



Fred R. OPPERDOES, Member
Paul A.M. MICHELS, Member



Véronique HANNAERT, Assistant Member
Jean-Pierre SZIKORA, Assistant Member
Muriel MAZET, FNRS Postdoctoral Fellow
Shreedhara GUPTA, de Duve Institute
Postdoctoral Fellow
Ana BRENNAND, Graduate Student
Melisa GUALDRON, Graduate Student
Nathalie GALLAND, Graduate Student
Emilie VERPLAETSE, Graduate Student
Johan DE JONCKHEERE, Guest
Investigator
Morena MAGNANI, Visitor (until
September 2009)
Simone PIERETTI, Visitor (from September
to December 2009)

Freddy ABRASSART, Technician
Nathalie CHEVALIER, Technician
Françoise MYLLE, Secretary

METABOLIC COMPARTMENTATION IN TRYPANOSOMES

Trypanosomatidae are parasitic protists that cause sleeping sickness, Chagas' disease and leishmaniasis in man, diseases that severely affect millions of people in tropical and subtropical parts of the world and cause over a hundred thousand of deaths each year. There is an urgent need for more efficacious and less toxic drugs than those currently in use. By molecular and cell biological investigation of these parasites we intend to provide a basis for the development of such new drugs. Trypanosomes rely on glycolysis for their ATP supply and are characterized by a unique form of glycolytic compartmentation where the majority of the enzymes of this pathway are sequestered inside peroxisome-like organelles called glycosomes. In the past we have validated many of the glycolytic enzymes as potential drug targets. Their structural information is used for the discovery of effective and selective inhibitors. Glycosome assembly and degradation, taking place during cell differentiation, are being studied as well. Many so-called peroxins, proteins involved in glycosome biogenesis, have also been identified and validated as excellent drug targets. Moreover, the availability of the genome sequences of three trypanosomatids in combination with various proteomic approaches has allowed to make a comprehensive inventory of the metabolic capacities of these organisms and to identify other essential differences between the respective parasites and their human host. Other potential drug targets that have been identified are the pentose-phosphate pathway, lipid metabolism and the biosynthesis of biopterin and reduced folate

METABOLIC PATHWAYS, ENZYMES AND DRUG DISCOVERY

Systems biology and glycolysis in glycosomes

P. Michels, in collaboration with H. Westerhoff (Vrije Universiteit Amsterdam, The Netherlands) and B. Bakker (Rijksuniversiteit Groningen, The Netherlands)

Trypanosoma brucei, the causative agent of sleeping sickness, is transmitted between mammalian hosts by tsetse flies. Glycolysis is the sole free-energy source for the parasite living in the mammalian bloodstream and is therefore a promising drug target. Previously, we demonstrated the importance of a systems biology approach for drug target validation when we investigated the contribution of each enzyme of the pathway to the control of the glycolytic flux. We determined that glucose transport into the cell exerts high control and hence the transporter is a promising target. In general, there is little awareness that the composition of a metabolic network may change in response to drugs. If the response is homeostatic (e.g. through upregulation of the target protein), this may neutralize the initial inhibitory effect. In this scenario the effect on cell growth and survival would be less than anticipated based on the affinity of the drug for its target. By our systems approach we recently showed that inhibition of the glucose transporter at high inhibitor concentrations causes cell death, as expected, but interestingly sublethal concentrations initiate a domino effect in which the metabolic network adapts via respectively (i) partial inhibition of the flux-controlling target protein, (ii) down-regulation of the expression of the target protein and other proteins in the same metabolic pathway and (iii) differentiation of the cells leading to expression of metabolic enzymes and immunogenic coat proteins characteristic for the procyclic life-cycle stage of trypanosomes living in the tsetse fly midgut

and not viable in humans. This ‘anti-homeostatic’ response may offer a possibility for efficient killing of parasites at an acceptable drug dosage.

