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Purine nucleotide biosynthesis pathway as a drug target: Identification of novel IMPDH and GMPR from *Trypanosoma congolense*, and an inhibitor screening study of *Cryptosporidium parvum* and human type II IMPDH

(薬物標的としてのプリンヌクレオチド合成： *Trypanosoma congolense* 由来の新規 IMPDH 及び GMPR の同定, 並びに *Cryptosporidium parvum* 及びヒト II 型 IMPDH 阻害剤の探索研究)

GENERAL INTRODUCTION

Purine nucleotide is an essential building block for genetic material syntheses in nearly all organisms. The simplest machinery of purine nucleotide biosynthesis pathway involves three essential enzymes: guanosine 5'-monophosphate dehydrogenase (GMPR) (EC 1.7.1.7), inosine 5'-monophosphate dehydrogenase (IMPDH) (EC 1.1.1.205), and GMP synthase (GMPS) (EC 6.3.5.2), with each catalyzing the conversion of GMP to IMP, IMP to XMP, and XMP back to GMP, respectively. These enzymes cooperatively regulate and maintain the intracellular balance of adenosine monophosphate (AMP) and guanosine monophosphate (GMP). This study focused on IMPDH and GMPR as a drug target (Fig. 1).

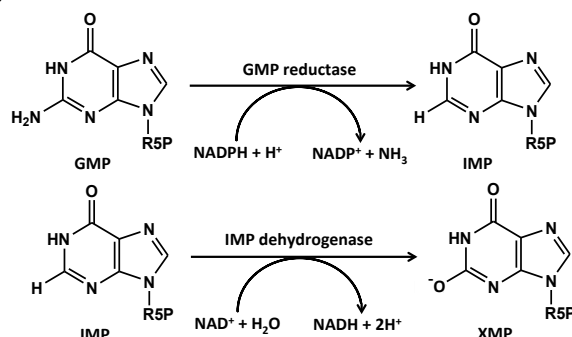


Fig. 1. Catalytic reaction of GMPR and IMPDH

Due to their essential role in nucleic acid biosynthesis, the enzymes in the purine nucleotide biosynthesis pathway are considered as an important drug targets. The inhibition of IMPDH, GMPR, or GMPS has been proved as one of the chemotherapeutic strategies against various medical conditions.

In human, the inhibitors of hIMPDH II specifically have been investigated as chemotherapeutic agents for controlling the progression of cancer cells, proliferation of lymphocyte cells, and replication of invading viruses. These bioactivities have been proven as the consequence of the depletion in guanine nucleotide pool. The broad application of hIMPDH II inhibitors therefore justified the screening effort for discovering more potent inhibitors against the enzyme.

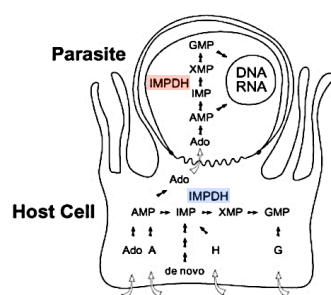


Fig. 2. Purine nucleotide biosynthesis in a protozoan infection model

Microbe from phylum Apicomplexa, such as pathogenic protozoan *Cryptosporidium* spp., relies solely on a very simple and streamlined salvage pathway to produce its guanosine nucleotides (Fig. 2). This difference lays the basis for the development of apicomplexan IMPDH as a molecular target for anti-protozoan agent.

IDENTIFICATION AND CHARACTERIZATION OF *TRYPANOSOMA CONGOLENSE* GMPR AND IMPDH

1. INTRODUCTION

T. congolense is an economically important pathogenic protozoan in tropical part of Africa. The protozoa infect a broad range of African animals, inflicting fatal animal trypanosomosis, which is also known as *nagana* disease.

Current chemotherapeutic compounds used against the infection consist of relatively old and low-efficacious compounds, resulting in the emergence of chemo-resistant *T. congolense* strains. The discovery of novel chemotherapeutic agents against trypanosomosis also suffered from limited attention and funding from major pharmaceutical companies.

Naturally, humans are resistant to *T. congolense* infection due to the trypanosome lytic factor in the serum. However, some cases of *T. congolense* infection in human have also been reported. While the exact mechanism underlying these cases is yet to be explained, the incidents have highlighted the possibility of future outbreak of infection in human. Therefore, it is of a high importance not only to discover new drug compounds but also to identify new druggable molecular targets.

