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Proteomic profiling and potential cellular target identification of K11777, a clinical cysteine protease inhibitor, in *Trypanosoma brucei*†

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We report herein the design, synthesis and application of K11777-derived activity-based probes (ABPs) allowing *in situ* profiling and identification of potential cellular targets of K11777 in *Trypanosoma brucei*.

More than a billion people suffer from neglected tropical diseases (NTDs), including malaria (caused by *Plasmodium falciparum*), sleeping sickness (aka Human African trypanosomiasis; caused by *Trypanosoma brucei*), Chagas' disease (aka American trypanosomiasis; caused by *Trypanosoma cruzi*), leishmaniasis, onchocerciasis, lymphatic filariasis and schistosomiasis.[1] Most drugs available to date however are limited by parasite resistance and marked host toxicity. New strategies and targets that can effectively combat these parasitic diseases are therefore needed urgently. Equally important, from the standpoint of drug efficacy and safety, comprehensive cellular target profiling of a given drug candidate is becoming an integral step in the process of drug discovery. The knowledge of potential on- and off-targets of a drug at the earliest stages of its development will not only shed light on its potential success (or failure) during the clinical trials, but also provide invaluable insight into its mode of action and further optimizations.

One promising strategy for discovering small-molecule therapeutics for parasitic diseases has been to target the cathepsin L subfamily of the papain-like (clan CA, family C1) cysteine proteases such as cruzain, rhodesain (also called brucipain or trypanopain) and falcipains (FP-2 and -3). Among various pharmacophores, vinyl sulfones have been widely studied as potential cysteine protease inhibitors and are entering the development pipeline as anti-parasitics (Fig. S1 in ESI†).[2] One of these, K11777 (Fig. 1), which was developed from the predecessor K11002 by replacing the morpholine–urea ring in K11002 with an *N*-methylpiperazine (*N*-Mpip) for increased oral bioavailability and solubility in intestinal fluids, is currently in late-stage preclinical trials for Chagas disease.[3] Although it is clear that K11777 also demonstrated efficacy against other parasites as diverse as

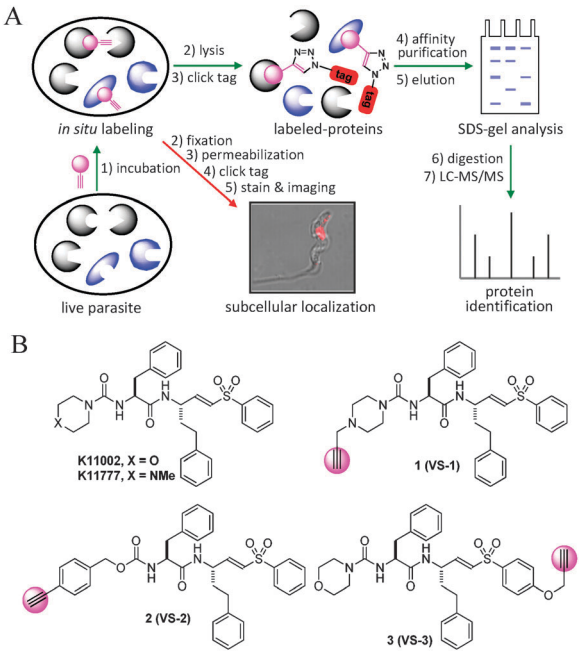

Fig. 1 (A) Workflow for proteome profiling of potential cellular targets of K11777 in living parasites using cell-permeable ABPs. (B) Structures of K11002/K11777 and corresponding cell-permeable and clickable probes (**1**, **2** and **3**; for synthesis, see ESI†).

trypanosomes (*i.e.*, *T. brucei*, *L. major*, *T. gondii*, *E. histolytica*) and schistosome bloodflukes (*i.e.*, *S. mansoni*),[4–6] its mechanism of action is not well understood except for inhibition of the cathepsin L and/or B-like cysteine proteases *in vitro*. Little information is available concerning its other potential cellular targets and distribution. Recently, we reported a chemical profiling approach that makes use of drug-like probes for proteome-wide profiling of cellular targets in living cells.[7] This method is based on activity-based protein profiling (ABPP),[8] and large-scale LC-MS/MS analysis, allowing rapid determination of potential cellular targets of bioactive small molecules.[8d,e] Herein, we report the first application of this method in live parasites. Several cell-permeable probes based on K11777 were designed, synthesized, and used to identify potential cellular targets of this drug candidate in *T. brucei*.

