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## Development of Chemical Proteomics for the Folateome and Analysis of the Kinetoplastid Folateome

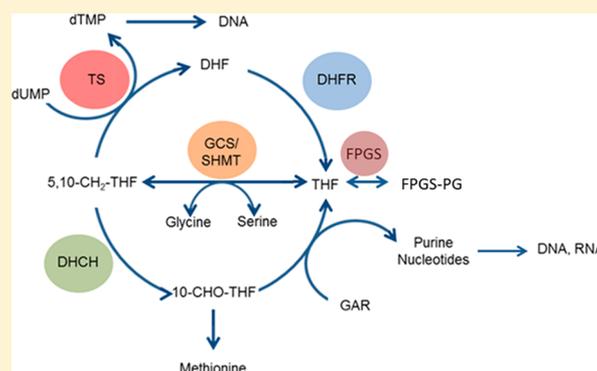
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### Supporting Information

**ABSTRACT:** The folate pathway has been extensively studied in a number of organisms, with its essentiality exploited by a number of drugs. However, there has been little success in developing drugs that target folate metabolism in the kinetoplastids. Despite compounds being identified which show significant inhibition of the parasite enzymes, this activity does not translate well into cellular and animal models of disease. Understanding to which enzymes antifolates bind under physiological conditions and how this corresponds to the phenotypic response could provide insight on how to target the folate pathway in these organisms. To facilitate this, we have adopted a chemical proteomics approach to study binding of compounds to enzymes of folate metabolism. Clinical and literature antifolate compounds were immobilized onto resins to allow for “pull down” of the proteins in the “folateome”. Using competition studies, proteins, which bind the beads specifically and nonspecifically, were identified in parasite lysate (*Trypanosoma brucei* and *Leishmania major*) for each antifolate compound. Proteins were identified through tryptic digest, tandem mass tag (TMT) labeling of peptides followed by LC-MS/MS. This approach was further exploited by creating a combined folate resin (folate beads). The resin could pull down up to 9 proteins from the folateome. This information could be exploited in gaining a better understanding of folate metabolism in kinetoplastids and other organisms.

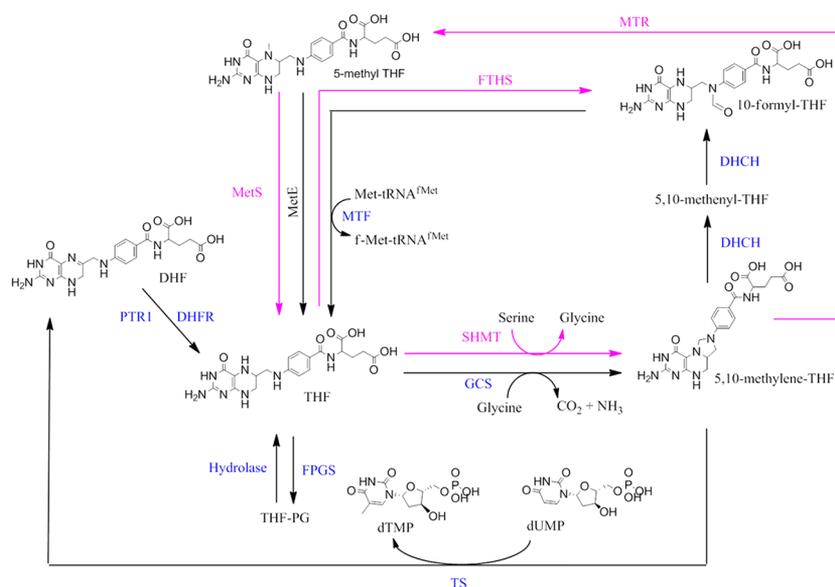


**KEYWORDS:** kinetoplastid, folate, chemical proteomics, pull down, *Trypanosoma brucei*, *Leishmania*

We and others are interested in gaining a better understanding of folate metabolism within kinetoplastids and using this information to identify potential drug targets for disease areas such as human African Trypanosomiasis (HAT), Leishmaniasis, and Chagas' disease. Folate metabolism (Figure 1) has been exploited for the development of drugs in a number of disease areas, including for the treatment of cancer, bacterial infections, malaria, and rheumatoid arthritis,<sup>1–6</sup> but there has been little success in the case of the kinetoplastids. It is known that folates are essential for kinetoplastid parasites since they are key cofactors in pyrimidine and purine biosynthesis and are also required for the production of essential amino acids (e.g., methionine, glycine, and serine).<sup>2</sup> Bacteria and plant species synthesize folates through condensation of pterins by dihydropteroate synthase, an enzyme already demonstrated as druggable. However, this process is absent in kinetoplastids and drug discovery programs have focused on targeting enzymes responsible for the subsequent metabolism of folic acid. Although some compounds show significant inhibition of the

parasitic enzymes, there is little translation of potency against the enzymes to cellular and animal models.<sup>7,8</sup> Understanding how the folate pathway operates in these organisms and which inhibitors bind to which enzymes under physiological conditions would be invaluable in identifying ways to both understand why current analogues are not effective against the parasites *in vitro* and to target this pathway for drug discovery. In this study, we use chemical proteomics to identify compounds capable of specifically binding to enzymes that constitute the folate metabolism of *Trypanosoma brucei* and *Leishmania major*, causative agents of HAT and cutaneous leishmaniasis. It is hoped the learnings can be used to develop more potent compounds capable of treating these devastating parasitic diseases.

Received: April 16, 2018



Enzymes	Abbreviation	Accession Number		
		<i>T. brucei</i>	<i>L. major</i>	Human
Dihydrofolate reductase - Thymidylate synthase	DHFR-TS	Tb927.7.5480	LmjF.06.0860	NA
Dihydrofolate reductase	DHFR	NA	NA	P00374
Thymidylate synthase	TS	NA	NA	P90463
Glycine Cleavage System GCV1 (T-subunit)	GCV	Tb927.11.9670	LmjF.36.3800	NA
Glycine Cleavage System GCV2 (P-subunit)	GCV2	Tb927.7.1910	LmjF.26.0030	NA
Glycine Cleavage System GCV3 H-subunit	GCVL-1	Tb927.3.4390	LmjF.29.1830	P23434
Dihydropyrimidinase Dehydrogenase	GCVL-2/DLDH	Tb927.11.16730	LmjF.32.3310	P23378
Aminomethyltransferase	AMT	NA	NA	P48728
Glycine Decarboxylase	GLDC	NA	NA	P23378
Methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase	DHCH1	Tb927.7.1600	LmjF.26.0320	NA
Formate--tetrahydrofolate ligase	FTHS	NP	LmjF.30.2600	NA
C1-Tetrahydrofolate synthase	MTFD1/MTFD1L	NA	NA	P11586 (cytoplasmic) Q6UB35 (mitochondrial)
Serine hydroxymethyl transferase	SHMT	NP / NP	LmjF.28.2370 (mitochondrial) LmjF.14.1320 (cytoplasmic)	P34897
Folylpolyglutamate synthase	FPGS	Tb927.10.7520	LmjF.36.2610	NP
Pterin-4-a-carbinolamine dehydratase	PCBD2	Tb927.11.7040	LmjF.11.0220	Q9H0N5
Methylene-THF-reductase	MTR	NP	LmjF.36.6390	Q99707
Pteridine reductase 1	PTR1	Tb927.8.2210	LmjF.23.0270	NP

**Figure 1.** Diagrammatic view of folate metabolism. Steps shown in black are common in human, leishmania, and *T. brucei*. Steps shown in purple are additional steps in human and leishmania. MTHFD (methylene tetrahydrofolate dehydrogenase), MTHCH (methenyl tetrahydrofolate cyclohydrolase), MTR (methylene tetrahydrofolate reductase), FTHS (10-formyl THF synthase), MetS and MetE (methionine synthase), MTF (methionyl-tRNA formyltransferase). NP, not present. NA, not applicable.

