Supporting Information (I)

Proteomic Profiling and Potential Cellular Target Identification of K11777, a Clinical Cysteine Protease Inhibitor, in *Trypanosoma brucei*

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1. General Procedures.

All chemicals were purchased as reagent grade and used without further purification, unless otherwise noted. Tetrahydrofuran (THF) was distilled over sodium benzophenone and used immediately. Dichloromethane (CH2Cl2) was distilled over CaH2. All non-aqueous reactions were carried out under nitrogen atmosphere in oven-dried glassware. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254, 250 µm thickness) and spots were visualized by basic KMnO4, UV light or iodine. 1H NMR and 13C NMR spectra were recorded on a Bruker model Avance 300 MHz or DPX-300 MHz or DPX-500 MHz NMR spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane (Si(CH3)4 = 0.00 ppm) or residual solvent peaks (CHCl3 = 7.26 ppm).

**Fig. S1.** (A) Structural representatives of vinyl sulfones which are anti-Trypanosomal agents (WRR-483,1 Cbz-Phe-Hph-VSCH2Ph2, and Cbz-Phe-Hph-VSOPh3), or anti-malarial agents (Mu-Leu-Hph-VSPh4a,5 N-Me-Pip-Leu-Hph-VSPh4b,5 and N-Me-Pip-Leu-Hph-VSNp-24c,5). (B) Structures of the two azide-containing reporter tags used in current study.6
2. Synthesis and Characterizations

2.1 Synthesis of compound 1 (VS-1).

Diethyl phenylthiomethylphosphonate (4)
To a cooled (0 °C) suspension of hexane-washed NaH (60% in mineral oil; 1.0 g, 24 mmol) in dry THF (100 mL) was added benzenethiol (2.0 mL, 20 mmol) drop-wise via syringe. The mixture was stirred for an additional 30 min at 0 °C until effervescence ceased. Diethyl iodomethylphosphonate (4.0 mL, 22 mmol) was added and the mixture was stirred for 12 h. A cold HCl solution (1 M) was added to break up the gelatinous emulsion until pH 6~7 was reached. Upon concentration in vacuo, the reaction was diluted with H2O (150 mL) and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with saturated aqueous NaHCO3 and brine, dried over Na2SO4, filtered and concentrated in vacuo. Purification by flash column chromatography (silica gel; using 20 to 50% EtOAc in hexanes) gave the product 4 as a colorless liquid (4.79 g, 92%). 1H NMR (300 MHz, CDCl3): δ 1.30 (t, J = 7.1, 6H), 3.20 (d, J = 14.0, 2H), 4.09-4.20 (m, 4H), 7.20-7.33 (m, 3H), 7.42-7.46 (m, 2H).

Diethyl phenylsulfonylmethylphosphonate (5)
To a solution of compound 4 (5.0 g, 19.2 mmol) in CH2Cl2 (100 mL) at 0 °C was added m-chloroperbenzoic acid (12.9 g of 77% m-CPBA, 57.2 mmol) over 1 h. The mixture was stirred overnight while being warmed to room temperature. The solution was then cooled to 0 °C and treated with NaOH (2 M) until pH 8~9. The organic phase was separated, dried over Na2SO4, filtered, and concentrated in vacuo. Purification by flash column chromatography (silica gel; using 20 to 50% EtOAc in hexanes) gave the product 5 as a colorless oil (5.6 g, 94%). 1H NMR (300 MHz, CDCl3): δ 1.29 (t, J = 7.1, 6H), 3.18 (d, J = 14.0, 2H), 4.09-4.20 (m, 4H), 7.20-7.33 (m, 3H), 7.42-7.46 (m, 2H).
MHZ, CDCl3): δ 1.30 (t, J = 7.1 Hz, 6H), 3.77 (d, J = 17.0 Hz, 2H), 4.11-4.21 (m, 4 H), 7.55-7.61 (m, 2 H), 7.65-7.71 (m, 1 H), 7.98-8.01 (m, 2 H).

(S)-tert-butyl [1-(methoxymethylcarbamoyl)-3-phenylpropyl]carbamate (6)

To a solution of (S)-Boc-Homophenylalanine (5.59 g, 20 mmol) in dry THF (100 mL) at 0 °C was added EDC (4.60 g, 24 mmol), HOBt (3.24 g, 12 mmol), N,N-dimethylhydroxylamine hydrochloride (2.34 g, 24 mmol) and DIPEA (5.2 mL, 30 mmol). The reaction was stirred at room temperature for 12 h, and concentrated in vacuo. Upon dilution with H2O (150 mL) and extraction with EtOAc (3 × 50 mL), the combined organic extracts were washed with 1 wt% HCl, 20 wt% Na2CO3 and brine, dried over Na2SO4, filtered and concentrated in vacuo. The crude product was purified by column chromatography (silica gel; using 20 to 50% EtOAc in hexanes), giving Boc-Hph-N(Me)OMe (6) as a white solid (6.20 g, 96%). 1H NMR (300 MHz, CDCl3): δ: 1.45 (s, 9H), 1.80-2.02 (m, 1H), 2.72 (m, 1H), 3.16 (s, 3H), 3.62 (s, 3H), 4.68 (br s, 1H), 5.23 (m, 1H), 7.15-7.31 (m, 5H).

(S)-tert-butyl (1-formyl-3-phenylpropyl)carbamate (Boc-Homophenylalaninal, Boc-HphH, 7)

To a solution of 6 (3.2 g, 10 mmol) in dry THF (50 mL) at 0 °C was added LiAlH4 (0.45 g, 12 mmol) over 10 min, with vigorous stirring. The mixture was stirred for an additional 20 min at 0 °C, whereupon cold water was carefully added until effervescence ceased. A cold HCl solution (1 M) was added to break up the gelatinous emulsion until pH 6~7. Upon dilution with H2O (150 mL) and extraction with EtOAc (3 × 50 mL), the combined organic extracts were washed with saturated aqueous NaHCO3 and brine, dried over Na2SO4, filtered and concentrated in vacuo. Purification by flash column chromatography (silica gel; using 20 to 50% EtOAc in hexanes) provided the product 7 as a white solid (1.92 g, 73%). 1H NMR (300 MHz, CDCl3): δ 1.46 (s, 9H), 1.83-1.95 (m, 1H), 2.22 (m, 1H), 2.67 (t, J = 7.6 Hz, 2H), 4.24 (m, 1H), 5.09 (br s, 1H), 7.17-7.32 (m, 5H), 9.55 (s, 1H).

