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Novel *β*-carboline-quinazolinone hybrid as inhibitor of *Leishmania donovani* Trypanothione Reductase: Synthesis, molecular docking and bioevaluation

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Abstract: Trypanothione reductase (TR) is a vital enzyme in the trypanothione based redox metabolism of trypanosomatid parasites. It is one of only a handful number of chemically validated targets for *Leishmania*. Herein, we report the synthesis of novel *β*-carboline-quinazolinone hybrids that are able to inhibit *Leishmania donovani* TR (LdTR) and subsequently inhibit cell growth. A molecular modeling approach based on docking studies and subsequent binding free energy estimation was performed in the active site of LdTR to understand their possible binding site. With the enzymatic assay on LdTR with compounds, we were able to identify six hit compounds (**8j-8o**) that were all found to be the competitive inhibitors of TR with K_i in the range of 0.8 - 9.2 μM. The whole-cell screening assay highlighted analogues **8k**, **8l** and **8n** as the most active compounds with IC_{50} 4.4, 6.0 and 4.3 μM respectively along with adequate selectivity index (SI) of >91, 36, 24 respectively.

Keywords: *Leishmania donovani*, *β*-Carboline, Quinazolinone, *In vitro/in vivo*, Trypanothione reductase

Introduction

The trypanosomatids are the etiological agents of several human and animal diseases, widespread in the third world and in the Mediterranean basin. The parasitic protozoan of genus *Leishmania*, belonging to the Trypanosomatidae family, is the causative agent of leishmaniasis, a severe tropical disease. There are mainly three forms of the disease namely, cutaneous, visceral, and mucocutaneous. Visceral leishmaniasis (VL) also known as kala azar is caused by *Leishmania donovani* and *Leishmania infantum* and is invariably fatal if left untreated. It is transmitted to humans by the bite of female phlebotomine sand fly and delivered as mammal-infective metacyclic promastigotes. In the human body, the parasites survive and multiply within phagolysosomes of macrophages as intracellular amastigotes.[1] According to the recent reports of WHO, 200 000 to 400 000 new cases of VL occur worldwide each year.[2] In addition, there is an important incidence of *Leishmania* co-infection in HIV patients, due to the opportunistic character of the parasite. With the spread of HIV/AIDS, co-infection with leishmaniasis has risen to epidemic proportions.[3] Since effective vaccines are not yet available, nowadays treatment of the disease relies exclusively on chemotherapy. However, existing chemotherapeutics, such as pentavalent antimony, paromomycin, amphotericin B and miltefosine show serious side effects and have several limitations that confines their extensive use.[4] Nonetheless, the paucity of new leads in the pipeline is raising concern about the near future. Therefore, the search for innovative drugs based on new molecular scaffolds is urgently needed to expand the treatment options available for the disease.

One of the strategies to develop potent antileishmanial agents is to exploit the metabolic pathways that are essential for the survival of the parasite in the host. Trypanothione Reductase (TR) is a vital enzyme for the antioxidant defenses in trypanosomatids which is analogous to the

mammalian redox defense system, based on the glutathione/glutathione reductase (GR*a*) couple. It is a unique NADPH-dependent flavoenzyme that fights oxidative stress by maintaining adequate levels of trypanothione $T[SH]_2$. The enzyme catalyzes the reduction of trypanothione disulfide $[T(S)_2]$ dithiol to trypanothione $[T(SH)_2]$. The reduction of trypanothione disulfide (TS_2) to $T[SH]_2$ catalyzed by TR, is homologous to the reduction of glutathione disulfide by glutathione reductase in mammals.[5-8] Although, being structurally and mechanistically similar to the mammalian enzyme glutathione reductase, trypanothione reductase sufficiently differs in its disulfide-binding site for selective chemotherapeutic attack.[9-12] The absence of the enzyme from mammalian cells and its vital role in the antioxidant defense of the parasite render trypanothione reductase as an attractive target molecule for a rational drug design.