## BACKGROUND

In our (Cellzome) laboratories, we have developed powerful methods to quantitatively analyze binding of compounds to proteins in cell extracts. As previously reported, this approach has been successfully used to interrogate target classes that share conserved binding pockets, such as kinases<sup>9</sup> and

dioxygenases.<sup>10</sup> This approach used an affinity matrix composed of nonselective inhibitors immobilized on beads that could effectively “pull down” a large number of different kinases or dioxygenases. Binding of cell extract proteins to these beads was studied in the presence or absence of free inhibitor. Following several washes to remove nonbinding

Table 1. Summary of Compounds Selected for Study and Their Hypothetical Target(s)<sup>a</sup>

ID	Hypothesized Target(s)							
	DHFR	TS	PTR-1	FPGS <sup>b</sup>	GCS	SHMT	DHCH	MTFT
MTX	✓ <sup>13</sup>		✓ <sup>8</sup>	✓				
RTX	✓ <sup>1,6</sup>	✓ <sup>1,6</sup>		✓				
PTX	✓ <sup>1,6</sup>	✓ <sup>1,6</sup>		✓				
Folic acid	✓	✓	✓	✓	✓	✓	✓	✓
LEU	✓ <sup>14-15</sup>					✓ <sup>14-15</sup>	✓ <sup>14-15</sup>	✓ <sup>14-15</sup>
1	✓ <sup>4</sup>							
2	✓ <sup>16</sup>							
NTX	✓ <sup>1,6</sup>	✓ <sup>1,6</sup>						
3			✓ <sup>7-8</sup>					
4				✓ <sup>17</sup>			✓	✓
THF	✓	✓	✓	✓	✓	✓	✓	✓
6				✓				
7				✓				

<sup>a</sup>FPGS, folypolyglutamate synthase; GCS, glycine cleavage system; SHMT, serine hydroxyl methyltransferase; DHCH MTFT, methionyl-tRNA-formyltransferase; MTX, methotrexate; RTX, raltitrexed; PTX, pemetrexed; LEU, leucovorin; NTX, nolatrexed; THF, tetrahydrofolate. <sup>b</sup>Compounds with a folate pharmacophore possessing a diglutamate moiety that are proposed to bind to FPGS (although it is unknown if these compounds are inhibitors of FPGS).<sup>18</sup>

proteins, specifically bound proteins were eluted from the beads, digested by trypsin, and analyzed by mass spectrometry. Quantification of the kinases binding to inhibitors was performed by isobaric tagging with isotope-containing reagents (TMT, tandem mass tags).

In this current study, we aimed to produce and develop an affinity matrix for enzymes involved in the folate metabolism of kinetoplastids, effectively the folateome of these parasites. This information could then be used to determine the molecular targets of compounds targeting folate metabolism and to correlate this to phenotypic responses. To achieve this, a small library of clinical and literature antifolate compounds was immobilized onto resins and used in “pull-down” experiments with and without test compound present, to ascertain proteins binding specifically to particular resins.<sup>9,11,12</sup> While the initial focus of this project was to establish the kinetoplastid folateome, the approach has the potential to be extended to other organisms.

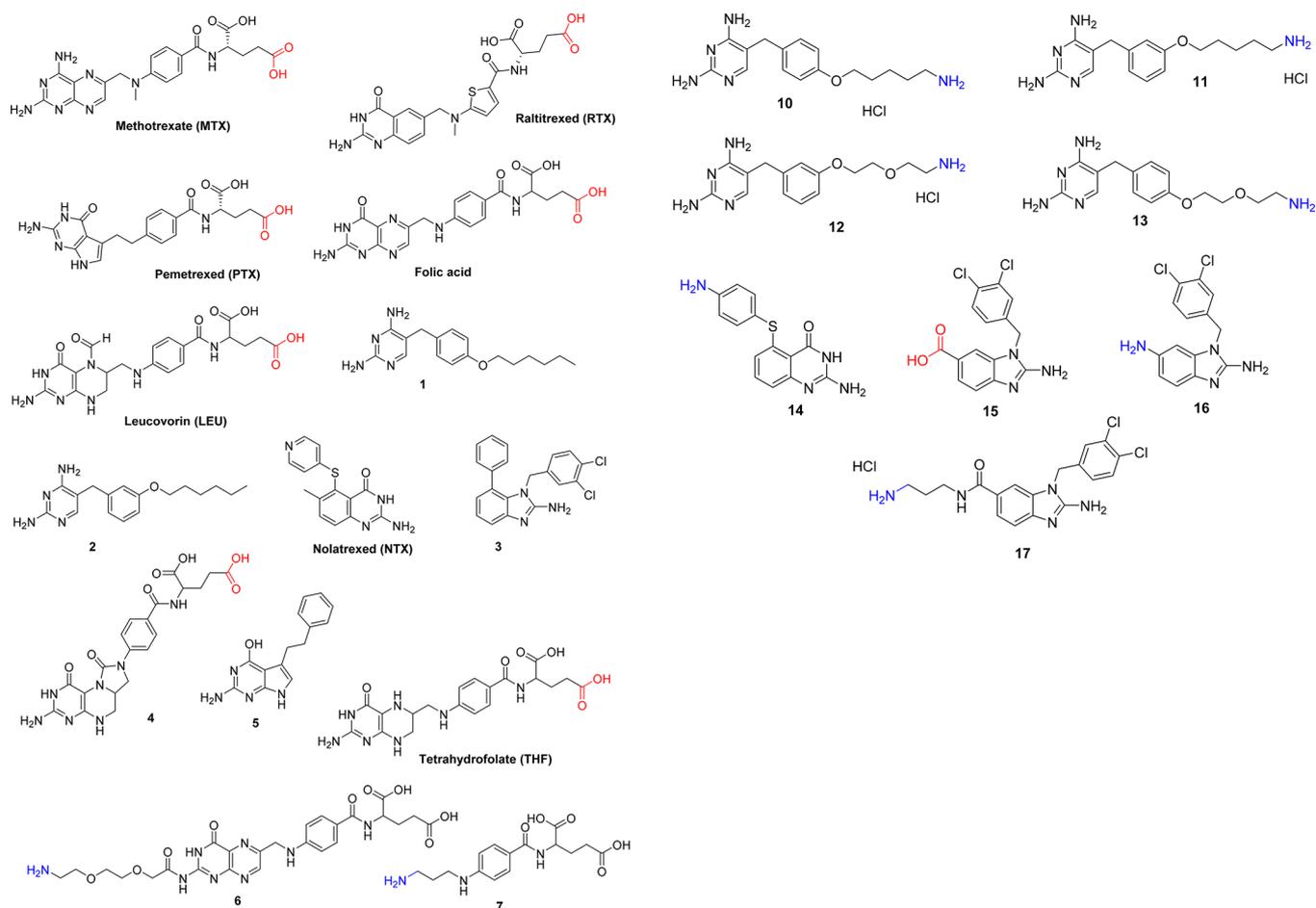
### ■ PREPARATION OF BEADS: SELECTION OF COMPOUNDS FOR IMMOBILIZATION

In order to prepare beads for the “pull-down” experiments, a number of compounds known to inhibit different enzymes in folate metabolism were selected. The known enzymes involved in folate metabolism are shown in Figure 1. It should be noted that there are some differences between folate metabolism in human, *T. brucei*, and *L. major* (Figure 1). Both clinically used

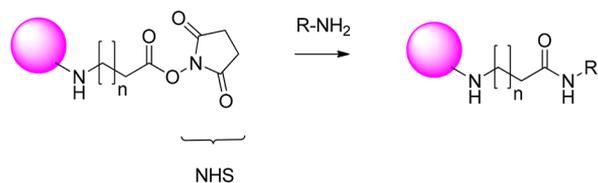
and experimental inhibitors of the following folate enzymes were used to cover as large percentage of the folateome as possible: DHFR, TS, PTR1, folypolyglutamate synthase (FPGS), and methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase (DHCH) inhibitors. Inhibitors were selected from the literature, where possible where there is literature data for inhibition of the kinetoplastid enzymes (see the references in Table 1 and further information in the Supporting Information).

Where appropriate, these inhibitors were modified for attachment to the beads (Table 1 and Figure 2). In addition, it was decided to include the substrate, folic acid, as it offered the potential to bind to a wide variety of folate-metabolizing enzymes. Folic acid should show good molecular recognition with multiple enzymes in the folateome. The folate ligand could of course undergo metabolism in some of the enzymes or bind but with a very weak binding constant. A peculiarity of folate metabolism in kinetoplastids is that dihydrofolate reductase (DHFR) and thymidylate synthase (TS) are found as a single bifunctional enzyme. In addition to the enzymes involved in folate metabolism, the kinetoplastid enzyme pteridine reductase 1 (PTR1) was included in this list, as it is known to metabolize dihydrofolate to tetrahydrofolate.

