Signal Transduction Pathways involving cyclic Adenosine Monophosphate in *Trypanosoma* parasites

How an old mechanism provides new possibilities for drug discovery research

Literature thesis as part of the Master Chemistry, track Molecular Design, Synthesis and Catalysis.

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1. Summary

*Trypanosoma brucei* and *Trypanosoma cruzi* are parasites that cause respectively African and American Trypanosomiasis diseases in humans. These diseases affect many hundreds of thousands in Africa and Latin America, and have a severe impact on society, economy and welfare. Current drugs are old, with the newest treatment officially registered as such in 1990. Also, current drugs have sub-optimal effectiveness and have severe side effects like liver damage, brain damage and/or arsenic poisoning.

To find new drug targets, the signal transduction pathway based on cyclic Adenosine Monophosphate (cAMP), which is the common signal transduction pathway in *Trypanosoma* species, is investigated. From this, phosphodiesterases (PDEs) have been validated as possible drug targets, which inhibit cAMP degradation. Other components of the cAMP-based signal transduction involve adenyl cyclase (AC, for cAMP production) and protein kinase A (downstream effector of cAMP), which may also prove to be drug targets, but have not been validated yet. Important factors for localisation and activity have been identified for several different *Trypanosoma* phosphodiesterases, among which are GAF-domains for activity and the N-terminus for localisation.

2. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenyl Cyclase, the protein responsible for cAMP production</td>
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<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanine Monophosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>5’-Adenosine Monophosphate (non-cyclic)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CVC</td>
<td>Contractile Vacuole Complex, a bladder-like structure for cell volume regulation</td>
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<tr>
<td>GAF</td>
<td>as in GAF-domain, an abbreviation of proteins containing this domain (cGMP-specific phosphodiesterases, Adenylyl cyclases and FhIA, a bacterial factor)</td>
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<tr>
<td>GDP</td>
<td>Guanyl Diphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanyl Triphosphaate</td>
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<td>HAT</td>
<td>Human African Trypanosomiasis, also known as (human) sleeping sickness</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase, the protein responsible for cAMP degradation</td>
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<tr>
<td>VSG</td>
<td>Variant Surface Glycoprotein, a dense coat surrounding <em>Trypanosomal</em> cells involved in immune system escape.</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>PK</td>
<td>Protein Kinase (A), the main target of cAMP molecules</td>
</tr>
<tr>
<td>PKI</td>
<td>Protein Kinase A Inhibitor</td>
</tr>
<tr>
<td>T.</td>
<td><em>Trypanosoma</em>, family name of species of kinetoplastid parasites</td>
</tr>
<tr>
<td>br</td>
<td><em>brucei</em>, subtype of <em>Trypanosoma</em>, often used in Tbr or T. br. abbreviations for gene or protein naming. Only found in Africa</td>
</tr>
<tr>
<td>cr</td>
<td><em>cruzi</em>, the same as <em>brucei</em>, though from a different part in the world. (South America)</td>
</tr>
<tr>
<td>Hs</td>
<td><em>Homo sapiens</em>, also known as ‘man(kind)’.</td>
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TbrPDEB1 *And similar*: Abbreviations for specific genes and proteins, built up from the species (*Tbr* = *trypanosoma brucei*) PDEB1 is the type and isozyme kind of the protein or gene
3. Introduction

According to the World Health Organisation (WHO), very little is known yet about unicellular parasites. The only well documented thing about parasites is the diseases they cause. Other information, like knowledge on their biochemistry is only limited at best. Insight into important topics, like signal transduction pathways, is in general very scarce, and for most kinds of parasites even unavailable at all. The two best known kinds of unicellular parasites, *plasmodium* and *trypanosoma* species, have become important research topics, as the WHO strives towards the invention of (new) drugs against them.

*Plasmodium* and *trypanosoma* parasites are relatively well known, mainly because they cause the most severe diseases caused by parasites. Annually, millions of people die because of *malaria* (caused by *plasmodium* species) and different kinds of *trypanosomiasis* (caused by different kinds of *trypanosoma* species).

Finding new treatments against the diseases is one of the main goals of several projects initiated by the WHO. Because of the limited amount of knowledge on the parasites, the research program is based on the parts of parasites that are best understood. For *trypanosoma* parasites, the main research field has been the signal transduction pathway of the parasite. This research resulted in the discovery of a cyclic Adenosine Monophosphate (cAMP)-based second messenger system that shows both similarities and differences to the cAMP signalling pathways in humans. Of all enzymes involved in the cAMP pathways, the phosphodiesterases (PDEs) are currently the ones of most interest for drug research. Phosphodiesterases are present in almost every organism, and are thus well conserved throughout evolution. However, their functionality is so specific that a total of 11 types have been identified in humans alone, with over 100 different polyproteins distributed amongst them.

The research on PDEs in humans has been going on for decades, and the knowledge of them is vast. Also, the knowledge on how to design new drugs aimed against human PDEs is very big. Normally, enzymes that are maintained throughout evolution are hard to target properly with drugs. The risk of finding an inhibitor that will accidentally inhibit similar enzymes of both the parasite and the host is high, and the side effects that will be caused by this render the drug useless. However, on the topic of PDEs, it was found that the highly similar structures can still be selectively inhibited.

The differences between human PDEs themselves are smaller than the differences between *trypanosoma* and human PDEs, which offers an opportunity. On one hand, it is known that PDEs have similar structures throughout species, and on the other hand, they can be inhibited selectively. Thus, with the knowledge of human PDEs and analogies of them with parasitic PDEs, new drug development programs should be relatively easy to set up. Also, it is likely that a number of compounds that resulting from these projects will inhibit parasitic PDEs very selectively, thus leaving the human PDEs virtually untouched, which will hopefully mean that the side effects are limited.

In this thesis, a literature study is performed to summarize the landscape of cAMP signal transduction in *trypanosoma* parasite species. Its production, degradation and main targets will be mapped, in conjunction with the search for new drugs against these parasites.
4. African and American Trypanosomiasis

4.1 General information about the diseases

The class of trypanosomiasis diseases covers a number of parasitic diseases. These parasites are found mainly in Africa and Latin America, as shown in figure 1. The name trypanosomiasis is derived from the parasite genus causing these diseases, the *trypanosoma*. *Trypanosoma* parasites are a member of the order of *trypanosomatids*, and belong to the *kinetoplastid* class of protozoa. Protozoa are highly motile and unicellular members of *eukaryota*, with much diversity amongst them. *Trypanosoma* certainly fit the character of high motility, for they have a tail-like part called the flagellum. This flagellum, which is clearly visible in figure 2, offers motility in ways quite similar to the tail or flagellum of respectively tadpoles and spermatozoons.

