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Titel der Diplomarbeit

„*In vitro* testing of substances derived from tropical plants
as possible drugs against *Entamoeba histolytica* and
Giardia intestinalis“

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Mirjana Drinić

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Summary

The protozoan parasites *Entamoeba histolytica* and *Giardia intestinalis* present important health problems worldwide. *E. histolytica* causes amoebic colitis and liver abscess with an incidence around 40 million infections per year and up to 100,000 deaths. *G. intestinalis* causes around 280 million symptomatic infections every year and due to the persistent diarrhea, has a high morbidity rate among children. The drug of choice for treatment of both parasites, for more than 40 years, is metronidazole. However, this drug also possesses adverse properties such as potential teratogenicity and central nervous system toxicity.

In order to identify new agents with activity against *E. histolytica* or *G. intestinalis*, fourteen compounds isolated from tropical plants, from Rutaceae, Meliaceae and Stemonaceae families, were tested for their activity against these parasites. The parasites were grown in 96 well microtiter plates, placed in air-tight plastic boxes, in which anaerobic conditions were established. The isolated compounds were examined in quick tests at concentrations of 1 µg/ml and 20 µg/ml. After 24 h and 48 h, life and dead cells were stained and counted.

Only one out of fourteen compounds showed activity against both parasites, methylgerambullin, a sulphur-containing amide, isolated from *Glycosmis mauritiana*. Additional testing showed, for example, an EC₅₀ of 6.09 µg/ml for anti-amoebic activity and 6.14 µg/ml for anti-giardial activity after 24 h of treatment. We found that the activity was lower in freshly prepared media, and linked this effect to the level of reduced cysteine in the medium. This led us to believe that chemical reaction of the compound with thiol groups in the medium and in the parasite may be important for its mode of action.

Furthermore, the effect of methylgerambullin on the *E. histolytica* proteome was analysed by two-dimensional gel electrophoresis, which revealed four newly induced proteins. The proteins were identified by tandem mass spectrometry as isomers of alcohol dehydrogenase and pyruvate:ferredoxin oxidoreductase. These proteins were shifted towards a more acidic isoelectric point (pI), possibly due to phosphorylation of the proteins. Taken together, a new active agent was identified and initial information on its mode of action was gathered.

Zusammenfassung

Die parasitischen Protozoen *Entamoeba histolytica* und *Giardia intestinalis* sind weltweit verbreitete Krankheitserreger. *E. histolytica* verursacht Amöbenruhr und Leberabzess mit etwa 40 Millionen Infizierten pro Jahr und bis zu 100.000 Todesfällen. *G. intestinalis* verursacht jedes Jahr etwa 280 Millionen symptomatische Infektionen und eine hohe Morbidität unter Kindern mit chronischem Durchfall. Seit über 40 Jahren ist Metronidazol das Therapeutikum der Wahl zur Behandlung der beiden Parasiten. Allerdings hat dieses Medikament auch unerwünschte Eigenschaften wie zum Beispiel mögliche Teratogenität und Neurotoxizität.

Das Ziel dieser Arbeit war es, die Aktivität von vierzehn neu isolierten Naturstoffen gegen *E. histolytica* und *G. intestinalis* zu testen, die aus tropischen Pflanzen der Familien Rutaceae, Meliaceae und Stemonaceae isoliert waren. Die Parasiten wurden in 96-Loch Mikrotiterplatten in luftdichten Plastikboxen unter anaeroben Bedingungen gezüchtet. Jede Substanz wurde zuerst in Schnelltests in den Konzentrationen von 1 µg/ml und 20 µg/ml getestet. Lebende und tote Zellen wurden nach 24 h und 48 h angefärbt und gezählt. Nur eine der Substanzen zeigte eine Aktivität gegen die beiden Parasiten, Methylgerambullin, ein Schwefel enthaltendes Amid, das aus *Glycosmis mauritiana* isoliert war. In zusätzlichen Tests wurde zum Beispiel eine EC50 von 6,09 µg/ml für anti-Amöben und 6,14 µg/ml für anti-Giardien Aktivität für eine Behandlung von 24 h gefunden. Die Aktivität des Methylgerambullins war in frischem Medium niedriger und wir vermuteten, dass dieser Effekt mit dem Gehalt von reduziertem Cystein in Medium zusammenhing. Deshalb könnte die chemische Reaktion zwischen Methylgerambullin und Thiolgruppen im Medium und in den Parasiten wichtig für den Wirkmechanismus sein.

Der Effekt des Methylgerambullins auf das *E. histolytica* Proteom wurde mittels zweidimensionaler Gelelektrophorese analysiert. Vier neue Spots in den behandelten Amöben wurden mittels Tandem-Massenspektrometrie als Isoformen von Alkoholdehydrogenase und Pyruvat:Ferredoxin Oxidoreduktase identifiziert. Beide Proteine waren in dem Gel zu einem saureren isoelektrischen Punkt verschoben, möglicherweise wegen Phosphorylierungen. Zusammenfassend wurde in dieser Arbeit eine neue aktive Substanz identifiziert und erste Informationen über ihren Wirkmechanismus wurden gewonnen.

1. Introduction

1. 1. Natural sources

Since the beginning of mankind, people were relying on nature. On the one hand they tried to restrain it in order to make it more predictable and to build habitats and acreage by destroying it. On the other hand they were dependent on natural goods as sources of food, shelter and healing remedies. The ancient traditional medicine systems were based on plants, which have been used as powders and botanical potions to cure and prevent diseases (Raskin et al., 2002; Gurib-Fakim 2006).

During the evolution, plants, being non-mobile organisms, had to develop defense mechanisms against predators and diseases. At the same time they had to attract symbionts and protect themselves against other plants which were immediate competitors for the same resources in an ecosystem (Gurib-Fakim, 2006; Cseke et al., 2006; Gale et al., 2007). In order to achieve all that, besides primary products, such as carbohydrates, lipids, proteins, chlorophyll and nucleic acids, which are used for building and maintaining the cells, the plants were able to synthesize so-called secondary metabolites with a crucial role in ecophysiology of the plants as recently shown (Briskin, 2000; Cseke et al., 2006). Most plant-derived bioactive compounds used in pharmacy, such as alkaloids, phenolics and terpenoids are, in fact, secondary metabolites of the plants (Raskin et al., 2002).

There is an estimation that 25% of the drugs prescribed nowadays are of plant origin as well as 11% of the 252 drugs, which are considered basic and essential by World Health Organization (WHO) (WHO, 1992; Rates, 2001). However, knowing that there are 250,000 living plant species, only 5000 of which have been screened for their pharmacological activities until 1991, make us aware that the higher plants as a source of new drugs are still underestimated and poorly studied (Payne et al., 1991; Rates, 2001).

