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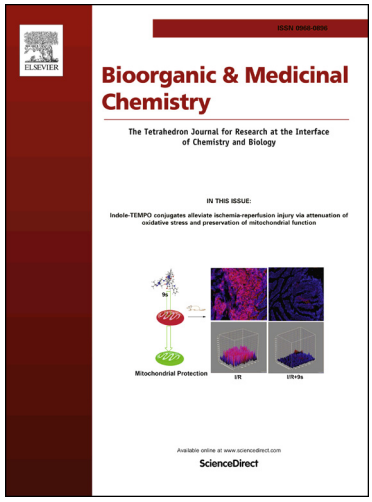

Synthesis and biological evaluation of selective tubulin inhibitors as anti-trypanosomal agents

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Synthesis and biological evaluation of selective tubulin inhibitors as anti-trypanosomal agents

Viharika Bobba[a], Vishal Nanavaty[b], Nethrie D. Idippily[a], Anran Zhao[a], Bibo Li[b]*, Bin Su[a]*

[a] *Department of Chemistry, Center for Gene Regulation in Health and Disease, College of Sciences and Health Professions, Cleveland State University, 2121 Euclid Ave., Cleveland, OH, 44115, USA*

[b] *Department of Biology, Geo. & Env. Sciences, Center for Gene Regulation in Health and Disease, College of Sciences and Health Professions, Cleveland State University, 2121 Euclid Ave., Cleveland, OH, 44115, USA*

TITLE RUNNING: Anti-trypanosomal agents

*To whom correspondence should be addressed:

Bin Su, Ph.D.
Center for Gene Regulation in Health and Disease
Department of Chemistry
College of Sciences and Health Professions
Cleveland State University
2121 Euclid Ave., Cleveland, OH 44115, USA
Phone: 216-687-9219
Fax: 216-687-9298
Email: B.su@csuohio.edu

Bibo Li, Ph.D.
Center for Gene Regulation in Health and Disease
Department of Biological, Geological & Environmental Sciences
College of Sciences and Health Professions
Cleveland State University
2121 Euclid Ave., Cleveland, OH 44115, USA
Phone: 216-687-2444
Fax: 216-687-6972
Email: b.li37@csuohio.edu

Abstract

African trypanosomiasis is still a threat to human health due to the severe side-effects of current drugs. We identified selective tubulin inhibitors that showed the promise to the treatment of this disease, which was based on the tubulin protein structural difference between mammalian and trypanosome cells. Further lead optimization was performed in the current study to improve the efficiency of the drug candidates. We used *Trypanosoma brucei brucei* cells as the parasite model, and human normal kidney cells and mouse macrophage cells as the host model to evaluate the compounds. One new analog showed great potency with an IC_{50} of 70 nM to inhibit the growth of trypanosome cells and did not affect the viability of mammalian cells. Western blot analyses reveal that the compound decreased tubulin polymerization in *T. brucei* cells. A detailed structure activity relationship (SAR) was summarized that will be used to guide future lead optimization.

Key words: trypanosomiasis; tubulin inhibitor; selective index; drug development

1. Introduction

Human African trypanosomiasis, also known as African sleeping sickness, is a vector-borne parasitic disease in sub-Saharan Africa where medical resources are limited.[1-3] Currently there are four drugs available for the treatment of human African trypanosomiasis, including Suramin, Pentamidine, Melarsoprol, and Eflornithine.[4] However, these drugs have many limitations: 1) high toxicity to the host; 2) only available as intramuscular or intravenous injections; 3) narrow anti-trypanosomiasis spectrum; and 4) high cost to administer the drugs to the patients. Overall, these drugs are not effective in the treatment of the disease, and it is urgently needed to develop more effective and inexpensive chemotherapeutic agents for the treatment of human African trypanosomiasis. [4-6]

Tubulin is a well-known target in anti-cancer drug discovery field, and several tubulin interfering agents are the first line chemotherapeutic drugs for cancer treatment in clinic. [7] Tubulin also plays an essential role during trypanosome cell division. The fast growing rate of trypanosome cells makes the parasites heavily depend on tubulin polymerization/depolymerization for proliferation.[8] More importantly, tubulin is important for the locomotion of trypanosome cells, which is an essential function for them to survive. Therefore, tubulin inhibitors have the potential activity to block the *T. brucei* cell division and decrease the locomotion function of the flagellum as well, which will lead to cell death.[9] These factors suggest that there are multiple advantages of tubulin inhibitors as a novel treatment to human African trypanosomiasis.

Tubulin is a highly conserved protein within different species. However, different susceptibility to antimitotic agents are known to exist among different organisms, indicating there are differences of tubulin structures among different species.[10] Based on the differences of the colchicine binding pocket between mammalian and *T. brucei* tubulins, selective tubulin inhibitors were developed that showed great *in vitro* potency to inhibit *T. brucei* cell growth without harming mammalian cells at the similar

concentrations. [11] Some compounds exhibited very specific inhibitory effect on *T. brucei* cell growth, with a selective index (IC_{50} inhibiting human cell growth/IC_{50} inhibiting *T. brucei* cell growth) beyond 100. [12] To our surprise, the pharmacophore of these compounds enhancing the mammalian cell growth inhibition is different to the pharmacophore improving the *T. brucei* cell growth inhibition. In addition, these compounds showed *in vivo* activity to decease *T. brucei* cell growth in the infected mice. However, the compounds were not potent enough to totally clear the infection yet. [12] Herein, we further lead optimized the compound based on the summarized structure activity relationship, and identified one compound with better potency and selectivity than previous ones.

2. Results and Discussion

2.1. Synthesis of the new tubulin inhibitors

In our previous studies, the developed selective sulfonamide tubulin inhibitors showed great *in vitro* activity to inhibit the growth of *T. brucei* cells. However, the potency of the compounds was not good enough to clear up the infections in the animals completely. [12] In the present study, further lead optimization was performed to develop more effective analogs. A total of 18 compounds were synthesized using combinatorial chemistry strategy to increase the selectivity and anti-parasite potency of the compounds. We modified the R_1, R_2, and R_3 moieties of the core structure with different substituents systematically (Figure 1).

