Examensarbete

Investigation of small molecules binding to UDP-galactose 4’-epimerase

- A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

Anders Jinnelöv
Examensarbetet utfört vid University of Dundee
11/08-06/09

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1 Abstract

African sleeping sickness is a parasitic infection spread by the protozoan parasite *Trypanosoma brucei*, and drugs used today are toxic and painful. Galactose metabolism is essential for the survival of *T. brucei* and without a functional UDP galactose 4’ epimerase (GalE) galactose starvation occurs and cell death will follow. In this Master thesis project two assays observing binding of small molecules to *Tb*GalE has been investigated in attempt to establish an assay that in the future could be used for screening for drugs.

*Tb*GalE was biotinylated through the Pinpoint Xa vector and expressed in *E. coli* cells. The protein was successfully immobilized to a Streptavidin chip for Surface Plasmon Resonance experiments and the binding of the substrates UDP-galactose and UDP-glucose was observed. Unfortunately, the assay was not optimal for screening due to low signal response. However, the established protocol for expressing biotinylated proteins that bind to Streptavidin surfaces could be used in further experiments with *Tb*GalE and other drug targets for African sleeping sickness.

The fluorescent sugar nucleotide analogue UDPAmNS, which is a known inhibitor for *E. coli* GalE, was synthesised and purified and then used to establish a displacement assay. IC\textsubscript{50} of UDPAmNS against *Tb*GalE was determined and a synergic effect in fluorescence between the protein and the inhibitor was proven. Further, evidence for a reduction in fluorescence by displacing UDPAmNS with UDP was obtained. This reduction in fluorescence was also shown by a predicted cofactor inhibitor. The IC\textsubscript{50} against *Tb*GalE for this compound was determined before the displacement assay, which showed that the cofactor inhibitor, at least partly, binds to the active site of *Tb*GalE. The UDPAmNS displacement assay could have the potential of becoming a robust screening assay for *Tb*GalE, in the effort to find a better drug for African sleeping sickness.
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3 Abbreviations

APS  Ammoniumpersulphate
C4  Fourth carbon
Da  Dalton
dNTP  Deoxynucleotide triphosphate
DTT  Dithiolthreitol
EDC  1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA  Ethylene diamine tetra acetic acid
GalE  UDP-galactose 4’-epimerase
GPI  Glycosylphosphatidylinositol
H-NMR  Proton Nuclear Magnetic Resonance
HPAEC  High Performance Anion Exchange Chromatography
IPTG  Isopropyl β-D-1-thiogalactopyranoside
Kb  kilo base pair
kDa  kilo Daltons
LB  Luria-Bertani medium
MS  Mass spectrometry
NHS  N-hydroxysuccinimide
PCR  Polymerase Chain Reaction
P-NMR  Phosphorus (P-31) Nuclear Magnetic Resonance
RU  Response Units
SDS-PAGE  Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis
TAE  Tris-acetate and EDTA
TbGalE  Trypanosoma brucei GalE
TEMED  Tetramethylethylenediamine
TRIS  Tris (Hydroxymethyl) Aminomethane
UCSF  University of California, San Francisco
UDP  Uridine diphosphate
UDPAmNS  Uridine-5’-diphosphoro-1-(5-sulfonic acid) naphthylamidate
UDP-Glc  Uridine diphosphate glucose
UDP-Gal  Uridine diphosphate galactose
VSG  Variant Surface Glycoprotein
WHO  World Health Organisation
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5 Introduction

5.1 Background

African sleeping sickness and cattle disease Nagana is caused by the protozoan parasite *Trypanosoma brucei*. This parasite is transmitted by the tsetse fly in the sub-Saharan part of Africa (fig. 1). West African trypanosomiasis, which represents 90% of the reported cases (1), leads to a chronic infection and is caused by the parasite *Trypanosoma brucei gambiense*. East African trypanosomiasis, which causes acute illness lasting several weeks, is caused by *Trypanosoma brucei rhodesiense*. Symptoms of the east African trypanosomiasis occur after one to four weeks and are often nonspecific initially. Usually fever, skin lesions, rash, oedema, or swollen lymph nodes on the back of the neck are the first symptom to show. These symptoms are usually referred to as the first stage. The infection then generally progresses to meningoencephalitis and then gives rise to other symptoms like personality change, weight loss, loss of concentration, progressive confusion, slurred speech, seizures, insomnia at night and sleeping long periods of the day. These more severe symptoms indicate that the infection has reached the second stage. The West African form of the disease can be symptomless for months and years before severe headaches, sustained fever, sleep

![Figure 1](image.png)

*Figure 1*. Depicting the countries effected by the Sleeping sickness disease epidemic, and the separation between *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. (1)
disturbances, alteration of mental state, and neurological disorders are noticed. By then the patient is often already in an advanced stage (second stage) where the central nervous system is affected. Untreated, death will occur for both forms within several weeks to months after the second stage is reached (1). In provinces of Angola, Congo and Southern Sudan African sleeping sickness is now the biggest killer, even surpassing HIV/AIDS (2).

Transmission of African sleeping sickness almost halted in 1960s (fig. 2) due to disease control programs that were set up by colonial administrations to deal with the negative impact of the disease on their territories. When the earlier troubled countries became independent, the disease was no longer a priority, which lead to a slow return of the African sleeping sickness. (1)

The situation today is improving and between 1995 and 2006 the total number of new cases

Figure 2. Graph showing all new cases of Sleeping sickness reported in Africa between 1927 and 1997 (1).
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reported was reduced with 68%, mainly due to reforming better control programs. Yet the WHO considered as late as 2006 that there are 36 countries endemic for African sleeping sickness giving rise to about 70,000 deaths every year, (2) and the four drugs used for treatment are far from good. Suramin is used for first stage rhodesiense, pentamidine for first stage gambiense, and eflorenithine for the second stage gambiense. Melarsoprol is used for the second stage of both of the forms disease, but kills 3-6% patients upon administration. Efolognithine was developed over 20 years ago, and even if it is less toxic than melarsoprol it has usual side effects such as fever, bleeding, diarrhoea, nausea, stomach pain and vomiting. In some cases even convulsions, loss of hearing, hair loss, headache, anaemia and thrombocytopenia have been observed (1). In addition to that Efolognithine requires 56 intravenous injections every 6th hour of two weeks.

Generally an infection leads to the triggering of a series of events, at first the innate immunity defence, later the specific immune response. Trypanosomes however have evolved to cope with host immune systems. The major reason for this is due to the fact that trypanosome parasites are nearly fully covered with a protective layer, the Variant Surface Glycoprotein (VSG). VSG forms a barrier between the host immune system and the membrane of the parasite and thereby inhibits macromolecules of the immune response, such as the components of the alternative complement pathway (3). However, small molecules such as nutrients are still able to diffuse to the underlying plasma membrane’s transporter system. This is not enough though to escape the specific immune response. Instead it is the effect of antigenic variation that protects the trypanosomes. The parasite genome contains about one thousand VSG genes, expressed one at a time by an individual cell, which encodes immunologically distinct VSGs (4). The sequential expression, and thus the antigenic variation, is the key factor to how the Trypanosoma brucei escapes the host specific immune response. In addition to protection the VSG also has several effects on the host immune system.