(S)-tert-butyl (3-benzenesulfonyl-1-phenethylallyl)carbamate (Boc-HphVSPh, 8)

To a cooled (0°C) suspension of hexane-washed NaH (60% in mineral oil; 0.24 g, 6 mmol) in dry THF (50 mL) was added drop-wise 5 (1.61 g, 5.5 mmol) in dry THF (10 mL) via syringe. The mixture was stirred for an additional 30 min at 0 °C and 7 (1.32 g, 5 mmol) in dry THF (10 mL) was added drop-wise. The stirring was continued for 1 h, before a cold 5 wt% NaHSO4 solution was added to break up the gelatinous emulsion until pH 6–7. The solution was concentrated in vacuo, diluted with water (100 mL) and extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with saturated aqueous NaHCO3 and brine, dried over Na2SO4, filtered and concentrated under vacuum. Purification by flash column chromatography (silica gel; using 20 to 50% EtOAc in hexanes) provided the product 8 as a white foam (1.4 g, 70%). 1H NMR (500 MHz, CDCl3): δ 1.40 (s, 9H), 1.83-1.94 (m, 2H), 2.62-2.70 (m, 2H), 4.36 (br s, 1H), 4.52 (br s, 1H), 5.43 (br d, J = 14.5 Hz, 1H), 6.43 (br, J = 14.5 Hz, 1H), 6.87-6.90 (m, 1H), 7.13-7.30 (m, 5H), 7.51-7.61 (m, 3H), 7.61 (d, J = 6.0 Hz, 2H); LC-IT-TOF/MS (m/z) calcd for C22H27NO4S [M+Na]+: 424.1661, Found: 424.1575.

(S)-3-benzenesulfonyl-1-phenethylallylamine trifluoroacetate (TFA•HphVSPh, 9)

To a cooled (0°C) solution of 8 (1.2 g, 3 mmol) in CH2Cl2 (15 mL) was added drop-wise TFA (5 mL) via syringe. After stirring for 2 h, the reaction was added Et2O (100 mL). The precipitate was filtered off, washed twice with Et2O, and finally dried in vacuo to give 9 (0.95 g, 76%). 1H NMR (300 MHz, CDCl3): δ 1.40 (br s, 2H), 1.83-1.94 (m, 2H), 2.62-2.70 (m, 2H), 4.36 (br s, 1H), 4.52 (br s, 1H), 6.43 (br d, J = 14.5 Hz, 1H), 6.87-6.90 (m, 1H), 7.13-7.30 (m, 5H), 7.51-7.61 (m, 3H), 7.61 (d, J = 6.0 Hz, 2H); LC-IT-TOF/MS (m/z) calcd for C22H27NO4S [M+Na]+: 424.1661, Found: 424.1575.

(S)-3-benzenesulfonyl-1-phenethylallylamine trifluoracetate (TFA•HphVSPh, 9)

To a cooled (0°C) suspension of hexane-washed NaH (60% in mineral oil; 0.24 g, 6 mmol) in dry THF (50 mL) was added drop-wise 5 (1.61 g, 5.5 mmol) in dry THF (10 mL) via syringe. The mixture was stirred for an additional 30 min at 0 °C and 7 (1.32 g, 5 mmol) in dry THF (10 mL) was added drop-wise. The stirring was continued for 1 h, before a cold 5 wt% NaHSO4 solution was added to break up the gelatinous emulsion until pH 6–7. The solution was concentrated in vacuo, diluted with water (100 mL) and extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with saturated aqueous NaHCO3 and brine, dried over Na2SO4, filtered and concentrated under vacuum. Purification by flash column chromatography (silica gel; using 20 to 50% EtOAc in hexanes) provided the product 8 as a white foam (1.4 g, 70%). 1H NMR (300 MHz, CDCl3): δ 1.40 (s, 9H), 1.83-1.94 (m, 2H), 2.62-2.70 (m, 2H), 4.36 (br s, 1H), 4.52 (br s, 1H), 6.43 (br d, J = 14.5 Hz, 1H), 6.87-6.90 (m, 1H), 7.13-7.30 (m, 5H), 7.51-7.61 (m, 3H), 7.61 (d, J = 6.0 Hz, 2H); LC-IT-TOF/MS (m/z) calcd for C22H27NO4S [M+Na]+: 424.1661, Found: 424.1575.

(S)-N-(4-chlorobenzylidene)phenylalanine methyl ester (HCl•Phe-OMe, 10)

To a cooled (0 °C) suspension of phenylalanine (16.5 g, 100 mmol) in dry MeOH (150 mL) was added drop-wise SOCl2 (9 mL, 120 mmol) over 1 h. The mixture was kept cool in an ice-bath throughout the whole duration in order to
keep the temperature < 5 °C. The clear solution was stirred for 12 h and subsequently heated at 50 °C for 2 h. Upon evaporation of the solvent under reduced pressure, Et₂O (100 mL) was added with stirring. The precipitate was filtered off, washed twice with ether, and finally dried in vacuo to give 10 (21.6 g; 100 %) as a white solid. This material was pure enough to be used in the next step without further purification.

Methyl (S)-2-isocyanato-3-phenylpropanoate (OCN-PheOMe, 11)
To a solution of 10 (5.5 g, 25.5 mmol) in CH₂Cl₂ (50 mL) at 0 °C was added saturated aqueous NaHCO₃ (50 mL) and triphosgene (2.52 g, 8.42 mmol) in a single portion with vigorous stirring. The reaction mixture was stirred at 0 °C for 15 min and then poured into a 250-mL separatory funnel. The organic layer was collected, and the aqueous layer is extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with brine, dried (Na₂SO₄), vacuum filtered, and concentrated at reduced pressure using a rotary evaporator to give the product 11 as a colorless oil. ¹H NMR (300 MHz, CDCl₃): δ 3.03 (dd, J = 7.8, 13.8 Hz, 1H), 3.16 (dd, J = 4.8, 13.6 Hz, 1H), 3.81 (s, 3 H), 4.27 (dd, J = 4.61, 7.8 Hz, 1H), 7.18-7.21 (m, 2H), 7.27-7.36 (m, 3H). This material was used in the next step without further purification, assuming a quantitative yield.

tert-Butyl 1-piperazinecarboxylate (12)
To a solution of ditert-butyl dicarbonate (5.80 g, 25.54 mmol) in 50 mL of dry MeOH was added drop-wise a solution of piperazine (4.0 g, 46.44 mmol) in 100 mL of dry MeOH at 0 °C. After 30 min, the mixture was warmed to room temperature and the reaction was continued for 2 d. Upon concentration under reduced pressure, the crude solid was dissolved in 200 mL of Et₂O, and the left-over white precipitate was filtered off. The aqueous solution obtained by extracting the organic solution with 1 M citric acid (aq) (3 × 100 mL) was washed with EtOAc (3 × 100 mL) and brought to pH ~ 11 by adding solid K₂CO₃. The turbid solution was extracted with EtOAc (3 × 100 mL) and dried over Na₂SO₄. The solution was concentrated under reduced pressure at 40 °C and stripped with CH₂Cl₂ to yield a clear oil which was recrystallized into a white solid upon drying under reduced pressure. Yield: 71%; ¹H NMR (300 MHz, CDCl₃): δ 3.45-3.33 (m, 4H), 2.88-2.74 (m, 4H), 1.57 (s, 1H), 1.46 (s, 9H).

