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Identification and characterization of guanosine 5'-monophosphate reductase of *Trypanosoma congolense* as a drug target

Running title: GMP reductase of *Trypanosoma congolense*

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Conflict of interest statement

The authors declare that there is no conflict of interest

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ABSTRACT

Trypanosoma congolense is one of the most prevalent pathogens which causes trypanosomosis in African animals, resulting in a significant economic loss. In its life cycle, *T. congolense* is incapable of synthesizing purine nucleotides via a *de novo* pathway, and thus relies on a salvage pathway to survive. In this study, we identified a gene from *T. congolense*, TcIL3000_5_1940, as a guanosine 5'-monophosphate reductase (GMPR), an enzyme that modulates the concentration of intracellular guanosine in the pathogen. The recombinant protein was expressed in *Escherichia coli*, and the gene product was enzymatically confirmed as a unique GMPR, designated as rTcGMPR. This enzyme was constitutively expressed in glycosomes at all of the parasite's developmental stages similar to other purine nucleotide metabolic enzymes. Mycophenolic acid (MPA) was found to inhibit rTcGMPR activity. Hence, it is a potential lead compound for the design of trypanocidal agents, specifically GMPR inhibitor.

KEYWORDS: African trypanosomosis; GMP reductase; purine metabolic pathway; *Trypanosoma congolense*

1. Introduction

The purine nucleotide is an essential building block of genetic materials in virtually all organisms. Guanosine 5'-monophosphate dehydrogenase (GMPR) (EC 1.7.1.7) is a regulatory enzyme for purine biosynthesis, which acts as a deamination catalyst of guanosine 5'-monophosphate (GMP) conversion to inosine 5'-monophosphate (IMP) (Fig. 1). This enzyme complements two other purine regulatory enzymes: inosine 5'-monophosphate dehydrogenase (IMPDH) (EC 1.1.1.205) and GMP synthase (GMPS) (EC 6.3.5.2), with each catalyzing conversion of IMP to xanthosine 5'-monophosphate (XMP) and XMP to GMP, respectively. Consequently, these enzymes cooperatively maintain the intracellular balance of adenine and guanine nucleotides. However, due to the similarity between GMPR and IMPDH at the primary structure level, misannotation of these two enzymes is common [1]. The similarities between these enzymes were even more pronounced in reported Kinetoplastida GMPR, which apparently featured a cystathione β-synthase (CBS) pair domain; previously it was only found in IMPDHs [2,3].

(Fig. 1)

Due to the lack of metabolic machinery for synthesis via *de novo* pathway, some protozoa rely on salvage pathway to produce purine nucleotides. On that account, the purine biosynthesis pathway in protozoan studies has been considered as an interesting target for drugs [4–6] . Purine nucleotide synthesis in Kinetoplastida parasites is also dependent on a salvage pathway, and most of the reactions catalyzed by the above enzymes are proceeded in the glycosomes [7]. Pathogenic protozoa *T. congolense* also possess this unique feature, which could be exploited as a novel chemotherapeutic target.

T. congolense itself is a pathogenic protozoan that infects a broad range of African animals, inflicting fatal animal trypanosomosis, which is also known as *nagana* disease. Up until recent time, the infection of domesticated animals in central Africa has resulted in severe economical loss to the society [8]. Currently, chemotherapeutic strategies against *T. congolense* infection consist of outdated and ineffective therapeutic compounds, resulting in the emergence of chemo-resistant *T. congolense* strains [9–11]. Generally, humans are resistant to *T. congolense* infection due to the trypanosome lytic factor in the serum. Several research groups, however, have reported some cases of viable *T. congolense* strains in human plasma, posing a future threat to human health [12–14].

This study provides the first enzymatic and cytological identification of a unique GMPR-encoding gene product in all life cycle stages of *T. congolense*. Firstly, by analyzing the primary structure of TcGMPR, we proposed a hypothesis on Kinetoplastida GMPR evolution lineage and distinctive amino acids for simple GMPR identification. Subsequently, the recombinant TcGMPR (rTcGMPR), expressed in *Escherichia coli*, was found to exhibit significantly different enzymatic parameters from those of mammalian GMPRs. In the protozoan cell, TcGMPR was continuously expressed in the glycosome throughout its life cycle. We also found that mycophenolic acid, a selective IMPDH inhibitor, was an inhibitor for rTcGMPR at micromolar levels.

2. Materials and Methods

2.1. Trypanosome in vitro culture

T. congolense IL3000, a Savannah subgroup strain isolated near the national border between Kenya and Tanzania in 1966, was used in this study. The blood stream form (BSF) of *T. congolense* IL3000 was propagated at 33ºC in 5% CO_2 with Hirumi's modified Iscove's medium (HMI)-9 [15]. Procyclic trypomastigotes (PCF) and epimastigotes (EMF) were propagated at 27ºC using *T. vivax* medium [16]. Metacycleic trypomastigote (MCF) were separated from the supernatant of confluently cultured EMF culture by a DE52 anion exchange column [17]. Trypanosome cultures were maintained by replacing the entire culture supernatant with fresh medium every other day.

2.2. Cloning of T. congolense GMP reductase

T. congolense GMP reductase (TcGMPR) (accession number TcIL3000_5_1940) was identified at TriTrypDB (http://tritrypdb.org/tritrypdb/). The full-length *TcGMPR* gene was PCR-amplified from *T. congolense* IL3000 strain genomic DNA using TcGMPR-F and TcGMPR-R primer sets (Table S1). A full-length *TcGMPR* gene was digested by *Bam* HI and *Eco* RI, and inserted into a pGEX 6P-2 plasmid (GE Healthcare Bio-Science Corp., UK).

2.3. Homology analysis and phylogenic analysis of GMPRs and IMPDHs

A homology analysis was performed using ClustalW ver. 2.1 (http://clustalw.ddbj.nig.ac.jp). A phylogenetic tree was constructed by neighbor-joining method using MEGA 5.2 software.