It has been noticed (5) that VSG promotes an induction of autoantibodies and cytokines, particularly Tumor Necrosis Factor-alpha, which if over expressed is involved in a variety of human diseases (6). An increase of parasite growth factors, Interferon-gamma and L-ornithine, is also a contribution of VSG in the bloodstream.
5.2 Biology of *Trypanosoma brucei*

*Trypanosoma brucei* is an extracellular parasite in both mammals and the tsetse fly. In mammals it lives in the bloodstream and lymph vessels, whereas in the tsetse fly it lives in the midgut and salivary glands. The bloodstream variant measures 25-40 µm in length (7). The parasite cell (fig. 3) have the same typical organells as an eukaryotic cell, for example, nucleus, mitochondria, endoplasmatic reticulum and Golgi apparatus. In addition to these it also contains kinetoplast, glycosome and a flagellum attached in a flagellar-pocket. The kinetoplast contains the mitochondrial DNA, which can be up to 25% of all cellular DNA, and is located adjacent to the flagellar pocket (7). The glycosomes are the organelles that, in *T. brucei* and other related parasites, compartmentalize the glycogenesis and the glycolysis. Therefore they contain all enzymes essential for these reactions. Yet the spectrum of enzymes within the glycosomes is changing during parasite development. Glycolytic enzymes are reduced in abundance in the insect midgut (procyclic) stage, in contrast to the mammalian bloodstream stage, where the parasites solely rely on glycolysis for the generation of energy. Evolutionarily the glycosomes are most related to the peroxisomes (9). The flagellar pocket is rich in glycoproteins and is the exclusive place for endocytosis and exocytosis in *Trypanosoma* (10).

![Figure 3](image-url)
5.3 Variant Surface Glycoprotein

In the mid-1970s George Cross established that the trypanosomes’ protective surface coat consisted of about $10^7$ VSG per cell (fig. 4) (11). VSG is a dense monolayer of homodimers where each monomer has a molecular weight of 55kDa, and typically contains one ore two N-linked oligosaccharides (12). Because of the formation of many similar dimers of elongated shape, and the denseness of this layer, B-cell-stimulating epitopes are limited and only found on the surface of the layer. The VSG molecules are attached to the plasma membrane through the Glycosylphosphatidylinositil anchor (GPI).

![Figure 4](image.png)

**Figure 4.** Top left shows an image from a scanning electron microscope of a bloodstream *T. brucei*. Top right shows how the parasite is coated with VSG dimers. Bottom right depicts the homodimer bound to two GPI anchors. Bottom left shows the GPI composition (13).
5.4 *Trypanosoma brucei* - lifecycle

The parasite undergoes a complex life cycle (fig. 5) and changes its morphological form from procyclic in the tsetse fly, to a slender shape in the mammalian bloodstream. In the bloodstream, after *T. brucei* has been injected by a tsetse fly bite, the parasite reproduces through binary fission. At high cell density the parasite differentiates into a more stumpy form. The stumpy parasite is non-proliferative unlike the two other forms of the parasite (except the metacyclic form in the salivary glands) and pre-adapted for transmission to tsetse flies. The parasite begins to express the immune-protective VSGs in the salivary glands of the tsetse fly, and stays protective until being ingested by a new tsetse fly’s blood meal (2).

![Figure 5](image-url) Figure 5. The lifecycle of *Trypanosoma brucei*, showing the different forms of the parasite, in host and in the vector tsetse fly (2).

5.5 Sugar nucleotides in *Trypanosoma brucei*

In *Trypanosoma brucei* and other trypanosomatid parasites the cell surface glycoconjugates, such as the VSG and GPI, are tightly connected to survival, infectivity, and virulence in both tsetse flies and mammalian hosts (14). Like in all cells, from bacteria to mammals, monosaccharides are being transformed to activated sugar-nucleotides, which are able to donate sugars to different acceptors in order to produce glycoconjugates in *T. brucei*.
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The long-time ongoing investigation of biosynthesis metabolism in T. brucei is trying to establish the sugar-nucleotide pool in the parasite. So far there are five known sugar nucleotides in Trypanosoma brucei: (14)

- **GDP-α-D-mannose (GDP-Man)**
- **UDP-α-D-N-acetylglucosamine (UDP-GlcNac)**
- **UDP-α-D-glucose (UDP-Glc)**
- **UDP-α-galactopyranose (UDP-Galp)**
- **GDP-β-L-fucose (GDP-Fuc)**

### 5.6 UDP- galactose 4’-epimerase

The enzyme responsible for the conversion between UDP-Gal and UDP-Glc (fig. 6) is UDP-galactose 4’-epimerase (GalE). Mammals have two ways to obtain galactose, either by the hexose transporter, or by the reaction catalyzed by GalE. T. Brucel’s hexose transporter does not recognize galactose and the parasite can therefore only obtain this sugar molecule through the work of TbGalE (15). It has been shown (15) that GalE is essential for T. brucei bloodstream form. Without a functional GalE a loss of galactose in glycoproteins as well as changes in morphology and metabolism are seen. In fact, after 96 hours of galactose starvation, from constructing a TbGalE conditional null mutant, cell division is halted and cell death occurs.

The TbGalE monomer contains 381 amino acids and consists of two domains. The larger N-terminal contains the cofactor-binding pocket and is formed by the 203 first amino acids and a stretch between 271 and 306. The C-terminal is smaller, residues 203 to 270 and 307 to 381, and contains the substrate-binding pocket. The catalytically active site is situated in the cleft between the two domains, shown in Figure 7 where the TbGalE dimer is depicted (16).
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**Figure 7.** *Tb*GalE dimer with the two monomers and their two domains highlighted in different colours. Substrate and cofactor are best seen in the green monomer, where NAD⁺ is binding to the N-terminal domain (the lower) in the lighter green region. The substrates bind to the C-terminal domain, the smaller region on top. In the figure UDP is binding to the active site.

The only difference between UDP-Gal and UDP-Glc is the change in position for the hydroxyl group at the fourth carbon. The OH-group is shifted from equatorial position in UDP-Glc to axial position in UDP-Gal through the work of *Tb*GalE and cofactor NAD⁺. Shaw *et al.* (16) described a mechanism in 2003 where they predicted that the substrate UDP-Glc binds to the *Tb*GalE-NAD⁺ complex. The nicotinamide abstracts a hydride from glucose’s fourth carbon simultaneously as Tyr173 (in *Tb*GalE) accepts a proton from the hydroxyl group, from the same carbon. This produces a 4-keto intermediate, and inversion can occur after a rotation of this intermediate. (17) The extra space at the sugar-binding pocket allows rotation and the substrate is retained in the catalytic center by the tight binding of the UDP moiety. (16) The rotation enables the NADH to transfer the hydride back to C4 at an axial position and Tyr173 reprotonate the hydroxyl group to produce UDP-Gal.

The Ferguson lab, University of Dundee, has been working with *Tb*GalE for several years, and since Roper *et al.* proved that the enzyme was a genetically validated drug target in 2002, several screening assays have been tested. So far though, the assays that have been approved
by the Drug Discovery Unit, which is the department performing all drug screening, has not been sufficient enough. The primary assay was not entirely reliable, and the secondary assay was to slow. Other activity assays have been tried, but in different ways failed.

5.7 Aim

My project focus has been to establish an assay that proves binding of small molecules to \( Tb\)GalE instead of activity assays that has been tried before. The first priority has been to investigate if small molecule binding could be analyzed with Surface Plasmon Resonance (SPR) by binding \( Tb\)GalE to a Streptavidin chip. Further, my project also included exploration of a fluorescent displacement assay, trying to establish and optimize the conditions in order to generate a usable assay.
6 Experimental

The wild-type TbGalE gene had prior to this project been cloned into a Pinpoint Xa vector and to initiate the expression the vector was transformed to an *E. coli* expression strain.

6.1 Transformation

Pinpoint Xa vector (Promega) with TbGalE-Wt gene incorporated was transformed into E. Coli BL21(DE3)pLysS expression cells (Novagen). Cells were taken from -80°C freezer and put on ice for 5 minutes prior to addition of 2.5µl of the plasmids and gentle mixing. The mixture was incubated on ice for 5 minutes, followed by a 42°C water bath for 30 seconds. Afterwards the solution was put on ice followed by sterile addition of SOC (Super Optimal broth with Catabolite repression). After being incubated (Infors HT multitron), shaking at 220 rpm at 37°C, the cells were spread on an LB agar plate containing the selectable marker Ampicillin. Plates were incubated at 37°C over night.