Similar to some other protozoa, *T. congolense* relies on a salvage pathway to produce purine nucleotides in its cell. Some reports have confirmed that an inhibition against one of the enzymes involved in the pathway could effectively inhibit the proliferation of pathogens relying on this pathway. This validates the pathway as a promising drug target for anti-trypanosomal chemotherapy. This study was aimed to identify and characterize the unique GMPR and IMPDH-encoding genes in *T. congolense* (TcGMPR and TcIMPDH).

Previously, a BLAST search of IMPDH analogues in *T. congolense* genome resulted in a match with a protein encoded by TcIL3000_5_1940. Interestingly, this particular enzyme has been annotated both as IMPDH and as GMPR, a typical annotation discrepancy on this enzyme family. Furthermore, BAT33662.1, an analogue of TcIL3000_5_1940, was also found in a sequencing study in *T. congolense* genome. Enzymatic activity of both enzymes was investigated in this study.

2. MATERIALS AND METHODS

The *E. coli* cells harboring plasmid encoding the gene were grown in 2xYT broth containing 100 µg/ml ampicillin at 37°C overnight. The recombinant enzyme, which fused

with glutathione *S*-transferase (GST), was induced with 0.1 mM isopropyl thio- β -D-galactoside (IPTG) for 12 h at 18°C. The cells were then harvested and sonicated.

The cell lysate was centrifuged, and the supernatant was applied to Glutathione Sepharose 4B overnight. The GST-tag was cleaved using PreScission protease according to manufacturer manual. The enzyme dialyzed overnight. All purification processes were carried out at 4°C. The protein was stored at –80°C as a 50% glycerol mixture until use.

The protein was subjected into both IMPDH and GMPR assay solution. The IMPDH assay solution contains 100 mM KCl, 3 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 250 μ M IMP, 800 μ M NAD⁺ in 50 mM Tris-HCl pH 8.0 (25°C). GMPR assay solution contains 100 mM KCl, 3 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 100 μ M GMP, 100 μ M NADPH in 75 mM Tris-HCl pH 7.8 (25°C). The reaction was started by the addition of an appropriate amount of enzyme. The production of NADH for IMPDH reaction or the consumption of NADPH for GMPR reaction was monitored.

The inhibition of MPA to TcGMPR was measured with an assay solution containing various concentrations of MPA at 30°C. DMSO was used as vehicle, with a final concentration of 1%. The IC₅₀ value was calculated using GraFit 7 software. The inhibition of MPA to TcIMPDH was measured in a similar manner.

3. RESULTS AND DISCUSSION

By subjecting the enzyme to both IMPDH and GMPR assay solution, the identity of the protein was determined. hIMPDH II was used as the IMPDH control. The result showed that TcIL3000_5_1940 is a GMPR from *T. congolense* (TcGMPR).

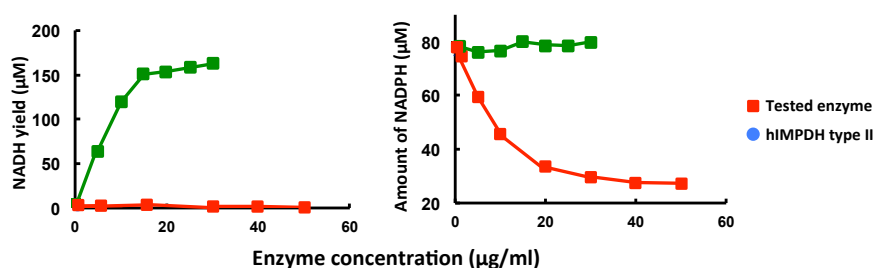


Fig. 3. Enzymatic identification of TcIL3000_5_1940

The values of K_m GMP, K_m NADPH, and k_{cat} were determined to be 91.6 ± 4.7 μ M, 11.3 ± 2.3 μ M, and 0.499 ± 0.014 s⁻¹, respectively. These values were quite similar to the reported values for TbGMPR (Table 1). But more importantly, they were significantly different from those of mammalian enzymes.

Table 1. Comparison of enzymatic parameters of TcGMPR to other GMPR

	k_{cat} (s ⁻¹)	K_m GMP (μ M)	K_m NADPH (μ M)
TcGMPR ^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 (Bessho *et al.*, 2016).