Glycolytic enzymes

V. Hannaert, P. Michels, in collaboration with L. Gilmore and M. Walkinshaw (University of Edinburgh, Scotland)

Previously, we have expressed and kinetically characterized trypanosomatid enzymes for all steps of the glycolytic pathway. Structures of most of these enzymes have become available through our collaboration with protein crystallographers elsewhere. Notably, coworkers at the University of Edinburgh have determined the structures of *T. brucei* phosphofructokinase, phosphoglycerate kinase and *Leishmania mexicana* phosphoglycerate mutase and pyruvate kinase with and without various ligands. This has provided insight into mechanisms of catalysis and in the conformational changes required for catalysis by these enzymes. Inhibitors are identified by using the enzymes in high-throughput screens of available large libraries of drug-like compounds and specifically designed libraries and by structure-based design and synthesis. To date, a series of hit compounds have already been found. Some of them inhibit growth of cultured bloodstream-form trypanosomes at concentrations in the micromolar range with no or less effect on cultured human fibroblasts. Detailed analysis of the structure-activity relationship of these compounds is now being used for improving the inhibitors by following medicinal chemistry principles to arrive at leads for anti-parasite drugs.

Pentose-phosphate pathway enzymes

S. Gupta, P. Michels, in collaboration with A. Cordeiro (Universidade de São Paulo, Brazil)

The pentose-phosphate pathway supplies the cells with precursors of nucleotides for DNA and RNA synthesis and the reduced co-factor NADPH for biosynthetic processes and

protection against oxidative stress. By knocking down the expression of the first enzyme of the pathway, glucose-6-phosphate dehydrogenase (G6PDH), by RNA interference (RNAi) growth of bloodstream-form *T. brucei* is slowed down and eventually the parasites die. Moreover, cells in which the G6PDH has been partially depleted by RNAi are more susceptible for oxidative stress caused by H₂O₂.

We have shown that G6PDH of *T. brucei* and *Trypanosoma cruzi* is uncompetitively inhibited by the human steroids dihydroepiandrosterone and epiandrosterone, with K_i values in the low-micromolar range. In contrast, *L. mexicana* G6PDH is not inhibited. Viability assays demonstrated that both steroids stunt growth of cultured *T. brucei* bloodstream-form cells, but not *Leishmania* cells. Importantly, trypanosomes became unsusceptible to the inhibitors when induced to express a transgene of *L. mexicana* G6PDH. Together these findings identified G6PDH as a drug target in trypanosomes and provide prospects for using the steroids to develop leads for of a new class of anti-trypanosomatid compounds.

Hexokinase (HK), the first enzyme of the glycolytic pathway, of trypanosomatids is located in their glycosomes. In addition, a glucokinase (GlcK) was found in the glycosomes of *T. cruzi* and *Leishmania* species, but not in *T. brucei*. The crystal structure of TcGlcK with bound glucose showed that the sugar was present in its β-anomeric form; this preference was confirmed in activity assays. In contrast, all known HKs have a preference for the α-glucose anomer. The enzymes following the HK in the glycolytic and pentosephosphate pathways, glucose-6-phosphate isomerase and G6PDH, are highly specific for respectively the α- and β-form of the glucose 6-phosphate. The presence of both a HK and GlcK in glycosomes could be related to these different specificities. In *T. brucei*, where GlcK is absent, a glucose-6-phosphate-1-epimerase (G6PE) would be required for the anomerization. Indeed, a homologue of yeast G6PE, with a glycosome-targeting signal, was identified in the

trypanosomatid genome databases. The activity was confirmed with recombinant G6PE. Knocking down its expression in bloodstream-form trypanosomes has no effect on their growth in regular HMI-9 medium. However, the cells are susceptible to oxidative stress in a non-reducing medium, very similar to results obtained for trypanosomes in which G6PDH has been depleted. These results suggest a role for G6PE in making glucose 6-phosphate available for the pentosephosphate pathway and NADPH production.

Fatty-acid desaturases

S. Gupta, M. Gualdrón, P. Michels, in collaboration with A. Uttaro (Universidad Nacional de Rosario, Argentina), P. Wallemacq (LCBM, UCL) and J.-P. Deboux (ANIM, UCL)