2.4. Expression and purification of recombinant TcGMPR protein

The *E. coli* cells harboring TcGMPR-pGEX 6P-2 plasmid were grown at 37ºC until the optical density at 600 nm reached 0.4. A recombinant TcGMPR fused with glutathione S-transferase (GST) was induced with 0.1 mM isopropyl thio-β-D-galactoside (IPTG) for 12 h at 18ºC. rTcGMPR was purified and cleaved using glutathione Sepharose 4B beads with PreScission protease (GE Healthcare Bio-Science Corp., UK) according to the manufacturer's protocol. rTcGMPR concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Japan, Yokohama, Japan) in accordance with the manufacturer's instructions. The protein was stored at -80ºC as a 50% glycerol mixture until use.

2.5. Effect of pH to rTcGMPR activity

Method 2.5, 2.6, and 2.8 were carried out according to a previous report, with some modifications [2]. Briefly, the activity of the recombinant protein was measured at 35ºC in 75 mM Tris-HCl buffer under various pH (7.0-8.7) conditions. The reaction solution contained 200 μM NADPH (Sigma Aldrich, St. Louis, MO, USA), 1 mM GMP (Sigma Aldrich Japan, Tokyo, Japan), 100 mM KCl, 3 mM EDTA, 0.1 mg/ml bovine serum albumin (BSA), and 1 mM dithiothreitol. The ionic strength of the solution was set to 0.2 with NaCl. The enzyme assay was started by the addition of rTcGMPR to the reaction solution. Activity of rTcGMPR was monitored at 340 nm using Biophotometer plus spectrophotometer (Eppendorf, Germany) to measure the conversion of NADPH to $NADP^+$. Activity for each pH value was expressed as percent difference of initial velocity (V_0) value from V_0 at pH 7.0.

2.6. Kinetic parameter determination of rTcGMPR

Enzymatic activity was measured with an assay solution containing 75 mM Tris-HCl pH 7.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, and 0.1 mg/ml BSA at 35ºC. The ionic strength of the solution was set to 0.2 with NaCl. Various concentrations of GMP were used with fixed dose of NADPH at 200 μM for measurement of steady-state kinetics for GMP. By contrast, various concentrations of NADPH were used with fixed dose of GMP at 1 mM for measurement of steady-state kinetics for NADPH. Measurements of V_{max} and K_m were conducted by fitting the acquired initial velocity data from three independent experiments to the Michaelis-Menten equation using GraFit 7 software (Erithacus software, U.K.).

2.7. Inhibition of rTcGMPR by MPA

MPA inhibition to rTcGMPR activity was measured with an assay solution containing 75 mM Tris-HCl pH 7.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 100 μM NADPH, 100 μM GMP, and various concentrations of MPA at 30ºC. DMSO was used as vehicle, with a final concentration of 1%. The reaction was started by adding 50 nM of rTcGMPR. Reaction was carried out for 1 h. Activity of rTcGMPR was monitored as a decrease of absorbance at 340 nm. Each point was expressed as a percentage of consumed NADPH in each treatment sample relative to the vehicle

control. The IC_{50} value was calculated using GraFit 7 software (Erithacus software, U.K.)

2.8. *Activation of rTcGMPR by monovalent cation*

Enzyme activation by monovalent cation was conducted with an assay solution containing 75 mM Tris-HCl pH 7.0, 3 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 200 μM NADPH, 1 mM GMP, and 100 mM monovalent chloride (either LiCl, NaCl, KCl, CsCl, or NH_4Cl) at 35ºC. Activity for each monovalent cation was expressed as a percent difference of the initial velocity (V_0) value from V_0 in the assay with NaCl.

2.9. *Polyclonal antibody production*

Two 8-weeks-old imprinting control region mice were immunized with 50 μg (100 μL) emulsion of equal volume of rTcGMPR and TiterMax® Gold (TiterMax USA Inc., Norcross, GA). Primary immunization and two boosters (at two-week intervals) were administered subcutaneously. One week after the second booster injection, blood was collected by cardiac puncture at terminal anesthesia. Sera were prepared by centrifugation at 5,000×g for 10 min at 4ºC. The animal experiments were carried out in accordance with the Standards Relating to the Care and Management of Experimental Animals of Obihiro University of Agriculture and Veterinary Medicine (No. 25-134).

2.10. *Western blot analysis*

To prepare the parasite cell extracts, each stage of trypanosome in four life cycles was suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, and 1.0% Triton X-100) supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics K.K. Tokyo, Japan). After 30 min incubation on ice, the extract was centrifuged at 21,500×g, 4ºC for 10 min. Five-μg of each extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred into a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare Co.). The membrane was blocked by 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), which was then incubated with a primary antibody (anti-TcGMPR serum) and further incubated with secondary antibody ECL™ anti-mouse IgG, horseradish peroxidase linked whole antibody from sheep (GE Healthcare Co., UK). The result was visualized using an ECL Western Blotting Detection System (GE Healthcare Co., UK) according

to the manufacturer's instructions. The relative quantity of TcGMPR was evaluated by measuring the signal densities of TcGMPR normalized by *T. congolense* α-tubulin signal densities using Quantity One software (Bio-Rad, Berkeley, CA) [18].

2.11. Immunofluorescence microscopy

T. congolense specimens corresponding to each developmental stage were suspended in PBS with 1% glucose. The cell suspensions were spread over glass slides printed with highly water-repellent mark (Matsunami Glass Ind., Ltd., Tokyo, Japan), air-dried, and fixed with 100% methanol for 10 min at room temperature. For blocking, the specimens were incubated with 5% skim milk in TBS-T for 1 h at room temperature. The specimens were incubated with a primary antibody (anti-TcGMPR serum), subsequently incubated with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG [H+L], Invitrogen, Life Technologies Japan, Tokyo, Japan) and Hoechest 33342 (Dojundo, Co. Ltd., Kumamoto, Japan). The specimens were observed using a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany).

