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Design and evaluation of *Trypanosoma brucei* metacaspase inhibitors

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Abstract

Metacaspase (MCA) is an important enzyme in *Trypanosoma brucei*, absent from humans and differing significantly from the orthologous human caspases. Therefore MCA constitutes a new attractive drug target for antiparasitic chemotherapeutics, which needs further characterization to support the discovery of innovative drug candidates. A first series of inhibitors has been prepared on the basis of known substrate specificity and the predicted catalytic mechanism of the enzyme. In this Letter we present the first inhibitors of TbMCA2 with low micromolar enzymatic and antiparasitic activity *in vitro* combined with low cytotoxicity.

In 2000, metacaspases (E.C. 3.4.22) were identified as a new family of caspase-like proteins.[1] Metacaspases, together with caspases and paracaspases, are endopeptidases that belong to family C14 in clan CD of the cysteine peptidases. They share the caspase fold[2] and their active site comprises a conserved His-Cys catalytic dyad, the cysteine being the active site nucleophile. All metacaspases examined to date have a basic P1 specificity towards Arg/Lys, whereas caspases have an acidic P1 specificity towards Asp.[2] This explains why metacaspases are not able to cleave known caspase substrates and are not inhibited by caspase inhibitors (zVAD, DEVD-CHO).[3] Metacaspases are not present in mammals and have little overall sequence homology with human caspases, making them good potential drug targets. In *Trypanosoma brucei*, five metacaspases have been identified denoted TbMCA1-5. Interestingly, TbMCA1 and TbMCA4 genes encode a serine in place of a cysteine in the catalytic dyad, thus cannot function as cysteine peptidases, although they may still have activity through the use of the substituent serine as a nucleophile. Of the other three TbMCAs, TbMCA2 and 3 have demonstrated activity and TbMCA5 is expected to have similar activity.[4] All three have been validated as drug targets by triple RNAi.[5] Triple RNAi analysis (simultaneous down-regulation) showed TbMCA2, TbMCA3 and TbMCA5 to be essential in the bloodstream form (BSF), with parasites accumulating pre-cytokinesis, meaning cell division failed. However, this effect could not be confirmed with triple null mutants (*Δmca2/3Δmca5*). After an initial slow growth phase following sequential gene deletion, they grew well under both standard *in vitro* and *in vivo* conditions, suggesting compensatory activation of alternative pathways. However, mutant strains remained highly

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sensitive to changes to the standard conditions *in vitro*. Overall, it seems metacaspases fulfil an important role in BSF *T. brucei*, but the likelihood of overlapping functions means that therapeutic targeting would require inhibition of multiple TbMCAs and/or enzymes of the alternative pathways.

The functions of metacaspases in protozoa remain unclear. It has been proposed that in trypanosomatids metacaspases function as caspase-like enzymes and are involved in Programmed Cell Death (PCD).6,7,8 However, there are still doubts about the existence of PCD in trypanosomatids and other unicellular organisms9,10 and some authors oppose the PCD function of metacaspases in protozoa by pointing out their different substrate specificity, which makes a similar function to caspases unlikely.2 Helms *et al.*5 suggest that MCAs have a function associated with RAB11 vesicles that is independent of known recycling processes of RAB11-positive endosomes. Clearly, the precise role of MCAs remains to be elucidated. Nonetheless, the fact that *T. brucei* metacaspases seem to play a vital role in the parasite, and are absent in humans makes them attractive drug targets.11

