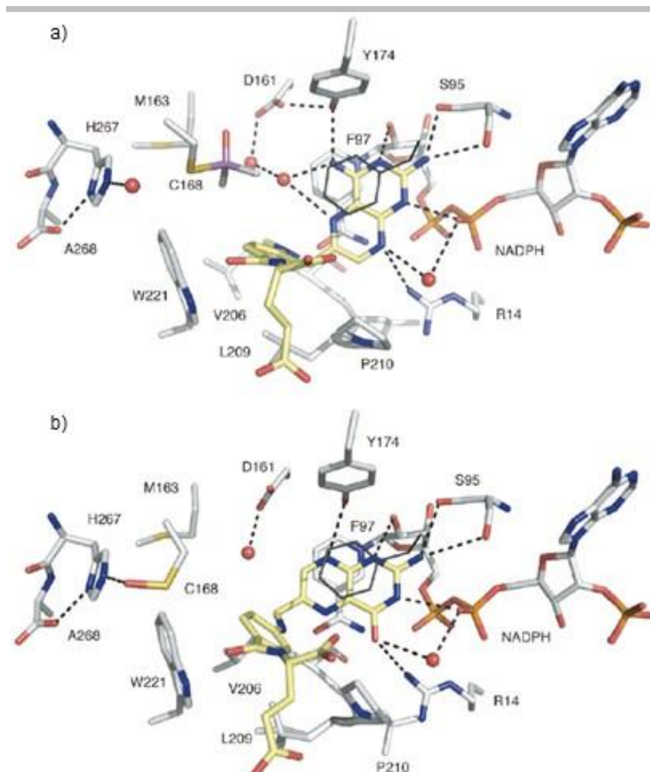


Figure 6. *Tb*PTR1 in complex with MTX (a) and folate (b) and NADPH cofactor. Reproduced from Tulloch et al.[23]

Pyrimidines and related heterocycles

Three different scaffolds (A-C, fig. 7) were assessed for their ability to inhibit the enzyme.[23] 2,6-Diaminopyrimidines (scaffold A) showed only a weak inhibition for *Tb*PTR1 when group R is an amino or an *N*-cyclopropylamino group (K_i > 35 μM). The activity is increased when R is an alkylthio group, with the most potent compound (**24**) showing a K_i of 3.2 μM. The introduction of a *p*-methoxy group on the benzyl ring of compound **24** reduced the activity for both the enzymes by a factor of ten. Pteridines (scaffold B) showed a good inhibitory activity for the enzyme, but their activity is higher against *Lm*PTR1 (aggiungi I dati qui). On the other hand, pyrrolopyrimidines (scaffold C) showed a preference for *Tb*PTR1 and a promising activity, with K_i in the micromolar or submicromolar range.[23,24] A series of analogues, characterized by the C core, were synthesized in an extensive study that led to a complete structure-activity relationships study (Table 5).[24] The substitution of the 4-O with an amino group was found favorable in some cases, but the most important remark is the need of a bulky hydrophobic substituent on the pyrrole ring, with the most interesting compounds bearing two phenyl rings in the 5 and 6 positions. Improvements in activity were obtained with the introduction of an halogen atom in the *para* or *meta* position of the aryl groups appended in position 5 or 6, while other modifications, such as the alkylation of the heteroatom at 4 position, the introduction of branched alkyl groups or a sulfone group on the aromatic rings, caused a loss of activity or worsen the solubility properties of the compounds, especially for the 4-oxo series. Crystal structures of the complexes inhibitor-NADPH-*Tb*PTR1 were generated, showing that most of the compounds bind the enzyme with a substrate-like pose.

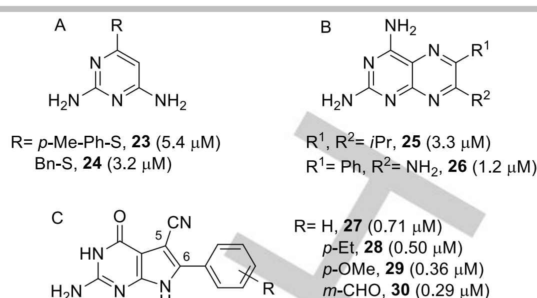


Figure 7. Selected examples of *Tb*PTR1 inhibitors characterized by scaffolds A-C. The relative K_i are reported in brackets.