In figure 1, *Leishmaniasis* is also indicated in the legend. Leishmaniasis is a group of related diseases caused by *Leishmania* parasites, which are related to *trypanosoma* parasites. They are, however, not treated any further by this thesis.

![Figure 1: The regions in which American and African trypanosomiasis are found. Also in blue are Leishmania infections, another subgroup of infections caused by kinetoplastida and trypanosoma.](image)

The best known trypanosomiasis diseases, and also the most dangerous for humans, are *human African trypanosomiasis (HAT)* and *American trypanosomiasis*. These diseases are also known respectively as African sleeping sickness and Chagas disease. According to the World Health Organisation and their main partners on trypanosomiasis diseases¹, HAT and Chagas are responsible for respectively 30,000 and 8,000,000 patients. For HAT, all of them will die if left untreated, for Chagas, the mortality is at 12,000 annually.
Figure 2: *Trypanosoma brucei* in bloodstream form\(^{38}\).

Trypanosomiasis infections are transferred by bug vectors, depending on the kind of parasite. For *Trypanosoma brucei*, the causative agent of African Sleeping sickness, the parasite is spread by the Tse Tse fly (figure 3l). Chagas disease, which is caused by *Trypanosoma cruzi*, is transferred by the ‘kissing bug’ (also known as triatomine or reduviid bug, figure 3r).

Figure 3: A Tse tse fly\(^{40a}\) (l) and a reduviid bug\(^{40b}\) (r)

The economic damage done by trypanosomes is very high, and often causes vicious circles in endemic or epidemic regions. A great part of the damage comes from trypanosomal infections of livestock, which is caused by different species from the *trypanosoma* genus. Examples of livestock-infecting trypanosomes are *Trypanosoma brucei brucei*, *T. congolense*, *T. simiae* and *T. vivax*, which all cause nagana, or animal African trypanosomiasis. The economic damage done this way is hard to estimate, because weak livestock means less welfare, and therefore fewer opportunities to escape poverty and get proper treatment for the diseases. It is safe, however, to predict that multiple millions of US dollars are lost due to this ongoing disease.

The life cycle of the parasite is of high importance for the disease development. The nature of the parasite requires it to go through several stages of proliferation before it can infect humans. The parasite takes a different form in each stage. Some of these forms can only survive within Tse Tse flies, whereas others can only survive in human beings. During the life cycle, and especially during the transfer from human to bug or vice versa, changing from one host to another brings environment-related shocks, mainly in the form of different osmolarity. To overcome these shocks, the parasites have developed ingenious systems to keep the cell volume under control, and even to use the osmotic changes to their advantage.
4.2 Physiology of the parasite

4.2.1 Life Cycles of the parasites

Trypanosoma cruzi and Trypanosoma brucei have life cycles that are similar on certain points. Most notably, both parasites have a cell cycle that needs both a bug and a mammalian vector to survive and flourish.

For Trypanosoma cruzi, the life cycle of the cell consists of four different stages, which are illustrated in figure 4. Each stage can be found in only one of both hosts. The first stage is that of epimastigote, in which the parasite resides inside the gut of its bug host. Epimastigote \( T. \) cruzi will replicate and differentiate into the metacyclic trypomastigote form of the parasite.

The trypomastigote can be encountered in two different forms, with different infectivity and during different stages of the cell cycle. The metacyclic trypomastigote form is released with the bugs faeces and will infect a mammalian host. It will then differentiate into amastigote form, the final form in the cycle. Amastigotes reside inside host cells and will divide by binary fission. After reaching a threshold density, amastigotes differentiate into bloodstream trypomastigotes. These parasites are released into the bloodstream (as the name already tells) and will be able to infect any nucleated cell in the body. In there, they will return to amastigote forms, and when sucked up by another bug, finally turn into epimastigotes inside the bug gut.

Figure 4: The life cycle of \( T. \) cruzi, with 1) The bite of the reduviid bug, infecting the human 2-4) stages of the bug inside a human body 5-8) stages inside the bug, after biting an infected human.\(^{41}\)
For *Trypanosoma brucei*, the life cycle consists of five different stages, of which two are found in the human bloodstream. When considering the moment of infection (or biting by the tsetse fly) as the start of the cell cycle, it is built up as follows. Upon infection of mammals, the parasite has taken the ‘bloodstream long slender’ form. In the mammalian bloodstream, also the short stumpy form can be found. The short stumpy stage is a differentiation from the long slender form, its ratio of occurrence is determined by the density of parasites in the blood. With high parasite density, the cells turn into short stumpy forms, which are unable to proliferate. The levels of short stumpy parasites build up over time, in order to increase the likeliness to infect another tsetse fly. Once being sucked up by the tsetse fly, the changes to the osmolarity induce parasite proliferation into the procyclic stage. This stage derives its name from the fact that a protective VSG (variant surface glycoprotein) coat that was present in the bloodstream forms, is shed and replaced by a procyclin-based coat. Procyclins are glycosyl proteins anchored by phosphatidylinositol groups, containing characteristic amino acid repeats at the C-terminus (either EP, a form of procyclin rich in Glu-Pro repeats, or GPEET, a form of procyclin rich in Glu-Pro-Glu-Glu-Thr repeats)\textsuperscript{52} Both cell coats are meant to protect the parasite from immune responses by the host, but insect immune responses are less severe than those found in mammals, so protection levels may be lowered by a factor 10. (10\textsuperscript{7} vs. 10\textsuperscript{6})

Figure 5: The life cycle of *Trypanosoma brucei* parasites, with the human stages (bloodstream, slender and stumpy) and Tsetse fly stages (midgut and salivary gland, procyclic, epimastigote and metacyclic) indicated. \textsuperscript{3} Graphical representations of the parasites are impressions of their real life phenotypes and are therefore not accurate.
Inside the tsetse fly, the procyclic form transfers from the midgut to the salivary gland of the fly, changing into *epimastigote* parasites on the go. This form attaches to the walls of the gland and first multiplies several times and subsequently receives the VSG coat again, which enables re-entry of the mammalian bloodstream. This last stage before restarting the cell cycle is called the *metacyclic stage*.