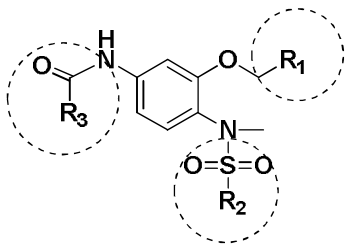
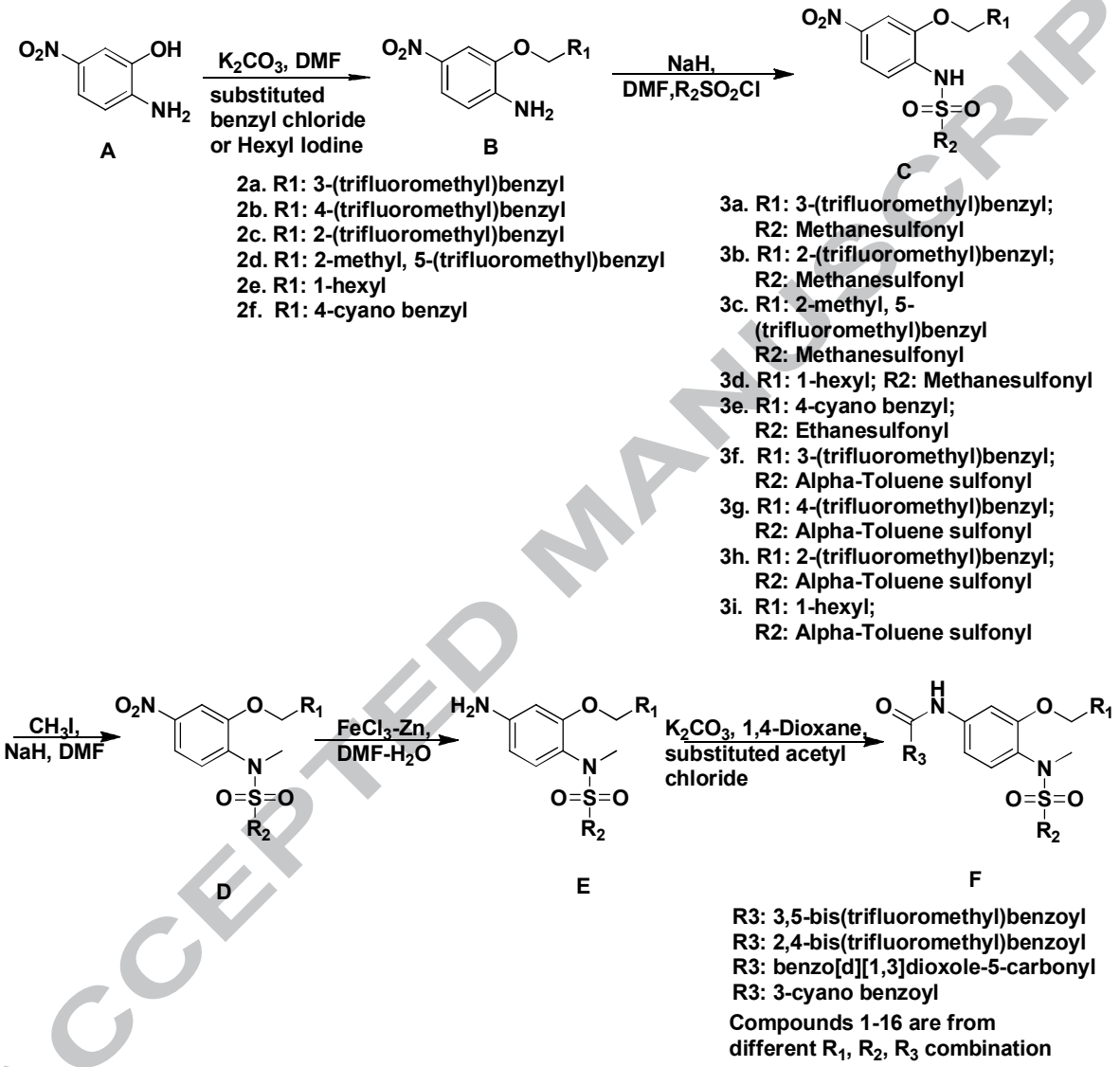


Figure 1. Core structure of the new derivatives

For these new compounds, we explored different structures at R_1 domain including aromatic rings and aliphatic groups. Next, the R_2 moiety of the scaffold was modified with different substituted

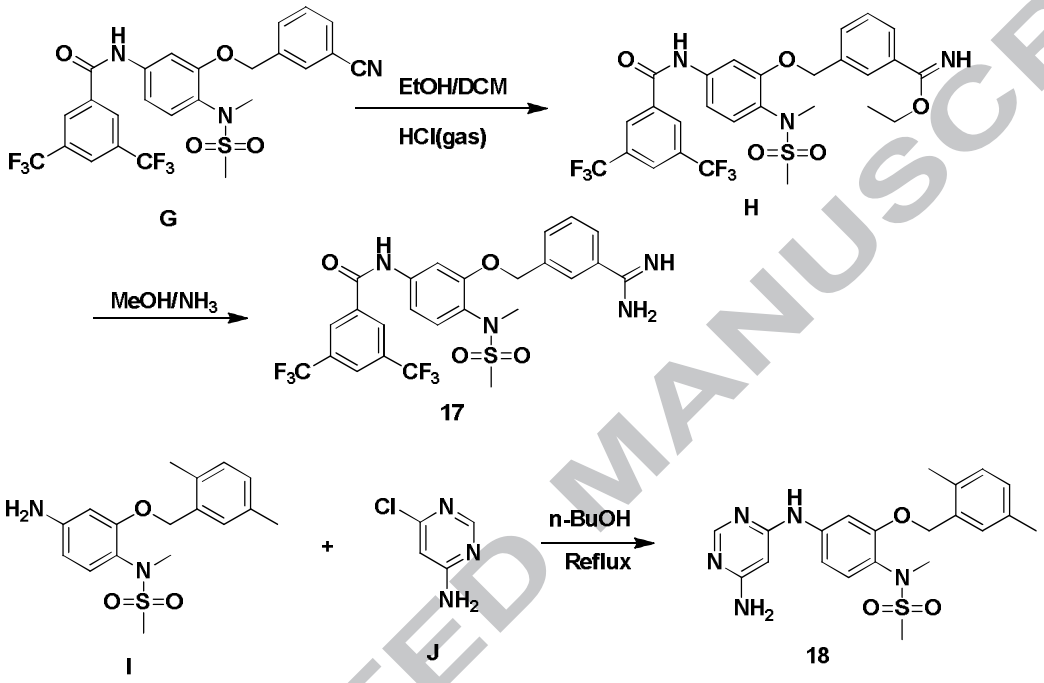
sulfonyl chlorides in order to generate different sulfonamide groups. Then, the nitro group was reduced to amino group in order to introduce R_3 group. Last, acylation of the amino group with different aromatic substituents resulted in different R_3 moiety. The synthesis is illustrated in Scheme 1.



