Mycophenolic Acid and Its Derivatives as Potential Chemotherapeutic Agents Targeting Inosine Monophosphate Dehydrogenase in *Trypanosoma congolense*

Keisuke Suganuma[a], Albertus Eka Yudistira Sarwono[b], Shinya Mitsuhashi[b], Marcin Jąkalski[c], Tadashi Okada[a,d], Molefe Nthatisi[a], Junya Yamagishi[e], Makoto Ubukata[b], Noboru Inoue[a,#]

Running Head: Trypanocidal Activity of MPA and Its Derivatives

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido, Japan[a]; Division of Applied Bioscience Research, Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido, Japan[b]; Institute of Bioinformatics, Faculty of Medicine, University of Münster, Münster, Germany[c]; Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Aichi, Japan[d]; Division of Collaboration and Education, Research Center for Zoonosis

17 Control, Hokkaido University, Sapporo, Hokkaido, Japan[e]

18

19 [#]Address correspondence to Prof. Noboru Inoue, D.V.M., Ph.D.,

20 ircpmi@obihiro.ac.jp

21 National Research Center for Protozoan Diseases, Obihiro University of

22 Agriculture and Veterinary Medicine, Inada, Obihiro, Hokkaido 080-8555, Japan

23 Tel.: +81-155-49-5647; Fax: +81-155-49-5643

24

ABSTRACT

This study aimed to evaluate the trypanocidal activity of mycophenolic acid (MPA) and its derivatives for *Trypanosoma congolense*. The proliferation of *T. congolense* was completely inhibited by adding less than 1 μM of MPA and its derivatives. In addition, the inosine monophosphate dehydrogenase in *T. congolense* was molecularly characterized as the target of these compounds. The results suggested that MPA and its derivatives have the potential to be new candidates as novel trypanocidal drugs.

Keywords: African trypanosomosis, Inosine monophosphate dehydrogenase, Mycophenolic acid, Trypanocidal drug, *Trypanosoma congolense.*

Trypanosoma congolense causes animal African trypanosomosis (AAT) in livestock. The lack of effective vaccines makes the use of chemotherapeutic agents the most effective measure for controlling AAT. Limited numbers of commercial drugs have long been used to treat AAT. The emergence of drug-resistant trypanosomes and cases of drug-refractory trypanosomosis have been reported (1-4), underscoring the need for the development of new drugs.

A candidate target for drug development is inosine monophosphate dehydrogenase (IMPDH). This enzyme is very important in the *Trypanosoma* spp. because it lacks a *de novo* purine synthesis pathway, which makes the purine nucleotide synthesis in these parasites solely dependent on a salvage pathway in the glycosomes (5-7). IMPDH converts inosine 5'-monophosphate (IMP) into xanthosine 5'-monophosphate (XMP) through this pathway, which is a rate-limiting step in the metabolism of guanine nucleotides (8). Mycophenolic acid (MPA, **1**) is a well-known IMPDH inhibitor (Fig. 1). Its enzymatic activity has already been proven in many protozoan parasites (9-14). The anti-protozoan activities of MPA against *Babesia* spp. have been reported in *in vivo* and *in vitro*

53 studies (9, 15). Thus, the activity of MPA against IMPDH is expected to lead to
54 a novel strategy for the development of trypanocides.
55 The novel *IMPDH* orthologue of *T. congolense* (*TcIMPDH*) (accession no.
56 LC094350) was identified from the *T. congolense* re-sequencing data
57 (unpublished data). The recombinant TcIMPDH showed IMPDH activity *in vitro*
58 (Supplemental Fig. 1-A and B). The nanomolar levels of MPA clearly inhibited
59 NADH production by TcIMPDH in a dose-dependent manner (IC_{50} = 26.2nM)
60 (Supplemental Figure 1-C). The expression profile and cellular localization of
61 TcIMPDH were analyzed by Western blotting and immunofluorescence
62 microscopy. TcIMPDH was expressed in glycosomes as granulated forms
63 throughout the life cycle stages of *T. congolense* (Supplemental Fig. 2).
64 TcIMPDH was expressed at similar levels in bloodstream form (BSF), procyclic
65 form (PCF), and epimastigote form (EMF). In contrast, TcIMPDH expression in
66 the metacyclic form (MCF) was significantly lower than in the other stages
67 (p<0.05, Tukey's multiple comparison test). This result suggests that purine

68 synthesis is highly important in the proliferative stages of the parasite, but not in
69 the non-proliferative MCF stage.

