Drug Discovery for Neglected Diseases: Molecular Target-Based and Phenotypic Approaches

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Ian H. Gilbert®

Drug Discovery Unit, Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee DD1 5EH, U.K.

ABSTRACT: Drug discovery for neglected tropical diseases is carried out using both target-based and phenotypic approaches. In this paper, target-based approaches are discussed, with a particular focus on human African trypanosomiasis. Target-based drug discovery can be successful, but careful selection of targets is required. There are still very few fully validated drug targets in neglected diseases, and there is a high attrition rate in target-based drug discovery for these diseases. Phenotypic screening is a powerful method in both neglected and non-neglected diseases and has been very successfully used. Identification of molecular targets from phenotypic approaches can be a way to identify potential new drug targets.

INTRODUCTION

Neglected tropical diseases represent a significant health burden in large parts of the world. The available medicines to treat these diseases by no means reflect the clinical need. However, there has been a significant effort to develop new drugs to treat these diseases over the past 10 years. This has been driven by funding from charities such as the Bill and Melinda Gates Foundation and the Wellcome Trust and through various governmental agencies. Public private partnerships such as the Drugs for Neglected Diseases Initiative (DNDi) and the Medicines for Malaria Venture (MMV) have been key for coordinating this work and establishing pathways for drug discovery. Also key to this progress has been the engagement of a number of pharmaceutical companies who have initiated work in this not-for-profit area. A significant number of academic groups have engaged in the basic biology, and a few groups have engaged in coordinated drug discovery, such as our own unit, the Drug Discovery Unit (DDU) at the University of Dundee.

The purpose of this perspective is to comment on strategies for drug discovery for neglected tropical diseases, considering both target-based (protein screening) and phenotypic (whole cell screening) approaches. The focus will be on human African trypanosomiasis (HAT) but will be of relevance for other neglected tropical diseases.

TARGET-BASED APPROACHES

Target-based approaches to drug discovery are extensively used in the pharmaceutical industry. This involves screening a library of compounds against a protein and then optimizing the compounds for potency against the enzyme, selectivity, cellular activity, and pharmacokinetic properties. However, there are relatively few validated drug targets across the disease spectrum, and this is particularly the case in infectious diseases. Arguably, a target is only fully validated when there is a registered drug for which it can be shown the principle mode of action is by inhibition of the target. Overington and colleagues carried out an extensive analysis (published in 2006) to assess how many different molecular targets are modulated by registered drugs. This was carried out by a literature review, where they sought to link evidence of modulation of a molecular target to cell or in vivo efficacy. They were able to do this for 1065 unique drugs. From this they estimated there were about 324 molecular targets for human diseases, of which 266 are targets in the human genome and the remainder (a minority) for pathogen targets.

In the case of parasitic diseases, there are very few validated molecular targets. By way of example, it is interesting to look at the mode(s) of action of currently registered drugs used for the treatment of kinetoplastid infections (Table 1). For most of them, the mode of action is poorly understood, and for many, it is likely to involve interaction with a number of different targets. The only drug for which there is a well-defined molecular target is eflornithine, which inhibits the enzyme ornithine decarboxylase.

With the notable exception of Trypanosoma brucei (the etiological agent of human African trypanosomiasis), there is a lack of genetic tools to validate drug targets in these parasites. Furthermore, there is often a disconnect between genetic validation (through methodologies such as RNAi and knock-out) and chemical modulation of targets in these organisms. Selection of molecular targets is key to success in target-based drug discovery. In Dundee, we triage drug targets by assessing...
them against a number of criteria: druggability, essentiality, assayability, toxicity, resistance potential, and structural information.12,13 Each criterion is scored using a traffic-light system. This helps to assess the level of validation of a target and also can highlight key experiments that need to be carried out to increase the level of validation. For example, it can highlight if more experiments need to be carried out to determine the essentiality of the target through chemical validation or whether a robust assay is required. In other cases, it can indicate that the target is not a valid drug target.

At the DDU in Dundee, we have carried out a number of target-based and phenotypic drug discovery programmes against HAT. By way of illustration, a few examples of target-based discovery will be discussed.