Both procyclic and bloodstream-form *T. brucei* are capable of *de novo* synthesis of fatty acids and the process is essential for parasite survival. Polyunsaturated fatty acids (PUFAs) are synthesized by enzymes known as desaturases. Two desaturase enzymes were identified in *T. brucei*: Δ9 desaturase that synthesizes oleate (C18:1Δ12) from stearate (C18) and Δ12 desaturase that converts oleate into linoleate (C18:2Δ9,12). Knocking down the expression of these desaturase enzymes by RNAi, in both procyclic and bloodstream-form *T. brucei*, caused a growth phenotype and also exerted a significant effect on the total fatty-acid composition of the parasite. Isoxyl and 9-thiostearate, known Δ9 desaturase inhibitors, showed an inhibitory effect on the growth of in vitro cultured bloodstream-form trypanosomes with EC₅₀ values of 0.1 μM and 1 μM, respectively. Moreover, in a preliminary experiment a significantly reduced parasitaemia was observed by treatment of *T. brucei* mice infected with Isoxyl. Two Δ12 desaturase inhibitors, 12- and 13-thiostearate, totally inhibited parasite growth with EC₅₀ of 2 μM and 7 μM, respectively. The results suggest that Δ9 and Δ12 desaturase are essential for both procyclic and bloodstream-form *T. brucei*. The complete

absence of $\Delta 12$ enzyme activity in mammalian cells and the significant structural differences between trypanosome and mammalian $\Delta 9$ desaturases, highlight these enzymes as promising targets for selective chemotherapeutic intervention against the parasitic disease.

Glycosomal solute transporters

M. Mazet, P. Michels, in collaboration with P Wallemacq (LCBM, UCL)

Previously, three half-size ABC transporters, designated GAT1-3, have been identified in the glycosomal membrane of *T. brucei*. GAT1 and GAT3 are expressed in both bloodstream and procyclic-form trypanosomes, whereas GAT2 is only present in bloodstream-form cells. In order to study the function of the transporters, procyclic RNAi cell lines for depletion of both GAT1 and GAT3 have been created. Expression knockdown of GAT1 and GAT3 resulted in a growth phenotype that is dependent on the nutritional conditions of the trypanosomes. In the presence of glucose, growth is not affected. When, however, for the GAT1 RNAi cell line, no glucose is available and proline forms the predominant source of free energy, the growth rate is reduced and eventually the trypanosomes die. Glucose-grown cells depleted of GAT1 show a significant increase in the content of the fatty-acid linoleate (C18:2). We hypothesize that GAT1 is fatty-acid transporter, like some of its homologues in the peroxisomal membrane of yeasts and mammalian cells. The fatty-acid uptake into glycosomes may be important either for the synthesis of ether-lipids, a process that is crucial for cells, and/or for β -oxidation. Previous work in TROP, involving cell fractionation in conjunction with enzyme activity assays, indeed showed the probable association of enzymes of both processes with glycosomes.

To address the question if GATs are involved in glycosomal ether-lipid synthesis, the RNAi cell lines were used that we have created for knocking down the expression of the

cytosolic enzymes $\Delta 9$ desaturase and $\Delta 12$ desaturase. Partial depletion of the respective desaturases caused the expected changes in cellular levels of their fatty-acid substrates and products. Interestingly, partial depletion of the $\Delta 9$ enzyme resulted in a decrease of GAT1 mRNA levels, whereas partial depletion of $\Delta 12$ desaturase caused an increase of the GAT1 transcript. This result strongly suggested that GAT1 is an oleate transporter and that oleate may be incorporated into ether-lipids. Relevant in this respect is that $\Delta 9$ desaturase depletion led also to a slight decrease of the ether-lipid biosynthetic enzyme dihydroxyacetone-phosphate acyltransferase. The notion that GAT1 is an oleate transporter has been tested. To that end, glycosomes purified from procyclic trypanosomes were incubated with radiolabelled oleate. Indeed, a time-, temperature-, oleate concentration- and ATP-dependent uptake of the fatty-acid was measured.

GLYCOSOME TURNOVER

Glycosome biogenesis in *Trypanosoma brucei*

M. Gualdrón, N. Galland, E. Verplaetse, P. Michels

So far, we have identified 11 proteins called peroxins (acronym PEX) involved in glycosome biogenesis in *T. brucei*; 10 of them are involved in the import of organellar matrix proteins. Using RNAi, we have shown the essentiality of most of the currently known peroxins for the viability of both cultured bloodstream and procyclic-form trypanosomes. In previous years, our work has been mostly focused on PEX5, 7, 13 and 14. PEX5 and PEX7 are unrelated cytosolic receptors for glycosomal proteins with a C-terminal and a N-terminal peroxisome-targeting signal (PTS1 and PTS2), respectively. PTS-bearing proteins associate with these receptors in the cytosol followed by interaction of the charged recep-

tor with a membrane-bound docking complex minimally comprising PEX13 and PEX14.