Antifolate compounds were immobilized onto NHS-activated sepharose beads/reverse sepharose beads through amide coupling via either an amino or carboxyl group (see Figure 3). The reaction was monitored by observing the disappearance of test compound by LCMS, after which the



**Figure 2.** Antifolate analogues under investigation. Areas highlighted in red/blue indicate where bead attachment occurs. In compounds where both free acidic and basic groups are available, specific beads were chosen to differentiate the two possible coupling sites.



**Figure 3.** Antifolates possessing amine points of attachment were coupled with NHS-activated sepharose beads.

unreacted coupling sites were capped with NHS-acetate or aminoethanol. Many “typical” antifolate analogues possess the common glutamic acid tail (e.g., MTX, folic acid) that enables these compounds to be directly coupled onto the beads. However, other antifolates (e.g., NTX, **1**, etc.) required modification to facilitate their attachment (see Figure 4).

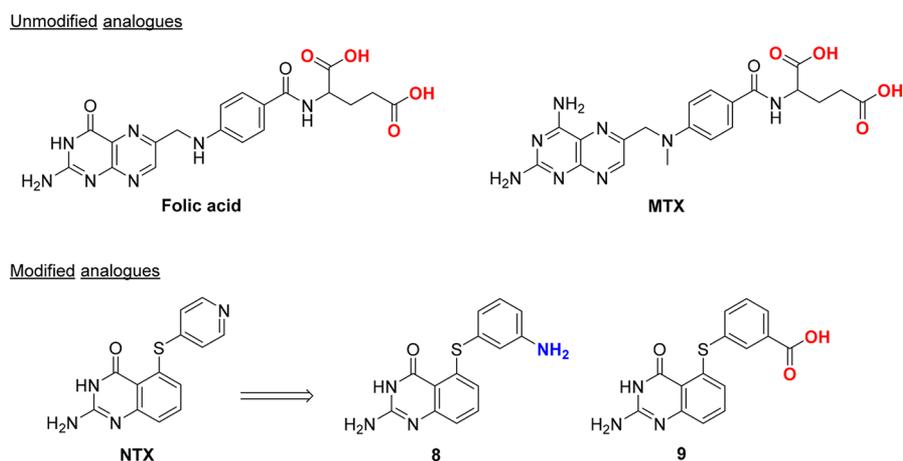
Folate metabolizing enzymes are not highly abundant proteins,<sup>19,20</sup> potentially making their identification by MS difficult. Beads will not only pull down the low abundant high affinity binders but also attract highly abundant, low affinity binders or so-called nonspecific binders. Sometimes, the nonspecific high abundant proteins can mask true binders. The target identification approach used in this study overcomes this issue by performing competition studies. Various concentrations of the free unmodified inhibitor (test compound) were preincubated with the lysate, prior to addition of the beads derivatized with immobilized compounds. Following washing of the bound proteins from the

beads and tryptic digest, the peptides were labeled using the TMTs and then pooled. Differences in peptide abundance were analyzed by fragmentation of the isotope-coded isobaric tags in the MS/MS spectra (Figure 5).<sup>9,11,12,21</sup> While the competition experiments with test compound confirm specific binding, it is also possible that some proteins will have a stronger binding interaction with the derivatized beads than the competing test compound, due to subtle differences in the interactions between the immobilized compound and the test compound with the target protein.

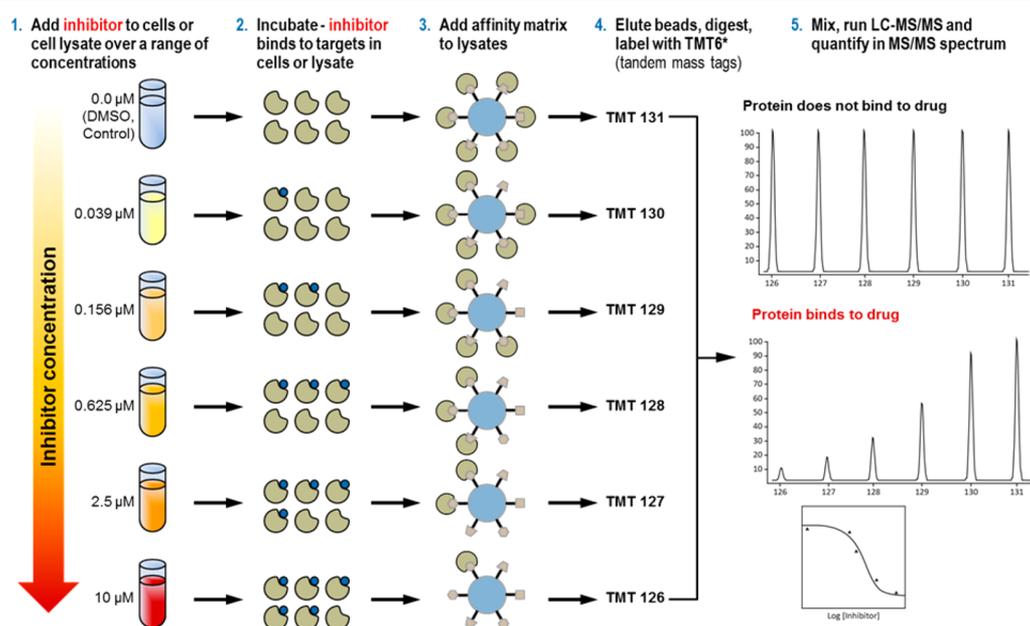
#### Identification of Protein Targets of Individual Beads.

Data from the pull-down and competition experiments is summarized in Table 2. In addition to identifying folate metabolizing proteins, these experiments also identified additional unrelated proteins that bind to the immobilized ligands, including proteins involved in ubiquitination and proteins that have been identified as potential drug targets in related parasites (*T. cruzi*) (see Supporting Information).

All clinically tested antifolates bound to their established target(s) (Table 2). It was surprising that many of the compounds tested here did not display binding to other proteins of the folateome, outside the reductive enzymes DHFR-TS and PTR1, given that the proteins in the folateome all bind relatively similar substrates. In the case of DHFR-TS, it is not known if the compounds bind to the DHFR or TS moiety; an example is the known TS inhibitor RTX (see Supporting Information for further discussion). Folic acid



**Figure 4.** Unmodified analogues MTX and folic acid both possess a glutamate tail which enables these compounds to be coupled to beads without modification. In the case of NTX, it does not possess suitable points of attachment and therefore these have to be chemically engineered into its synthesis. Where possible, crystallography was used to guide the synthetic design.



**Figure 5.** Proteomic work flow employed to identify binding proteins of antifolate derivatives using TMT to quantify the samples.

shares a common pharmacophore with many substrates of the folate pathway, and we expected that it might be able to pull down a large percentage of the folateome. Individual bead testing revealed that folic acid-derivatized beads were only able to bind to DHFR-TS (from both parasites) and PTR1 from *T. brucei* (Table 2). There are slight differences in the active sites of the *T. brucei* and *L. major* PTR1 enzymes that may explain this difference.<sup>22</sup> The fact that the folic acid-derivatized beads did not specifically pull down more of the folateome could be due to several scenarios: some of the enzymes may have very specific binding pockets, the linker is in the wrong place, the linker is too short, or there is a very weak binding of the enzymes to folic acid. Another possibility is that the folic acid attached to the beads is being metabolized by the enzymes and the product (attached to the beads) has a relatively weak binding affinity to the enzymes. Folic acid derivatized-beads could therefore not be used solely as an affinity resin for screening potential antifolates due to its poor protein coverage of this pathway. Compound 4 was the only compound found

to pull down (when resin bound) and compete for the bifunctional enzyme DHCH (dehydrogenase-cyclohydrolase) from *L. major* lysates; however, *T. brucei* DHCH was not detected. There are several binding pockets within this enzyme complex, and it is uncertain which of these is interacting with the compound.

The glycine cleavage system (GCS), a multiprotein complex,<sup>23</sup> was also identified with the DHFR-TS modified compounds. Beads derivatized with compounds 10 or 11 were found to bind to one of the proteins making up the GCS (<50% inhibition, LMJF. 32.3310). This was also evident with beads derivatized with compound 9 (a functionalized TS inhibitor). There was only weak competition with the competing ligands (see Table 2), implying that the derivatized beads have a stronger interaction with the protein than the competing free ligand. GCS pull down was only evident with the above-mentioned beads. There could be a number of explanations why this was the case, in addition to lack of potency of the ligand for the enzyme.