The natural product inspired molecular hybridization approach[13] for the synthesis of new scaffolds can help to overcome the limitations associated with the known standard drugs.[14] Natural products have served as a potential source of TR-inhibitors.[15] *β*-Carboline and quinazolinone also forms the building block of some most interesting class of naturally occurring compounds associated with a wide range of biological activities.[16-21] From the literature search it was revealed that several natural product scaffolds including *β*-carboline alkaloid, harman and its derivatives have shown potential inhibition of TR.[22] A great deal of work has been done in our lab for the synthesis of *β*-carboline, tetrahydro-*β*-carboline and quinazolinone derivatives that have shown promising antileishmanial activity.[23-26]

Encouraged by these inspiring facts, we designed and synthesized an entirely new series of *β*-carboline-quinazolinone hybrid and investigated their activity against TR of *L. donovani* (LdTR). The investigation included *in silico* modeling and enzymatic assay of these compounds

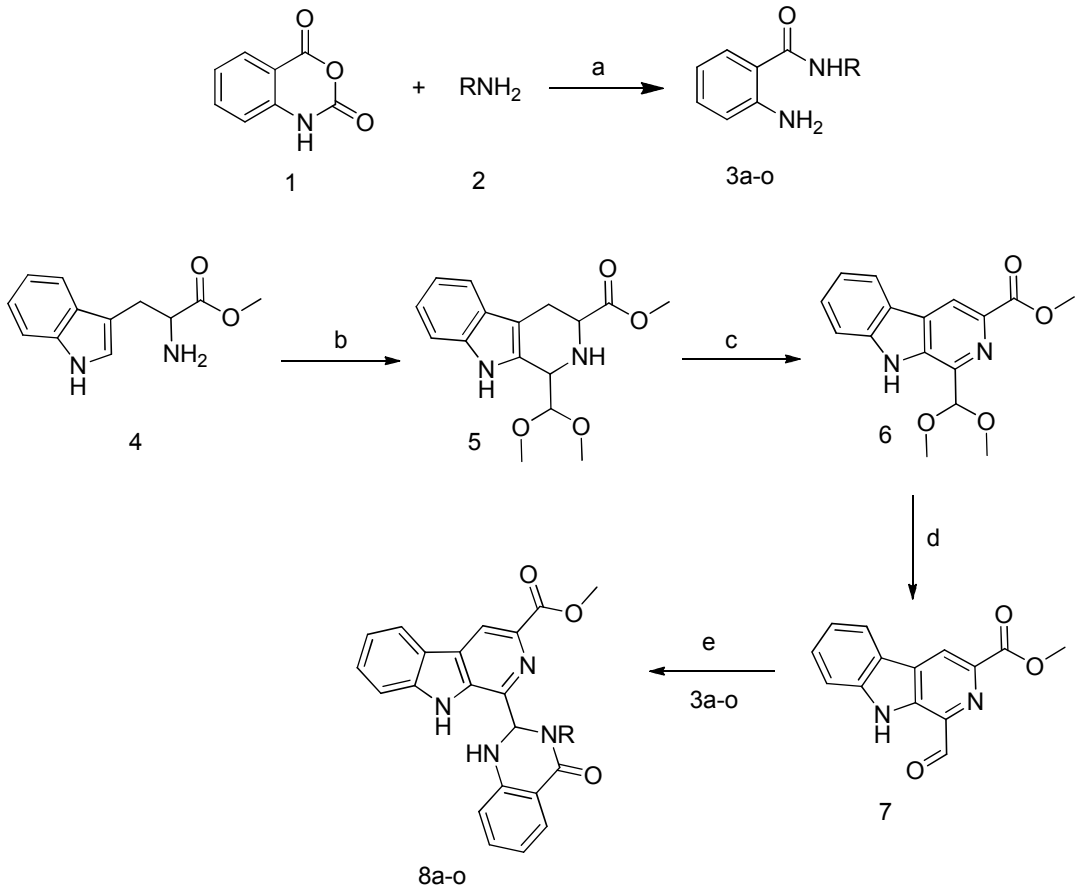
against LdTR. We identified few compounds that showed potent TR inhibition and also promising antileishmanial activity against intracellular amastigote form of *L. donovani*.