6.2 Expression and purification of biotinylated TbGalE

6.2.1 Biotinylated TbGalE expression

After picking colonies from the agar plates cells were grown in 5ml LB-Amp media at 37°C in 220 rpm shaking incubator for 4 hours. 3ml was inoculated to 1L LB-Amp and once again shaken at 37°C until OD_{600} was about 0.5 when expression of TbGalE gene was induced by addition of 100µM IPTG and the cells grown shaking (220 rpm) at 22°C over night. The cell culture was then centrifuged (Beckman J6-MC) at 4000 x g for 20 minutes at 4°C to harvest cells. After discarding the supernatant the pellet was resuspended in 10ml lysis buffer (50mM TRIS, 200mM NaCl, pH 8) and put on ice. 100mg lysozyme was added and after 20 minutes on ice EDTA-free protease inhibitors (Complete EDTA-free protease inhibitor cocktail tablets (Roche)) were added to reduce protein degradation. A French press (American instrument company) was used to lyse cells and thereafter the solution was centrifuged (Beckman J2-21) at 30000 x g for 30 minutes at 4°C. The supernatant was then incubated with activated Softlink™ soft release avidin resin beads (Promega) for 2 hours slowly rotating at 4°C.
In the case of the crosslinker reaction, *Tb*GalE was purified in phosphate buffer (50mM phosphate, 200mM NaCl, pH 7.6).

### 6.2.2 Softlink™ soft release avidin resin capturing - protein purification

Prior to capturing of biotynlated *Tb*GalE the soft release avidin resin beads were activated and regenerated in a column of appropriate volume at room temperature. 2ml of the beads were first equilibrated with 0.1M NaPO₄ (pH 7), then non reversible binding sites were pre-absorbed with 5mM biotin in the phosphate equilibration buffer, to a total volume of 5 ml. The flow was stopped to allow binding of biotin. The beads were then regenerated by washing initially with 8 column volumes of 10% acetic acid, then with 8 column volumes of 100mM NaPO₄ (pH 7.0). pH of eluate was monitored until it reached 6.8 and then the flow was stopped for 30 minutes to allow the avidin to refold. The beads were then equilibrated in lysis buffer which was also used as washing buffer (50mM TRIS, 200mM NaCl, pH 8) and stored in 4°C fridge.

The biotynlated *Tb*GalE bound to the beads was captured by first pouring the incubated solution containing the bound protein in a column. The bigger beads stayed in the column while the solution ran through. The flow through was collected. The beads with the bound proteins were then washed with 50ml wash buffer. Also the wash solution was collected. The proteins were eluated by the addition of 5mM biotin to the wash buffer. Three eluations of 1.5ml were collected. In the second eluation the flow was stopped for 30 minutes to promote a higher protein concentration in the eluent.

### 6.3 Characterization of biotynlated *Tb*GalE

#### 6.3.1 SDS-PAGE gels

10% SDS-PAGE gels were cast (Table I) and run using Mini Protean II™ (BioRad) system and PowerPack300 (BioRad) in 1x running buffer. Gels were run at 200 volts until dye front had run off.
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Table I. Components of the 10% SDS-PAGE gels. Solutions highlighted in red were added last.

<table>
<thead>
<tr>
<th>Components</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>4.1ml</td>
<td>4.1ml</td>
</tr>
<tr>
<td>1M TRIS-HCl pH 6.8</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>1.5M TRIS-HCl pH 8.8</td>
<td>2.5ml</td>
<td>-</td>
</tr>
<tr>
<td>Acrylamide/Bis 30%</td>
<td>3.3ml</td>
<td>3.3ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1ml</td>
<td>0.1ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>50µl</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

6.3.2 Coomassie blue staining
One gel was stained with coomassie blue for 45 min and then destained (40% EtOH, 40% H₂O and 10% AcOH) until gel was transparent.

6.3.3 Western blot
The second gel was blotted to a Hybond™-ECL™ Nitrocellulose membrane, which is optimized for use with ECL Plus Western Blotting System (GE Healthcare). After blocking with BSA-blocking buffer over night ExtrAvidin peroxidase antibodies (that bind the biotin tags, diluted 1:100000) (Sigma) were added, 2µl in 10ml blocking buffer, for one hour. The membrane was then washed again for 45 minutes in blocking buffer, followed by applying an ECL mix, containing 1ml solution A, 25µl solution B (both included in the ECL kit (GE Healthcare)) and 1ml phosphate-buffered saline, on the membrane. Immediately after, the membrane was put in darkness for 2 minutes, and then taken to a dark room for generating films (Amersham Hyperfilm ECL generated in a Compact X4, X-ograph imaging systems).

6.3.4 Tryptic mass fingerprinting
Purified protein was submitted to the Proteomics Facility, University of Dundee, for tryptic mass fingerprinting. The protein was digested by trypsin and the peptide masses were measured by nano-Liquid Chromatograph MS/SM, using a C18 reverse-phase coupled to a 4000 QTRAP (Applied Biosystems) with an electrospray ion source. Data was processed with MASCOT software.
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6.4 Activity assay

6.4.1 High performance anion exchange chromatography (HPAEC)
Activity measurements were performed on HPAEC (Dionex) under basic conditions. The column used was a CarboPac PA1 (Dionex) 2x 250mm. 1000ml H₂O (A) and 1M NaAcetat (C), both with 1mM NaOH, were used as running buffers. Gradient used for separation: 5 minutes 20% C and 80% A. 15 minutes 60% C and 40% A. 10 minutes 100% C and a finishing 7.5 minutes step with 20% C and 80% A. Detection was achieved by an UV-detector at 260nm. 100µM substrate, UDP-Gal or UDP-Glc, were mixed with 100µM cofactor NAD⁺ to a total volume of 100µl. An appropriate concentration (about 150nM) of biotinylated *Tb*GalE was added, the mixture was vortexed and then incubated 30 minutes in water bath at 37°C. The reaction was then stopped with the addition of 10µl 10mM NaOH, and the samples were thereafter analysed on the HPAEC. As standards, UDP-Gal and UDP-Glc (50µM) was added.

6.5 Surface Plasmon Resonance (SPR)

6.5.1 SPR setup
Before immobilizing the biotinylated *Tb*GalE to a StreptAvidin chip (Biacore) the excess free biotin from the eluation was removed by dialysis with 0.5ml ImmunoPure Immobilized Streptavidin beads (Pierce) added to the dialysis buffer of one litre. The dialysis buffer was the same as the buffer the protein had been purified in. A Slide-A-Lyzer dialysis cassette (Thermo scientific) with a cut off value of 10kDa was used for dialysis.

All SPR measurements were made on Biacore 3000 at room temperature and at 4°C. When using Biacore CM5 chip the dextran layer was enlarged by washing with HCl, NaOH and SDS, then activated by EDC/NHS. After activation Streptavidin was bound to the chip by adding it in acetic acid (pH 4.5) to all flowcells. Afterwards *Tb*GalE was immobilized by adding protein in 50mM TRIS, 200mM NaCl, 100µM NAD⁺ with 10% glycerol buffer to a chosen flowcell until saturation was achieved. Adding of substrate, UDP-Gal or UDP-Glc, was done in three-fold dilutions, with a highest concentration of 200µM. When using Biacore Streptavidin (SA) chip, once again biotinylated *Tb*GalE was added until saturation was achieved. Concentration gradients of substrates, three serial three-fold dilutions starting with...
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for Trypanosoma brucei, the parasite responsible for African Sleeping Sickness.

