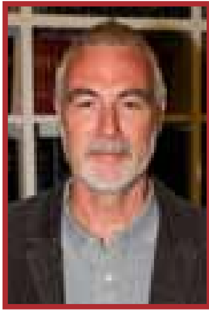

Paul A.M. MICHELS, Member
Fred R. OPPERDOES, Emeritus Member

Véronique HANNAERT, Assistant Member
Jean-Pierre SZIKORA, Assistant Member
Muriel MAZET, FNRS Postdoctoral Fellow
Shreedhara GUPTA, de Duve Institute Postdoctoral Fellow (until November 2010)
Marcelo ALVES-FERREIRA, Postdoctoral Fellow (from August 2010)
Nathalie GALLAND, Graduate Student (until January 2010)
Emilie VERPLAETSE, Graduate Student (until March 2010)
Ana BRENNAND, Graduate Student
Melisa GUALDRON-LOPEZ, Graduate Student
Davide GUERRERI, Visitor (from March to October 2010)
Freddy ABRASSART, Technician
Nathalie CHEVALIER, Technician
Françoise MYLLE, Secretary

METABOLISM OF TRYPANOSOMATID PARASITES AND DRUG DISCOVERY

Trypanosomatidae are parasitic protists that cause sleeping sickness in Africa, Chagas' disease in Latin America and various manifestations of leismaniasis in man, in many tropical and subtropical parts of the world. These diseases threaten a few hundreds of millions of people worldwide, affect millions and cause many thousands of deaths each year. The parasites responsible for these diseases are transmitted between humans by insects: tsetse flies, triatomine bugs and sand flies, respectively. There is an urgent need for new, adequate, affordable drugs to treat these diseases, because those currently in use are often toxic and not efficacious, sometimes difficult to administer or too expensive and increasing resistance has been reported against some of them. By a molecular and cell biological investigation of these parasites we intend to provide a basis for the development of such new drugs. African trypanosomes rely entirely on glycolysis for their ATP supply. Interestingly, trypanosomatids are characterized by a unique form of glycolytic compartmentalization by which 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 potent 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 been identified and also validated as excellent drug targets. Moreover, the availability of the genome sequences of different species of the trypanosomatid parasites causing the three groups of diseases, 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

Glycolytic enzymes

V. Hannaert, M. Mazet, P. Michels, in collaboration with L. Gilmore and M. Walkinshaw (University of Edinburgh, Scotland), A. Cavalli and M.-L. Bolognesi (Università di Bologna), O. Thiemann and A. Cordeiro (Universidade de São Paulo, Brazil) and W. Quiñones, L. Avilán and J.-L. Concepción (Universidad de los Andes, Mérida, Venezuela)

Glycolytic enzymes are very important for all trypanosomatids. For *Trypanosoma brucei*, the parasite that causes sleeping sickness when infecting people in sub-Saharan Africa, glycolysis is even the sole free-energy source when the parasite resides in the human blood. Therefore the glycolytic enzymes of these parasites are considered as promising drug targets. Over the years, we have expressed and kinetically characterized trypanosomatid enzymes for all 10 successive steps of the pathway for breakdown of glucose into pyruvate and two additional enzymes present in the glycosome, glycerol-3-phosphate dehydrogenase and glycerol kinase, which also play a crucial role in this process. Structures of most of these enzymes have become available through our collaboration with protein crystallographers elsewhere. All these analyses have revealed important differences between the enzymes of the parasites and the corresponding ones from the human host, offering prospects for developing parasite-enzyme specific potent inhibitors that may be used as lead drugs.