Although there is a high predominance of single-ingredient drugs or so called new chemical entities (NCEs) in modern pharmaceutical industry, one has to keep in mind that there is a much greater diversity of bioactive compounds in the variety of plant species than in any artificially made chemical library. Therefore, natural compounds should be screened in order to find alternative treatments to failing medicinal products as well as to detect new drug candidates (Balandrin et al., 1993; Balunas

and Kinghorn 2005; Rocha et al., 2005; Chin et al., 2006; Gale et al., 2007; Butler, 2004; Schmidt et al., 2007).

1. 1. 2. Historical and current use of plant-derived substances

Alkaloids

The first written record about medicinal plants can be found around 5000 years B.C., on the cuneiform Mesopotamian tablet of the old Sumerians (Swerdlow, 2000). For centuries the surgeons have used extracts of opium and mandrake (*Mandragora officinarum*) as anesthetics prior to surgery or for the treatment of pain and sleeplessness (Carter, 1996, 2003). The opium is mentioned among other ancient medical texts, in the Ebes Papyri, around 1500 B.C. and texts of Greek's pharmacologist and botanist Pedanius Dioscorides (40-90 A.D.) as well (Scholl, 2002; Carter, 2003). It was used in combination with mandrake all over the Old and New World, until 1874 when they were replaced with ether and then other modern anesthetics (Carter, 1996). Morphine, isolated from opium poppy (*Papaver somniferum*), was the first pure natural product used for therapy introduced for a wide commercial use by Merck Company in 1826 (Newman et al., 2000). Together with the other alkaloids isolated from the poppy plants narcotine, codeine, thebaine, papaverine and narceine, it is still applied as analgesic in modern medicine (Goldstein et al., 1974).

Many other alkaloids, in use today, have been used for centuries in traditional medicine, for example, galantamine, an acetylcholinesterase inhibitor, isolated from *Galanthus nivalis* (Howes et al., 2003). This plant was in use for neurological conditions by people in Caucasus Mountains in Russia and other Eastern European countries (Heinrich and Lee Teoh, 2004). Galantamine is currently one of only four drugs applied in treatment of Alzheimer's disease (Chin et al., 2006; Prvulovic et al., 2010).

Atropine, hyoscyamine and scopolamine, which are isolated from *Atropa belladonna*, *Datura* spp., *Mandragora* spp. and *Brugmansia* spp., the plants of which the sedative powers were described even in Shakespeare's plays (Carter, 1996; Griffin and Lin, 2000), are in use as anticholinergics in western medicine (Balandrin

et al., 1993; Schmidt et al., 2007). Until replaced with tropicamide or phenylephrine, atropine was broadly used in ophthalmology as mydriatic, for a pupil dilatation, and cyclopegic, for a temporary paralysis of the accommodation reflexes (Kedvessy et al., 1950). However, the atropine earned its place on the WHO's Model list of essential medicines from 2005 because it is used for the treatment of bradycardia and asystole in cardiac arrest (Vincent, 1997; WHO, 2009).

Rauwolfia serpentina (Indian snakeroot) was for centuries an important part of Indian traditional medicine. The active alkaloids of the plant, namely, reserpine and rescinnamine reduce blood pressure (Thompson, 1956).

Psychotria ipecacuanha (coca bush), member of the Rubiaceae family, had been brought to Europe in 1658 from Brazil and ever since was used in treatment of some types of dysentery. In 1817 an active compound, the alkaloid emetine, was isolated from the root of the plant (Craig, 1934; Imperato, 1981). Despite negative side effects on the heart, emetine and its synthetically produced dehydroemetine, were the gold standard in treatment of amoebiasis caused by *Entamoeba histolytica* (Bianchi et al., 1965; Brink et al., 1969), before the efficacy of metronidazole against the parasite was proven in 1966 (Powell et al., 1966).



Figure 1: *Psychotria ipecacuanha* (Photo Pharmawiki.ch)

Other members of the Rubiaceae family that saved millions of lives, during three centuries of use, are *Cinchona* spp. The extracts from the bark of the cinchona tree

have antimalarial properties, due to the alkaloid quinine and its stereoisomer quinidine (Duran-Reynals, 1947; Bruce-Chwatt, 1987; Greenwood, 1992). Although isolated at the beginning of 19th century, quinine was not chemically synthesizable until 1944, so the whole amount of the treatment drug came directly from the tree barks (Kyle and Shampe, 1974).

One area of the pharmaceutical research that relies heavily on plants as possible sources of new drugs and treatments is anti-cancer research. It is estimated that over 60% of anti-cancer agents, which are in use, are of natural origin. They are derived not only from plants, but from marine animals as well as from microorganisms (Cragg et al., 2005; Newman et al., 2003). The breakthrough has happened when in the 1950s so-called vinca alkaloids, vinblastine and vincristine, were isolated from *Catharanthus roseus* (Madagascar periwinkle). They were shown to have anti-mitotic and anti-microtubule activity in cancer chemotherapy (Cragg et al., 1997; Takimoto and Calvo, 2008).

Terpenoids

In the field of anti-malaria treatment Artemisinin (qinghaosu) and its derivatives are the most important drugs in use today. Artemisinin, a terpenoid, was isolated in 1972 from *Artemisia annua*. The beneficial activity of *Artemisia* had been mentioned as early as 200 B.C. in “Prescriptions for Fifty-two Diseases” found in a Mawangdui Han dynasty tomb. Extract of *Artemisia annua* was one of the cornerstones in traditional Chinese medicine (TCM) and its anti-malarial application was first reported in the fourth century in the “Handbook of Prescriptions for Emergencies” by Ge Hong. Although isolated in 1972 by Tu Youyou and proven to be the best and fastest existing antimalarial agent, it was not until 1979, when the findings were published in the Chinese medical journal that the rest of the world found out about it (Q.A.C.R.G., 1979).

The terpenoids digitoxin and digoxin are secondary metabolites from *Digitalis* spp. (foxglove). As early as 1775, William Withering had identified foxglove as the active component of a polyherbal mixture able to reduce edema in patients with congestive heart failure, and foxglove and its active constituents have been in use ever since (Dewick, 2002).

Other compounds

The National Cancer Institute (NCI) of the USA has founded a collection program with the U.S. Department of Agriculture (USDA), during which around 20,000 plant extracts were tested for anti-tumor activity in the period from 1957 to 1981.

Unfortunately these samples were not screened for other pharmacological activities (Hamburger and Hostettmann, 1991; Rates, 2001). However, within the program, in 1967, Monroe E. Wall and Mansukh C. Wani have isolated antineoplastic paclitaxel (Taxol) from *Taxus brevifolia* (Pacific yew tree) (Wall et al., 1995; Wani et al., 1971; Cragg, 1998; Cragg and Newman, 2005). Paclitaxel is used for the treatment of ovarian, breast, lung, as well as head and neck cancer. It has also shown efficacy against Kaposi sarcoma and ability to prevent restenosis of coronary stents as anti-proliferative agent (Heldman et al., 2001).