Scheme 1: Synthesis of sulfonamide derivatives

T. brucei cells salvage adenosine from their mammalian host via two transporters, P1 and P2. P1 is specific for adenosine and inosine, whereas P2 transports adenosine, adenine, melaminophenyl arsenicals, and diamidines. [13-16] These two proteins are responsible for the active uptake of Pentamidine

and Melasoprol by *T. brucei*, which contributes to the higher trypanocidal activity of the compounds. [16] Based on this information, we also introduced adenosine or diamidine like structures to our analogs to examine if the drug uptake could be improved. Two new compounds with such moieties were synthesized based on the following scheme (Scheme 2).



Scheme 2. Synthesis of analogs containing adenosine or diamidine like structures

2.2. Biological evaluation of the new derivatives

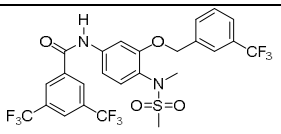
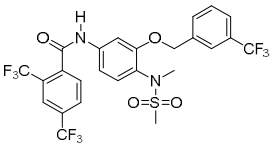
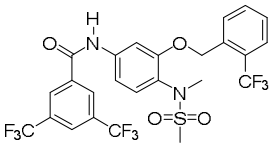
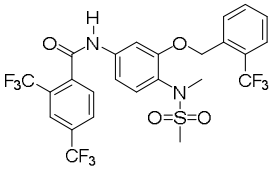
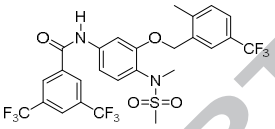
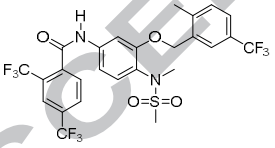
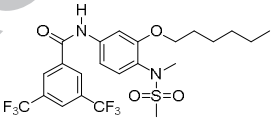
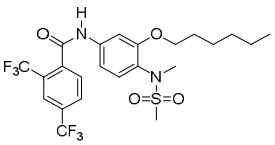
The biological activity of the synthesized analogs was determined with cell proliferation assays. *T. b. brucei* Lister 427 cells, which are the bloodstream form of the cells, were used as the parasite model, and human normal kidney HEK293 cells and mouse macrophage RAW267.4 cells were used as the mammalian host model. Results of the cell growth inhibition by the compounds are listed in Table 1. The selective index is calculated by dividing the IC_{50}s of the mammalian cell growth inhibition with the IC_{50}s of the *T. brucei* cell growth inhibition. Overall, these compounds show higher activity to inhibit the growth of parasite cells than mammalian cells.

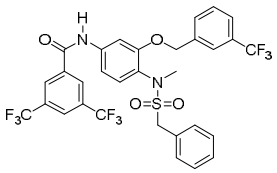
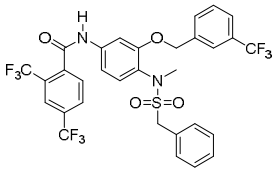
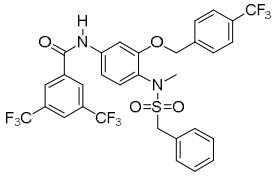
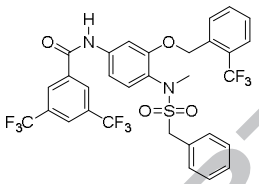
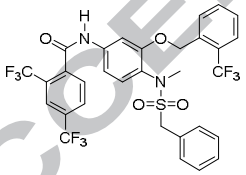
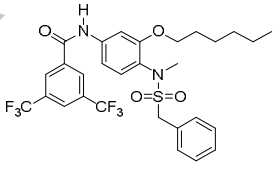
For the three moieties (R_1 – R_3) of the compound scaffold (Figure 1), various functional groups were introduced to enhance the anti-trypanosomal activity and decrease the cytotoxicity to mammalian cells. For R_1 domain, the benzyl moiety with the ortho and meta trifluoride methyl groups overall decreases the activity to inhibit mammalian cell growth (compounds **1**-**6**). However, the inhibition of the parasite cell growth was not promising. Most compounds have IC_{50} values above 5 μM, which is a concentration difficult to reach in the blood circulation system. The alkyl group hexane at the R_1 moiety (compounds **7-8**) significantly decreases the inhibition to mammalian cell growth, but their activity against *T. brucei* cell proliferation is not promising either.

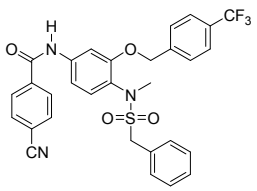
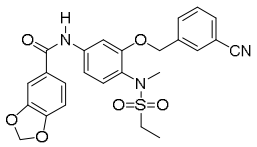
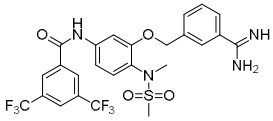
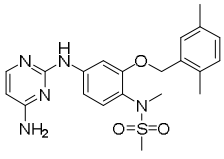
For R_2 moiety, it seems that a bulky aromatic group promotes the activity to inhibit the growth of *T. brucei* cells. Compounds **9**, **10**, **11** and **15** all have IC_{50}s below 5 μM. In addition, these compounds show weaker activity to affect mammalian cell growth, which significantly improve the selectivity of the compounds.