Notably, it was also observed that compounds bearing a formyl group on the phenyl ring at position 6 establish a thioester linkage with Cys168 (probably arising from an initial thioacetal intermediate), suggesting the opportunity for the design of covalent inhibitors.[25] Six compounds (**31**-**36**, table 5) with improved pharmaceutical properties were selected for *in vivo* evaluation in mice. At the selected dose of 30 mg/kg, compound **32** did not show curative effects while compound **31** induced chronic toxicity. The other compounds were tolerated up to 4 days when administered once daily and reduction of parasitaemia from 10^8 to below detection limits was demonstrated. Mice survived after treatment with compounds **34** and **35**, but unfortunately, showed a relapse of parasitaemia on day 10.[24]

Aminobenzimidazoles

5-Chloro-aminobenzimidazole **37** emerged as an interesting inhibitor of *Tb*PTR1 (K_i^{app} 10.6 μM) from a research campaign focused on compounds characterized by a non-classical scaffold (not related to folate and known antifolates) and by favorable pharmaceutical properties.[26] The substitution of the N1 of aminobenzimidazole with a benzyl group (compound **38**) was not detrimental (K_i^{app} 16 μM). In contrast, when the benzyl group was linked to the 2-amino group a drastic drop in activity was observed. Optimization of the substituents on the phenyl ring led to compound **39** (K_i^{app} 0.4 μM), which was further modified at the 4 or 7 positions: position 4 tolerates only small substituents, while position 7 can be decorated with bulkier groups, such as a phenyl ring as in compound **40**, the most potent inhibitor of *Tb*PTR1 described so far (K_i^{app} 0.007 μM).[27] The structures of benzimidazole inhibitors are reported in figure 8.

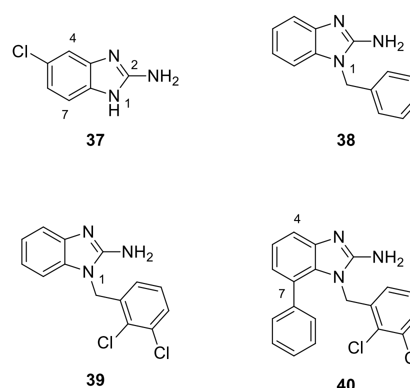
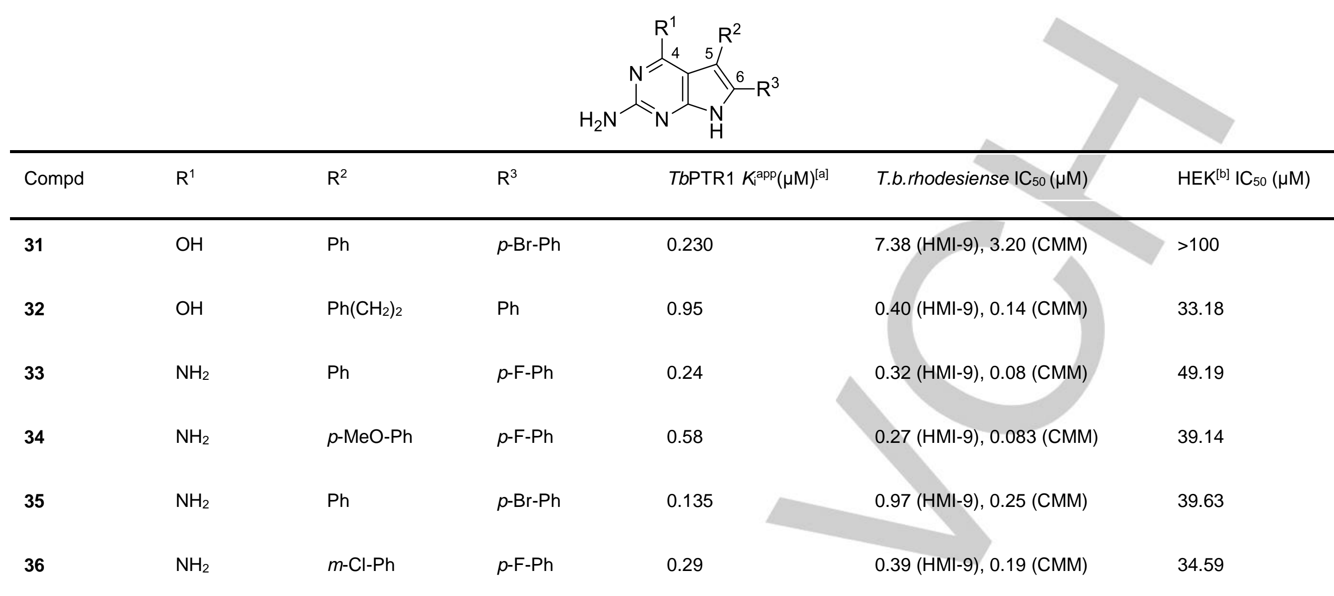


Figure 8. Structures of *Tb*PTR1 aminobenzimidazole inhibitors.