In the absence of crystallographic data and full knowledge of the natural substrates, we describe the application of a rational design where the substrate specificity of TbMCA2 was used to design and synthesise the first specific MCA inhibitors. It is known that for peptides the S1 subsite selectivity of TbMCA2/3 is limited to Arg/Lys2 (Fig. 1A), leading to initial substrate-based inhibitor design with Arg/Lys in the P1 position. Previously, potent cysteine peptidase inhibitors have been obtained by replacing the scissile amide bond by an electrophile (P1′). Reversible competitive inhibitors were designed by using a nitrile12 or α-ketoheterocyclic warhead13. For the latter, the formation of a hydrogen bond between the sp2 nitrogen of the ketoheterocycle and protonated histidine from the His-Cys dyad was hypothesized. Irreversible inhibitors were developed with a vinylsulfone warhead. Irreversible peptidase inhibitors can be advantageous for the treatment of antiparasitic diseases, as these infections usually only require short courses of treatment; thus avoiding tissue accumulation and long term safety issues. In addition, irreversible inhibitors often show more efficient *in vivo* antiparasitic activities than reversible inhibitors.11

Our strategy was to develop a small diversity set of inhibitors in order to select a first hit from a screening assay on TbMCA2. Different parameters were tested: a set of warheads was investigated and peptidomimetic inhibitors were designed using a peptoid backbone. In addition, an attempt to influence basicity at P1 position was made. From this first screen a hit was selected which was then subjected to a range of optimization efforts. Several Arg mimetics were introduced in P1 and also P2 modification was addressed in order to obtain more potent inhibitors and gain insight in the scope of the active site of MCAs.

For a first set of inhibitors we started from the α-amino protected arginine with a nitrile warhead **1** (Fig. 1B). In the P2 position the protecting group benzyloxycarbonyl (Cbz) was used. Diversification was carried out at the level of the P1 position (Fig. 1B, **2** and **3**). To circumvent possible membrane permeability issues which are known for highly basic residues such as amidines and guanidines, we selected functional groups in the P1 side chain with lower basicity. First, aromatic functions were introduced in the P1 tail (**2** and **3**). A second set of inhibitors was then designed with a peptoid backbone (Fig. 2, **4**-**10**) and a variety of functional groups in the P1 position which were intended to have a similar basicity-lowering effect.

A third set of inhibitors (Fig. 3) was derived from α-amino protected arginine with a α-ketoheterocyclic warhead (P1′). Different inhibitors **11-14** were screened and resulted in our first hit **11** (IC_{50} TbMCA2 = 0.6 μM).

One inhibitor with a warhead known to have irreversible binding properties towards cysteine peptidases was included in our diversity set, derived from α-amino protected arginine with a vinylsulfone warhead **15** (Fig. 4).

The synthesis of inhibitors **1-15** is shown in schemes 1-6. For compound **1**, commercially available Z-ArgPmc-OH (**21**) was converted to the corresponding amide (**22**) in the presence of *N*-hydroxysuccinimide, dicyclohexyl carbodiimide (DCC) and ammonia. The amide was then dehydrated with trifluoroacetic anhydride (TFAA) to the corresponding nitrile. TFA deprotection afforded target compound **1** (scheme 1).

4-Nitro-L-phenylalanine **23** was first protected with a Cbz group. Then the nitro group was reduced with $SnCl_2$ and the obtained amine function was protected with a *tert*-butyloxycarbonyl (Boc) group (**24**). This intermediate was converted to the final compound **2** following the same synthetic steps as described for **1** (scheme 2).

For target compound **3**, intermediate **25** was first Boc-deprotected in order to introduce a Boc-protected guanidine function, then a similar synthetic strategy was followed.

For target compound **4**, synthesis was started from the aminoacetonitrile derivative **27** which was synthesised from *tert*-butyl 4-aminopiperidine-1-carboxylate **26** and bromoacetonitrile. After Cbz-protection of **27** followed by a final TFA deprotection, the desired compound was obtained (scheme 3).

Target compound **5** was synthesised from commercially available 4-aminobutanol (**28**) and bromoacetonitrile, followed by a Cbz-protection of the free amine (scheme 4). Compound **5** was then converted via a mesylate to intermediate **29** using a Finkelstein reaction.14 A nucleophilic substitution with different amino moieties afforded target compounds **6-10** (scheme 4).