There are some other plant-derived compounds, which are in clinical development as possible anti-cancer agents. Triptolide from *Tripterygium wilfordii*, a plant well known in traditional Chinese medicine for treatment of variety of syndromes (Schmidt et al., 2007), has high cytostatic activity in different cell lines (Yang et al., 2003). Furthermore there is the group of combrestatins, isolated from *Combretum cafferum* (South African bush willow). *C. cafferum* was used in African and Indian traditional medicine, among others, for treatment of hepatitis and malaria. The combrestatins behave as anti-angiogenic agents, which by destroying the tumor's vascular system cause its necrosis (Li and Sham, 2002; Pinney et al., 2005; Cragg and Newman, 2005).

1. 1. 3. Obstacles and challenges in drug discovery from plant-derived substances

As discussed above, plants and plant-derived substances played an enormous role in drug discovery. Some of the most important medicines today, such as quinine, artemisinin, atropine, morphine, and digoxin as well as the anti-cancer drugs paclitaxel, vincristine and vinblastine are of plant origin. Even if the isolated substances do not serve as a drug, they very often provide a lead and serve as a model for development of the novel agents (Cragg and Newman, 2005). One of the

world wide best known drug, Bayer's Aspirin, is a synthetic derivate of salicylic acid, originally isolated from the willow bark in 1897 (Raskin et al., 2002).

However, many pharmaceutical companies have dismissed their departments for natural product research, because the drug discovery from medicinal plants was regarded as more time and money consuming than other drug discovery methods (Butler, 2004; Koehn and Carter, 2005; Balunas and Kinghorn, 2005). It is estimated that only 1 in 10,000 tested compounds is promising for further development and only 1 in 4 promising compounds is indeed approved as a new drug. This process takes on average 10 years (Williamson et al., 1996; Rates, 2001; Reichert, 2003) and costs even more than 800 million dollars per successful development (Dickson and Gagnon, 2004; Balunas and Kinghorn, 2005). Furthermore, some drugs, even if they were active could not be chemically synthesized and had to be isolated from the plants. For example, before paclitaxel (Taxol) was chemically produced, 12,000 trees of *T. brevifolia* needed to be cut down in order to obtain 2.5 kg of Taxol (Hamburger and Hostettman, 1991; Wall and Wani, 1996; Rates, 2001).

Even with all these obstacles the experts predict that the plant-derived compounds will remain an essential component as basis for drug development, because it is believed that the diversity, complexity and richness of plant-isolated secondary metabolites are by far greater than those of chemically synthesized products (Balunas and Kinghorn, 2005; Schmidt et al., 2007). Nevertheless, considerable improvements in the field have to be made. Better and faster methodologies for plant collection, new technologies for compound isolation, as well as high-throughput bioassays are just some steps of the process, which need to be developed (Do and Bernard, 2004; Koehn and Carter, 2005; Butler, 2004; Balunas and Kinghorn, 2005).

1. 1. 4. Current knowledge about the plants used in this study

In this study, fourteen plant-derived substances were tested for their activity against the microaerophilic protozoa *Entamoeba histolytica* and *Giardia intestinalis*. Biologically they have been isolated from three different plant families: Asteraceae, Rutaceae and Meliaceae and chemically belong to six different classes of substances, namely sulphur-containing amides, a flavagline, acridones, a quinazoline, quinolines, and coumarines. Some of the compounds had been tested before, mostly for anti-cancer, anti-fungal or insecticidal activity.

Sulphur-containing amides, namely, methylgerambullin, methyldambullin and sakambullin are isolated from the genus *Glycosmis*, family Rutaceae, which consists of around 40 species, all rich in various secondary metabolites (Greger et al., 1994; Hinterberger et al., 1998; Hofer et al., 2000). Some of these sulphur-containing amides have shown anti-fungal (Greger et al., 1993) or anti-cancer activity (Abdullah et al., 2006), so it is a fair assumption that they could have anti-parasitic activity as well. This has already been shown within our project for *Trypanosoma cruzi* (Astelbauer et al., 2010) and *Leishmania infantum* (Astelbauer et al., 2011).

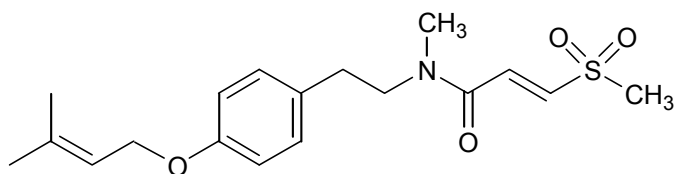
Flavaglines, isolated from *Aglaia* species, family Meliaceae, were shown to have cytostatic activity (Proksch et al., 2005; Wu et al., 1997), and insecticidal activity (Greger et al., 2001; Schneider et al., 2000). The essential antifilarial drug ivermectin (WHO, 2009) has shown insecticidal activity as well (James et al., 1980), so it is worth analyzing is it vice versa with flavaglines.

Other compounds derived from *Glycosmis* spp. are the quinazoline arborine, the furoquinoline kokusagenine and the acridone arborinine, which all showed cytostatic activity (Rethy et al., 2008; Roseghini et al., 2006; Sohrab et al., 2004), as well as the furoquinolines dictamnine and iso-gama-fagarin, which showed anti-microbial activity (Bautz, 1994). 5-hydroxynoracronycine and yukocitrine are acridones isolated from both *Glycosmis trichanthera* and *Atlantia rotundifolia*, which previously have shown anti-plasmodial (Weniger et al., 2001) and anti-allergic activity (Chukaew et al., 2008).

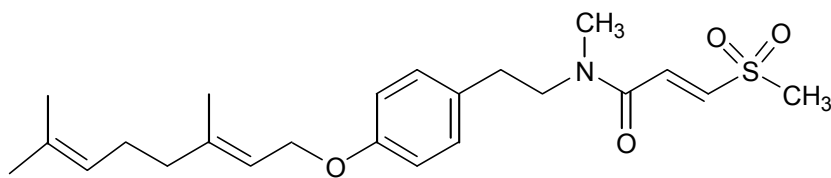
The coumarine microminutine derived from *Micromelum minutum*, another member of Rutaceae family, demonstrated cytostatic activity *in vitro* (Tantivatana et al., 1983). The coumarine, methyllacarol, isolated from *Artemisia laciniata*, Asteraceae family, and the pyranoquinoline zanthobungeanine, isolated from *Glycosmis* have not been analyzed prior to this study.

