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„The effect of active substances on mitochondrial function in Leishmania tarentolae“

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Alexandra Lacková

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1 Background

Leishmaniasis is a protozoal disease that represents a major health problem for humans in (sub-) tropical countries. This disease caused by *Leishmania*, which belong to the Trypanosome protozoa, is usually transmitted through sand flies (Phlebotominae).The preferred hosts of *Leishmania* are vertebrates, including humans. In endemic areas dogs infected with *Leishmania* represent a reservoir for transmission [1]. In general the treatment of leishmaniasis is very difficult due to different pathogenic species, different clinical manifestations and resistance development [2]. Due to climate change the endemic area extends also north to new areas, such as southern Europe [1]. Depending on the *Leishmania* species different clinical manifestations are observed. Antimonials used for the treatment of canine and human leishmaniasis show as major adverse effects nephrotoxicity [3] and so far no commercial vaccine against *Leishmania* is available for humans [4]. Therefore, new drug targets and new compounds for these targets need to be identified. It is known that many drugs influence mitochondrial functions in the *Leishmania* parasite, however, it is often not clear whether the drugs target mitochondria in these organisms directly [5]. For these reasons this study focused on the potential antileishmanial effects of a panel of compounds (drugs, food ingredient and synthetic compounds) and whether these are mediated by mitochondrial inhibition.

2 Introduction

2.1 Leishmaniasis as a health problem

Leishmaniasis is a vector-borne disease that is transmitted by sand flies and caused by obligate intracellular protozoa of the genus *Leishmania*. It is one of the major

diseases in 88 developing countries mostly in tropical and subtropical areas. There are 12 million people infected and about 350 million of people live in areas with high risk of infection. Leishmaniasis was ranked as second in mortality and forth in morbidity caused by neglected tropical diseases. More than 90% of reported cases are situated in India, Bangladesh, Nepal and other developing countries. However, the climate has changed and the human migration caused, that leishmaniasis extended also to developed countries. There are reported cases from 16 European countries as France, Italy, Greece, and Malta [6]. *Leishmania spp*. are dimorphic organisms which exist as flagellated promastigotes in the sand fly gut and as aflagellated amastigotes in mammalian macrophages. The parasite may survive for decades in asymptomatic infected people, who are of great importance for the transmission since they can spread visceral leishmaniasis indirectly through the sand flies.

2.2 Infective cycle

The life cycle of *Leishmania* involves two stages, promastigotes in the vector and the amastigotes in the vertebrate host (Figure 1). In the insect vectors, the parasite takes a promastigote form which is characterized by an elongated and flagellated form, therefore it is motile and persists in the extracellular environment. In vertebrate hosts the parasites are found as amastigote form, which is ovoid, non-flagellated, non-motile and reproduces in different phagocytic cells as obligatory intracellular pathogen.

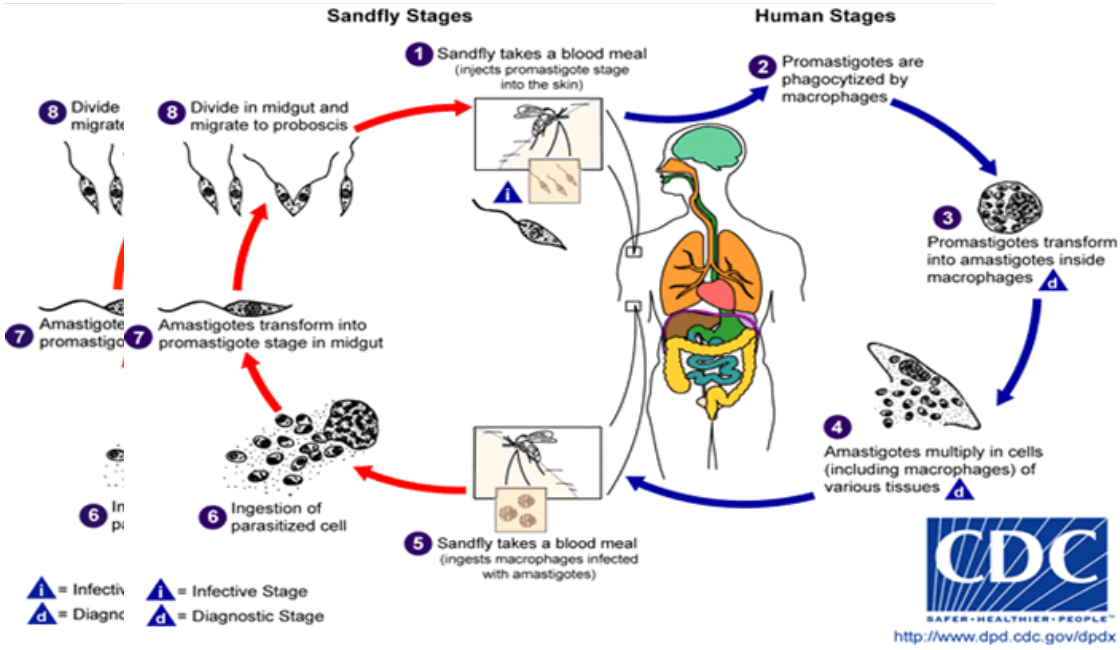


Figure 1. Cycle of life of *Leishmania* parasites including sand flies as a vector and humans as mammalian hosts (the image was taken from http://www.dpd.cdc.gov/dpdx/HTML/Leishmaniasis.htm).

During the first blood uptake (tick), vectors aspirate intramacrophagic amastigotes from infected host, releasing them in its stomach. The transformation of the amastigote form into promastigote form starts within hours of ingestion and occurs exclusively in the sand fly gut. The promastigotes keep on dividing by binary division in the midgut of the sand fly until they are transformed into non-dividing infectious metacylcic promastigotes [7]. A few days after the infective meal, the infectious promastigotes migrate to the pharynx and sand flies bite at this time lead to the transfer of promastigotes to the new host. After inoculation into the vertebrate host, the promastigotes are phagocytosed by mainly macrophages and dendritic cells and transform again into amastigotes where they multiply by binary fission until the host cell gets packed up with the parasites leading to rupture of the host cells. The amastigotes are liberated into the lymphatic circulation and have the opportunity to infect additional macrophages either locally or in distant tissues after dissemination.

The risk of Leishmaniasis infections is generally increased by co-infections and in certain life stages. It has been shown that the parasites can be also transmitted directly from person to person through the sharing of infected needles which is often the case with the *Leishmania*/HIV co-infections. Transplacental vertical transmission is also a possibility that needs to be explored, because of reported cases in newborns [8].

Depending on the parasite species and the cellular immune system of the patient the severity of the disease and the outcome can strongly vary. There are many species of *Leishmania* with different geographical distributions that cause different clinical manifestation of leishmaniasis.

2.3 Leishmania species and clinical manifestations

Human infections by *Leishmania* are caused by about 21 of 30 different species (Table 1). The three main subgenera of *Leishmania* consist of the *L. donovani* complex with three species, the *L. mexicana* complex with three main species and the subgenus Viannia with four main species. The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, molecular methods or monoclonal antibodies [8].

Table 1. Classification of species from *Leishmania* genus and basic clinical manifestations.

Subgenus	***Complex***	***Species***	***Clinical form***
Leishmania	Donovani	Donovani	VL, PKDL
		Archibaldi	VL, CL
	Infantum	Infantum	VL, CL
		Chagasi	VL ,CL
	Tropica	Tropica	CL, RL
		Killicki	CL
	Major	Major	CL
	Aethiopica	Aethiopica	CL
	Turanica	Turanica	N
	Gerbil	Gerbil	N
	Arabica	Arabica	N
	Mexicana	mexicana	CL, MCL
		Pifanoi	CL, DCL
		Venezuelensis	CL
		Amazonensis	CL, DCL
		Garnhami	CL
		Aristidesi	N
		Forattini	N
	Enrietti	Enrietti	N
	Hertigi	Hertigi	N
		Deanei	N
Vianna	Braziliensis	Braziliensis	CL, MCL
		Peruviana	CL
		Equatorensis	N
	Guyanesis	Guyanesis	CL, MCL
		Panamensis	CL, MCL
	Shawi	Shawi	CL
	Naiffi	Naiffi	CL
	Lainsoni	Lainsoni	CL

CL: Cutaneous leishmaniasis MCL: Mucocutaneous leishmaniasis VL: Visceral leishmaniasis
PKDL: Post-Kala azar dermal leishmaniasis DCL: Diffuse cutaneous leishmaniasis
N: No reports about infection

The taxonomic classification into subgenera does not easily predict infectivity and clinical signs upon infection. Therefore, leishmaniasis is divided in three major diseases that have different clinical manifestations in humans and are caused by specific *Leishmania* species: cutaneous (CL), mucocutaneous MCL) and visceral

(VL) form. For example *Leishmania braziliensis* is mostly associated with cutaneous leishmaniasis, while *L. tropica, L. etiopica, L. major, L. amazonenis, L. panamensis* and *L. guyanensis* present high risk of mucosal leishmaniasis [6,9]. This specific association is also responsible for the geographical distribution of the disease. The most common and most dangerous form is visceral leishmaniasis, also known as a kala-azar, caused by *Leishmania donovani* infection (Figure 2. A). Typical signs are bouts of fever, swelling of spleen and liver, weight loss, as anemia and hepatosplenomegaly [10,11]. The most affected organs in visceral leishmaniasis are liver, spleen and bone marrow. From there the infection can develop to a systemic disease and ends fatal if it stays untreated. For cutaneous leishmaniasis typical topic signs are ulcers on all body parts, mostly at face, arms and legs (Figure 2. B). The disease begins with small erythema and exacerbates to raised indurated borders. Spontaneous healing can occur but is very slow and it leaves scarves and disfigurings [6,12]. Least frequent is the mucocutaneous leishmaniasis (Figure 2.C) that effects mucous tissues [10].

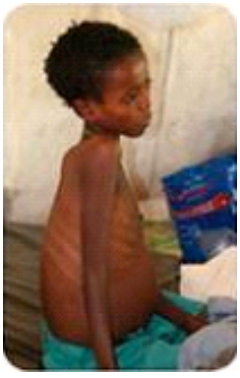
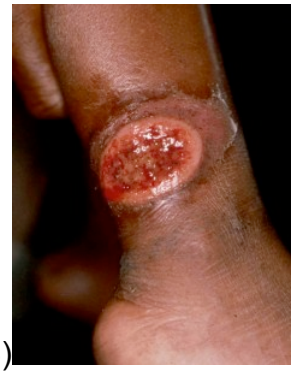
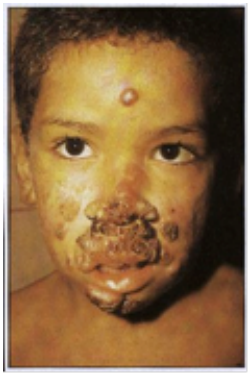
(A)  (B)  (C) 

Figure 2. (A) Typical signs of visceral leishmaniasis (kala azar) http://www.who.int/leishmaniasis/visceral_leishmaniasis/en/index.htm,(B)Disfigurings from cutaneous leishmaniasis (http://www.primehealthchannel.com/leishmaniasis-picturessymptomsdiagnosis-and-treatment.html)and(C)mucocutaneous leishmaniasis infacearea(http://www.stanford.edu/class/humbio103/ParaSites2006/Leishmaniasis/Mucocutaneous.htm).

2.4 *Current drugs* for leishmaniasis

Because of the high increase of cases of leishmaniasis and the high amount of people living at risk, both prevention and treatment of the disease is required. Leishmaniasis often occurs in developing countries where the current drugs are not affordable for all. In addition, current drugs have serious side effects and with so far reliable drugs also the emergence of drug resistant parasites causes problems. The set of currently available drugs include antimonials, amphotericin B, miltefosine, paromomycin, pentamidine, sitamaquine, imiquimod.

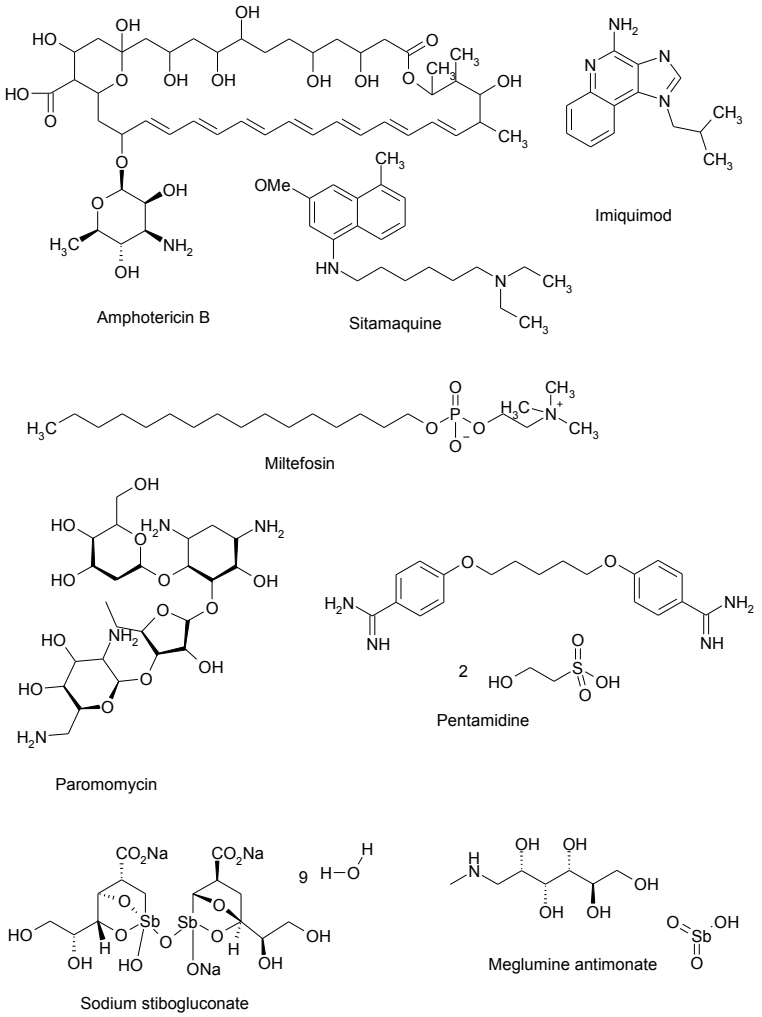


Figure 3. Molecular structure of the currently used antileishmanial drugs: antimonials (sodium stibogluconate and meglumine antimonite), amphotericin B, miltefosine, paromomycin, pentamidine, sitamaquine, imiquimod.

Pentavalent antimonials (meglumine antimonate and sodium stibogluconate) are the oldest drugs used to treat leishmaniasis. They have quite good treatment results in areas without previous medication. The biggest disadvantage is the increasing resistance. A major adverse effect of these drugs is cardiotoxicity. Nevertheless the antimonials are frequently used as a first line therapy.

Amphotericin B deoxycholate was primarily developed as a systemic antifungal, but has the major disadvantage of being acutely toxic and therefore administration must be clinically monitored. In areas with resistance to antimonials, it is used as a first line therapy or as a second line therapy in other cases. Side effects as fever, chills, thrombophlebitis, hypokalemia, nephrotoxicity and myocarditis were reported. High costs of therapy are not only connected with the drug administration itself, but also with the hospitalization of patients because of possible side effects. There are some new lipid formulations of amphotericin B on the market. The highest effectivity reveals the liposomal amphotericin B registered as AmBisome®, used for visceral leishmaniasis, but its use in endemic regions is limited by cost. Other commercial amphotericin B lipid formulations, as amphotericin B lipid complex (Abelcet®) or an amphotericin B colloidal dispersion (Amphocil®) are available but their cost-benefit ratio is not as good as for AmBisome®.