Recently, most of our work has focused on a few selected glycolytic enzymes: phosphofructokinase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase from different trypanosomatid parasites. The structures of these enzymes have been determined both without and with various ligands. This has provided insight into mechanisms of catalysis and in the conformational changes required for catalysis by these enzymes. Inhibitors for these enzymes are searched by using them in high-throughput screens of available large libraries of drug-like compounds –mainly at the NIH Chemical Genomics Center, Rockville, USA– and specifically designed libraries and designed by structure-based approaches followed by their synthesis. To date, a series of hit compounds have already been obtained. 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. Moreover, our Venezuelan collaborators identified enolase also at the surface of *Trypanosoma cruzi*, the parasite that causes Chagas' disease in Latin-America and *Leishmania* species. In these parasites, which live mainly intracellularly in the human host, this isoform of enolase seems to function as plasminogen receptor, playing a role in the parasites' invasiveness and virulence. This additional location and probable function of enolase offer perspectives to use the enzyme not only as a drug target, but also for vaccination.

For the human pathogenic form of *Leishmania* species not only glucose uptake is important, but also the reverse process of glycolysis, gluconeogenesis; both processes share most of their enzymes. We have also identified and characterized the key enzyme of the gluconeogenic pathway, fructose-1,6-bisphosphatase, of both *T. brucei* and *L. mexicana*. It is present in glycosomes and its activity in *T. brucei* was shown to be silenced by a still unknown mechanism when glycolysis is active.

A small focused library of naphtoquinone-carrying compounds that showed anti-parasite effects in in vitro growth assays with different parasites was synthesized by colleagues at the University of Bologna. Several compounds exhibited potency in the nanomolar range, with up to 80-fold less activity on human cells. By means of a chemical proteomics approach several *T.*

brucei molecular targets of the most promising compound, 2-phenoxy-1,4-naphtoquinone, were identified. Amongst these targets were the glycosomal enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycerol kinase. Subsequently, these two targets were expressed in bacteria and purified, and then used for inhibition assays. The selected compound was able to inhibit both enzymes with IC_{50} values in the low micromolar range. Supporting the notion that GAPDH is indeed an in situ target was the observation that glucose-grown procyclic trypanosomes, for which the GAPDH activity is crucial, were 28-fold more susceptible to the inhibitor than cells relying much less on GAPDH when they use proline as energy and carbon source. Moreover, it was observed that the compound was able to generate oxygen radicals in mitochondrial fractions of bloodstream-form trypanosomes, but much less so in human mitochondrial preparations, probably related to the highly different respiratory systems of both organisms. This mechanism of inducing the production of oxygen radicals could be additionally responsible for the compound's trypanocidal activity. Indeed, we could show that growth of trypanosomes in which glucose-6-phosphate dehydrogenase (G6PDH) was depleted by RNA interference (RNAi), causing them to be susceptible to oxidative stress, was inhibited at about 100-fold lower concentration of the compound than growth of wild-type trypanosomes. Overall, the molecule showed a multitarget mechanism of action, likely forming the basis of its promising anti-trypanosomatid profile.

Pentose-phosphate pathway enzymes

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

The pentose-phosphate pathway (PPP) supplies the cells with precursors of nucleotides for DNA and RNA synthesis and the reduced cofactor NADPH for biosynthetic processes and protection against oxidative stress. By knocking down the expression of the first enzyme of the pathway, G6PDH, by 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 to oxidative stress caused by H_2O_2.

We have shown that G6PDH of *T. brucei* and *T. cruzi* are inhibited in an uncompetitive way by the human steroids dihydroepiandrosterone and epiandrosterone and derivatives, with K_i values in the lower micromolar range. In contrast, *L. mexicana* G6PDH is not inhibited. Viability assays demonstrated that the steroids stunt growth of cultured bloodstream-form *T. brucei* and insect-form *T. cruzi,* but not *Leishmania* cells. Importantly, trypanosomes became unsusceptible to the inhibitors when transfected with the *L. mexicana* G6PDH gene and expressing it. Together these findings identified G6PDH as a drug target in trypanosomes and provide prospects for using the steroids to develop leads for a new class of trypanocidal compounds.