70 The aim of this study was to reveal the trypanocidal activities of MPA
71 derivatives for developing an effective trypanocidal drugs. Various inhibitory
72 activities and the cell-differentiation activity of MPA derivatives against
73 mammalian cells have been reported *in vitro*. Some MPA derivatives (**2**, **4**, **9**
74 and **10**) have shown particularly significant inhibitory activities against human
75 IMPDH and were observed to induce erythroid differentiation in K562 cells (16,
76 17). These previous reports suggested that some MPA derivatives might be
77 specific inhibitors for *Trypanosoma*. The chemical structures of the MPA
78 derivatives in this study are shown in Figure 1. We evaluated the trypanocidal
79 activity against *T. congolense*, *T. b. brucei* and *T. evansi* using an ATP-based
80 luciferase viability system (18). To evaluate the trypanocidal activity of **1** and its
81 derivatives *in vitro*, BSFs were cultivated with 1μM of each compound. At 1μM,
82 nine derivatives showed less than 10% anti-*T. congolense* activity (Table 1). In
83 contrast, only three compounds, **1**, **2**, and **4**, inhibited *T. congolense* growth by

99.60±0.38%, 94.46±3.89% and 98.87±0.78% at 1μM, respectively (Table 1). Although **1** showed high trypanocidal activity against *T. b. brucei* and *T. evansi*, **2** and **4** showed lower inhibitory activities at 1μM against *T. b. brucei* and *T. evansi* than against *T. congolense* (Table 1). The low plasma membrane permeability **of 3**, **5**, **6**, **7**, **8**, **11** and **12** might account for their low trypanocidal activity; while the low trypanocidal activity of **9** and **10** against all of the tested trypanosome species and of **2** against *T. brucei* and *T. evansi* suggest their low affinity with these trypanosome IMPDHs or the deactivation of these compounds by other species-specific enzymes in cytosol. The IC_{50} of **1**, **2**, and **4** to *T. congolense* were 0.10±0.04μM, 0.56±0.21 μM, and 0.16±0.04μM, respectively (Table 2). The IC_{50} values of these three compounds to MDBK cells were 0.52±0.12, 1.40±0.18, and 0.84±0.21μM, respectively. The selectivity indices of MPA and the two derivatives in *T. congolense* were 5.14, 2.62, and 5.10, respectively (Table 2). However, the higher IC_{50} values and lower selectivity indices of these 3 compounds were shown in *T. b. brucei* and *T. evansi* (Table 2). The cytotoxicity of these compounds was higher than that of commercial drugs (19).

However, the IC_{50} values of **1** and **4** for *T. congolense* BSF were comparable to those of two commercially available trypanocides (pentamidine [0.17μM] and diminazene [0.11μM]) against *T. congolense* (18). These results suggested that **1**, **2**, and **4** might be potential lead compounds in the development of trypanocides, especially against *T. congolense.*

To clarify the mode of action of **1** and **4** in trypanosomes, the effects of guanosine and xanthine supplementation on the trypanocidal effects of these compounds were examined. The IC_{50} values of **1** and **4** were increased by guanosine in a dose-dependent manner (Table 3), while xanthine supplementation did not alter the IC_{50} values of either **1** or **4** in *T. congolense* BSF (Table 3). These results suggest that guanosine was transported into the *T. congolense* BSF and converted into GMP as a purine nucleotide source, while no xanthine was transported or converted into XMP by hypoxanthine-gunaine phosphoribosyltransferase in *T. congolense*. We therefore concluded that the proliferation inhibitory effects of MPA against *T. congolense* BSF were caused by the inhibition of intracellular TcIMPDH.