tert-Butyl 4-propargylpiperazine-1-carboxylate (13)
To a solution of 12 (1.86 g, 10 mmol) and diisopropylethylamine (1.9 mL, 11 mmol) in CHCl₃ (50 mL) at 0 °C was added drop-wise a solution of propargyl bromide (80% in toluene, 1.2 mL, 10mmol) in CHCl₃ (50 mL). After the mixture was stirred for 24 h at room temperature, the solution obtained was washed with 5% NaHCO₃ (3 × 50 mL), brine (2 × 50 mL), and then dried over Na₂SO₄. The solution was filtered and evaporated to provide a brown oil. Purification by flash column chromatography (silica gel; using 50% EtOAc in hexanes) provided the product 13 as a yellow oil (1.4 g, 86%), which ultimately crystallized uponstanding. ¹H NMR (500 MHz, CDCl₃): δ 1.49 (s, 9H), 2.26 (t, J = 2.5 Hz, 1H), 2.51 (t, J = 5.0 Hz, 4H), 3.32 (d, J = 2.55 Hz, 2H), 3.47 (t, J = 5.0 Hz, 4H); 13C NMR (125 MHz, CDCl₃): δ 29.10, 47.67, 52.32, 74.10, 79.10, 80.40, 155.39.

N-Propargylpiperazine•TFA salt (14)
To a solution of 13 (1.1 g, 5 mmol) in CH₂Cl₂ (25 mL) at 0 °C was added trifluoroacetic acid (25 mL). The solution was stirred at room temperature overnight, and then evaporated to dryness in vacuo. The residue was suspended in 20 mL of THF and used immediately in the next step without further purification.

(S)-methyl 3-phenyl-2-(4-(prop-2-yn-1-yl)piperazine-1-carboxamido)propanoate (15)
To a solution of 14 (0.7 g, 2 mmol) in dry THF (10 mL) at 0 °C was added drop-wise a solution of DIEA (0.7 mL, 4 mmol) in 10 mL of dry THF. After 10 min, a solution of 11 (0.68 g, 2.4 mmol) in dry THF (10 mL) was added. The mixture was stirred for 12 h and concentrated in vacuo to give a brown oil, which was subsequently diluted with water
(100 mL) and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered and concentrated in vacuo. Purification by flash column chromatography (silica gel; using 5 to 10% methanol in CH₂Cl₂) provided the product 15 as a white solid (0.53 g, 80%).

**1H NMR (500 MHz, CDCl₃):** δ 2.49-2.56 (m, 4H), 3.08-3.16 (m, 2H), 3.31-3.43 (m, 6H), 3.72 (s, 3H), 4.77-4.80 (m, 1H), 4.81-4.91 (m, 1H), 7.10-7.11 (m, 2H), 7.23-7.30 (m, 3H); **13C NMR (125 MHz, CDCl₃):** δ 38.97, 44.24, 47.53, 52.02, 52.87, 55.00, 74.23, 78.88, 127.68, 129.17, 129.96, 136.87, 157.07, 173.74; **LC-IT-TOF/MS (m/z) calcd for C₁₈H₂₃N₃O₃ [M+Na]+:** 352.1739, Found: 352.1738.

**(S)-3-phenyl-2-(4-(prop-2-yn-1-yl)piperazine-1-carboxamido)propanoic acid hydrochloride (16)**

To a solution of 15 (0.6 g, 1.8 mmol) in THF (30 mL) at 0 °C was added drop-wise a solution of LiOH•H₂O (0.23 g, 5.5 mmol) in 10 mL of H₂O. The mixture was stirred for 4 h, and 4 N HCl in dioxane was then added slowly to adjust the pH of the mixture to ~ 2 at 0 °C. The resulting solution was evaporated in vacuo. The residue was washed with Et₂O (2 × 25 mL), dried in vacuo, and then lyophilized overnight to give the crude product 16, along with a small amount of LiCl, which was used directly in the following reaction without further purification, assuming a quantitative yield.

**N-((S)-1-oxo-3-phenyl-1-((((S,E)-5-phenyl-1-(phenylsulfonyl)pent-1-en-3-yl)amino)propan-2-yl)-4-(prop-2-yn-1-yl)piperazine-1-carboxamide (1)**

To a solution of 16 (215 mg, 0.6 mmol) in DMF (5 mL) was added EDC/HCl (115 mg, 0.6 mmol), HOBt (81 mg, 0.6 mmol) and DIEA (0.4 mL, 2.4 mmol). After 10 min, TFA•HphVSPh (9; 208 mg, 0.5 mmol) in DMF (5 mL) was added drop-wise. The reaction was stirred at rt for 21 h. The resulting solution was evaporated in vacuo to give a brown oil, which was diluted with water (50 mL) and extracted with DCM (3 × 50 mL). The combined organic extracts were washed with saturated aqueous NaHCO₃ and brine, dried over Na₂SO₄, filtered and concentrated in vacuo. Purification by flash column chromatography (silica gel; using 5 to 10% methanol in CH₂Cl₂) provided the product 1 as a white solid (165 mg, 55%).

**2.2 Synthesis of compounds K11002 and K11777**

![Scheme S2. Synthesis of K11002 and K11777.](image)

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(S)-benzyl-2-isocyanato-3-phenylpropionate (OCN-PheOBzl, 17)

To a solution of (S)-Benzyl-2-amino-3-phenylpropionate hydrochloride (HCl•PheOBzl) (3.72 g, 12.75 mmol) in CH2Cl2 (50 mL) at 0 °C was added saturated aqueous NaHCO3 (50 mL) and triphosgene (1.25 g, 4.21 mmol) in a single portion with vigorous stirring. The reaction mixture was stirred at 0 °C for 15 min and then poured into a 250-mL separatory funnel. The organic layer was collected, and the aqueous layer is extracted with CH2Cl2 (3 × 15 mL). The combined organic layers were washed with brine, dried (Na2SO4), vacuum filtered, and concentrated at reduced pressure using a rotary evaporator to give a colorless oil. The product, OCN-PheOBzl was used in the next step without further purification, assuming a quantitative yield.

(S)-benzyl 2-(morpholine-4-carboxamido)-3-phenylpropanoate (18)

To a solution of 19 (3.59 g, 12.75 mmol) in dry THF (50 mL) at 0 °C was added morpholine (1.1 mL, 12.75 mmol). The mixture was stirred for 1 h and was concentrated in vacuo to a pale orange oil, and diluted with water (100 mL) and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with HCl (1 M), saturated aqueous NaHCO3 and brine, dried over Na2SO4, filtered and concentrated under vacuum. Purification by flash column chromatography (silica gel) using 10 to 20% EtOAc in hexanes to give the product (S)-benzyl 2-(morpholine-4-carboxamido)-3-phenylpropanoate (Mu-PheOBzl, 18) as a white solid (3.9 g, 83% over two steps). 1H NMR (300 MHz, CDCl3): δ 3.11 (d, J = 5.3 Hz, 2H), 3.27-3.31 (m, 4H), 3.62-3.65 (m, 4H), 4.81-4.90 (m, 2H), 5.15 (dd, J = 12.3, 27.8 Hz, 2H), 6.99 (dd, J = 3.5, 7.0 Hz, 2H), 7.19-7.22 (m, 3H), 7.29-7.38 (m, 5H); 13C NMR (75 MHz, CDCl3): δ 38.22, 43.92, 54.28, 66.41, 67.20, 127.01, 128.51, 128.58, 129.34, 135.19, 136.01, 156.66, 172.43.