The PPP enzyme ribose-5-phosphate isomerase (RPI) catalyses the isomerization of ribulose 5-phosphate and ribose 5-phosphate, to provide the precursors for nucleotide synthesis. Two non-homologous types of RPI exist in nature: RPI-A, that is broadly distributed among most eukaryotic organisms, including humans and some prokaryotes, and RPI-B that is found in most prokaryotic organisms. Also trypanosomatids possess the isoform RPI-B, making it an interesting potential drug target. The *T. brucei* enzyme was expressed in *Escherichia coli*, purified and functionally characterized. The active form of the enzyme is a homodimer and has a $K_m = 1.26 \pm 0.49$ for ribose 5-phosphate, similar to the affinity of the corresponding enzyme from *T. cruzi* and some bacteria. Site-directed mutagenesis was performed to confirm the predicted catalytic role of residue Cys69. Knockdown of the expression of the enzyme by RNAi showed a slight reduction in growth rate of both the bloodstream and procyclic forms of the parasite under usual culture conditions.

Folate metabolism

S. Gupta, V. Hannaert, D. Guerrieri, P. Michels, in collaboration with S. Ferrari and M.-P. Costi (Università di Modena e Reggio Emilia, Italy) and S. Henrich and R. Wade (Heidelberg Institute for Theoretical Studies, Germany)

Enzymes involved in the provision of reduced folate cofactors, such as dihydrofolate reductase (DHFR) and enzymes that use these cofactors, like thymidylate synthase (TS) have been validated as drug targets for cancer and certain bacterial infections and parasitic diseases such as malaria. When DHFR is inhibited, DNA replication is impaired, resulting in cell death. Trypanosomes are auxotrophic for folates and pterins, and inhibition of the enzymes involved in the salvage pathways like the bifunctional DHFR-TS used by trypanosomatids should provide effective treatment. However, antifolates are currently not employed in therapy of trypanosomatid infections, mainly because of the presence of a pteridine reductase (PTR1) –absent from human cells– that is able to carry out successive reductions of both conjugated (folate) and unconjugated (biopterin) pterins and therefore might provide a bypass for DHFR. While DHFR can only reduce folic acid, PTR1 can act on a broader range of substrates. Under physiological conditions, PTR1 is responsible for 10% of the folic acid required by the cell, but inhibition of DHFR often leads to overexpression of PTR1. It was anticipated that folate analogue inhibitors of *Leishmania* PTR1 are potential drugs for combined therapy with DHFR inhibitors. Based on a virtual screening of the Available Chemicals Directory (ACD) database and the known crystal structure of *L. major* PTR1, followed by two rounds of structure-based design to optimize the compounds, 18 drug-like molecules were identified that displayed low micromolar affinities for *L. major* PTR1 and high in vitro specificity when compared to *Leishmania* and human DHFR. The compounds were tested as growth inhibitors of cultured *L. mexicana* and *L. major* promastigotes and human fibroblasts, without and in combination with the DHFR inhibitor pyrimethamine (PYR). Six compounds showed efficacy in combination with PYR, one was active alone and several compounds showed low toxicity for human cells. Interestingly, one compound, Riluzole, a known drug approved for pathologies of the central nervous system, was active in combination and is suitable for early preclinical evaluation of its potential as an antiparasitic drug. Riluzole is notably effective on proliferating parasites. Moreover, the compound was shown to also increase the parasite's susceptibility to oxidative stress.

Thiolase

M. Mazet, P. Michels, in collaboration with R. Wierenga (University of Oulu, Finland) and F. Bringaud (Université Bordeaux Segalen, France)

Thiolases are enzymes that remove an acetyl-CoA group from acyl-CoA in the catabolic β-oxidation of fatty acids, or catalyze the reverse condensation reaction for anabolic processes such as the biosynthesis of sterols and ketone bodies. In humans, six homologous isoforms of thiolase have been described, differing from each other in sequence, oligomeric state, substrate specificity and subcellular localization. Using the sequences of these six isoforms as queries in searches of databases of African trypanosomes, *T. cruzi* and *Leishmania* species, one of these isoforms (called SCP2-thiolase) was found in each of them, another one (TFE-thiolase) in *T. cruzi* and *Leishmania* and a third one (AB-thiolase) only in *T. cruzi*. The role of the different isoforms in the specific metabolism of each the distinct parasites is a topic of study in the TROP unit. The single *T. brucei* SCP2-thiolase has been expressed as recombinant enzyme in *E. coli*, purified and its activity determined. Interestingly, it shows activity in both the degradative and synthetic reaction, with the latter one being 3-fold higher, whereas the synthetic activity of SCP2 from other organisms studied is negligible. mRNA of the thiolase was detected in both bloodstream form and procyclic trypanosomes, but translation of the transcript into