**N-Myristoyltransferase as a Drug Target for HAT.**

N-Myristoyltransferase (NMT) is an enzyme responsible for the cotranslational myristoylation of the N-terminal glycine of a number of different peptides. This enzyme is essential in eukaryotic organisms, and it has been extensively investigated as a drug target in fungal diseases.14−26

We were interested in NMT as a potential drug target for HAT. Smith’s group in York had carried out RNAi studies that indicated that the enzyme was essential in *T. brucei*.27,28 To further establish the essentiality of the target, an in vivo RNAi experiment was carried out. Mice were infected with *T. brucei*; when the RNAi was induced, these parasites were not viable, neither were the ones that we tested showed activity against the parasite, presumably acting as a Michael acceptor. This probably accounts for some or all of the activity of nifurtimox.30

We looked at: replacing the pyrazole ring with a number of potential aromatic replacements, substituents on the pyrazole ring; replacement of the sulphonamide, and variations around the phenyl ring. The outcome of these studies (Figure 2) were that the aromatic replacements to the pyrazole were less active, the aromatic sulphonamide could not be replaced (data not shown). We prepared a number of different phenyl derivatives to probe this criterion, except that having a 2,6-dichloro substituent enzyme over the human enzyme.22 We were unable to deduce how the hit bound in the active site. However information that it is possible to prepare compounds selective for the fungal enzyme over the human enzyme.22

**Table 1. Mode of Action of Currently Used Drugs for Kinetoplastids**

| drug       | disease | mode of action                                                                 |
|------------|---------|--------------------------------------------------------------------------------|
| suramin    | HAT     | Not fully known.2 Probably polypharmacology. There is some linkage to the endocytic pathway, lysosomal function, spermidine, and N-acetylglucosamine biosynthesis.3 |
| pentamidine| HAT     | The drug is selectively accumulated in the parasite by the action of various transporters.4 The precise molecular target(s) are unknown. Pentamidine is known to interact with DNA and to affect mitochondrial function.5 |
| melarsoprol| HAT     | Selectively concentrated by the parasite.3 The target(s) are not known - possibly multiple targets.2 |
| efomithine | HAT     | Inhibits ornithine decarboxylase (suicide inhibition). |
| nifurtimox | HAT/Chagas | This is activated by a type I nitro-reductase.6,7 The product of this reaction was thought to generate radicals which generate oxidative stress on the parasites. Recent evidence indicates that upon reduction, nifurtimox produces an unsaturated nitrile compound which is active against the parasite, presumably acting as a Michael acceptor. This probably accounts for some or all of the activity of nifurtimox.30 |
| benznidazole | Chagas | Free radical generation after being activated through a type I nitroreductase.6,9 |
| amphotericin B | leishmaniasis | The drug targets the cell membrane, disrupting the ion gradients across the membrane. |
| miltefosine | leishmaniasis | Multiple modes of action, including effects on lipid metabolism, induction of apoptosis, and mitochondrial function.10 There also is some suggestion of immunomodulatory effects. |
| paromomycin | leishmaniasis | In bacteria this inhibits protein biosynthesis by binding to the 30S subunit of the ribosome. The precise mode of action in leishmania is not known.11 |
| stibogluconate | leishmaniasis | Activated to a trivalent form within the macrophage. Molecular targets unknown.11 |

**Table 2. Target Assessment of *T. brucei* at the Outset of Our Project**

| criterion | score | comment |
|-----------|-------|---------|
| essentiality | amber | Genetic validation (RNAi studies in *T. brucei*). |
| druggability | green | Drug-like inhibitors known for the fungal enzymes. |
| assayability | green | High throughput assay reported. Protein available. |
| resistance | green | No known isoforms within *T. brucei* and no known escape mechanisms. |
| potential | amber/red | Human homologue present. No information as to whether it is possible to obtain selectivity for *T. brucei*. However information that it is possible to prepare compounds selective for the fungal enzyme over the human enzyme.12 |
| structural information | amber | Opportunity to build a homology model. |

Figure 1. Screening hit for *T. brucei* NMT.