Throughout all different stages in the life cycles, differentiation to another stage can only occur if stresses can be overcome by the parasite. Such stresses include changes in osmolarity when going from one host to another, or when switching localisation within one host (intra- or extracellular). Thus, the different stadia of the life cycle all come with physiological restrictions and implications, and therefore, often an altered amount of one or more components of the signal transduction pathway is present, such as increased Adenyl cyclase or decreased phosphodiesterases density. Also, activity of these and other important proteins varies between different life cycle stages.
Figure 6: Schematic overview of an acidocalcisome, with ion, water and phosphate transporters depicted. Ca\(^{2+}\)-ATPase, H\(^{+}\), Na\(^{+}\) and H\(_2\)O pumps are mentioned in the text.

To be able to cope with osmotic shock situations, *Trypanosoma* parasites contain acidocalcisomes to regulate the cell volume. Acidocalcisomes are organelles that are found in bacteria, trypanosoma parasites and humans. It is therefore that these organelles are either conserved throughout evolution, or were evolved multiple times by convergent evolution. Dying with different dyes showed the presence of both polyphosphates and acidic parts, which yielded the name ‘poly P granules’ in 1947. (Later, between 1994 and 1996, proton and calcium pumps were discovered, after which the name was changed to acidocalcisomes.)

Acidocalcisomes have been identified in trypanosomatids as early as 1908.\(^7,8\) The organelle serves as a store for important molecules and ions like ortho-, pyro- and polyphosphates, H\(^{+}\), Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\), Mg\(^{2+}\), Zn\(^{2+}\) and Fe\(^{2+}\). Also, basic amino acids are found inside the acidocalcisome.

To allow the flow of these molecules and ions in and out of the organelle, the organelle contains a number of proteins and pumps. Calcium ATPase was positively identified by both analysis of the organelle and the implementation of the corresponding gene into Calcium ATPase deficient yeasts. Also, two proton pumps were identified, as well as...
other cation pumps and a water channel protein (aquaporin)\textsuperscript{4,5,6} All these pumps are necessary for the main job of the acidocalcisome, maintaining the cell volume of the trypanosome. Trypanosomes find themselves under changing conditions due to host and life cycle stage variations. During the transition period from one environment to another, the contents and pumps of acidocalcisomes maintain the right cell volume, by an immediate flow of water and ions into or out of the cell body.

However, it was found that the acidocalcisome is not the only organelle responsible for maintaining the right cell volume. Although the acidocalcisome has the major role in this process under changing osmotic pressures, in a steady state, the volume is predominantly maintained by the contractile vacuole complex, or CVC. The CVC will contract when under normal circumstances, the inlet of water by aquaporins and osmosis has caused the cell to swell beyond its preferable size. This pumping effect, which could be superficially compared to a heart beat (both processes move fluids from one bladder or room into another), occurs about every one minute.

The CVC was also shown to contain transporter proteins that may be transferred to the membrane of the cell in case of need. Examples of such transporters are polyamine transporters found by Hasne et al\textsuperscript{9,10} and aquaporins\textsuperscript{5,9}. Also, the \textit{Trypanosoma cruzi} phosphodiesterase PDE C (TcrPDEC) has been identified on the CVC complex, as well as vacuolar proton pyrophosphatase, which is a proton pump working on pyrophosphate hydrolysis. All these proteins are involved mainly in the maintaining of the proper CVC volume. Upon hypotonic stress, cAMP levels are increased and aquaporins translocate from the acidocalcisomes to the contractile vacuole complex\textsuperscript{6,9}. Together with hydrolysis of polyphosphate, which creates an osmotic pressure inside the CVC, water is thus osmotically driven into the CVC\textsuperscript{4,9}. This process ends when the TcrPDEC located on the CVC hydrolyses the cAMP signal molecule that caused the response.

\textbf{4.2.3} Drug treatment and drug design

\textbf{4.2.3.1} Current treatments

The current medication available for treatment of trypanosomiasis diseases is one of the main reasons why the research carried out on the Trypanosoma parasites has increased a lot. In the treatment of American trypanosomiasis, the compounds suramin, pentamidine, melarsoprol and efloarlithine (see figure 7) are used\textsuperscript{11a}, depending on the stage of the disease. In the first stage, suramin and pentamidine are used. However, these drugs cause side effects like kidney, liver and heart failure, and can only be used if the parasite has not yet entered the central nervous system (CNS). In a later stage, melarsoprol and efloarlithine are used. Melarsoprol is an arsenic-containing drug and is thus highly toxic (5-10\% of the patients die of arsenic poisoning), and efloarlithine is very hard to administer properly, due to the fact that specialized equipment and staff are needed. All these negative issues associated with the current treatments show the need for newer and better drugs, with less side effects and lower drug-related mortality rates.
According to the WHO, suramin was discovered in 1920 and pentamidine in 1940. The newest drug, eflornithine, dates back to 1990. All available drugs are (very) old, and the need for newer drugs is high. Trypanosome parasites are diploid, just as humans, meaning they have two homologous sets of chromosomes. Because of this, resistance against drugs has not built up much; it is easier to acquire resistance when only one gene has to be mutated to achieve different protein expression. Diploid organisms have the ‘risk’ of switching off the mutated gene in favour of the not yet mutated copy on the homologue chromosome. Natural selection for the mutated (but silenced) gene will not occur, making it harder to implement the newly acquired resistance in the species in general. However, new drugs will be needed in the future, because cases of resistance have been reported more and more.

4.2.3.2 New strategies in drug design

Recently, a new tactic has been developed to come up with possible drugs against trypanosomiasis infections. Traditionally, drug discovery was based on more or less random compounds which were tested against organisms, or on the principle of ‘the magic bullet’, which would yield a compound that only harms the target organism and not the surrounding tissue from the host. However, this procedure functions best if host and guest differ considerably from an evolutionary point of view. The larger the genetic differences are, the less alike will be the proteins involved in vital cell biology like signal transduction.

Trypanosoma parasites and humans do, however, not differ much from an evolutionary point. This meant that old procedures for drug development were useless, and new ones would have to be developed before compounds could actually be tested. This kept many industrial parties from investing into the research.

New tactics that have been developed in the past few years are both simple and elegant, and have already caused a surge in projects aimed against trypanosoma and other parasites. The new tactics involve the use of genetic similarities, rather than avoiding them. The best example of this is also the example on which new research against trypanosomes is based. It takes the similarity of the phosphodiesterase (PDE) proteins present in both humans and trypanosomes, and uses it to speed up the research. Phosphodiesterases are proteins that hydrolyze one phosphate bond in 3’-5’-AMP (or cyclic AMP), yielding 5’-AMP, which ends the signal transduction of which the cAMP was part. Also, phosphodiesterases are present in almost every organism, and the human ones are well known. Although 11 families of human phosphodiesterases exist, and an estimated 100 polypeptides can be found in the human body, inhibitors against them are both abundant and very selective. Possibly the best known example of this is the compound sildenafil (better known under its trade name Viagra), which
inhibits the human phosphodiesterase hPDE5 specifically. The compound is not free of side effects, but the severity of them is only limited. Side effects of drugs are generally associated with off-target interactions. This means that not only the targeted protein shows affinity for the drug molecules, but other proteins have some affinity as well. Whenever they are activated (or inhibited) by this molecule, side effects may occur. Side effects of available PDE-inhibitors are generally mild. This shows that even for a class of proteins that is both well preserved throughout evolution and abundant in many different forms, inhibitors can still be very selective.