protein only occurred in procyclics as revealed by western blot analysis. The encoded protein has both a predicted N-terminal signal peptide for routing to the mitochondrion and a C-terminal candidate type 1 peroxisome-targeting signal for sorting it to glycosomes. However, both fluorescence analysis of the thiolase with the Green Fluorescent Protein (GFP) fused to either its N- or C-terminus and cell fractionation by differential centrifugation followed by western blot analysis showed only a mitochondrial localization for procyclic cells, irrespective whether the cells were grown with glucose or amino acids as carbon and energy source. When the expression of the thiolase in procyclic cells was knocked down by RNAi, no significant change in growth rate occurred, whether the cells were grown with or without glucose. This absence of a growth phenotype indicates that the metabolic pathway involving this enzyme is not essential for the parasite under either of these growth conditions. Further research is currently being performed to determine the role of this enzyme in the metabolism of the trypanosomes.

Translocation of solutes across the glycosomal membrane

M. Mazet, M. Gualdrón-López, P. Michels, in collaboration with P. Wallemacq (LCBM, UCL) and V. Antonenkov (University of Oulu, Finland)

The glycosomal membrane of *T. brucei* possesses three half-size ABC transporters, designated GAT1-3. GAT1 and GAT3 are expressed in both bloodstream and procyclic-form trypanosomes, whereas GAT2 is only present in bloodstream-form cells. Expression knockdown of GAT1 and GAT3 by RNAi 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 modification of the total cellular fatty-acid composition; no or only minor changes were observed in the levels of most fatty acids, including oleate (C18:1), but the linoleate (C18:2) abundance was significantly increased. We hypothesized that GAT1 is a fatty-acid transporter, like some of its homologues in the peroxisomal membrane of yeasts and mammalian cells. 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. Cell fractionation in conjunction with enzyme activity assays, indeed confirmed the association of enzymes of both processes with glycosomes. Glycosomes purified from procyclic wild-type trypanosomes incorporate the activated fatty acid oleoyl-CoA in a temperature-,

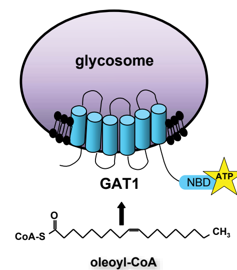

Fig. 1. Model of the half-size glycosomal ABC transporter GAT1 that binds ATP to its nucleotide-binding domain (NBD) to mediate the uptake of oleoyl-CoA into the organelle.

concentration- and ATP-dependent manner, whilst this incorporation was severely reduced in glycosomes from cells in which GAT1 levels had been decreased. This result strongly suggested that GAT1 is an oleate transporter. The increase in linoleate rather than oleate levels upon GAT1 depletion is probably due to the conversion of the latter into the former fatty acid by the high activity of oleate desaturase found in trypanosomes, as described in the report of 2010.

Previously, channel forming activities have been detected for proteins from membranes of mammalian and yeast peroxisomes. We

have now shown similar activities also for glycosomal membrane proteins. When such proteins were solubilized from membranes of highly purified glycosomes and subsequently incorporated into planar lipid bilayers, channels were produced as could be detected by electrophysiological approaches. Preliminary experiments showed the formation of different conductance channels. We hypothesize, by analogy to results obtained for peroxisomes of other organisms, that these channels allow the permeation of small solutes, but restrict the exchange of more bulky compounds such as NAD(H), NADP(H), ADP, ATP, CoA and acetyl/acyl derivatives, etc. Future experiments should lead to the molecular characterization of the channel components and provide information about the assembly and selectivity of the channels.