Results and discussion

Chemistry

The detailed synthetic route to synthesize compounds **(8a-o)** is outlined in Scheme 1. The synthesis was achieved by an efficient and facile methodology.[27] We started the study with synthesis of 2-aminobenzamides **3a-o** from isotoic anhydride **1**. For the synthesis of 2-aminobenzamide derivatives with alipahatic amines and benzyl amines a simpler and greener protocol was followed similar to those described in literature.[28,29] An equivalent amount of isatoic anhydride **1** and amines **2** were reacted in water at room temperature for two hours. After completion of the reaction, the resulting precipitates were filtered off and used for the next reactions without further purifications. But in case of aromatic amines this procedure was not facile therefore for the synthesis of 2-aminobenzamide derivatives **8a**, **8f**, **8k** and **8l,** isatoic anhydride **1** and amines **2** were refluxed in acetonitrile (ACN) in the presence of catalytic amount of triethyl amine (TEA). Next, the desired hybrid compound **8** was synthesized in four steps. The synthetic strategy started with the Pictet–Spengler reaction of tryptophan ester **4** with dimethoxyglyoxal, to afford the tetrahydro-*β*-carboline derivative **5**, which was oxidized with $KMnO_4$ at room temperature overnight, resulting in acetal **6**. The acetal **6** was deprotected in aqueous acetic acid (30% aq. solution) to give the corresponding aldehyde **7**.[30] Aldehyde **7** was coupled with 2-amino-alkyl benzamides **3** using cyanuric chloride as a catalyst to provide the desired product **8** in moderate to good yield. The compounds were well characterized by spectroscopic methods such as IR, NMR, and HRMS.

Scheme 1:



Reagents and conditions: (a) water, rt, for aliphatic amines and ACN, TEA, 80°C for aromatic amines; (b) $OHCCH(OMe)_2$, 5% TFA, CH_2Cl_2, rt, 17 h; (c) KMnO4, THF, rt, 17h; (d) $AcOH/H_2O$ (2:3 ratio), 120°C, 1 h; (e) cyanuric chloride, ACN, 1-2 h, 60-70°C .

Molecular Modeling and Docking

In silico modeling offers immense potential in identification of novel drug candidates. A docking study for compounds **8a-8o** in the active site of LdTR was performed to recognize their potential binding modes. The crystal structure of LdTR is not available; therefore the homology modeling was performed to get the protein structure of LdTR (see supplementary information for detailed methodology). The LdTR sequence has 98% identity with template used and the superimposition of the modeled protein with template shows a RMSD of 0.269Å (Supplementary

Fig 1). Validation of the resulting model was done with the SAVS server.[31] The majority of the residues (91.7%) occupy the most favored region of Ramachandran Plot generated by PROCHECK [32] and 7.6%, 0.7% and 0.0% residues lie in additional allowed region, generously allowed and disallowed region respectively (Supplementary Fig 2). The docking studies were performed with the use of the Autodock 4.2. All the relevant protocol for the docking studies is provided in the 'Supporting Information'.

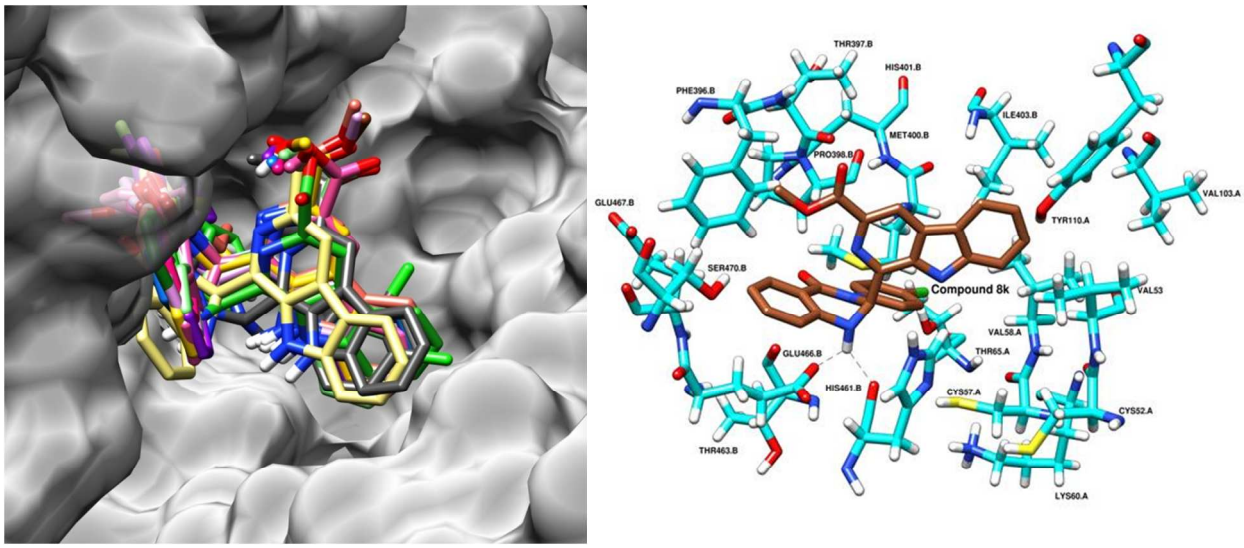


Fig. 1: (a) Overlay of inhibitors in the binding site of modeled LdTR protein, (b) Interaction of Compound 8k (*Brown*) with LdTR model protein (*Cyan*)