(S)-2-(morpholine-4-carboxamido)-3-phenylpropanoic acid (19)

A solution of Mu-PheOBzl (18) (3.9 g, 10.6 mmol) in 1% HOAc/ethanol (100 mL) was charged with 10% palladium on active charcoal (Aldrich: 0.4 g). The solution in the Parr bottle was exposed to hydrogen on a Parr shaker (50 psi) for 12 h, filtered through Celite, and concentrated in vacuo. The residue was triturated with ether (100 mL) to remove residual ethanol and was reprecipitated from CH2Cl2/ether to give 2.94 g (99%) of (S)-2-(morpholine-4-carboxamido)-3-phenylpropanoic acid (Mu-PheOH, 19). 1H NMR (300 MHz, DMSO-d6) δ 2.86-2.94 (m, 1H), 3.00-3.16 (m, 1H), 3.18-3.28 (m, 4H), 3.41-3.48 (m, 4H), 4.19-4.27 (m, 1H), 6.72 (d, J = 8.2 Hz, 1H), 7.17-7.30 (m, 5H). 13C NMR (75 MHz, DMSO-d6) δ 36.60, 43.94, 65.87, 126.24, 128.08, 129.15, 138.44, 157.37, 174.24. Without further purification, the mixture was used directly in the next step.

N-((S)-1-oxo-3-phenyl-1-(((S,E)-5-phenyl-1-(phenylsulfonyl)pent-1-en-3-yl)amino)propan-2-yl)morpholine-4-carboxamide (K11002)

Prepared according to the similar procedure mentioned above by using 19 (290 mg, 1.04 mmol), 9 (420 mg, 1.0 mmol), EDC/HCl (190 mg, 1.0 mmol), HOBT (140 mg, 1.0 mmol) and DIEA (0.34 mL, 2 mmol) in DMF (5 mL). Purification by flash column chromatography (silica gel) using 25 to 50% EtOAc in hexanes to give K11002 as a white solid (450 mg, 80%). 1H NMR (500 MHz, CDCl3): δ 1.74-1.90 (m, 2H), 2.55-2.60 (m, 2H), 3.25 (d, J = 4.1 Hz, 2H), 3.25-3.34 (m, 4H), 3.59-3.65 (m, 4H), 4.51 (m, 1H), 4.62 (m, 1H), 5.06 (m, 1H), 6.10 (dd, J = 1.65, 15.1 Hz, 1H), 6.79 (dd, J = 4.85, 15.1 Hz, 1H), 7.07 (d, J = 7.65 Hz, 2H), 7.15-7.28 (m, 8H), 7.57 (t, J = 7.8 Hz, 2H), 7.65 (t, J = 7.4 Hz, 1H), 7.87 (d, J = 7.5 Hz, 2H); LC-IT-TOF/MS (m/z) calcd for C31H35N3O5S [M+H]+: 562.2297, Found: 562.2629.

(S)-benzyl 2-(4-methylpiperazine-1-carboxamido)-3-phenylpropanoate (20)

Prepared according to the similar procedure mentioned above by using (S)-benzyl-2-amino-3-phenylpropionate hydrochloride (HCl•PheOBzl) (5.84 g, 20 mmol), triphosgene (1.98 g, 6.67 mmol), and N-methylpiperazine (2.2 mL, 20 mmol). Purification by flash column chromatography (silica gel) using 5 to 10% methanol in DCM to give the
product [(S)-benzyl-2-[(4-methylpiperazine-1-carbonyl)amino]-3-phenylpropionate, MePip-PheOBzl, 20] as a pale orange oil.

(S)-2-(4-methylpiperazine-1-carboxamido)-3-phenylpropanoic acid (21)\(^7\)

Prepared according to the similar procedure mentioned above by using MePip-PheOBzl (20) (7.5 g, 19.7 mmol), 10% Pd/C (0.75 g) in 1% HOAc/ethanol (50 mL) under 50 psi for 12 h. The compound was obtained as white solid (5.61 g, 98%). \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 2.15 (s, 3H), 2.18 (m, 4H), 2.84-2.94 (dd, \(J\) = 10.8, 15.1 Hz, 1H), 2.95-3.04 (dd, \(J\) = 5.0, 15.1 Hz, 1H), 3.14-3.30 (m, 4H), 4.17 (m, 1H), 6.65 (d, \(J\) = 8.0 Hz, 1H), 7.17-7.27 (m, 5H).

4-Methyl-N-((S)-1-oxo-3-phenyl-1-(((S,E)-5-phenyl-1-(phenylsulfonyl)pent-1-en-3-yl)amino)propan-2-yl)piperazine-1-carboxamide (K11777)\(^7\)

Prepared according to the similar procedure mentioned above by using 21 (291 mg, 1.0 mmol), 9 (420 mg, 1.0 mmol), EDC/HCl (190 mg, 1.0 mmol), HOBt (140 mg, 1.0 mmol) and DIEA (0.34 mL, 2 mmol) in DMF (5 mL). Purification by flash column chromatography (silica gel) using 5 to 10% methanol in DCM to give K11777 as a white solid (260 mg, 45%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 1.76-1.88 (m, 2H), 2.27-2.35 (m, 7H), 2.55-2.60 (m, 2H), 3.05 (d, \(J\) = 7.5 Hz, 2H), 3.29-3.37 (m, 4H), 4.51 (m, 1H), 4.63 (m, 1H), 5.02 (m, 1H), 6.12 (dd, \(J\) = 1.55, 15.2 Hz, 1H), 6.79 (dd, \(J\) = 4.95, 15.1 Hz, 1H), 7.07 (d, \(J\) = 7.6 Hz, 2H), 7.15-7.30 (m, 8H), 7.57 (t, \(J\) = 7.9 Hz, 2H), 7.65 (t, \(J\) = 7.25 Hz, 1H), 7.87 (d, \(J\) = 1.3 Hz, 2H); LC-IT-TOF/MS (m/z) calcd for C\(_{32}\)H\(_{38}\)N\(_4\)O\(_4\)S [M+H]\(^+\): 575.2614, Found: 575.2600.

2.3 Synthesis of compound 2 (VS-2)

\((S)-methyl\ 2-(((4-ethynylbenzyl)oxy)carbonyl)amino)-3-phenylpropanoate (22)

To a solution of 11 (2.46 g, 12 mmol) in anhydrous toluene (25 mL) was added (4-ethynylphenyl)methanol (1.32 g, 10 mmol). The resulting solution was heated to 100 °C for 6 h and concentrated \textit{in vacuo} to give a pale orange oil, which was diluted with water (100 mL) and extracted with ether (3 \times 50 mL). The combined organic extracts were washed with HCl (1 M), saturated aqueous NaHCO\(_3\) and brine, dried over Na\(_2\)SO\(_4\), filtered and concentrated \textit{in vacuo}.