It is this very knowledge that is now the hope for drugs against trypanosomal infections. Drug design is nowadays often aided by the use of computational studies. In these studies, a (small) molecule is simulated inside the cavity of a protein that it should inhibit. All structural, physical or chemical interactions are simulated, and the molecule is best fitted into the protein cavity. Because the crystal structure of parasitic phosphodiesterases is getting known better and better, they are more easily inserted into the simulation programs. By combining this knowledge of the target protein and the knowledge on inhibitors of highly similar proteins (the human PDEs), the search for new drugs or drug-like compounds is much more effective. The suggested compounds are generally synthesised and tested against the targeted organism or protein, and the knowledge built up this way is used to both improve the simulations and drug design. The resulting information on which functional groups may or may not be desirable within the molecule, provides many leads for optimization of both the compounds and the simulation programs.

This new method of research to date has not yet provided new drugs against either African or American human trypanosomiasis. However, one of the biggest players in the field, the Drugs for Neglected Diseases Initiative (or DNDi) reports on their website that more than a dozen compounds are in clinical or pre-clinical trials. Considering the fact that the newest of all drugs is already over 20 years old, this seems to provide both hope and potential for the future.

Recently, the first validated newly developed inhibitors of TbrPDEB1 and TbrPDEB2 have been reported. TbrPDEB1 and TbrPDEB2 are isozymes of the Trypanosoma brucei phosphodiesterase B. This PDE is the most common and important one in T. brucei. (More information on this and other PDEs is found in ‘signal transduction’ section of this review.) This validated PDEs from trypanosomatids as potential drug targets, and also provided a lead compound for future research and database expansion. All reported inhibitors were of the tetrahydrophthalazinone or hexahydro-phthalazinone groups, which are shown in figure 8.

Compounds like the tetra- and hexahydrophthalazinones are currently the main focus of database expansion and lead compound synthesis projects. During screening of such compounds, all are screened for affinity to HsPDE4, which is the human PDE with the highest similarity to TbrPDEBs, making it the primary off-target for these compounds inside humans. As HsPDE4 is distributed throughout the entire body, with a relatively high concentration in the brain, this off-target may cause possible drugs to have all sorts of side effects if they show affinity to the new compounds. One of the HsPDE4 variations, HsPDE4A10, is mainly found in heart and small intestines. Off-target affinity for this and other HsPDE4A variants, which are also found mainly in nervous systems, could thus lead to interference with many different processes in the body, and might cause serious intestine problems. Also, PDE4 inhibition has been showed to affect the immune system and respiratory functions.
5. Signal transduction

5.1 General Signal transduction pathway

Almost every cell of any organism receives a nearly constant stream of information, or signals, from its surroundings. The cells or organisms have to react to these signals properly to stay healthy. For example, a threatening situation causes human adrenal glands to release epinephrine (also known as adrenaline), which will lead to increased performance of the heart and temporary increase of muscle performance due to the release of stored energy. This sudden boost of energy increases the survival chances, by enabling the individual to either fight or flee. This example is only one of many in which a signal causes a response. However, the response is not directly due to the signal, the signal must first be properly processed by the body.\textsuperscript{14} The processing of the signal is also known as signal transduction. Through a number of conversions, the original signal is translated into the proper response by the appropriate part of the organism. A visual representation of the signal transduction pathway is given in figure 9.
Figure 9: General signal transduction based on cyclic adenosine monophosphate (cAMP). The signal, or external stimulus, is received by the N-terminal part of adenyl cyclase on the outside of the cell. On the inside, the C-terminal part is structurally altered to be catalytically active, and produce cAMP from ATP. cAMP molecules diffuse through the cell to be recognised by Protein Kinases (PK) and other components as acidocalcisomes. Upon hydrolysis of the cAMP molecule by phosphodiesterases, the signal is terminated (red, crossed arrow) and PK and other activities cease.

Signal transduction is often a process in which many components are involved and in which many branches along the signal transduction pathway are available. Although this causes a risk of confusion and overlapping of several routes for signal transduction, some principles, tactics or techniques are maintained throughout the different paths of the transduction routes.

The process of signal transduction starts of with a stimulus. (Figure 9, top left) This can either be external or internal, (though relative to the cell that receives the signal, it is always external, hence the ‘external stimulus’ in figure 9) depending on the organism, its environment and the pathway under investigation. For the example of a threatening situation, the stimulus is external. In case of a freshly ingested meal, the stimulus is internal for an organism as a whole (e.g. a human being), but external for individual cells involved in the digestion. No matter what stimulus has occurred, it will induce the release of a signal molecule, which is called the primary messenger molecule. The primary messenger molecule has to transport the ‘message’ of the stimulus to the proper cells (e.g. adrenal glands) to process it as intended. However, the primary messenger is in most cases unable to pass the cell membrane. To send the message through the cell membrane, the messenger molecule binds to receptor proteins. These proteins are embedded in the cell membrane, and contain both an extracellular and an intracellular part. Upon binding of the messenger molecule at the outside of the cell, the tertiary or quaternary structure of the protein changes. This change induces conformational changes on the inside of the cell membrane, at the other end of the receptor. At this point, the signal transduction in mammals and trypanosoma parasites
deviates. Adenyl Cyclases in trypanosomes resemble those found in mammals, though the topology differs greatly. For example, the ACs found in humans have both the N- and C-terminus on the inside of the cell membrane, whereas trypanosomal ACs have an extracellular N-terminus. The protein extends from the N-terminus onward in a similar fashion in both cases, crossing the cell membrane. However, in case of the mammalian ACs, the membrane is crossed multiple times and intracellular loops make up the catalytic domain, whereas in trypanosomes, the membrane crossing is only observed once, and the C-terminus of the enzyme has the catalytic site in it. This is also depicted in figure 11.23,43,44

In mammals, the signal is received and transported over the membrane to a G protein (so called because it binds guanyl nucleotides). The signal sent from the receptor to the G protein is a hormone, which stays active for a relatively long duration. Because of its life time, this hormone, created by only one receptor that was stimulated by only one molecule, can interact with many G proteins, thus amplifying the signal up to a hundredfold. The G-protein normally binds GDP (guanyl diphosphate), but upon receiving the signal from the receptor, its binding site opens up and allows GTP (guanyl triphosphate) to take GDP’s place. The binding of GTP simultaneously releases an α-subunit of the G-protein, which activates the synthesis of the second messenger by adenyl cyclase. In trypanosoma parasites, however, the G protein does not exist, and the adenyl cyclase has a structure that crosses the cell membrane. (see figure 11) In this, it is similar to the receptors in mammalian cells, but the number of membrane spans differs from 1 for the trypanosoma receptor to 7 for the mammalian receptor. It is, however, unclear whether this similarity is due to the evolution of one structure from the other, or evolution of both from the same ancestral receptor structure.