Table 2. Summary Table of Individual Bead Pull Downs with Competing Free Ligand (100  $\mu$ M) in Both *T. brucei* and *L. major*<sup>a</sup>

Immobilized compound	Competing Ligand	% Inhibition of protein binding to bead							
		DHFR-TS		PTR-1		DHCH		GCS	
		<i>T. brucei</i>	<i>L. major</i>	<i>T. brucei</i>	<i>L. major</i>	<i>T. brucei</i>	<i>L. major</i>	<i>T. brucei</i>	<i>L. major</i>
10	10 <sup>^</sup>	84	< 50*	77	< 50*	/	/	/	< 50
11	11	87	N.D	56	N.D	/	/	/	< 50
12	12 <sup>^</sup>	79	< 50*	51	< 50*	/	N.D	/	N.D
13	13	75	N.D	51	N.D	/	N.D	/	N.D
8	8	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
14	14	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
9	NTX	/	< 50*	/	< 50*	/	/	/	/
15	3	< 50*	< 50*	< 50*	< 50*	/	/	/	/
16	3	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
17	17	/	/	/	/	/	/	/	/
MTX	MTX	95	93	90	75	/	/	/	/
RTX	RTX	51	81	83	/	/	/	/	/
Folic acid	Folic acid	77	85	92	/	/	/	/	/
H <sub>4</sub> F	H <sub>4</sub> F	/	/	/	/	/	/	/	/
4	4	/	/	/	/	/	96	/	/

<sup>a</sup>N.D. = not determined. (/) = no affinity was recorded for the chosen target. (^) for beads 10 and 12 in *L. major*, 1 and 2 were used as the competing ligand, respectively. \* designation in the table: protein was identified on bead but was not sufficiently bound by competing ligand for target identification.

- The immobilized derivatives, 10 and 11, bind more strongly to the GCS than the free compounds 1 and 2. This could be due to, for example, the amide in the linker introducing potential polar interactions with the protein and conformational changes of the ligand.
- Folate metabolizing enzymes require cofactors (i.e., NADP<sup>+</sup> or NADPH) for reactions to occur and also for substrate binding. Since the lysate was not enriched with saturating amounts of cofactor, it is difficult to say how much cofactor was present in the active site(s) of the enzymes involved in folate metabolism. Therefore, competition may not have been evident due to the absence of key interactions between ligand and cofactor.

Other factors contributing to the lack of competition with the test compound could be due to structure related issues of both ligand and enzyme. Enzymes are often physiologically found as oligomers. The number of active sites available for competition is dependent on structure of the enzyme complex. For example, the test compound may be able to bind to DHFR, but as DHFR-TS is found as a bifunctional homodimer (*L. major*<sup>24</sup>), there are multiple active sites that can bind to ligand and or bead.

The majority of proteins that bound to beads belonged to families associated with the folate metabolism and the synthesis of its products (i.e., purine and pyrimidine metabolism). This finding led to the possibility of creating

“folate–beads” to analyze the folateome. The bead set would be comprised of beads that had the ability to pull down a large percentage of the folateome, thereby enriching this low abundant pathway in trypanosomatids.<sup>19</sup> This is analogous to kinobeads, a set of immobilized kinase inhibitors that display little selectivity for particular protein kinases and interact with kinases of different classes. Screening of compounds against the kinobeads aids in determining their kinase-inhibition profile.<sup>9</sup>

Individual bead testing revealed five folate metabolizing enzymes (the bifunctional DHFR-TS, PTR1, DHCH, and GCS) that could be captured by the beads. Work commenced on the development of a mixed bead set that had the ability to pull down a large percentage of the available folateome in the kinetoplastid parasites. These beads should (i) compete with test compound; (ii) not have a high background signal (i.e., clean binding profiles containing minimal nonspecific binding proteins); (iii) ideally interact with several proteins from the folateome.

**Multitargeted Bead Mixture.** A bead mixture was generated which combined beads from MTX, folic acid, 4, and 10 (3:11:11:11). In *T. brucei*, the bead mixture covered DHFR-TS and PTR1, while in *L. major*, the beads covered a wider area of the folateome, with the addition of GCS and DHCH to the list of targeted proteins.

The beads were initially tested against *T. brucei* lysate using as free ligands for competition, 3, NTX, 5,<sup>21</sup> MTX, and RTX

**Table 3. Multitargeted Bead Mixture Preliminary Experiments against a Small Subset of Compounds Whose Targets Are Known Either from the Literature or Have Been Identified during Individual Bead Testing<sup>a</sup>**

TB#	Name	5	NTX	3	MTX	RTX
TB927.8.2210	PTERIDINE REDUCTASE	82	16	89	83	84
TB427.07.5480	DIHYDROFOLATE REDUCTASE-THYIMIDYLATE SYNTHASE	5	-5	-11	85	-9
TB427.05.3680	HYPOTHETICAL PROTEIN, CONSERVED	70	38	68	69	65
TB427.04.1870	TB427.04.1870	50	52	47	58	51
TB427TMP.18.0017	CHOLINE KINASE	49	34	-1	57	12
TB11.03.0290	TB11.03.0290	61	77	10	57	1
TB427.06.4300	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE, GLYCOSOM	15	18	3	56	7
TB427.10.3890	PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE, PUTATIV	49	16	-23	56	19
TB927.8.6460	TB927.8.6460	65	68	15	54	8
TB09.211.1690	TB09.211.1690	-34	-7	39	51	-21
TB927.7.1340	TB927.7.1340	-41	11	40	51	-24
TB927.8.6760	TB927.8.6760	-71	8	20	50	-29
TB427.03.2670	HYPOTHETICAL PROTEIN, CONSERVED	54	11	62	50	35
TB427TMP.211.1350	PEPTIDYL-PROLYL CIS-TRANS ISOMERASE (CYCLOPHILIN-	-24	1	38	50	-30
TB427.02.4590	BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE, PUTATI	-22	9	51	34	0
Tb427tmp.02.5000	NGG1 INTERACTING FACTOR 3-LIKE	6	87	-6	31	13
Tb427.08.3310	ACETYLTRANSFERASE, PUTATIVE	61	29	37	31	44
Tb427.10.6100	HYPOTHETICAL PROTEIN, CONSERVED	77	33	67	23	17
Tb427.03.2230	SUCCINYL-COA SYNTHETASE ALPHA SUBUNIT, PUTATIVE	51	27	10	18	-11
Tb427.02.6180	IRON/ASCORBATE OXIDOREDUCTASE FAMILY PROTEIN, PUTA	80	52	0	17	14
Tb427.03.1570	PROTEIN KINASE, PUTATIVE	60	11	-2	9	2

<sup>a</sup>Table has been displayed as a heatmap which indicates percentage of competition from bead binding.

(Table 3). Preliminary work on bead mixtures suggested careful selection of the bead composition was required to obtain the correct balance between “pull down” of the folateome and competition with test compounds. For example, too high a percentage of MTX–beads led to fewer known DHFR inhibitors being identified (previously identified to bind during individual bead testing), presumably due to very tight binding of the MTX–beads to the enzyme. The new bead mixture showed confirmed targets being pulled down and outcompeted for. The only artifact was that the multitargeted bead mixture did not show DHFR-TS as a target for RTX, although experimentally we were able to show that RTX inhibited (albeit weakly compared to MTX) the enzyme biochemically (*Tb*DHFR-TS IC<sub>50</sub> 5.1 μM) in a direct assay (see Supporting Information). Chemical proteomics showed that RTX was able to outcompete for PTR1 (83% inhibition of binding) as were the other PTR1 compounds 3 and 5, while in nonoptimized bead mixtures, less than 50% inhibition of PTR1 binding was observed. This previously unreported inhibition of *Tb*PTR1 by RTX was also confirmed in direct enzyme assays, showing that RTX inhibited *Tb*PTR1 (IC<sub>50</sub> 22 μM (K<sub>i</sub> *Tb*PTR1 0.8 μM) (see Supporting Information). The multi-target bead mixture was not only able to pull down and allow competition of target proteins but also obtained cleaner binding profiles than previous bead mixtures (better signal-to-noise). However, this could in part be due to increased concentration of competing ligand (100 μM) compared with previous bead mixtures.