Purification by flash column chromatography (silica gel; using 10% EtOAc in hexanes) provided 22 as a white solid (2.65 g, 79%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 3.03-3.16 (m, 2H), 3.72 (s, 3H), 4.62-4.69 (m, 1H), 5.03-5.12 (m, 2H), 5.34 (br d, \(J\) = 12.65 Hz, 2H), 7.08-7.11 (m, 2H), 7.21-7.31 (m, 5H), 7.46 (d, \(J\) = 13.7 Hz, 2H); \(^13\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 38.90, 53.01, 55.49, 67.02, 78.19, 83.98, 122.60, 127.85, 128.46, 129.30, 129.92, 132.93, 136.32, 137.71, 156.12, 172.59.

\((S)-2-(((4-ethynylbenzyl)oxy)carbonyl)amino)-3-phenylpropanoic acid (23)
To a solution of 15 (3.4 g, 10 mmol) in THF (60 mL) at 0 °C was added drop-wise an aqueous solution of LiOH (0.72 g, 30 mmol) in 20 mL of H2O. The reaction was stirred for 2 h) of the starting ester, then acidified with 2 M HCl (to pH 2) and extracted with EtOAc (3 × 50 mL). Upon drying over Na2SO4, filtration and evaporation of the organic phase, the compound was used directly in the following reaction without further purification (assuming quantitative yield). 1H NMR (300 MHz, DMSO-d6): δ 2.84 (m, 1H), 2.88-3.11 (m, 1H), 4.17-4.23 (m, 2H), 4.99 (s, 2H), 7.20-7.31 (m, 2H), 7.45 (d, J = 13.45 Hz, 2H), 7.68 (d, J = 13.95 Hz, 1H); 13C NMR (75 MHz, DMSO-d6): δ 36.49, 55.52, 64.73, 80.86, 83.27, 121.01, 126.39, 127.54, 128.18, 129.09, 131.65, 137.86, 138.00, 155.88, 173.26.

4-Ethynylbenzyl((S)-1-oxo-3-phenyl-1-(((S,E)-5-phenyl-1-(phenylsulfonyl)pent-1-en-3-yl)amino)propan-2-yl)carbamate (2)

Prepared according to the same procedure mentioned above by using 23 (162 mg, 0.5 mmol), 9 (208 mg, 0.5 mmol), EDC/HCl (115 mg, 0.6 mmol), HOBt (81 mg, 0.6 mmol) and DIEA (0.2 mL, 1.2 mmol) in DMF (5 mL). Purification by flash column chromatography (silica gel; using 20% EtOAc in hexanes) provided 2 as a white solid (258 mg, 85%). 1H NMR (500 MHz, CDCl3): δ 1.74-1.79 (m, 1H), 1.86-1.89 (m, 1H), 2.52-2.57 (m, 2H), 2.95-3.03 (m, 2H), 3.09 (s, 1H), 4.27 (s, 1H), 4.64 (dd, J = 3.5, 5.0 Hz, 1H), 5.05 (s, 2H), 5.23 (br d, J = 6.25 Hz, 1H), 5.79 (br d, J = 7.55 Hz, 1H), 6.04 (dd, J = 0.9, 15.1 Hz, 1H), 6.75 (dd, J = 4.7, 15.1 Hz, 1H), 7.03 (d, J = 7.2 Hz, 2H), 7.11 (d, J = 7.1 Hz, 2H), 7.16-7.28 (m, 8H), 7.44 (d, J = 8.1 Hz, 2H), 7.55 (t, J = 7.8 Hz, 2H), 7.63 (t, J = 7.35 Hz, 1H), 7.85 (d, J = 7.65 Hz, 2H); LC-IT-TOF/MS (m/z) calcd for C36H34N2O5S [M+H]+: 607.2188, Found: 607.2078.

2.4 Synthesis of compound 3 (VS-3)

Diethyl (((4-(prop-2-yn-1-yloxy)phenyl)sulfonyl)methyl)phosphonate (24)

A mixture of 4-hydroxy-thiophenyl-methyl-diethylphosphonate sulfone 8 (3.08 g, 10 mmol) and anhydrous K2CO3 (1.66 g, 12 mmol) in dry acetone (50 mL) was stirred at rt for 2 h. 80% of propargyl bromide in toluene (1.25 mL, 11 mmol) was added drop-wise. The mixture was then stirred for 12 h, and TLC analysis indicated all the starting materials had been consumed. Upon removal of acetone under reduced pressure, the reaction mixture was poured into water (50 mL) and extracted with EtOAc (3 × 50 mL). The combined organic layer was washed successively with 1 M HCl, water, and brine. Upon drying over Na2SO4, filtration and evaporation of the organic phase, the compound was purified by flash column chromatography (silica gel; using 50% EtOAc in hexanes) provided 24 as a white solid (3.01 g, 87%). 1H NMR (500 MHz, CDCl3): δ 1.30 (t, J = 7.0 Hz, 6H), 2.57 (t, J = 2.4 Hz, 1H), 3.74 (d, J = 16.8 Hz, 2H), 4.15 (m, 4H), 4.78 (d, J = 2.4 Hz, 2H), 7.10-7.23 (m, 2H), 7.93-7.95 (m, 2H).

Scheme S4. Synthesis of probe 3 (VS-3)
(S,E)-tert-butyl (5-phenyl-1-((4-(prop-2-yn-1-yloxy)phenyl)sulfonyl)pent-1-en-3-yl)carbamate (25)

Prepared according to the same procedure mentioned above by using 24 (3.0 g, 8.67 mmol), 7 (2.1 g, 7.88 mmol), and NaH (60% in oil, 0.38 g, 9.5 mmol) in anhydrous THF (100 mL). Purification by flash column chromatography (silica gel; using 20% EtOAc in hexanes) provided 25 as a white solid (2.67 g, 75%). 1H NMR (500 MHz, CDCl3): δ 1.40 (s, 9H), 1.78-1.86 (m, 1H), 1.89-1.96 (m, 1H), 2.55 (t, J = 2.5 Hz, 1H), 2.62-2.70 (m, 2H), 4.35 (br s, 1H), 4.52 (br s, 1H), 4.76 (d, J = 1.9 Hz, 2H), 6.40 (d, J = 15.1 Hz, 1H), 6.84 (dd, J = 3.8, 14.5 Hz, 1H), 7.08 (t, J = 3.15 Hz, 2H), 7.14 (d, J = 6.95 Hz, 2H), 7.18-7.30 (m, 3H), 7.81 (dd, J = 2.55, 11.35 Hz, 2H); 13C NMR (125 MHz, CDCl3): δ 28.23, 31.89, 35.96, 50.62, 56.03, 76.53, 77.30, 80.23, 115.44, 126.32, 128.32, 128.61, 129.84, 131.09, 132.67, 140.75, 145.23, 154.88, 161.42, 184.26.