Miltefosine, initially developed as an anticancer drug, with the structure of an alkyl-phosphocholine was the first antileishmanial drug for oral application. Clinical trials reported side-effects in gastrointestinal and transient moderate elevation of hepatic enzymes as well. Because of it teratotoxicity miltefosine is contraindicated in pregnancy and during therapy mandatory contraception for woman in child-bearing age is required. Beyond the teratotoxicity and high potential of resistance development, miltefosine exhibits a small therapeutic window.

Paromomycin, an aminoglycoside antibiotic, is one of the newest drugs used to treat visceral leishmaniasis. It can be used as a topical application and eliminate the side-effect as fever and high costs connected with systemic treatment. Side-effects of injection therapy are pain around the injected area, ototoxicity and nephrotoxicity.

Pentamidine, an aromatic diamidine, is used in visceral and cutaneous leishmaniasis as well. The major disadvantage of its use is the induction of insulin depended diabetes mellitus.

Sitamaquine (8-aminoquinoline) is the new hope of pharmaceutical research with high cure rate. As side-effects abdominal pain, headache, vomiting, dyspepsia, cyanosis and mild methemoglobinemia were reported [13].

Imiquimod is an antiviral substance used toward genital warts caused by human papilloma virus. It is an immunomodulator that activates a local immune system at the site of application, which in turn resolves the infection. It was recommended to combine the imiquimod with antimonials, which are responsible for elimination of systemic amastigote. Topical treatment with imiquimod causes activation of local macrophages and killing of parasites.

There is also hope in new applications forms of amphotericin B or combination therapies which are already in use for other diseases, such as malaria, tuberculosis or leprosy [13-15].

2.5 Subcellular organization of Leishmania

Leishmania parasites exist in two different morphologic forms. In the insect vectors, the parasite takes a promastigote form, which is characterized by elongated, flagellated and motile shape. In vertebrate hosts, the parasite is found as amastigote

form, which is ovoid, non-flagellated, non-motile and reproduced in different phagocytic cells as obligatory intracellular organism. *Leishmania* are eukaryotes and have their typical organelles but also some unique intracellular structures.

Mitochondria

Leishmania and other Trypanosomatids have only one highly structured mitochondrion extending through the protozoan cell. This organelle is present in all developmental stages of the Trypanosomatids [16]. Although the mitochondrial structure is not drastically changed in different developmental stages of *Leishmania* it has been shown that there are quantitative and qualitative differences in the protein pattern in these stages [17]. Antileishmanial agents frequently trigger swelling of mitochondria.

Nucleus

In *Leishmania* the nucleus shows a typical nuclear membrane with pores. In the nuclei chromatin is organized in 16-23 chromosomes or chromosome sized molecules. Nuclei of *Leishmania* parasites are surrounded by the endoplasmatic reticulum, that is punctuated by pores [18]. Depending on the antileishmanial drug ultrastructural changes of the nucleus are observed indicating the death pathway triggered.

Surface

The surface of *Leishmania* (Trypanosomatids) consists of: the cell coat (or glycocalyx), the cell membrane and the subpellicular microtubule layer. The composition of cell coat is based on oligosaccharides associated with membrane lipids and proteins. It is in direct contact with the extracellular medium. The cell

membrane is characterized by chemically distinct domains: the membrane lining the cell, the flagellar membrane and the flagellar pocket membrane. Drug effects on the surface were observed leading to rupture and detachment of cellular and flagellar membranes.

Cytoskeleton

The Trypanosomatids possess a variety of microtubules immediately below the cell membrane, the subpellicular microtubule layer. They extend throughout the protozoan cell. Direct damage of this cytoskeleton by drugs is difficult to observe however shape changes of the whole parasitic cell indicate at least partial effects.

Golgi Complex and Endoplasmic Reticulum

The Golgi complex (GC) and the endoplasmic reticulum (ER) have the same basic structure as observed in other eukaryotic cells. The localization of GC varies in different developmental forms, but remains near the flagellar pocket. It has been shown that drugs affect the ultrastructure of the ER/GC system, such as inhibitors of the sterol biosynthesis.

Kinetoplast

The kinetoplast is a network of the mitochondrial DNA (kDNA) of the protozoa, which represents about 30% of the total cellular DNA. It is accumulated in a specialized region of the mitochondrion and its shape and its localization relative to the nucleus varies in different Trypanosomatids. In *Leishmania* a compact rod- or bar-shaped structure is observed. Major drug effects involve an impaired organization of the DNA filaments in the kDNA network, fragmentation of the kDNA, and compaction as well as multiple kDNAs.

Endocytosis/ Exocytosis Related Organelles

For survival of Trypanosomatids it is essential that exogenous molecules are internalized by fluid phase and receptor-mediated endocytosis. In promastigotes of *Leishmania* molecules are internalized from the flagellar pocket pass through a network of tubules and vesicles extending through the whole parasite. Drugs such as pentamidine may significantly change these endo-/exo-cytotis pathways.

Acidocalcisomes

The acidocalcisome is an acidic organelle enclosed by a single membrane that is found in all Trypanosomatids. The number and size of theses organelles vary. They are observed in all developmental forms of *Leishmania*. They contain metal ions such sodium, magnesium, potassium, calcium, zinc, iron and phosphorous in the form of inorganic phosphates. In addition, these organelles are also involved in pH homeostasis and osmoregulation. Some drugs were observed to accumulate in acidosomes.

Glycosomes

Glycosomes are spherical or elongated organelles with a uniform matrix surrounded by a single membrane. The glycolytic pathway of Trypanosomatids is performed in this organelle playing an important role in cellular ATP supply. Other metabolic activities involve isoprenoid and sterol biosynthesis. Enzymes involved in these pathways are in part different from mammalian cells therefore representing potential drug targets.

2.6 Mitochondrial Functions

Mitochondria belong to key organelles of the cells. With its many functions it plays an important role in energy production, programmed cell death, storage of Ca^{2+} or non-shivering thermogenesis and others. The most important functions of mitochondria is the electron transport chain (ETC), which is dominant of the energy metabolism of the cell. In higher eukaryotic organisms, the electron transport chain in the inner mitochondrial membrane is composed of five integral membrane enzyme complexes:

Complex I: NADH:ubiquinone oxidoreductase

Complex II: succinate:ubiquinone oxidoreductase

Complex III: ubiquinol:cytochrome c oxidoreductase

Complex IV: cytochrome c oxidase (reduces oxygen to water).

ATP synthase represents complex V and uses the proton gradient across the inner mitochondrial membrane produced by complexes I-IV to produce ATP. This process is termed oxidative phosphorylation (Figure 4).

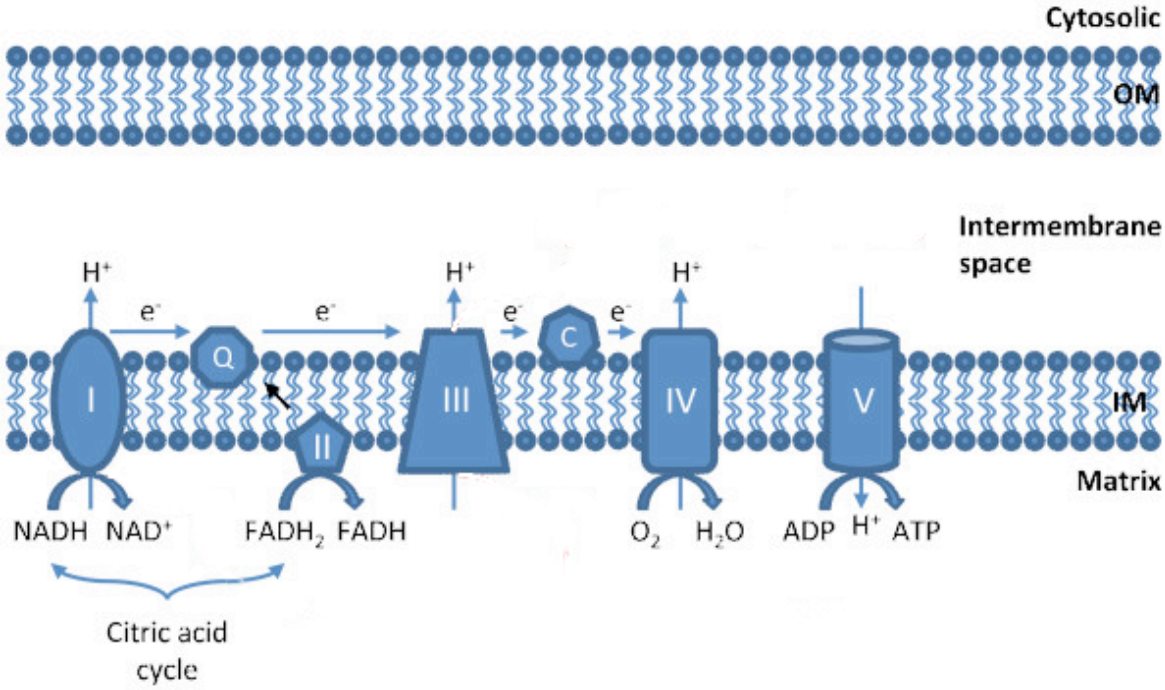


Figure 4. Electron transport chain. Electrons donated from NADH and $FADH_2$ pass through the ETC and reduction of O_2 to H_2O at complex IV. Complex V, ATP synthase uses proton gradient across inner membrane to produce ATP. Q, ubiquinone; c, cytochrome c; IM, inner membrane; OM, outer membrane.(http://www.jhoonline.org/content/6/1/19/figure/F1)

Complex I, initial complex of the ETC is responsible for reduction of ubiquinone (Q) to ubiquinol, that diffuse within membrane and it comes to translocation of four protons across the membrane. This transfer results in a proton gradient. Complex II add another electron to the quinone pool newly formed from succinate and transformed to ubiquinol.

Complex III is also responsible for proton translocation based on proton cycling between ubiquinone, cytochromes b and c_1 and iron sulfur protein.[19]

2.7 Similarities and differences to mammalian mitochondria

Mammalian cells contain multiple mitochondria, to ensure the compensation for injured mitochondria in multicellular organism, but *Leishmania* parasites life depends only on the function of a single mitochondrion.

Leishmania mitochondria has an alternative complex I (NADH: quinone oxidoreductase). The mammalian mitochondrial complex I activity is highly sensitive and is already inhibited by low concentrations of rotenone, what is different to leishmanial complex I [5]. So far results published by others suggest the presence of a classical pathway from complex II to complex III in *Leishmania* parasites.

Another difference that can be used as drug target is fumarate reductase. This important component of intermediate metabolism catalyzes the reduction of fumarate to succinate and is a key enzyme in the anaerobic energy metabolism for many organisms respiring with fumarate as terminal electron acceptor. This enzyme is totally absent in the mammalian host [20].

Next important role of mitochondria in *Leishmania* is the lipid metabolism. It has been published that inhibition of three involved enzymes (CΔ14-demethylase, squalene synthase, and Δ24(25)-sterol methyltransferase) for ergosterol biosynthesis causes damage of mitochondria. The fatty acid production is essential for survival of the parasites [21].

Due to two different stages of parasites there are also various activities in each stage. Some sources declare the increased activity of mitochondria in amastigote form [5].

2.8 Mitochondrial superoxide production

Inside of the cells mitochondria, produce most reactive oxygen species (ROS) via electron transfer in the respiratory chain. The relevance of mitochondrial ROS production within a cell is indirectly evident from the results obtained from mitochondria deficient of antioxidant enzymes (p.e. superoxide dismutase, SOD). SOD function is vital in the normal cell for preventing the toxic effect of superoxide radicals generated in mitochondria. Similarly, the lack of mitochondrial glutathione peroxidase is deleterious leading to the accumulation of hydrogen peroxide. In the presence of reduced metal ions this in turn leads to the extremely toxic hydroxyl radical [22],[23].

For mammals it is known that dominant role in superoxide production of the respiratory chain play complexes I and III. There is evidence that the one-electron donor to oxygen in complex I is a non-physiological quinone reduction site different from the physiological site. It is assumed that this hydrophilic site reduces several quinones to the corresponding semiquinone forms, which are unstable and can reduce oxygen to superoxide radicals as primary ROS species. Because of antioxidant enzymes it is often difficult to identify the exact location of ROS production in mitochondria. It is known for its short half-life and is converted fast into hydrogen peroxide (H_2O_2). This reaction occurs spontaneously by disproportionation or alternatively is supported by SOD. In addition superoxide radicals can act as electron donors (reductants) and attack other molecules. In already converted form (H_2O_2) it can escape from the mitochondrion [22]. Normally during oxidative phosphorylation, the release of ROS in the form of superoxide anions occurs to an extent of 3 to 5% of total oxygen consumed. In case that drugs inhibit the oxidative phosphorylation, the rate of ROS production can be increased greatly [24]. About

specific sites and amount of superoxide production in *Leishmania* little is known and the few data published so far were obtained with fluorescence methods not specific for superoxide radicals.

2.9 Role of mitochondria in cell death

Cell death is a natural process in all organisms. It depends on triggers, whether the process is carried out as programmed as cell death (apoptosis) or necrosis.

Apoptosis, also called cell suicide, triggers a cascade of biochemical reactions that results in cell death. During apoptosis all cell organelles fall apart in apoptotic bodies, but during the collapse the cell maintains the production of proteins and adenosine triphosphate. In contrast, necrosis is characterized by destructed cell organelles and loss of metabolic functions.

Programmed cell death provides an elimination of cells when their biological function has come to end or when the cell was damaged or mutated to such an extent that its further existence might be highly risky for the survival of the whole organism. Apoptosis has an important meaning in the development of each organism and occurs in every part of live of each organ from embryogenesis to growth and maturation of individual organs. Two partially interdependent routes lead to apoptosis. The first one is initiated by ligation of the so-called death receptors at the cell surface whereas the second involves mitochondria. Mitochondria play a major function in controlling life and death and are important cellular source for generation of reactive oxygen species inside cells [24]. In cases in which mitochondria are involved, one of the early events leading to apoptosis is the release of cytochrome c from mitochondria. Along with another mitochondrial protein, the apoptosis-inducing factor (AIF), it activates in the cytosol a cascade of events leading to the activation of

intracellular proteases of the caspase family and, eventually, to a partial self-digestion of the cell. The mechanism by which cytochrome c is liberated from mitochondria to the cytosol is not completely clear yet.

Mitochondria can be seen as a target that controls cell death. An important mechanism in mitochondria that can contribute to apoptosis is the mitochondrial permeability transition pore (PTP). A physiological factor that regulates the opening probability of mitochondrial permeability transition pore is the calcium ion. Many observations have revealed an increased cytosolic Ca^{2+} concentration during apoptosis. In this context also ROS are considered as apoptosis-promoting factor. Excessive production of ROS in the cell can be induced by a number of xenobiotics, transition metal ions, and ultraviolet and ionizing radiations. ROS action on mitochondria can result in the opening of PTP and thus triggers the mitochondria-related apoptotic pathway [25].

This knowledge was acquired mostly in higher eukaryotic (mammalian) cells and whether all of these effects are similarly important in *Leishmania* needs to be demonstrated. However, it has been shown that *Leishmania* undergo apoptosis upon inhibition of the mitochondrial respiratory chain [26]. Little is known about superoxide production in *Leishmania.*

2.10 Model systems for testing antileishmanial drugs

To study mitochondrial drug effects useful for antileishmanial therapy several model systems were established in research. Mice infected with *Leishmania amazonensis* are an ultimate checkpoint for drug development of antileishmanial agents for human treatment. However, in the lead finding phase of such research even these in vivo experiments are not necessary since there are sufficiently predictive cellular and

subcellular in-vitro models which do not or require less animal experiments. Macrophages infected with *Leishmania* amastigotes are a good in vitro model, which however, still requires some animal experiments to harvest p.e. intraperitoneal macrophages from mice. For these reasons simple in vitro model systems are established by the culture of *Leishmania* promastigotes and axenic amastigotes. Furthermore, for model systems of infective protozoal diseases always the questions of the safety of such models in the lab play an important role.