The design of K11777-like probes was based on the general structure of several trypanocidal vinyl sulfones (*e.g.*, K11777, K11002 and Cbz-Phe-Hph-VSCH$_2$Ph) and our previous experience with orlistat-like probes.[7a] We took advantage of key properties of

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vinyl sulfones in our rational design of ABPs: (1) vinyl sulfones inhibit cysteine proteases *via* a Michael addition to form a covalent bond with the active-site cysteine residue (*e.g.*, Cys 25 in cruzain/rhodesain and Cys 51 in FP-3);[2,9] (2) recent crystal structures of rhodesain with K11777 and K11002 have shown that the *N*-Mpip/morpholine–urea ring did not form any specific polar interactions with rhodesain and thus was assumed non-essential for inhibitor binding.[2,9] Accordingly, probes **1** and **2**, in which a C≡C triple bond was introduced at the P_3 position, were synthesized. We also synthesized probe **3** by introducing a C≡C triple bond at the P_1' position. Our experience was that such extremely conservative modifications would not compromise the native biological properties of mother compounds, whilst allowing subsequent target identification by downstream conjugation of reporter tags using bioorthogonal click chemistry. The three analogues were synthesized by using appropriate modifications of previously described methods, as shown in the ESI† (Schemes S1–S4). The resulting probes were fully characterized (MS and NMR) before being used in subsequent biochemical/biological experiments.

We first evaluated the trypanocidal activities of vinyl sulfones **1**, **2** and **3**, together with K11002 and K11777 in cell culture of both the bloodstream form (BSF) and the procyclic form (PCF), which represent two distinct stages of *T. brucei* during its complex life cycle alteration between mammalian hosts and insect vectors. In this context, it is worth noting that the BSF and PCF parasites differ extensively in morphology and metabolism. All vinyl sulfones blocked the parasite growth to a similar extent in a dose-dependent manner with ED_{50} values between 4 and 8 μM (Fig. 2A). Additionally, there was no noticeable difference in inhibition between BSF and PCF trypanosomes by all five compounds. These data show that the introduction of a terminal alkyne handle at the indicated positions did not noticeably affect its trypanocidal activity. Therefore, all three probes (**1**, **2** and **3**) should be suitable chemical probes for proteome profiling and cellular target identification of K11777. Only **1** (**VS-1**), however, was chosen for further comprehensive proteome profiling, LC-MS/MS and cellular bioimaging experiments based on its closest resemblance to K11777.

Next, we compared the *in situ* proteome reactivity profiles of our probes against their cellular targets in live BSF and PCF (Fig. 2B). Generally, the three probes showed comparable *in situ* proteome reactivity profiles, thereby indicating that they have similar cellular targets in the parasites. However, the reactivity profiles between BSF and PCF for **VS-1** (lanes 1 and 5 in Fig. 2B), though similar, showed noticeable differences, suggesting the existence of both common and unique targets in the two forms. In addition to the expected rhodesain and TbCatB bands (~41 kDa and ~31 kDa indicated by arrows, respectively), which were subsequently verified by affinity pull-down/western blotting with the corresponding antibodies (Fig. 2C), a number of other proteins were also covalently labeled by the three probes. These labeled bands were K11777-sensitive, that is, their labeling was blocked by the presence of a competing K11777 (lanes 2 and 6 in Fig. 2B). Also evident in Fig. 2B and C is that, **VS-1** labeled only the mature active enzyme form of rhodesain (~41 kDa), but not its proform (~48 kDa).[10] In addition, TbCatB labeled by **VS-1** was mostly detected in BSF, which is consistent with previous findings that TbCatB was up-regulated in BSF.[11] Subsequently, we performed