The metabolic proteome of *Crithidia fasciculata*

F. Opperdoes, in collaboration with S. Beverley (Washington University, St. Louis, USA)

Crithidia fasciculata is a member of the family of Trypanosomatidae and is related to the genera *Leishmania* and *Trypanosoma*. It has a single host life cycle within the mosquito. A limited number of biochemical studies of *Crithidia*, as model organism for the study of the pathogenic trypanosomatids, have been restricted to the cultured choanomastigote stage of the life cycle, which can easily be grown in large quantities in semi-defined culture medium. From these studies it has emerged that *C. fasciculata* has a fully developed mitochondrion with a tricarboxylic acid (TCA) cycle, a complete respiratory chain and it seems to contain essentially the same set of metabolic enzymes as all other trypanosomatids analyzed. Recently its genome has been sequenced and sequence information was placed in the public domain. We have analyzed 406131 translated sequences from open-reading frames and compared them with an in-house database of protein sequences representing 431 metabolic enzymes from previously sequenced trypanosomatid genomes.

The genome analysis of *C. fasciculata* confirms the presence of a fully functional mitochondrion and respiratory chain, as well as the presence of functional glycosomes and a set of peroxins involved in its biogenesis. A classical glycolytic pathway, which partly takes place inside glycosomes, is responsible for the metabolism of exogenous sugars. Carbohydrate metabolism is characterized by an incomplete aerobic oxidation because one of the mitochondrial TCA cycle enzymes is absent and therefore the cycle cannot function as a true cycle. However, the TCA-cycle enzymes present can be used for the interconversion of metabolic building blocks for biosynthetic purposes. Fatty acids are oxidized in the mitochondrion by β-oxidation, while cytosolic fatty-acid synthesis occurs by a number of cytosolic elongases, rather than by Type-I fatty-acid synthetase. Ether-lipid synthesis occurs in part inside glycosomes. Although *C. fasciculata* is able to synthesize its own pyrimidines, it depends on the presence of external purines. It lacks the capacity to oxidize aromatic amino acids and requires an external supply of most of the essential amino acids and vitamins for its growth.

Some trypanosomatids (e.g. *T. brucei*) have an RNAi pathway, while others, such as *T. cruzi and L. major*, have not. The Argonaut protein AGO1 and the Dicer proteins DCL1 and DCL2 are responsible for this activity. In the genome of *C. fasciculata* AGO1 and one of the dicer proteins, DCL2, were detected. A homologue of DCL1 was detectable only at low probability (E = 7e-13). Thus it is likely, but not certain, that *Crithidia* has RNAi capacity.

GLYCOSOME TURNOVER

Glycosome biogenesis in Trypanosoma brucei

M. Gualdrón-López, A. Brennand, P. Michels in collaboration with D. Rigden (University of Liverpool, UK) and R. Erdmann and W. Schliebs (Ruhruniversität Bochum, Germany)

So far, 12 candidate peroxins (acronym PEX), proteins involved in glycosome biogenesis in *T. brucei* have been identified; for the majority of them, their role in glycosome biogenesis has been experimentally confirmed and, using RNAi, their essentiality for the viability of both cultured bloodstream- and procyclic-form trypanosomes has been demonstrated. Most of these peroxins are involved in the transport of newly synthesized glycosomal proteins from the cytosol to the organellar matrix. In recent years, our work has been mostly focused on a detailed investigation of 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 loaded receptor with a membrane-associated docking complex minimally comprising PEX13 and PEX14.

Much of our current research addresses the cycling of PEX5 and the role of PEX4 and PEX22 in that process. In yeast and mammalian peroxisomes it was found that receptor PEX5, after delivery of its cargo into the peroxisomal matrix, is retrieved in a mono- or di-ubiquitination dependent process and cycled back to the cytosol. Non-cycled 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 mono-ubiquitination 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 orthologue and no PEX22 homologue are present, but there another cytosolic E2 enzyme (UbcH5) that is responsible for ubiquitination of the receptor.