(S,E)-5-phenyl-1-((4-(prop-2-yn-1-yloxy)phenyl)sulfonyl)pent-1-en-3-amine trifluoroacetate (26)

Prepared according to the same procedure mentioned above by using 25 (2.28 g, 5.0 mmol) in 100 mL of TFA/DCM (1/1). Upon completion of the reaction, the mixture was precipitated with Et2O, filtered off, washed twice with Et2O, and finally dried in vacuo to give 2.3 g (98%) of 26. This material was pure enough to be used in the next step without further purification.

N-((S)-1-oxo-3-phenyl-1-(((S,E)-5-phenyl-1-((4-(prop-2-yn-1-yloxy)phenyl)sulfonyl)pent-1-en-3-yl)amino)prop-2-yl)morpholine-4-carboxamide (3)

Prepared according to the same procedure mentioned above by using 19 (139 mg, 0.5 mmol), 26 (234 mg, 0.5 mmol), EDC/HCl (115 mg, 0.6 mmol), HOBt (81 mg, 0.6 mmol) and DIEA (0.2 mL, 1.2 mmol) in DMF (5 mL). Purification by flash column chromatography (silica gel; using 20% EtOAc in hexanes) provided 3 as a white solid (265 mg, 86%). 1H NMR (500 MHz, CDCl3): δ 1.64-1.85 (m, 2H), 2.41 (t, J = 7.55 Hz, 2H), 2.59 (t, J = 1.8 Hz, 1H), 3.03-3.11 (m, 2H), 3.22-3.31 (m, 4H), 3.60-3.66 (m, 4H), 4.46 (dd, J = 7.6, 15.2 Hz, 1H), 4.60-4.62 (m, 1H), 4.78 (d, J = 2.55 Hz, 2H), 5.03 (d, J = 7.55 Hz, 1H), 6.39 (br s, 1H), 6.50 (br d, J = 15.15 Hz, 1H), 6.80 (dd, J = 5.0, 15.1 Hz, 1H), 7.05 (d, J = 6.95 Hz, 2H), 7.08 (dd, J = 1.9, 6.95 Hz, 2H), 7.20-7.30 (m, 8H), 7.80 (dd, J = 1.85, 6.9 Hz, 2H); 13C NMR (125 MHz, CDCl3): δ 31.68, 35.48, 37.97, 43.92, 49.15, 56.04, 56.20, 66.27, 76.62, 77.32, 115.41, 126.25, 127.15, 128.32, 128.53, 128.79, 129.22, 129.84, 131.16, 132.63, 136.68, 140.37, 144.48, 157.23, 161.41, 171.64; LC-IT-TOF/MS (m/z) calcd for C34H37N3O6S [M+H]+: 616.2403, Found: 616.2293.

3. Biological and Other Experiments.

3.1 General.

Anti-cathepsin L (ab6314) was from Abcam. Anti-rhodesain, and anti-TbCatB were generous gifts from James H. McKerrow (University of California, San Francisco). Other reagents are from commercial sources, unless otherwise indicated. For Cell Cultures, T. brucei procyclic cells YTAT 1.1 were grown at 28 °C and 5% CO2 in Cunningham's medium supplemented with 15% heat-inactivated fetal bovine serum (FBS). T. brucei BSF cells were grown at 37 °C and 5% CO2 in HMI-9 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS). HepG2 cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in a humidified 37 °C incubator with 5% CO2.

3.2 Molecular modeling.
Fig. S2. Molecular docking experiments were carried out as previously described. Superimposition of rhodesain•K11777 (PDB entry 2P7U) and rhodesain•K11002 (PDB entry 2P86) are shown. Images were generated with PyMOL.

3.2 Guava ViaCount anti-trypanocidal assay.

Parasite number and percentage viability were determined in 96-well plate format using the Guava ViaCount assay on a Guava PCA-96 system (Guava Technologies, USA) following the manufacturer's instructions. Briefly, BSF and PCF trypanosomes were harvested in exponential growth phase and adjusted to a concentration of $1 \times 10^5$ cells/mL in complete growth medium. Diluted trypanosomes were dispensed manually using a multichannel pipette. After 24 h of incubation with compounds, the final DMSO concentration in the assay never exceeded 1% in cultivation medium, and medium containing 1% DMSO was used as a negative control. Cell density and viability were evaluated using ViaCount assay on the Guava PCA-96 system. ED$_{50}$ values were calculated by sigmoid curve fitting with GraphPad Prism 5.0 software (San Diego, USA). All data were collected in triplicate.

3.3 *In situ* proteomic profiling and in-gel fluorescence scanning.

*T. brucei* parasites were plated into 6-well plates (PCF, 2 mL at $\approx 1 \times 10^5$ cells/mL) or 25-mL cell culture flasks (BSF, 10 mL at $\approx 2 \times 10^6$ cells/mL), and incubated with probe for 2 h at culture temperature with or without a competing inhibitor, K11777. All compounds were solubilized in DMSO. To avoid adverse effects on parasite growth, the final DMSO concentration in the assay never exceeded 1% in cultivation medium. After incubation, the parasite cells were pelleted at 2,000 rpm for 10 min, washed twice with PBS and re-suspended in PBS ($100 \mu$L). Cells were homogenized by sonication, and diluted to $\approx$ 1 mg/mL with PBS. To initiate the click chemistry reaction, 20 $\mu$L of freshly premixed solution containing rho-azide (100 $\mu$M final concentration), TCEP (1 mM final concentration), ligand (100 $\mu$M final concentration), and CuSO$_4$ (1 mM final concentration) was added. The reaction was incubated at 10 °C for 4 h with gentle mixing. Termination of the reaction was done by addition of pre-chilled acetone (0.5 mL). The resulting solution was then placed at -20 °C for 30 min, followed by centrifugation (13000 rpm × 10 min) at 4 °C. The supernatant was discarded and the precipitated protein pellets were washed with pre-chilled methanol (2 × 200 $\mu$L), air-dried for 10 min, resuspended in 1 × standard reducing SDS-loading buffer (25 $\mu$L) then heated for 10 min at 95 °C. Finally, the protein sample ($\approx$ 20 $\mu$g/lane) was loaded onto 12% SDS-PAGE gel, separated followed by in-gel fluorescence scanning with a Typhoon 9410 Variable Mode Imager scanner (GE Amersham).

Fig. S3 Dose-dependent *in situ* proteome profiling of *T. brucei* in BSF and PCF with VS-1.
3.4 Affinity pull-down and LC/MS-MS experiments.