Hypoxanthine and inosine were predicted to be the main purine sources in *T. brucei* (20). Hypoxanthine and inosine have also been shown to be present in the blood at higher concentrations than other purines (21), suggesting their roles as the main purine sources in trypanosomes and that they are supplied via the salvage pathway. The concentration of purine bases and nucleosides in the extracellular environment is lower than that in the intracellular environment (21). *T. brucei* spp. proliferate in blood circulation and then invade the central nervous system through the blood-brain barrier (22, 23), while *T. congolense* only proliferates in blood circulation by adhesion to the vascular endothelium (24). In conclusion, MPA and its derivatives might therefore also inhibit trypanosome proliferation *in vivo,* particularly in *T. congolense.*

ACKNOWLEDGMENTS

We thank Ms. Yoko Matsushita for her excellent technical assistance. This study was financially supported by the Japan Society for the Promotion of Science (JSPS), Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and the AMED/JICA SATREPS.

References

1. **Baker N, de Koning HP, Maser P, Horn D.** 2013. Drug resistance in African trypanosomiasis: the melarsoprol and pentamidine story. Trends Parasitol **29:**110-118.
2. **Delespaux V, Geysen D, Van den Bossche P, Geerts S.** 2008. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. Trends Parasitol **24:**236-242.
3. **Delespaux V, Dinka H, Masumu J, Van den Bossche P, Geerts S.** 2008. Five-fold increase in *Trypanosoma congolense* isolates resistant to diminazene aceturate over a seven-year period in Eastern Zambia. Drug Resist Updat **11:**205-209.
4. **Pinder M, Authie E.** 1984. The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. Acta Trop **41:**247-252.
5. **Hammond DJ, Gutteridge WE.** 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Mol Biochem Parasitol **13:**243-261.
6. **Boitz JM, Ullman B, Jardim A, Carter NS.** 2012. Purine salvage in Leishmania: complex or simple by design? Trends Parasitol **28:**345-352.
7. **Vertommen D, Van Roy J, Szikora JP, Rider MH, Michels PA, Opperdoes FR.** 2008. Differential expression of glycosomal and mitochondrial proteins in the two major life-cycle stages of *Trypanosoma brucei*. Mol Biochem Parasitol **158:**189-201.
8. **Shu Q, Nair V.** 2008. Inosine monophosphate dehydrogenase (IMPDH) as a target in drug discovery. Med Res Rev **28:**219-232.
9. **Cao S, Aboge GO, Terkawi MA, Zhou M, Luo Y, Yu L, Li Y, Goo Y, Kamyingkird K, Masatani T, Suzuki H, Igarashi I, Nishikawa Y, Xuan X.** 2013. Cloning, characterization and validation of inosine 5'-monophosphate dehydrogenase of *Babesia gibsoni* as molecular drug target. Parasitol Int **62:**87-94.
10. **Umejiego NN, Li C, Riera T, Hedstrom L, Striepen B.** 2004. *Cryptosporidium parvum* IMP dehydrogenase: identification of

functional, structural, and dynamic properties that can be exploited for drug design. J Biol Chem **279:**40320-40327.

11. **Sullivan WJ Jr., Dixon SE, Li C, Striepen B, Queener SF.** 2005. IMP dehydrogenase from the protozoan parasite *Toxoplasma gondii*. Antimicrob Agents Chemother **49:**2172-2179.

12. **Dobie F, Berg A, Boitz JM, Jardim A.** 2007. Kinetic characterization of inosine monophosphate dehydrogenase of *Leishmania donovani.* Mol Biochem Parasitol **152:**11-21.