T. brucei contains genes coding for 15, highly diverse E2-like enzymes. One of them stands out as a candidate PEX4, it is orthologous to the yeast PEX4, although they only share about 30% sequence identity; it has conserved the characteristic region near the C-terminus containing the cysteine residue that is critical for catalytic activity. Similarly, a trypanosomatid orthologue of yeast and plant PEX22 was identified by homology searches in spite of very low sequence conservation. The residues of yeast PEX4 and PEX22 responsible for their interaction have been conserved in the *T. brucei* candidates.

PEX4 is expressed in bloodstream- and procyclic-form cells as observed by RT-PCR and western blot analysis. By confocal immunofluorescence microscopy 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. Only a minor growth phenotype could be observed when its expression was partially knocked down by RNAi or when both alleles of the gene were deleted. Possibly one of the other E2-like proteins could compensate for the PEX4 loss. This is currently under study.

Previously, *T. brucei* PEX13 has been characterized; a glycosomal membrane protein with a Tyr-Gly rich N-terminal region of low sequence complexicity, two transmembrane segments, a SH3 domain in its C-terminal half and, uniquely for the trypanosomatid PEX13, terminating with a PTS1-like sequence. Recently, another candidate PEX13 has been identified in the trypanosomatid databases. Although this new candidate lacks the SH3 domain of

PEX13s, it has a higher overall sequence identity with PEX13s of other organisms and possesses the typical PEX13 N-terminal half with Tyr-Gly motifs and transmembrane segments. It does not contain a PTS1. Indeed, when expressed in procyclic trypanosomes as a GFP-fusion protein, the fluorescence pattern colocalizes with that of glycosomal marker proteins. Current research is devoted to determining its role in glycosome biogenesis. It should be noted that different isoforms of PEX13 have not been found so far in any other organism.

In collaboration with colleagues in Germany, very recently also a PEX16 candidate has been identified with very low sequence identity with its orthologues in other organisms, a peroxisomal membrane protein involved in the integration of other proteins into this membrane. GFP-fusion constructs, expressed in procyclic trypanosomes, colocalized with glycosomal marker proteins. Functional studies of this peroxin are in progress.

Glycosome degradation in *Trypanosoma brucei*

A. Brennand, M. Gualdron-Lopez, P. Michels, in collaboration with E. Pays (Université Libre de Bruxelles), 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 into the procyclic 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. Furthermore, we performed a bioinformatics analysis that allowed us to identify in the trypanosomatid databases orthologues of about 20 of the 40 known yeast proteins known to be involved in autophagy (ATGs = AuTophaGy-related proteins). Also orthologues specifically required for pexophagy were recognized in trypanosomatids. Recently, this bioinformatics analysis was extended to a taxonomically diverse range of other protists. This analysis, together with similar analyses performed by others for plants, animals and fungi 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 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. With regard to the process in parasitic protists, this may offer opportunities for drug design targeting autophagy proteins in these organisms.

Experimental support for degradation of glycosomes in trypanosomes by autophagy, both under conditions of nutrient deprivation and during differentiation –from long-slender bloodstream forms to short stumpy forms and from short stumpy to procyclic forms– has previously been obtained by immuno-electron and -fluorescence microscopy. Furthermore, several of the *T. brucei* homologues of yeast proteins involved in autophagy, notably that of peroxisomes, have been cloned and sequenced: VPS34, ATG7, ATG8, ATG24 and VAC8. Recombinant forms of ATG8, ATG24 and VAC8, as well as a control protein (actin) have been produced in *E. coli* for production of antisera to be used for immuno-blot and immunofluorescence studies of cells. Also for labeling of organelles (lysosome and autophagosomes) involved in autophagy, procyclic-form trypanosome cell lines expressing an ATG24-myc fusion construct has recently been created, as