Mycophenolic acid (MPA) is a potent inhibitor of mammalian IMPDH. However, since IMPDH and GMPR have similar substrates and primary structure, it is possible that a same inhibitor could inhibit both enzymes. The TcGMPR inhibition assay of MPA showed a clear inhibitory activity with IC_{50} of $239.6 \pm 26.7 \mu M$ (Fig. 4). MPA also showed a 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. Similar finding on GMPR inhibition by MPA was also reported with *Leishmania major* GMPR, which exerted a K_i value of 20 μM .

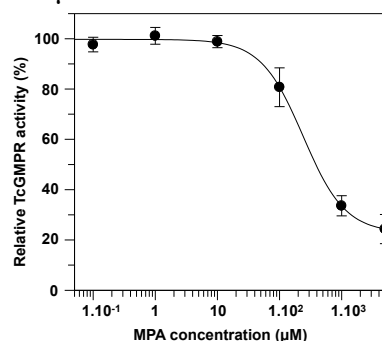


Fig. 4. Inhibitory activity of MPA against TcGMPR

In the same manner, BAT33662.1 was found to be an IMPDH from *T. congolense* (TcIMPDH).

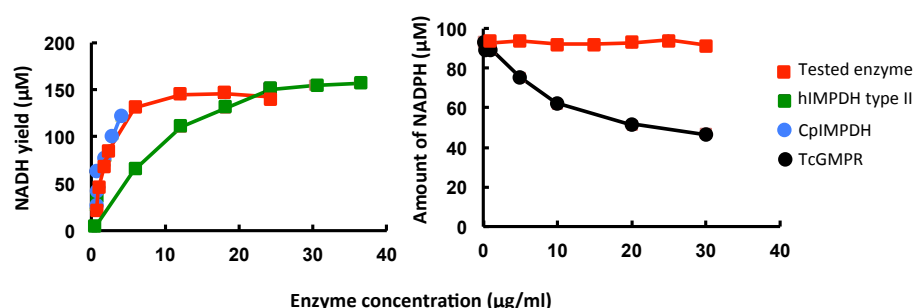


Fig. 5. Enzymatic identification of BAT33662.1

As expected MPA showed potent inhibition to TcIMPDH, with calculated IC_{50} of MPA against TcIMPDH was 26 nM.

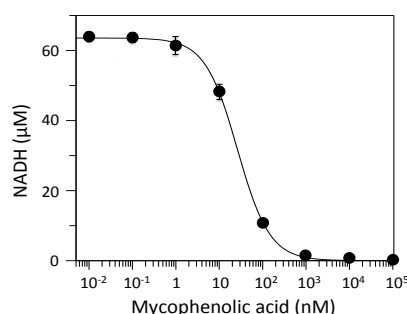


Fig. 6. Inhibitory activity of MPA to TcIMPDH

4. CONCLUSION

In conclusion, the present study revealed that TcIL3000_5_1940 is a GMPR-encoded gene of *T. congolense*. Additionally, a protein encoded by BAT33662.1 was identified as an IMPDH from *T. congolense*.

HIGH THROUGHPUT SCREENING FOR IMPDH INHIBITORS

1. INTRODUCTION

As mentioned earlier, IMPDH is one of the enzymes involved in the purine nucleotide biosynthesis. Human IMPDH type II (hIMPDH II) has been found to expressed mainly in rapidly replicating cells. This renders hIMPDH II as a drug target for various puposes, such as suppressing the replication of cancer cells, lymphocytes, and virus. Therefore, the discovery of hIMPDH II inhibitors is necessary.

Cryptosporidium parvum is a waterborne pathogen, which causes watery or mucoid diarrhea and abdominal pain in many mammal species, including human. In the last decade, the IMPDH of *C. parvum* was found to play an essential role in guanine nucleotide production in *C. parvum*. Several studies have proven that the inhibition of the enzyme could effectively disrupt the nucleic acid synthesis in the protozoa. Therefore, this enzyme is considered as a chemotherapeutic target against *C. parvum* infection.

Previously, several high-throughput studies have been conducted to screen IMPDH inhibitors. However, some limitations were present in the assays: (1) the measurements of IMPDH activity were by OD₃₄₀ or fluorescence, thus prone to false negative detections, (2) the assays were conducted with a relatively high amount of enzyme, and (3) there was no report of the repurposing of known compounds.