13. **Bessho T, Morii S, Kusumoto T, Shinohara T, Noda M, Uchiyama S, Shuto S, Nishimura S, Djikeng A, Duszenko M, Martin SK, Inui T, Kubata KB.** 2013. Characterization of the novel *Trypanosoma brucei* inosine 5'-monophosphate dehydrogenase. Parasitology **140:**735-745.

14. **Digits JA, Hedstrom L.** 1999. Kinetic mechanism of *Tritrichomonas foetus* inosine 5'-monophosphate dehydrogenase. Biochemistry **38:**2295-2306.

15. **Cao S, Aboge GO, Terkawi MA, Zhou M, Kamyingkird K, Adjou Moumouni PF, Masatani T, Igarashi I, Nishikawa Y, Xuan X.** 2014. Mycophenolic acid, mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole inhibit propagation of Babesia parasites by targeting inosine 5'-monophosphate dehydrogenase. J Parasitol **100:**522-526.

16. **Sunohara K, Mitsuhashi S, Shigetomi K, Ubukata M.** 2013. Discovery of N-(2,3,5-triazoyl)mycophenolic amide and mycophenolic epoxyketone as novel inhibitors of human IMPDH. Bioorg Med Chem Lett **23:**5140-5144.

17. **Mitsuhashi S, Takenaka J, Iwamori K, Nakajima N, Ubukata M.** 2010. Structure-activity relationships for inhibition of inosine monophosphate dehydrogenase and differentiation induction of K562 cells among the mycophenolic acid derivatives. Bioorg Med Chem **18:**8106-8111.

18. **Suganuma K, Allamanda P, Hakimi H, Zhou M, Angeles JM, Kawazu S, Inoue N.** 2014. Establishment of ATP-based luciferase viability assay in 96-well plate for *Trypanosoma congolense*. J Vet Med Sci **76:**1437-1441.

19. **Sykes ML, Baell JB, Kaiser M, Chatelain E, Moawad SR, Ganame D, Ioset JR, Avery VM.** 2012. Identification of compounds with anti-proliferative activity against *Trypanosoma brucei brucei* strain 427 by a whole cell viability based HTS campaign. PLoS Negl Trop Dis **6:**e1896.

20. **de Koning HP, Bridges DJ, Burchmore RJ.** 2005. Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. FEMS Microbiol Rev **29:**987-1020.

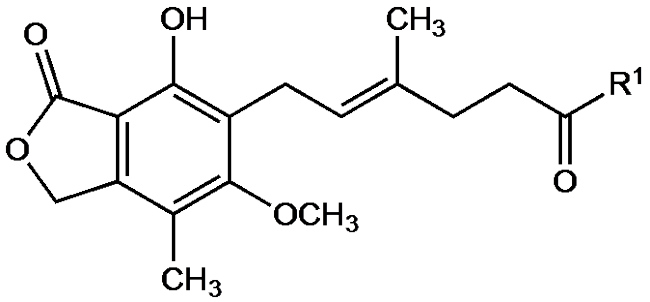
21. **Traut TW.** 1994. Physiological concentrations of purines and pyrimidines. Mol Cell Biochem **140:**1-22.

22. **Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S.** 2003. The trypanosomiases. Lancet **362:**1469-1480.

23. **Mulenga C, Mhlanga JD, Kristensson K, Robertson B.** 2001. *Trypanosoma brucei brucei* crosses the blood-brain barrier while tight junction proteins are preserved in a rat chronic disease model. Neuropathol Appl Neurobiol **27:**77-85.

24. **Shakibaei M, Milaninezhad M, Risse HJ.** 1994. Immunoelectron microscopic studies on the specific adhesion of *Trypanosoma congolense* to cultured vascular endothelial cells. J Struct Biol **112:**125-135.