For proteomic experiments, BSF and PCF trypanosomes (≈2 × 10⁹ cells, ≈5 mg each), labeled in Cunningham’s media (1 × 10⁷ cells/mL) with VS-1 (25 µM) or DMSO (negative control), were harvested, washed and homogenized in PBS. CuAAC reagents were added at the same concentrations as described above, except that biotin-azide was substituted for rho-azide. Acetone-precipitated and methanol-washed protein pellets were solubilized in PBS containing 0.1% (w/v) SDS by brief sonication. Insoluble materials were precipitated by centrifugation (13,000g × 10 min) at 4 °C. The supernatants were then incubated with gentle shaking at 4 °C overnight with Neutravidin agarose beads (50 µL/mg protein, Prod # 29204, Thermo Scientific, USA) which have been pre-washed twice with PBS. After centrifugation, the bead/complexes were washed extensively 8 times with 1% (w/v) SDS in PBS, three times with PBS and twice with 250 mM of ammonium bicarbonate (ABC). Elution of bound proteins from beads was then performed twice using the boiling buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% (w/v) SDS), then pooled. Protein samples were concentrated using an YM-10 Centricon spin column (Millipore, USA). Following SDS-PAGE separation, protein bands were visualized by Coomassie blue staining. Gel lanes corresponding to both DMSO- and VS-1-treated samples were then each cut into 10 slices. Subsequent trypsin digestion (using In-Gel Trypsin Digestion Kit, Pierce Co., USA) and peptide extraction (with 50% acetonitrile and 1% formic acid) generated a total of 10 LCMS samples for each pull-down experiment. All samples were dried in vacuo and stored at -20 °C until future LCMS analysis.

Each LCMS sample was resuspended in 0.1% formic acid for mass spectrometry analysis as previously described. Briefly, peptides were separated and analyzed on a Shimadzu UFLC system (Shimadzu, Kyoto, Japan) coupled to an LTQ-FT Ultra (Thermo Electron, Germany). Mobile phase A (0.1% formic acid in H₂O) and mobile phase B (0.1% formic acid in acetonitrile) were used to establish the 60 min gradient comprised of 45 min of 5-35% B, 8 min of 35-50% B and 2 min of 80% B followed by re-equilibrating at 5% B for 5 min. Peptides were then analyzed on LTQ-FT with an ADVANCE™ CaptiveSpray™ Source (Michrom BioResources, USA) at an electrospray potential of 1.5 kV. A gas flow of 2 L/min, ion transfer tube temperature of 180°C and collision gas pressure of 0.85 mTorr were used. The LTQ-FT was set to perform data acquisition in the positive ion mode as previously described except that the m/z range of 350-1600 was used in the full MS scan. The raw data were converted to mgf format as previously described. The database (76708 sequences, 33362815 residues) used for Mascot search was a concatenated T. brucei protein database. The database search was performed using an in-house Mascot server (version 2.2.07, Matrix Science, UK) with MS tolerance of 10 ppm and MS/MS tolerance of 0.8 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification, and oxidation (M) and phosphorylation (S, T and Y) were set as variable modifications.

LCMS results obtained from above experiments (with VS-1 as well as with DMSO as a negative control) were processed as above. As in the case of most large-scale LCMS experiments, a large number of proteins were identified from each LCMS run, many of which were “sticky” and/or highly abundant proteins. These proteins were excluded. For those proteins that appeared in the “negative” run (i.e. pull-down/LCMS experiments with DMSO in place of VS-1), they were automatically removed from the list as well. The final list was shown in SI_2. From this list, we placed our focus on those proteins that might be potential K11777 targets, and they were shown in Table S1 (in ESI) and Table 1 in the main text.

3.5 Pull-down and western blotting analysis.

Pull-down samples from in situ labeling with VS-1 (25 µM) were separated on 12% SDS-PAGE gel together with pull-down sample from DMSO-treated (negative control). After SDS-PAGE gel separation, proteins were then transferred to a PVDF membrane and subsequently blocked with 3% (w/v) BSA/PBST overnight at 4°C. Membranes were incubated for 1 h at room temperature with the respective antibodies (anti-cathepsin L for HepG2; anti-rhodesain, or anti-TbcatB for T. brucei), and washed with PBST (3 × 15 min with gentle agitation), then followed by incubation
with an anti-mouse-IgG conjugated secondary antibody in the blocking buffer mentioned above. After washing with PBST (3 × 15 min with gentle agitation), the SuperSignal West Pico kit (Pierce) was used to develop the blot.

3.6 Fluorescence Microscopy.

For drug uptake analysis, trypanosomes (1 × 10^5 cells/mL for both forms) were incubated in growth medium containing different concentrations of VS-1 at culture temperature and 5% CO₂ for 2 h. Medium containing 1% DMSO was used as a negative control. The parasites were then washed twice with PBS, and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed with PBS (2 × 5 min with gentle agitation), and then sedimented to poly-L-lysine-coated coverslips. Fixed cells were permeabilized with 0.25% Triton-X 100 in PBS for 15 min at room temperature, and washed with PBS (2 × 5 min with gentle agitation). The cells were blocked with 3% BSA in PBS for 30 min at room temperature, and washed with PBS (2 × 5 min with gentle agitation). The cells were then treated with a freshly pre-mixed click chemistry reaction solution [rhodamine-azide (10 µM final concentration from a 10 mM stock solution in DMSO), TCEP (1 mM final concentration from a 50 mM freshly prepared stock solution in deionized water), TBTA (100 µM final concentration from a 10 mM stock solution in DMSO), and CuSO₄ (1 mM final concentration from a 100 mM freshly prepared stock solution in deionized water)] in PBS for 1 h at room temperature. The cells were washed with PBS (1 × 5 min with gentle agitation), and cold methanol (1 × 5 min with gentle agitation), followed by 1% Tween-20 and 0.5 mM of EDTA in PBS (3 × 2 min with gentle agitation), and with PBS (2 × 5 min with gentle agitation). The cells were then incubated in PBS containing 2 µg/mL of DAPI for 15 min at room temperature to stain the kinetoplast and nuclear DNA, and washed with PBS (2 × 5 min with gentle agitation) and a final wash with deionized water (1 × 5 min with gentle agitation) before mounting onto the Fluoromount G (Emulsion, USA). For immunofluorescence (IF) analysis, cells were then incubated for 1 h in PBS with anti-rhodesain and washed with PBS (3 × 5 min with gentle agitation), followed labeled with FITC-conjugated anti-rabbit IgG (1:500) and a final wash with PBS (3 × 5 min with gentle agitation) before mounting. Confocal images were taken on a Leica TCS SP5X Confocal Microscope System equipped with Leica HCX PL APO 100×/1.40 oil objective, 405 nm Diode laser, White laser (470 nm to 670 nm, with 1 nm increments, with 8 channels AOTF for simultaneous control of 8 laser lines, each excitation wavelength provides 1.5 mV, PMT detector range from 420 nm to 700 nm for steady state fluorescence. DAPI, FITC and rhodamine were excited with a krypton/argon laser at 405, 488 nm and 554 nm, respectively, and the emission was collected through a 420–470, 500–550 and 565–650 nm filters, respectively. Images were processed with Leica Application Suite Advanced Fluorescence (LAS AF).