Figure 2: Overview of the fourteen compounds used in this study

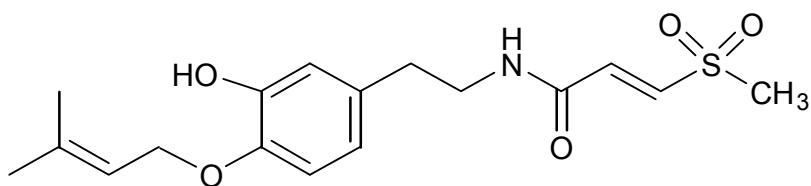
Sulphur-containing amides:



Methyl dambullin, isolated from leaves of *Glycosmis angustifolia* (Greger et al., 1994).

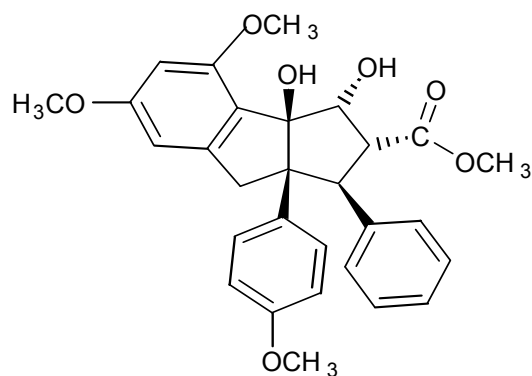


Methyl gerambullin, isolated from leaves of *Glycosmis mauritiana* (Greger et al., 1994; Hinterberger et al., 1998).

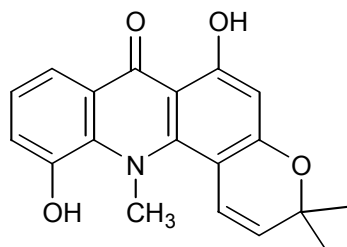


Sakambullin, isolated from leaves of *Glycosmis chlorosperma* (Hofer et al., 2000).

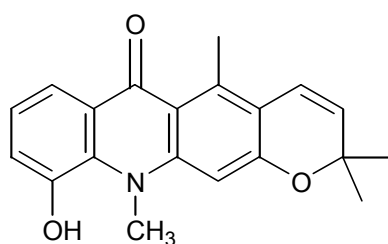
Flavagline and acridones:



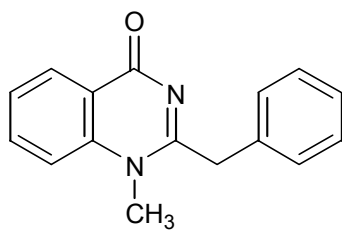
Aglafoline belongs to the flavagline class of compounds, isolated from rootbarks of *Aglaia odorata*, *Aglaia australiensis* and *Aglaia coriacea* (Engelmeier et al., 2000; Greger et al., 2001).



5-hydroxynoracronycine, acridone isolated from stembarks of *Atalantia rotundifolia* and *Glycosmis trichantera* (Vajrodaya et al., 1998).

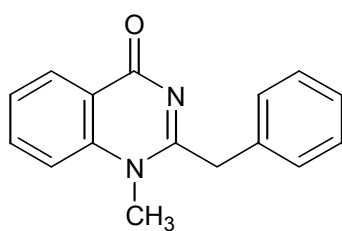


Yukocitrine, acridone isolated from stembarks of *Atalantia rotundifolia* and *Glycosmis trichantera* (Vajrodaya et al., 1998).

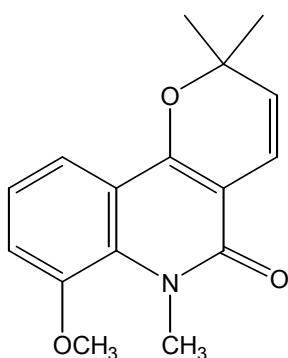


Arborinine, acridone isolated from leaves of *Glycosmis pentaphylla*, *Glycosmis sapindoides*, *Glycosmis parva* and *Glycosmis mauritiana* (Greger et al., 1993; Mester, 1983).

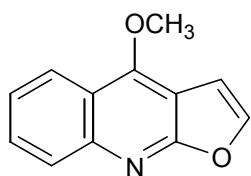
Quinazoline and quinolines:



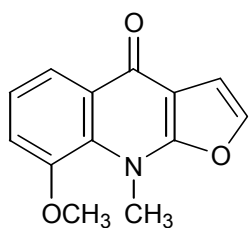
Arborine, quinazoline isolated from leaves of *Glycosmis pentaphylla* (Rahmani et al., 1992).



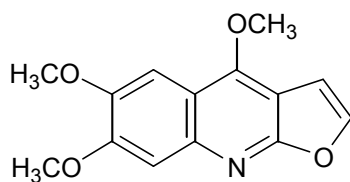
Zanthobungeanine, pyranoquinoline isolated from stembarks and rootbarks of *Glycosmis puberula*, *Glycosmis cyanocarpa*, *Glycosmis pseudoracemosa* and *Zanthoxylum simulans* (Brader et al., 1993).



Dictamnine, furoquinoline isolated from rootbarks of *Glycosmis trichantera* (Vajrodaya et al., 1998).

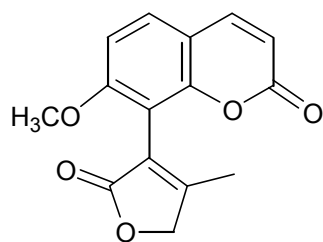


Iso-gamma-fagarine, furoquinoline isolated from leaves of *Glycosmis citrifolia* and *Ruta graveolens* (Bautz et al., 1994; Ito et al., 2000).

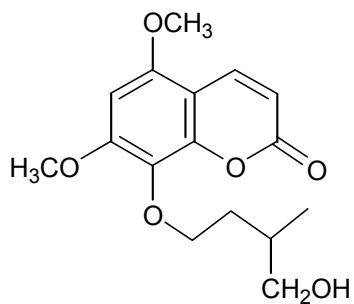


Kokusagenine, furoquinoline isolated from leaves of *Glycosmis cyanocarpa*, *Glycosmis mauritiana*, *Glycosmis chlorosperma* and *Glycosmis sapinodoides* (Greger et al., 1992; Pacher et al., 2001).

Coumarines:



Microminutine, isolated from fruits, leaves and stem bark of *Micromelum minutum* (Tantivatana et al., 1983).



Methyllacarol, isolated from leaves of *Artemisia laciniata* (Hofer et al., 1986; Szabo et al., 1985).

1. 2. *Entamoeba histolytica*

Entamoeba histolytica is a microaerophilic protozoan and the causative agent of amoebiasis.