Although we could develop a homology model of TnNMT, we were unable to deduce how the hit bound in the active site. Therefore we set about a chemistry program systematically varying the structure of the hit.20 We looked at: replacing the pyrazole ring with a number of potential aromatic replacements, substituents on the pyrazole ring; replacement of the sulphonamide, and variations around the phenyl ring. The outcome of these studies (Figure 2) were that the aromatic replacements to the pyrazole were less active, the N-methyl group on the pyrazole was essential for activity, and the sulphonamide could not be replaced (data not shown). We prepared a number of different phenyl derivatives to probe this position. We found relatively flat SAR with simple phenyl modification, except that having a 2,6-dichloro substituent appeared to increase activity by about 10-fold. Substitution was tolerated in the para-position of the phenyl group, which led us...
to investigate derivatives in this position in more detail (Figure 3). From our studies in substitution at the para-position of the phenyl ring, there were two compounds which particularly stood out: an N-methyl piperazine derivative 12 (IC\textsubscript{50} = 0.14 μM) and the corresponding morpholine analogue 11 (IC\textsubscript{50} = 38 μM) (Figure 3). These had over a 100-fold difference in potency; the key difference in the structure was the presence of a basic nitrogen in the more active substituent. Reported inhibitors of fungal NMT have a basic group which forms an electrostatic interaction with the C-terminal carboxylic acid residue of NMT. This is where the N-terminal glycine of peptides to be myristoylated binds. We hypothesized that the basic nitrogen on our compound in Figure 3 was binding in this pocket, explaining the increase in binding potency. Further work focused on constraining the amine, giving the lead molecule DDD85646 (13).\textsuperscript{30,32} This compound had very high levels of potency against both the \textit{T. brucei} NMT (IC\textsubscript{50} = 0.002 μM) and bloodstream form \textit{T. brucei} parasites (EC\textsubscript{50} = 0.002 μM).

The lead compound 13 showed good oral pharmacokinetics in mice, commensurate with obtaining sufficient oral levels for treatment of HAT. Efficacy studies with mouse models of stage 1 infection with \textit{T. brucei brucei} and \textit{T. brucei rhodesiense} showed cure when treated orally with 12.5 and 50 mg/kg bid for four days, respectively.\textsuperscript{30,32}
To give further evidence that NMT is a good drug target in *T. brucei*, we carried out experiments to confirm that these compounds were inhibiting NMT in the cell, and it was through inhibition of NMT that the parasites were dying. These experiments have been reported in detail, but the key points are summarized herein: (1) There was an excellent correlation between inhibition of *T. brucei* NMT and growth inhibition of *T. brucei* across a range of analogues of the hit with a range of potencies. (2) We were able to obtain a cocrystal structure of 13 with the homologous *Leishmania major* NMT, showing the compound binding in the peptide binding site of enzyme, with the basic nitrogen of the piperazine binding interacting with the C-terminal carboxylic acid of NMT. (3) Compound 13 caused rapid cell death in the parasite. (4) When NMT was overexpressed in the parasite, this gave an increase in the EC₅₀ value for inhibition by NMT. (5) Compound 13 prevented incorporation of radiolabeled myristic acid into proteins of *T. brucei*.

So why is NMT a good drug target in *T. brucei*? In terms of biology, inhibition of the enzyme causes rapid death of the parasite. The reasons for this are not fully understood, but it must block some vital pathway(s) for the parasite. Bioinformatic analysis predicts there to be at least 60 peptides/proteins which are myristoylated by NMT in *T. brucei*. Therefore inhibition of the enzyme is likely to have pleiotropic effects, inhibiting multiple pathways. In terms of chemistry, the enzyme active site is about the correct size and balance of hydrophilicity/hydrophobicity to obtain potent small molecule drug-like inhibitors.

**Other Successful Target-Based Approaches.** Successful target-based approaches have been reported for neglected diseases by a number of other groups, where targets have been validated and progressed through into clinical trials. For example, inhibitors of sterol 14-α-demethylase have been shown to be active against Chagas disease; posaconazole and E1224 (a prodrug of ravuconazole) have been advanced into clinical trials. Other target-based approaches are also being investigated against malaria. In some cases, potential targets are highlighted through the identification of the molecular targets of phenotypic hits; for example, the mitochondrial bc1 complex has been highlighted as the target of atovaquone.

One antimalarial target currently being pursued is dihydroorotate dehydrogenase, an enzyme involved in pyrimidine biosynthesis. Elegant work by Phillips and co-workers has shown the power of structure-based design approaches, using X-ray crystallographic information to increase the potency of hits to give very potent compounds.