200µM and six serial three-fold dilutions starting with 400µM were added. Also three serial three-fold dilutions of five known inhibitors, were added with the highest concentration of 200µM.

6.5.2 Crosslinking
The crosslinking reaction was performed in an amine free phosphate buffer at room temperature for 30 minutes. Crosslinker Bis(sulfosuccinimidyl)suberate (BS³, Thermo scientific) was dissolved to 25mM in H₂O immediately before reaction started. Protein concentration was kept low (0.35 mg/ml) to favour dimer crosslinking. His-tagged (purified in lab prior to this project) and biotinylated TbGalE were mixed 10:1 and sonicated 2x 10 seconds to attempt formatting heterodimers. Reaction (Table II) was performed with 30-50 molar excess of crosslinker with a final crosslinker concentration of 0.25-5mM as instructions said. Reaction was quenched by adding 1M TRIS-HCl pH 7.5 buffer and incubated for 15 minutes, thereafter dialysed to remove non-reacted BS³ reagent.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
<th>Volume</th>
<th>Added BS³ (µl)</th>
<th>Tot. conc BS³</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS³</td>
<td>25mM</td>
<td>140µl</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Biotinylated TbGalE</td>
<td>6.2µM</td>
<td>200µl</td>
<td>2.2</td>
<td>0.27mM</td>
</tr>
<tr>
<td>His-Bt tagged TbGalE</td>
<td>9.8µM</td>
<td>1000µl</td>
<td>16</td>
<td>0.39mM</td>
</tr>
</tbody>
</table>

6.6 Chromotrop 10 B – a NAD⁺ inhibitor?

6.6.1 Chromotrop IC₅₀ against TbGalE
Chromotrop 10 B (2,7-Naphthalenesulfonic acid-4,5-dihydroxy-3-(1-naphthalenylazo) was predicted by the McCommon group (UCSF, USA) to bind and inhibit TbGalE by blocking NAD⁺ from binding and participating in the reaction. In order to determine an IC₅₀ for Chromotrop 10 B, inhibition curves were obtained by using the activity assay on the HPAEC. The total volume of 100µl was incubated at 37°C water bath for 30 minutes, before the reaction was quenched by addition of 10µl 10mM NaOH. Fixed components are shown in Table III and the Chromotrop 10 B concentration gradient is shown in Table IV.
Investigation of small molecules binding to UDP-galactose 4'-epimerase - A validated drug target for Trypanosoma brucei, the parasite responsible for African Sleeping Sickness.

Table III. Condition used for Chromotrop 10 B IC₅₀ binding curves. *Concentration could vary a bit for different protein batches.

<table>
<thead>
<tr>
<th>Fixed components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TbGalE (H₆)</td>
<td>0.16µM*</td>
</tr>
<tr>
<td>UDP-Gal</td>
<td>100µM</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>100µM</td>
</tr>
</tbody>
</table>

Table IV. Concentration of Chromotrop 10 B used to establish IC₅₀.

<table>
<thead>
<tr>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03µM</td>
</tr>
<tr>
<td>0.1µM</td>
</tr>
<tr>
<td>0.3µM</td>
</tr>
<tr>
<td>1.0µM</td>
</tr>
<tr>
<td>3.0µM</td>
</tr>
<tr>
<td>10µM</td>
</tr>
<tr>
<td>30µM</td>
</tr>
<tr>
<td>100µM</td>
</tr>
</tbody>
</table>

6.7 Fluorophore UDPAmNS

6.7.1 UDPAmNS synthesis
UDPAmNS (uridine-5'-diphosphoro-1-(5-sulfonic acid) naphthylamidate) was synthesized according to the procedure of Dhar et al. (18) with some modifications: 223.5mg (1mmol) of AmNS (Flukta) was added to 10ml of H₂O, and dissolved by adjusting the pH to 5.8 with 0.1M NaOH, followed by filtration and sonication. 4ml of 12.5mM UDP and 2ml of 1M EDC (Flukta) (pH 5.8) were added to a reaction vessel maintained at 20°C. The reaction was initiated by adding 10ml of AmNS. The reaction pH was kept between 5.65 and 5.75 by periodic addition of 0.1M HCl until end of reaction after 3.5 hours. The mixture was then neutralized with 0.1M NaOH and made 0.05M in ammonium bicarbonate. The solution was filtered and sonicated and then loaded onto a DEAE-cellulose column (Omnifit) (about 22ml) equilibrated with 0.05M ammonium bicarbonate. After a washing step (0.05M ammonium bicarbonate) and a concentration gradient up to 0.5M ammonium bicarbonate with a flow rate of 1ml/min, the fractions collected were checked for blue fluorescence under UV-lamp and 260/320nm absorption ratios were calculated.

6.7.2 UDPAmNS purity control
To verify that the synthesized UDPAmNS was the correct molecule and that it was pure, samples were submitted to P-NMR, H-NMR and mass spectrometry.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

6.7.3 UDPAmNS concentration calculations
After separation of UDPAmNS from unreacted AmNS the concentration of the reaction product was calculated from a specific absorption at 315 nm, due to the naphthalene ring in UDPAmNS. The absorption coefficient for the naphthalene ring is $\approx 5580 \text{ M}^{-1} \text{ cm}^{-1}$. (19)

$$\text{Conc}_{\text{UDPAmNS}} = \frac{\text{Abs}_{320}}{\text{absorption coefficient}}$$

6.7.4 UDPAmNS IC\textsubscript{50} against TbGalE
UDPAmNS IC\textsubscript{50} inhibition curves were collected on the HPAEC machine. The amount of enzyme (His-tagged *Tb*GalE was used), substrate (UDP-Gal) and cofactor NAD$^+$ was kept the same under all tests (Table V). Different concentrations of UDPAmNS (Table VI) were added to obtain curves from which the IC\textsubscript{50} could be calculated. The total volume that was incubated at 37°C water bath for 30 minutes, before reaction was quenched by addition of 10µl 10mM NaOH, was always 100µl.

Table V. Setup used to calculate IC\textsubscript{50} for UDPAmNS. *Concentration could vary a bit for different protein batches.

<table>
<thead>
<tr>
<th>Fixed components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tb</em>GalE (H$_6$)</td>
<td>0.16µM*</td>
</tr>
<tr>
<td>UDP-Gal</td>
<td>100µM</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>100µM</td>
</tr>
</tbody>
</table>

K$_i$ for UDPAmNS against *E. coli* GalE had earlier been determined to 200µM by Bhattacharyya *et al.* J (1999) (20).
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

**Table VI.** Concentration gradients of UDPAmNS used to obtain IC$_{50}$ of the inhibitor. Left to right is showing first to last setup.

<table>
<thead>
<tr>
<th>UDPAmNS µM</th>
<th>UDPAmNS µM</th>
<th>UDPAmNS µM</th>
<th>UDPAmNS µM</th>
<th>UDPAmNS µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>5</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>44</td>
<td>11</td>
<td>0.3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>87</td>
<td>22</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>174</td>
<td>44</td>
<td>3</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>348</td>
<td>66</td>
<td>10</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>522</td>
<td>87</td>
<td>30</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>696</td>
<td>174</td>
<td>100</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>870</td>
<td>348</td>
<td>300</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

6.7.5 Fluorescence assay with UDPAmNS
Envision 2102 multilabel fluorescence plate reader (PerkinElmer) and 96 well plates (Greiner Bio-one) were used for all experiments. PBS buffer was used to dilute all samples to 100µl. To investigate whether a signal good enough for an assay could be obtained with lower concentrations than values from literature (20), concentration ranges 0.4, 0.8 and 1.6µM, of both TbGalE and UDPAmNS were tested, in the presence of 10µM NAD$^+$. Displacement of UDPAmNS was investigated by the addition of UDP. To a mixture of 1.0, 2.0 or 4.0µM TbGalE and 1.0µM UDPAmNS, 1mM UDP was added. No NAD$^+$ was added in this experiment.