3. Results

3.1. *Identification, bioinformatics analysis, and expression of TcGMPR*

By utilizing a BLAST search, a GMPR/IMPDH family gene, TcIL3000_5_1940, was identified in *T. congolense* as the homolog with the highest similarity to our previously discovered *T. congolense* IMPDH [18]. The similarity of amino acid sequence between GMPRs and IMPDHs, however, often results in misleading differentiation between enzymes if it is carried out only by computational gene prediction methods [1]. Phylogenetic analysis revealed that IMPDHs and GMPRs were classified into different clades. However, while GMPR from *L. donovani* and *T. brucei* clearly exhibited a GMPR enzyme activity [2,19], they were not classified into the GMPR clade, but to in the IMPDH related clade instead. TcIL3000_5_1940 itself was classified in the same clade as *L. donovani* and *T. brucei* GMPR as shown in Fig. 2. Amino acid sequences of GMPR homologs were aligned to examine the conservation of its sequence in Fig. 3.

(Fig. 2)

(Fig. 3)

The predicted secondary structure of the TcIL3000_5_1940 protein consisted of the TIM_phosphate_binding superfamily and two tandem repeats of CBS pair domains. To date, it has been generally accepted that the CBS pair domain was contained in IMPDH but not in GMPR [1], whereas this domain was found in *L. donovani* GMPR, *T. brucei* GMPR, and TcIL3000_5_1940 (Fig. 3). TcIL3000_5_1940 itself shares 72% of amino acid similarity with *L. donovani* GMPR and 80% of amino acid similarity with *T. brucei* GMPR. However, TcIL3000_5_1940 shares only 32-35% gene similarity to *Homo sapiens* GMPR1 (HsGMPR1), HsGMPR2, and *Bos taurus* GMPR1 (BtGMPR), and BtGMPR2 as indicated in Table 1 [2,20–23]. In similar order, we observed up to 44% of shared identity in the primary structures between TcIL3000_5_1940 and the IMPDHs in this study (Table S2).

(Table 1)

A full length TcIL3000_5_1940 candidate was cloned and a recombinant protein of TcIL3000_5_1940 was expressed in *E. coli*. The purified protein was detected as a single band with a molecular weight of 52 kDa on SDS-PAGE (Fig. 4). The protein did not show any IMPDH activity (Fig. S1), while the activity of GMPR was confirmed as described in the following sections.

(Fig. 4)

3.2. *Effect of pH to rTcGMPR activity*

To determine the optimum assay condition for steady-state kinetics measurement, activity of the recombinant protein was evaluated at several pH values under neutral to basic conditions. In agreement with a previous report on *T. brucei* GMPR, activity of rTcGMPR was found to be highest at pH 7.0, and gradually decreased as the pH of the assay solution increased (Fig. 5) [2]. Acidic conditions are generally avoided for GMPR studies due to the instability of co-factor NADPH under such conditions.

(Fig. 5)

3.3. *Enzymatic analysis of rTcGMPR*

The apparent steady-state kinetics parameters of rTcGMPR were characterized with varying either GMP or NADPH, and the acquired initial velocity was charted into a Lineweaver-Burk plot (Fig. S2). The values of $K_{m\,GMP}$, K_{mNADPH}, and k_{cat} were determined to be 91.6 ± 4.7 μM, 11.3 ± 2.3 μM, and 0.499 ± 0.014 s^{-1}, respectively. These values were quite similar to the reported values for TbGMPR, but the enzymatic properties were significantly different from those of HsGMPR1, HsGMPR2, BtGMPR1, and BtGMPR2 as indicated in Table 2 [2]. The values of these parameters indicated that rTcGMPR has at least 4-fold lower affinity to GMP, but up to 5-fold stronger affinity to NADPH, compared to those of mammalian GMPRs. Moreover, the k_{cat} value of rTcGMPR indicated that the *Trypanosomatidae* enzyme is up to 1.7-fold more efficient compared to its mammalian counterparts for catalyzing GMP conversion to IMP.

(Table 2)

3.4. *Inhibition of rTcGMPR by MPA*

MPA is a known inhibitor of IMPDH. The structural similarities at the primary level between IMPDH and GMPR make MPA an intriguing candidate as a multi-target inhibitor. The rTcGMPR inhibition assay of MPA showed a clear activity with IC_{50} of 239.6 ± 26.7 μM (Fig. 6). MPA also showed potent inhibition activity in the *T. congolense* culture, where the presence of 1 μM inhibitor resulted in a 99.6% decrease in free-living protozoa [18].

(Fig. 6)

3.5. *Activation of rTcGMPR by monovalent cation*

The results suggested that the cation effect for rTcGMPR is correlated with the radius size of the corresponding cation. In the presence of Li^+ (0.60 Å), the enzyme activity was lowest at 3.8 ± 0.2 nM/s. Subsequently, the addition of larger radius cations: Na^+ (0.95 Å), K^+ (1.33 Å), Cs^+ (1.69 Å), and

NH_4^+ (1.48 Å) enhanced the enzyme activity to 5.8 ± 0.2 nM/s, 28.5 ± 1.0 nM/s, 9.7 ± 0.8, and 19.5 ± 2.5 nM/s, respectively (Fig. 7).

(Fig. 7)

3.6. *Expression pattern and cellular localization of TcGMPR*

Previous studies have revealed that purine metabolic enzymes of Kinetoplastida were mostly located in glycosomes [7,24]. In addition, further amino acid sequence analysis of TcGMPR indicated a C-terminal Ser-Lys-Leu peroxisomal (glycosomal) targeting signal similar to that of *L. donovani* GMPR (Fig. 3) and other glycosomal proteins [7,24,25]. TcGMPR was expressed in all stages of the life cycle of *T. congolense* as shown in Fig. 8A and 8C. By Western blot analysis, TcGMPR was found to be expressed up to 2-3 fold higher in EMF than in BSF, PCF, and MCF as shown in Fig. 8B. This result was consistent with the previous report concerning the differential protein expression analysis of all stages of the *T. congolense* lifecycle [26]. TcGMPR was expressed in the cytosol as a granular localization, which was consistent with the glycosomes localization of *T. brucei* GMPR and *T. congolense* IMPDH (Fig. 8C) [2,7,18,24].