Figure 3: *E. histolytica* trophozoite

1. 2. 1. Historical overview

It was reported at the beginning of the 19th century that Indian physicians can differentiate between several types of dysentery (Ballingall, 1818; Imperato, 1981), and though there were numerous reports about parasites in the stool of dysenteric patients, it wasn't until 1875 that *Entamoeba histolytica* was studied in detail by a Russian physician Fedor Lösch. He examined a patient with intestinal amoebiasis, the content of the patient's stool, and gave a detailed description of the parasite, which he named *Amoeba coli*, its structure, size, motility, vacuoles, nucleus, and he documented his observations by drawings. He also performed an experiment in which four dogs were infected, and one of them became ill. Because of the different course of the dog's disease, which was rather mild in comparison to patient's

disease, Lösch concluded wrongly that amoebae sustain the disease, but are not causing it (Lösch, 1875; Imperato, 1981).

In 1893 Quincke and Roos described the cyst form of the amoebae and also noticed that the vegetative form causes dysentery when injected into the rectum of kittens, but does not cause the disease when its given orally (Quincke and Roos, 1893). Huber in 1903 first noticed four nuclei in amoebic cysts (Huber, 1903; Imperato, 1981).

Strong and then Schaudinn differentiated between pathogenic and non-pathogenic amoebae. The disease-causing amoeba was named *Entamoeba histolytica* by Schaudinn because of its tissue destroying ability and the harmless one *Entamoeba coli* Lösch (Schaudinn, 1903; Imperato, 1981).

This was confirmed by Walker and Sellards in 1913, which demonstrated not only the differences between *E. histolytica* and *E. coli*, but also the fact that infection with *E. histolytica* does not always develop into clinical disease and that infection is caused by the cyst of the parasite (Walker and Sellards, 1913).

In 1925 Emile Brumpt suggested that humans can be infected with two different but morphologically identical species, one pathogenic, namely *Entamoeba histolytica* and the other non-pathogenic, which he named *Entamoeba dispar* (Brumpt, 1925). However, there was no way to distinguish the two species, so the theory was dismissed until 53 years later when Sargeant and Williams (1978) showed that the two species can be differentiated on the basis of isoenzyme typing. Since then other biochemical, immunological and genetic evidence was emerging, and in 1993 a formal redescription of *E. histolytica* by Diamond and Clark and separation from *E. dispar* occurred (Diamond and Clark, 1993). This was formally accepted by the World Health Organization in 1997 (WHO, Report of the Expert Consultation on Amoebiasis, 1997).

In 2005 the sequencing of the *Entamoeba histolytica* genome was reported, as a result of cooperation of a large number of *Entamoeba* research groups (Loftus et al., 2005).

The first *in vitro* cultivation of *E. histolytica* was preformed with blood agar plates, which contained a single species of bacteria. The cultivation was done by Musgrave and Clegg, which also established the term “amoebiasis” for the disease caused by the parasite (Musgrave and Clegg, 1904). Boeck and Drbohlav succeeded in cultivating the amoeba on an artificial medium in 1925 (Boeck and Drbohlav, 1925),

and Diamond in 1961 first established the axenic cultivation of *Entamoeba* and finally in 1978 introduced the monophasic medium, TYI-S-33, which is still in use (Diamond, 1961; Diamond et al., 1978).

1. 2. 2. Epidemiology

Entamoeba histolytica has a global distribution and it is a significant health risk in developing countries and everywhere where poor sanitary conditions predominate (Stanley, 2003). The countries with highest incidence are in Africa, Middle and South America, the Indian subcontinent and tropical Asia. In developed countries, as well, the parasite can cause outbreaks, when the cysts of the parasite contaminate drinking water, as happened in Chicago in 1933 when union between sewage and tap-water pipes caused at least 800 cases of amoebiasis among hotel guests. A similar outbreak happened in Tbilisi, Republic of Georgia, in 1998, when due to contaminated municipal water supplies 177 patients had either liver abscess or amoebic colitis and four of them died. In developed countries most infections are caused by *E. dispar* and there are certain populations with higher predominance, such as travelers and immigrants from endemic areas, homosexual males, as well as the institutionalized populations (Barwick et al., 2002; Stanley, 2003; Fauci et al., 2008; Petri, 1996; Ali et al., 2008).

It is estimated that 500 millions or approximately 10% of the human population is infected with *Entamoeba*, however the majority of those infections, around 90%, are due to non-pathogenic *Entamoeba dispar*. Report of the World Health Organization (WHO) from 1997 revealed that up to 100,000 people annually die from invasive amoebiasis, what makes *E. histolytica* the third leading cause of death due to a parasitic disease, after schistosomiasis and malaria (Walsh, 1986; WHO, 1997; Stauffer and Ravdin, 2003; Fauci et al., 2008; Petri, 2008).

It has been shown that amoebic liver abscess is predominantly a male disease, affecting men between 18 and 50 years of age with rates 3-20 times higher than in other populations. Amoebic colitis, however, is still considered an equal-opportunity disease, affecting both sexes, adults as well as the children (Acuna-Soto et al., 2000; Stanley, 2003).

1. 2. 3. Life cycle

E. histolytica has a simple two-stage life cycle. Infection is caused by ingestion of fecally contaminated food or water containing cysts of the parasite (Fig. 3 ②). The cysts are round, 10 –15 µm in diameter, contain four nuclei and are enclosed with a refractile wall. After surviving the stomach acid, the cysts travel through the small intestine to the terminal ileum or colon, where the excystation takes place (Fig. 3 ④). Each of the nuclei of the quadrinucleate cyst divides once more, so one cyst forms eight highly motile, pleomorphic shaped trophozoites (Stanley, 2003; Diesfeld et al., 2003; Haque et al., 2003) (Fig. 3 ④). The size of the trophozoites varies from 10 to 50 µm in diameter. They ingest food particles and intestinal bacteria, reproduce by a clonal expansion. Their encystation (Fig. 3 ⑤) and the excretion of the new cysts completes the life cycle of the parasite. The trophozoites can be found in the stool, however are not able to survive outside the human body for a long time (Fig. 3 ①) and they are not infectious (Stanley, 2003; Stauffer and Ravdin, 2003).

In 90% of the cases the trophozoites aggregate in the intestinal mucin layer and then encyst so the infection is not causing any symptoms. In 10%, however, the infection takes an invasive course, when the trophozoites adhere to the colonic epithelium and induce cell lysis. In most cases the infection stays in the intestine causing amebic colitis but quite frequently *Entamoeba* trophozoites spread extraintestinally to the liver where they cause large abscesses. In very rare cases, further spread to the brain is observed (Haque et al., 2003).