This study aimed to discuss the discovery and the characterization of IMPDH inhibitors discovered from a bioluminescence-based high-throughput screening of 3,200 compounds.

2. MATERIAL AND METHODS

The HTS assay was established using NAD(P)H-Glo Assay. Briefly, 10 µl of substrate solution containing 50 mM Tris-HCl pH 8.0, 200 mM KCl, 0.1 mg/ml BSA, 1.6 mM β-NAD⁺, 100 µM IMP, and the assay kit was added to white 384-well plates. Mycophenolic acid or chemical library compounds were also added to the wells in concentration of 10 µM. Reaction was started by addition of enzyme. The production of NADH was monitored by the measurement of luminescence (RLU) using either VERITAS Microplate Luminometer or EnSpire Multimode Reader.

The counter assay was carried out by directly incubating assay kit, the library compounds, and appropriate amount of NADH. The produced luminescence was monitored. IC₅₀ values were calculated by plotting the recorded NADH yield against log of compound concentration in GraFit ver. 7.

The kinetic of irreversible inhibition was carried out as following: Standard IMPDH assay solution contained 50 mM Tris-HCl pH 8.0, 100 mM KCl, 3 mM EDTA, 0.1 mg/ml BSA, and an appropriate amount of inhibitor with a total volume of 270 µl incubated at 30°C. Substrate concentrations in the solution were 500 µM NAD⁺ and 250 µM IMP for CpIMPDH, 100 µM NAD⁺ and 250 µM IMP for hIMPDH II. Reaction was started by the addition of 30 µl enzyme solution. The activity of the enzyme was measured by monitoring NADH production in either absorbance at 340 nm or fluorescence emissions at 465 nm.

The exponential inactivation was quantified by fitting the decay progress curve to Eq. 3:

$$R_t - R_0 = \frac{V_0}{k_{\text{obs}}} (1 - e^{-k_{\text{obs}} \cdot t}) \quad (\text{Equation 1})$$

where R_t is the absorbance (OD₃₄₀) or fluorescence at time t , R_0 is the initial absorbance or fluorescence at time 0, V_0 is the initial reaction rate, and k_{obs} is the observed rate constant of enzyme inactivation. Acquired k_{obs} values then fitted into Eq. 4 to obtain k_{on} value:

$$k_{\text{obs}} = \frac{k_{\text{on}} [I]}{1 + \frac{[S]}{K_m}} \quad (\text{Equation 2})$$

where k_{on} is the apparent second-order rate constant for IMPDH inactivation, $[I]$ is inhibitor concentration, $[S]$ is IMP concentration, and K_m is Michaelis-Menten constant for IMP. Plot fitting of inhibitory study was carried out using GraphPad Prism 7.0.

The kinetic of reversible inhibition was carried out as following: Assay was carried out with standard IMPDH assay solution as mentioned previously. Data were fitted into noncompetitive inhibition (Eq. 5) or mixed model inhibition (Eq. 6):

$$v = \frac{V_m[S]}{\left(K_m\left(1 + \frac{[I]}{K_i}\right) + [S]\left(1 + \frac{[I]}{K_i}\right)\right)} \quad (\text{Equation 3})$$

$$v = \frac{V_m[S]}{\left(K_m\left(1 + \frac{[I]}{K_{is}}\right) + [S]\left(1 + \frac{[I]}{K_{ii}}\right)\right)} \quad (\text{Equation 4})$$

where v is reaction velocity, V_m is the maximal velocity, $[S]$ is substrate concentration, K_m is the Michaelis-Menten constant for the substrate, K_i is inhibition constant when the inhibitor shows equal affinity to free enzyme and enzyme-substrate complex, K_{is} is the slope inhibition constant, and K_{ii} is the intercept inhibition constant. Plot fitting of inhibitory study was carried out using GraphPad Prism 7.0.

3. RESULTS AND DISCUSSION

The developed system has a Z' -factor value of 0.7, indicating an excellent assay system for screening. CpIMPDH was used for all the screening process.

First screening was conducted on 3,200 compounds, comprised of 1,600 novel synthesized compounds of Hokkaido University and 1,600 known bioactive compound. From the first screening, a total of 79 compounds were selected as “hits”.