Much of our current research is on the role of PEX4 and PEX22. In yeast and mammalian peroxisomes it was found that receptor PEX5, after delivery of their cargo in the peroxisomal matrix, are recovered in a mono- or di-ubiquitination dependent process and cycled back to the cytosol. Non-recycled PEX5 is degraded in proteasomes after its poly-ubiquitination. PEX4, belonging to the family of ubiquitin-conjugating E2 enzymes, is the peroxin that in yeast is responsible for the monoubiquitination of PEX5. PEX4 is a cytosolic enzyme that in

yeasts and plants is associated with the peroxisomal membrane by binding to the integral membrane protein PEX22. In mammalian cells no PEX4 and PEX22 homologues are present, but there a cytosolic E2 enzyme (UbcH5) is responsible for receptor ubiquitination.

The *T. brucei* candidate PEX4 displays only low overall sequence identity (30%) with yeast PEX4, but has a conserved region near the C-terminus that contains the cysteine residue that is critical for catalytic activity. It is expressed in bloodstream and procyclic-form cells as observed by RT-PCR and western blot analysis. By confocal immunofluorescence microscopy

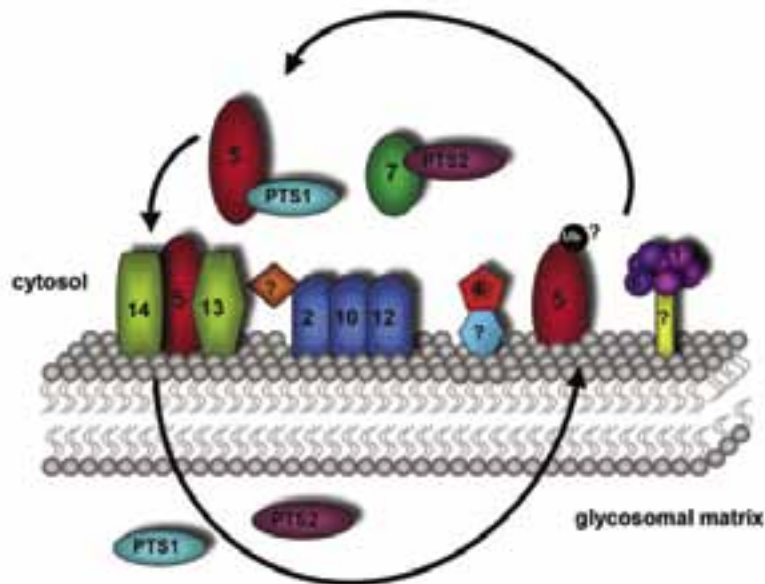


Fig. 1. Model of the import of proteins into the matrix of trypanosomatid glycosomes. Peroxins which have been shown to mediate import of proteins with a type-1 or -2 peroxisome-targeting signal (PTS) into yeast peroxisomes are indicated. The numbered peroxins are the corresponding *Trypanosoma brucei* peroxins identified and currently being studied in the Research Unit for Tropical Diseases. Newly synthesized PTS1 and PTS2 containing proteins are recognized in the cytosol by receptors PEX5 and PEX7, respectively. The cargo-loaded receptors interact with a docking complex (PEX13 and PEX14) at the glycosomal membrane, resulting in the release of the PTS proteins in the organelle's matrix. Receptor PEX5 is cycled back to the cytosol through a ubiquitination process involving E2 ubiquitin-conjugating enzyme PEX4 and E3 ubiquitin-ligase enzymes PEX2, PEX10 and PEX12, as well as AAA-ATPases PEX1 and PEX6.

a N-terminal GFP-tagged PEX4 was shown to be mainly associated with glycosomes of bloodstream-form trypanosomes. Biochemical analysis showed that it was predominantly localized in the membrane fraction of both life-cycle stages and by protease treatment it appeared to be present on the cytosolic face of the organelles. No significant growth phenotype was observed when its expression was partially knocked down by RNAi. However, this partial depletion appeared to affect the abundance of PEX5.

A trypanosomatid orthologue of yeast and plant PEX22 was identified by homology searches in spite of very low sequence conservation. Its partial depletion by RNAi caused some growth retardation in procyclic trypanosomes but not in bloodstream forms. Currently we are developing trypanosome cell lines in which PEX4 and PEX22 can be depleted more severely, for further characterization of their role in glycosome biogenesis. Moreover, additional studies to confirm the glycosomal localization of PEX22 are in progress.