Adenyl cyclase is a protein that converts ATP (adenosine triphosphate) into cyclic adenosine monophosphate, or cAMP. (figure 9, centre) cAMP is known as the second messenger molecule of many signal transduction pathways, because it took over the role from the primary messenger on the inside of the cell. cAMP has a number of possible targets to carry the signal on to, the most notable of which is Protein Kinase A. This enzyme phosphorylates other enzymes, changing their activity and/or selectivity.

The altered activity is, among others, found in a transcriptional activator called cAMP-response element. This enhances the expression rates of specific genes. This enhancement has important implications. On one hand, it shows us that the signal transduction can carry as far as into the cell nucleus, which is a hard to penetrate barrier. On the other hand, it implies a role of cAMP during processes associated with cell proliferation. This role was confirmed by cell lysate analysis studies, which showed an increase in cAMP levels during proliferation of several stages.
5.2 Adenyl Cyclases

Components of the signal transduction pathway have been thoroughly investigated. In order of activity during the signal transduction, Adenyl Cyclase (AC), Protein Kinase A (PKA) and Phosphodiesterases (PDEs) will be discussed.

One of the major components of cAMP based signal transduction is the adenyl(yl) cyclase protein, abbreviated as AC. This protein uses ATP and cyclises it to cAMP. It is thus the first step in the cAMP signal transduction pathway where cyclic AMP is actually involved.

![Figure 10: The conversion of ATP into cAMP, as done by Adenyl Cyclases](image)

![Figure 11: The structural difference between mammalian and trypanosomal Adenyl Cyclase.](image)

In mammals, Adenyl Cyclase proteins are always G-protein coupled, and belong to the class I ACs. In trypanosomes, the ACs are not G-protein coupled. This is underlined by the fact that trypanosomal AC units lack the binding region for G-proteins and do not need them for bioactivity. Also, in the completed genomes, there is no evidence of G-proteins or related structures.

AC activity from *trypanosoma* parasites was first reported on *T. brucei gambiense* in 1974. By genome studies, it was found that adenyl cyclase genes were present on at least six chromosomes of the protozoan parasites. It was found in the 1990’s that *T. brucei brucei* also showed adenyl cyclase activity.

In 1981, Mancini and Patton showed that cAMP levels vary between different stages of the parasites life cycle. Although this may be due to higher metabolic activity during the
long slender stage than during the short stumpy stage, it suggests an influence of cAMP on the life cycle, or the other way around.

In *T. brucei*, an increase of Adenyl cyclase activity has been measured by Rolin et al. during the proliferation of bloodstream forms, where it coincides with the release of the VSG, a protein coat to avoid immune system detection. However, by mutant species it was found that there is no apparent relation between these observations.

About fifty Adenyl cyclases putative genes have been identified in *T. brucei* as well as about twenty in *T. cruzi* and although not all have been identified as encoding for fully functional proteins, it is likely that some will be true AC-expressing genes. Yet, it is unclear if all identified Adenyl cyclases are truly meant for cAMP production, or act as some sort of receptor with different purposes.

Although the high number of putative AC genes provides a large target for possible AC inhibitors, it also poses the problem of finding a drug that will inhibit multiple dozens of Adenyl cyclases, without inhibiting the human ACs as well. This problem is partly caused by the fact that the exact way in which the cyclases are activated, is still unknown. This is also true for inhibition, and in both cases, this uncertainty and lack of insight mean that drug development programs aimed against AC complexes have yet to prove feasible. Yet, some general features are clear, like that some trypanosomal ACs show calcium-dependency. This dependency is likely due to some down-regulation of the Ca$^{2+}$-signal, rather than direct Ca$^{2+}$ recognition.

5.3 *Protein Kinases A*

Protein Kinases are enzymes with an extremely important role within organisms. PKAs (cAMP-dependant protein kinases) are often part of signal transduction pathways based on cAMP, and are also the main target of this molecule and pathway. Upon activation, protein kinases phosphorylate specific residues of other proteins, altering their function. Some of the known PKA targets involve ATPase, aquaporins located in *Trypanosoma cruzi* and DERP, DNA Excision Repair Protein. These are all important for survival of the organism throughout different stages of the life cycle.

The enzyme in inactive state is a heterotetramer with two regulatory and two catalytic subunits each, which will dissociate upon ligand binding, thus freeing the catalytic subunits. Because the catalytic subunits are catalytically active on their own, this dissociation induces PKA activity. Protein Kinases can have affinity for both cAMP and cGMP, and in mammals the specificity is governed by the amino acid residue at position 319 of the regulatory subunit, or nucleotide-binding region. For cAMP specific kinases, the residue is an alanine always, whereas for cGMP the residue is serine or threonine. However, in *T. brucei*, the residue found on this position is a valine. Possible implications of this difference have not been made, but work by Shalaby et al. showed PKA activity that was cGMP dependant. It might therefore be that not only the cyclic nucleotide is important for PKA activity, and that some other (unknown) cofactors have a major role as well. cGMP-dependant activity could thus be explained by cGMP-dependant cofactor production.

PKAs have been showed to be inhibited by PKI (Protein Kinase A Inhibitor), a natural inhibitor with pseudo-substrate functionality. This PKI factor contains a specific R-R-N-A amino acid sequence, which is important for PKA binding. Because the PKI ‘ligand’ lacks a phosphorylatable residue, it is however unable to undergo normal phosphorylation by PKA, thus it resides on the catalytic site of PKA, effectively inhibiting it. This inhibition ultimately
leads to several enzymes or other PKA targets no longer being phosphorylated, causing them to dysfunction. This leads to cell death eventually.