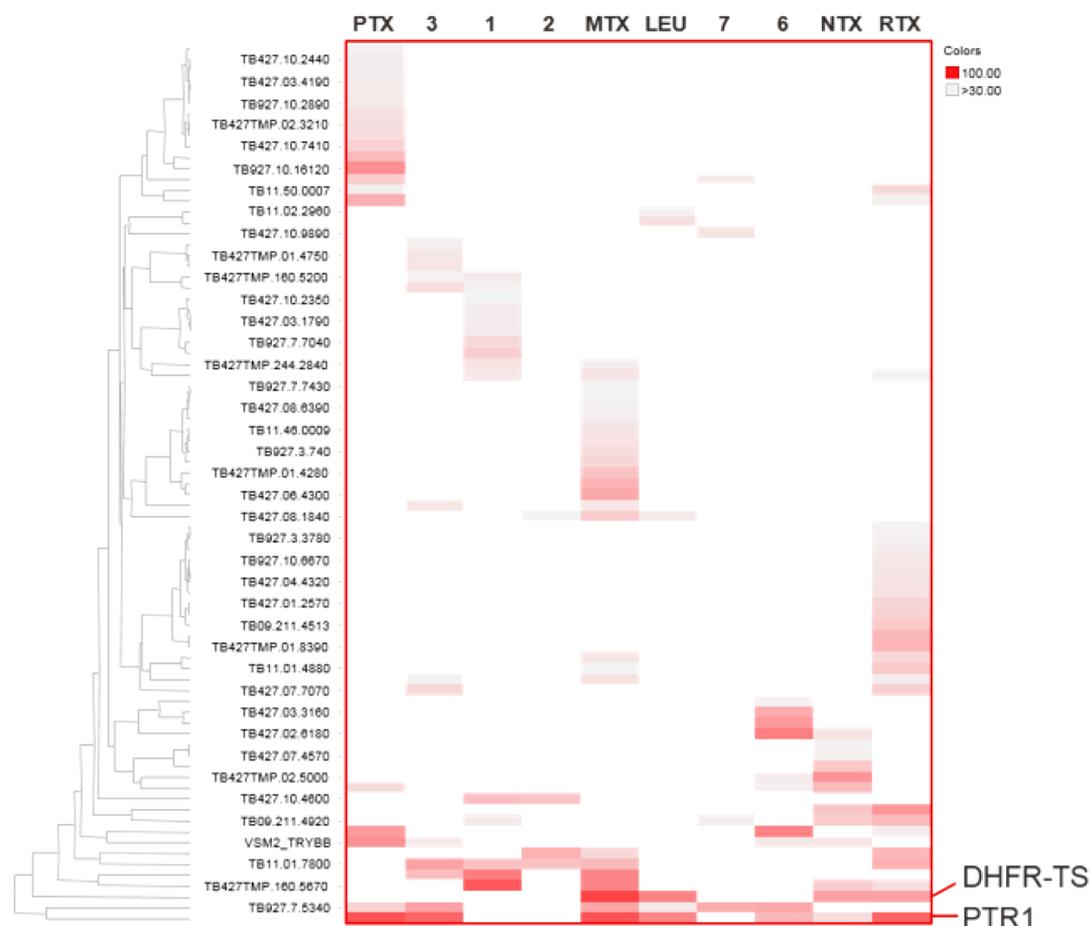
Competition studies were undertaken with 10 compounds using lysates from *T. brucei* (Figure 6), *L. major* (Figure 7), and HeLa cells (Figure 7). The 10 compounds were: MTX (DHFR and PTR1 inhibitor); RTX (TS inhibitor, but also found to inhibit *T. brucei* PTR1); 1 and 2 (parasite DHFR inhibitors); 3 (*T. brucei* PTR1 inhibitor); NTX (TS inhibitor); LEU (reported to inhibit DHFR, SHMT, MTF, MTHFD, and MTHCH);<sup>14,15</sup> PTX (DHFR inhibitor); 6 and 7 (potential FPGS inhibitors).

In *T. brucei* lysate (see Figure 6), only MTX was found to compete for DHFR-TS; the other known DHFR inhibitors investigated did not successfully compete for DHFR-TS. This may be due to the tight binding of MTX–beads to the DHFR.

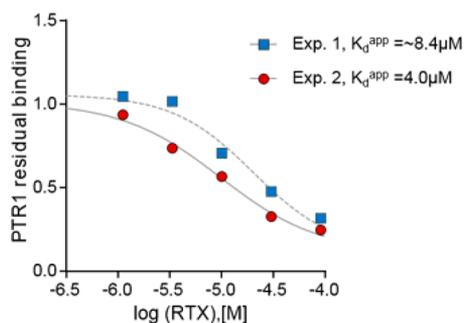
Other known DHFR inhibitors were not such strong binders in direct enzyme assays, including 1,<sup>15</sup> 2,<sup>4</sup> and PTX (*Tb*DHFR K<sub>i</sub> 290 ± 20 nM, *Tb*TS K<sub>i</sub> 20,500 ± 200 nM<sup>1,6</sup>). Likewise, the TS inhibitors, RTX and NTX, did not compete for DHFR-TS. MTX, RTX, PTX, and 3 competed for PTR1. Compound 3 was designed to be a highly selective *T. brucei* PTR1 inhibitor, and this was born out of these studies.

In the case of *L. major* (Figure 8a), MTX, RTX, and 1 competed for DHFR-TS, with MTX also outcompeting for PTR1. Interestingly, 1 competed for its hypothesized target DHFR, despite its derivative bead not being able to pull down this target. This suggested that the derivatized bead interfered with DHFR recognition, and if repeated, a different linker position would be used to link the inhibitor to the bead. Compound 3 showed no inhibition of PTR1 in *L. major*; however, this compound was designed for *T. brucei* PTR1 inhibition, and there are structural differences between the *L. major* and *T. brucei* PTR1s. An interesting observation was made with both 3 and PTX showing binding and competition to phenylalanine-4-hydroxylase (PAH), showing an additional target for PTX. PAH is a H<sub>4</sub>B dependent nonessential enzyme found in *Leishmania spp.* and a target not known for these compounds. None of the compounds from the screen showed affinity toward DHCH, although it was pulled down by the bead mixture, through inclusion of beads derivatized with compound 4 in the mixture.

In both *T. brucei* and *L. major* data sets (Figures 6 and 8), a number of enzymes were identified that appear to interact specifically with our compounds of interest but that are not known to directly interact with folates or pterins. For instance, in *T. brucei* lysates, PTX appeared to bind to inosine-5'-monophosphate dehydrogenase (Tb927.10.16120), involved in purine biosynthesis but not known to require folate or pterin cofactors. In addition, PTX also bound specifically to a dynein associated protein (Tb09.211.4920) and the highly abundant variant surface glycoprotein 221 (VSM2.TRYBB). Indeed, MTX bound to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Tb427.06.4300), also known to be one of the most abundant proteins in the cell. In *Leishmania* lysates, PTX strongly associated with glutamate 5'-kinase (LmjF.26.2710), while compound 1 bound to methylthioadenosine phosphor-

*Trypanosoma brucei*

**Figure 6.** Bead immobilized compounds MTX, folic acid, **4**, and **10** were incubated with *T. brucei* lysate. To evaluate specific binding, the indicated compounds were added to the extracts at a concentration of 100  $\mu\text{M}$ . Target proteins would be expected to bind to the beads only in the absence of excess competing compound. Proteins captured by the beads were quantified following tryptic digestion, isobaric peptide tagging, and LC-MS/MS analysis. Heatmaps were created in the data visualization software Spotfire (TIBCO software). Proteins were clustered with the clustering method UPGMA using the Euclidean distance measure as implemented in Spotfire. Shown are all proteins identified in 2 replicate experiments with at least 2 unique quantified peptides and with >30% inhibition by the indicated compounds.