Table 5. Biological data for pyrrolopyrimidines **31**-**36**.



Compd	R[1]	R[2]	R[3]	*Tb*PTR1 K_i^{app}(μM)[a]	*T.b.rhodesiense* IC_{50} (μM)	HEK[b] IC_{50} (μM)
31	OH	Ph	*p*-Br-Ph	0.230	7.38 (HMI-9), 3.20 (CMM)	>100
32	OH	$Ph(CH_2)_2$	Ph	0.95	0.40 (HMI-9), 0.14 (CMM)	33.18
33	NH_2	Ph	*p*-F-Ph	0.24	0.32 (HMI-9), 0.08 (CMM)	49.19
34	NH_2	*p*-MeO-Ph	*p*-F-Ph	0.58	0.27 (HMI-9), 0.083 (CMM)	39.14
35	NH_2	Ph	*p*-Br-Ph	0.135	0.97 (HMI-9), 0.25 (CMM)	39.63
36	NH_2	*m*-Cl-Ph	*p*-F-Ph	0.29	0.39 (HMI-9), 0.19 (CMM)	34.59

[a] The K_i^{app} is the apparent K_i before correction for the inhibition modality-specific influence of substrate concentration relative to K_m. K_i can be derived from the equation $K_i^{app} = K_i / (1 + S \cdot K_m^{-1})$, where S and K_m refers to the pterine substrate. [b] HEK: human embryonic kidney cells. HMI-9: Hirumi's Modified Iscove's medium 9. CMM: Creek's Minimal Medium.

Remarkably, X-ray crystallography analysis revealed that the binding mode of these inhibitors completely differs from the one of folates and MTX. Although compound **37** is locked into the catalytic pocket, **38** and **40** are located in a perpendicular area, away from the nicotinamide of the cofactor (fig. 9). Amino acidic residues that participate to create this pocket are Phe97, Asp161, Met163, Cys168, Phe171, Tyr174 and Gly205. The protonated N3 and the 2-NH_2 group of the ligand form a bidentate interaction with the carboxylate moiety of Asp161; the amino group also interacts with the backbone carbonyl of Gly205. A second pocket, formed by Val206, Trp221, Leu263, Cys168 and Met163, closed by His267 and Asp268 of a neighboring subunit, accommodates the dichlorophenyl ring of **40**. Unfortunately, the high inhibitory activity of derivative **40** did not translate in an improved inhibitory activity toward *T. brucei* (EC_{50} 9.9 μM) and some of these compounds, unfortunately, showed toxicity toward human cell lines. The authors pointed the attention to the low K_m/K_i ratio for **40** (only 10) to explain the observed low *in vitro* antiproliferative activity. The inhibitory activity of this compound should be then increased at least of two orders of magnitude to produce an effective drug candidate.

3. Enzymes involved in THF functionalization

3.1. *N*[5],*N*[10]-Methylene-THF

N^5,N^{10}-Methylenetetrahydrofolate is one of the more important active form of THF. It functions as a donor of a C_1 unit in the synthesis of thymidylate (catalyzed by TS). In trypanosomatids, the monocarbon unit can be, generally, donated to THF by serine or by glycine: the first reaction is catalysed by the PLP-dependent Ser hydroxymethyltransferase (SHMT) with release of Gly, while in the other case the glycine cleavage system (GCS), a tetrameric complex, degrades the amino acid to ammonia, CO_2 and donate the C_1 unit to THF.[28] *T. brucei* completely lacks of any SHMT-encoding gene, thus the GCS appears to be the only responsible for the synthesis of N^5,N^{10}-CH_2-THF.[29] GCS is a multimeric complex formed by three enzymes, proteins P, T and L, and a carrier protein, protein H, to which lipoic acid is covalently bound. Protein P, also known as glycine dehydrogenase (EC 1.4.4.2), decarboxylates glycine and transfers the aminomethyl radical to lipoic acid (bound to protein H). Protein T (or aminomethyl transferase, EC 2.1.2.10) transfers the C_1 unit from lipoic acid to tetrahydrofolate converting it to N^5,N^{10}-CH_2-THF. The reduced lipoic acid is oxidized from protein L (or dihydrolipoamide dehydrogenase, EC 1.8.1.4) with reduction of NAD^+ and a new cycle can begin.[30]