For compounds **11**, **12** and **14** the synthesis described by Costanzo *et al.* was followed.13 For compound **13**, the protocol reported by Lin *et al.* was used.15 The synthesis is shown for **11-14** starting from Z-Arg-Pmc-OH (**21**) (scheme 5).

To obtain compound **15**, commercially available Z-Orn-Boc-OH (**31**) was converted to the corresponding Weinreb amide. The amine function was deprotected and converted to Boc-protected guanidine (**32**). Then the protected arginine Weinreb amide was reduced to the aldehyde (**33**) in which a Horner-Emmons reaction was executed in the presence of diethyl phenylsulfonylmethylphosphonate **34** to give the desired vinylogous arginine.16 A final deprotection in acidic medium provided target compound **15** (scheme 6).

All target compounds were biochemically evaluated by *in vitro* testing as competitive inhibitors of TbMCA2 (table 1). Six compounds showed significant inhibition (IC_{50} < 2.2 μM), compound **11** was the most potent inhibitor with an IC_{50} of 0.6 μM. In addition, compounds **1** and **11-14** were tested against TbMCA3 and the overall inhibition profile was similar to that for TbMCA2 (data not shown). TbMCA2 inhibitors would be expected to show inhibition of TbMCA3 as they share 89% sequence identity.17 As simultaneous inhibition of all catalytically active TbMCAs is probably necessary, inhibition of multiple TbMCAs by an inhibitor would be advantageous. The specificity of inhibitors **1** and **11-14** was assessed using human caspase-3, a member of the same peptidase family as the MCAs. Caspase-3 activity was unaffected by any of these inhibitors (data not shown), demonstrating their selectivity for TbMCAs.

Next, a biological evaluation was performed *in vitro* against the following protozoa (table 1): *T. b. brucei* (BSF), *T. cruzi* and *Leishmania infantum* intracellular amastigotes, and

Plasmodium falciparum (ring stage and schizont). *Leishmania* parasites have a single metacaspase gene18, whilst *P. falciparum19* and *T. cruzi20* both have 2 MCAs. Cytotoxicity was assessed by testing the compounds on human lung fibroblasts (MRC-5). Compound **1** has an IC_{50} value of 1.9 μM for TbMCA2 but showed no antiparasitic activity. Modifications of the P1 tail (**2** and **3**) in order to lower the basicity combined with the introduction of a peptoid backbone (**4**-**10**) resulted in inactive compounds. Introduction of α-ketoheterocyclic warheads with retention of the Arg P1 tail increased enzymatic inhibition, especially for benzothiazole (**11**, TbMCA2 IC_{50} = 0.6 μM) and thiazole (**13**, TbMCA2 IC_{50} = 2.2 μM). For **11** and **14**, antiparasitic activity although very modest, was observed against *P. falciparum*. Finally, irreversible inhibitor **15** also was found to be a low micromolar inhibitor (TbMCA2 IC_{50} = 3.9 μM) with modest antiparasitic activity towards *P. falciparum*.

Compound **11** was considered as a valuable hit, the first to be developed towards TbMCA2, with low micromolar activity and without cytotoxicity. To obtain more potent compounds, optimisations were carried out at both P1 and P2 level (Fig. 5). At the P1 level, the arginine side chain was substituted for arginine mimetics. Many arginine mimetics are able to interact with carboxylic groups in the active site21 such as homoarginine **17** and less basic lysine **16** and ornithine. At the P2 level, the Cbz protecting group was substituted for a mimic of the P2 amino acid, which is known to be a hydrophobic uncharged aliphatic amino acid.22 This resulted in dipeptide analogues **18** and **19**.

Compound **16** was synthesised as per the reported procedure by McGrath *et al.23* Compound **17** was synthesised in the same way as **11**-**14** (scheme 5). For this compound, the amine function of *N*-ε-Boc-N-α-Z-L-Lysine was first deprotected and converted to a Boc-protected guanidine. The ornithine analogue could not be obtained due to formation of the inactive enamine after cyclisation. For compounds **18** and **19** the first synthetic steps are comparable to scheme 5: commercially available Boc-Arg-Pbf-OH was transformed to intermediate **35** (scheme 7). After a selective Boc deprotection and activation of Z-Leu/Z-Val in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxybenzotriazole (HOBt), a coupling reaction took place (**36**).24 A final TFA deprotection yielded compounds **18** and **19** (scheme 7).