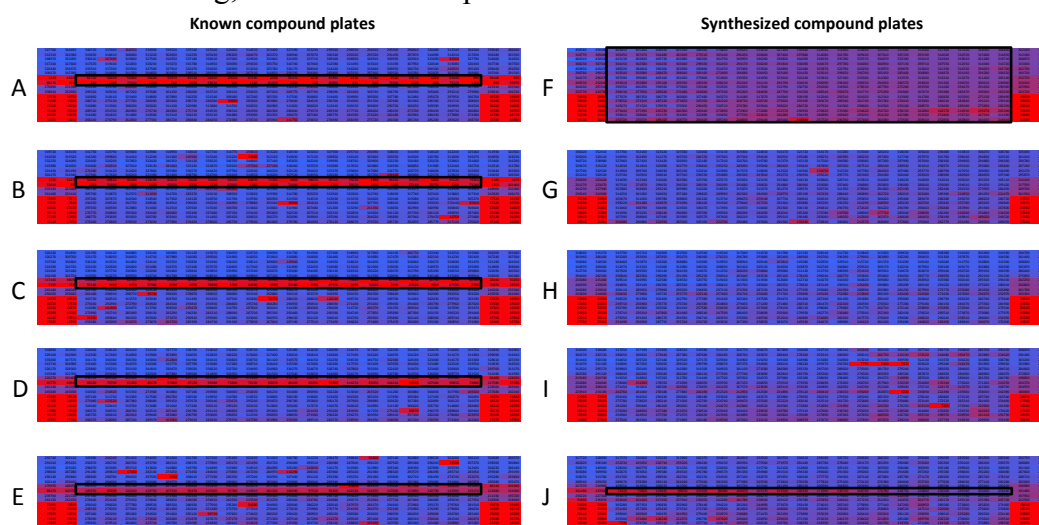


Fig. 7. Heatmap of the first screening

The 10 screening plates for screening of 3,200 library compounds. Known compound library were assayed in plate A-E, while synthesized compounds were assayed in plate F-J. Forty compounds of each plate A-E, all compounds in plate F, and 20 compounds from plate J were disqualified from the study. Disqualified wells were marked by black boxes. Blue and red represents high and low luminescence, respectively.

Inhibition of a certain compounds to an enzyme is not always a result of stoichiometric enzyme-inhibitor interaction. Some references have shown that some compounds might form a micelle and act as detergent-like inhibitor to the enzyme. These compounds usually show weak or very steep dose-dependency inhibition. The inhibitory activity of each compound was measured in a 5-points of increasing concentration, with the highest concentration of 50 μ M. The 32 compounds from the known library and 19 compounds from the synthesized library were subjected to this assay.

In total, 60 compounds were tested in this stage. All of the compounds were found to display a dose-dependent inhibition. Twenty-nine compounds were found to inhibit in the dose-dependent manner with IC_{50} values less than 10 μM (Fig. 8).

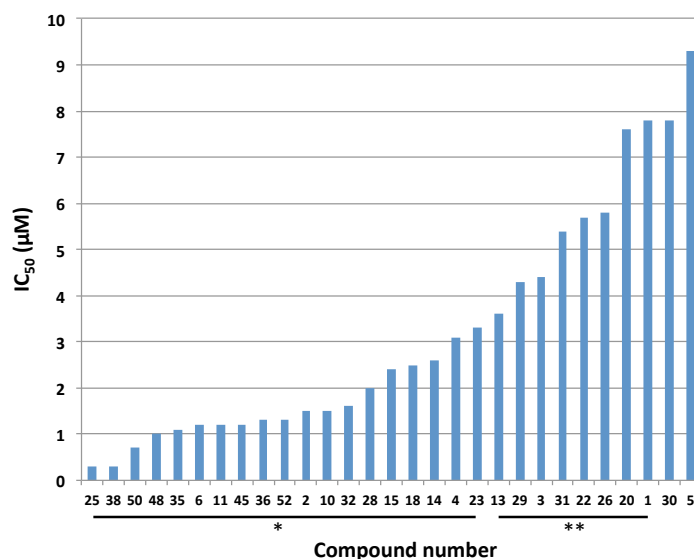


Fig. 8. The twenty-nine compounds with IC_{50} less than 10 μM . Known compounds were numbered 1-32, synthesized compounds were numbered 33-60. *: 2 asymptotes in dose-dependency assay, **: 1 asymptote, inhibit completely at concentration of 50 μM .