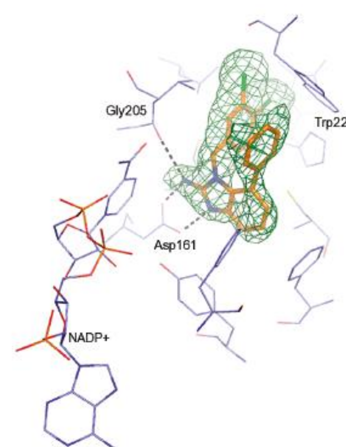
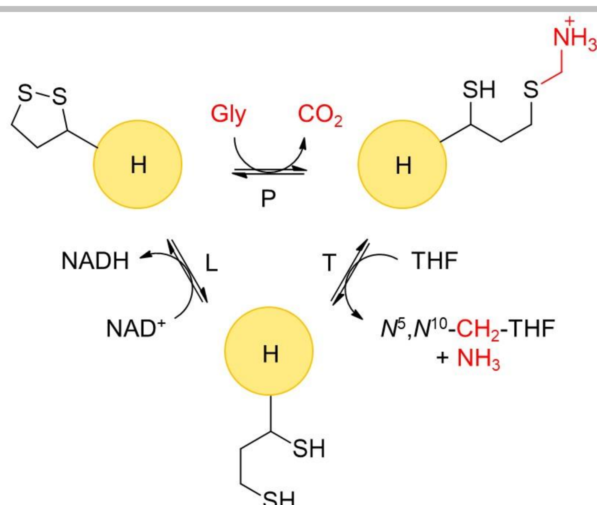


Figure 9. Crystallographic binding mode of compound **40**. Enzyme is depicted in purple, the inhibitor in orange. Reproduced from Mpamhanga et al.[26]



Scheme 2. Mechanism of GCS.

The mechanism is resumed in scheme 2. The structures of some of these proteins have been described for some bacteria, such as *E. coli*[31] and *T. thermophiles*,[32] and, in particular, the structure of *T. cruzi* dihydrolipoamide dehydrogenase has been reported in the protein data bank (PDB 2QAE). The role of the same component of the GCS has been studied in *T. brucei*. Both double null mutation and silencing of protein L expression by RNAi resulted in alterations in the morphology and in the cell cycle distribution of the parasites. Moreover, double null mutants were not able to cause infection in mice. For the bloodstream form of *T. brucei* (the form present in the human host), these effects have been related with GCS activity defects.[33] Despite the evidence of the importance of GCS for the parasite, only few information are available, especially from a structural point of view. Given the central position of GCS in *T. brucei* folate metabolism, advances in this area might prompt the development of new effective agents against the parasite.

3.2. *N*10-formyl-THF

In N^{10}-CHO-THF, the C_1 unit is present as a formyl group. In trypanosomatids, which are auxotrophs for purines, this cofactor participates only in the formylation of the methionyl-*t*RNA^{Met} to fMet-*t*RNA^{Met} (the initiator aminoacyl-*t*RNA in protein synthesis).

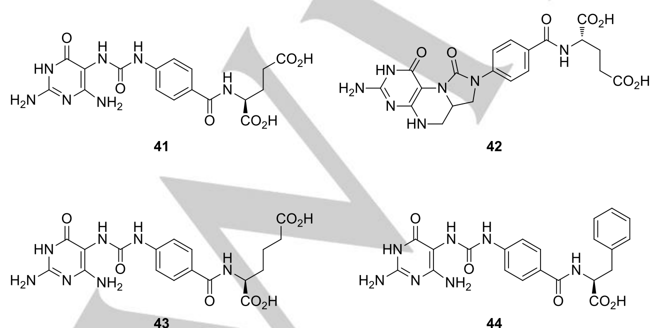


Figure 10. Selected inhibitors of *Tb*DHCH.