Leishmania species infective for humans are mostly classified as biosafety level 2 or even higher requiring specialized labs to handle these organisms. In contrast *Leishmania tarentolae* (a lizard parasite) is not infective for humans and has a biosafety level of 1 and therefore can be handled in any lab. Comparative studies have shown that promastigotes and axenic amastigotes of *Leishmania* are well suited to predict drug effects in *Leishmania* species infective for humans [27].

For testing mitochondrial functions, cells and subcellular fractions are suitable. While oxygen consumption of intact cells and subsequent drug effects can be monitored in cell culture media, identification of the mitochondrial targets often requires isolated mitochondrial fractions. Typical model systems which can be used are *Leishmania tarentolae* promastigote cells and isolated mitochondrial fractions from these cells.

To explore differences between *Leishmania* and mammals correspondingly mammalian cells and mammalian mitochondria are suitable. A reliable test system are submitochondrial particles (SMP) which can be obtained from bovine heart. SMP consist of the inner mitochondrial membrane of heart mitochondria and contain all electron transfer complexes and therefore can be studied for drug actions in comparison to leishmanial mitochondria.

2.11 Analytical methods

Major important methods to study drug effect on *Leishmania* and mammalian cells involve tests for viability, mitochondrial metabolism and oxygen radical formation.

Viability

Alamar Blue (Resazurin:7-hydroxy-3H-phenoxazin-3-one 10-oxide) in the viability test has been used for many years to monitor bacterial and yeast contamination of milk or assessing the semen quality. The main idea of this test is the reduction of resazurin (blue and non-fluorescent) to resorufin (pink and highly fluorescent) which is further reduced to hydroresorufin (uncolored and non-fluorescent). Possible targets of resazurin are mitochondria and cytosolic quinone reductases. This very simple and sensitive method can be used to test drug actions on cells. A prerequisite is that these drugs do not interfere with resazurin itself. By measurement of colorimetric or fluorimetric changes after a defined incubation time it is possible to characterize drug effects on viability and cell proliferation.

Oxygen consumption

The oxygen consumption of cells provides an easy measure for the mitochondrial function since most oxygen is consumed by mitochondria. By this way information about the metabolism of mitochondria under normal cellular conditions, e.g. feeding on substrates from the cell culture medium is obtained. For isolated mitochondrial fractions also non-cell-permeable substrates can be added, therefore allowing discrimination of different mitochondrial targets of drug actions. Technical background of oxygen consumption measurement is based on an electrochemical reduction of O_2 at negatively polarized electrode of an electrochemical detector. The pO_2 Clark electrode is oxygen sensitive and consists of an anode and cathode that

are in contact with an electrolyte solution. It is covered by a semi-permeable membrane, which is permeable to gases but not to contaminants and reducible ions of the sample. The cathode is in a glass envelope in the body of the electrode.

Reactive oxygen species production

Reactive oxygen species (ROS) and especially superoxide radical production triggered by drugs in *Leishmania* can be important to trigger apoptosis in these cells. Important ROS taking part on mitochondrial metabolism are superoxide radical $O_2^{\cdot-}$,hydrogen peroxide H_2O_2, hydroxyl radicals $OH^{\bullet}$ and others.

Initial radical released from mitochondria is $O_2^{\cdot-}$ as outcome of monoelectronic reduction in complexes I and III. Then $O_2^{\cdot-}$ is transformed by two different enzymes into H_2O_2,that is more stable. CuZnSOD is active in intermembrane space and MnSOD works in matrix. In mitochondria produced H_2O_2 can permeate the membrane so there are several fates for it. It can be removed by cytosol antioxidants as catalase, glutathione peroxidase or thioredoxin peroxidase. Next it can also adopt the function of a signaling molecule in cytosol and interfere in cell cycle, stress response, energy metabolism or redox metabolism. If H_2O_2 is not metabolized by mitochondrial antioxidants it may form the hydroxyl radicals ($OH^{\bullet}$) as result of iron-catalyzed Fenton reaction. Hydroxyl radicals possess the ability to destroy the cell. To prevent the formation of these radicals mitochondria provides protective mechanisms [23]. Univalent O_2 reduction with its intermediates is shown in figure 5.

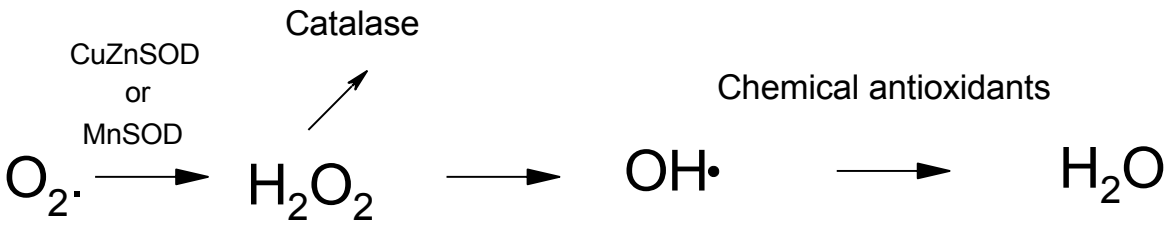


Figure 5. Univalent reduction steps of oxygen

In many studies superoxide radicals / or ROS have been measured by oxidation of fluorescence dyes. However, these methods have been repeatedly shown to be unspecific and unreliable. In contrast, detection of radicals by ESR spectroscopy (electron paramagnetic resonance) has some advantages because of its inherent specifity. The principle of ESR spectroscopy is based on absorption of microwave rays by paramagnetic substances in a magnetic field. To detect superoxide radicals addition of detection reagents is required (spin traps or cyclic hydroxyl amines). These reaction partners can react and accumulate to enable detection of $O_2^{\cdot-}$ radicals. The radical concentration in the sample is proportional to the amplitude of the ESR signal. This method can measure $O_2^{\cdot-}$ radicals inside and outside of cells depending on the detection reagent used.

3 Materials and Methods

3.1 Chemicals and biological

Table 2. Used chemicals and biologicals

Structure	*Source*
Albumin from bovine serum ess. Fatty acid free	Sigma
AmBisome	Gilead
Amentoflavone	IPK,Havanna
Antimycin A	Sigma
Ascaridole	IPK,Havanna
Atovaquone	Glaxo
Carvacrol	SARC
Caryophyllene Oxid	SARC
cis Oxachromanol	UNRLS,Vienna
CMH (1-hydroxy-3methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCl)	Noxygen
CPH (1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidin)	Noxygen
D-(+)-Glucose Monohydrat	Sigma
Desferal (Desferoxamin)	Novartis Pharma
Dicumarol	Sigma
Di-potassium hydrogen phosphate	Merck
DMSO Dimethylsulfoxid	Röth
DTPA Diethylenetriamine Pentaacetic Acid	Merck
Duroquinone	Merck
EDTA Ethylenediaminetetraacetic acid	Merck
EO Arteminisia absinthi	IPK,Havanna
EO Bixa orellana	IPK,Havanna
EO Chenopodium ambrosoides	IPK,Havanna
EO Piper auritium	IPK,Havanna
Guttiferone A	IPK,Havanna
Hemin	Sigma
Idabenone	Smart Nutrition Ltd. Dublin Ireland
M8	Uni Vienna
Myxothiazol	Sigma
NADH	Sigma
Penicillin-Streptomycin Solution	Sigma
Pentamidine isothionate	Sigma
Potassium cyanide	Merck
Potassium dihydrogen phosphate	Merck
Potassium hydroxide	Merck
Quercetin	UniWien
Rapanone	IPK,Havanna
Resazurin	Sigma

Table 2. Continuation

Structure	*Source*
Resveratrol	Uni Vienna
Rotenone	Sigma
Silibinin	Uni Vienna
Stigmatelin	Fluka analytical
Succinate	Merck
Tetramethylchroman-2-one	UNRLS,Vienna
Tetramethylchroman-2-one ester 001	UNRLS,Vienna
Tetramethylchroman-2-one ester 002	UNRLS,Vienna
Tetramethylchroman-2-one ester 003	UNRLS,Vienna
Tetramethylchroman-2-one ester 004	UNRLS,Vienna
Tetramethylchroman-2-one ester 005	UNRLS,Vienna
Tetramethylchroman-4-one	UNRLS,Vienna
trans Oxachromanol	UNRLS,Vienna
TRIS (Tris(hydroxymethyl)aminomethan	Merck
Xanthin (2,2 Dihydroxypurine) sodium salt	Sigma
Xanthohumol	Uni Vienna
Yeast extract Bacteriological	Amresco
α-tocopherol acetat	Calbiochem
α-tocopherol amine succinate	UNRLS,Vienna
α-tocopherol succinate	Fluka analytical

UNRLS (University of Natural Resources and Life Sciences), UniVienna (Department of Clinical Pharmacy and Diagnostics, University of Vienna), IPK (Institute of Tropical Medicine Pedro Kourí)

3.2 Leishmania tarentolae cell culture

Cell culture stocks of LtP (Strain P10, Jena Bioscience GmbH) were stored in cryopreservation medium (consisting of 10 % Fetal Bovine Serum and 5 % DMSO) as stock in liquid nitrogen. For the cultivation we used sterile 50 ml tubes (Sarstedt, 62.547.254) and 1000 ml polycarbonate bottles (VWR 89042-772). Bottles, tubes, magnetic stir bars, tweezers, pipette tips were sterilized in an autoclave (Certoclav Multi Control) for 15 minutes at 125°C. The medium was based on yeast extract and other ingredients diluted with high-purity water from a Mili-Q-Plus Advantage A10

from Millipore. This water was also used for all components of the medium that were prepared separately. For yeast extract stock solution 24 g of yeast extract powder (Amresco Nr.0151C058) were dissolved in 1000 ml water. Stock solution of potassium phosphate was made of 2.3 g potassium dihydrogen phosphate and 12.5g di-potassium hydrogen phosphate dissolved in 1000 ml water and pH was adjusted to 7.4 by 4 N KOH. Glucose stock solution was prepared of 55 g glucose-monohydrate and dissolved in 500 ml high purity water. Stock solutions of glucose, potassium phosphate and yeast extract were sterilized. Hemin stock solution was prepared by dissolving 2.5 g/l in DMSO and xanthine was dissolved to 100 mM with water. Hemin and xanthine were filtered sterile trough disposable filters with a pore size of 0.2 µm. To prevent the contamination an antibiotics (Sigma Nr: P4458 5000 Units penicillin/ml and 5 mg streptomycin/ml) was added to the medium. An appropriate volume of new medium was prepared immediately before every passage. This medium contained: 20.7 g/l yeast extract, 2.9 g/l glucose, 1.2 g/l K_2HPO_4 , 0.2 KH_2PO_4, 50 U/ml Penicillin-Streptomycin, 10 mg/l Hemin. At the beginning the parasites were cultured in 10-15 ml medium in 50 ml tubes and shaked to supply oxygen. After 2 or 3 days cultures were expanded to an about 3- to 5-fold volume. For the passage of parasites the cultures were centrifuged directly in the culture vessels (50 ml Sarstedt 62.547.254 tubes using a rotor inset, or in the VWR 1000 ml polycarbonate bottles fitting exactly to the rotor buckets) for 10 minutes at 3000 RPM in a SH-3000 rotor (Sorvall) at 20-25°C. The supernatant was disposed and the cell pellet was resuspended in an appropriate volume of fresh medium which was distributed into the new culture vessels. Later on with the growing volume the parasites were transferred from to 50 ml tubes to 1000 ml bottles. Agitation of cultures in 1000 ml bottle was achieved by a magnetic stirrer rotating at 100 – 200

RPM at a temperature of 26-27 °C. Every day the cell density was determined by measuring the optical density (OD) at a wavelength of 600_{nm} in a 1 cm cuvette. From OD_{600nm} cell density was obtained according to the following formula [28]:

Formula : Cells $_{(Mio/ml)} = OD_{600nm} \times 0.969 \times 124$

Formula contains an conversion factor of 0.969 g/l dry weight/OD_{600nm} in yeast extract and 1 g DW/l correspond approximately 0.124 $*10^9$ cells/ml. Alternatively the cell count was determined in a Neubauer chamber and the quality was checked trough a microscope.

3.3 Isolation of Leishmania tarentolae mitochondria

After growing up to 3 liter of culture with OD_{600nm} between 1 and 1.5 OD *Leishmania* promastigote culture was centrifuged (rotor Sorval SH-3000) for 10 min at 3000 RPM and 20-25 °C. The pellet was resuspended with buffer (10 mM TRIS-HCl, 0,3 M sucrose, 0.2 mM EDTA, 0.2 % BSA, pH 7.4), transferred to SS34 centrifuge tubes and again centrifuged with a rotor Sorvall SS34 again for 10 min at 3000 RPM and 20-25 °C. This was repeated until the suspension volume was below 35 ml. This suspension was again centrifuged under the same conditions. The pellet was resuspended in a volume of 10-20 ml 5 mM TRIS-HCl (pH 7.4) and incubated for 10 min at 20°C to lyse the cells and liberate mitochondria. To support this process the suspension was homogenized with a potter Elvehjem homogenizer using 20 stokes manually. Afterwards the suspension was centrifuged for 10 min at 3000 RPM and 20-25 °C to sediment cell debris. The supernatant was transferred to new tubes and centrifuged in a SS34 rotor (Sorvall) for 20 minutes at 10500 RPM and 20-25.°C to sediment mitochondria. At the end the pellet was resuspended in as little buffer as possible (250 mM sucrose, 50 mM KH_2PO_4, 0.2 mM EDTA, pH 7.2) and

homogenized. Mitochondrial fractions were divided into aliquots and stored in liquid nitrogen. Protein concentration was determined according to Biuret [29].

3.4 Submitochondrial particles

For preparation of bovine heart submitochondrial particles (SMP) a bovine heart (1.5 kg) was obtained from the slaughterhouse in Hollabrunn (Austria); immediately (ca. 20 min) after slaughtering, it was cooled down to 4 °C; and after 60 min, it was immersed in preparation buffer (4 °C). Beef heart mitochondria were isolated according to in a preparation buffer consisting of 250 mM sucrose, 10 mM TRIS, 0.5 mM EDTA, pH 7.4. The mitochondria were washed twice (resuspension of the mitochondrial pellet with subsequent centrifugation for 35 min at 11600 × g and 4 °C). The final mitochondrial pellet was resuspended in the preparation buffer. SMPs were prepared from bovine heart mitochondrial suspensions similar to the method described in [30]. Mitochondrial fractions were sonicated 10 times for 30 s with 1 min interruption at 4°C in buffer (250 mM sucrose, 10 mM TRIS, 1 mM EDTA, pH 9.0) using a Branson Sonifier set to 40 W energy output. Remaining mitochondria were removed by centrifugation at 6500 × g for 10 min at 4 °C. SMPs were sedimented by centrifugation at 95,000 × g for 30 min. SMPs were resuspended in buffer (250 mM sucrose, 10 mM TRIS, 1 mM EDTA, pH 7.4) and stored at 77 K until use. Protein concentrations were assayed by the Biuret method using bovine serum albumin (BSA) as standard.