Modification of P1 caused inhibition in the same range as the hit (**16**, TbMCA2 IC_{50} = 1.6 μM and **17**, TbMCA2 IC_{50} = 4.0 μM) with modest antiparasitic activities. P2 modification (**18** and **19**) scored comparable enzymatic and antiparasitic activities (table 2).

We report the first MCA inhibitors with low micromolar activity. These compounds inhibit TbMCA2 and TbMCA3, possess modest antiparasitic activity, but have excellent selectivity when compared to mammalian caspases. Optimization efforts are ongoing.

Acknowledgments

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25. All newly synthesized compounds have full characterisation data (see Supplementary Data), for example, compound **13**: MS (ESI) *m/z* 376.0 $[M+H]^+$; LC-MS Rt 11.6 min, *m/z* 376.0 $[M+H]^+$; ^{1}H-NMR (400 MHz, MeOD) d δ 1.70-1.77 (m, 4H), 3.21-3.24 (m, 2H), 5.10 (s, 2H), 5.39-5.41 (m, 1H), 7.23-7.35 (m, 5H), 8.03 (d, J = 3.0 Hz, 1H), 8.09-8.11 (m, 1H); ^{13}C-NMR (100 MHz, MeOD) d δ 26.4, 30.0, 41.8, 57.6, 67.8, 116.4 (q, CF$_3$COOH), 128.8, 129.1, 129.5, 146.3, 158.7, 162.8 (q, CF$_3$$C$OOH), 166.1, 192.8

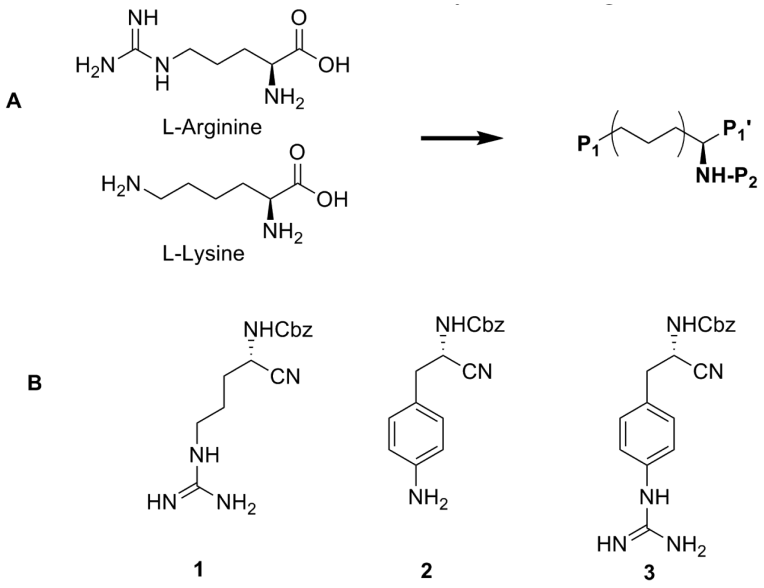

Figure 1.
A) Schematic representation of TbMCA inhibitors based on L-Arginine or L-Lysine. B) First set of TbMCA inhibitors derived from α-amino protected arginine with nitrile P1′ warhead and modifications in P1.