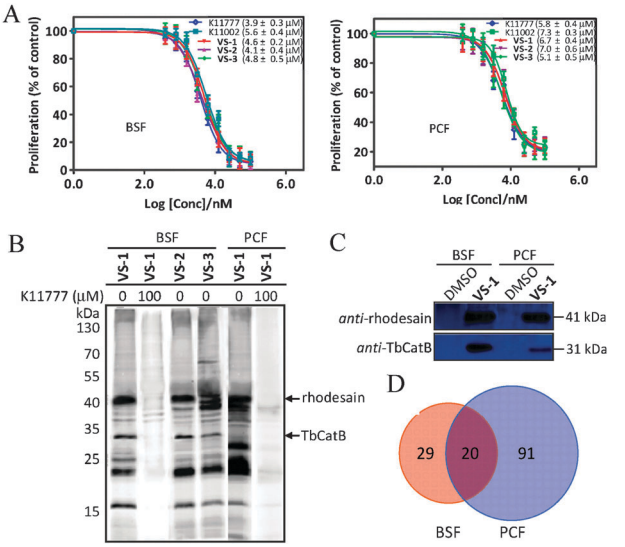

Fig. 2 (A) ED_{50} curves of K11777, K11002 and three probes showing *T. brucei* killing after 24 h. (B) *In situ* proteome profiling of probes (25 μM) against *T. brucei* BSF and PCF. K11777 was added to selected lanes in competition experiments. Putative bands of labeled rhodesain and TbCatB are indicated (with arrows). (C) Western blotting validation of rhodesain and TbCatB labeled with **VS-1** (25 μM), following *in situ* labeling and affinity pull-down/WB experiments (see ESI† for details). Null pull-down experiments (with DMSO) were used as negative controls. (D) Venn diagram illustrating the number of proteins identified from *T. brucei* after *in situ* labeling and large-scale pull-down/LC-MS/MS experiments.

large-scale proteomic analyses using **VS-1** to identify potential cellular targets of K11777 in both parasite forms by affinity pull-down/LC-MS/MS experiments; all proteins were identified with a minimum protein score of 40 as well as at least two unique peptides, and results are summarized in Fig. 2D, Table 1 and in ESI.† In total, 49 and 111 proteins were identified from BSF and PCF, respectively, 20 of which were from both parasite forms (see SI_2, ESI†, for detailed protein ID). Among these proteins, some were inevitably non-specific protein binders caused by their "sticky" nature as well as their high endogenous expression level (*e.g.* cytoskeletal proteins and carbohydrate-metabolism-related proteins). We focused our attention on other candidates which possess known nucleophilic cysteine residues, because they are more likely true cellular targets of K11777 (Table 1); in addition to the expected rhodesain and TbCatB, other proteins such as BS2, MCA4, AOX and two proteasome subunits were identified. It is worth noting that vinyl sulfones had previously been identified as potent and irreversible inhibitors of proteasomes (through modifications of their active-site threonine).[12] Thus, it is not surprising that two proteasome subunits were detected in PCF. It is also interesting to note that many more proteins were identified from PCF alone (91) than from BSF (29), and many of them were cytosolic (SI_2 in ESI†).