Glycosome degradation in *Trypanosoma brucei*

A. Brennand, P. Michels, in collaboration with E. Pays (Université Libre de Bruxelles) and D. Rigden (University of Liverpool, UK) and M. Ginger (Lancaster University, UK)

Trypanosomes encounter highly different environments during the successive stages of their life cycle and have to adapt their metabolism accordingly. Previously we have provided strong indications that, when the trypanosomes develop from the bloodstream form into the procyclic insect form, the adaptation involves a drastic degradation of glycosomes by a selective form of autophagy called pexophagy and the synthesis of new glycosomes with a different repertoire of metabolic enzymes. A previously performed bioinformatics analysis enabled us to identify in the trypanosomatid databases orthologues of about 20 of the approximately 40

ATGs (= AuTophagy-related proteins) known to be involved in autophagy in yeast, suggesting a functional but highly streamlined version of the process in these parasites. Also orthologues specifically required for pexophagy were recognized in trypanosomatids. This bioinformatics analysis was very recently extended to a taxonomically diverse range of other unicellular eukaryotes. This analysis confirmed autophagy as an ancient eukaryotic invention, utilizing a conserved core machinery but also with lineage-specific moderation (specific losses of ATGs) and elaboration (expansion of a paralogous repertoire of some ATGs). This was indeed also observed in trypanosomatids. Some protists seem to have undergone a secondary loss of macroautophagy, the best understood of the autophagy pathways. This is possibly due to adaptation to a very constant

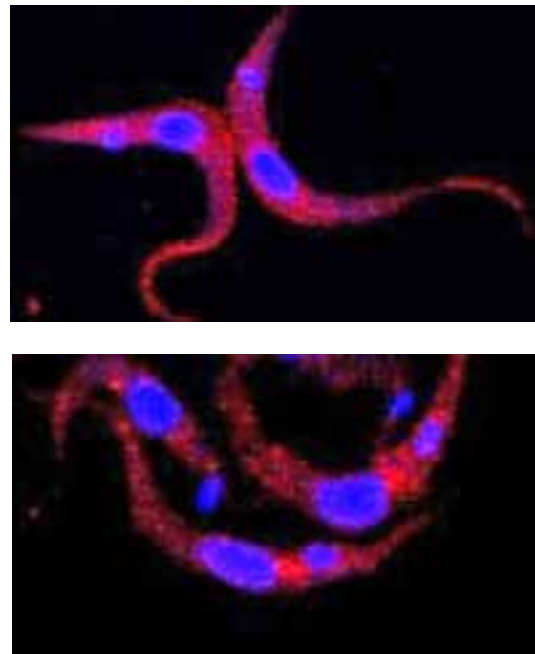


Figure 2. Subcellular localisation of autophagy-related proteins (detected by immunofluorescence; stained in red) in *T. brucei*. (a) cytosolic distribution of TbATG8 in procyclic cells. (b) cytosolic and punctate distribution of TbATG24 in procyclic cells. The puncta are mainly located between the nucleus and kinetoplast (the large and small structures, respectively, stained in blue by TOPRO) and possibly colocalize with components of the endosomal system.

niche, two of the three examples found being parasites with a very simple life cycle and one free-living organism adapted to an extreme environment. Furthermore, although pexophagy is a conserved process in all organisms having peroxisomes, it seems to involve at least some proteins that are not widely conserved.

Several of the *T. brucei* homologues of yeast proteins involved in autophagy, notably that of peroxisomes, were cloned and analyzed: VPS34, ATG7, ATG8, ATG24 and VAC8. When autophagy was induced in procyclic cells by nutrient deprivation, the putative ATG8 was found in punctuate structures reminiscent of autophagosomes, but only a rather disperse ATG7 signal was recognized. For different pexophagy-related proteins (ATG8, VAC8, ATG24 and VPS34) bloodstream-form and procyclic RNAi-mutant cell lines have been constructed and analyzed under standard culturing conditions and under conditions where an increased rate of glycosome turnover is expected. RT-PCR experiments confirmed in each case a decrease of the respective mRNA level upon induction of RNAi. However only a partial depletion was achieved and under those conditions no important growth phenotype was observed. We are currently repeating the experiments with cell lines transfected with RNAi-constructs prepared with a different vector in which we expect a more efficient decrease of the targeted mRNAs.