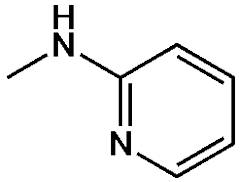
Fig. 1. The structures of mycophenolic acid and its derivatives



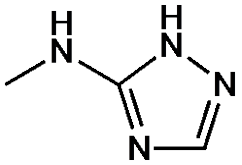
MPA (1): R^1 = OH

2: R^1 = NHOH

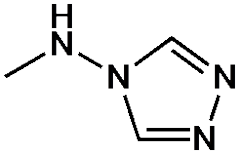
3: R^1 =



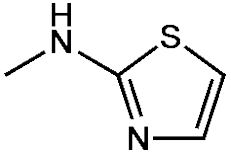
4: R^1 =



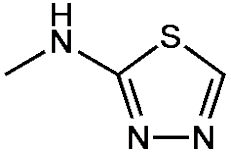
5: R^1 =



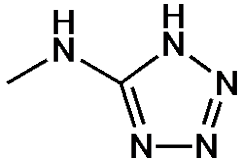
6: R^1 =



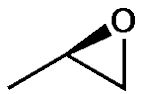
7: R^1 =



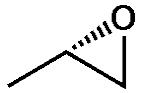
8: R^1 =

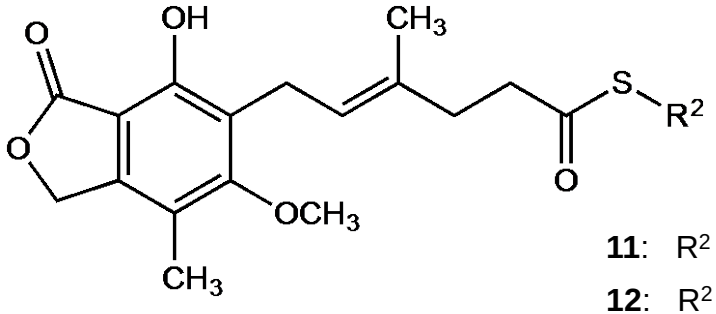


9: R^1 =



10: R^1 =





11: $R^2 = CH_3CO$

12: R^2 = H

Table 1. The trypanocidal activity of MPA (1) and its derivatives at 1 μM

Compound	Inhibition rate (%)		
	T. congolense	*T. b. brucei*	*T. evansi*
1	99.60±0.38	82.99±2.82	90.53±1.22
2	94.46±3.89	5.24±13.12	14.21±8.64
3	2.36±8.64	7.83±10.35	16.66±5.55
4	98.87±0.78	46.13±5.21	42.79±4.58
5	4.65±15.29	14.29±34.17	32.43±4.88
6	1.45±10.94	22.27±4.81	17.11±6.14
7	4.59±15.12	14.50±13.76	29.44±10.03
8	3.59±14.06	22.99±12.90	19.94±8.44
9	0.06±8.66	9.28±5.15	11.99±1.59
10	3.15±8.43	9.03±7.91	9.49±6.13
11	6.51±14.38	16.47±6.97	12.79±4.49
12	3.03±12.91	11.56±4.17	13.61±8.67
Pentamidine	99.93±0.07	99.96±0.06	99.94±0.07
Control	0.00±1.74	0.48±1.58	-0.24±2.25

The trypanocidal activity of MPA (**1**) and 11 MPA derivatives (see Fig. 1) at a concentration of 1μM was evaluated for *T. congolense, T. b. brucei* GUTat 3.1 strain and *T. evansi* Tansui strain. Five hundred ng/mL of pentamidine was used as a 100% inhibition control (Pentamidine). HMI-9 media with 0.25% DMSO was used as a 0% inhibition control (Control). The inhibition rate (%) was calculated from 3 independent experiments, and expressed as the mean inhibition rate (%) ± standard deviation.