The predicted cofactor inhibitor Chromotrop 10 B was also used for displacement trials. If the prediction was to be correct no displacement should be seen. 1.6µM TbGalE and 0.4µM UDPAmNS were mixed and the fluorophore was attempted to be displaced by two different Chromotrop 10 B concentrations, 100 and 500µM respectively. Once again 10µM NAD$^+$ was present.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for Trypanosoma brucei, the parasite responsible for African Sleeping Sickness.

7 Results

7.1 Expression of biotinylated TbGalE

Since earlier attempts of analysing binding to TbGalE by Surface Plasmon Resonance (SPR) through a His-tag linkage had given poor results, new efforts was made to immobilize the protein through biotin-Streptavidin binding.

7.1.1 Pinpoint Xa vector
To be able to attach TbGalE to a Streptavidin SPR chip for investigation of inhibitor binding the protein needs to be biotinylated. The Pinpoint Xa vector introduces a fusion protein tag to the N-terminus of the protein. The fusion tag is recognised by native E. coli biotin ligase, and during protein expression bacteria biotin is ligated to the fusion tag. The biotinylated fusion tag is 13 kDa and thus considered to be large enough so that it does not affect the native protein conformation upon biotin-Streptavidin binding. Viewing the crystal structure of TbGalE (16) in Pymol shows the protein as a dimer where the two N-terminals are located on the surface, separated from the binding pockets for the substrates and the cofactor.

The protein is captured by Softlink™ soft release avidin resin beads, which are monomeric avidins mutated to give a weaker binding that can be broken under non-denaturing conditions. The E. coli bacteria also produce another biotinylated protein, but this does not bind to the avidin resin beads.

7.1.2 Coomassie blue gel and Western blot
Expression yield was about 3mg/ml, and in range with the suggested yield of 1-5mg/ml. Further optimization of expression was therefore not considered. Two SDS-PAGE gels were made, the first Coomassie blue stained (fig. 8) and the other Western blotted (fig. 9). TbGalE without fusion tag has a molecular weight of 43 kDa, which gives the biotinylated TbGalE a molecular weight of about 57 kDa.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

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Figure 8. The SDS-PAGE gel after Coomassie blue staining showing clear bands in the correct weight region of biotinylated *Tb*GalE (biotinylated *Tb*GalE = 57 kDa). Lane 1; supernatant, Lane 2; pellet, Lane 3; flow through, Lane 4; 1\textsuperscript{st} wash, Lane 5; weight marker, Lane 6; 1\textsuperscript{st} eluation, Lane 7; 2\textsuperscript{nd} eluation, Lane 8; elutions pooled, Lane 9; elutions pooled and desalted, Lane 10; elutions pooled, desalted and spun.

Figure 9. The Western blot visualizing the 5 different eluations, comparable to bands in lane 6-10 in figure 8.

7.1.3 *Tryptic mass fingerprinting*

The results from the tryptic mass fingerprinting confirmed that the purified protein was *Tb*GalE. Sequence coverage when using NCBI database was 29%, and when using the *T. Brucei* database the coverage was as high as 52%. Both sequence coverages were high enough to conclude that the correct protein, *Tb*GalE, had been purified.
7.2 Activity assay

Since the fusion biotin tag was almost a third of the molecular weight of TbGalE, questions were raised whether the activity was affected. To investigate this, activity assays were set up as described in the experimental section. Even though the only difference between UDP-Glc and UDP-Gal is the change in the stereochemistry of the hydroxyl group on the fourth carbon of the hexose moiety, two defined peaks can be separated by using High Performance Anion Exchange Chromatography (HPAEC) (fig. 10).

Figure 10. Graph showing the peaks of the mixture of UDP-Gal and UDP-Glc used as standards.

Figure 11 shows the peaks from the activity control, proving that the biotinylated TbGalE was active.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

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### 7.3 Surface Plasmon Resonance

After the purified biotinylated *Tb*GalE was confirmed active the SPR experiments were prepared. Capture of *Tb*GalE on a Streptavidin (SA Biacore) chip at 4°C in the presence of NAD$^+$ gave the highest response unit result (fig. 12).

**Figure 11.** A) When UDP-Gal is added as substrate without the presence of *Tb*GalE only the peak for UDP-Gal is visible. When *Tb*GalE is present a peak for UDP-Glc is also seen, proving that *Tb*GalE has converted UDP-Gal to UDP-Glc. B) When UDP-Glc is added as substrate without the presence of *Tb*GalE only the peak for UDP-Glc is visible. When *Tb*GalE is present a peak for UDP-Gal is also seen, proving that *Tb*GalE has converted UDP-Glc to UDP-Gal.

**Figure 12.** Graph depicting the capturing of *Tb*GalE to the SA StreptAvidin chip. The chip was saturated at about 5000 RU.

The capture of the protein to the chip surface was a significant step towards the investigation of SPR analysis of molecules binding to *Tb*GalE. Saturation of the chip was achieved after three immobilizations as seen in the figure above. The first immobilization gave about 4000 RU, and the two following attempts increased the signal to 5000 RU. After
attaching *TbGalE* to the chip surface, substrates and known inhibitors from precedent screenings were added to the flowcell to test if binding could be observed. The capturing of 5000 RU of protein should be good enough to detect binding of the small molecules (500-600 Da). The substrates were detected binding the enzyme with $K_D$ (average) of 105µM (calculations a courtesy of Dr. Iva Navratilova, fig. 13 and 14), but gave much weaker RU than expected. The $K_m$ for UDP-Gal and UDP-Glc are known to be about 80µM, so the results are comparable. However the $K_D$ results from the second run were much higher than in the first run. Furthermore, the inhibitors, which were added after the fist two substrate runs, unfortunately gave no binding. The most plausible reason for this would be that the enzyme loses its activity very quickly when bound to the chip. This would explain the higher $K_D$ values in the second run, and also why no inhibitor binding could be seen in the following run.
Investigation of small molecules binding to UDP-galactose 4'-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

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**Figure 13.** Illustrates the results of binding UDP-Gal to *Tb*GalE. The first run, A), gave a $K_D$ of 29µM, whereas the following run, B), gave a much higher value of 180µM. C) shows the results from a reference surface with no added UDP-Gal.
The experiment that gave the binding curves in Figure 13 and 14 was performed at 4°C. The substrates were added just after the protein capturing and after finishing that experiment new concentration gradients were made instantaneously, with even higher concentration of substrates and inhibitors included. Since no binding could be seen in that experiment one could imply that the protein seems very unstable when bound to the chip surface. From the response value we calculated that only 7% of the immobilized protein had bound any substrate. The following day a new immobilization was performed, attempting to bind the higher concentrations of substrate and inhibitors immediately after protein capture. From this attempt much lower response unit values were received. After a second immobilization with equally low capturing, it seemed like the SA Biacore chip had lost most of its activity.

7.3.1 Crosslinking
Because almost all proteins seem unable to bind when attached to the chip the question about dimer breakage was raised. When looking at the 3D-structure of the dimer (fig. 15) it is evident that the two N-terminals and thus the biotin tags, are in opposite positions in space.

Figure 14. Illustrates the results of binding UDP-Glc to TbGalE. The first run, A), gave a $K_D$ of 46µM, whereas the following run, B), gave a much higher value of 165µM. C) shows the results from a reference surface with no added UDP-Gal.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

This “head to tail” dimerization can perhaps be a reason for the low binding of substrates. In order to bypass this possible problem thoughts about crosslinking arose. *Tb*GalE contains 17 lysines in each monomer (fig. 16). Therefore an amine crosslinker was used in order to introduce a covalent bond between the two monomers. Attempts were also made to form a heterodimer, His-tagged *Tb*GalE bound together with biotin-tagged *Tb*GalE (100 kDa), which would be preferable because it only contains one biotin tag.