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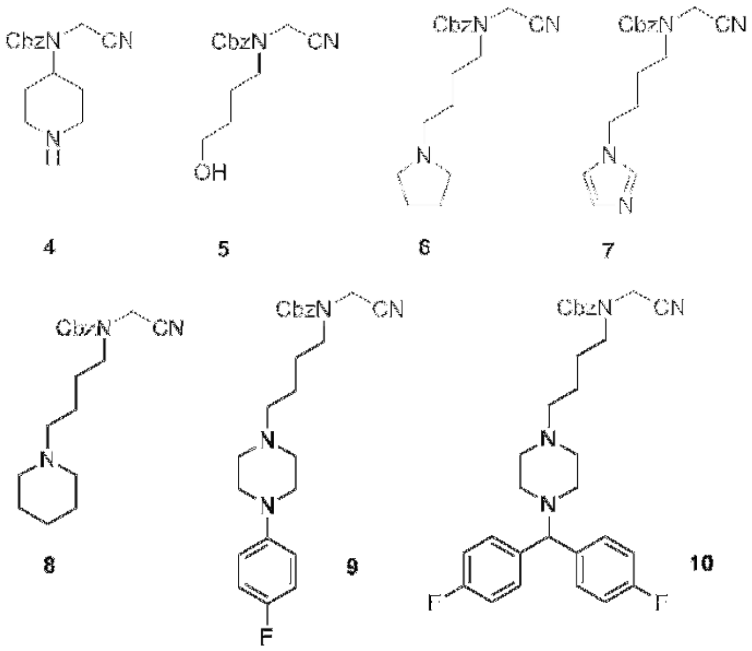

Figure 2.
TbMCA inhibitors with peptoid backbone, nitrile P1′ warhead and modifications in P1.

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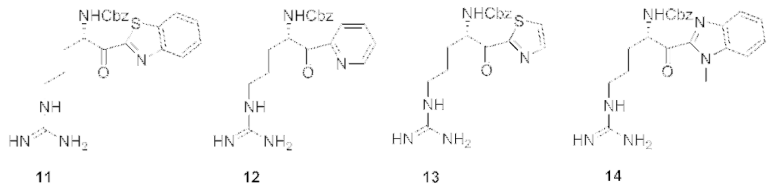

Figure 3.
TbMCA inhibitors derived from α-amino protected arginine with α-ketoheterocyclic P1′ warhead.

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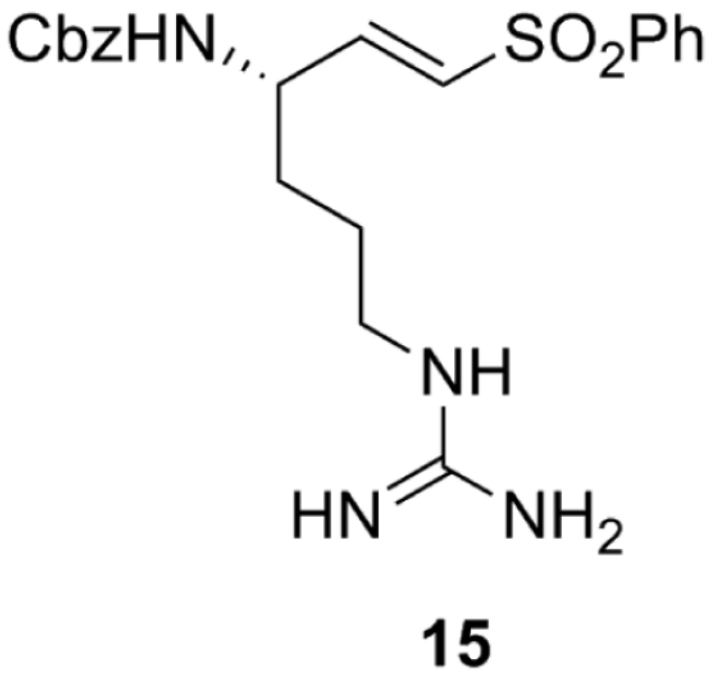
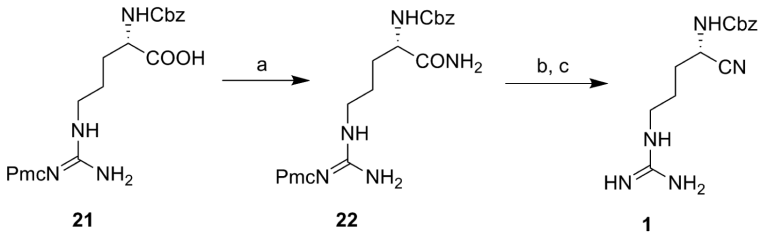
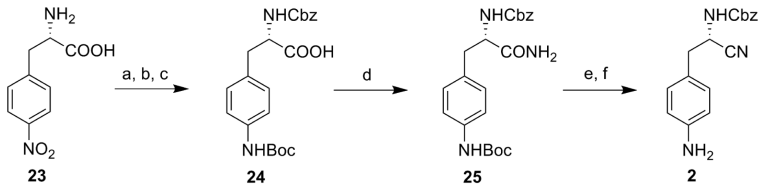