3.5 Cell viability

Cell viability was assessed by reduction of resazurin. For these experiments 96 well culture micro plates (Kawasaki, flat bottom) were used. Assays were performed in the yeast extract medium (YEM) and a control row on the plate was loaded with YEM.

Other wells were loaded with 200 µl LtP cell culture using a stepper (Eppendorf Multipette Xstream) giving an amount of 500 cells/ ml in the wells. One row of cells was used as positive control (100 % viable). In the next row with cells an additional amount of cell suspension was placed to compensate for serial dilution steps. Then the highest inhibitor concentration was placed in these wells by adding the appropriate volume of DMSO stock solutions (below 5 µL). In a next step serial dilution was performed using a multi-channel pipette (Biohit 8-channel) and transferring 100 or 66 µl (depending on the intended dilution 1:3 or 1:4) of suspension to the rows below. Finally 50 µl of resazurin stock was added to each well using a stepper giving a final concentration of 100 µM resazurin in the wells. Immediately after the preparation of micro plates an image was taken as zero point and then after certain time interval pictures were taken to follow the resazurin reduction (color change from blue (resazurin) to pink (resorufin)). Plates were incubated at 27°C. After about 48 hours the endpoint of the measurement was reached. The reduction of resazurin correlates directly with the number of living *Leishmania* in the experiments. The reduction level was quantified the PLReader Software using the absorption of the red channel minus the absorption in the green channel in analogy to [31]. From these data logarithmic viability drug concentration plots were prepared and the IC_{50} was calculated by non-linear regression according to a four parameter logistic model using origin.

3.6 Measurement of superoxide radicals

Detection of $O_2^{\cdot-}$ was performed using cyclic hydroxyl amines 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCl (CMH) and 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidin (CPH) as reaction partners and measuring the formed stable nitroxyl radicals by ESR spectroscopy. Measurements were performed in

PBS-buffer (136 mM NaCl, 1.15 mM KH_2PO_4, 14 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) containing 400 µM CPH or CMH as well as 100 µM DFO (Desferal) and 25 µM DTPA (diethylenetriaminepentaacetic acid). For measurements of cell suspensions LtP cells were washed twice with PBS to remove the yeast extract medium (YEM). SMP and leishmania mitochondria were directly suspended in the PBS buffer. Typical experiments contained 1.08 µg/ml SMP or 0.85 µg/ml leishmania mitochondria or 500 Mio LtP cells/ml. Samples with cells contained in addition 16 mM glucose. For samples with mitochondria as substrates 12 mM succinate or 3 mM NADH were added. Simultaneously drug stock solutions in DMSO were added. Prior to the ESR measurement 400 µM CPH (for mitochondria) and 400 µM CMH (for cells) was added. For ESR measurements 17 µl of suspension was aspirated in a gas permeable Teflon tube (ø 0.7mm). This capillary tube was placed in resonator of the ESR Instrument (Bruker EMX, split ring MD5) and 10 sequential measurements were performed. Following instrument settings were used: microwave frequency 9.682 GHz, modulation frequency 100 kHz, modulation amplitude 1 G, time constant 0.082 sec, center field 3446 G, scan rate 71 G/min, sweep width 100 G, scan time 84 sec and attenuation CPH: $2x10^4$ CMH: $7.96\ x10^3$. From the ESR spectra the middle peak intensity was retrieved and concentrations of oxidized CPH and CMH were obtained by comparison with a standard curve prepared from 3-carboxy-proxyl ($CP^{\cdot}$) solutions with defined concentrations.

3.7 Complex I and II activity

Most activity assays were performed in 96 well micro plates. For these assays usually a premix and a start mix were prepared.

For complex I assay the premix contained 75 µM DCPIP, 62.5 µM idebenone (hydroxy decyl ubiquinone), 4.375 mg/ml BSA and mitochondrial fractions (10 µg/ml BH-SM 170 µg/ml LtPMit). Each 200 µl of this start mix were distributed to each well and then appropriate amounts of drug stock solutions in DMSO were added. The start mix contained 1.5 mM NADH and 5 mM KCN. The reaction was started by addition of 50 µl start mix to the wells.

For complex II assay the premix contained 75 µM DCPIP, 62.5 µM idebenone (hydroxy decyl ubiquinone), 1.25 mg/ml BSA and mitochondrial fractions (10 µg/ml BH-SMP.0.065 µg/ml LtP-Mit). Each 200 µl of this start mix were distributed to each well and then appropriate amounts of drug stock solutions in DMSO were added. The start mix contained 20 mM succinate and 5 mM KCN.

After start of the reaction, images of the plates were recorded with a Canon EOS 300D in 2 min intervals. Using PLReader software the images were analyzed for the slope of the absorption decay in the red channel. Results were expressed as percent inhibition with reference to the non-inhibited control wells.

3.8 Oxygen consumption

Oxygen consumption was followed in a reaction vessel (volume ca. 1 ml) equipped with a Clark-type electrode. For measurements of LtP cell suspensions cells concentrations were adjusted 100 Mio/ml in YEM containing 16 mM glucose and 1 ml suspension was placed in the electrode vessel. After equilibration the basal oxygen consumption rates were measured (100% activity). Subsequently stock solutions of drugs were added in an incremental fashion and after each addition the altered oxygen consumption rate was measured. The result was defined either as

percentage inhibition at the highest drug concentration or an IC_{50} value was calculated using rates of non-inhibited cells as reference.

4 Results

4.1 Leishmania tarentolae promastigote cell culture

Cell culture of LtP was grown as described and the culture volume was expanded according to the cell density. The following figure represents the growth curve showing the cumulative number of cells in each step.

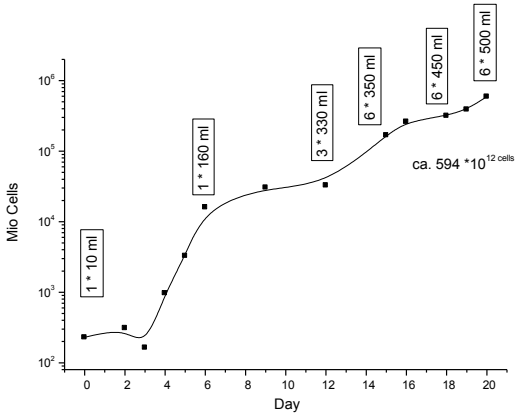


Figure 6. Growth curve of LtP cells for mitochondrial isolation

Before every passage and each experiment a visual control was taken. Sign for healthy growth was an elongated-round shape and proper motility. This control was also used to monitor bacterial contamination.

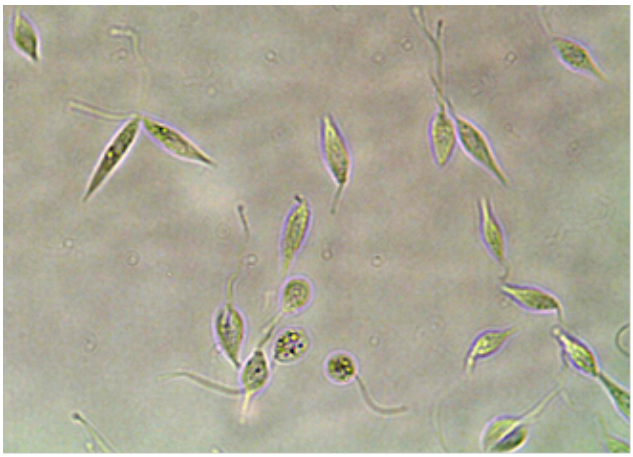


Figure 7. Typical picture of LtP cells as quality control before experiments and passage.

4.2 Compounds studied (groups similarities differences origin)

Several compound groups were studied in this work for their antileishmanial activity.

A first group which was studied were tocopherol derivatives which were described to have anticancer activity. These compounds are derived from α-tocopherol succinate (α-TOS) and also an acetate was included for reference purposes.

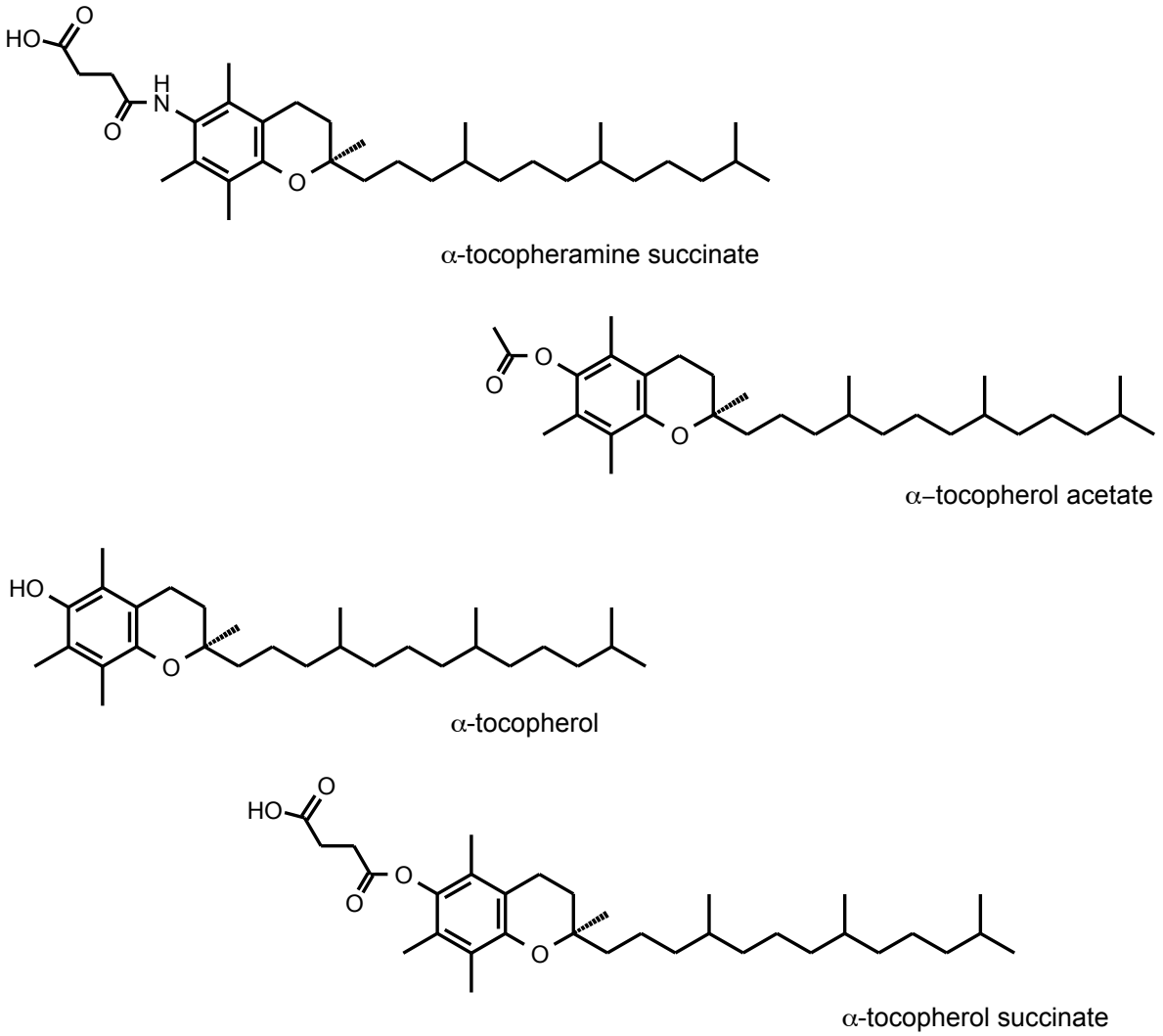


Figure 8. Structures of tocopherol derivatives: α-tocopheramine succinate (αTNS) ,α-tocopherol (αTOH), α-tocopherol acetate (αTOA), α-tocopherol succinate (αTOS).

A second group of compounds is derived from chromanol structures with different substitution patterns.

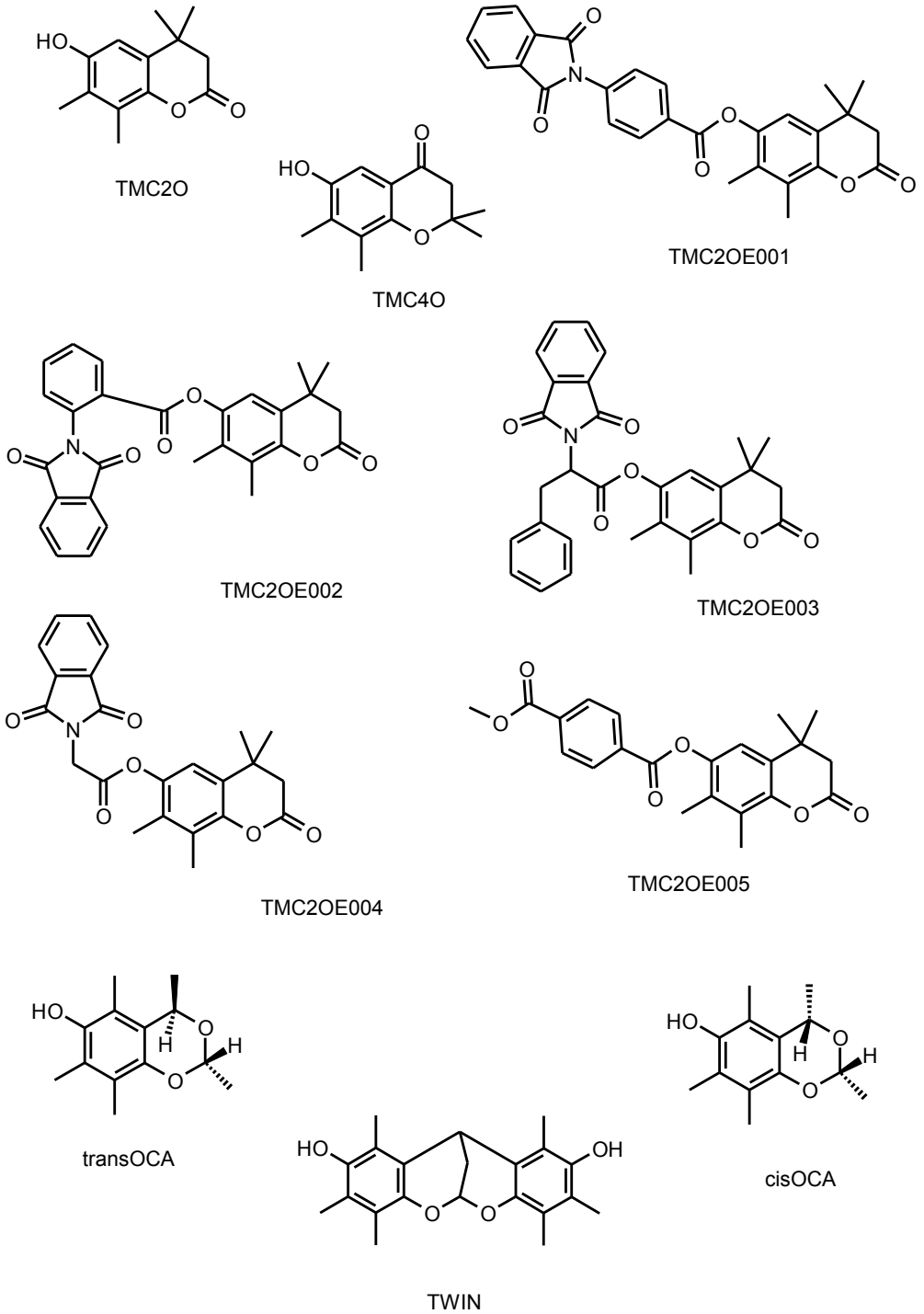


Figure 9. Structures of chromanol derivatives: TMC2O (tetramethylchroman-2-one), TMC2OE001 (tetramethylchroman-2-one ester E001), TMC2OE002 (tetramethylchroman-2-one ester E002), TMC2OE003 (tetramethylchroman-2-one ester E003), TMC2OE004 (tetramethylchroman-2-one ester E004), TMC2OE005 (tetramethylchroman-2-one ester E005), TWIN (twin chromanol), transOCA (trans-oxachromanol), and cisOCA (cis-oxachromanol).