(Fig. 8 – Colored Figure)

4. Discussion

Due to the lack of *de novo* purine synthesis pathway in trypanosomes, the protozoa is entirely dependent on a salvage pathway, thus making this pathway an interesting drug target against any pathogen of this genus [2,5,27–29]. GMPR is one of the enzymes in the pathway that maintains intracellular adenine and guanine balance, along with IMPDH and GMPS [1]. More recent reports have confirmed that an inhibition to one of the enzymes in the streamlined pathway effectively inhibits the growth and proliferation of pathogens having a similar purine synthesis pathway, thus validating the pathway as a promising drug target for anti-infection chemotherapy [3,27,28,30–34]. Therefore, holistic understanding of this pathway is essential for designing novel therapeutic strategies against such pathogens.

In gene data banks, an IMPDH/GMPR gene TcIL3000_5_1940 of *T. congolense* was annotated as either GMPR or IMPDH, allegedly as a result of identity determination based on genomic sequence analysis. This sort of discrepancy is common in the IMPDH/GMPR family gene, and has caused confusions [1]. We have recently discovered a protein in the IMPDH/GMPR family from *T. congolense*, which was later identified as an IMPDH [18]. Therefore, to fully understand the purine biosynthesis pathway in *T. congolense*, further analysis was conducted to accurately identify the

TcIL3000_5_1940 gene product as a possible GMPR candidate.

To date, it was assumed that IMPDHs and GMPRs were classified into different clades, with only IMPDHs having CBS pair domain. However, GMPRs of Kinetoplastida contain a CBS pair domain despite clearly exhibiting GMPR activity. These results suggest that GMPR is a polyphyletic group, and Kinetoplastida GMPR evolved independently, separated from other GMPRs in the earlier evolutionary process. While it is understood that the CBS pair domain is not a core enzyme domain, it may act as a regulatory domain to bind ATP, AMP, AdoMet, and so on [35]. Moreover, in a report on *Leishmania* GMPR, CBS has been linked to biological responses of GMPR at the intracellular level of GTP and ATP, which subsequently controls the state of the enzyme complex to modulate its catalytic activity [3].

Specific amino acid residues have actually been proposed as distinctive points between GMPR and IMPDH at the primary structure level. One proposal was the consensus sequence Tyr303-Arg304 and Glu307 (Y-R-X-X-E) of GMPR (human GMPR2 numbering) [1]. This proposal was derived from the non-conserved region of IMPDHs at position 303-304 and a Glu to Gln substitution at position 307.. However, this might not be the case because Tyr303-Arg304 dyad is also non-conserved in the reported *L. donovani* and *T. brucei* GMPR, while Glu431 (*Tritrichomonas foetus* IMPDH numbering) is also present in several IMPDHs instead of Gln. Surprisingly, all GMPRs that were included in this study are were conserved in the catalytic loop region in residue Leu217-Ser218 (Fig. S3). Due to the fact that TcIL3000_5_1940 was also conserved in this position, we were confident that the gene was in fact a GMPR-encoded gene in *T. congolense* (TcGMPR). The role of this conserved region for GMPR activity might be necessary to investigate in further studies.

The kinetics parameters of rTcGMPR were found to be similar to those of TbGMPR. In contrast, these values were significantly different from those of *Bos taurus* GMPR (BtGMPR), one of the susceptible animals to *T. congolense* infection (Table 2) [2]. Calculated K_m values against GMP and NADPH suggested that rTcGMPR exerted up to 6-fold higher affinity for GMP and conversely 5-fold lower affinity for NADPH, compared to BtGMPR. These differences implied two key takeaways: that the development of a TcGMPR-targeting drug might also be useful as a TbGMPR-targeting drug, and that an anti-trypanosomal drug for infected cattle or humans might be developed by targeting trypanosome GMPRs, such as TbGMPR and TcGMPR.

Currently, the discovery of novel chemotherapeutic agents against trypanosomosis has been thwarted by limited attention and funding from major pharmaceutical companies [36,37]. Consequently, infections were treated with outdated drugs, most of which have been tolerated by some protozoan strains. While GMPRs could be an attractive drug target, at present no potent GMPR inhibitor has been proposed as having a broad therapeutic window and minimal adverse effects. Bessho et al. suggested ribavirin as a possible chemotherapeutic compound or a lead compound for the development of new GMPR inhibitors targeting the GMP-binding site of TbGMPR [2]. *L. donovani* GMPR was also reported to be inhibited by IMP analogs [38,39]. Our current investigation revealed that mycophenolic acid, a selective nicotinamide-binding site inhibitor of IMPDH, showed a moderate inhibitory activity against rTcGMPR. Similar finding on GMPR inhibition by MPA was also reported with *Leishmania major* GMPR, which exerted a K_i value of 20 μM [3]. However, MPA is also known as a potent IMPDH inhibitor, with higher affinity to mammalian enzymes than to protozoan enzymes [31]. In our previous publication, we showed that MPA has relatively potent inhibitory activity against rTcIMPDH [17]. Therefore, it is expected that MPA potently inhibited growth of *T. congolense* in cultures but showed less potent activity against rTcGMPR *in vitro*. By considering these facts, appropriate derivatization of MPA and further drug designs are necessary for the development of anti-infective agents for mammalian hosts [18,32,40]. Nevertheless, our findings re-highlight the possibility of a new class of *Trypanosomatidae* GMPR inhibitor.

T. congolense GMPR was found to exhibit higher activity in the presence of larger radius cations, especially K^+ and NH_4^+. A similar activation pattern was also reported in IMPDHs, where the presence of smaller ions such as Li^+ and Na^+ could cause a negative (inhibition), positive (activation), or neutral effect (no effect) on enzyme activity, but larger ions (K^+, Rb^+, Cs^+, Tl^+, and NH_4^+) always activate the reaction [32,41,42]. Mammalian GMPRs on the other hand, showed no activation in the presence of K^+ and NH_4^+ in comparison with Na^+ [2]. This is the first report on a similar response between GMPR and IMPDH to a broad range of cations.