In the study, the luminescence produced by luciferase was measured as a parameter of IMPDH activity. In the assay kit, two enzymes are necessary: reductase and luciferase. Therefore, it is also possible that the compounds were inhibiting the luciferase or reductase instead of IMPDH. The aim of the counter assay experiment was to exclude compounds that inhibit the assay-kit reaction instead of IMPDH reaction. Sixty compounds from the previous screening were subjected into this stage.

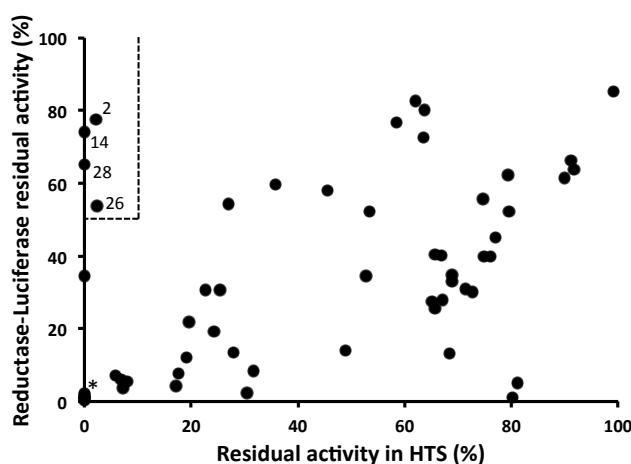


Fig. 9. Inter-relation model of counter assay. The counter assay was performed on 60 compounds to exclude reductase-luciferase inhibitors from hit compounds. Dashed line showed the selection criteria, which eliminated inhibitors that inhibit less than 90% of control IMPDH activity, and more than 50% of control reductase-luciferase activity in the concentration of 10 μM . Four compounds were selected: compound number 2, 14, 26, and 28. *: A cluster of compounds from synthesized library.

The compounds that showed selectivity to IMPDH were prioritized for the study: disulfiram (2), thiram (14), ebselen (26), and bronopol (28). All of these compounds are from the known compound library. In contrast, the novel compounds were found to have

relatively low inhibition specificity between IMPDH and reductase-luciferase. Therefore, the selection was conducted based on the IC_{50} value alone: compound **35**, **36** (a replication of compound **45**), **38**, and **48** (a replication of compound **50**) (Fig. 32). These synthesized compounds inhibit both IMPDH and reductase-luciferase (Fig. 31, * symbol). Hits from synthesized compound library are not discussed further. In the subsequent toxicology analysis, thiram (**14**) were removed from the hit list due to toxicology concerns.

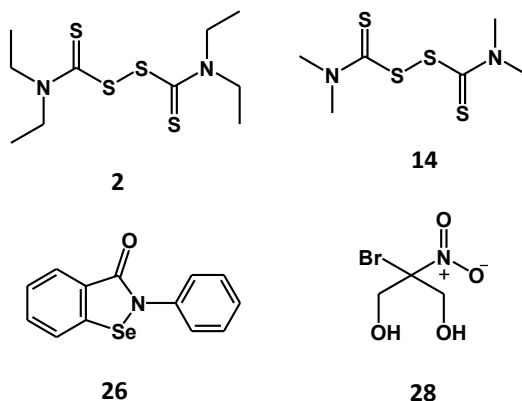


Fig. 10. The structures of the hit compounds

By subsequent dialysis experiment, compounds **2**, **26**, and **28** were found to be irreversible. The time-dependent inactivation of IMPDH in the presence of various inhibitor concentrations was also fitted into Eq. 1 (Fig. 11). The acquired k_{obs} values were then fitted into Eq. 2 (Fig. 12). Both compounds were found to be potent inhibitors of IMPDH, compared to other known irreversible IMPDH inhibitors (Table 8). However, both inhibitors showed a low selectivity between mammalian and protozoan IMPDH with k_{on} CpIMPDH:hIMPDH II value of 0.78 and 3.1 for disulfiram and bronopol, respectively. Inhibition of IMPDH by disulfiram and bronopol showed linear k_{obs} plots, which implied that the inactivation was progressed in a one-step inactivation mechanism (Fig. 40).