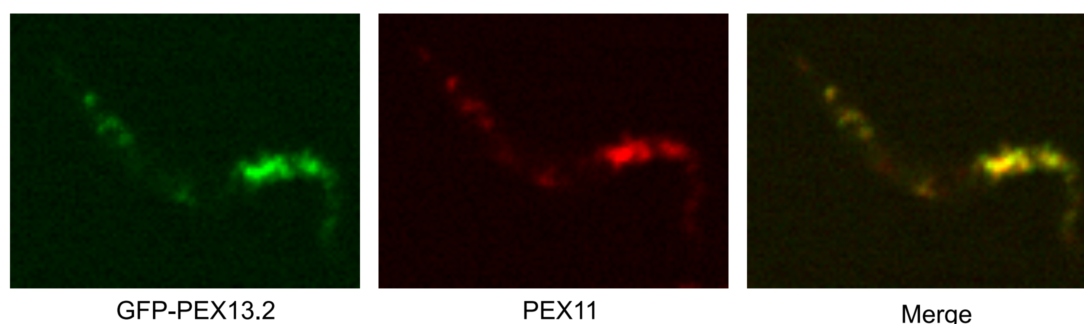

Fig. 2. Subcellular localization of the second isoform of peroxin 13 (PEX13.2) in *T. brucei* by expression of a fusion construct of PEX13.2 with green fluorescent protein (GFP-PEX13.2) in *T. brucei*. The green fluorescence of GFP colocalizes with the red signal of marker protein PEX11, a peroxin present in the glycosomal membrane, as detected by immunofluorescence, giving the yellow merged signal.

well as procyclic cells expressing ATG7-GFP. Using these tools, the localization of the candidate ATG7 and ATG8 has already been analysed. 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 (VAC8, ATG24 and VPS34) bloodstream-form and procyclic RNAi-mutant cell lines have been constructed and are currently being analyzed under standard culturing conditions and under conditions where an increased rate of glycosome turnover is expected. The subcellular localisation of ATG24 and VAC8 is currently being determined as well as the mechanism by which these proteins associate with intracellular membranes.

NATURAL PRODUCTS AS TRYPANOCIDAL DRUGS

V. Hannaert, in collaboration with J. Bero and J. Quetin-Leclercq (Pharmacognosy Research Group, UCL) and I. Ngantchou and B. Nyasse (Université de Yaoundé, Cameroun)

Nature is a potential source of new drugs since it contains a countless quantity of molecules with a great variety of structures and pharmacological activities. The potential of natural products in the treatment of diseases can be seen in traditional medicines. For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases. Several well established human antiprotist drugs have their origins in nature, such as quinine and artemisinin used to treat malaria. In Africa, plants have been used traditionally for centuries and are still widely employed to treat sleeping sickness. However, no trypanocidal drug molecule from natural origin is currently used against *T. brucei* infections.

In our search for more effective trypanocidal drugs, we investigated plants from different African countries (Benin, Cameroon, Ghana) that are traditionally used as antiparasitics. The selection was based on information obtained from traditional practitioners. Plant extracts were prepared and evaluated in vitro on *T. brucei* bloodstream forms and their selectivity analysed on MRC-5 mammalian fibroblasts. Active extracts were further fractionated to isolate pure compounds. Some of them have been shown to inhibit the growth of trypanosomes in vitro with EC_{50} values in the micromolar range and to have moderate cytotoxicity values towards mammalian cells. Their chemical structures were determined and showed a great diversity. Further investigations have to be made directly on the parasite to study in detail

the action mechanisms of these antitrypanosomal molecules and to determine if they constitute suitable candidates for drug development.

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Paul Michels
de Duve Institute
TROP - B1.74.01
Av. Hippocrate 74-75
B - 1200 Brussels
[T] +32 02 764 74 73
[F] +32 02 762 68 53
[E] paul.michels@uclouvain.be
[W] http://www.deduveinstitute.be/trypanosome_metabolism.php

Fred Opperdoes
de Duve Institute
TROP - B1.74.01
Av. Hippocrate 74-75
B - 1200 Brussels
[T] +32 02 764 74 55
[F] +32 02 762 68 53
[E] fred.opperdoes@uclouvain.be