GMPR could also catalyze a reverse reaction, the direct conversion of IMP to GMP. This activity is directly dependent on the concentration of ammonium ion in the reaction [43]. The activation of the deamination reaction, however, could be observed in a much lower ammonium concentration than the progression of the aforementioned reverse reaction. While K^+ has been described as a molecular "lubricant" that facilitates the conformational changes in IMPDH reaction, the

role of ammonium ion may be more complex, since it may play a very different role in the forward and reverse reaction. Nevertheless, the ability of GMPR to catalyze the conversion between GMP and IMP in both directions suggests that it might have an important role in the early development of IMPDH/GMPR family, since the earliest forms of life are believed to have emerged in an ammonia rich environment [44]. The opposite reaction by rTcGMPR was observed in an enzyme-concentration-dependent manner. However, the reaction progressed very slowly even at high concentrations of enzyme and substrates (Fig. S4). Trypanosome itself has been known as an ammonotelic organism, and is able to tolerate relatively high ammonium concentrations [45–47]. Therefore, the protozoa might be able to survive when there is a high concentration of ammonium ion in the blood and under repressed activity of other enzymes, provided that TcGMPR can produce sufficient GMP for its survival [43].

TcGMPR was constitutively expressed and localized in cytosol as a granular localization during the developmental stages. In addition to our experimental data, previous reports also suggested that purine metabolic enzymes was localized in glycosomes of Kinetoplastida [7,48]. These results implied that TcGMPR was also expressed in glycosomes, similar to other purine metabolic enzymes.

In conclusion, the present study revealed that TcIL3000_5_1940 is the first enzymatically analyzed GMPR-encoded gene in *T. congolense*. Although rTcGMPR showed remarkably similar kinetic parameters to TbGMPR, it differs significantly to mammalian GMPRs. In addition, Leu217-Ser218 dyad was identified by the amino acid alignment as a practical distinction point to identify GMPRs from IMPDHs. Lastly, a selective IMPDH inhibitor, mycophenolic acid, was found to inhibit rTcGMPR activity, demonstrating its potential as a lead compound for the development of effective chemotherapeutic agents against *Trypanosoma* infection.

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Fig. 1. Deamination reaction of GMP catalyzed by GMPR.

Fig. 2. Phylogenetic tree based on the GMPRs and IMPDHs amino acid sequence**.** The phylogenetic tree was generated using the neighbor-joining method incorporated into the MEGA 5.2 software.

Fig. 3. Amino acid alignment of TcGMPR and other GMPRs. The square indicates the predicted CBS pair domain amino acid residues. The dashed square indicates the catalytic loop. The bold characters indicate the C-terminal Ser-Lys-Leu peroxisomal (glycosomal) targeting signal. Star symbol indicates the catalytic Cys204 residue (HsGMPR2 numbering). Diamond symbols indicate Leu217-Ser218, the conserved dyad that differentiates a GMPR from IMPDHs. Protein sequence of TcGMPR (TriTrypDB: TcIL3000_5_1940); *T. brucei* GMPR [(TbGMPR) TriTrypdDB: Tb927.5.2080]; *L. donovani* GMPR [(LdGMPR) Uniprot: E9BDA8]; *B. taurus* GMPR1 and 2 [(BtGMPR 1) Uniprot: AAI23864.1; (BtGMPR2) NCBI: NP_001033208]; *H. sapiens* GMPR1 and 2 [(HsGMPR1) NCBI: NP_006868.3; (HsGMPR2) NCBI: NP_057660.2] were aligned using ClustalW (http://clustalw.ddbj.nig.ac.jp).

Fig. 4. SDS-PAGE of recombinant TcGMPR. Protein expression in the *E. coli* culture was monitored from the induction until purification steps. Lane M: protein marker, 1: *E. coli* culture before IPTG addition, 2: *E. coli* culture 12 h after IPTG addition, 3: purified rTcGMPR fused with GST, and 4: purified rTcGMPR with cleaved GST.

Fig. 5. Determination of optimum pH for rTcGMPR. Each rTcGMPR activity was plotted against the corresponding value of experimental pH. The activity at pH 7.0 was set to 100% relative activity. Acidic conditions were excluded from the experiment due to NADPH instability. Each data point represents mean ± standard deviation from three independent experiments.

Fig. 6. Inhibitory effect of MPA against rTcGMPR. Each data point represents mean ± standard deviation from three independent experiments.

Fig. 7. Effect of monovalent cation on rTcGMPR activity. rTcGMPR activity was evaluated in the presence of each monovalent cation in the form of a chloride salt. The activity in the presence of NaCl was set as 100%. Each bar represents mean ± standard deviation from three independent experiments.

Fig. 8. Expression profile and localization of TcGMPR. (A) Western blot analysis was performed with five μg of total cell protein extracted from BSF, PCF, EMF and MCF using anti-TcGMPR and anti-Tcα-tubulin antibody. (B) The graph showed the relative quantity (each stage/BSF) of TcGMPR in each

stage. Each bar represents mean ± standard deviation from three independent experiments. (C) Indirect immunofluorescence staining using anti-TcGMPR antibody was observed by confocal laser scanning microscopy. Nucleolus and kinetoplast DNA were stained by Hoechest 33342 and are shown in red. For the microscopy, each of the images was captured using the same photomultiplier tube gain and voltage.

Fig. S1. IMPDH activity assay of TcGMPR. Unlike human IMPDH (closed circles), TcIL3000_5_1940 (open circles) did not show any IMPDH activity. IMPDH activity assay was conducted with assay solution containing 50 mM Tris-HCl pH 8.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 800 μM NAD^+, and 250 μM IMP. Reaction was initiated by adding enzyme solutions to the assay solution. The reaction was carried out at 30ºC for 1 h. IMPDH activity was monitored as an increase of absorbance in 340 nm, indicating formation of NADH. Each point was expressed as the amount of NADH formed during reaction.

Fig. S2. Steady-state kinetic analysis of TcGMPR. (a) The $K_{m\ NADPH}$ was determined at a fixed GMP concentration of 1.0 mM and varying concentration of NADPH. (b) The $K_{m\ GMP}$ was determined at a fixed NADPH concentration of 200 μM and varying concentration of GMP. The initial velocity values of the reactions were fitted to the Michaelis-Menten equation using GraFit 7 software (Erithacus software, U.K.). Each point represents mean ± standard deviation from three independent experiments.