By immunoblotting, the PKA was identified in both procyclic and bloodstream forms of *T. brucei*, with procyclic forms showing higher levels. In *T. cruzi*, the PKA was identified in all life cycle stages, although in different concentrations.

In other experiments, a number of (putative) genes with phosphorylatable sites were identified in *T. cruzi*, among which are some that code for proteins of high importance to the regulation of the cell and life cycle of *T. cruzi*. They are a PI3-kinase associated with immune system escape, a phosphodiesterase important for maintaining cAMP levels, a protein kinase, an ATPase and an aquaporin, important to maintain the cell volume. This diverse set of genes associated with PKAs is a clear demonstration of the fine interdependent relation between all the factors from the signal transduction pathways of cAMP, as well as its effectors.

5.4 Phosphodiesterases

In the chain of cAMP signalling, proteins like adenyl cyclases and Protein Kinases have important roles. Yet, the signal transduction system would not function without a system for self-containment. This is the role of proteins called phosphodiesterases. Phosphodiesterases, or PDEs, bind cyclic nucleotide molecules like cAMP or cGMP, and hydrolyse their 3'-phosphorus-oxygen bond, but leave the 5'-P-O bond intact, hence the name diesterase. This hydrolysis terminates the signal transduction functionality of the cAMP or cGMP molecule, and thus prevents an overload of signals building up inside the cell.

![Figure 12: Hydrolisis of cAMP yields 5'-AMP](image)

5.4.1 General structure and function of PDEs

Phosphodiesterases are common proteins in a wide variety of organisms. They are found in both mammals and bacteria, and perform similar reactions in every species. However, phosphodiesterases can be divided into at least three different classes, based on homologies (or differences) in amino acid sequence. Also, PDEs may be classified in different families when multiple forms are found within one species. For example, human PDEs are divided into 11 different families, based on substrate specificity, localisation and biological impact (or its roles). In humans, both cAMP and cGMP specific phosphodiesterases have been discovered, localised throughout the body. The best example of a cGMP specific phosphodiesterase in humans is hPDE5, which is inhibited by sildenafil, and is situated in intestinal tissues. It is perhaps the most famous human PDE, for it is targeted by Viagra (sildenafil) to treat male erectile dysfunction.
Compared to human phosphodiesterases, the number and isozyme-forms of *Trypanosoma* PDEs is much smaller. There are only four families known to date, compared to 11 for humans, and the number of identifies isozymes is also significantly lower, with no family containing more than three identified isozymes. The main task of PDEs is the same in the trypanosoma parasites as it is in humans, i.e. hydrolyzing the cyclic nucleotide messenger molecule. However, trypanosoma parasites are much smaller organisms than humans, and thus require less site-specific enzymes. In trypanosomatids, the main role of the identified phosphodiesterases is to either control intracellular levels of cAMP or process rising levels of cAMP from the outside of the cell. The intracellular PDEs are localised in such a way that the cell is ‘compartmentalised’ to prevent flooding with cAMP. The cell volume is so small that a mere 30,000-40,000 molecules of cAMP are sufficient for the signal transduction pathways to provide the entire cell with cAMP messages.

5.4.2 Specific domains present in trypanosomal PDEs

![Figure 13: A very simplified representation of phosphodiesterases in *trypanosoma* parasites. On the left, a representation of TcrPDEC, on the right a representation of all other PDEs. The N-terminus, in green, is responsible for proper localisation of the protein. The red and pink GAF-domains are for substrate recognition and binding, and thus affect activity and selectivity. In TcrPDEC (l), the yellow FYVE domain is also involved in localisation and activity. The true catalytic site is located midway through the protein, in blue. In other PDEs than TcrPDEC (r), no FYVE-domain is found, and the catalytic site is located at the C-terminus of the protein.](image)

Phosphodiesterases are in general non-specific enzymes. They are part of a signal transduction system which is widely used, and to achieve this amount of versatility, simple molecules are most convenient. Although the cyclic nucleotides are greatly limiting the complexity of the system, a lot of fine tuning is done at the production (adenyl/guanydyl cyclases) and termination ends of the transduction line. In case of phosphodiesterases, the first piece of fine tuning is localisation. For example, the two isozymes of the phosphodiesterase B from *Trypanosoma brucei* (TbrPDEB1 and TbrPDEB2) are located in different parts of the parasite. The TbrPDEB1 enzyme can only be found in the flagellar region of the parasite. The isozyme TbrPDEB2 however, is located mainly in the cell body, and is found in only slight amounts in the flagellar region. PDEB proteins are only active when in dimer form. The formation of heterodimers of TbrPDEB1 and TbrPDEB2 explains why the TbrPDEB2 is also found in the flagellum The specific localisation of TbrPDEB1 is regulated by one of the
functional domains of the phosphodiesterases, namely the N-terminus, which is the ‘start’ of a polypeptide chain (see figure 13, the green parts). In the case of TbrPDEB1, the N-terminus contains a specific amino acid sequence with a T28E29 (Threonine at position 28 and Glutamic Acid at position 29) motive present. Upon mutation, turning the T28E29 into two prolines, the helix that was located in this part of the N-terminus was ripped apart.57 This change in 3D configuration also changed the localization of the enzyme, as it ‘moves’ from the flagellum to the cell body, although remaining near the flagellum. Unfortunately, the mutation experiments only reported the effects of proline introduction, whereas other amino acids with a less pronounced impact on the 3D structure were not tested. For the other isozyme, mutations at two specific points in the polypeptide (L10 and Y45 L=Leucine, Y=Tyrosine) were shown to have a similar, yet opposite effect. Normally, the TbrPDEB2 localizes to the cell body, but upon the mutations, the isozyme becomes located at the flagellum. However, it is not incorporated into the flagellar skeleton, shown by the fact that TbrPDEB2* (the mutated form) is still extractable by Triton X, whereas the normal TbrPDEB1 is also located at the flagellum, but not extractable. It was found that the first 70 amino acids play an important role in localisation by experiments with GFP (green fluorescent protein) constructs of a polypeptide of the first 70 amino acids from different trypanosomal species.57 These constructs located and integrated into the cell exactly like their parent proteins did, showing the importance of the first 70 amino acids. This is also supported by the mutation sites discussed, which are all located in the first 70 amino acids of the protein.