While in humans and in other parasites the synthesis of N^{10}-CHO-THF is catalyzed by different enzymes starting from different substrates,[34] in *T. brucei* only DHCH (or FolD), a bifunctional enzyme, catalyzes the biosynthesis of the cofactor.[34a]DHCH is a bifunctional enzyme that converts N^5,N^{10}-CH_2-THF into N^{10}-CHO-THF in two steps through the formation of N^5,N^{10}-methenyl-THF (N^5,N^{10}-CH^+-THF). In the first step (dehydrogenase, DH, EC 1.5.1.5) a $NADP^+$-dependent oxidation takes place, then N^5,N^{10}-CH^+-THF is hydrolyzed by an activated water molecule thanks to the cyclohydrolase activity (CH, EC 3.5.4.9). The enzyme has been described as essential for *Pseudomonas aeruginosa*,[35] *L. major*,[34b] and we have recently assessed the *T. brucei* ortholog as potential antiparasitic target.[36] We have described a series of compounds derived from compound **41** (K_i 1.1 μM) in which we studied the effect of the substitution of the amino acidic tail (fig. 10). Compound **41** (EC_{50} 49 μM) showed better *in vitro* selectivity than compound **42** (EC_{50} 53 μM), a known inhibitor of DHCH,[37] and allowed us to solve the structure of the ternary crystal **41**-$NADP^+$-*Tb*DHCH (fig. 11a). Most of the analogues showed micromolar K_i values or, in the case of **43** (K_i 0.48 μM) and **44** (K_i 0.54 μM), even lower, possibly thanks to the enhanced interaction of the amino acid tails of these compounds with the enzyme (as predicted by binding studies, fig. 11b and 11c). The same compounds were assayed for their trypanocidal activity but, with the exception of compound **41**, they did not show antiparasitic activity, probably due to their unfavorable pharmacokinetic properties, such as poor solubility and high hydrophilicity.

4. Other potential enzymatic targets

Other underexplored possible approaches in the design of new antifolates exist. Although we do not know yet their importance for the parasite, FPGS, TS, FMT, and MS could also be interesting targets. Surely, data available nowadays are too scarce to invite to a drug discovery campaign, but these enzymes, once they will be isolated and characterized, could be included in multitarget assays.

4.1. Thymidylate synthase (TS)

Gibson et al.[11] studied the effect of nolatrexed (NTX), pemetrexed (PMX), and raltitrexed (RTX), three known inhibitors of *h*TS, towards *Tb*TS and *Tb*DHFR (table 6). NTX is the most potent *Tb*TS inhibitor (K_i 39.4 nM), while the other two are more active towards *Tb*DHFR. However, the *in vitro* activities of RTX and PMX are higher, probably due to the activity of FPGS (polyglutamyl derivatives are known to be more potent inhibitors of the enzyme).[11] The high efficacy of these compounds (although more active towards *h*TS) encourages the discovery of *Tb*TS inhibitors. Nonetheless, the high similarity between the human and *T. brucei* ortholog (60%) and the identical amino acid composition of their active sites anticipate the difficulty in reaching selectivity.

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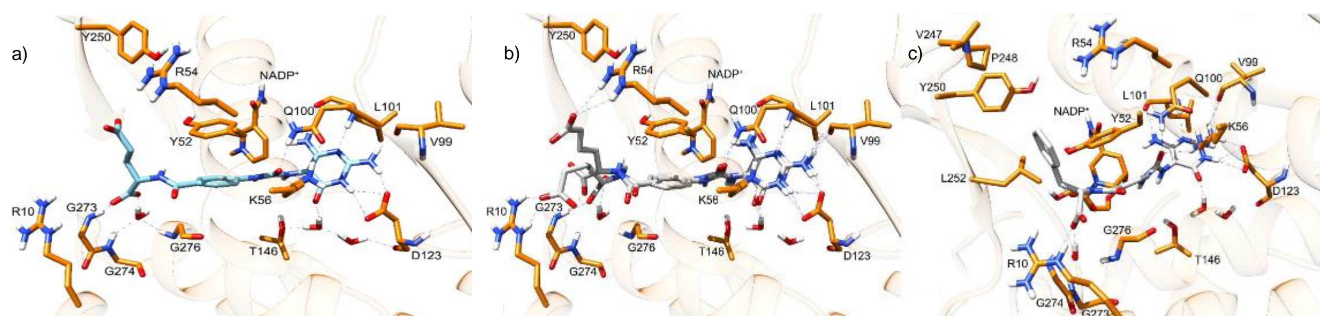
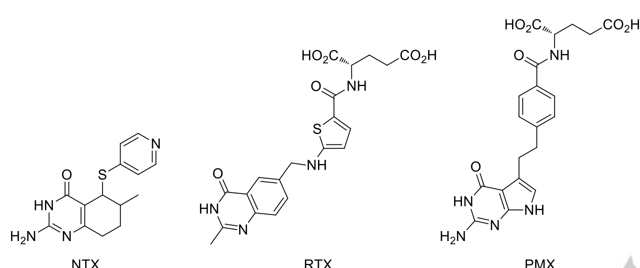


Figure 11. Co-crystal structure of *Tb*DHCH-**41**-NADP+ (a) and predicted binding mode of **43** (b) and **44** (c). The enzyme polypeptide is depicted as off-white ribbon, the interacting residues and the NADP+ nicotinamide ring are depicted as orange sticks, the inhibitors as cyan sticks.