The enzyme TR in its active form is a homo dimer consisting of two identical chains each consists of 491 residues.[29] The catalytic site of the enzyme include two redox active cysteine residues (Cys52 and Cys57) and one histidine residue (His461) that are involved in anchoring of trypanothione disulfide at the active site.[33,34] The trypanothione binding site that is involved in the binding of an inhibitor[35] was also selected as the active site for docking of these molecules. The top scoring docked conformations of all the ligands in the active site of homology model of LdTR were analyzed in terms of key residues involved in the interaction and their preferred

mode of binding. The docking energies of docked conformation are given in supplementary table1. Figure 1a shows that all the compounds are in the binding pocket of protein. It can be seen from the figure that all the ligands are docked bound inside a cavity which is formed at the interface of chain A and chain B. To illustrate the probable mechanism of the protein–ligand interaction, docking of compound **8k** with LdTR is described in detail in figure 1b. It can be seen in the figure that the compound is involved in hydrogen bond formation with His461 and Glu466. These interactions are known to play important role in substrate binding and might therefore contribute to the inhibitory activity of these compounds.

Biological activity

Enzymatic activity

Inspired with the docking results, all the compounds were further tested for their inhibitory activity against *L. donovani* TR enzyme. A systematic approach to investigate the potential LdTR inhibitory agent was taken and the study was designed *via* exploring the quinazolinone nucleus of the hybrid molecule with different cyclic/acyclic aliphatic and aromatic groups, consequently establishing the structure–activity relationships (SAR). All the synthesized compounds were found to inhibit the LdTR enzymatic activity except compounds **8e**, **8f**, **8g** and **8h.** Under the selected assay conditions (see Supporting Information), the compounds (at 100 μM) exhibited upto 99% inhibition of TS_2 reduction (Table 1). From these results, we could speculate that the *β*-carboline-quinazolinone hybrid is a good motif for TR recognition. However, the presence of a suitable capping fragment is essential for activity. The activity of these molecules depended upon the substitution pattern at the amide NH of the quinazolinone.

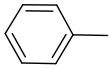
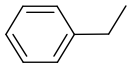
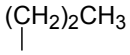
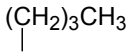
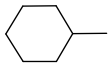
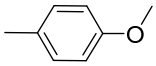
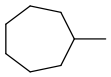
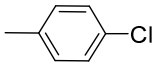
Within the set of aliphatic group substituted compounds, potency of compounds with acyclic aliphatic groups were found to be more than the compounds with cyclic aliphatic groups,

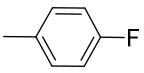
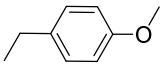
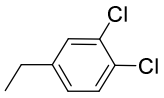
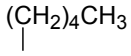
with the exception of compound **8c** that showed negligible inhibitory activity (9%) in comparison to other compounds of the series **8d**, **8j** and **8o**. The cyclic aliphatic group substituted compounds (**8e**, **8g, 8h** and **8i)** were found to be inactive except compound **8i** that showed only a mild inhibitory activity of 30%.

The substitution with aromatic group like unsubstituted phenyl (**8a**) and benzyl (**8b**) resulted to insignificant inhibition of LdTR with 18% and 11% respectively. While the substitution of phenyl group of compound **8a** with electron withdrawing groups (**8k** and **8l**) was tolerated with high potency (67% and 96% respectively), the substitution with electron donating group (**8f**) resulted into a complete loss in inhibition potency. On the other side substitution of the benzyl group in compound **8b** with both electron donating group (**8m**) and electron withdrawing group (**8n**) were tolerated with high efficacy showing inhibition of 60% and 85% respectively, although in this case also electron withdrawing group showed higher inhibition than the electron donating group.