Fig. S3. Leu217-Ser218 as a distinctive residue dyad of GMPR. Fraction of active loops in GMPR and IMPDH were aligned. Diamond symbols indicate Leu217-Ser218 (HsGMPR2 numbering), the residue that differentiates GMPRs from IMPDHs. Star symbol indicates Cys204, an important catalytic residue for GMPR and IMPDH.

Protein sequence was taken from NCBI database unless stated otherwise. Protein sequences for *H. sapiens* (accession number of HsIMPDH1: NP_000874.2, HsIMPDH2: NP_000875.2, HsGMPR1: NP_006868.3, HsGMPR2: NP_057660.2), *B. taurus* (BtGMPR1: AAI23864.1 [Uniprot], BtGMPR2: NP_001033208), *L. donovani* (LdIMPDH: AAA29253.1 [Uniprot], LdGMPR: E9BDA8 [Uniprot]), *T. brucei* (TbIMPDH: P50098.1 [Uniprot], TbGMPR: Tb927.5.2080 [TriTrypDB]), ***T. congolense*** **(TcIMPDH: BAT33662.1 [Uniprot], TcGMPR: TcIL3000_5_1940 [TriTrypDB])**, *B. gibsoni*

(BgIMPDH: AFL46337.1), *P. falciparum* (PfIMPDH: XP_001352079.1), *T. gondii* (TgIMPDH: XP_002368170.1), *T. foetus* (TfIMPDH: AAB01581.1 [Uniprot]), *C. parvum* (CpIMPDH: XP_625342.1), *E. coli* (EcIMPDH: NP_417003.1, EcGMPR: NP_414646.1), and *S. pyrogenes* (SpIMPDH: NP_270113.1) were aligned using ClustalW program.

Fig. S4. Reverse reaction of TcGMPR. TcGMPR was able to convert IMP to GMP in the presence of NH_4^+. The assay of GMPR reverse reaction activity was conducted with solution containing 75 mM Tris-HCl pH 8.0, 100 mM KCl, 3 mM EDTA, 1 mM DTT, 1 mM $NADP^+$, 50 mM NH_4Cl, and various IMP concentrations. Reaction was initiated by adding enzyme to the assay solution. The reaction was carried out at 25ºC for 20 h. The reverse GMPR activity was monitored as an increase of absorbance in 340 nm, indicating formation of NADPH. Each point was expressed as the mean amount of NADPH formed during reaction ± standard deviation from three independent experiments.