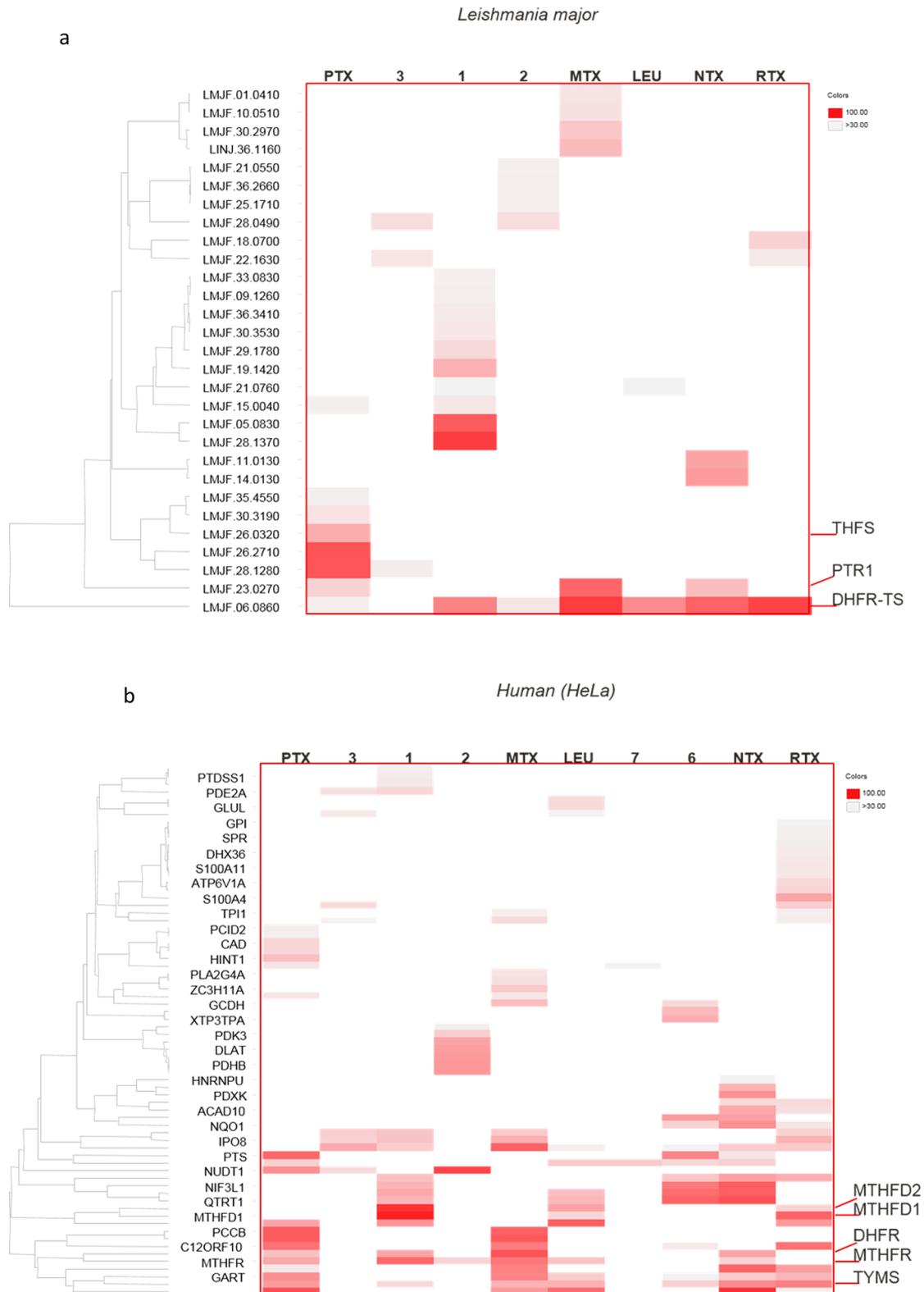


**Figure 7.** Generation of  $\text{IC}_{50}$  value for PTR1/RTX. The capturing experiment was performed as in Figure 6 but over a range of concentrations of the competing free inhibitor RTX (90–1.1  $\mu\text{M}$ ). Taking into account the depletion of PTR1 by the beads, the apparent dissociation constant was determined as 8.4  $\mu\text{M}$  in experiment 1 and 4.0  $\mu\text{M}$  in experiment 2. Quantification determined using TMT tagging.

ylase (LmjF.05.0830), an enzyme involved in purine salvage. This compound also interacted strongly with haloacid

dehalogenase-like hydrolase (LmjF.28.1370). Some of these interactions may simply be explained by our compounds interacting with highly abundant proteins within parasites, while other associations are more difficult to explain and could represent other molecular targets of the compounds, which may or may not have a phenotypic effect.

In the HeLa cell extract (Figure 7b), the known DHFR inhibitors MTX and **1** were found to bind DHFR. However, PTX did not appear to bind DHFR, which may also be an artifact of the strength of binding of the MTX-derivatized beads. Interestingly, **1** targeted all subunits of the MTHF complex (DHCH protein in humans) with >70% inhibition. To the authors' knowledge, the MTHF complex has not previously been described as a target of the diaminopyrimidine compound. MTX also bound methylene-THF-reductase (77% inhibition), which is an unreported activity of MTX. NTX, LEU, and PTX also bound MTHFS, the 5-formyl-THF-cyclo-ligase. PTX, NTX, LEU, and RTX were all inhibitors of TS (>56% inhibition); this was expected for NTX and RTX which are designed TS inhibitors. However, it is not expected for LEU and PTX.



**Figure 8.** (a, b) Bead immobilized compounds MTX, folic acid, 4, and 10 were incubated with the indicated protein extracts. Images were created using data visualization software Spotfire (TIBCO software) in the same manner as Figure 6. In *L. major*, compounds 6 and 7 are not shown (no protein inhibition >30%). MTHFD1/MTHFD2, methylenetetrahydrofolate dehydrogenase 1/2; MTHFR, methylenetetrahydrofolate reductase; TYMS, thymidylate synthase.

**DISCUSSION**

We have successfully derived a set of beads that pull down the majority of enzymes in the folateome. This provides a very

important tool for understanding folate metabolism and the effects of inhibitors on folate metabolism. In addition to pulling down proteins in the folateome, these beads also pull down a variety of other proteins. Included in these are proteins

from pterin metabolism, which is important given the parallel nature of the folate and pterin metabolic pathways.

There are potent inhibitors described in the literature for some enzymes of the folateome (DHFR, TS, PTR1, DHCH), that can be used to derivatize beads. However, in some cases, attaching inhibitors to the beads may well have caused a loss in their binding affinity. Potent inhibitors are not known for some of the other enzymes in the folateome (e.g., the GCS, SHMT, FPGS); even immobilized folic acid was poor at pulling down these proteins. Nevertheless, the vast majority of the proteins of the folateome were pulled down by our multibead panel.

A careful balance of affinity of the beads for the protein must be achieved. If the affinity is too low, the protein will not bind effectively to the bead (probably as observed with compound 11-derivatized beads in *L. major* extract). Conversely, if the bound ligand has a very high affinity for the protein, then it may be difficult for other ligands to prevent binding of the protein to the beads. A particular case in point is that of MTX, which has a very potent interaction with the protein. Reducing the proportion of MTX-beads in the mixture allowed detection of DHFR-TS and PTR1 binding by other compounds. At higher proportions of MTX-beads, compound 3 did not compete successfully with the MTX-beads for PTR1, although it did so successfully at lower proportions of MTX-beads. The concentration of MTX-beads used in the bead mixture is critical to allow for folate identification (see Table 4), especially when the protein is not present in vast quantities.

**Table 4. Comparison of the Potential Targets That Can Be Screened for on MTX/Folic Acid and Multitargeted Beads**

	DHFR	TS	DHFR-TS	DHCH	SHMT	GCS	MTHF	PTR-1	Pterin 4- $\alpha$ carbinolamine dehydratase
MTX : Folic acid beads	✓	✓	✓					✓	
Multi-targeted bead mixture	✓	✓	✓	✓	✓	✓	✓	✓	✓

## CONCLUSION

This approach has been successfully used to confirm the molecular targets of clinically used and literature antifolates in *T. brucei* and *L. major*. By using a mixture of antifolates attached to beads, we were able to pull down the majority of the folateome, despite the relatively low abundance of enzymes in the folateome. Care was needed in the selection of the composition and ratio of different beads, to obtain a good coverage of the folateome and to ensure that proteins were not too strongly bound to the immobilized ligands. There were also some limitations caused by the lack of suitable inhibitors of some proteins in the folateome. Nonetheless, the majority of the folateome was successfully pulled down by the beads. In addition to pulling down proteins of the folateome, additional proteins were pulled down. It is clear from Figures 6 and 8 that the folate compounds actually interact with multiple proteins within the cell, both those involved in folate metabolism and other enzymes with no involvement in folate metabolism. Some of these may also be important in the activity of the compounds, and the work described here may contribute to understanding the mode of action of these compounds. It may be that the key to antiparasite activity is getting the correct profile of enzymes being inhibited. While we focused our study on the trypanosomes, we expect our folateome beads are not

restricted to the organisms/species tested here but can be used in a number of tissue/lysate extracts.