A third group of compounds is derived from resveratrol, which is a natural antioxidant and belongs to the group of polyphenols.

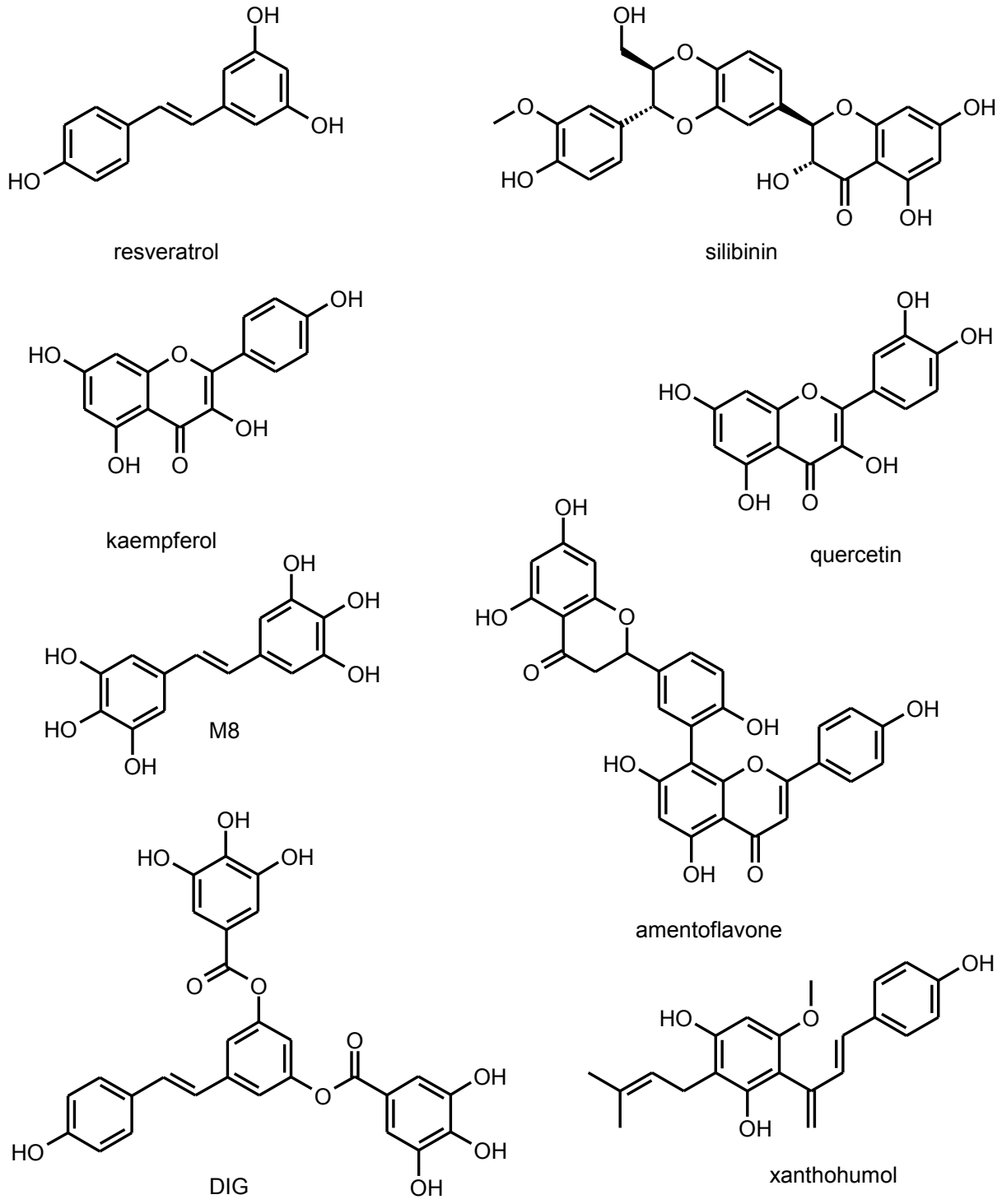


Figure 10. Structures of polyphenols: resveratrol (RV), silibinin (Slb), kaempferol (Kpf), M8 , digalloylresveratrol (DIG), amentoflavone (Ame), and xanthohumol (Xtl).

A forth group contains approved drugs and other bioactive molecules which possess potential antiprotozoal activity.

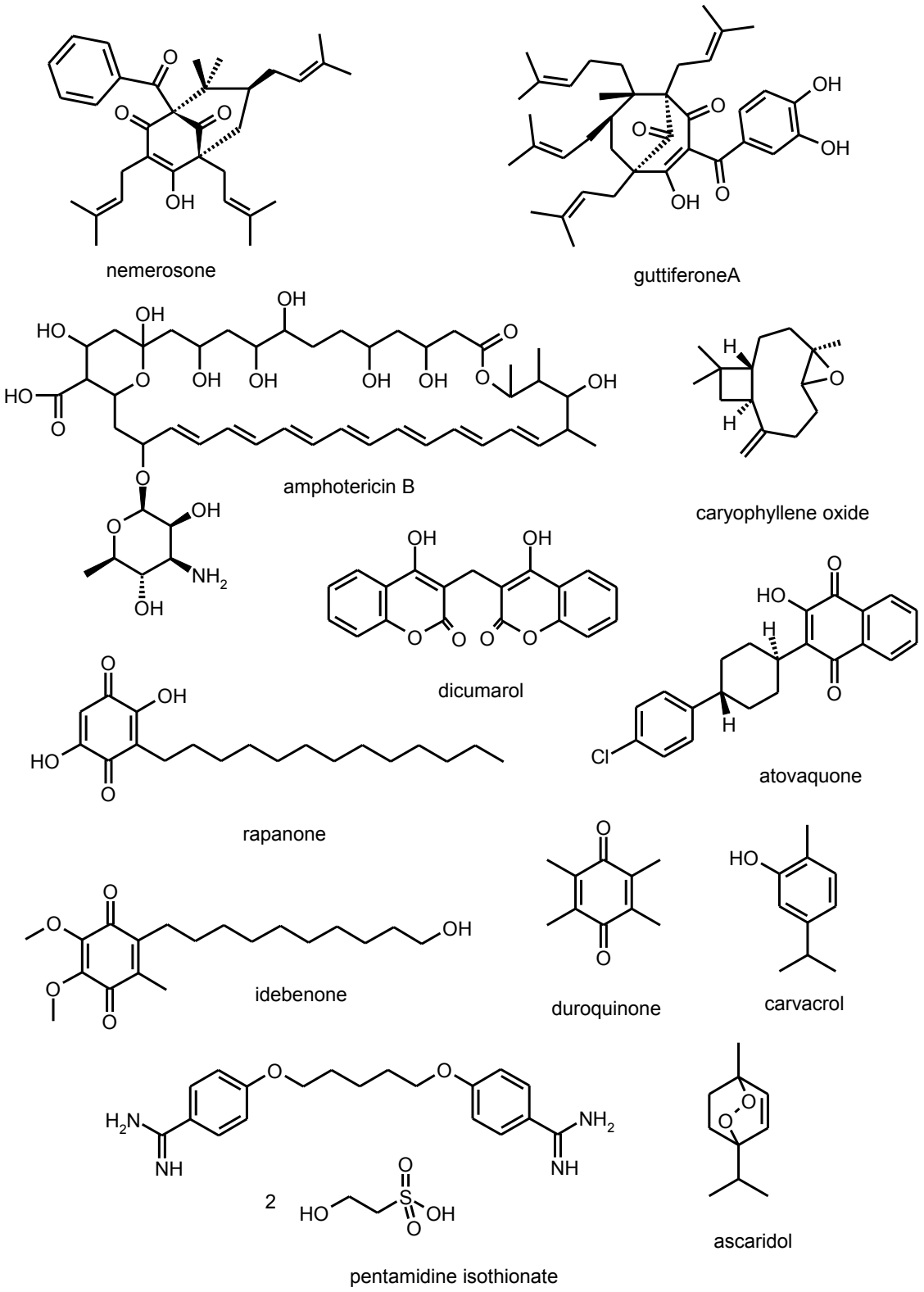


Figure 11. Structures of approved drugs and other drug candidates contains compounds as nemerosone (NEM), guttiferone A (GutA), amphotericin B (AmB), caryophyllene oxide (CO), dicumarol (DiCu), rapanone (Ben), ascaridol (Asc) , duroquinone (DQ), carvacrol (Car) and pentamidine isothionate (Pen).

The fifth group of tested compounds consist of familiar known antibiotics that operate as mitochondrial inhibitors in mammals.

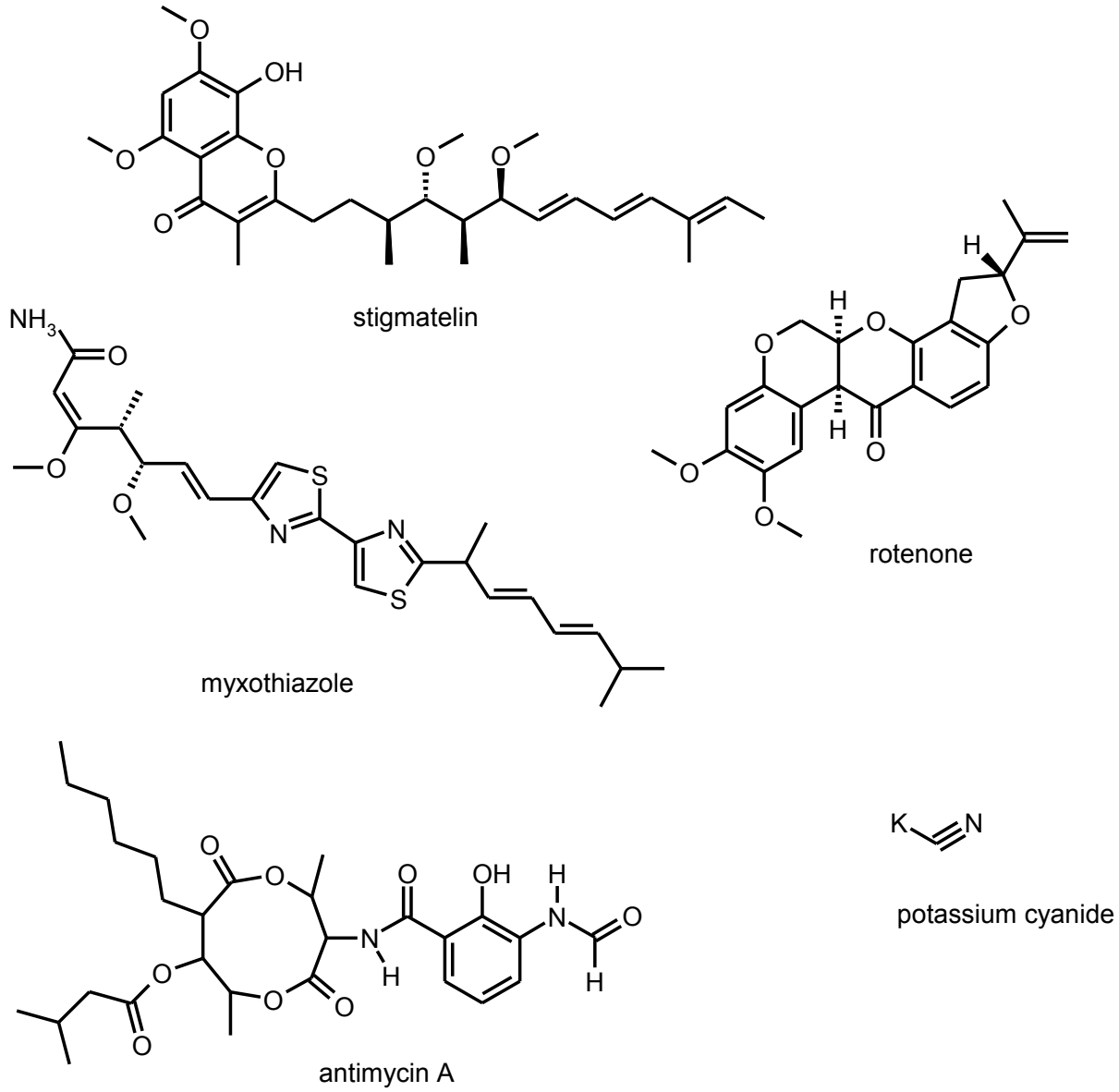


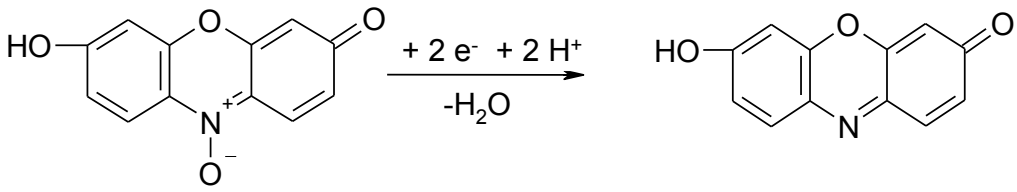
Figure 12. Structures of mitochondrial inhibitors as stigmatellin (Stig), rotenone (Rot), myxothiazol (Myx), potassium cyanide (KCN), and antimycin A (AA).

The sixth group contained essential oils from several medicinal plants and Surfacen

EO Artemisia absinthi	EO Chenopodium ambrosoides
EO Bixa orellana	EO Piper auritum
Surfacen	

4.3 Viability

The viability of *Leishmania tarentolae* promastigote cells was tested by the ability of cells to reduce resazurin to resorufin.



Resazurin (blue) Resorufin (red)

Figure 13. Reaction of resazurin upon reduction by intact cells

The reduction indicates an intact cell metabolism and can be used to monitor cytotoxic activity of drugs and drug candidates.

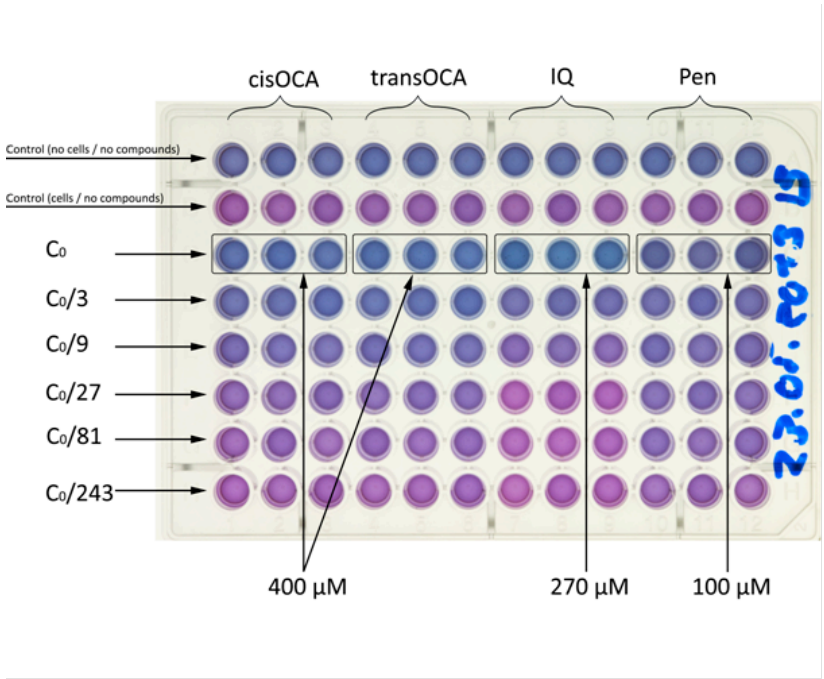


Figure 14. Typical picture of a 96-well plate for the resazurin assay in the presence of drugs after 48 h incubation.