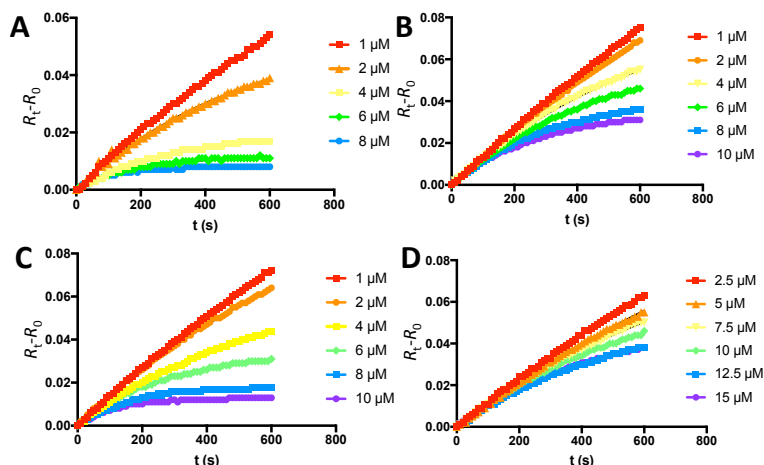


Fig. 11. Decay of enzyme activity in the presence of disulfiram and bronopol
Inhibition of CpIMPDH (A) and hIMPDH II (B) by disulfiram. The inhibition of CpIMPDH (C) and hIMPDH II (D) by bronopol. OD₃₄₀ values were fitted to Eq. 3 to acquire k_{obs} values. Concentration values represent concentration of inhibitor compounds in the assay solution

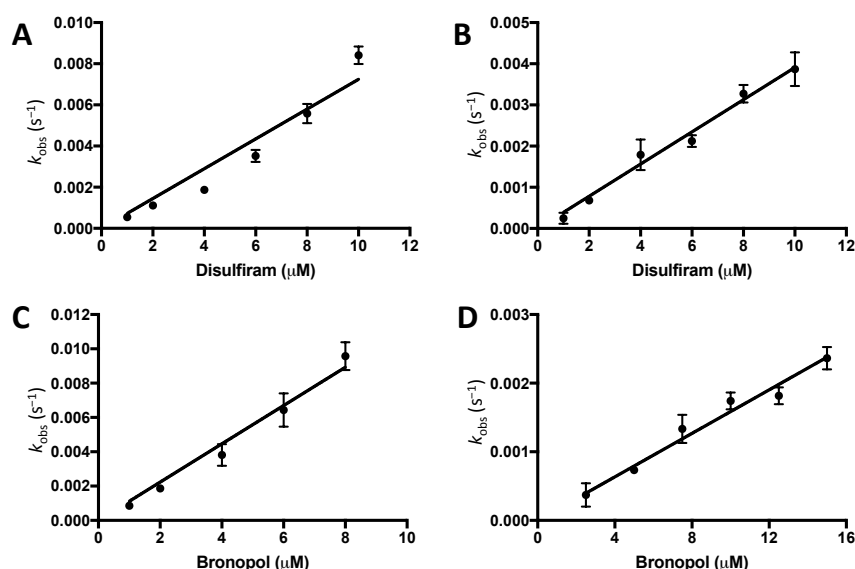


Fig. 12. The inactivation of IMPDH by disulfiram and bronopol. Inhibition of CpIMPDH (A) and hIMPDH II (B) by disulfiram. The inhibition of CpIMPDH (C) and hIMPDH II (D) by bronopol. Values are fitted to Eq. 4. Values are mean $\pm$ SD from two independent experiments.

This study is the first report on disulfiram and bronopol as potent IMPDH inhibitors. Previously, disulfiram has been known as an alcohol aversion drug which acts as inactivator of acetaldehyde dehydrogenase. Taking disulfiram with alcohol resulted in accumulation of acetaldehyde, causing discomforting symptoms thus discouraging further intake. Bronopol on the other hand was utilized as antiseptic and antifungal compound in aquatic veterinary settings and preservative in cosmetic products.

Ebselen however, showed a quite different inhibition property compared with the other two inhibitors. Ebselen did not show any irreversibility against CpIMPDH, even after decreasing the amount of enzyme and extending the measurement time. The inhibition to CpIMPDH was then regarded as a reversible inhibition. This finding is particularly interesting, because previously ebselen has shown irreversible inhibition in the dialysis experiment.