## METHODS

**Synthesis of Chemical Probes and Competing Ligands.** Chemicals and anhydrous solvents were purchased from commercial sources and were used without further purification. <sup>1</sup>H NMR spectra were recorded on either a Bruker Avance DPX 500 or a Bruker Avance 300 spectrometer. Chemical shifts ( $\delta$ ) are expressed in ppm. Signal splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), multiplet (m), or combinations thereof. LC-MS analyses were performed with either an Agilent HPLC 1100 series connected to a Bruker Daltonics MicrOTOF or an Agilent Technologies 1200 series HPLC connected to an Agilent Technologies 6130 quadrupole LC/MS; both instruments were connected to an Agilent diode array detector. LCMS chromatographic separations were conducted with a Phenomenex Gemini C18 column, 50  $\times$  3.0 mm, 5  $\mu$ m particle size; mobile phase, water/acetonitrile +0.1% HCOOH 80:20 to 5:95 over 3.5 min and then held for 1.5 min; flow rate, 0.5 mL min<sup>-1</sup>. High resolution electrospray measurements were performed on a Bruker Daltonics MicrOTOF mass spectrometer. TLC was performed on Kieselgel 60 F<sub>254</sub> (Merck) with detection under UV light and by charring with KMnO<sub>4</sub> or ninhydrin for visualization. Column chromatography was performed using RediSep 4, 12, 24, 40, or 80 g silica prepacked columns. Full experimental details in the [Supporting Information](#).

**Preparation of Kinetoplastid Lysate.** *T. brucei brucei* variant 117 were purified from infected blood over DE52 cellulose as described previously.<sup>19</sup> The cells were centrifuged at 800g for 10 min at 4 °C, and the supernatant was discarded. The pellet was resuspended at 1  $\times$  10<sup>9</sup> cells/mL in ice-cold Buffer I (water containing 0.1  $\mu$ M 1-chloro-3-tosylamido-7-amino-2-heptone (TLCK), 1 mM benzamidine, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1  $\mu$ g/mL leupeptin, and 1  $\mu$ g/mL aprotinin), and hypotonic lysis was allowed to proceed for 10 min on ice (NB, *T. brucei* cells spontaneously lyse under these conditions). An equal volume of ice-cold Buffer II (100 mM Tris pH 7.5, 10% glycerol, 300 mM NaCl, 50 mM NaF, 3 mM MgCl<sub>2</sub>, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 1.6% NP40, 2 mM DTT, 0.1 mM TLCK) was added to the cell extract. The lysate was centrifuged at 145 000g for 1 h at 4 °C (40 000 rpm, Beckman Type 50.2 Ti rotor). The supernatant was aliquoted into 15 mL tubes, frozen in liquid nitrogen, and stored at -80 °C. The BCA assay (Pierce) was used to determine the total protein content (BSA standard). The final cell extracts are 0.5  $\times$  10<sup>9</sup> cells/mL or 2 mg/mL total protein content (5  $\times$  5 mL; 50 mg).

*L. major* (Friedlin) promastigotes were grown in M199 media (1) until they reached late log (cell density 4  $\times$  10<sup>7</sup> cells/mL). Cells were centrifuged at 1200g for 10 min at 4. The resulting pellet was washed in phosphate buffered saline, centrifuged as above, and resuspended in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM DTT, 60 mM MgCl<sub>2</sub>, 0.2% (v/v) NP40, complete EDTA-free protease inhibitor cocktail (Roche), phosphatase Inhibitor Cocktail II (Calbiochem)). Parasites were then biologically inactivated by three cycles of freeze-thawing. The organisms were then lysed under pressure (30 kpsi) using a one-shot cell disruptor (Constant Systems). The lysate was centrifuged at 30 000g for 30 min at 4 °C, and the resulting supernatant was collected. The BCA assay

(Pierce) was used to determine protein content, and lysates were diluted with lysis buffer to a final concentration of 5 mg/mL. The supernatant was aliquoted into 15 mL tubes, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

**Chemoproteomics.** The chemoproteomic inhibition binding experiments were performed as previously described.<sup>11,25</sup> These references include a detailed description of the LC-MS/MS procedures (including instruments used, the method setup, filter criteria, acceptance of peptides, and FDR rate). Briefly, sepharose beads were derivatized with compounds MTX, folic acid, 4, and 10. The four bead types were mixed, washed, and equilibrated in lysis buffer (50 mM Tris-Cl pH 7.4, 0.4% Igepal-CA630, 1.5 mM  $\text{MgCl}_2$ , 5% glycerol, 150 mM NaCl, 25 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM DTT, and 1 complete EDTA-free protease inhibitor tablet (Roche) per 25 mL). They were incubated at  $4^{\circ}\text{C}$  for 1 h with *L. major*, *T. brucei*, or HeLa cell extract, which was preincubated with compound or DMSO (vehicle control). Beads were transferred either to Filter plates (Durapore (PVDF membrane, Merck Millipore)) or to disposable columns (MoBiTec), washed extensively with lysis buffer, and eluted with SDS sample buffer. Proteins were alkylated, separated on 4–12% Bis-Tris NuPAGE (Life technologies), and stained with colloidal Coomassie.

Gel lanes were cut into three slices and subjected to in-gel digest using trypsin for 4 h. Digestion, labeling with TMT isobaric mass tags, peptide fractionation, and mass spectrometric analyses were performed. Proteins were quantified by isobaric mass tagging and LC-MS/MS.<sup>11,25</sup>

For experiments generated with *Leishmania major* extract, MS spectra were searched using Mascot (Matrix Science) against a sequence nonredundant database consisting of two species of *Leishmania*: *L. infantum* and *L. major* (Fredlin strain) which were download from TriTrypDB release 4.1 (30 June 2012): <http://tritrypdb.org/common/downloads/release-4.1/Linfantum/fasta/>. The following files were utilized: LinfantumAnnotatedProteins\_TriTrypDB-4.1.fasta and LmajorFredlinAnnotatedProteins\_TriTrypDB-4.1.fasta.

To ensure as wide a coverage as possible, additional *Leishmania* protein sequences from the NCBI nr (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/>) database were added, as well as known contaminant sequences such as keratins and trypsin. The resulting database consisted of 8312 sequences from *L. infantum* and 8416 from *L. major*.

For experiments generated with *Trypanosoma brucei* extract, MS spectra were searched using Mascot (Matrix Science) against a nonredundant, in-house compiled database of *Trypanosoma brucei* 927 and 427 strains obtained from TriTrypDB 3.0 (9 February 2011): <http://tritrypdb.org/common/downloads/release-3.0/Tbrucei/fasta/>. The following files were utilized: Tbrucei427AnnotatedProteins\_TriTrypDB-3.0.fasta and TbruceiTreu927AnnotatedProteins\_TriTrypDB-3.0.fasta.

Additional *T. brucei* protein sequences from SwissProt ([www.uniprot.org](http://www.uniprot.org)) and RefSeq (<http://www.ncbi.nlm.nih.gov/refseq/>) databases, as well as known contaminant sequences such as keratins and trypsin, were added to ensure greatest protein coverage. From these two strains, a total of 18 389 protein sequences were extracted. To assess the false discovery rate (FDR), “decoy” proteins (reverse of the protein sequence) were created and included in the databases.

Protein identification and quantification was performed.<sup>25</sup> Proteins identified with >1 unique peptide matches were considered for further data analysis. Raw data tables for the

chemoproteomics experiments can be found in the Tables S1 to S5. The protein identification numbers and descriptions on these tables were updated to the version 33 of TryTripDB using the TryTripDB database conversion tools.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsinfecdis.8b00097](https://doi.org/10.1021/acsinfecdis.8b00097).

Additional information on enzymes and inhibitors selected, details of the biochemical assays with PTR1, and full synthesis and characterization of intermediates and final compounds (PDF)

Table S3 proteomic data for experiments with *T. brucei* (XLS)

Table S3 proteomic data for experiments with *T. brucei* (XLS)

Table S4 proteomic data for experiments with *L. major* (XLS)

Table S5 proteomic data for experiments with *L. major* (XLS)

Table S6 proteomic data for experiments with HeLa cells (XLS)

Table S7 proteomic data for experiments with *T. brucei* (XLS)

Table S8 proteomic data for experiment with *T. brucei* (XLS)

Table S9 proteomic data for experiment with *T. brucei* (XLS)

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### Notes

The authors declare the following competing financial interest(s): S.G.-D., M.B., and G.D. are employees and shareholders of GSK. The company funded part of the study. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository<sup>26</sup> with the data set identifiers PXD010405, PXD010406, PXD010407, PXD010408, and PXD010409.