**Figure 15.** *Tb*GalE dimer with UDP and NAD⁺ bound to both monomers. The N-terminals of both monomers are located on the surface of the protein, here highlighted in red.

**Figure 16.** Picture of *Tb*GalE dimer where the lysines are highlighted in red. An approximate measurement from Cα to Cα of two lysines in separate monomers is highlighted in blue. Substrate and cofactor are highlighted in magentas.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for *Trypanosoma brucei*, the parasite responsible for African Sleeping Sickness.

The crosslinker BS³ (Bis[sulfosuccinimidyl] suberate) (fig. 17) used to covalently attach the two monomers is a homobifunctional, water-soluble, non-cleavable and membrane impermeable crosslinker.

![BS³ crosslinker](image)

Figure 17. BS³ crosslinker.

After the crosslinking reaction the sample was added to an SDS-PAGE gel to confirm that a covalent bond had been introduced (fig. 18). Results from a following Western blot are shown in Figure 19. The possible dimers formed from crosslinking are homodimeric His-tagged (86 kDa), homodimeric biotin-tagged (114 kDa) and heterodimeric *Tb*GalE (100 kDa).

![Western blot results](image)

Figure 18. 1; biotinylated *Tb*GalE, 2; biotinylated *Tb*GalE after Streptavidin dialysis, 3; His-tagged *Tb*GalE, 4; mixture of biotin- and His-tagged *Tb*GalE, 5; empty, 6; biotinylated *Tb*GalE after crosslinking, 7; biotinylated *Tb*GalE after crosslinking and dialysis, 8; mixture of biotin- and His-tagged *Tb*GalE after crosslinking and dialysis.

![Western blot results](image)

Figure 19. 1; biotinylated *Tb*GalE, 2; biotinylated *Tb*GalE after Streptavidin dialysis, 3; His-tagged *Tb*GalE, 4; mixture of biotin- and His-tagged *Tb*GalE, 5; mixture of biotin- and His-tagged *Tb*GalE after crosslinking, 6; biotinylated *Tb*GalE after crosslinking, 7; biotinylated *Tb*GalE after crosslinking and dialysis, 8; mixture of biotin- and His-tagged *Tb*GalE after crosslinking and dialysis.
The two first bands on the gel and the Western blot were, as expected, the monomeric biotinylated \( Tb\)GalE that have a weight of 57 kDa. In the second lane free biotin has been removed by dialysis. Lane three shows the His-tagged protein with its lower molecular weight of 43 kDa. It can be seen in the gel but not in the Western blot since antibodies against biotin have been used. In lane four two bands should be seen on the gel, one at 43 kDa (His-tagged \( Tb\)GalE) and one at 57 kDa (biotinylated \( Tb\)GalE). Only one is seen though and instead in the Western blot, where only one band for biotinylated \( Tb\)GalE should be seen, both differently tagged proteins are visualized. In lane five three bands can be seen in the Western blot, one for His-tagged \( Tb\)GalE, one for monomeric biotinylated \( Tb\)GalE, and one for the crosslinked, dimeric \( Tb\)GalE at about 114 kDa. In lane six and seven more proof for a successful crosslinking is visualized. Both the gel and the Western blot show two bands, one for the monomeric unreacted biotinylated \( Tb\)GalE at 57 kDa, and one for the crosslinked dimeric biotinylated \( Tb\)GalE at 114 kDa. The bands on the very top of lane six and seven in the Western blot were protein stuck in the stacking gel and are therefore not of interest. The bands in lane eight on the gel is really faint but they could (with good will) be seen lower than the bands in lane six and seven. It is plausible that these bands represent the monomeric His-tagged protein at 43 kDa as well as the crosslinked dimeric His-tagged protein at 86 kDa.

Bands in lane eight in the Western blot are clearer and are probably representing the biotinylated monomer and crosslinked dimer. It seems like the attempt to construct a heterodimer failed, although it is hard to distinguish between homodimeric biotinylated \( Tb\)GalE (114 kDa) and heterodimeric \( Tb\)GalE (100 kDa).

When looking at the Coomassie blue gel in Figure 18 the highest bands in lane six and seven seem to be approximately 150 kDa, which is higher than the expected value 114 kDa. The samples in both gels ran strangely though, for example protein was caught in the stacking gel as seen in lane six and seven in Figure 19. Further, the lower bands in lane six and seven in Figure 18 are coming out higher up than in lane one and two, even though they are probably the same protein. Therefore the molecular weight estimation is quite approximate.

One strange feature with the Western blot is, as previously noticed, that bands at the approximate weight for His-tagged monomer are seen in lane four and five. The method for visualizing the bands is ExtrAvidin antibodies conjugated to Horseradish peroxidase. The ECL Plus is only enhancing the signal, which thus should be specific for the biotin on the
fusion tag. Therefore the expected result in lane four and five would be not to see the lower bands, similar to lane three.

The conclusions to be drawn from the gel and the Western blot results are that the crosslinking reaction worked with about 50% efficiency. The attempt to form crosslinked heterodimers seems to have failed, and the antibodies used for the Western blot were, to some extent, binding unspecifically and thus envisaged the His-tagged TbGalE in lane four and five. The activity of the crosslinked biotinylated protein was tested on the HPAEC activity assay and compared to the activity prior to the reaction. The specific turnover (moles turned over/molar protein/hour) dropped to around 30% and therefore the work on the crosslinked protein was not continued.

7.4 Chromotrop 10 B – a NAD\(^+\) inhibitor?

A scientific group in USA (McCommon, UCSF) has through docking screening predicted that the compound Chromotrop 10 B (also named NCS (cas:5850-63-5), originally from Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda), binds to the cofactor pocket, blocking NAD\(^+\) and thus inhibits TbGalE (fig. 20).

The structure of Chromotrop 10 B is seen in Figure 21.

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**Figure 20.** 3D picture showing binding of UDP (1) and NAD\(^+\) (2) and also the predicted binding of Chromotrop 10 B (3).

**Figure 21.** Predicted inhibitor Chromotrop 10 B.
Investigation of small molecules binding to UDP-galactose 4'-epimerase - A validated drug target for Trypanosoma brucei, the parasite responsible for African Sleeping Sickness.

7.4.1 Chromotrop 10 B IC₅₀ against TbGalE
By using the HPAEC activity assay it could be concluded that Chromotrop 10 B inhibits TbGalE and, after setting up the concentration gradients mentioned in the experimental section, IC₅₀ was determined to 0.75µM (fig. 22).

![Chromotrop 10 B IC₅₀ binding curve](image)

**Figure 22.** Graph showing the IC₅₀ curve for Chromotrop 10 B. The IC₅₀ was determined to 0.75µM.

7.5 Fluorophore UDPAmNS

UDPAmNS is a sugar nucleotide analogue known to bind the active site of E. coli GalE with an affinity of 200µM, according to Bhattacharyya et al. (1999) (19). They also showed that UDPAmNS is a fluorescent compound when stretched out in non-polar solvents or when bound to a surface, such as E. coli GalE. When in polar solution the two ring systems stack over each other and the fluorescence is self-quenched (fig. 23).

![UDPAmNS bound to E. coli GalE](image)

**Figure 23.** UDPAmNS bound to E. coli GalE is fluorescent, but when in (polar) solution the two ring systems stack and self-quenche.
Investigation of small molecules binding to UDP-galactose 4’-epimerase - A validated drug target for Trypanosoma brucei, the parasite responsible for African Sleeping Sickness.