Table 2. Kinetics of ebselen inhibition against CpIMPDH

C: competitive, UC: uncompetitive, NC: noncompetitive, MM: mixed model inhibition

	CpIMPDH
	706 (NC, NAD^+)
Ebselen (nM) ^a	64.6 ± 16 (K_{is} , MM, IMP) 2808 ± 927 (K_{ii} , MM, IMP)
MPA (nM) ^b	9300 (UC, NAD^+)
GMP (μM) ^b	46 (C, IMP)

^a: Activity was measured at 30°C, 50 mM Tris-HCl buffer pH 8.0, 100 mM KCl, 3 mM EDTA, and 0.1 mg/ml BSA. Assay against NAD^+ , constant IMP at 250 μM , varied NAD^+ . Assay against IMP, constant NAD^+ at 500 μM , varied IMP.

^b: Activity was measured at 25°C, 50 mM Tris-HCl buffer pH 8.0, 100 mM KCl, 3 mM EDTA, and 1 mM DTT. Assay against NAD^+ , constant IMP at 250 μM , varied NAD^+ . Assay against IMP, constant NAD^+ at 500 μM , varied IMP (Umejiego *et al.*, 2004).

In contrast to CpIMPDH, inhibition of ebselen to hIMPDH was clearly showing enzymatic decay. Therefore, the inhibition was characterized in the same manner as disulfiram and bronopol.

Irreversible inhibition of ebselen to hIMPDH II was very interesting, due to the hyperbolic k_{obs} versus inhibitor concentration plot. Forcing this plot into Eq. 2 yielded k_{on} value of $9.3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 13 and Table 3).

Therefore, the mode of IMPDH inhibition by ebselen seemed to be that of mixed mechanisms, alternating between reversible and irreversible, depending on the assay condition. Lower concentration of inhibitor might have reversible inhibition as the main mode, hence the low k_{obs} value, and the irreversible inhibition mode was progressively become more dominant as the inhibitor concentration increase. Ebselen inactivates hIMPDH II at sub-micromolar level, and might be the most potent irreversible inhibitor of IMPDH reported to date.

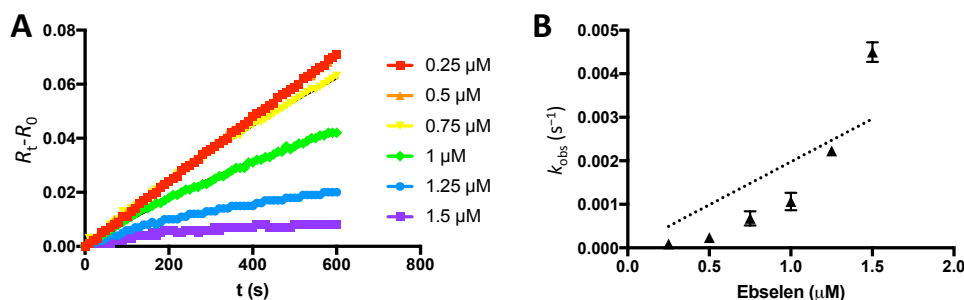


Fig. 13. The inactivation of hIMPDH II by ebselen

(A) Decay of hIMPDH II activity in the presence of ebselen. Concentration values represent concentration of ebselen in the assay solution. OD_{340} values were then fitted into Eq. 3. (B) Plot of second-order rate of inactivation (k_{on}) was acquired by fitted k_{obs} values and inhibitor concentration to Eq. 4.

Table 3. k_{on} values of ebselen against hIMPDHs ($\text{M}^{-1} \cdot \text{s}^{-1}$)

hIMPDH II	
Ebselen ^a	$> 9.3 \times 10^4$

^a: Activity was measured at 30°C, 50 mM Tris-HCl buffer pH 8.0, 100 mM KCl, 3 mM EDTA, and 0.1 mg/ml BSA.

Previously, ebselen has been known as an organoselenium compound with broad reported activities, such as anti-inflammatory, anti-atherosclerotic, antibacterial, and anticancer. This compound has been subjected into clinical trials as a treatment for acute ischemic stroke and a prophylactic neuroprotective agent. Generally regarded as a low toxicity selenium-containing compound, ebselen is exerting adverse effects only in the high concentrations.

3. CONCLUSION

In conclusion, three known compounds have been repurposed from the screening of 2,660 compounds as potent novel IMPDH inhibitors: disulfiram, bronopol, and ebselen. These compounds provide novel moiety for future design of IMP binding site inhibitor, in particular to improve the selectivity of the inhibition.