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## REFERENCES

- (1) Gibson, M. W. (2009) *Characterisation of Trypanosoma brucei dihydrofolate reductase-thymidylate synthase*, University of Dundee, Dundee.
- (2) Sienkiewicz, N., Jaroslowski, S., Wyllie, S., and Fairlamb, A. H. (2008) Chemical and genetic validation of dihydrofolate reductase-thymidylate synthase as a drug target in African trypanosomes. *Mol. Microbiol.* 69, 520–533.
- (3) Sirawaraporn, W., Sertsrivanich, R., Booth, R. G., Hansch, C., Neal, R. A., and Santi, D. V. (1988) Selective-inhibition of leishmania dihydrofolate-reductase and leishmania growth by 5-benzyl-2,4-diaminopyrimidines. *Mol. Biochem. Parasitol.* 31, 79–85.
- (4) Chowdhury, S. F., Villamor, V. B., Guerrero, R. H., Leal, I., Brun, R., Croft, S. L., Goodman, J. M., Maes, L., Ruiz-Perez, L. M., Pacanowska, D. G., and Gilbert, I. H. (1999) Design, synthesis, and evaluation of inhibitors of trypanosomal and leishmanial dihydrofolate reductase. *J. Med. Chem.* 42, 4300–4312.
- (5) Bello, A. R., Nare, B., Freedman, D., Hardy, L., and Beverley, S. M. (1994) PTR1 - a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. *Proc. Natl. Acad. Sci. U. S. A.* 91, 11442–11446.
- (6) Gibson, M. W., Dewar, S., Ong, H. B., Sienkiewicz, N., and Fairlamb, A. H. (2016) *Trypanosoma brucei* DHFR-TS revisited: characterisation of a bifunctional and highly unstable recombinant dihydrofolate reductase-thymidylate synthase. *PLoS Neglected Trop. Dis.* 10, e0004714.
- (7) Mpamhanga, C. P., Spinks, D., Tulloch, L. B., Shanks, E. J., Robinson, D. A., Collie, I. T., Fairlamb, A. H., Wyatt, P. G., Frearson, J. A., Hunter, W. N., Gilbert, I. H., and Brenk, R. (2009) One Scaffold, Three Binding Modes: Novel and Selective Pteridine Reductase 1 Inhibitors Derived from Fragment Hits Discovered by Virtual Screening. *J. Med. Chem.* 52, 4454–4465.
- (8) Spinks, D., Ong, H. B., Mpamhanga, C. P., Shanks, E. J., Robinson, D. A., Collie, I. T., Read, K. D., Frearson, J. A., Wyatt, P. G., Brenk, R., Fairlamb, A. H., and Gilbert, I. H. (2011) Design, Synthesis and Biological Evaluation of Novel Inhibitors of *Trypanosoma brucei* Pteridine Reductase 1. *ChemMedChem* 6, 302–308.
- (9) Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., Mathieson, T., Perrin, J., Raida, M., Rau, C., Reader, V., Sweetman, G., Bauer, A., Bouwmeester, T., Hopf, C., Kruse, U., Neubauer, G., Ramsden, N., Rick, J., Kuster, B., and Drewes, G. (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat. Biotechnol.* 25, 1035–1044.
- (10) Joberty, G., Boesche, M., Brown, J. A., Eberhard, D., Garton, N. S., Humphreys, P. G., Mathieson, T., Muelbaier, M., Ramsden, N. G., Reader, V., Rueger, A., Sheppard, R. J., Westaway, S. M., Bantscheff, M., Lee, K., Wilson, D. M., Prinjha, R. K., and Drewes, G. (2016) Interrogating the Druggability of the 2-Oxoglutarate-Dependent Dioxxygenase Target Class by Chemical Proteomics. *ACS Chem. Biol.* 11, 2002–2010.
- (11) Bantscheff, M., Hopf, C., Savitski, M. M., Dittmann, A., Grandi, P., Michon, A.-M., Schlegl, J., Abraham, Y., Becher, I., Bergamini, G., Boesche, M., Delling, M., Duempelfeld, B., Eberhard, D., Huthmacher, C., Mathieson, T., Poekkel, D., Reader, V., Strunk, K., Sweetman, G., Kruse, U., Neubauer, G., Ramsden, N. G., and Drewes, G. (2011) Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. *Nat. Biotechnol.* 29, 255–265.
- (12) Ross, P. L., Huang, Y. L. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 1154–1169.
- (13) Shanks, E. J., Ong, H. B., Robinson, D. A., Thompson, S., Sienkiewicz, N., Fairlamb, A. H., and Frearson, J. A. (2010) Development and validation of a cytochrome c-coupled assay for pteridine reductase 1 and dihydrofolate reductase. *Anal. Biochem.* 396, 194–203.
- (14) Vickers, T. J., Beverley, S. M., and Docampo, R. (2011) Folate metabolic pathways in *Leishmania*. *Essays Biochem.* 51, 63–80.
- (15) Stover, P., and Schirch, V. (1993) The Metabolic Role of Leucovorin. *Trends Biochem. Sci.* 18, 102–106.
- (16) Pez, D., Leal, I., Zuccotto, F., Boussard, C., Brun, R., Croft, S. L., Yardley, V., Perez, L. M. R., Pacanowska, D. G., and Gilbert, I. H. (2003) 2,4-diaminopyrimidines as inhibitors of leishmanial and trypanosomal dihydrofolate reductase. *Bioorg. Med. Chem.* 11, 4693–4711.
- (17) Tonkinson, J. L., Habeck, L. L., Toth, J. E., Mendelsohn, L. G., Bewley, J., Shackelford, K. A., Gates, S. B., Ray, J., and Chen, V. J. (1998) The antiproliferative and cell cycle effects of 5,6,7,8-tetrahydro-N-5,N-10-carbonylfolate, an inhibitor of methylenetetrahydrofolate dehydrogenase, are potentiated by hypoxanthine. *J. Pharmacol. Exp. Ther.* 287, 315–321.
- (18) El Fadili, A., Kundig, C., and Ouellette, M. (2002) Characterization of the folylpolyglutamate synthetase gene and polyglutamylation of folates in the protozoan parasite *Leishmania*. *Mol. Biochem. Parasitol.* 124, 63–71.
- (19) Urbaniak, M. D., Guthrie, M. L. S., and Ferguson, M. A. J. (2012) Comparative SILAC Proteomic Analysis of *Trypanosoma brucei* Bloodstream and Procyclic Lifecycle Stages. *PLoS One* 7, e36619.
- (20) Panigrahi, A. K., Ogata, Y., Zikova, A., Anupama, A., Dalley, R. A., Acestor, N., Myler, P. J., and Stuart, K. D. (2009) A comprehensive analysis of *Trypanosoma brucei* mitochondrial proteome. *Proteomics* 9, 434–450.
- (21) Rix, U., and Superti-Furga, G. (2009) Target profiling of small molecules by chemical proteomics. *Nat. Chem. Biol.* 5, 616–624.
- (22) Tulloch, L. B., Martini, V. P., Iulek, J., Huggan, J. K., Lee, J. H., Gibson, C. L., Smith, T. K., Suckling, C. J., and Hunter, W. N. (2010) Structure-Based Design of Pteridine Reductase Inhibitors Targeting African Sleeping Sickness and the Leishmaniases. *J. Med. Chem.* 53, 221–229.
- (23) Scott, D. A., Hickerson, S. M., Vickers, T. J., and Beverley, S. M. (2008) The role of the mitochondrial glycine cleavage complex in the metabolism and virulence of the protozoan parasite *Leishmania major*. *J. Biol. Chem.* 283, 155–165.
- (24) Knighton, D. R., Kan, C. C., Howland, E., Janson, C. A., Hostomska, Z., Welsch, K. M., and Matthews, D. A. (1994) Structure of and Kinetic Channeling in Bifunctional Dihydrofolate Reductase-Thymidylate Synthase. *Nat. Struct. Biol.* 1, 186–194.
- (25) Werner, T., Sweetman, G., Savitski, M. F., Mathieson, T., Bantscheff, M., and Savitski, M. M. (2014) Ion coalescence of neutron encoded TMT 10-plex reporter ions. *Anal. Chem.* 86, 3594–3601.
- (26) Vizcaino, J. A., Cote, R. G., Csordas, A., Duanes, J. A., Fabregat, A., Foster, J. M., Griss, J., Alpi, E., Birim, M., Contell, J., O’Kelly, G., Schoenegger, A., Ovelheiro, D., Perez-Riverol, Y., Reisinger, F., Rios, D., Wang, R., and Hermjakob, H. (2013) The PRoteomics IDentifications (PRIDE) database and associated tools: status in 2013. *Nucleic Acids Res.* 41, D1063.