Overall, the compounds having aromatic group with electron withdrawing substituent exhibited better inhibition of LdTR enzyme as compared to unsubstituted aromatic group or the one with electron donating substituent. The acyclic aliphatic group displayed significantly higher potency when compared with compounds substituted with cyclic aliphatic group.

Table 1 Percentage inhibition of *L. donovani* TR and Inhibition constant of compounds **8a-8o**

Compound	R	Percent Inhibition[a]		K_i[b] (μM)
		100μM	50μM	
8a		18 ± 0.5	16 ± 1.7	ND[d]
8b		11 ± 0.9	6 ± 0.8	ND
8c		9 ± 0.5	7 ± 0.6	ND
8d		35 ± 1.9	28 ± 1.2	ND
8e		1 ± 0.2	NI	ND
8f		NI[c]	NI	ND
8g		NI	NI	ND
8h		NI	NI	ND
8i		30 ± 1.3	20 ± 0.9	ND
8j		75 ± 0.4	58 ± 0.5	6.5 ± 0.2
8k		67 ± 0.9	64 ± 0.4	7.2 ± 0.5

8l		96 ± 0.8	81 ± 1.6	3.9 ± 0.1
8m		60 ± 0.5	56 ± 0.8	9.2 ± 0.2
8n		85 ± 0.5	77 ± 0.5	3.7 ± 0.7
8o		99 ± 0.6	90 ± 0.9	0.8 ± 0.1

[a]5μM TS_2, 100μM and 50μM inhibitor, 1m unit *L. donovani* TR
[b]Inhibition constant. All inhibitors are of competitive nature
[c]NI – no inhibition
[d]ND – not determined

The type of inhibition and their respective inhibition constants (K_i) of the best active compounds (**8j-8o**) was determined by Dixon plot graphical method at varying concentration of inhibitor and two fixed substrate ($T[S]_2$) concentration (50 and 100μM). The compounds showed good inhibition constant with K_i values in the range of 0.8 - 9.2 μM. All the six potential inhibitors were found to be competitive type of inhibitors (Figure 2).