Table 2. The IC_{50} value and selectivity index of MPA (1) and MPA derivatives 2 and 4 against *T. b. brucei* and *T. evansi*

	IC_{50} (μM)				Selectivity index[a]		
Compound	*T. congolense*	*T. b. brucei*	*T. evansi*	MDBK cell	*T. congolense*	*T. b. brucei*	*T. evansi*
1	0.10±0.04	0.62±0.05	0.61±0.002	0.52±0.12	5.14	0.84	0.85
2	0.56±0.21	>2.5	>2.5	1.4±0.18	2.62	ND	ND
4	0.16±0.04	1.26±0.009	1.38±0.10	0.84±0.21	5.10	0.67	0.61

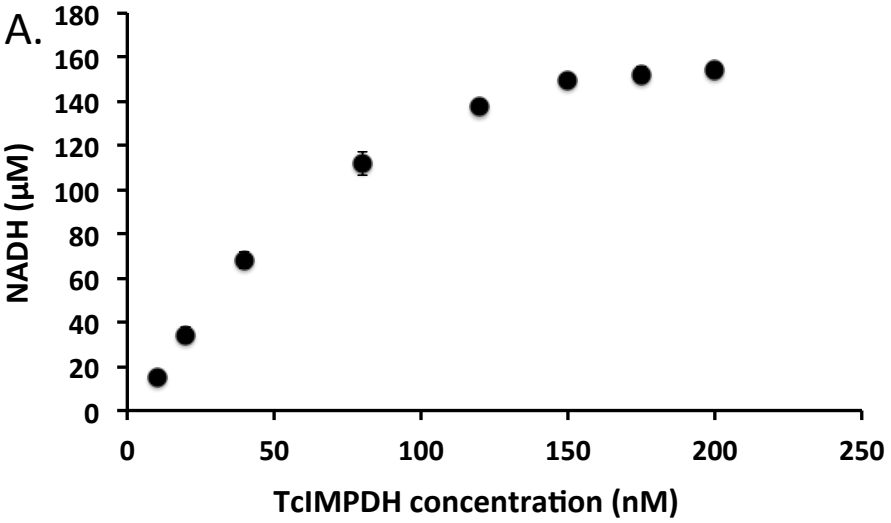
All of the values were calculated from 3 independent experiments and expressed as the mean ± standard deviation. IC_{50}: 50% inhibitory concentration; [a], the mean IC_{50} of MDBK cells/the mean IC_{50} of trypanosomes; ND, not determined.

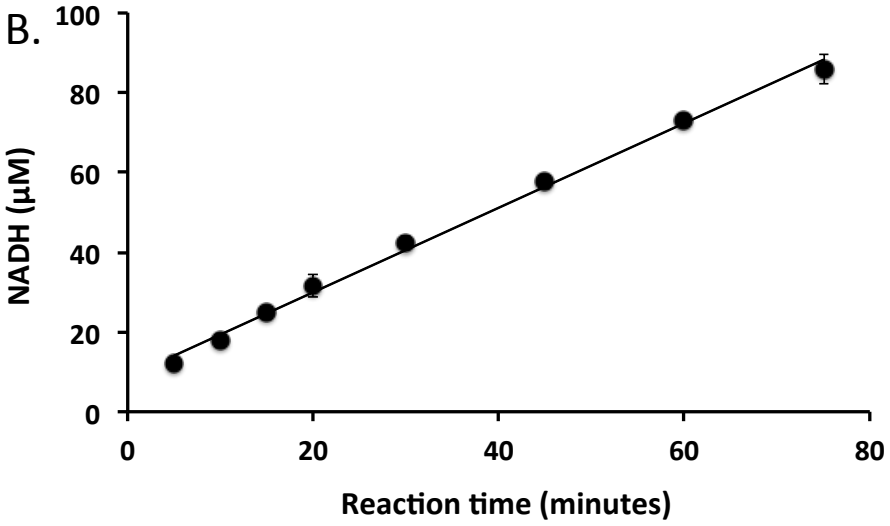
Table 3. The effects of guanosine and xanthine on parasite proliferation under the IMPDH inhibition by MPA (1) and *N*-(2,3,5-triazolyl) mycophenolic amide (4)