**Care in Selection of Target-Based Discoveries.** However, care has to be taken with target-based drug discovery, particularly in the selection of molecular targets. There is likely to be a high attrition rate in drug discovery programmes. Target selection is very difficult, given our incomplete understanding of the complex biology underlying these pathogens and the lack of both genetic and chemical validation. This will be illustrated with some examples. In many cases, given our lack of knowledge, the large effort to develop chemical tools is required to validate or otherwise potential molecular targets.

**Pteridine Reductase 1 (PTR1).** Pteridine reductase (PTR1) has been highlighted as a potential drug target in *T. brucei*. Recently, Fairlamb’s group has genetically validated this as a drug target in *T. brucei* by RNAi knockdown. It proved impossible to carry out gene knockout studies; whenever this was attempted, the gene for PTR1 was either retained at the same site in the genome or found elsewhere. However, RNAi studies led to complete knockdown of protein and cell death within four days. The RNAi cell lines had greatly reduced virulence. Most known inhibitors of PTR1 are also inhibitors of dihydrofolate reductase (DHFR). However, we managed to develop some inhibitors which showed no inhibition of *T. brucei* dihydrofolate reductase (TbDHFR). We then attempted to develop new inhibitors which showed no inhibition of *T. brucei* dihydrofolate reductase (TbDHFR) but were potent inhibitors of TbPTR1 (IC₅₀ for TbPTR1 = 7 nM; IC₅₀ for TbDHFR = > 30 μM). These were developed by initially carrying out an in silico fragment screen. One hit based on a benzimidazole scaffold was identified. We were able to obtain cocrystal structures of three examples of this scaffold (compounds 14–16, Figure 4), which all bound in slightly different locations within the active site. We then optimized one of these benzimidazole scaffolds, using a structure-guided approach, to give the potent inhibitor 17 (Figure 4). These compounds occupy a subpocket of the substrate (biotin) binding pocket. However, although we had some very potent inhibitors of the enzyme, there was no correlation between inhibition of TbPTR1 and growth inhibition of the parasite *T. brucei*.

There could be a number of reasons why there is a lack of correlation between inhibition of the enzyme and inhibition of parasite growth. This includes lack of cellular penetration, efflux of compound, sequestration of the compound into lipid compartments (it is quite lipophilic), high nonspecific protein binding, alterations in protonation state of the molecule in the enzyme active site, or bypass through pterins/nutrients in the media.

However, we have made an additional hypothesis based on kinetic properties of the enzyme. The *T. brucei* enzyme has a very low *Kₐ* for the substrate (25 nM). Given the physiological levels of the substrate for TbPTR1 (around 480 nM) and the
need to have substantial inhibition of the enzyme (estimated to be >90% inhibition), this indicates PTR1 inhibitors will require very high levels of inhibition ($K_i$ less than 1 nM) in order to see an appreciable effect against the intact parasite.\textsuperscript{38,39} To achieve this level of enzyme potency, will probably require a significantly larger molecule than the current lead in order to develop more interactions with the enzyme. This increase in size of compound may well be incompatible with the required blood–brain barrier penetration.

Further work is required to understand the lack of cell activity of the above aminobenzimidazoles, although interestingly the author has been unable to find a report in the literature of good correlation between inhibition of $T. brucei$ PTR1 and growth of parasites. More potent PTR1 inhibitors may well have an effect on the intact parasites. Additionally, some classes of compounds are known to be accumulated in $T. brucei$ (for example using the P2 transporter\textsuperscript{4}), and if this occurred with PTR1 inhibitors, it would facilitate cell efficacy. Nonetheless, enzyme kinetics are an important characteristic that needs to be taken into account when selecting molecular targets.

**Trypanothione Synthase (TryS).** In the kinetoplastids, the thiol trypanothione is required for prevention of oxidative stress. The enzyme trypanothione synthase is required for biosynthesis of this unique thiol. Work by the Fairlamb group has shown that knockout of TryS in $T. brucei$ using a tetracycline conditional double knockout of the enzyme gives parasite death after about eight days. Mice treated with these parasites and fed with tetracycline did not develop disease.\textsuperscript{41} This is very strong genetic evidence for the validation of TryS as a drug target.