Figure 4.
TbMCA inhibitor derived from α-amino protected arginine with α-vinylsulfone P1′ warhead.



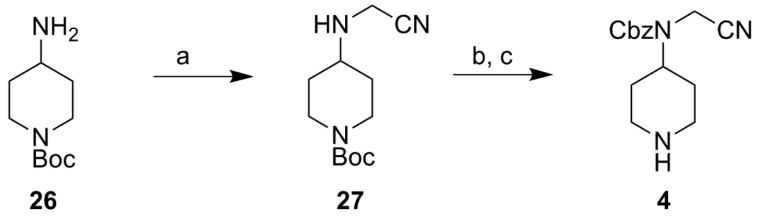
Scheme 1.

Reagents: (a) 1. *N*-(OH)-Suc, DCC, THF, rt, 1 h; 2. NH_3/MeOH 7N, rt, 1 h (68%); (b) TFAA, pyridine, THF, rt, 2 h; (c) TFA:DCM, rt, 1 h (19%).



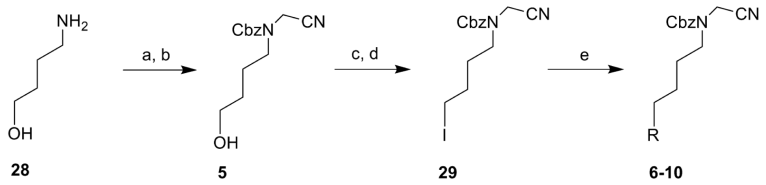
Scheme 2.

Reagents: (a) benzylchloroformate, NaOH, water/dioxane, 0°C, 3 h (95%); (b) $SnCl_2$, EtOH, reflux, 3 h (98%); (c) $(Boc)_2O$, water, dioxane, Et_3N, 0°C to rt, 12 h (17%); (d) 1. *N*-OH-Suc, DCC, THF, rt, 1 h; 2. NH_3/MeOH 7 N, rt, 1 h (47%); (e) TFAA, pyridine, THF, rt, 3 h (8%); (f) TFA:DCM, rt, 1 h (65%).



Scheme 3.

Reagents: (a) bromoacetonitrile, K_2CO_3, 0°C to rt, 12 h (95%); (b) benzylchloroformate, NaOH, dioxane, 0°C, 3 h (43%); (c) TFA:DCM, rt, 1 h (26%).



Scheme 4.

Reagents: (a) bromoacetonitrile, K_2CO_3, 0°C to rt, 12 h (95%); (b) benzylchloroformate, DCM, Et_3N, 0°C, 3 h (45%); (c) MsCl, Et_3N, rt, 2 h (96%); (d) NaI, dry acetone, 60°C, 12 h (59%); (e) R = pyrrolidine (**6**), imidazole (**7**), piperidine (**8**), 1-(4-fluorophenyl)piperazine (**9**), 1-(bis(4-fluorophenyl)methyl)piperazine (**10**), DCM, rt, 1 h-12 h (**6**, 43%; **7**, 99%; **8**, 79%; **9**, 17%; **10**, 26%).