GENOME ANALYSIS

F. Opperdoes, in collaboration with J. de Jonckheere and M. Ouelette, Quebec, Canada

The amoeboid flagellate *Naegleria gruberi* is a free-living protist which belongs to the phylum Heterolobosea, to which also the Euglenozoa (with the euglenoids and the Trypanosomatidae) belong. Early in eukaryotic evolution the Heterolobosea separated from all other eukaryotes. While the Trypanosomatidae lost many of their metabolic capacities as the result of an adaptation of the parasite to its host, *N. gruberi* is free living and was expected to provide a

more complete blueprint of the metabolic capacities present in the ancestral eukaryotic cell.

The *N. gruberi* proteome, predicted from 15753 gene models was analyzed and the results are presented here.

Protein sequence analysis revealed that *N. gruberi* has all the necessary enzymes to feed on oligosaccharides as well as extracellular peptidoglycans from bacterial origin. The resulting monosaccharides are metabolized via a slightly modified glycolytic pathway where hexokinase has been replaced by fructokinase and glucokinase and the classical ATP-dependent phosphofructokinase (PFK) by a PPi-dependent PFK. In addition to a pyruvate kinase, *N. gruberi* has a PPi-dependent pyruvate-phosphate dikinase (PPDK), an enzyme that catalyzes the conversion of PEP into pyruvate similarly as pyruvate kinase except that this enzyme utilizes PPi rather than ATP as phosphoryl donor. Due to the absence of fructose-1,6-bisphosphatase the reversed pathway, called gluconeogenesis, utilizes the enzymes PPDK and a reversed PPi-dependent PFK. *N. gruberi* also has a homologue of the glucokinase regulatory protein which in bacteria has been shown to have etherase activity and catalyzes the delactoylation of bacterial N-acetylmureate 6-phosphate. The products are D-lactate and N-acetylglucose. Together with D-lactate dehydrogenase this allows the protist to degrade bacterial mureine to N-acetylglucoseamine and pyruvate.

Although there are no reports in the literature suggesting the presence of peroxisomes in *Naegleria*, 287 protein sequences with a C-terminal peroxisome-targeting signal were identified. Therefore, the presence of peroxisomes, involved in the detoxification of ROS and the degradation of fatty acids is more than likely.

In addition to a full set of enzymes involved in aerobic mitochondrial metabolism, *Naegleria* is predicted to be able to survive under microaerophilic or anaerobic conditions. Electrons may not only be passed on to oxygen but also nitrate and nitrite, as electron ac-

ceptors. Moreover, the organism is capable of malate dismutation and contains a gene coding for a hydrogenase, an enzyme typically found in the hydrogenosomes of anaerobic protists and present in many anaerobic bacteria. The *Naegleria* mitochondrion thus seems to be the long sought intermediate in mitochondrial evolution that unites biochemical properties of both aerobic and anaerobic mitochondria and hydrogenosomes. Although it is not known whether *N. gruberi* is able to thrive under anaerobic conditions, its genome predicts that it should be able to adapt to a great variety of life styles.

A start has been made with the genome project of the lizard parasite *Leishmania tarentolae*.

In order to get a first impression of the metabolic capacities of *L. tarentolae*, over 700 sequences, all of metabolic enzymes from the *Leishmania major* genome database, which were previously analyzed in detail were selected and used in a BlastP search against the *L. tarentolae* predicted proteome. All *L. major* sequences were found to have orthologues in *L. tarentolae*. Also the sequences that previously were found to be specific for *Leishmania* and being absent from e.g. *T. brucei*, are all present in the *L. tarentolae* genome. These include enzymes of sugar metabolism, the urea cycle, folate metabolism and haem synthesis. Therefore, it can be concluded that the metabolic capacities of *L. tarentolae* are essentially identical to that of *L. major*, despite the fact that the natural host of *L. tarentolae* is the lizard rather than the human. *L. tarentolae* therefore may be a good model organism for the study of *Leishmania* metabolism and drug screening.

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Fred Opperdoes

de Duve Institute
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 74 55

[F] +32 02 762 68 53

[E] fred.opperdoes@uclouvain.be

[W] http://www.deduveinstitute.be/trypanosome_metabolism.php

Paul Michels

de Duve Institute
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 74 73

[F] +32 02 762 68 53

[E] paul.michels@uclouvain.be