For the R_3 moiety, we mainly focused on the electron withdrawing groups, which was based on the structure activity relationship summarized in our previous studies. [12,17] When electron donating group was introduced (compound **16**), the inhibition to *T. brucei* cell growth was decreased. We did not observe any improvement on the activity of compounds **17** and **18**, even the adenosine or diamine like structures were introduced to enhance their cell uptake. It is possible that the uptake of the compounds has been increased, but their targeting effect has been diminished due to this structural modification. Compound **15** so far shows the best activity against *T. brucei* cells with an IC_{50} of 70 nM. It also shows great selectivity with a selective index more than 7000, which is far better than the compounds from previous studies. [12]

Table 1. Comparison of the growth inhibitory effects of the tubulin inhibitors on mammalian and *T. brucei* cells

Entry	Structure	IC_{50} against *T. brucei* cells (μM)	IC_{50} against macrophage RAW 267.4 cells (μM)	IC_{50} of macrophage cells/IC_{50} of *T. brucei*	IC_{50} against HEK293 cells (μM)	IC_{50} of HEK293 cells/IC_{50} of *T. brucei*
1		8.58±3.82	>500	>58	53.95±6.81	6
2		8.42±4.13	>500	>59	80.63±3.05	10
3		9.85±4.83	>500	>51	>500	>51
4		13.33±6.64	>500	>38	34.77±7.65	3
5		15.58±7.17	>500	>32	>500	>32
6		13.01±6.75	>500	>38	102.1±22.95	8
7		23.04±11.76	>500	>22	>500	>22
8		30.13±15.52	>500	>17	>500	>17

9		2.08±1.01	59.81±1.53	29	69.54±4.63	33
10		1.78±0.72	175.57±2.04	99	20.51±1.77	12
11		3.39±1.22	293.1±18.23	86	>500	>147
12		7.13±3.27	>500	>70	>500	>70
13		10.46±5.23	>500	>48	158.1±39.91	15
14		7.81±3.83	69.85±0.9	9	13.26±2.88	2

15		0.07±0.02	>500	>7143	>500	>7143
16		23.47±12.88	>500	>21	>500	>21
17		>50	>500	>10	>500	>10
18		>50	>500	>10	>500	>10

2.3. Biological evaluation of the tubulin targeting effect of compound 15

Our previous study reveals that these compounds interfere with the microtubule dynamics. [12] To test if the new compound is still a tubulin inhibitor, we determined the amount of the polymerized and the un-polymerized tubulin after treating *T. brucei* cells with compound **15**. We found that the compound decreased the amount of polymerized tubulin in a dose-dependent manner (Figure 2), indicating that after the treatment, the tubulin polymerization in the cells was inhibited by the compound. Therefore, the new compound is still a tubulin inhibitor and selectively targets the tubulin dynamic regulation in *T. brucei* cells. It is a potent and selective tubulin inhibitor to against trypanosomiasis.

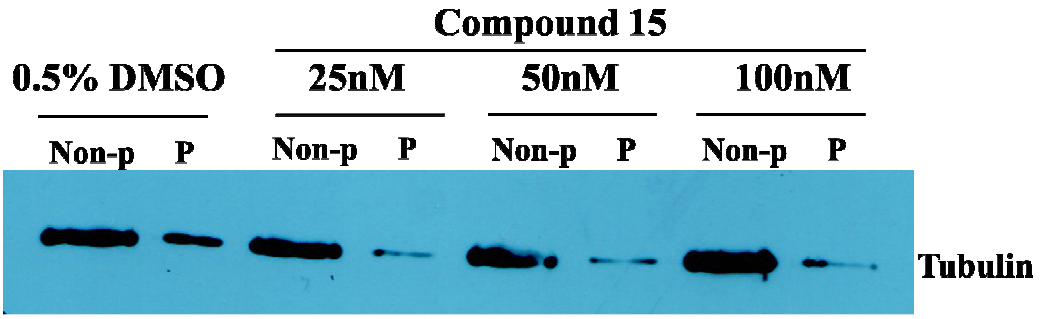


Figure 2. Tubulin inhibitor compound **15** inhibits the tubulin polymerization in *T. brucei* cells. After treatment, the level of polymerized tubulin is significantly decreased. The experiment was independently repeated for three times and the representative one is presented.

3. Conclusion

Due to the limitations of the current anti-trypanosomiasis drugs, the development of new medicines for the treatment of African trypanosomiasis is still urgently needed. We focus on the development of selective tubulin inhibitors for the treatment of this disease. Based on the inhibitory effects of the compounds on *T. brucei* cell proliferation and mammalian cell growth, the selective index is determined in order to identify more potent and selective drug candidates. The current study results in a compound with an IC_{50} of 70 nM to inhibit the growth of *T. brucei* cells, and does not affect the viability of mammalian cells even with a concentration that is more than 7,000 fold higher. Furthermore, the selective tubulin inhibitor interferes with tubulin polymerization in *T. brucei* cells and decreases the polymerized tubulin in cytosol. However, the solubility of this compound limits the formulation for the *in vivo* study. We are currently working on further structural modification to increase the solubility of the compound and expect to remain or improve its potency and selectivity.

4. Experimental Section

4.1. Chemistry

Chemicals were commercially available and used as received without further purification unless

otherwise noted. Moisture sensitive reactions were carried out under a dry argon atmosphere in flame-dried glassware. Thin layer chromatography was performed on silica gel TLC plates with a fluorescence indicator at 254 nm (Fluka). Flash column chromatography was performed using silica gel 60Å (BDH, 40-63 μm). Mass spectra were obtained on a Bruker Ion-Trap Mass Spectrometer at Cleveland State University MS facility Center. All the NMR spectra were recorded on a Bruker 400 MHz spectrometer using $CDCl_3$ or DMSO-d6 as the solvent.

Reversed-phase HPLC analysis of compounds was conducted on a Beckman HPLC system with an Auto Sampler. The chromatographic separation was performed on a C18 column (2.0 mm × 150 mm, 5 μm) from Phenomenex (Torrance, CA). The mobile phase was employed for isocratic elution with a flow rate of 0.2 mL/min. The injection volume was 20 μL and the UV detector was set up at 260 nm.