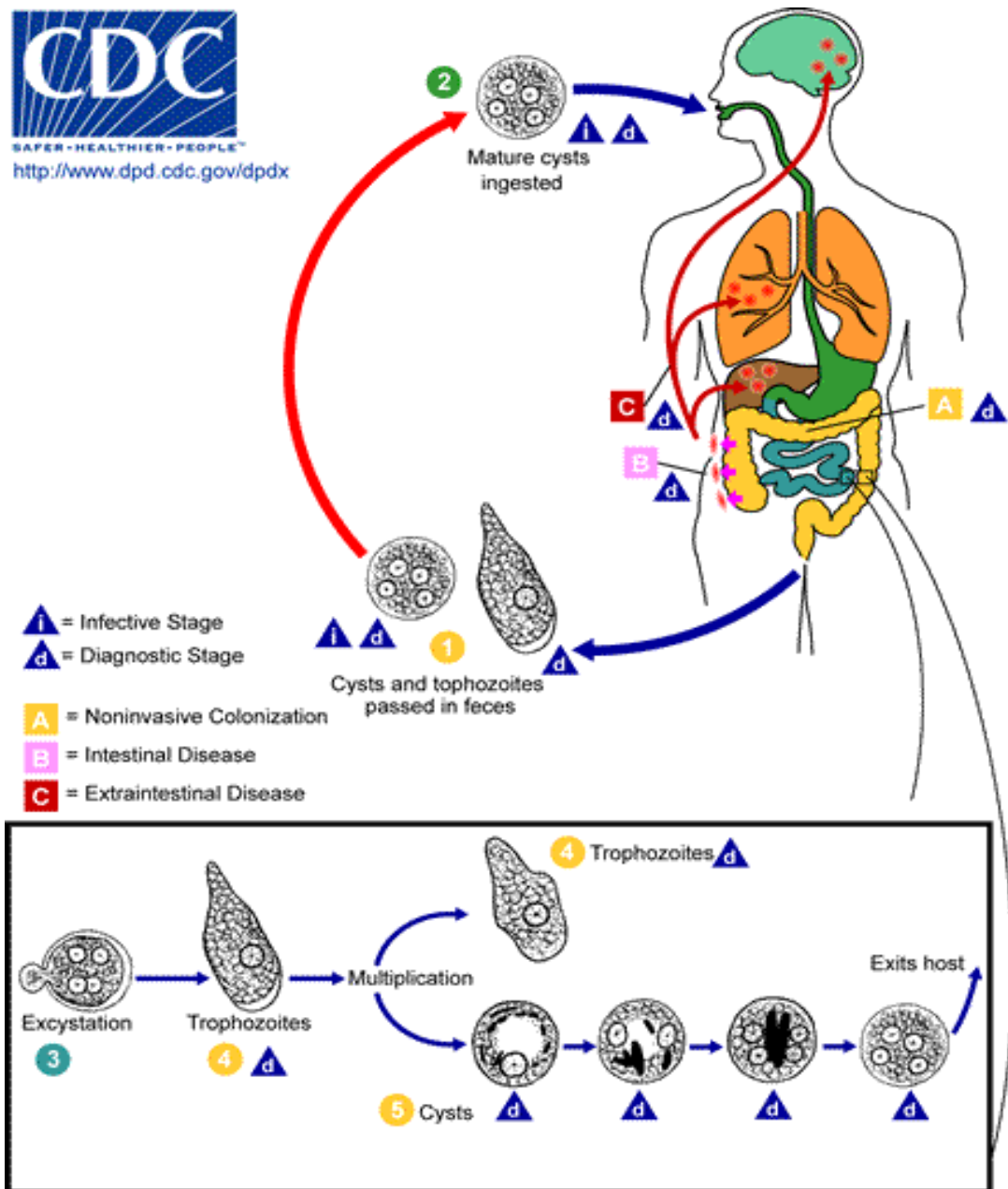


Figure 4: Life cycle of *Entamoeba histolytica*. From CDC (Centers for Disease Control and Prevention, Atlanta, GA, USA; www.dpd.cdc.gov/dpdx)

1. 2. 4. Disease and diagnostics

Although, as previously mentioned, 90% of *E. histolytica* infections are asymptomatic and self-limited, in 4–10% of cases the parasite causes amoebic colitis or liver abscess, rarely with pulmonary complications or very rarely with a brain abscess (Stanley, 2003; Haque, 2003). Patients with amoebic colitis present with abdominal pain and tenderness, as well as bloody diarrhea, and after a few days with significant

weight loss. Patients may also develop so-called toxic megacolon, which is characterized by severe bowel dilation with intramural air (Stanley, 2003; Fauci et al., 2008). The liver abscess is a result of haematogenous spread of trophozoites through the portal circulation to the liver and presents with fever, right upper quadrant pain and hepatic tenderness (Stanley, 2003). These patients can also develop complications if the amoebic liver abscess ruptures through the diaphragm, causing the pleuropulmonary amoebiasis. The illness presents with pleuritic chest pains, cough and respiratory distress (Stanley, 2003). Brain amoebic abscess is a very rare complication of liver abscess with a variety of symptoms, like headache, vomiting and seizures. Almost half of the reported cases had a deadly outcome (Orbison et al., 1951; Stanley, 2003).

The diagnosis of *E. histolytica* colitis relies on three consecutive microscopic examinations of properly and freshly prepared stool samples of a patient. This method, however, cannot distinguish between *E. histolytica* and non-pathogenic *E. dispar*, so it is not appropriate as a screening technique in epidemiological studies (Healy, 1971; Krogstadt, 1978; González-Ruiz, 1994). There are commercially available ELISA assays that identify *E. histolytica* antigen in the stool and in this way also distinguish between pathogenic and non-pathogenic *Entamoeba* species (Haque et al., 2000; Pillai et al., 1999; Stanley, 2003). PCR is more commonly used in research and field studies, however, it can be used as a diagnostic method as well (Clark and Diamond, 1993; Stanley, 2003). The methods of choice for diagnosis of amoebic liver abscess are ultrasound or CT scan. However, although both methods are sensitive, they are not absolutely specific for this disease (Stanley, 2003).

1. 2. 5. Metabolism

Carbohydrate metabolism

E. histolytica has been classified as amitochondriate type I protist (Martin and Müller, 1998), so the energy generation takes place in cytosol and not in mitochondria, although, there is a mitochondrial remnant, called mitosome. The function of the mitosome has not been clarified with certainty even after complete genome sequencing (Clark et al., 2007).

Entamoeba uses glucose and to a lesser extent some amino acids as energy source (Loftus et al., 2005; Clark et al., 2007). Reeves has pointed out in 1984 that *Entamoeba* generates energy by various types of substrate level phosphorylation (Reeves, 1984; Clark et al., 2007). Being a gut parasite it has limited oxygen availability, so the pathways for ATP synthesis do not have oxygen as a terminal electron acceptor on disposal (Tielens et al., 2010). The complete genome sequencing revealed, indeed, absence of tricarboxylic acid cycle protein genes, as well as, mitochondrial electron transport chain (Loftus et al., 2005; Clark et al., 2007). One of the most important enzymes is pyruvate:ferredoxin oxidoreductase (PFOR) for a conversion of pyruvate in acetyl-CoA, by utilization of ferredoxin as electron acceptor (Reeves et al., 1977; Clark et al., 2007). In mitochondriate organisms after the glucose has been broken down to acetyl-CoA, the molecule further enters the tricarboxylic acid cycle. Since *Entamoeba* lacks this pathway the cleavage energy of the thioester bond of acetyl-CoA is used by acetyl-CoA synthetase (acetate thiokinase) to generate one ATP from ADP and PPi (Reeves et al., 1977; Clark et al., 2007). The end products of glucose breakdown in *Entamoeba* are acetate, ethanol and carbon dioxide (Tielens et al., 2010). The energy is stored in glycogen granules that can be seen in the cytosol (Takeuchi et al., 1977; Clark et al., 2007).