The properties of UDPAmNS could be used in a displacement assay, where other compounds that bind to TbGalE will reduce the fluorescence by displacing bound fluorescent UDPAmNS into free solution where the fluorescence is quenched.

7.5.1 UDPAmNS synthesis
The first step towards such an assay is to synthesize the fluorophore UDPAmNS. The compound is produced by a one step carbodiimide reaction where AmNS and UDP are the substrates and EDC the imide that catalyses the reaction. Figure 24 depicts the general carbodiimide reaction, the only difference in the UDPAmNS reaction (fig. 25), is that the second phosphorus atom, with its double bonded oxygen is acting as the carboxyl carbon in the general reaction. The catalyzing imide is seen in Figure 26.

![Figure 24. The carbodiimide reaction. A bond between the nitrogen and the carboxyl carbon is introduced and an imide is catalyzing the reaction.](image)

![Figure 25. UDPAmNS reaction.](image)

![Figure 26. The imide EDC used in the UDPAmNS reaction in figure 25.](image)
7.5.2 UDPAmNS purity control

After synthesis the 260/320nm absorption ratios were calculated and fractions with a ratio of 1.6 to 1.7 were pooled. Fractions giving a ratio of about 0.8 was the unreacted AmNS, which gives a green fluorescent light under a UV-lamp, compared to the blue light fluorescing from the pooled product (fig. 27).

![Figure 27](image)

**Figure 27.** Pooled product UDPAmNS seen to the left, giving a blue fluorescent light. In the middle tube the unreacted AmNS and its distinguished green light is seen. To the right, pooled fractions containing only eluation buffer is seen giving no fluorescent light.

The purified UDPAmNS was also submitted to P-NMR (H-NMR results are seen in Appendix) and mass spectrometry to verify that the product was correct and pure. The mass spectrometry result (fig. 28) is showing a big peak at 608 Da, the correct weight for UDPAmNS. Also a smaller peak at 385 Da is seen, consistent with the UDP moiety after cleavage from AmNS. Results from the P-NMR show two defined doublet peaks belonging to the two phosphorus atoms in UDPAmNS (fig. 29).

![Figure 28](image)

**Figure 28.** Results from mass spectrometry. The biggest peak belongs to UDPAmNS and the smaller one belongs to the UDP part after the bond to AmNS is broken. AmNS has a molecular weight of 223 Da.
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Reviewing the results together it can be concluded that the purified molecule is UDPAmNS and that it is uncontaminated.

### 7.5.3 UDPAmNS concentration calculations

The naphthalene ring in UDPAmNS has a specific absorption at 315-320 nm, which was used to calculate the concentration of the purified compound. Concentration was calculated using the equation in the experimental part.

\[
\text{Abs}_{320} = 0.546 \times 50 = 27.3 \quad \text{(diluted 50 times)}
\]

\[
\text{Conc}_{\text{UDPAmNS}} \approx 5\text{mM}
\]

A concentration of 5mM gives a yield of about 10%, which compared to the 30-40% yield in the literature (20) was considered to be a satisfactory amount.

### 7.5.4 UDPAmNS IC\textsubscript{50} against TbGalE

The concentration gradient of UDPAmNS, seen in the experimental section, gave a bit confusing results (fig. 30 and 31). It seems like low concentrations of the inhibitor UDPAmNS are actually stimulating the reaction.
Figure 30. Graph showing the inhibition curves obtained by adding UDPAmNS. At lower concentrations negative inhibition can be seen.

Figure 31. Graph showing the averaged data with standard deviation error bars at the concentrations that were used more than once. The negative inhibition is still seen.

No decisive conclusions about the IC$_{50}$ value can be drawn, but from the graph above it seems plausible that UDPAmNS have a somewhat stronger binding to $Tb$GalE than to $E. coli$ with an IC$_{50}$ of about 30-60µM.
7.5.5 Fluorescence assay with UDPAmNS

The first aim was to decide the conditions for the best fluorescent signal before trying to go into the displacement assay. Literature (20) describes a concentration ratio of 3:1 TbGalE and UDPAmNS, but with rather high (28:9µM) concentration of both compounds. The first setup described in the experimental section gave answer to whether an enhancement, bigger than the additive effect (individually fluorescence for TbGalE and UDPAmNS added together), can be seen by mixing TbGalE and UDPAmNS. From Figure 32 it is evident that this is the case for the 4:1-1:1 ratio (1.6µM protein and 0.4-1.6µM UDPAmNS), TbGalE over UDPAmNS. The change in the observed value from the expected value of TbGalE and UDPAmNS (when not binding) is distinguished. The “signal to background” in (Table VIII) shows values from 2.5 to 5.

![Figure 32](image.png)

**Figure 32.** Showing the difference in the expected additive signal and the observed signal when mixing TbGalE and UDPAmNS.

<table>
<thead>
<tr>
<th>S/B</th>
<th>0.4uM TbGalE</th>
<th>0.8uM TbGalE</th>
<th>1.6uM TbGalE</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPAmNS</td>
<td>0.4µM</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>UDPAmNS</td>
<td>0.8µM</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>UDPAmNS</td>
<td>1.6µM</td>
<td>5.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

**Table VIII.** Showing signal to background.

In Figure 33 the graph is showing the fluorescence depending on TbGalE and UDPAmNS concentration. The steeper slopes seen after added UDPAmNS proves a synergic effect between the two compounds, explained by the stretching of UDPAmNS upon binding.
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Displacement of UDPAmNS should yield in a decrease of the fluorescence intensity, due to the self quenching ring systems. When adding a fixed concentration of UDP to a mixture of varying concentrations of *Tb*GalE and a set concentration of UDPAmNS, the graph in Figure 34 was obtained.

The bars with added UDP are almost as low as the expected additive value. This proves that the UDP is displacing UDPAmNS almost completely, with a large reduction in fluorescence.
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Figure 20 shows how Chromotrop 10 B is believed to bind to the NAD\(^+\) pocket, not to the active site of *Tb*GalE. The displacement results however show (fig. 35) that Chromotrop 10 B is able to displace UDPAmNS from the active site.

**Figure 35.** Showing that the predicted NAD\(^+\) inhibitor Chromotrop 10 B is actually displacing UDPAmNS from the active site.
8 Discussion

To be able to investigate whether small molecules binding to *Tb*GalE could be detected by SPR the protein had to be biotinylated. Biotinylation of proteins with the Pinpoint Xa vector had never been done in the Ferguson lab prior to this project. By using this vector system it was possible to express active biotinylated *Tb*GalE, even though the fusion tag was almost a third of the molecular weight of the protein. One positive aspect with this technique is the fact that only one N-terminal biotin is added, which enables the method to be very suiting for e.g. SPR. By having a single biotin attached at the same spatial position, identical binding to Streptavidin surfaces is facilitated. Unspecific biotinylation to lysines and free amines can possibly interfere with the activity of proteins, if any lysines are situated close to the active site. Further it makes the Streptavidin binding more random, which could lead to active sites being less exposed for further binding. The protocol established during this project for purifying biotin-tagged proteins from the PinPoint vector and binding them to a Streptavidin chip, has since been tried successfully in other projects in the Ferguson lab.