The synthesis of compounds **1**-**16** followed the procedure described in previous studies. [18-20]

The reaction procedure is illustrated in Scheme 1.

The final step of the reaction and the product characterization are listed below.

N-(4-(*N*-methylmethylsulfonamido)-3-((3-(trifluoromethyl)benzyl)oxy)phenyl)-3,5-bis(trifluoromethyl) benzamide (**1**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 2 hrs. Yield 80.3%, Pale Yellow solid. ^{1}H NMR (400MHz, $CDCl_3$) δ_H 8.36 (s, 3H), 8.09 (s, 1H), 7.93 (d, 1H), 7.74 (s, 1H), 7.66 (d, 2H), 7.59 (t, 1H), 7.32 (d, 1H), 6.97 (q, 1H), 5.19 (s, 2H), 3.26 (s, 3H), 2.88 (s, 3H); ^{13}C NMR (100MHz, $CDCl_3$) δ_C 162.97,155.23, 138.78, 136.73, 136.47, 132.62, 132.50, 132.28, 130.95, 129.42, 127.58, 125.68, 125.39, 124.46, 124.22, 113.01, 105.51, 69.84, 38.33, 37.76, 29.71. ESI-MS calculated for ($C_{25}H_{19}N_2SO_4F_9$) $[M - H]^-$: 613.0; Molecular Weight (calculated from structure): 614.48. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.31 mins, purity: >99%

N-(4-(*N*-methylmethylsulfonamido)-3-((3-(trifluoromethyl)benzyl)oxy)phenyl)-2,4-bis(trifluoromethyl) benzamide (**2**). 2,4-bis(trifluoromethyl)benzoyl chloride was used and stirred for 2 hrs. Yield 92.6%,

Pale Yellow solid. ^{1}H NMR (400MHz, DMSO) δ_H 10.84 (s, 1H), 8.23 (d, 2H), 7.98 (d, 1H), 7.84 (d, 1H), 7.73 (d, 1H), 7.63 (d, 2H), 7.32 (d, 1H), 7.23 (d, 1H), 7.21 (d, 1H), 5.27 (s, 2H), 3.14 (s, 3H), 2.92 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 164.83, 155.54, 139.94, 138.38, 132.29, 131.97, 130.50, 130.06, 129.15, 129.75, 126.15, 125.98, 125.73, 125.22, 124.87, 112.46, 105.34, 69.33, 38.21, 37.96. ESI-MS calculated for ($C_{25}H_{19}N_2SO_4F_9$) [M - H]$^-$: 613.02; Molecular Weight (calculated from structure): 614.48. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.14 mins, purity: >99%

N-(4-(*N*-methylmethylsulfonamido)-3-((2-(trifluoromethyl)benzyl)oxy)phenyl)-3,5-bis(trifluoromethyl) benzamide (**3**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred at room temperature for 3 hrs. Yield 78.1 %, Pale Yellow solid. ^{1}H NMR (400MHz, DMSO) δ_H 10.78 (s, 1H), 8.60 (s, 2H), 8.40 (d, 1H), 7.88 (d, 1H), 7.83 (d, 1H), 7.77 (t, 1H), 7.69 (s, 1H), 7.62 (t, 1H), 7.43 (d, 1H), 7.35 (d, 1H), 5.32 (s, 2H), 3.12 (s, 3H), 2.87 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 163.24, 155.34, 140.03, 137.40, 134,71, 133.49, 131.54, 130.84, 129.40, 129.10, 126.59, 125.82, 124.93, 122.22, 113.69, 106.12, 66.85, 38.17, 37.95. ESI-MS calculated for ($C_{25}H_{19}N_2SO_4F_9$) [M - H]$^-$: 612.92; Molecular Weight (calculated from structure): 614.48. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.40 mins, purity: >99%

N-(4-(*N*-methylmethylsulfonamido)-3-((2-(trifluoromethyl)benzyl)oxy)phenyl)-2,4-bis(trifluoromethyl) benzamide (**4**). 2,4-bis(trifluoromethyl)benzoyl chloride was used and stirred for 2.5 hrs. Yield 67.1%, pale yellow solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.83 (s, 1H), 8.23 (d, 2H), 7.98 (d, 1H), 7.87 (q, 2H), 7.76 (t, 1H), 7.62 (t, 1H), 7.51 (s, 1H), 7.34 (s, 2H), 5.30 (s, 2H), 3.13 (s, 3H), 2.88 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 164.81, 155.45, 140.01, 134.76, 133.46, 132.20, 130.63, 130.53, 129.30,

126.57, 125.70, 112.64, 105.18, 66.76, 38.15, 37.95. ESI-MS calculated for ($C_{25}H_{19}N_2SO_4F_9$) [M - H]$^-$: 612.86; Molecular Weight (calculated from structure): 614.48. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.18 mins, purity: >99%

N-(3-((2-methyl-5-(trifluoromethyl)benzyl)oxy)-4-(*N*-methylmethylsulfonamido)phenyl)-3,5-bis(trifluoromethyl)benzamide (**5**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 71.3%, white solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.80 (s, 1H), 8.60 (s, 2H), 8.39 (s, 1H), 7.90-7.36 (m, 6H), 5.25 (s, 2H), 3.51 (s, 3H), 3.13 (s, 3H), 2.91 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 163.21, 141.76, 139.89, 137.31, 136.39, 131.49, 131.18, 129.03, 126.08, 125.15, 124.92, 113.60, 106.46, 67.81, 66.81, 38.18, 38.05. ESI-MS calculated for ($C_{26}H_{21}N_2SO_4F_9$) [M - H]$^-$: 626.86; Molecular Weight (calculated from structure): 628.51. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.63 mins, purity: >99%