Although localisation of PDEs is an important part of the fine tuning in the cAMP/cGMP based signal transduction systems, there are examples where different localisation is no issue. The best example is given by the TbrPDEB1 and TbRPDEB2 (again). These enzymes were found to be completely compatible with each others tasks, by performing inhibition and knock-down studies.20,58 In these studies, only one isozyme was selectively inhibited or knocked down, either by RNA interference or by mutations (scission of the specific genes or alleles). It was shown that this procedure did not influence the phenotype of the parasite cells. However, inhibition or knock-down of both isozymes at the same time resulted in cell death. These studies show that one isozyme is able to take over the roles of the other in case it gets knocked down. Although the localisation of the isozymes is different, this did not have any noticeable effect on the parasites, which shows that, at least in small organisms, localisation is more important for fine tuning than it is for environment control.

The domain responsible for the binding of the cyclic nucleotide, and therefore the selectivity of the phosphodiesterase, is called the GAF domain. In figure 13, the GAF domains are depicted in red and pink. The name GAF domain comes from proteins it is found in (cGMP-specific phosphodiesterases, Adenyl cyclases and FhIA, a bacterial transcription factor50). However, because GAF domains are also encountered in cAMP specific phosphodiesterases (e.g. those in trypanosomatids), the name is not entirely correct. GAF domains have been identified in many human PDEs, in which they exhibit different cyclic nucleotide affinities. Some of the human PDEs are cGMP selective, others only hydrolyse cAMP, and a couple of human PDEs have similar affinities for both cAMP and cGMP. In trypanosoma parasites, the GAF domain has only been identified in PDEBs of Trypanosoma brucei and cruzi. The first of these two is best investigated. Trypanosoma brucei has been shown to contain two GAF domains (GAF-A and GAF-B), of which GAF-A is the regulatory subunit, and GAF-B is the sensor for cyclic nucleotides.

GAF domains have certain amino acid called ‘critical residues’ for binding of cGMP, these are aspartate-289 and a phenylalanine residue.47 However, these residues are also found in GAF domains of parasite phosphodiesterases, which convert cAMP instead of cGMP. This
indicates an important role for the rest of the protein, instead of just the GAF domains determining affinity and selectivity.

GAF domains have furthermore been suggested as allosteric regulation sites. In affinity studies of phosphodiesterases against cAMP and cGMP\textsuperscript{51}, it has been shown that the catalytic domain of PDEs have affinity for either cAMP or cGMP over the other, which was increased when testing the whole protein. Here might be a link to the fact that cAMP levels act as allosteric factors for human PDE inhibition by PDE-specific drugs. In both cases, the cAMP or cGMP available would have cooperative interactions with the protein, presumably via the GAF domain.

GAF domains provide the regulation and binding of cAMP to the phosphodiesterases of the parasite, making sure the substrate is close to the catalytic domain of the protein. The catalytic domain is located near the C-terminus in most of the parasitic PDEs. However, PDEs type C in \textit{T. brucei} and \textit{T. cruzi} have their catalytic domain situated in the middle of the protein chain.\textsuperscript{28,55}

Recent computational studies have developed more insight into the ‘size’ of the catalytic pocket of the protein. It has been shown that, although human and parasite PDEs are very similar, they differ substantially near the catalytic domain.

In the parasite PDEs, a ‘pocket’ is open, whereas in the human PDEs, this same region is closed by the amino acids in the chain. (figures 14 and 15) The open ‘P-pocket’ (P from parasite) encountered in the parasite’s PDEs has the opportunity of allowing bigger molecules into the catalytic pocket than human PDEs would do, which would allow for selective inhibitors of parasite’s PDEs.

The final functional domain is the FYVE domain (figure 13, the yellow part of the left picture), which can only be found in \textit{T. cruzi} PDEC. The FYVE domain is 60 amino acid residues long, is stabilised by Zn\textsuperscript{2+} ions and recognises Phosphatidylinositol-3-phosphate in membranes, which makes it a possible localisation regulating domain. Beside localisation, the FYVE domain also enables dimerization of the TcrPDEC enzyme. The use or need of this phenomenon is unknown, although substrate recognition may require the catalytic site or the entire enzyme to be dimerized. Docampo \textit{et al.} concluded in 2011 that the enzyme TcrPDEC is essential for \textit{T. cruzi} epimastigote form survival and plays an important role in the contractile vacuole complex volume recovery after osmotic shock.\textsuperscript{28}
Figure 14: A representation of the P-pocket in trypanosomal PDE catalytic site simulations. On the left is trypanosomal PDEB simulated, on the right is mammalian PDE4 (the off-target).\textsuperscript{45}

Figure 15: Another representation of the P-pocket, more zoomed in and from a different angle. Also, a different inhibitor is used in the simulation.\textsuperscript{46}
6. Discussion

The signal transduction in trypanosomes has become clearer in the last 10-15 years. Understanding of the signal transduction has improved much, with increased knowledge on specific proteins involved. The localisation of different proteins has been shown to depend on the amino acid sequence in the N-terminus of the protein. This has been shown by mutation experiments incorporating proline residues into the protein, and although prolines make a very different 3D structure due to the ‘bend’ or ‘turn’ they introduce, it has been shown to completely change the localisation for both isozymes of TbrPDEB1 and TbrPDEB2, effectively switching their location. Intuitively, such a disruption of 3D structures could lead to loss of discrimination in localisation, but because the localisation of the proteins is switched rather than ruined, the obtained results are still valuable.

Also, the knowledge of GAF-domains and PDE activity has improved, which ultimately led to the first new compounds that show PDE inhibition, and could go on to become fully approved drugs. The use of computer models for protein simulation has identified a ‘subpocket’ near the catalytic domain of trypanosomal PDEs, which could fit molecules that human PDEs could not fit. This, and extensive research into the PDEs functional domains, has made these results possible. It is likely that more results will follow in the coming years, with more crystal structures becoming available, also for proteins related to TbrPDEs, like TcrPDEC and others.

7. Additional information on Trypanosoma parasites

The signal transduction within trypanosomes is important for the development of new drugs, but is not the only field in which disease-fighting progress is made. For example, the mechanisms the parasite carries to avoid the hosts immune system may also be of high importance in the battle against parasite-caused diseases. Therefore, although slightly off-topic to the signal transduction, some information on these fields is provided below.

7.1 Variant Surface Glycoproteins and Immune system evasion.

When mammals become infected by parasitic organisms, their immune system will try to get rid of this infection as soon as possible. To avoid the immune system of the host, parasitic organisms like Trypanosoma brucei and T. cruzi have developed different mechanisms.