To visualize cellular localization of **VS-1** and potential cellular targets of K11777, live parasites were directly treated with **VS-1**, fixed, and reacted *in situ* with the rho-azide reporter (ESI†), then imaged. Overall, no noticeable difference was observed in the probe uptake between BSF and PCF (Fig. S4, ESI†). As shown in Fig. 3, **VS-1** signals were detected mostly in the lysosome of BSF, where endogenous rhodesain resides (panels b–d). In contrast, the

Table 1 Representative proteins identified by **1 (VS-1)** in *T. brucei*[a]

T. brucei gene	Protein name	Localization	Detection
Tb927.6.1000	Cysteine peptidase precursor (CP), Clan CA, family C1, cathepsin L-like*	L	Both
Tb927.10.8230	Protein disulfide isomerase, bloodstream-specific protein 2 precursor (BS2)*	C	BSF
Tb927.5.1810	Lysosomal/endosomal membrane protein p67 (p67)*	L	BSF
Tb10.70.5250	Metacaspase MCA4, cysteine peptidase, Clan CD, family C13 (MCA4)**	N	BSF
Tb927.10.7090	Alternative oxidase (AOX)**	M	BSF
Tb927.10.290	Proteasome alpha 2 subunit*	C	PCF
Tb927.10.6080	Proteasome beta 5 subunit (PRCE)*	C	PCF
Tb09.160.4250	Tryparedoxin peroxidase (TRYP1)**	C	PCF
Tb927.5.3350	Iron superoxide dismutase**	M	PCF
Tb927.10.7410	Succinyl-CoA ligase [GDP-forming] beta-chain*	M	PCF

[a] L, C, N, M and G represent lysosome, cytoplasm, nucleus, mitochondrion and glycosome, respectively. Symbols in the protein name column: (*) sensitive to RNA interference; (**) putative drug target.

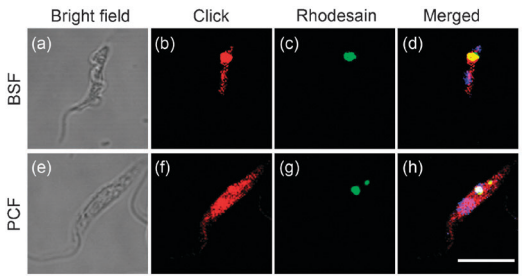

Fig. 3 Confocal microscopy of **VS-1** localization in BSF (top) and PCF (bottom). Live parasites were treated with **VS-1** (25 μM) followed by imaging as described in ESI.† Panels (a) and (e): bright-field images. Panels (b) and (f): 554 nm channel (pseudocolored in red) detecting cellular localization of **VS-1**. Panels (c) and (g): immunofluorescence (IF) staining at 488 nm channel (pseudocolored in green) to detect cellular localization of rhodesain. Anti-rhodesain primary antibody and FITC-conjugated anti-rabbit IgG secondary antibody were used. Panels (d) and (h): merged images of panels (b) and (c), (f) and (g) together with nuclei (stained with DAPI; pseudocolored in blue). All images were acquired under the same settings. Scale bar = 10 μm. Cells treated with DMSO are shown in Fig. S5 (ESI†).

probe was shown to be evenly distributed throughout PCF parasites with no specific subcellular localization (panels f–h; similar results were obtained with HepG2 mammalian cells as shown in Fig. S7, ESI†). These results correlate well with our above-described proteome profiling and affinity pull-down/LC-MS/MS data where different cellular targets were labeled in the two parasite forms, and many more cytosolic proteins were identified in PCF. The partial colocalization observed between **VS-1** and rhodesain in BSF presumably also reflected the presence of other side targets of K11777. Collectively, these results established that **VS-1** could be used for *in situ* proteome profiling and identification of potential cellular targets of K11777 in live parasites. As an imaging probe, it may also find potential applications to study drug localization.

In conclusion, we have successfully synthesized and evaluated K11777-like probes for their trypanocidal activities against both BSF and PCF *T. brucei*. Subsequent *in situ* proteome profiling of **VS-1** enabled us to tentatively identify previously unknown cellular targets of K11777 in both parasite forms. Furthermore, we demonstrated the utility of our probe for live-parasite labeling and visualization of potential K11777-responsive targets (*e.g.* rhodesain). Our probes should be useful in assisting future investigations of K11777 as an anti-parasitic drug in both parasites and human cells. Further studies are underway to validate some of the potential cellular targets identified from the present study.

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