Several genes coding for enzymes of amino acid catabolism were found in the *Entamoeba* genome (Anderson and Loftus, 2005), which is consistent with Reeves' observations that *Entamoeba* is capable of taking up amino acids (Reeves, 1984) and using them for energy generation (Zuo and Coombs, 1995; Clark et al., 2007).

Biosynthesis of amino acids

In the human intestine *E. histolytica* has access to many host-derived as well as bacteria-produced organic compounds, so it lacks any pathway for amino acid biosynthesis other than serine and cysteine (Ali et al., 2003, 2004; Nozaki et al., 1998, 1999; Clark et al., 2007). The two amino acids are probably used for production of cysteine, which is the essential intracellular thiol (Loftus et al., 2005; Fahey et al., 1984). The high levels of cysteine in the parasite are also likely to make up for the lack of glutathione, a major part of oxidative stress resistance in many organisms (Fahey et al., 1984; Loftus et al., 2005).

Lipid metabolism

As mentioned above *Entamoeba* lacks oxidative phosphorylation, so the fatty acids cannot be exploited as source of energy. The ability of de novo fatty acid synthesis is also lost, but the abilities to elongate fatty acids and to synthesize some phospholipids are maintained. However, phospholipids as well as the cholesterol, which are membrane components, are usually acquired from the food or from the human host (Das et al., 2002; Sawyer et al., 1967; Clark et al., 2007; Loftus et al., 2005).

1. 2. 6. The genome

Completion of the *E. histolytica* genome in 2005 (Loftus et al., 2005) showed that the genome consists of approximately 24 Mb and around 10,000 genes with an average size of 1.7 kb. Pulse-field gel analysis indicates 14 chromosomes in *E. histolytica* and a possibly ploidy of four (Willhoeft and Tannich, 1999). Although the majority of genes comprise a single exon, there are still 25% of them with introns and 6% that contain two or more introns, so the amoeba genome must encode the machinery for mRNA splicing, as well. One further unique feature of the *Entamoeba* genome is the organization of tRNA into distinct arrays, the function of which remains still unclear (Tawari et al., 2008).

1. 2. 7. Pathogenicity

There are three main virulence factors in *E. histolytica*: the Gal/GalNAc lectin, amoebapores and cysteine proteinases.

Gal/GalNAc lectin

Maybe the most important factor in *E. histolytica* virulence is the Gal/GalNAc lectin, a protein that is responsible for adherence to the colonic wall, as well as cytolysis, invasion and resistance to the human complement (Mann, 2002; Petri, 2008).

It is a heterodimer composed of heavy (170 kDa) and light (35/31 kDa) subunits (Petri et al., 1989), as well as a noncovalently linked intermediate subunit (150 kDa) (Cheng et al., 2001). The protein binds to N-acetyl-D-galactosamine containing structures on the surface of the host cell, as the first step of the process ultimately leading to its death (Ravdin and Guerrant, 1981; Petri et al., 1989). It attaches to the colonic epithelium and also to the human neutrophils, human erythrocytes and to certain bacteria (McCoy et al., 1994; Ravdin et al., 1985; Petri, 2008).

Amoebapores

Cytoplasmic vesicles contain, among others, amoebapores, which are channel-forming peptides of 77 amino acid residues (Leippe et al., 1992). There are three isoforms of amoebapores, A, B and C with a ratio of 35: 10: 1 and a 35% to 57% amino acid sequence identity (Leippe et al., 1992; 1994; 1997; Petri, 2008).

The amoebapores manifest their cytolytic activity by formation of channels within the plasma membrane of the target cell, through which water, ions and small molecules pass (Leippe et al., 1994). It is also suggested, after *in vitro* investigations, that *E. histolytica* without amoebapore A is unable to cause pathological effects (Bracha et al., 2003). Furthermore, *in vivo*, it has been demonstrated that the avirulent *E. histolytica* strain G3 indeed lacks amoebapore A and this is believed to be cause of its lack of virulence (Bujanover et al., 2003).

Cysteine proteinases

Cysteine proteinases belong to the papain superfamily and occur in a wide range of organisms. *E. histolytica* has 44 genes encoding cysteine proteinases, whose molecular weight lie between 16 kDa and 96 kDa (Clark et al., 2007). It appears that most important for *E. histolytica* pathogenesis are cysteine proteinase-1 (CP1), cysteine proteinase-2 (CP2) and cysteine proteinase-5 (CP5), whose functional orthologs are absent in non-virulent *E. dispar*. CP5 is the only proteinase present on the surface of amoebae (Bruchhaus et al., 1996, 2003; Jacobs et al., 1998; Willhoeft et al., 1999), and it has been shown that it binds to a receptor on colonic cells, an $\alpha_v\beta_3$ integrin, and stimulates a pro-inflammatory response via NF κ B (Hou et al., 2010; Ralston and Petri, 2011). The proteinases of *E. histolytica* have a strong

cytopathic effect on the target cells. By degradation of the components of the extracellular matrix, such as laminin, fibronectin and collagens, they release adherent cells from monolayers and facilitate invasion into the colonic mucosa and they also degrade secretory IgA and serum IgG (Keene et al., 1986; Stanley, 2003; Haque et al., 2003).

1. 2. 8. Host immune response

The host responds to *E. histolytica* by activating both cell-mediated and humoral immune response, which can result in either asymptomatic or symptomatic course of disease (Stauffer and Ravdin, 2003). When the parasite breaks through a physical barrier, the mucin layer, which is the first line of defense, it causes an inflammatory response by activating nuclear factor κ B (NF κ B), which activates intestinal epithelial cells to produce interleukin-1 β , interleukin-8 and cyclooxygenase-2. These mediators attract neutrophils and macrophages to the site of infection. However, *E. histolytica* has the ability to suppress the macrophage respiratory burst and to lyse neutrophils. This results in the release of mediators, which cause diarrhea and additional tissue damage (Seydel et al., 1997; Stenson et al., 2001; Haque et al., 2003).