However the actual response from the SPR measurements upon binding was very low. This is a bit puzzling since the satisfactory protein capturing in Figure 12 is sufficient to give higher responses. Also, we could only detect binding of the two substrates, not of the known inhibitors. Because almost all proteins seem unable to bind when attached to the chip the question about dimer breakage was raised. We had previously shown that *Tb*GalE is functional as a dimer through a size exclusion experiment (data not shown). Even though the dextran layer is quite flexible, if the two tags bind to Streptavidins far apart, perhaps the dimerization is broken? If that is the case the activity of the protein could be lost. The Streptavidin-biotin binding is the strongest non-covalent binding there is, so the risk seems possible. The crosslinker BS$_3$ was used in order to investigate if we could surmount this possible problem. A fair conclusion is to estimate that the crosslinking seemed to work to an extent of about 50%. Unfortunately, the activity of the crosslinked protein batches was reduced to a large extent. The activity actually seen could come from both the crosslinked and the unreacted protein, implying that an introduced covalent bond only reduces, not reset the activity fully. Yet, it seems more plausible that the activity originates from the unreacted protein in the sample. For example each monomer contains 17 lysines and a few of them close to the binding sites (Fig. 36) In the figure below the lysines are highlighted in red. Three
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Lysines are positioned near the active site, where UDP is bound, centered in the figure. One could argue that if the crosslinker binds to any of these three, or even links two of them together, the active site would be partly blocked.

*Figure 36.* Showing UDP bound to the active site, centred in the figure. Three lysines, highlighted in red, surround the active site cavity. In the top left corner NAD$^+$ is seen.

Trying to optimize the crosslinking reaction, in order to obtain less binding to lysines close to the active site, seemed both difficult and time consuming. By diluting the protein concentration, the risk of tetra- and octamerization is reduced. Lowering of the crosslinker concentration implies a lesser binding to the protein. That would mean that mostly the easy accessed lysines on the surface would bind a crosslinker. This is favorable because less crosslinker would bind the lysines in the binding pocket. Yet the ratio between intra-molecular and inter-molecular (dimer) binding will not be affected. This is due to the fact that the distance between lysines in the same monomer is similar to the distance of lysines in the adjacent monomer. Blocking of the active site is not the only possible way to lose activity with intra-molecular crosslinking. By crosslinking two lysines in the same monomer the conformation could change, which can also reduce the activity.

This problem, combined with the fact that the protein seemed to lose the little activity there was very quickly (when bound to the chip), were strong reasons for not continuing with the assay. In addition, the chip used for the experiment was rather expensive and seemed to lose activity fairly rapid. Therefore the SPR assay was put on hold.
Subsequent projects in the Ferguson lab group have purified a biotinylated monomeric protein and proved successful protein capture. Still, they also got low binding of substrate and inhibitors (about 10%). This could shine light on the possible dimerization breakage not being the problem, or at least not the only problem. In fact, when discussing these problems with SPR expert Dr. Iva Navratilova we are told that almost 50% of all proteins analyzed with SPR are problematic, and similar problems and low responses are seen for many other proteins.

The other assay investigated in this project work is the UDPAmNS assay. Preliminary data in the Ferguson lab had shown that a fluorescence enhancement, bigger than the additive effect, could be seen by mixing TbGalE and UDPAmNS. Displacement had also been tested and proven, but without knowing the correct concentration or purity of UDPAmNS.

The yield of the synthesized UDPAmNS and the purity of the compound (showed by mass spectrometry and P-NMR) were satisfactory. The yield could perhaps be somewhat improved by quicker handling of the reactive EDC immediately before starting the reaction. Figure 33 shows evidence for a synergic effect between TbGalE and UDPAmNS, and Figure 34 shows an evident displacement of the fluorophore by UDP. This was done with about 20 times lower concentration than the prior published data (20) and could be a ground for subsequent work with the UDPAmNs displacement assay. In the future, perhaps this assay could work as a secondary screening assay, confirming displacement and correlate the displacement to activity loss seen with a primary screening assay.

The UDPAmNS IC₅₀ curves (fig. 30 and 31) showed negative inhibition at lower concentrations. This was most unexpected and no similar results with UDPAmNS have been found in the literature consulted. Many vague explanations were discussed but no good theories emerged. After discussing the data with Prof. Alan Fairlamb a new idea about UDPAmNS’ inhibition curves developed. In the book “Enzymes, third edition” (21) different modifiers were described, showing different Rate/Conc_modifier curves. Two of the curves were showing rate stimulation at low concentrations and inhibition at higher concentrations. When transferring the IC₅₀ data to the general equation for the different curves in the book (courtesy of Prof. Alan Fairlamb) it was evident that the numbers obtained from UDPAmNS binding curves fitted the equation (fig 37).
Therefore a hypothetical explanation for the increased rate at lower concentration could perhaps be that UDPAmNS binds one monomer and activates the other monomer sufficient enough to obtain an increase in rate. As the UDPAmNS concentration increases the other monomer will also bind an UDPAmNS molecule and the rate will rapidly decrease. This theory is not proven though.

Chromotrop 10 B was according to the McCommon group (UCSF) predicted to inhibit TbGalE by binding the cofactor NAD\(^+\) pocket. Initially the activity assay confirmed that the compound inhibits TbGalE with an IC\(_{50}\) of 0.75\(\mu\)M. But when investigating Chromotrop 10 B in the UDPAmNS displacement assay clear evidence of displacement was seen (fig. 35). One possible explanation could perhaps be that Chromotrop 10 B is binding the cofactor pocket, but also blocking the active site partly. Another explanation could be that Chromotrop 10 B is promiscuous, and thus able to bind both pockets.

The work with TbGalE in Fergusons lab has been going on for several years and is still one of the prioritized projects. The research carried out during this project has provided a useful
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Protocol for how to biotinylate proteins and immobilize them to an SPR chip. Even though the SPR experiment with TbGalE unfortunately provided poorer results than expected this protocol can be used for other proteins that is interesting as drug targets. Further the conditions established for the UDPAmNS displacement assay present good opportunities for designing a robust assay, which perhaps, in the long run, could be used for screening compounds binding to the validated drug target TbGalE.
9 Conclusion

Galactose metabolism is essential for the survival of Trypanosoma brucei, the protozoan parasite responsible for African sleeping sickness. Loss of galactose metabolism leads to galactose starvation with the result of ceased cell division and cell death. In T. brucei the only production of UDP-galactose is through the epimerization of UDP-glucose catalyzed by UDP-glucose 4’ - Epimerase (GalE).

In this Master thesis project two TbGalE binding assays have been investigated to evaluate if a reliable screening assay could be established. The Pinpoint Xa vector system have been used to successfully biotinylate TbGalE expressed in BL21(DE3)pLysS E. coli cells. Despite the low signal from the Surface Plasmon Resonance experiment with the biotinylated TbGalE, binding of substrates UDP-galactose and UDP-glucose could be observed with a $K_D$ of 105µM, comparable to the known $K_m$ of 80µM. The protocol established for expression and purification of biotinylated proteins and binding them to Streptavidin surfaces could be used in the future in other projects at Dundee University, in the effort of finding a better drug for African sleeping sickness.

Further the fluorescent sugar nucleotide analogue UDPAmNS, which was known to bind to the E. coli GalE active site, was synthesised and purified successfully. The IC$_{50}$ against TbGalE was approximated to 30-60µM. Conditions for an UDPAmNS displacement assay were established, with about 20 times lower concentrations than previously seen in literature. Evidence showing that TbGalE and UDPAmNS mixed together give a synergetic effect on the fluorescence was obtained and also that the fluorescent signal could be reduced by displacing UDPAmNS with UDP. In this way a predicted cofactor inhibitor, that we proved inhibited TbGalE with an IC$_{50}$ of 0.75µM, was demonstrated to be able to displace UDPAmNS. This shines light on the fact that the compound at least partly binds the active site of TbGalE. This displacement assay could, in the long run, have the potential of becoming a usable screening assay for TbGalE drug hits.
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11 References


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Structural characterization of the asparagine-linked oligosaccharides from *Trypanosoma brucei* type II and type III variant surface glycoproteins. *J. Biol. Chem.*, 266, 20244-20261.


12 Appendix

Appendix. Results from UDPAmNSH-NMR.