We developed potent inhibitors of this enzyme at the DDU (eg Figure 5, 18, $IC_{50} = 0.045$ μM).\textsuperscript{42} Mode of action studies indicate that using these compounds led to a loss of thiols (trypanothione and glutathionylspermidine) within $T. brucei$, as would be expected from the role of the TryS in biosynthesis of trypanothione.\textsuperscript{41} Furthermore, it was possible to show that chemical inhibition of TryS gave a reduction in the intracellular levels of the reaction product trypanothione and an increase in the levels of the enzyme substrate, glutathione.\textsuperscript{43} The TryS single knockout $T. brucei$ was more sensitive to inhibition with compound 18 than wild-type ($SKO EC_{50} = 1.2$ μM; WT $EC_{50} = 7.0$ μM). Similarly, $T. brucei$ lines which overexpressed TryS were less sensitive to inhibition by compound 18 ($EC_{50} = 23$ μM).\textsuperscript{41} All this is very strong evidence that our TryS inhibitors are acting on target and that chemical inhibition of TryS gives growth inhibition of $T. brucei$.

However, despite the strong genetic and chemical validation of TryS, there was a very large drop in potency on going from enzyme to cell. What is the explanation for this? Interestingly, when the conditional double knockout parasites were treated with tetracycline, the knockout of the enzyme has no effect on growth rate of the parasite for the first three days and the growth rate only decreased after that. Analysis of the levels of TryS indicated that levels of enzyme were still detectable up to six days after removal of the tetracycline in the conditional double knockouts.\textsuperscript{41} Therefore, $T. brucei$ can survive with very low levels of TryS (or trypanothione). This is probably the explanation for the relatively poor cellular effect of the TryS inhibitors; compound pressure would have to be applied for considerably longer in our standard cellular assay to cause sufficient depletion of TryS and trypanothione levels to have a very significant trypanocidal effect. The longer time course would probably mean that compounds targeted against TryS would require a longer time to show a therapeutic benefit to the patients and require longer dosing. Hence, although TryS is a genetically and chemically validated target within $T. brucei$, compounds targeted against it may not fulfill the target product profile on their own. There is a possibility that TryS inhibitors could be used in combination with other compounds as therapeutic agents.\textsuperscript{41}

**CRK3 (Cdc2-Related Kinase 3).** Protein kinases represent potential drug targets in kinetoplastid diseases.\textsuperscript{44} The cyclin-dependent cdc2-related kinase, CRK3, has been proposed as a potential drug target in kinetoplastids. CRK3 forms a complex with cyclin 6 (CYC6), which is thought to have a similar role to the human cyclin dependent 1—cyclin B complex which is involved in the control of the cell cycle. There is genetic evidence reported with *Leishmania mexicana* which suggested that CRK3 is essential for cell cycle progression and involved in the G2/M transition.\textsuperscript{45,46} To further validate the target, we carried out a screen against the *Leishmania* CRK3—CYC6 complex using our focused kinase set.\textsuperscript{31} This led to identification of eight chemical series. Three of these series were optimized to give high potency and high selectivity compared to mammalian cyclin dependent kinase 2—cyclin A

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Figure 5. Inhibitor of TbTryS Developed in the DDU.

Figure 6. Examples of compound series active against CRK3.
interaction (CDK2–CYCA) (Figure 6). Examples of compounds from these series however were not active against *Leishmania major* promastigotes. Given the high degree of similarity in the sequences of CRK3 between the *Leishmania* and *Trypanosoma* enzymes, selected compounds were also evaluated against *T. brucei* brucei but likewise were not active.

There could be a number of reasons for the lack of cellular activity. First, the enzyme may not be essential for the parasite; there may be bypass mechanisms. Second, by analogy to our experience with PTR1, we may not have a sufficient level of inhibition; this is a potential problem with protein kinases where millimolar levels of ATP are present in the cell which can out-compete ATP-competitive inhibitors. Third, the physiological cyclin partner for CRK3 may be different from CYC6, which may not be inhibited by our compounds. Other possibilities might be lack of cell penetration or efflux mechanisms for compounds. However, this lack of correlation between inhibition of enzyme and parasite extended over a number of different chemotypes, all with reasonable physicochemical properties, suggesting these latter causes are not likely to be the reason.