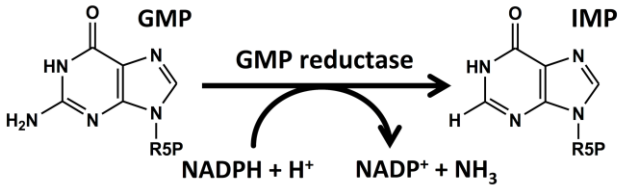

Figure 1

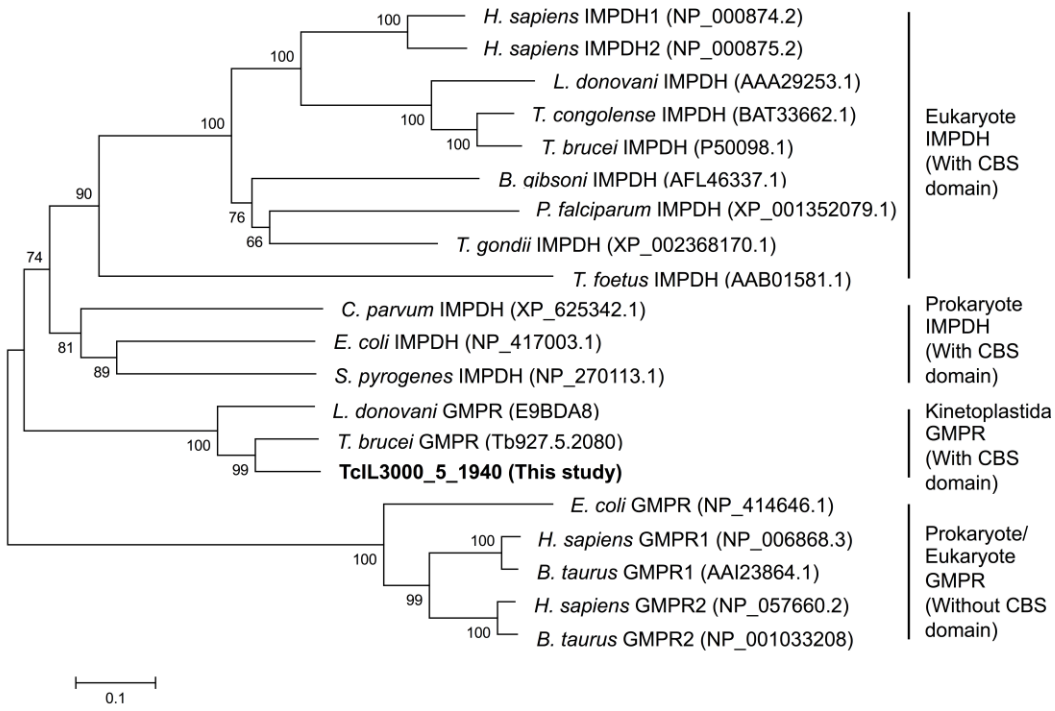

Figure 2

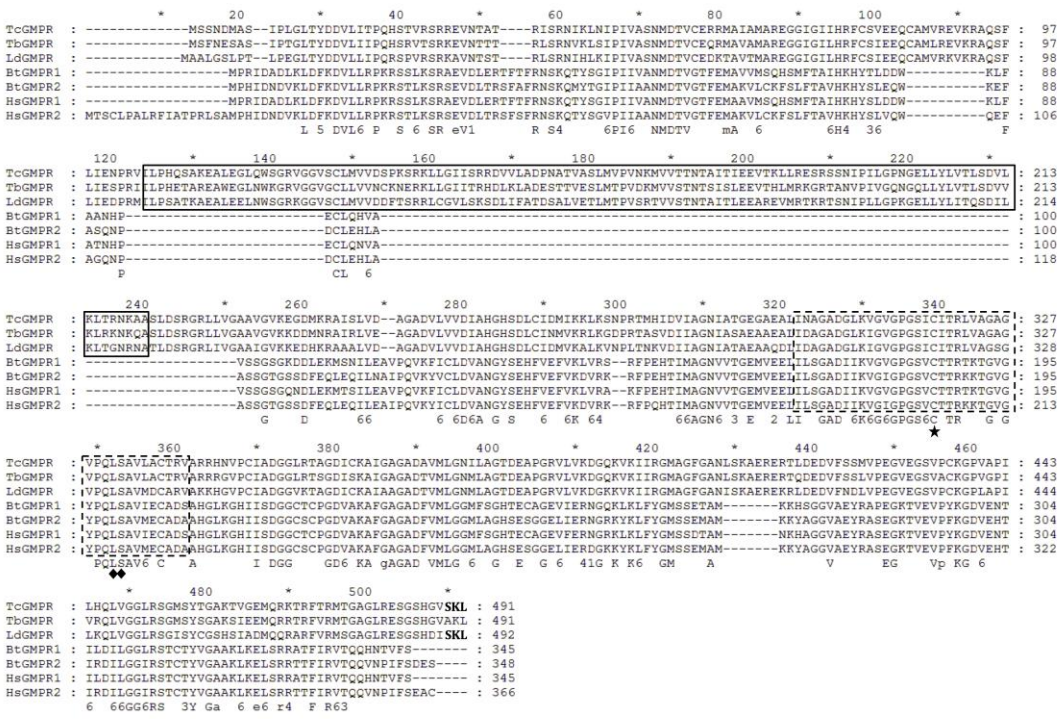

Figure 3

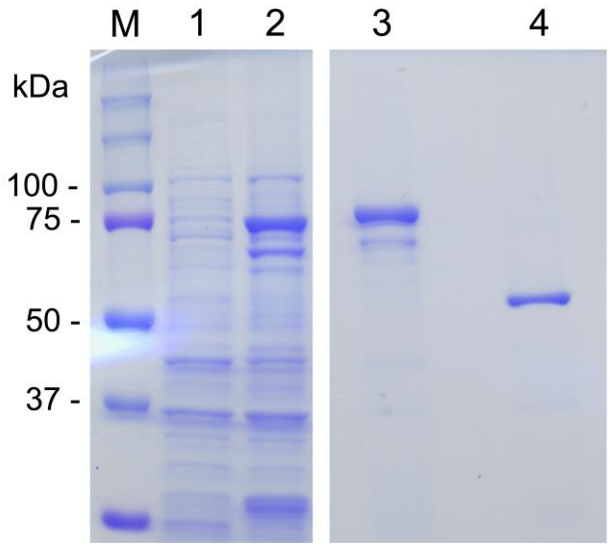

Figure 4

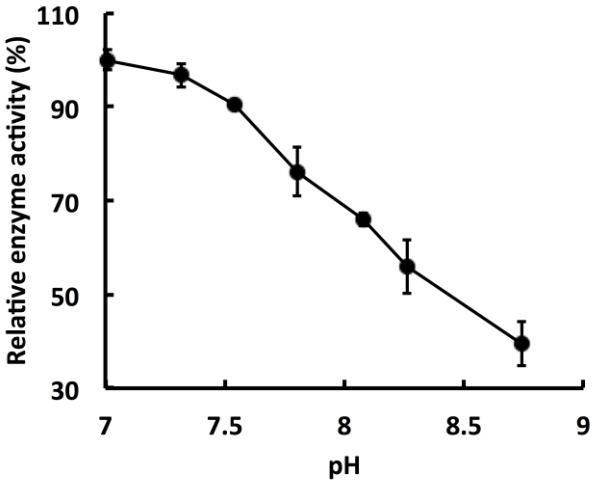

Figure 5

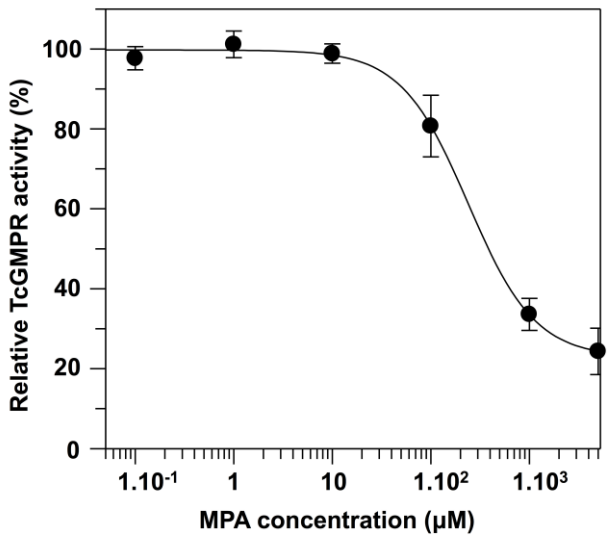

Figure 6

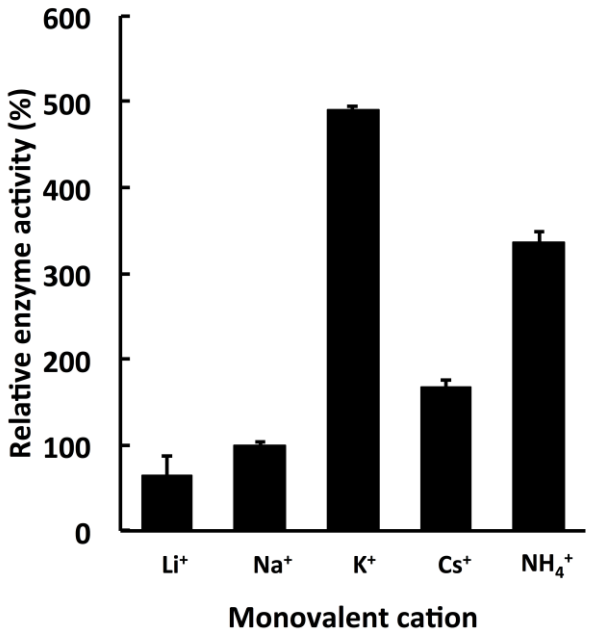

Figure 7

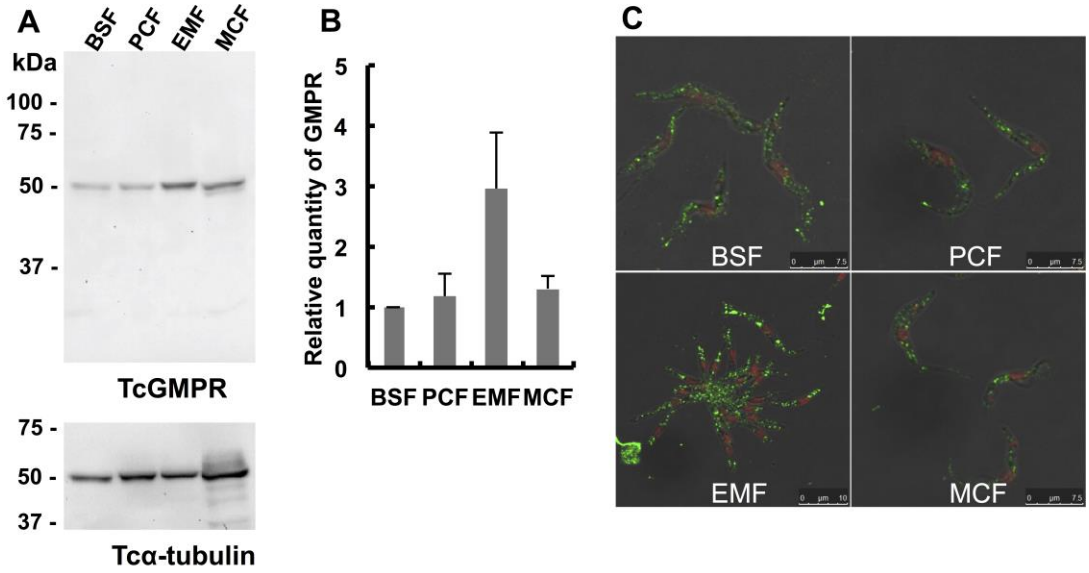

Figure 8

Graphical abstract:

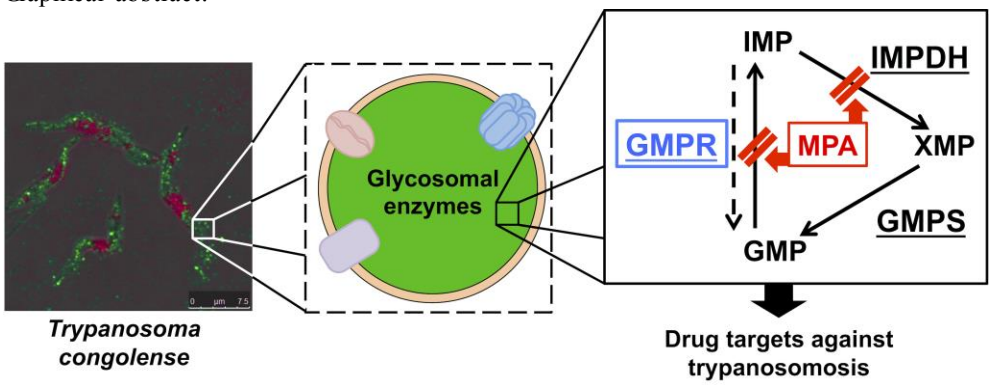

Table 1

Amino acid sequence identities among GMPRs

	Identity (%)						
	T. congolense TcIL3000_5_1940	*T. brucei* GMPR	*L. donovani* GMPR	*H. sapiens* GMPR1	*H. sapiens* GMPR2	*B. taurus GMPR1*	*B. taurus* GMPR2
TcIL3000_5_1940	100	80	72	34	32	35	34
T. brucei GMPR		100	72	34	32	35	33
L. donovani GMPR			100	32	31	32	31
H. sapiens GMPR1				100	79	95	79
H. sapiens GMPR2					100	80	94
B. taurus GMPR1						100	79
B. taurus GMPR2							100