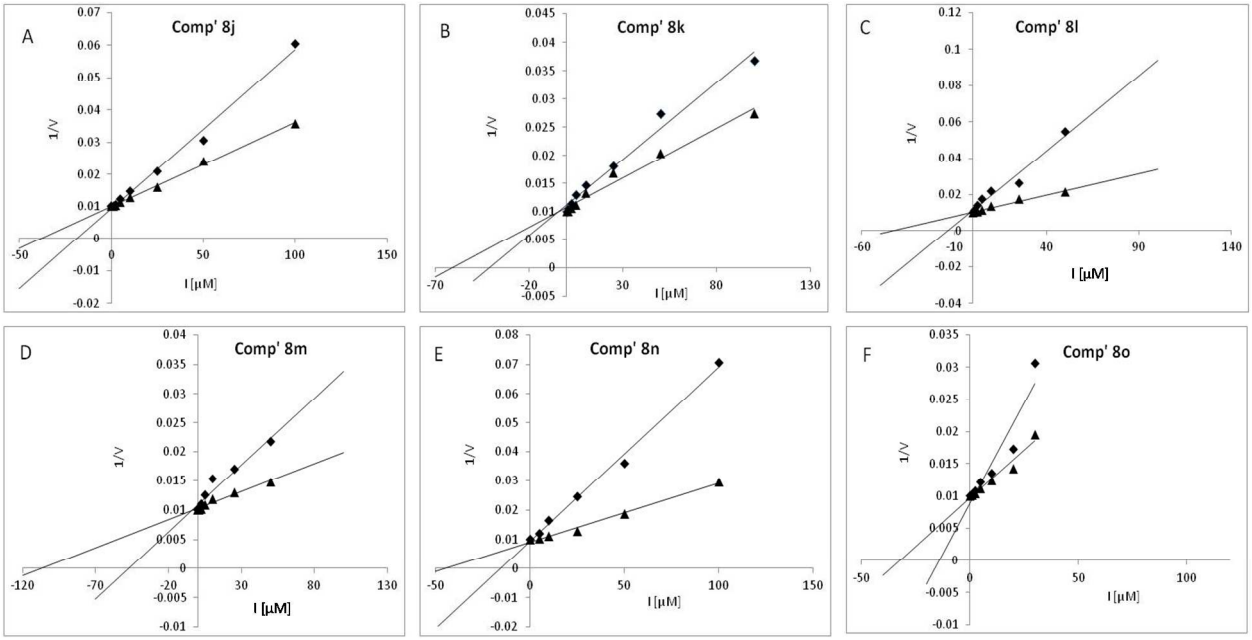


Figure 2: A Plot of reciprocal of velocity as function of concentration of inhibitor(s) at two substrate concentrations [50 µM (♦) and 100 µM (▲)] to determine type of inhibition and K_i values. **A**: Dixon plot for compound **8j**; **B:** Dixon plot for compound **8k**; **C:** Dixon plot for compound **8l**; **D:** Dixon plot for compound **8m**; **E:** Dixon plot for compound **8n**; **F:** Dixon plot for compound **8o**. Points are experimental; lines are theoretical for linear competitive inhibition.

Antileishmanial activity and cytotoxicity

As a subsequent step, the compounds **8j-8o** were tested against the extracellular promastigotes of *L. donovani*. All the compounds showed potent activity with IC_{50} in the range of 3.3 to 9.9 µM (Table 2). Based on these results, compounds were further tested against the intracellular amastigotes of *L. donovani* and their cytotoxicity on Vero cells was also determined. The results (table 2) showed a good correlation between enzyme inhibition and antileishmanial activity of compounds **8k**, **8l** and **8n** which also potent TR inhibitors. The compounds **8k**, **8l** and **8n** showed IC_{50} of 4.4, 6.0 and 4.3 µM respectively against the intracellular amastigotes.

Table 2 Antileishmanial activities of synthetic analogues **8j-8o** against extracellular promastigotes and intracellular amastigotes of *Leishmania donovani* and their cytotoxicities

Compound	Antipromastigote activity IC_{50} (μM)[a]	Antiamastigote activity IC_{50} (μM)	CC_{50} (μM)[b]	SI[c]
8j	8.5 ± 1.0	>40	ND[d]	NA[e]
8k	3.3 ± 0.7	4.4 ± 0.8	>400	>91
8l	4.6 ± 0.6	6.0 ± 1.0	217.3 ± 13.0	36
8m	5.4 ± 0.7	35.2 ± 4.0	105.8 ± 8.7	3
8n	4.8 ± 0.7	4.3 ± 0.5	103.7 ± 8.7	24
8o	9.9 ± 1.2	>40	ND	NA
Miltefosine	1.2 ± 0.4	8.1 ± 0.5	56.5 ± 4.0	7
SSG	Not active	54.4 ± 1.8	>400	7

[a]50% inhibitory concentration (IC_{50}) and the values are the average of two independent assays done in duplicates expressed as average ± standard deviation.
[b]50% cytotoxicity concentration (CC_{50}) and the values are the average of two independent assays done in duplicates expressed as average ± standard deviation.
[c]Selectivity index (SI) for each compound was calculated as ratio between, CC_{50} in vero cells and IC_{50} against *Leishmania* amastigotes.
[d]Not done; [e]Not available
Miltefosine and SSG (Sodium stibo-gluconate) were used as standard antileishmanials.

Further, the cytotoxicity data in the range of 103.7 - >400 μM also suggested that these compounds were non toxic to the mammalian cells. The *in vitro* results illustrate that the compounds were superior and less toxic than the known standard drugs miltefosine and sodium stibogluconate. Unfortunately, compounds **8j**, **8m** and **8o** that showed good activity against promastigotes did not showed the similar results against amastigotes of *L. donovani*. Compounds **8j** and **8o** were found to be completely inactive while **8m** showed only a slight antileishmanial

activity of IC_{50} = 35.2 μM. The possible explanation for the inactivity of these compounds could be the selective uptake or their inability to cross the host's cell membranes to reach their intracellular target.

Conclusion

The parasite-specific flavoenzyme trypanothione reductase is an attractive target for antileishmanial drug development. In this scenario, this small library holds promise for further exploration of these hybrid compounds for interaction with TR, with the potential to generate new antileishmanial agents. The present study introduces *β*-carboline-quinazolinone hybrid as a new class of compounds that are efficient competitive TR inhibitor and showed good antiparasitic activity against *L. donovani.* The clear correlation between the enzyme inhibition constants and antileishmanial activity of compounds suggests that the compounds probably act by inhibiting the enzyme TR. These investigations revealed that this group of compounds is extremely useful leads to further explore the trypanothione pathway in kinetoplastids and in the development of new antiparasitic agents.

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