N-(3-((2-methyl-5-trifluoromethyl)benzyl)oxy)-4-(*N*-methylmethylsulfonamido)phenyl)-2,4-bis(trifluoromethyl)benzamide (**6**). 2,4-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 59.3%, white solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.86 (s, 1H), 8.23 (d, 2H), 7.98-7.27 (m, 7H), 5.23 (s, 2H), 3.12 (s, 3H), 2.89 (s, 3H), 2.44 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 164.85, 155.67, 149.50, 148.15, 141.82, 139.94, 136.35, 131.69, 131.47, 130.49, 125.88, 125.15, 112.49, 105.36, 67.75, 66.81, 38.13, 38.04. ESI-MS calculated for ($C_{26}H_{21}N_2SO_4F_9$) [M - H]$^-$: 626.91; Molecular Weight (calculated from structure): 628.51. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.21 mins, purity: >99%

N-(3-(hexyloxy)-4-*N*-methylmethylsulfonamido)phenyl)-3,5-bis(trifluoromethyl)benzamide (**7**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 80.2%, pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.53 (s, 1H), 8.38 (s, 2H), 8.07 (s, 1H), 7.80 (d, 1H), 7.20 (d, 1H), 6.79 (q, 1H), 4.06 (t, 2H), 3.26 (s, 3H), 2.97 (s, 3H), 1.82 (m, 2H), 1.47 (m, 2H), 1.36 (m, 4H), 0.93 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 163.03, 155.76, 138.81, 136.57, 132.59, 132.25, 127.69, 125.47, 125.07, 124.26, 121.55, 112.48, 105.27, 68.55, 38.11, 37.67, 31.45, 29.19, 25.77, 22.55, 13.96. ESI-MS calculated for ($C_{23}H_{26}N_2SO_4F_6$) $[M - H]^-$: 539.00; Molecular Weight (calculated from structure): 540.52. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.79 mins, purity: >99%

N-(3-(hexyloxy)-4-*N*-methylmethylsulfonamido)phenyl)-2,4-bis(trifluoromethyl)benzamide (**8**). 2,4-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 74.0%, pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.95 (s, 1H), 7.92 (t, 2H), 7.78 (d, 2H), 7.28 (t, 1H), 6.67 (q, 1H), 4.09 (t, 2H), 3.26 (s, 3H), 2.90 (s, 3H), 1.85 (q, 2H), 1.61-1.35 (m, 6H), 0.94 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 164.44, 155.92, 138.60, 132.86, 132.82, 132.53, 129.43, 129.25, 128.58, 125.23, 124.16, 123.90, 121.44, 111.91, 104.88, 68.55, 37.97, 37.59, 31.45, 29.19, 25.78, 22.55, 13.97. ESI-MS calculated for ($C_{23}H_{26}N_2SO_4F_6$) $[M - H]^-$: 539.01; Molecular Weight (calculated from structure): 540.52. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.37 mins, purity: >99%

N-(4-(*N*-methyl-1-phenylmethylsulfonamido)-3-((3-(trifluoromethyl)benzyl)oxy)phenyl)-3,5-bis(trifluoromethyl)benzamide (**9**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 71.2%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.35 (d, 3H), 8.07 (s, 1H), 7.69-7.28 (m, 10H), 6.96 (q, 1H), 6.88 (d, 1H), 5.00 (s, 2H), 4.32 (s, 2H), 3.11 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 155.49,

138.56, 136.90, 136.47, 131.55, 130.77, 130.68, 129.29, 129.02, 128.70, 127.61, 126.26, 125.53, 125.10, 112.96, 105.63, 69.60, 58.12, 38.54. ESI-MS calculated for ($C_{31}H_{23}N_2SO_4F_9$) [M - H]$^-$: 689.01; Molecular Weight (calculated from structure): 690.58. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.73 mins, purity: >99%

N-(4-(*N*-methyl-1-phenylmethylsulfonamido)-3-((3-(trifluoromethyl)benzyl)oxy)phenyl)-2,4-bis(trifluoromethyl)benzamide (**10**). 2,4-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 67.5%, white solid. ^{1}H NMR (400 MHz, DMSO) δ_H 7.88 (s, 1H), 7.80-7.63 (m, 6H), 7.56 (t, 2H), 7.35 (d, 5H), 7.08 (d, 1H), 6.76 (q, 1H), 5.28 (s, 2H), 4.26 (s, 2H), 3.16 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 155.70, 139.87, 139.15, 138.75, 138.41, 132.15, 131.66, 131.36, 130.24, 130.00, 129.15, 128.75, 128.50, 127.75, 126.15, 125.05, 124.95, 112.40, 69.32, 57.08, 38.46. ESI-MS calculated for ($C_{31}H_{23}N_2SO_4F_9$) [M - H]$^-$: 688.94; Molecular Weight (calculated from structure): 690.58. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.36 mins, purity: >99%

N-(4-(*N*-methyl-1-phenylmethylsulfonamido)-3-((4-(trifluoromethyl)benzyl)oxy)phenyl)-3,5-bis(trifluoromethyl)benzamide (**11**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 98.3%, white solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.74 (s, 1H), 8.59 (s, 2H), 8.40 (s, 1H), 7.69 (q, 5H), 7.37 (q, 6H), 7.08 (d, 1H), 5.32 (s, 2H), 4.46 (s, 2H), 3.12 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 155.57, 141.80, 139.83, 137.32, 131.40, 131.17, 130.84, 130.24, 129.05, 128.76, 128.58, 128.54, 126.15, 125.87, 125.83, 113.45, 106.30, 69.42, 57.12, 38.54. ESI-MS calculated for ($C_{31}H_{23}N_2SO_4F_9$) [M - H]$^-$: 688.87; Molecular Weight (calculated from structure): 690.58. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 3.01 mins, purity: >99%

N-(4-(*N*-methyl-1-phenylmethylsulfonamido)-3-((2-(trifluoromethyl)benzyl)oxy)phenyl)-3,5-bis(trifluoromethyl)benzamide (**12**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 99%, white solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.76 (s, 1H), 8.60 (s, 2H), 8.39 (s, 1H), 7.96-7.12 (m, 12H), 5.34 (s, 2H), 4.39 (s, 2H), 3.08 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 163.21, 155.48, 139.98, 137.40, 134.78, 133.44, 131.67, 131.34, 131.14, 130.81, 130.78, 130.20, 129.34, 129.08, 128.74, 128.55, 127.18, 126.88, 126.55, 126.49, 126.18, 126.05, 125.76, 124.93, 122.22, 113.62, 106.02, 66.82, 57.11, 38.50. ESI-MS calculated for ($C_{31}H_{23}N_2SO_4F_9$) [M - H]$^-$: 688.96; Molecular Weight (calculated from structure): 690.58. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.92 mins, purity: >99%