From these colorimetric plate data, viability vs. drug concentration plots were obtained. These plots were used to calculate the IC_{50} values for the individual drugs.

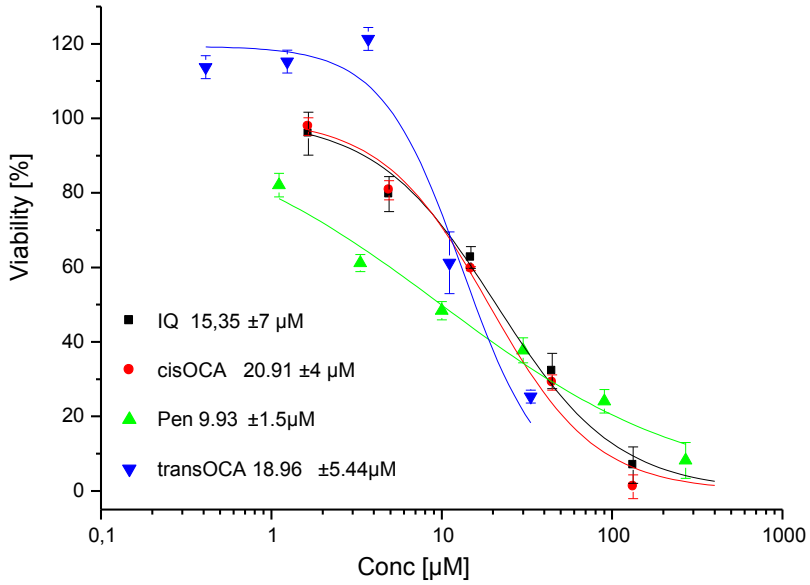


Figure 15. Typical plot of the viability vs. drug concentration and nonlinear regression curves for calculation of IC_{50}.

To characterize the activity, the percentage of resorufin formation in comparison to the control row (with cells only and without drugs) was calculated. For compounds which showed no inhibition or chemical interference in this assay no value was determined. For some compounds percentages higher than 100 % were obtained suggesting chemical reduction of resazurin by the drugs without interference of cells.

The data obtained for the resazurin viability assay are summarized in following table.

Table 3. IC_{50} values for the resazurin viability assay in *Leishmania tarentolae* promastigotes

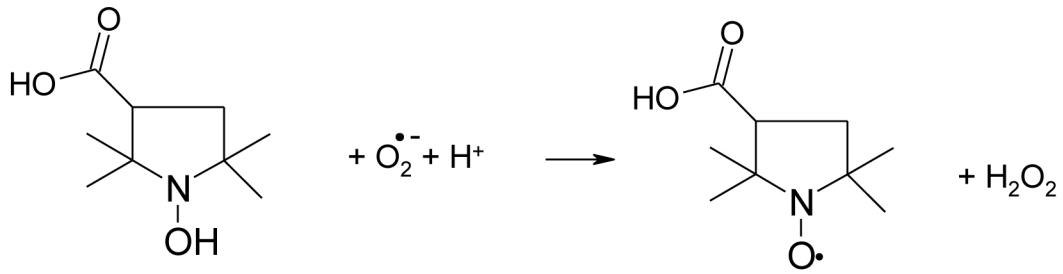
Compound	***IC50*** ***mean ± SD Unit***	***other activity***	***Replicates*** ***(plates x columns)***
α-Tocopheramine succinat	2695.7 ± 3090.1 µM		3 x 3
α-Tocopherol acetat	2284.0 ± 1671.0 µM		1 x 3
α-Tocopherol succinate		1.16 % Inhibition	
Tetramethylchroman-2-one	69.4 ± 30 µM		1 x 3
Tetramethylchroman-2-one ester E001	2281.0 ± 947.0 µM		1 x 3
Tetramethylchroman-2-one ester E002	3207.0 ± 3340.0 µM		1 x 3
Tetramethylchroman-2-one ester E003	399.0 ± 103.0 µM		1 x 3
Tetramethylchroman-2-one ester E004	38.03 ± 9.8 µM		2 x 3
Tetramethylchroman-2-one ester E005	567.5 ± 112.4 µM		2 x 3
Tetramethylchroman-4-one	133.8 ± 11.6 µM		1 x 3
cis Oxachromanol	28.2 ± 7.7 µM		3 x 3
trans Oxachromanol	17.8 ± 1.9 µM		3 x 3
TWIN	56.9 ± 32.3 µM		3 x 3
Resveratrol	161.5 ± 52.5 µM		3 x 3
Silibinin	496.3 ± 135 µM		3 x 3
Quercetin		654 % Stimulation	1 x 3
M8		213 % Stimulation	1 x 3
Amentoflavone	801.5 ± 966.6 µM		4 x 3
Digalloylresveratrol	131.7 ± 47.9 µM		1 x 3
Xanthohumol	23,8 ± 10.6 µM		3 x 3
Myxothiazol	5.3 ± 3.2 µM		3 x 3
Rotenone	21.7 ± 26.0 µM		2 x 3
Stigmatellin	5.7 ± 1.5 µM		2 x 3
Potassium cyanide	2192.5 ± 1908.5 µM		2 x 3
EO Arteminisia absinthi	611.0 ± 509.1 µg/ml		2 x 3
EO Bixa orellana	12.4 ± 7.2 µg/ml		3 x 3
EO Chenopodium ambrosoides	19.1 ± 6.1 µg/ml		4 x 3
EO Piper auritum	38.7 ± 9.7 µg/ml		2 x 3
Surfacen	1358 ± 722 µg/ml		1 x 3

Table 3. Continuation

Compound	IC_{50} mean ± SD Unit	other activity	Replicates (plates x columns)
Nemerosone	1.6 ± 1.6 µM		3 x 3
Guttiferone A	6.2 ± 2.6 µM		4 x 3
Ascaridol	143.1 ± 27.9 µM		3 x 3
Atovaquone	5.7 ± 5.0 µM		4 x 3
AmBisome		15% Inhibition	1 x 3
Dicumarol	142.5 ± 68.7 µM		3 x 3
Carvacrol	97.9 ± 10.5 µM		3 x 3
Caryophyllene Oxide	72.2 ± 18.3 µM		3 x 3
Duroquinone	142.5 ± 68.7 µM		3 x 3
Idebenone	10.4 ± 4.5 µM		3 x 3
Rapanone	74.6 ± 62.6 µM		6 x 3

4.4 Electron paramagnetic resonance $O_2^{\cdot-}$ measurements in Leishmania mitochondria and submitochondrial particles

Superoxide radical production in mitochondria of LtP or of Bovine heart SMP triggered by tested compounds was measured using the hydroxyl amine (1-hydroxy-3-carboxy-2,2,5,5 tetramethylpyrrolidin (CPH). For these experiments directly with organelles we used this non-cell-permeable CPH. To start respiration an addition of succinate or NADH was required. The reaction of radical transfer is shown below.



CPH
(hydroxyl amine)

CP·
(spin probe)

Figure 16. Reaction of the superoxide under the formation of stabile nitroxyl radical which can be detected by ESR spectroscopy.

The measurement proceeded in ten scans from which the increase of the amplitude was calculated. A sample spectrum is presented below.

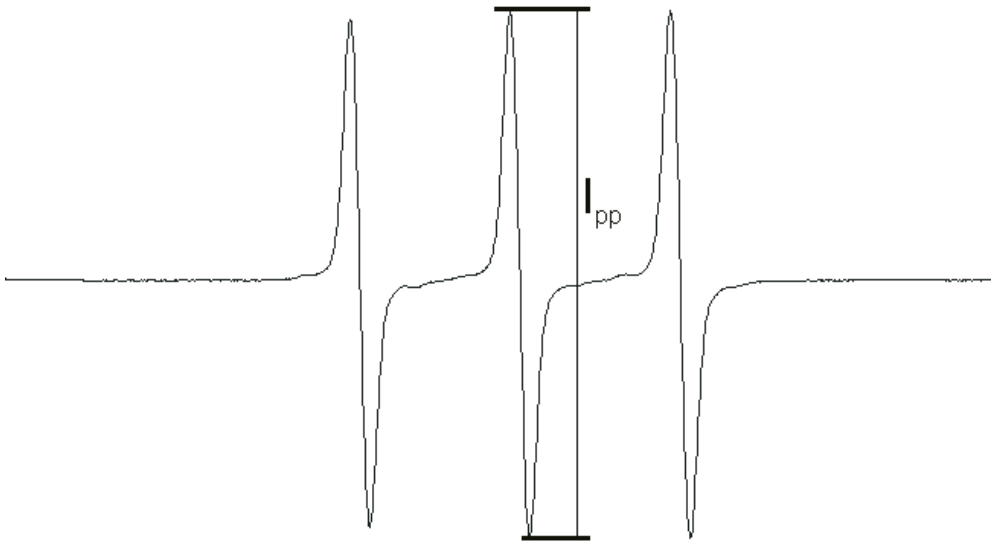


Figure 17. Image of an ESR spectrum obtained by one electron oxidation of CPH in biological systems. The intensity of central peak (Ipp) was used for quantification.

The obtained data from SMP are listed below.

Table 4. Radical production in SMP in presence of NADH. Data are expressed in percentage of radical production of control samples respiring the same substrate.

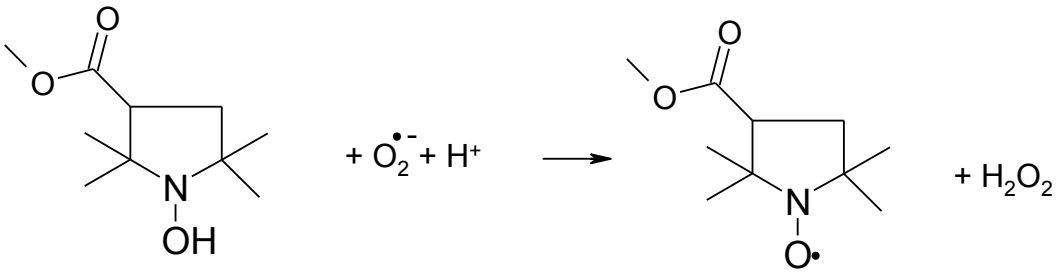
Compound	***Radical production*** ***Mean ± SD***	***Concentration***
Antimycin A	196 ± 17 %	5 µM
Ascaridol	109 ± 9 %	100 µM
Atovaquone	112 ± 13 %	100 µM
EO Chenopodium ambrosioides	126 ± 0 %	100 µg/ml
Guttiferone A	98 ± 5 %	100 µM
Nemerosone	106 ± 7 %	100 µM
Pentamidine isothionate	104 ± 9 %	100 µM

Table 5. Radical production in SMP in presence of succinate. Data are expressed in percentage of radical production of control samples respiring the same substrate

Compound	*Radical production* *Mean ± SD*	*Concentration*
Antimycin A	377 ± 71 %	5 µM
Ascaridol	123 ± 3 %	100 µM
Atovaquone	207± 14 %	100 µM
EO Chenopodium ambrosioides	157 ± 14 %	100 µg/ml
Guttiferone A	106 ± 2 %	100 µM
Nemerosone	165 ± 58 %	100 µM
Pentamidine isothionate	99 ± 8 %	100 µM

4.5 Electron paramagnetic resonance $O_2^{\cdot-}$ measurements in Leishmania cells

For the superoxide radicals production in LtP cells triggered by tested compounds the hydroxyl amine 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCl (CMH) was used, which has the ability to passage the cell membrane. The measurement process and analysis correspond to the measurements in SMP or mitochondria of *Leishmania*.



CMH
(hydroxyl amine)

CM·
(spin probe)

Figure 18. Reaction of the hydroxyl amine CMH with superoxide radicals.

The data obtained from ESR measurements and plotted via ESRView are summarized in the table below.

Table 6. Superoxide radical production (CMH Oxidation) in *Leishmania tarentolae*

Compound	***Radical production*** ***Mean ± SD***	***Concentration***
cis Oxachromanol	93 ± 13 %	100 µM
trans Oxachromanol	87 ± 6 %	100 µM
Tetramethylchroman-2-one	238± 185 %	100 µM
Tetramethylchroman-2-one ester E002	133 ± 119 %	100 µM
Tetramethylchroman-2-one ester E004	78 ± 16 %	100 µM
Tetramethylchroman-2-one ester E005	61 ± 14 %	100 µM
Twin chromanol	337 ± 266 %	100 µM
Antimycin A	145 ± 27 %	5 µM
Ascaridol	104 ± 11 %	100 µM
EO Chenopodium ambrosioides	225 ± 93 %	100 µg/ml
Pentamidine isothionate	84 ± 12 %	100 µM
Resveratrol	72 ± 5 %	100 µM
Xanthohumol	32 ± 2 %	100 µM
Nemerosone	257 ± 43 %	100 µM
Guttiferone A	130 ± 22 %	100 µM
Atovaquone	68 ± 3 %	100 µM
AmBisome	136 ± 8 %	100 µM
Dicumarol	108 ± 6 %	100 µM
Idebenone	291 ± 77 %	100 µM
Rapanone	107 ± 25 %	100 µM

4.6 Complex activities

The activity of complex I and II were measured in *Leishmania* mitochondria (LtP Mit) according to the principle in figure 19.

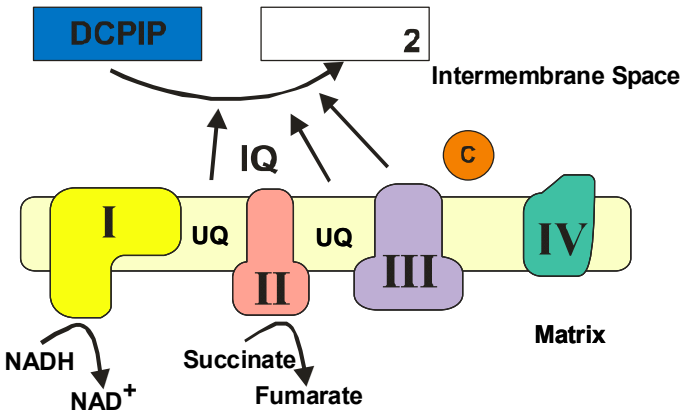


Figure 19. Measurement principle of complex I and II activities for mitochondria.

In this assay dichlorophenol-indophenol (DCPIP, blue) is reduced by the mitochondrial complexes via ubiquinone (UQ) and idebenone (IQ) to colorless reduced DCPIP (DCPIP-H_2). Complex specifity is achieved by using NADH or succinate as substrate. The rate of the DCPIP activity was used as a measure for the inhibition of the respective complexes by drugs as shown in figure 20.

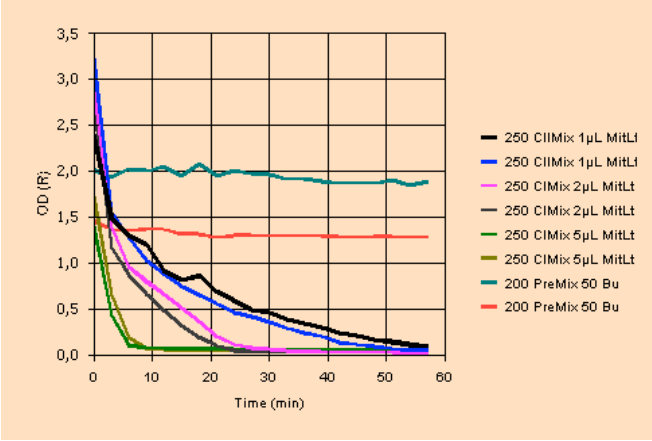


Figure 20. Typical DCPIP reduction kinetics during the measurement of mitochondrial complex activities.