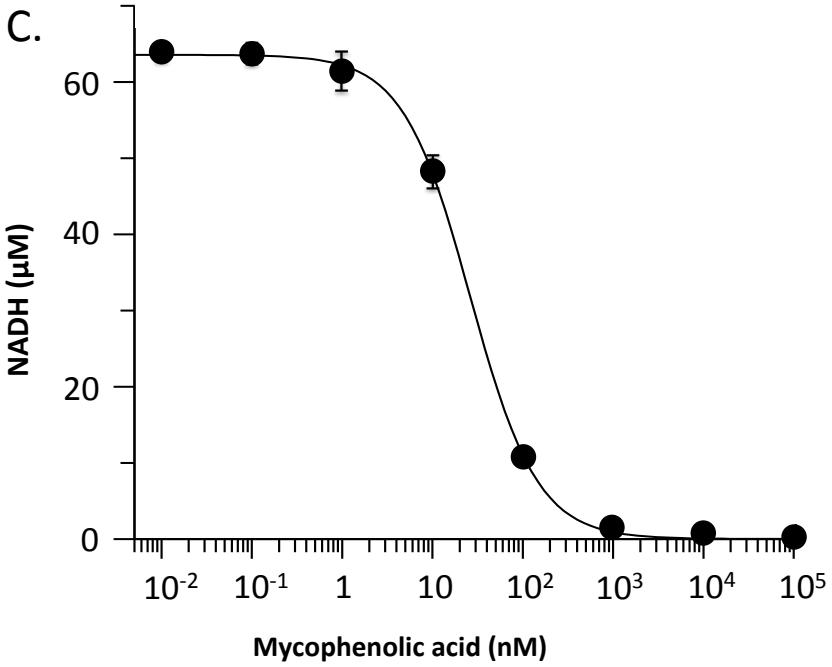
Guanosine or	IC_{50} (μM) with guanosine		IC_{50} (μM) with xanthine	
xanthine (μM)	**1**	**4**	**1**	**4**
250	>5.0	>5.0	0.09±0.001	0.21±0.01
50	0.29±0.19	0.50±0.31	0.09±0.003	0.22±0.01
0	0.07±0.006	0.13±0.02	0.09±0.004	0.22±0.02

The IC_{50} values of MPA (**1**) and **4** supplemented with 250, 50 and 0μM of guanosine or xanthine. All of the values were calculated from 3 independent experiments and are shown as the mean ± standard deviation. IC_{50}, 50% inhibitory concentration.

Supplemental Fig. 1. The enzymatic activity of TcIMPDH and the inhibition of TcIMPDH by mycophenolic acid







Supplemental Figure 1. The enzymatic activity of TcIMPDH and the inhibition of TcIMPDH by mycophenolic acid