N-(4-(*N*-methyl-1-phenylmethylsulfonamido)-3-((2-(trifluoromethyl)benzyl)oxy)phenyl)-2,4-bis(trifluoromethyl)benzamide (**13**). 2,4-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 98%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.57-7.28 (m, 14H), 7.05 (d, 1H), 6.96 (q, 1H), 5.31 (s, 2H), 4.18 (s, 2H), 3.10 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 164.41, 155.53, 138.45, 134.10, 132.46, 132.05, 130.75, 130.05, 129.42, 129.00, 128.62, 128.58, 128.53, 127.88, 126.21, 112.90, 105.29, 66.85, 57.87, 38.42. ESI-MS calculated for ($C_{31}H_{23}N_2SO_4F_9$) [M - H]$^-$: 688.92; Molecular Weight (calculated from structure): 690.58. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.34 mins, purity: >99%

N-(3-(hexyloxy)-4-(*N*-methyl-1-phenylmethylsulfonamido)phenyl)-3,5-bis(trifluoromethyl)benzamide (**14**). 3,5-bis(trifluoromethyl)benzoyl chloride was used and stirred for 3 hrs. Yield 99%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 8.10 (s, 2H), 7.89 (s, 1H), 7.70 (s, 1H), 7.60-7.22 (m, 4H), 6.95 (q, 1H),

6.72 (s, 1H), 4.34 (s, 2H), 4.01 (t, 2H), 1.68 (q, 2H), 1.59 (s, 3H), 1.35 (quin, 8H), 0.92 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 134.15, 132.75, 130.68, 128.87, 128.74, 19.15, 105.75, 100.15, 31.48, 28.15, 25.60, 22.52, 13.98. ESI-MS calculated for ($C_{29}H_{30}N_2SO_4F_6$) [M - H]$^-$: 615.56; Molecular Weight (calculated from structure): 616.61. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 2.84mins, purity: >99%

4-cyano-*N*-(4-(*N*-methyl-1-phenylmethylsulfonamido)-3-((4-(trifluoromethyl)benzyl)oxy)phenyl)benzamide (**15**). 4-cyano benzoyl chloride was used and stirred for 2.5 hrs. Yield 96%, Pale brown solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.57 (S, 1H), 8.06 (t, 4H), 7.73 (d, 5H), 7.36 (d, 6H), 7.07 (d, 1H), 5.31 (s, 2H), 4.46 (s, 2H), 3.12 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 206.97, 164.75, 156.25, 142.25, 140.45, 139.15, 132.99, 131.39, 130.27, 129.00, 128.75, 128.56, 125.86, 113.19, 106.07, 69.38, 57.11, 38.25, 31.14. ESI-MS calculated for ($C_{30}H_{24}N_3SO_4F_3$) [M - H]$^-$: 578.12; Molecular Weight (calculated from structure): 579.59. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 3.12 mins, purity: >99%

N-(3-((3-cyanobenzyl)oxy)-4-(*N*-methylethylsulfonamido)phenyl)benzo[*d*][1,3]dioxole-5-carboxamide (**16**). Benzo[*d*][1,3]dioxole-5-carbonyl chloride was used and stirred for 2 hrs. Yield 94%, white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.56-6.81 (m, 11H), 6.09 (d, 2H), 5.20 (s, 2H), 3.27 (s, 3H), 3.08 (q, 2H), 1.32 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 148.38, 139.75, 137.64, 132.14, 132.09, 131.15, 129.68, 128.55, 125.25, 121.78, 113.25, 112.34, 108.26, 107.65, 105.11, 101.98, 69.42, 46.38, 38.36, 8.12. ESI-MS calculated for ($C_{25}H_{23}N_3SO_6$) [M - H]$^-$: 492.34; Molecular Weight (calculated from structure): 493.53. HPLC mobile phase: 80% ACN with 0.5% Formic acid, retention time: 1.98 mins, purity: >99%

Compound **17** was synthesized as described below.

The precursor compound was dissolved in dichloromethane (DCM) and then ethanol (EtOH) was added. The reaction was bubbled with HCl gas for 15-20 minutes and the mixture was stirring at room temperature overnight until the precursor was gone. Then the reaction was quenched with water and the solid product was filtered. This intermediate product was dissolved in 10 ml of methanol (MeOH) and bubbled with ammonia for 10-15 mins, the mixture was stirring for hours at room temperature until the intermediate was gone. The formed solid product was filtered, washed, and dried.

N-(3-((3-carbamimidoylbenzyl)oxy)-4-(*N*-methylmethylsulfonamido)phenyl)-3,5-bis(trifluoromethyl)benzamide (**17**). Yield 65%. Pale yellow solid. ^{1}H NMR (400 MHz, DMSO) δ_H 9.23 (s, 1H), 8.62 (s, 2H), 8.39 (s, 1H), 7.96 (s, 2H), 7.75 (s, 2H), 7.46 (t, 2H), 7.36 (m, 3H), 5.23 (s, 2H), 3.17 (s, 4H), 2.94 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 163.16, 162.90, 155.50, 140.26, 137.56, 136.78, 131.52, 130.63, 129.33, 128.82, 126.61, 125.70, 124.95, 122.24, 119.75, 113.40, 106.45, 70.11, 38.20. ESI-MS calculated for ($C_{25}H_{22}N_4O_4SF_6$) $[M - H]^-$: 587.61; Molecular Weight (calculated from structure): 588.52. HPLC mobile phase: 83% ACN with 0.5% Formic acid, retention time: 7.23 mins, purity: >99%

Procedure for the synthesis compound **18**:

The precursor amine (1 eq) was refluxed with 2-chloropyrimidin-4-amine (1.5 eq) in the presence of n-BuOH and a drop of trifluoroacetic acid (TFA) for 4-5 hours. The solid product formed was filtered, washed, and dried.