**PHENOTYPIC SCREENING**

Whole cell parasite screening has become feasible in high-throughput screening mode, allowing screens of large libraries. Phenotypic screening has the advantage of identifying compounds which are active against the whole cell, meaning issues such as cell uptake and cell efflux have already been addressed. It is also straightforward to carry out counter-screens against mammalian cells to obtain an idea of selectivity, allowing rapid filtering of compounds showing general cytotoxicity. There has been a major emphasis on phenotypic approaches to drug discovery for neglected diseases and a number of notable successes reported in the literature, particularly in the areas of malaria and HAT. In the area of HAT, two compounds have recently been progressed into clinical trials by DNDi (www.dndi.org): the nitroimidazole, fexinidazole, and the oxaborole, SCYX-7158. Both of these were discovered through phenotypic screening. In the case of malaria, MMV (Medicines for Malaria Venture, www.mmv.org) have a number of novel compounds in preclinical and clinical development, most of which were discovered through phenotypic screening. Efforts are being made to understand the mode of action of these compounds and to identify their molecular targets. However, this is a retrospective study.

**EXPERIENCE IN THE PHARMAEUTICAL SECTOR**

Recently, a very interesting review of the experience in the pharmaceutical sector has been published by Swinney and Anthony.47 Essentially, in this review, they summarized all the new molecular entities (NMEs) that were registered between 1999 and 2008; there were 176 small molecule NMEs registered during this period. The majority of these NMEs were discovered through target-based approaches, where the isolated protein was screened. However, a significant proportion were also discovered through phenotypic approaches (Figure 7). What is particularly interesting is to look at this information for *First in Class* molecules. For these molecules, a bigger proportion were discovered through cellular (phenotypic screening) compared to screening of isolated proteins. Identification and validation of the target through phenotypic approaches may then lead to subsequent target-based (protein) approaches against these targets. Thus in the pharmaceutical industry, drug discovery is conducted using both target-based (protein) screens and phenotypic (cellular) screens.

**CONCLUSION**

At this time, a balanced portfolio of carefully selected target-based approaches together with phenotypic approaches is probably the best strategy for drug discovery for the neglected tropical diseases. However, care has to be taken in selection of molecular targets for target-based drug discovery approaches. This can be problematic in the case of neglected tropical diseases, where there are very few fully validated molecular targets.

One approach being taken to select molecular targets is to identify the molecular targets of phenotypic hits. This can be a very effective approach; however, it is not without its difficulties and pitfalls. Despite recent advances such as next generation sequencing and chemical proteomics, discovering the molecular targets of phenotypic hits can still be very challenging and in some cases these molecular targets may not be proteins. Furthermore, many phenotypically active compounds may well work by inhibition of several different proteins/enzymes within a cell.48

**AUTHOR INFORMATION**

**Corresponding Author**

*Phone: +44 1382 386 240. E-mail: i.h.gilbert@dundee.ac.uk.*

**Notes**

The authors declare no competing financial interest.

**Biography**

Ian H. Gilbert did his Ph.D. in synthetic organic chemistry at the University of Cambridge. After lecturing chemistry at the University of Zambia and postdoctoral research, he set up a medicinal chemistry research group at the Welsh School of Pharmacy, Cardiff University, with extensive interests in neglected tropical diseases. In 2005, he moved to a chair in medicinal chemistry at the College of Life Sciences in the University of Dundee. He is head of chemistry in the Drug Discovery Unit.

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### Abbreviations Used

CRK3, Cdc2-related kinase 3; DHFR, dihydrofolate reductase; CYC6, cyclin 6; CDK2–CycA, cyclin dependent kinase 2–cyclin A interaction; DDU, Drug Discovery Unit at the University of Dundee; DNDi, Drugs for Neglected Diseases Initiative; HAT, human African trypanosomiasis; MMV, Medicines for Malaria Venture; NME, new molecular entities; NMT, N-myristoyltransferase; PTR1, pteridine reductase 1; TbNMT, Trypanosoma brucei dihydrofolate reductase; TbNMT, Trypanosoma brucei N-myristoyltransferase; TBPTR1, Trypanosoma brucei pteridine reductase 1; TryS, trypanothione synthetase.

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