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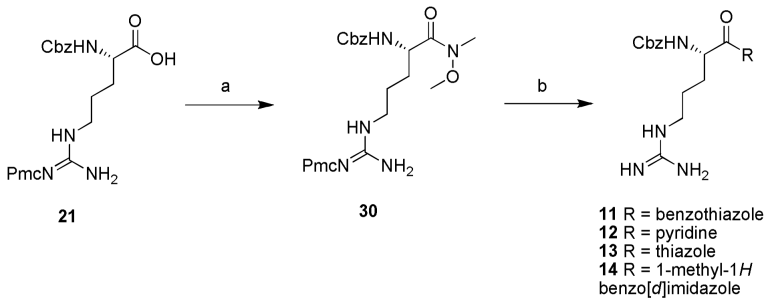
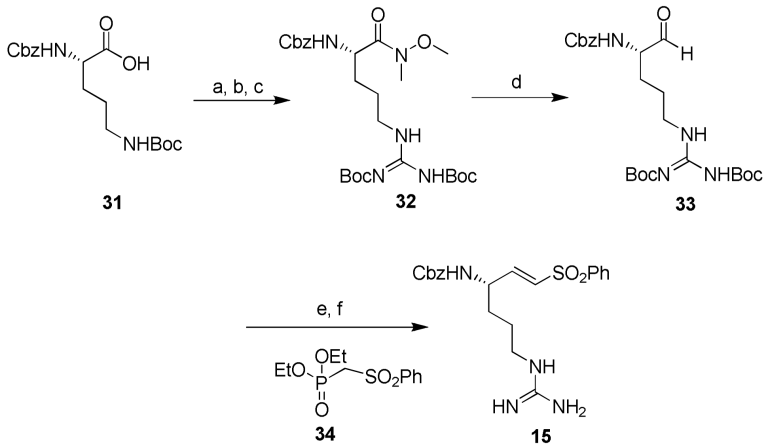


Scheme 5.

Reagents: (a) *N,O*-dimethylhydroxylamine hydrochloride, TBTU, Et_3N, DMF, 0°C, 12 h (96%); (b) 1. THF, n-BuLi, heterocycle, −78°C, 2 h; 2. TFA:DCM, rt, 1 h (**11**, 17%; **12**, 29%; **13**, 17%; **14**, 26%).



Scheme 6.

Reagents: (a) *N,O*-dimethylhydroxylamine hydrochloride, TBTU, Et_3N, DMF, 0°C, 3 h (95%); (b) TFA:DCM, rt, 1 h (99%); (c) *tert*-butyl (1*H*-pyrazol-1-yl)methylenedicarbamate, rt, 20 h (15%); (d) $LiAlH_4$, THF, 0°C, 30 min (81%); (e) NaH, THF, 0°C, 1.5 h (14%); (f) TFA:DCM, rt, 1 h (70%).