**Fig. S4** Cellular uptake of VS-1 within *T. brucei*. Parasites (2 × 10^5 cells) were incubated with VS-1 (at 0, 10 and 25 µM, respectively) for 2 h, reacted with 10 µM of rho–azide under CuAAC conditions, and then imaged. DAPI stained (with nucleus and/or kinetoplast pseudocolored in Blue); Rhodamine channel showing cellular distribution of VS-1 (pseudocolored in Red). Scale bar represents 10 µm.
Fig. S5 Confocal microscope images of rhodesain in BSF (top) and PCF (bottom) treated with DMSO and immunofluorescence staining. Panel (a) and (e): Bright field images of the corresponding parasites. Panel (b) and (f): 554 nm channel (pseudocolored in red). Panel (c) and (g): immunofluorescence staining at 488 nm channel (pseudocolored in green) using anti-rhodesain primary antibody and FITC-conjugated anti-rabbit IgG secondary antibody detecting cellular localization of rhodesain. Panel (d) and (g): merged images of panels (b) and (c), (f) and (g) together with stained nuclei (with DAPI; pseudocolored in blue). All images were acquired under the same settings. Scale bar = 10 μm.

4. In Situ Proteomic Profiling and Fluorescence Microscopy in HepG2 Mammalian Cells.

For in situ proteomic profiling and cellular imaging of HepG2 live cells using VS-1, our previous published procedures were followed. Briefly, cells were grown to 80-90% confluence in 24-well plates, and medium was removed, washed twice with cold PBS, then treated with 0.5 mL of DMEM-containing probe for 2 h (the final DMSO concentration in the assay never exceeded 1% in cultivation medium) as previously described. After incubation, the growth medium was aspirated, and cells were washed twice with PBS to remove the excessive probe, trypsined, and pelleted at 1,000 rpm for 10 min, washed twice with PBS and re-suspended in PBS (100 μL). Cells were homogenized by sonication, and diluted to ~1 mg/mL with PBS, then followed by click chemistry, SDS-PAGE gel analysis, and in-gel fluorescence scanning (Fig. S6). For cellular imaging, cells were grown to ~50% confluence in 24-well plates containing sterile glass coverslips, and medium was removed, washed twice with cold PBS, then treated with 0.5 mL of DMEM-containing probe for 2 h. After incubation, the growth medium was aspirated, and cells were washed twice with PBS. Cells were fixed, permeabilized, and blocked, then followed by click chemistry, washing, staining (for IF, using mouse anti-cathepsin L; 1:100) and mounting mentioned above. Confocal images were taken as above described using a Leica TCS SP5X Confocal Microscope System equipped with Leica HCX PL APO 63x/1.20 W CORR CS (Fig. S7).

Fig. S6 In situ proteome-profiling of VS-1 against HepG2 live cells and Western blotting analysis of pulled-down fractions treated with VS-1 (25 μM), or DMSO as negative controls with anti-cathepsin L antibody.
**Fig. S7** Confocal microscope images of cathepsin L in HepG2 cells treated with DMSO (top) or VS-1 (bottom) and immunofluorescence staining. Panel (a) and (e): Bright field images of the corresponding cells. Panel (b) and (f): 554 nm channel (pseudocolored in red) detecting cellular localization of VS-1. Panel (c) and (g): immunofluorescence staining at 488 nm channel (pseudocolored in green) using anti-cathepsin L primary antibody and FITC-conjugated anti-rabbit IgG secondary antibody detecting cellular localization of cathepsin L. Panel (d) and (h): merged images of panels (b) and (c), (f) and (g) together with stained nuclei (with Hoechst; pseudocolored in blue). All images were acquired under the same settings. Scale bar = 10 μm.

5. Affinity Pull-Down and LC/MS-MS Results

Details are described in the main text and key proteins (i.e., putative drug targets) were summarized in Table 1. Table S1 provides a list of the rest of functional proteins (some of them are also putative drug targets), many of which are high-abundance proteins (such as proteins involved in carbohydrate metabolism), and some are sensitive to RNA interference. Though they only appeared in our positive pull-downs, some of them could be due to non-specific bindings (as a result of high abundance). The complete list is shown in SI_2.

<p>| <strong>Table S1.</strong> Representative proteins identified in <em>Trypanosoma brucei</em>&lt;sup&gt;a&lt;/sup&gt; |
|---|---|---|---|
| <strong>T. brucei</strong> | <strong>protein name</strong> | <strong>M&lt;sub&gt;w&lt;/sub&gt;/kDa</strong> | <strong>location</strong> | <strong>detection</strong> |
| <strong>Carbohydrate metabolism</strong> |
| Tb10.70.5820 | hexokinase1 (HK1)** | 51.3 | G | both |
| Tb10.70.1370 | fructose-biphosphatase aldolase (ALD)** | 41.07 | G | both |
| Tb927.3.3270 | ATP-dependent phosphofructokinase (PFK)** | 53.52 | G | both |
| Tb11.02.3210 | trisephosphate isomerase (TIM)** | 26.82 | G | both |
| Tb927.6.4280 | glyceraldehyde 3-phosphate dehydrogenase (GAPDH)** | 39.05 | G | both |
| Tb927.8.3530 | glyceraldehyde 3-phosphate dehydrogenase [NAD+]** | 37.81 | G | both |
| Tb927.1.700 | phosphoglycerate kinase (PGK)** | 47.25 | G | BSF |
| Tb09.211.3550 | glycerol kinase | 56.37 | G | both |
| Tb10.70.4740 | enolase** | 46.59 | G | PCF |
| <strong>Amino acid metabolism</strong> |
| Tb927.6.4840 | S-adenosylhomocysteine hydrolase | 43.54 | n/a | PCF |
| Tb09.160.4560 | arginine kinase | 44.72 | G | PCF |
| Tb927.8.6060 | 2-amino-3-ketobutyrate coenzyme A ligase | 43.74 | M | PCF |
| <strong>Protein synthesis</strong> |</p>
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**Cytoskeletal proteins**

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<tr>
<td>Tb927.3.4290</td>
<td>73 kDa paraflagellar rod protein (PFR1)*</td>
<td>68.68</td>
<td>flagellum both</td>
</tr>
</tbody>
</table>

* G, M and n/a represent, respectively, glycosomal, mitochondrial and not available. Symbols in the protein name column: *, sensitive to RNA interference; **, putative drug target.

### 6. References

N-Me-Pip-Phe-Hph-V8Ph in CDCl3 1H AMX500

General

SI = 16384
SF = 500.13
SW_p = 10330.579

K11002
**Electronic Supplementary Material (ESI) for Chemical Communications**

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*** Current Data Parameters ***
NAME : ag19py
EXPNO : 2
PROCNO : 1

*** Acquisition Parameters ***
DATE_t : 05:05:51
DATE_d : Aug 19 2009
DBPNAM0 : 
INSTRUM : spect
LOCNUC : 2H
NS : 400
NUCLEUS : off
O1 : 7924.11 Hz
SFO1 : 75.4756731 MHz
SOLVENT : CDCl3

*** 1D NMR Plot Parameters ***
NUCLEUS : off

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py0422 Allyne-Cbz-Fle-Hph-VSp in CDCl3 1H Allyx500

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**Proteins who were also detected in procyonal forms are shaded in Turquoise.**