In analogy to other assays for strongly inhibiting substance an IC_{50} value was calculated and for weakly inhibiting substances the percentage of inhibition at the highest concentration was given in tables 6 and 7.

Table 7. Inhibition of complex I activity in LtP Mit.

Compound	**IC_{50}** **mean ± SD Unit**	***Inhibition*** ***mean ± SD***	***Concentration***
AmBisome		31.5 ± 3.6 %	100 µM
Amentoflavone		1.1 ± 0.7 %	100 µM
Ascaridol		1.9 ± 0.3 %	100 µM
Atovaquone	0.1 ± 0.01 µM		
Carvacrol		-33.3 ± 15 %	100 µM
Caryophyllene Oxide		8 ± 1 %	100 µM
EO Artemisia absinthium		50.4 ± 4.3 %	100 µg /ml
EO Bixa orellana	0.8 µg/ml		
EO Chenopodium ambrosoides		-81 ± 6.3 %	100 µg /ml
EO Piper auritum		-12.6 ± 2.3 %	100 µg/ml
Guttiferone A		-63.1 ± 11.9 %	100 µg/ml
Nemerosone		-4.4 ± 2.8 %	100 µM
Pentamidine isothionate		-24.4 ± 6.3 %	100 µM
Rapanone		-45.1 ± 8.3 %	100 µM

Table 8. Inhibition of complex II activity in LtP Mit.

Compound	**IC_{50}** **mean ± SD Unit**	***Inhibition*** ***mean ± SD***	***concentration***
AmBisome		22.2 ± 7.4 %	100 µM
Amentoflavone	46.1 ± 6.4 µM		
Ascaridol		49.7 ± 2 %	100 µM
Atovaquone		33.6 ± 6.4 %	100 µM
Carvacrol		-14.7± 2.1 %	100 µM
Caryophyllene Oxide		-16.1 ± 6.9 %	100 µM
EO Artemisia absinthium	5 ± 0.2 µg/ml		
EO Bixa Orellana	41.9 ±0.6µg/ml		
EO Chenopodium ambrosoides		-1.2 ± 0.9 %	100 µg /ml
EO Piper auritum	0.2 µg/ml		
Nemerosone	36.5 ± 7.2 µM		
Pentamidine isothionate		20.6 ± 4.8 %	100 µM
Rapanone		-2.8 ± 4.81 %	100 µM

4.7 Oxygen consumption

To evaluate drug effects on mitochondrial functions within LtP cells we measured the oxygen consumption of LtP cells directly in the cell culture medium (YEM) which contained glucose as an energy source. In figure 21 a typical oxygen consumption experiment is shown.

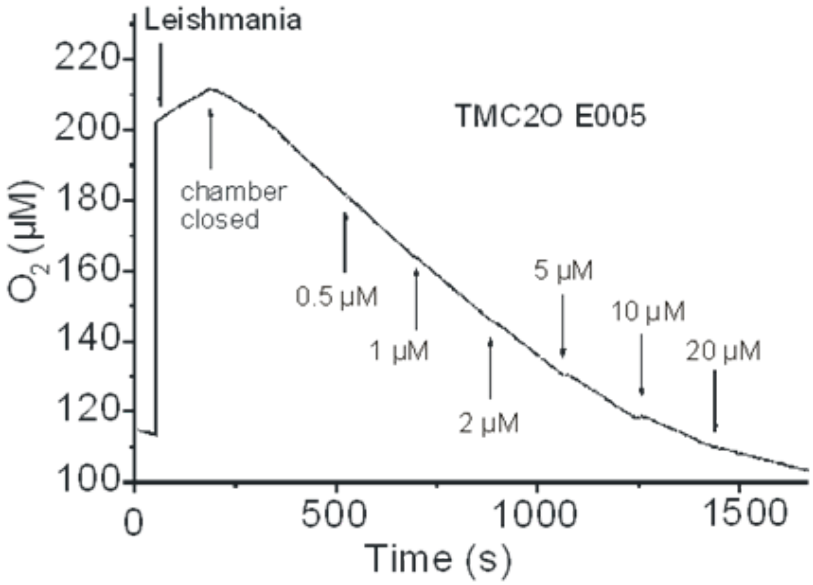


Figure 21. Oxygen consumption of LtP cells and inhibition by increasing concentrations of TMC2O.

In a typical experiment LtP (ca.100 Mio cells/ml) cell suspensions were added to the chamber of the oxygen electrode. After equilibration the chamber was closed and the basal consumption rate was measured. Then stepwise aliquots of the drug (TMC2OE005) stock solution in DMSO were added giving the indicated concentrations and the resulting rates were monitored.

From the oxygen consumption rates in the absence (100 %) and in the presence of drugs the activity – drug concentration curves were calculated. For compounds with strong inhibition (> 50%) in the chosen concentration range IC_{50} values were calculated (Tab. 8). For minor active compounds the inhibition at the highest concentration was given.

Table 9. Inhibition of oxygen consumption of LtP cells by tested compounds. For strongly inhibiting substance the IC_{50} for half inhibition of oxygen consumption is given. For less active compounds the percentage of inhibition at the highest concentration is reported.

Compound	**IC50** mean ± SD Unit	*Inhibition* mean ± SD	*Concen-tration*
Antimycin A	0.0067 ± 0.0002µM		
Ascaridol		11.9 ± 5.5 %	
KCN	39.60 ± 5.40 µM		
Myxothiazole	5.21 ± 0.55 µM		
Stigmatellin	0.238 ± 0.06 µM		
Rotenone		39.0 ± 4.6 %	200 µM
α-Tocopheramine succinate		4.81 ± 0.92 %	200 µM
α-Tocopherol acetate		8.69 ± 0.69 %	200 µM
α-Tocopherol succinate		5.47 ± 0.83 %	200 µM
Carvacrol		28.8 ± 28.5 %	400 µM
Atovaquone	2.46 ± 2.54 µM		
Caryophyllene Oxide	123 ± 32.3 µM		
Dicumarol		56 ± 8.3 %	200 µM
Guttiferone A	144 ± 19 µM		
Idebenone	33.1 ± 24.70 µM		
Nemerosone	42.7 ± 8.7 µM		
EO Bixa orellana	9.81 ± 1.26 µg/ml		
EO Chenopodium ambrosioides	66.6 ± 6.4 µg/ml		
EO Piper auritum	34.6 ± 1.1 µg/ml		
Rapanone	416 ± 119 µM		
Amentoflavone		15.8 ± 14.9 %	400 µM
Resveratrol		16.4 ± 0.9 %	200 µM
Xanthohumol	28.1 ± 5.5 µM		
Tetramethylchroman-2-one		63.5 ± 5.2 %	200 µM
Tetramethylchroman-2-one ester E001		29.1 ± 3.5 %	200 µM
Tetramethylchroman-2-one ester E002		23.4 ± 4.6 %	200 µM
Tetramethylchroman-2-one ester E003		61.4 ± 6.4 %	200 µM
Tetramethylchroman-2-one ester E004	63.9 ± 19.3 µM		
Tetramethylchroman-2-one ester E005	9.2 ± 1.6 µM		
Tetramethylchroman-4-one		19.7 ± 2.3 %	200 µM
Trans-Oxachromanol		17.9 ± 6.5 %	200 µM
Twin chromanol	55.00 ± 4.4 µM		
Cis-Oxachromanol		3.67 ± 23.29 %	200 µM

5 Discussion

5.1 Studied compounds

A variety of compounds was studied in this work. Due to the different complexity of the analytical methods applied not all compounds were studied with all analytical methods applied. For all compounds the inhibition of viability of LtP cells was studied. These results were used as selection guide for the other analytical methods which were aimed at elucidating the role of the mitochondrial function in drug action.

The compound classes studied originate from previous research work in our lab or from other labs. Tocopherol succinate and related compound were studied in the past as anticancer agents. Several groups have shown that α-TOS and also related amino analogs triggering apoptosis in certain cancer cell lines [32-34]. It was postulated that these compounds target mitochondrial complex II (succinate:ubiquinone oxidoreductase) in cancer cells and trigger mitochondrial ROS production [35]. Since anticancer and antimicrobial substances often share some mechanistic aspects, it was of interest how these compounds act on *Leishmania* cells.

The second class of compounds contains synthetic chromanol derivatives. Some of these compounds were synthesized and tested for improvement of antioxidant functions [36,37]. For example Twin has been shown in mitochondrial model systems of oxidative stress to be a highly effective antioxidant [38]. Furthermore, it was reported that low molecular chromanols and degradation products partially inhibit the mammalian complex III in mitochondria [39]. Chromanone derivatives in this group were synthesized to obtain structure activity information with respect to inhibition of mitochondrial complex III in mammals and *Leishmania*. It has been shown that some

of these derivatives inhibit complex III in leishmanial mitochondria with IC_{50} values of about 5 µM (TMC2OE005) while the mammalian complex was only inhibited at ten times higher concentrations [40].

The third group of resveratrol and related polyphenols are mostly natural food ingredients and synthetic analogues. For these compounds it has been shown that they are excellent antioxidants and interfere in multiple signaling pathways mediating beneficial effects in the mammalian organisms, such as antiinflammatory action [41,42]. Due to their effects on signaling cascades involved in cancer development and the observation of antiproliferative and proapoptotic effects in cancer cell lines benefits of such compounds in cancer therapy were suggested [41,43]. This may in part not only be mediated by the native polyphenols but also by their metabolites [43]. Furthermore, direct inhibition of ATP synthase in mammalian mitochondria was described [44]. Due to the molecular structure with many hydroxyl groups often for these compounds the oral bioavailability is questioned [41]. As many of these compounds are produced in plants as defense agents against fungi [45] their effect on *Leishmania* and their mitochondria was of interest.

The forth group of compounds contains a set of drugs which is approved in some countries for antimicrobial therapy. Pentamidine, amphotericin B, atovaquone are in use as antiprotozoal and/or antifungal drugs and were here included as reference compounds. Idebenone was used as therapeutic agent for neurological disorders, such as Friedreich ataxia [46]. Dicumarol is an obsolete anticoagulant agent which was used in human medicine. Other compounds (nemerosone, guttiferone A, caryophyllene oxide, carvacrol and duroquinone are compounds which occur in nature and are under study in many different pharmacological contexts, p.e. as antileishmanial, anticancer and antifungal agents.

Finally the fifth group of compounds are well known inhibitors of mammalian mitochondria and were included for reference purposes. All these compounds block respiration in mitochondria. Stigmatellin and myxothiazole inhibit the mitochondrial complex III at the Q_o binding site. In SMP for both compounds IC_{50} values for complex III inhibition around a few micromoles/l were reported [47]. In contrast, antimycin A inhibits the Q_i binding site in complex III with IC_{50} values also in the nmolar range [48]. While stigmatellin and myxothiazole inhibit $O_2^{\cdot-}$ release from complex III antimycin A is a strong trigger for $O_2^{\cdot-}$ formation at this complex. Rotenone binds to the ubiquinone binding site of mammalian complex I and inhibits respiration. For this compound IC_{50} values in the lower nanomolar range were reported [49]. Whether rotenone triggers $O_2^{\cdot-}$ release from complex I is controversial. Most likely low concentrations of rotenone which incompletely block complex I may increase autoxidation of iron-sulfur clusters in complex I giving rise to increased $O_2^{\cdot-}$ release. Potassium cyanide (KCN) blocks complex IV and prevents oxygen consumption at this terminal complex. In contrast to the other inhibitors KCN requires around 1 mM in mammalian mitochondria for inhibition [48].

Major components of EO Chenopodium ambrosioides are ascaridole, caryophyllene oxide and carvacrol [50]. In EO from Bixa orellana ishwarane and geranyl geraniol were identified as major components, while however, the major portion is composed of 71 other components [51]. Major components of EO Artemisa absinthi were found to be monoterpenes, β-pinene and β-thujone as well as at least 26 other compounds [52]. In EO from Piper auritum the most abundant compound was safrol with 87 % and more than 60 other compounds [53]. For these essential oils it has to be taken into account that the major components are not necessarily the most active

pharmacological ingredients. Therefore, analysis of these EOs is only the first step to identify the active principle. Surfacen showed no strong activity in our assays.

5.2 Mitochondria in Leishmania as drug targets

Mitochondria provide important targets for drug action since: (i) they are essential for the energy metabolism of the cell, (ii) they are a major source of ROS, (iii) they are involved in the intrinsic pathway of apoptosis, (iv) and there are significant differences between mitochondria from mammals and *Leishmania*. In contrast to mitochondria in mammalian cells, which have rather stable environmental conditions, the function of mitochondria in protozoal parasites is obviously strongly modulated by the various extracellular environments during their life cycle. For *Leishmania,* only limited information about the importance of mitochondria during their life cycle is available. At least the existence and function of the mitochondrial cytochrome bc_1 complex (complex III) [54], succinate: ubiquinone oxidoreductase (complex II), and cytochrome oxidase (complex IV) was confirmed in promastigotes [55,56]. Likewise, NADH was shown to feed electrons into the respiratory chain [56] which, however, is different from the mammalian complex I [57]. The partial efficiency of atovaquone and antimycin A against *Leishmania*, shows that energy metabolism is one of the indispensable systems for the survival of the parasite [58,59]. The mitochondrial effects of other antileishmanial drugs, such as: licochalcone A and naphthoquinones, on the parasite mitochondrion, indicated that mitochondrial metabolism in kinetoplastid parasites could provide promising chemotherapeutic targets. In protozoa, the electron transfer chain has several particularities. One of them is an alternative complex I NADH: quinone oxidoreductase that is rotenone-insensitive [20,60]. Studies demonstrated that an enzyme corresponding to

the mammalian rotenone-sensitive complex I may be absent or not very active. The characteristic rotenone-insensitive NADH: quinone oxidoreductase has been observed in Trypanosoma and *Leishmania* and has no counterpart in humans [20,60].

Also complex II is present in mammals and *Leishmania*. Succinate: ubiquinone oxidoreductase (complex II) is entirely coded by nuclear DNA and is not involved in proton translocation. Basic biochemical studies suggested that in protozoa a classical complex II is present. Since the parasite has a limited electron transport between complexes I-III, succinate might be a primary electron donor for energy production. In addition, in *Leishmania* parasites succinate can be recycled from fumarate by fumarate reductase [60]. The structure of complex II has recently been studied for Trypanosoma and consisted of six hydrophilic and six hydrophobic subunits [61]. The complex III occurs also in mammalian and leishmanial mitochondria. The cyt bc_1 complex (mitochondrial complex III) is a dimeric protein with a mass of 250 kDa. Each monomer consisting of 11 subunits in mammals. For Trypanosoma, belonging like *Leishmania* to Trypanosomatids, three subunits with catalytic groups and in total six units were recently identified [62]. The prosthetic groups involved in electron transfer are located in the cyt b subunit (chain C, cyt b_L and cyt b_H), in the Rieske iron sulfur protein (chain R, FeS cluster) and in chain Q (cyt c_1) [63]. The cyt b subunit of the cyt bc_1 complex includes b cytochromes and is the binding site of ubiquinone and ubiquinol. The Q_{in} binding site for ubiquinone close to the matrix side is responsible of UQ reduction and the Q_{out} pocket in the proximity of the intermembrane space catalyzes ubiquinol oxidation and are both part of the cyt b subunit. Autoxidizing ubisemiquinone intermediates formed at the cyt bc_1 complex are a major source of reactive oxygen species in mitochondria [64,65]. This process can be modulated by

inhibitors of the cyt bc_1 complex. Essentially this principle of function is also observed in cyt bc_1 complex from yeast and Trypanosomatids including *Leishmania*. However, the comparison of sequences from the cyt b subunits reveal a high degree of conservation for functional important residues but a rather strong variance of amino acid sequences for the binding pocket surrounding these residues. This suggests species specific substrate or inhibitor binding as a target for this complex.