Identities were calculated using ClustalW program.

Table 2

Comparisons of enzymatic parameters of rTcGMPR to other GMPRs

	k_{cat} (s^{-1})	$K_{m\,GMP}$ (μM)	$K_{m\,NADPH}$ (μM)
rTcGMPR[a]	0.499 ± 0.014	91.6 ± 4.7	11.3 ± 2.3
TbGMPR[b]	0.519 ± 0.012	89.3 ± 9.0	12.3 ± 0.8
HsGMPR1[b]	0.284 ± 0.006	22.1 ± 2.2	34.8 ± 4.3
HsGMPR2[b]	0.265 ± 0.016	17.8 ± 3.5	29.3 ± 3.2
BtGMPR1[b]	0.243 ± 0.004	13.5 ± 1.3	54.1 ± 5.0
BtGMPR2[b]	0.296 ± 0.009	22.6 ± 4.2	62.1 ± 7.0

[a]: activity was measured at 35°C, 75 mM Tris-HCl buffer (pH 7.0), 100 mM KCl, 3 mM EDTA, 1 mM DTT, and 0.1 mg/ml BSA.
[b]: activity was measured at 35°C, 50 mM sodium phosphate buffer (pH 7.0), 100 mM KCl, 3 mM EDTA, and 1 mM DTT [18]

Highlights

- A unique GMPR was analyzed from *Trypanosoma congolense* (TcGMPR).
- TcGMPR has different characteristics compared to mammalian (host) GMPR.
- Mycophenolic acid inhibits both TcGMPR and TclMPDH.