Table 6. Activity of *h*TS inhibitors towards *T. brucei*.



Compd	*Tb*TS K_i (nM)	*Tb*DHFR K_i (nM)	*T. brucei* EC_{50} (µM)
NTX	39.4	348	33.8
RTX	215	93.1	0.038
PMX	20500	290	0.020

4.2. Methionine synthase (MS)

Methionine synthase uses N^5-CH_3-THF, the major form of folates in human plasma, to convert hCys to Met, regenerating THF. This enzyme represents an important connection between folate and methionine metabolism. This last is linked to polyamine metabolism, a highly important target for the treatment of HAT.[38] It is tempting to assume that inhibitors of MS could perturb several biosynthetic pathways at the same time.

5. Transporters

Recently, three functional folate transporters (*Tb*FT1-3) have been identified in *T. brucei*. These allow folate and analogues to cross cell membrane. The *p*ABA-glutamyl moiety (present in folate and MTX) is essential for binding *Tb*FT1-3. In the same study, evidences suggested the involvement of the mitochondrial carrier protein 2 (MCP2) in the import of folates to the mitochondrion, highlighting that one or more steps of the pathway happen in this organelle.[10] Considering the impact that these transporters might have on the availability and activity profile of the target molecules, it appears of primary importance to study possible interactions with folate transporters already in the early phases of drug discovery programs. Although not listed among essential transporters for the parasite,[39] they can indeed modify the distribution of the drugs in the cell and in its organelles, allowing or not the inhibitors to reach their targets. Moreover, as for other drugs,[5a, 5d] transporters might be involved in resistance mechanisms, a possibility that should be taken into account for the development of new successful therapies

6. Conclusions and perspectives

In the last years, researches have been focused on the inhibition of the reductive metabolism of folate presenting a massive quantity of data. Several remarkable results have been obtained, allowing a detailed knowledge of *Tb*DHFR and *Tb*PTR1 as targets. Much more has to be done in the study of the modification of THF into its two “charged” forms, N^5,N^{10}-CH_2-THF and N^{10}-CHO-THF. Although sometimes highly potent enzyme inhibitors have been obtained, the *in vitro* and *in vivo* activities of the compounds have never been good enough to be considered a valuable clinical candidate. Unfortunately, a discrepancy between the activities in biochemical assays and *in vitro* cell assays is often observed during the drug discovery of antiparasitic drugs. As mentioned above, it would be also of great interest to describe the transporters that could help the inhibitors to reach the intracellular environment and the early evaluation of the capabilities of the active molecules to cross the cell membrane through these systems would greatly aid in the pursuit of effective drugs. Another important aspect to take into account is that, in the literature, there are many sound research works performed following a phenotypic approach. This is a powerful direct approach, but sometimes it could be interesting to include a qualitative target deconvolution study, in particular when recurrent structures of classical antifolate drugs (*e.g.*, pyrimidines) are present in the set of molecule under investigation. It is not indeed excluded that inhibitors might inhibit different enzymes involved in folate metabolism.

With this minireview, we hope to have produced a useful resume of the data collected up to now and to recall the attention to the promising and fascinating field of antifolate drugs.

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Keywords: folates, *Trypanosoma brucei*, Human African Trypanosomiasis, neglected diseases, structure-activity relationships

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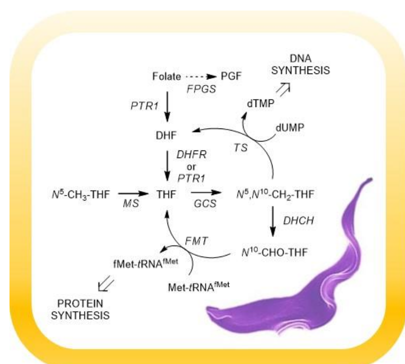

Targeting the folate pathway has been a valuable strategy for the treatment of different diseases and, in particular, antimicrobial infections. Despite the simple metabolism of folates in *Trypanosoma brucei*, no effective antifolate to treat its infections has been described so far. In this minireview, we analyze the folate pathway of this parasite, showing a selection of the most important results obtained to date in this research field.

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