5.3 Conclusions from our results

The determination of the drug action on the viability of LtP cells has been reported to be an indicator of potential antileishmanial activity [27]. The resazurin assays is an established assay for the viability of cells including *Leishmania* [66]. This was also confirmed in our experiments with a few exceptions. According to the chosen experimental protocol in our study LtP cells, drugs and resazurin were co-incubated in our assays. In this setup we observed problems with some polyphenols (Quercetin, M8) causing chemical resazurin reduction much faster than cellular reduction of resazurin. Therefore, the results of these compounds do not reflect their influence on viability.

The first group of compounds, the tocopherol succinate derivatives (α-TOA, α-TOS, α-TNS), show almost no or very weak inhibition of viability. Likewise these compounds show at 200 µM also only weak inhibition of mitochondrial oxygen consumption in *Leishmania*. In contrast to the potential anticancer activity of these compounds they are not active in *Leishmania*. A possible explanation could be a high esterase/peptidase activity in *Leishmania* decomposing these compounds before they can inhibit mitochondria.

The second group of compounds, the synthetic chromanols, are a rather heterogenous group of compounds. Remarkable IC_{50} values for viability were obtained for TMC2O (69.4 µM), TMC2OE004 (38 µM), cis-oxachromanol (28.2 µM), trans-oxachromanol (17.8 µM) and Twin (56.9 µM). Obviously not all these effects are based on mitochondrial inhibition since oxygen consumption in LtP cells ís only affected by Twin and TMC2OE004. Surprisingly TMC2OE005 effectively inhibits mitochondrial oxygen consumption (IC_{50} = 9.2 µM) but not viability. A possible explanation could be a lacking long term stability of this compound in the viability assay (48 h). Furthermore, significant stimulation of $O_2^{\cdot-}$ release in LtP cells is only achieved by Twin, which shows also inhibition of viability and O_2 consumption.

In the third group of compounds, the polyphenols, the highest inhibition of viability was observed with xanthohumol at an IC_{50} of 23.8 µM. Other polyphenols show effects on viability at much higher concentrations or even stimulation of resazurin reduction without cells. This suggests that the current resazurin viability assay with co-incubation of polyphenols and resazurin is not well suited for this compound class and should be modified or backed up by a viability assay with a different principle. An interesting finding however is that xanthohumol inhibits LtP cell oxygen consumption (IC_{50} = 28.1 µM), while resveratrol is ineffective (at 200 µM only 16.4 % inhibition). This suggests a direct action of xanthohumol on leishmanial mitochondria. However, for both compounds no strong stimulation of $O_2^{\cdot-}$ formation in LtP cells was observed. These findings show that xanthohumol is interesting for further studies in this field.

The forth group of compounds, drugs and other bioactive natural products, show variable effects on LtP cell viability. Best compounds are nemerosone (1.6 µM), atovaquone (5.7 µM), guttiferone A (6.2 µM) and idebenone (10.4 µM). For

idebenone this result has to be verified by other viability assay to exclude a possible direct interaction with resazurin due to its strong redox activity. Atovaquone is a well-known antiplasmodial drug, therefore, it is interesting that it is also active in LtP cells. It also strongly inhibits oxygen consumption in LtP cells (IC_{50} = 2.46 µM) and stimulates $O_2^{\cdot-}$ formation in SMP. In LtP cells this stimulation of $O_2^{\cdot-}$ release by atovaquone was not observed, an effect which however could have various reasons. In contrast, nemerosone significantly stimulated $O_2^{\cdot-}$ release in LtP cells and SMP suggesting a specific mitochondrial target of nemerosone. This is supported by its effects on oxygen consumption of LtP cells: nemerosone (IC_{50} = 42.7 µM) and idebenone (33.1 µM). Although ascaridole and liposomal amphotericin B show interesting results in other studies the effect on viability in our assay are only moderate or weak. Therefore, from this group of compounds nemerosone, idebenone and guttiferone A are interesting for further studies besides established compounds as atovaquone, amphotericin B and ascaridole.

The fifth group of compounds contains the known inhibitors of mammalian mitochondria. For this group we saw strong effects of the complex III inhibitors stigmatellin and myxothiazol on viability and oxygen consumption in LtP cells. In contrast rotenone is less effective in inhibiting viability and O_2 consumption in LtP cells. This is possible due to the alternative complex I in leishmanial mitochondria. Furthermore antimycin A is a strong trigger of $O_2^{\cdot-}$ formation in LtP cells and mammalian SMP. Therefore, in general complex I and III inhibitors seem to be promising for further studies.

In the group of essential oils EO Bixa orellana (12.4 µg/ml) and EO Chenopodium ambrosioides (19.1 µg/ml) had the strongest effects LtP cell viability. The strong

effects of EO Bixa orellana (9.81 µg/ml) on LtP cell oxygen consumption suggest mitochondrial targets of their ingredients. On the other hand EO Chenopodium ambrosioides stimulated $O_2^{\cdot-}$ release in SMP and LtP cells. Interestingly, EO Bixa orellana gave a rather strong inhibition of LtP Mit complex I activity. With the exception of EO Artemisia absintum other EOs showed significant effects on LtP Mit complex II activity. Further studies of EO Bixa orellana and EO Chenopodium ambrosioides should reveal their potential as antileishmanial agents.

Although not all compounds were studies with all methods this study revealed interesting candidates from each compound group which deserve further studies on their mechanism and their effectivity as antileishmanial agents as well as the role of leishmanial mitochondria as drug target.

6 Summary (english)

Leishmaniasis represents a major health problem for people living in developing countries. There are 12 million people infected and about 350 million of people live in area with high risk of infection. The current drugs used for treatment or prevention of leishmaniasis are insufficient because of developing resistance or side effects. Therefore, the development of new antileishmanial drugs is inevitable.

In these diploma thesis diverse compounds were studied with different analytical methods. Inhibition of viability of *Leishmania tarentolae* promastigote (LtP) cells was used as a screening method to select compounds for further testing. These results were used as indicator of potential antileishmanial activity of tested drugs. With effective compounds further assays, such as the influence on superoxide radical formation in submitochondrial particles from bovine heart (SMP) or LtP cells, oxygen

consumption of LtP cells and complex I and II activities in LtP mitochondria, were performed. From these methods we obtained the following results: The tocopherol succinate derivatives showed low inhibition of viability and also weak inhibition of mitochondrial oxygen consumption in *Leishmania*. The synthetic chromanols showed very variable IC_{50} values for viability. Only for few compounds this was based on mitochondrial inhibition. The polyphenol xanthohumol showed highest inhibition of LtP cell viability (IC_{50} = 23.5 µM). The influence of xanthohumol on oxygen consumption (IC_{50} = 28.1 µM) point at a direct action on leishmanial mitochondria. In contrast, resveratrol was ineffective in this assays. From the group of drug molecules best results in viability assays were achieved with nemerosone (IC_{50} = 1.6 µM), atovaquone (IC_{50} = 5.7 µM), guttiferone A (IC_{50} = 6.2 µM), idebenone (IC_{50} = 10.4 µM). Atovaquone inhibits oxygen consumption strongly in LtP cells and stimulates $O_2^{\cdot-}$ formation in SMP, but this stimulation was not achieved in LtP cells. In contrast, nemerosone significantly stimulated $O_2^{\cdot-}$ release in SMP and LtP cells and the oxygen consumption of LtP cell was moderately inhibited. For inhibitors of mammalian mitochondrial complex III stigmatellin and myxothiazole strong effects were shown on viability and oxygen consumption of LtP cells. In contrast rotenone showed a low effectivity on viability and oxygen consumption. Antimycin A proved to be a very effective as trigger of $O_2^{\cdot-}$ formation in both SMP and LtP cells.From the group of essential oils the EO Bixa orellana (12.4 µg/ml) and EO Chenopodium ambrosioides (19.1µg/ml) had the strongest effects on LtP cell viability. The strong effects of EO Bixa orellana (9.81 µM) on LtP cell oxygen consumption suggest mitochondrial targets of their ingredients. On the other hand EO Chenopodium ambrosioides stimulated $O_2^{\cdot-}$ release in SMP and LtP cells. EO Bixa orellana inhibits complex I activity in LtP Mit, strongly. Beside of EO Artemisia absintum all other EOs

showed significant effects on LtP Mit complex II activity. EO Bixa orellana and EO Chenopodium ambrosioides should reveal their potential in consecutive studies.In summary, the results of this study show the potentials of compounds in antileishmanial activity and demonstrate that for some compounds this is based on inhibition of mitochondrial function in *Leishmania*.

7 Summary (german)

Leishmaniasis stellt ein bedeutendes Gesundheitsproblem in vielen Entwicklungsländern dar. Etwa 12 Millionen Menschen sind infiziert und 350 Millionen Menschen leben in Regionen mit erhöhtem Infektionsrisiko. Die Medikamente die derzeit angewendet werden sind aufgrund von Nebenwirkungen und Resistenzentwicklung nicht ausreichend. In dieser Diplomarbeit werden verschiedene aktive Verbindungen gegen *Leishmanien* mit unterschiedlichen Methoden getestet. Die Hemmung der Viabilität von Zellen der *Leishmania tarentolae* Promastigoten wurde als Selektionsmethode verwendet. Die erhaltene Ergebnisse wurden als Indikator für die potentielle antileishmaniale Aktivität verwendet. Die wirksamen Substanzen wurden weiter charakterisiert mit folgenden Methoden: Einfluss auf die Sauerstoffradikalfreisetzung in submitochondrialen Partikeln und Leishmanien Zellen, Hemmung des Sauerstoffverbrauchs der Leishmanien und der Einfluss auf die Aktivität des Komplex I und II in Leishmanien getestet. Diese Methoden ergaben, dass die Tocopherolsuccinat Derivate eine schwache Hemmung der Viabilität sowie eine geringe Hemmung des mitochondrialen Sauerstoffverbrauches in Leishmanien zeigten. Die synthetischen Chromanole zeigten unterschiedliche IC_{50} Werte für die Viabilität, aber nur für einige Verbindungen ist dies ein Zeichen von mitochondrialer Hemmung. Auf der anderen

Seite zeigt das Polyphenol Xanthohumol die höchste Viabilitätshemmung (IC_{50}= 23.5 µM). Der Einfluss auf den Sauerstoffverbrauch von Leishmanien durch Xanthohumol (IC_{50} = 28.1 µM) beweist die direkte Wirkung auf Mitochondrien von Leishmanien. Resveratrol war nicht effektiv als Hemmstoff des Sauerstoffverbrauchs und der Viabilität von Leishmanien. Die besten Ergebnisse bei der Viabilitäts-Hemmung zeigten Nemerosone (IC_{50} = 1.6 µM), Atovaquone (IC_{50} = 5.7 µM), Guttiferone A (IC_{50} = 6.2 µM), Idebenone (IC_{50} = 10.4 µM). Atovaquone hemmt stark den Sauerstoffverbrauch in *Leishmanien* und stimuliert $O_2^{\cdot-}$-Freisetzung in submitochondrialen Partikeln, aber in Leishmanien Promastigoten wurde diese Stimulation nicht beobachtet. Nemorosone dagegen bewirkte eine wesentliche $O_2^{\cdot-}$-Freisetzung in SMP und Leishmanien sowie eine moderate Sauerstoffverbrauchshemmung in Leishmanien Zellen. Stigmatellin und Myxothiazol zeigten eine starke Hemmung der Viabilität und des Sauerstoffverbrauches in Leishmanien. Rotenon zeigte eine schwache Wirkung in der Viabilitäts-Prüfung und eine moderate Hemmung des Sauerstoffverbrauchs in Leishmanien Zellen. Antimycin A fungiert als effektiver $O_2^{\cdot-}$ Auslöser sowohl in SMP als auch in Zellen von Leishmanien. Aus der Gruppe von essentiellen Öle zeigen EO Bixa orellana (12.4 µg/ml) und EO Chenopodium ambrosioides (19.1 µg/ml) die stärkste Aktivität auf die Viabilität von Leishmanien Zellen. EO Bixa orellana zeigte eine starke Wirkung (9.81 µM) auf den Sauerstoffverbrauch was auf die Hemmung der Mitochondrien hindeutet. EO Chenopodium ambrosioides stimulierte die $O_2^{\cdot-}$ Freisetzung sowohl in SMP als auch in Leishmanien. EO Bixa orellana inhibiert stark die Aktivität von Complex I in Mitochondrien von Leishmanien. Außer EO Artemisia absintum zeigten alle essentiellen Öle signifikante Wirkung auf Aktivität von Komplex II in Mitochondrien von Leishmanien. Daraus ergibt sich, dass das Wirkungspotenzial von

EO Bixa orellana und EO Chenopodium ambrosoides weiter untersucht werden sollten. Zusammenfassend, zeigen die Ergebnisse dieser Studie die Potenziale der Wirkung der getesteten Verbindungen gegen Leishmanien und die Rolle der Hemmung mitochondrialer Funktionen dabei.

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10 List of Abbreviations

Abbreviation	Description
BSA	bovine serum albumin (fatty acid free)
c	cytochrome c
CL	cutaneous leishmaniasis
CMH	hydroxylamines 1-hydroxy-3methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-HCl
CPH	1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidin
DFO	Desferal
DMSO	dimethylsulfoxide
DTPA	Diethylenetriamene pentaacetate
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
EO	essential oil
ER	Endoplasmatic reticulum
ESR/EPR	electron spin resonance/electron paramagnetic resonance
ETC	electron transport chain
GC	Golgi Complex
IC_{50}	half minimal inhibitory concentration of a substance
Ipp	Intensity peak to peak
kDNA	kinetoplast deoxyribonucleic acid
LtP	Leishmania tarentolae promastigotes
LtPMit	Leishmania mitochondria
MCL	mucocutaneous leishmaniasis
NADH	Coenzym Q Oxidoreduktase
OD	optical density
Q	Ubiquinone
ROS	reactive oxygen species
RPM	revolutions per minute
SMP	submitochondrial particles
SOD	superoxide dismutase
TRIS	tris(hydroxylmethyl)aminomethan
VL	visceral leishmaniasis
YEM	yeast extract medium

11 Curriculum vitae

Education

1999-2002 European Middle School ,Neustiftgasse 100,1070 Vienna, Austria

2002-2006 Bilingual Grammar School, Bilikova 24, 84419 Bratislava, Slovakia

30.Mai 2006 State examinarion C1 (natural scientific-german)

1.Juny 2006 School leaving examination

since September 2006 University of Vienna, Pharmacy

2010-20111 Englishcourse at Canadian Bilingual Institute, Bratislava

July-September 2012 GEOS, English School (USA-California)

Work Experience

July/August 2004 Intership at Allianz Insurance Group, Slovakia

July/August 2006 Intership at Lufthansa, Slovakia

2006-2008 Activity Leader (Basketball Coach) at Vienna International School,Austria

July/August Intership Adrianmed Bratislava, Slovakia (office work)

July 2009 Intership at University Pharmacy in Bratislava, Slovakia

2011-2013 Temporary job in Aevitas Pharmacy, Dunajska Streda,Slovakia

July 2013 Intership at Department of Parasitology*, Institute* of Tropical Medicine *"*Pedro Kouri*",*Havanna *City, Cuba*