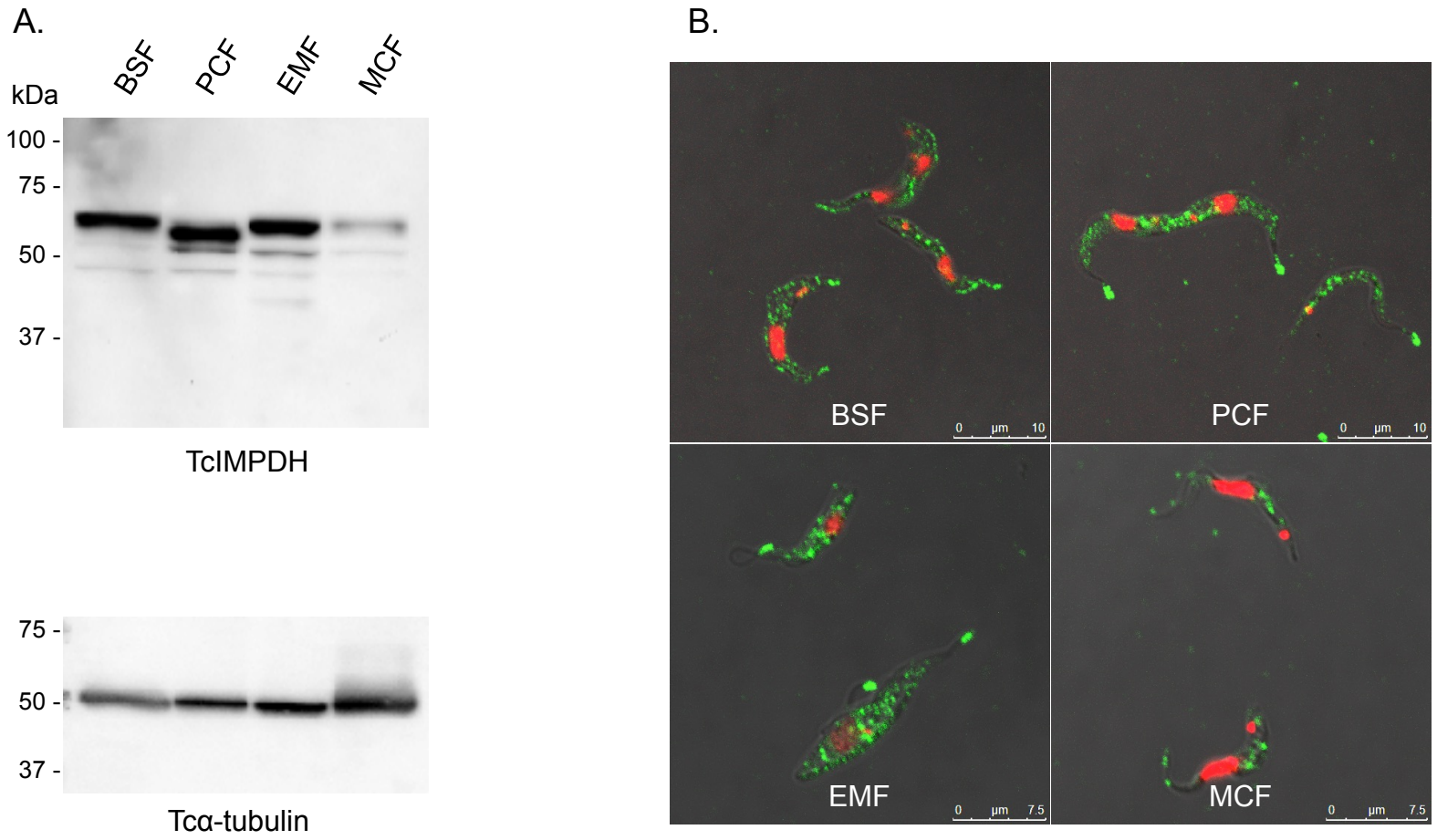
The activity of IMPDH was evaluated, based on the detection of the enzymatic product NADH, as the increasing absorbance at 340 nm. The data represent the mean values of three independent experiments.

(A) The dose-dependent assay of TcIMPDH. The assay was carried out for 60 minutes in standard IMPDH buffer with various concentrations of enzyme.

(B) The time-course assay of TcIMPDH. The assay was carried out for various reaction times in standard IMPDH buffer with a TcIMPDH concentration of 40 nM.

(C) MPA has been known as a specific noncompetitive inhibitor of IMPDH. A dose-dependent assay of MPA was carried out in the presence of 250 μM IMP, 800 μM β-NAD^+, and 40 nM TcIMPDH. The data represent the mean values of three independent experiments.

Supplemental Fig. 2. The expression profile and localization of TcIMPDH



Supplemental Fig. 2. The expression profile and localization of TcIMPDH

(A) Western blotting was performed with 5 μg of total cell protein extracted from BSF, PCF, EMF, and MCF using anti-TcIMPDH and anti-Tc α-tubulin antibodies.

(B) Indirect immunofluorescence staining using anti-TcIMPDH antibody was observed by confocal laser scanning microscopy. Nucleolus and kinetoplast DNAs were subjected to Hoechest 33342 staining and are shown in red. The images were constructed by merging a fluorescence image and a differential interference contrast image. Each of the microscopy images was captured using the same photomultiplier tube gain and voltage.