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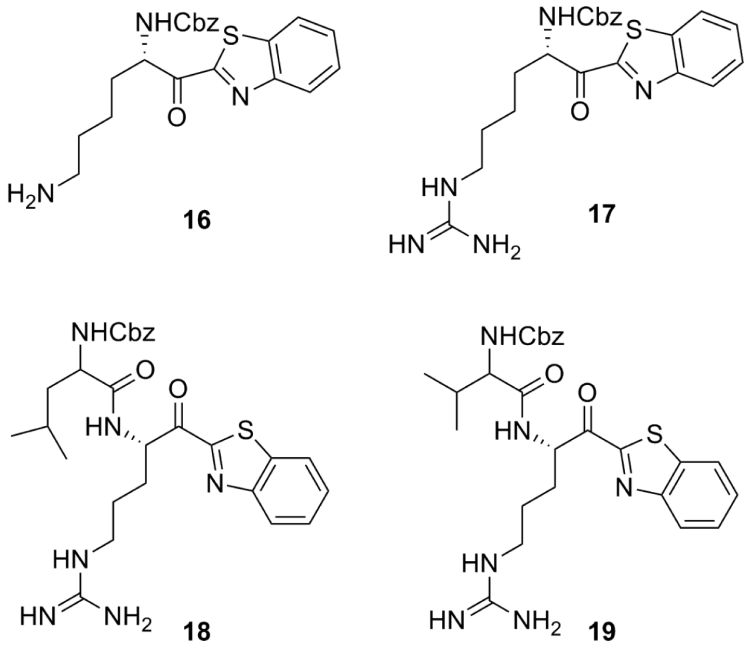
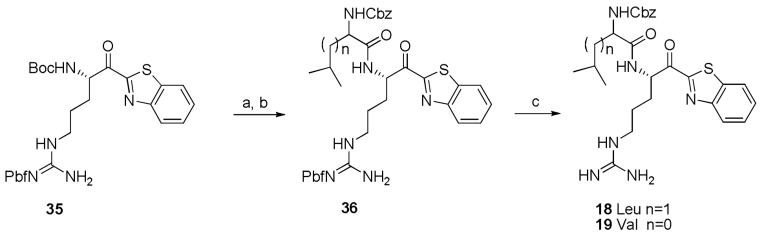


Figure 5.
*Tb*MCA inhibitors derived from hit **11** with modifications at P1 and P2 level.



Scheme 7.

(a) TFA:DCM (10%, 4 eq), rt, 3-5h (85%); (b) 1. Z-Leu/Z-Val, HOBt, EDC, DCM, rt, 2 h; 2. Et_3N, rt, 12 h; (n = 1, 19%; n = 0, 13%) (c) TFA:DCM, rt, 1 h (**18**, 76%; **19**, 63%).

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Table 1

Inhibition of TbMCA2 and *T. b. brucei, T. cruzi, L. infantum* and *P. falciparum* (IC_{50}) for **1-15**. Cytotoxicity was measured against human fibroblasts (MRC-5).

Compound	IC_{50} *Tb MCA2* (μM)	IC_{50} *T. b. brucei* (μM)	IC_{50} *T. cruzi* (μM)	IC_{50} *L. infantum* (μM)	IC_{50} *P. falciparum* (μM)	IC_{50} MRC-5 (μM)
1	1.9	> 64	> 64	> 64	50.2	> 64
2	> 100	30.3	28.6	> 64	16.6	> 64
3	> 100	> 64	27.2	> 64	25.4	> 64
4	> 100	32.5	34.6	> 64	28.0	> 64
5	> 100	> 64	> 64	> 64	> 64	> 64
6	> 100	52.1	> 64	> 64	20.5	> 64
7	> 100	> 64	35.7	> 64	17.2	> 64
8	> 100	> 64	> 64	> 64	6.9	> 64
9	> 100	22.6	7.0	> 64	> 64	> 64
10	> 100	2.1	2.6	5.9	7.6	11.3
11	0.6	32.9	20.9	> 64	8.0	> 64
12	62	> 64	> 64	> 64	> 64	> 64
13	2.2	> 64	38.7	> 64	24.7	> 64
14	38.4	32.5	28.3	> 64	7.1	> 64
15	3.9	45.3	19.0	> 64	3.4	> 64

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Table 2

Inhibition of TbMCA2 and *T. b. brucei, T. cruzi, L. infantum* and *P. falciparum* (IC_{50}) for **16-19**. Cytotoxicity was measured against human fibroblasts (MRC-5).

Compound	IC_{50} *Tb MCA2* (μM)	IC_{50} *T. b. brucei* (μM)	IC_{50} *T. cruzi* (μM)	IC_{50} *L. infantum* (μM)	IC_{50} *P. falciparum* (μM)	IC_{50} MRC-5 (μM)
16	1.6	10.6	10.8	22.8	3.00	20.0
17	4.0	11.4	5.5	45.4	5.6	19.0
18	1.4	5.7	5.6	36.8	2.6	29.0
19	1.1	11.2	7.3	> 64	3.3	32.6