Trypanosoma cruzi avoids the immune system by entering (blood) cells of the host, thus preventing recognition by antigen-based immune systems. However, trypanosoma brucei has a different mechanism. It stays in the blood and lymph circulation after infection, and may even enter the cerebro-spinal fluid. Therefore, exposure to antigen-based immune systems is constant, and the protection against it is delivered by the variant surface glycoprotein coat. This coat hides away the invariant surface antigens from the parasite, and expresses many millions of copies of one single glycoprotein, with constantly changing antigenic specificity. This ensures evasion of the B- and T-cell based immune system from the host, since the antigenic recognition is no longer available after the recognition site has been changed. Because of this, prolonged infection is ensured, and chances of survival and infection of new hosts are increased.32
The variant surface glycoprotein (VSG) is a glycoprotein, anchored by glycosyl-phosphatidyl inositol (GPI), of around 60 kDa and two functional domains. The N-domain is rod-shaped and exposes some variable loops at the surface of the VSG layer. On these loops, the antigenic recognition site of the protein is located. The true nature of this recognition site is governed by only a few key residues, but almost 1700 different possibilities exist. The N-terminus shows only very limited identity between different VSGs, but considering the mode of action of the VSG, this is no surprise. The C-terminus, on the contrary, is more conserved, and contains a number of cysteine moieties that form disulfide bridges. Also, the C-terminus is anchored to the plasma membrane by a GPI-bridge. The region between the N- and C-termini is made by a region that is very prone to proteolytic cleavage.33

From the 1700 possibilities, the majority are pseudogenes, which are essentially misshaped genes, which for example lack promoters, have premature stop codons or other defects. However, recombination of the pseudogenes with active VSG genes allows for accessibility of the pseudogenes for antigenic variation. The VSGs are all situated near the telomeric parts of the chromosomes. Many are found at the extremities of the telomeric regions, but most have been identified in clusters at the subtelomeric arrays. Variant Surface Glycoproteins are only transcribed by large VSG-containing chromosomes, with a VSG expression site at one of their telomeres, however, smaller chromosomes co-exist, but are believed to only provide many different VSGs. The most important part of VSG transcription and expression is the activity of the VSG expression sites. Only one of the identified 15 will be fully active at any time, allowing for the expression of only one type of surface protein, to provide the protective monolayer. Although multiple VSGs are initially transcribed simultaneously, only one of the VSG expression sites will be fully transcribed. The mechanism of this transcription, and especially the factors that decide which VSG to transcribe, are unknown. The VSG that is turned ‘on’ can switch every instant, and the timing, or probability of a switch, is partly determined by the sequence homology between recombining (pseudo)genes, in which a better homology leads to increased probability of recombination, thus increasing the chance the VSG will be changed.32,33

Humans are one of the few mammals that have developed immunity against Trypanosoma brucei brucei, the prototype of African trypanosomes. Human blood contains a lytic factor against trypanosoma brucei species, which was identified to be apolipoprotein L-I (apoL-I).48 This protein enters the trypanosome in an unknown way. The protein is targeted to lysosomal membranes by a pH-sensitive domain, where it triggers a chloride ion flux, resulting into osmotic swelling of the lysosome. The pressure that is built up by this swelling will compromise the membrane, which results into cell death. Unfortunately, the Trypanosoma brucei gambiense and T. b. rhodesiense have acquired resistance against this lytic protein, allowing them to cause African sleeping sickness in respectively Western and Eastern Africa. In T. b. rhodesiense, resistance to the human lytic protein was found to be linked to antigenic variation by a specific VSG expression site that is selected during residence in human blood. This expression site is the only one to contain one or multiple genes associated with serum resistance. More intriguingly, this specific resistance-causing expression site was only selected when the parasite is in contact with human serum. Laboratory tests have shown that T. b. rhodesiense cultured inside animal tissue does not express the specific resistance-related gene, and is therefore sensitive to the lytic properties of apoL-I. This mode of action indicates that T. b. rhodesiense normally has its natural propagation in animals, but has mechanisms to survive in humans as well.

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The specific gene identified for resistance against human trypanolytic factors was transfected into T. brucei brucei and showed to render this transfected species fully resistant
against human serum, whereas its original, non-transfected counterpart would effectively be removed by apoL-I.

7.2 The importance of vector control

Although an increasing amount of knowledge on trypanosome parasites has been gained and drug research programs have emerged in numbers, one of the important parts of the WHO program to tackle the diseases caused by these parasites is vector control, or stopping the infected bugs that carry the parasites from biting and infecting humans. By successfully implementing vector control, the WHO hopes to break the vicious circle of poverty in which the affected regions live. To reach this goal, the WHO and partners provide local communities in Africa and Latin America with education on both personal hygiene and the use of insecticides. By providing mosquito nets, sufficient knowledge about bugs and insecticides, plagues and swarms are controlled, and local people are better able to prevent getting bitten by trypanosomatid-carrying vectors. Also, if the livestock is treated equally well (and thus gets infected less), an increase in welfare may be expected, resulting in more income and resources to provide a family with medical care. The project that deals with this education and prevention started in 2009 and is expected to show its effects in the coming years.

7.3 Research on malaria parasites

One of the advantages of vector control is that it applies to more than only trypanosomiasis diseases. It also has a great beneficial effect on the most devastating parasitic disease, malaria. Malaria is the only disease beside the trypanosomal ones that is somewhat investigated. Knowledge of the Plasmodium parasites (that cause malaria) on the subject of signal transduction is limited. It is believed that plasmodium parasites make use of similar systems as their trypanosome counterparts, based on the identification of multiple PKA and putative PDE genes. However, the exact build-up of the signal transduction in plasmodium is unknown. Also, the parasites are evolutionary unrelated, they differ already on the Kingdom level, with chromalveolata (malaria) versus excavata (trypanosomiasis diseases) under the Eukaryote domain. Although the parasites are thus unrelated and may differ substantially in their signal transduction systems and proteins involved, the compounds created and tested against trypanosoma parasites are mostly tested against plasmodium parasites as well. This is likely only to rule out the possibility of a missed hit, in case the compound is inactive against T. brucei and/or T. cruzi, but not against Plasmodium species. However, this approach has not yet yielded any hit compounds.
8. Conclusions

Investigations of signal transduction in *Trypanosoma brucei* and *cruzi* parasites has come a long way since it started, and especially after 2000 a lot of progress has been made. Several Adenyl Cyclase, Protein Kinase and Phosphodiesterase structures have been identified to all play a vital role in the parasites, as well as organelles like the acidocalcisome. New possible targets for drug development have been identified, including the first report of tetrahydro and hexahydro phthalazinone derivative inhibitors against TbrPDEB1 and TbrPDEB2. More is likely to follow in the coming years. Phosphodiesterases have proved both an important protein for parasite survival (including precise activity and localisation regulation) and for drug development, as the first new lead compounds are all derived from potential PDE inhibiting structures.
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