N-(4-((4-aminopyrimidin-2-yl)amino)-2-((2,5-dimethylbenzyl)oxy)phenyl)-N-methylmethanesulfonamide (**18**): 2-chloropyrimidin-4-amine was used and refluxed for few hours. Yield 86%. Yellow solid. ^{1}H NMR (400 MHz, DMSO) δ_H 10.46 (s, 1H), 8.29 (s, 2H), 7.90 (d, 1H), 7.62 (s, 1H), 7.28 (t, 2H), 7.13

(m, 3H), 6.19 (d, 1H), 5.13 (s, 2H), 3.10 (s, 3H), 2.85 (s, 3H), 2.32 (s, 3H), 2.27 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ_C 164.98, 155.89, 153.75, 139.03, 135.23, 134.63, 133.92, 131.63, 130.31, 129.31, 125.46, 113.53, 106.80, 98.51, 68.99, 60.85, 38.27, 38.03, 21.05, 18.46. ESI-MS calculated for ($C_{21}H_{25}N_5O_3S$) $[M - H]^-$: 426.01; Molecular Weight (calculated from structure): 427.52. HPLC mobile phase: 83% ACN with 0.5% Formic acid, retention time: 8.38 mins, purity: >99%

4.2. Cell culture

HEK293 kidney cells and mouse macrophage RAW267.4 cells were obtained from ATCC (Rockville, MD) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-Glutamine, 1 mmol/L sodium pyruvate, and 100 U/mL penicillin-streptomycin. FBS was heat inactivated for 30 min at 56 ºC. Mammalian cells were grown at 37 ºC in a Heraeus water-jacketed incubator with 5% CO_2. Bloodstream form *T. b. brucei* Lister 427 cells were cultured in HMI-9 medium [21] with 10% FBS at 37 ºC in a Heraeus water-jacketed incubator with 7.5% CO_2. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS) reagents were ordered from Promega life science (Madison, WI). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was ordered from Sigma-Aldrich (Milwaukie, WI).

4.3. Mammalian cell viability analysis

The MTT assay was used to examine the effect of tubulin inhibitors on the growth of HEK293 and RAW267.4 cells in four replications. 3000 cells per well were seeded with RPMI1640 medium in 96-well flat-bottomed plates for 24 hrs and were then exposed to various concentrations of test compounds dissolved in DMSO (final concentration ≤0.1%) in medium for 48 hrs. Controls received DMSO at a same concentration as that in drug-treated cells. Cells were incubated in 200 µl of 0.5 mg/ml of MTT reagent diluted in fresh media at 37°C for 2 hrs. Supernatants were removed from the wells, and

the reduced MTT dye was solubilized with 200 µl/well DMSO. Absorbance at 570 nm was determined on a SpectraMax Plus384 spectrophotometer (Molecular Devices). Data obtained with quadruplication were normalized and fitted to a dose–response curve using GraphPad Prism v.5 (GraphPad).

4.4. *T. brucei* cell viability analysis

The (MTS) assay was used to examine the effect of tubulin inhibitors on *T. b. brucei* cell viability [11]. 5,000 cells of *T. brucei* were seeded in 96 well plates and treated with 0.1% DMSO and tested agents at various concentrations for 48 hrs at 37 °C. Subsequently, 20 µL of MTS (5% PMS) from the CellTiter Cell Proliferation Assay (Promega) was added to 200 µL of *T. b. brucei* culture in each well and incubated at 37 °C for 3 hrs. Soluble formazan, produced by viable cells due to reduction of MTS, was measured at 490 nm with a SpectraMax Plus384 spectrophotometer (Molecular Devices). Data obtained with quadruplication were normalized and fitted to a dose-response curve using GraphPad Prism v.5 (GraphPad).

4.5. *T. brucei* cell lysate preparation and western blot assay

T. brucei cells were incubated with 0.5% DMSO and different doses of compound **15** for 7 hrs. Cell pellets were harvested by centrifugation at 1,500 rpm for 10 mins at 4°C, washed twice with 1X TDB buffer (5 mM KCl, 80 mM NaCl, 1 mM $MgSO_4$, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 20 mM glucose, pH 7.4) with protease inhibitor (Roche), and lysed with 300 µl of lysis buffer (80 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% Triton X-100, 10% glycerol and protease inhibitor (Roche)) at 30°C for 5 mins. The cell lysate was centrifuged at 12,000 rpm for 10 mins at 4°C, and the supernatant was transferred into a fresh Eppendorf tube. Pellets were re-suspended in 50 µl of lysis buffer and sonicated. 50 µl of 2 x SDS buffer was added subsequently, and the sample was boiled at 95°C for 5 mins. Protein lysates from equal number of cells were separated on 10% polyacrylamide gels by electrophoresis.

Proteins were transferred onto nylon hyblot CL membranes. Tubulin antibody (TAT-1, a gift from Dr. K. Gull) was used in the following western analysis.

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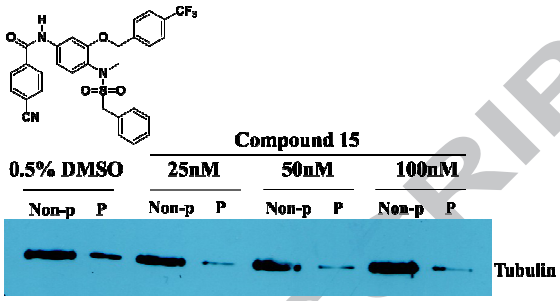
Graphical Abstract

Synthesis and biological evaluation of selective tubulin inhibitors as anti-trypanosome agents

Viharika Bobba, Vishal Nanavaty, Nethrie D. Idippily, Anran Zhao, Bibo Li, Bin Su



- A series of sulfonamide tubulin inhibitors were synthesized
- The compounds were examined with mammalian cells and trypanosomal cells to identify selective drug candidates
- A new analog showed high selectivity and potency against the growth of trypanosome cells
- The compound decreased tubulin polymerization in trypanosome cells