In how the disease unfolds, amoebic virulence factors have more roles. Due to its sequence similarity and cross reactivity to the human leukocyte antigen CD59, the Gal/GalNAc-specific lectin is able to inhibit the assembly of the complement C5b-C9 membrane attack complex (Braga et al., 1992). Amoebic cysteine proteinases inactivate and degrade the anaphylatoxins C3a and C5a (Reed et al., 1995) and protect amoebae from opsonization by degrading secretory IgA and serum IgG (Haque et al., 2003). The cell-mediated response is characterized by a lymphocyte proliferation and lymphokine secretion, which are proven to be amoebicidal *in vitro* (Salata et al., 1986; Haque et al., 2003). Some studies suggest a role of CD4⁺ T-cells in the outcome of illness in the way that depletion of these cells decreases the severity of the disease (Arellano et al., 1996; Houpt et al., 2002).

1. 3. *Giardia intestinalis*

Giardia intestinalis (syn. *Giardia lamblia*, *Giardia duodenalis*) is a flagellated, unicellular, binucleated protozoan (Fig. 5) that commonly causes diarrheal disease all over the world (Adam, 2001; Ali and Hill, 2003).

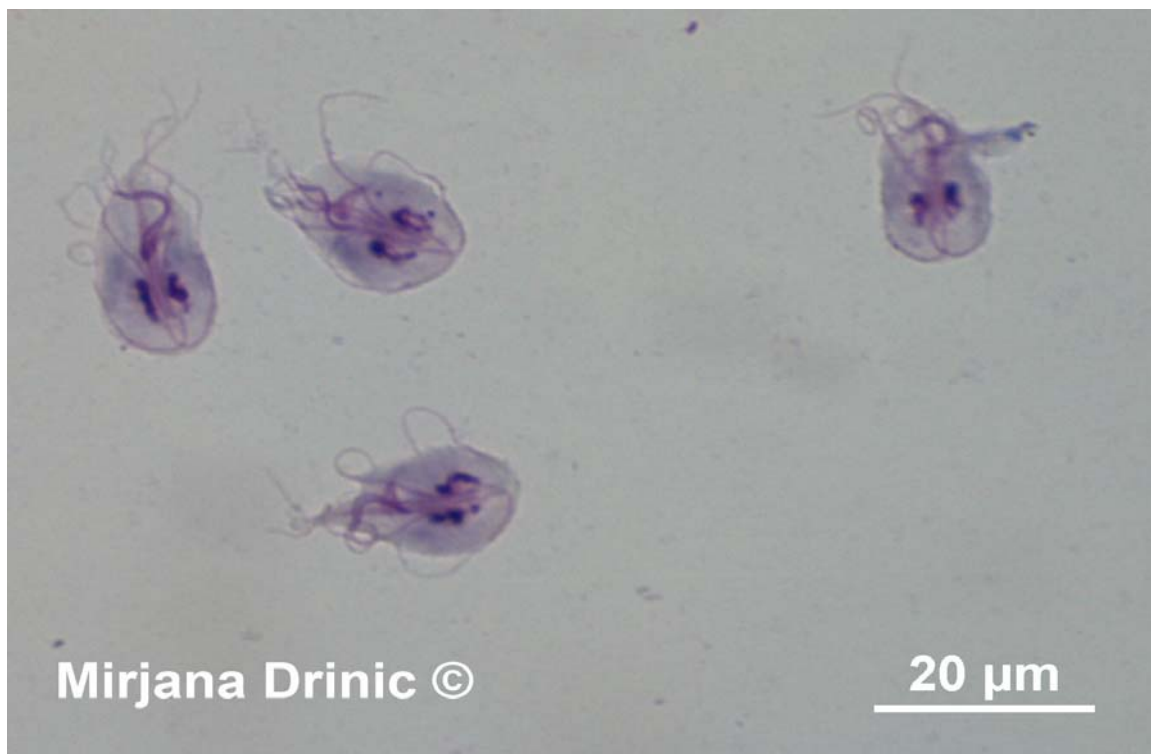


Figure 5: *G. intestinalis* trophozoites

1. 3. 1. Historical overview

G. intestinalis was first described in 1681 by Antonie van Leeuwenhoek, as he was conducting microscopical examination of his own diarrheal stool (Dobell, 1920).

The parasite was more thoroughly described by Vilem Lambl in 1859, however, he named it *Cercomonas intestinalis*, because he was certain that it belongs to the genus *Cercomonas* (Lambl, 1859). First time *Giardia* was used as genus name, when in 1882 Kunstler described a parasite in tadpoles, presumably *Giardia agilis* (Adam, 2001). The name of the parasite was changing, from *Lamblia intestinalis* proposed by Blanchard in 1888, to *Giardia duodenalis* by Stiles in 1902 and *Giardia*

lamblia by Kofoid in 1915 (Blanchard, 1888; Stiles, 1902; Kofoid, 1915). Today the parasite is most commonly called *Giardia intestinalis*.

G. intestinalis was cultivated axenically for the first time in 1970 by Meyer with the trophozoites acquired from the rabbit, chinchilla and cat (Meyer, 1970). Today, the most commonly used medium for cultivation is Keister's modified TYI-S-33 medium (Keister, 1983).

1. 3. 2. Epidemiology

Giardiasis occurs throughout the world, but especially in regions with poor sanitary conditions and with an estimated 2,800,000 cases per year (Wolfe, 1992; Ali and Hill, 2003). There is still a high morbidity rate, especially among children (Boreham, 1991; Upcroft and Upcroft, 1998). Infection may be caused by ingestion of as few as 10 cysts of *G. intestinalis* (Rendtorff, 1954). Cysts are very resilient. They can survive for several months in a humid environment with a temperature between 4– 10°C (Wolfe, 1992). The infection with *G. intestinalis* is most commonly the result of ingestion of contaminated water, however it can be caused by direct fecal-oral transmission, ingestion of contaminated food or person to person transmission (Hill, 1993; Ortega and Adam, 1997). Although *Giardia* infections and especially waterborne outbreaks of diarrhea are more common in developing countries, they are frequent in developed countries as well (Hill, 1993; Ortega and Adam, 1997). The prevalence of *G. intestinalis* in patients with diarrhea is about 20% in developing countries (Islam, 1990; Flanagan, 1992), and from 3–7% in developed countries, where is linked mainly with waterborne outbreaks or traveling to countries where the parasite is endemic (Hoogenboom-Verdegaal et al., 1989; Adam, 1991; Farthing, 1994; Kortbeek et al., 1994; Flanagan, 1992), with peaks at the age groups 1– 4 and 20– 40 (Flannagan, 1992).

Giardia cysts are relatively resistant to the chlorination and ultraviolet light and the only effective method to remove them from water is filtration (Hill, 1993; Ortega and Adam, 1997).

1. 3. 3. Taxonomy and life cycle

Giardia is regarded as one of the most primitive eukaryotic organisms (Sogin et al., 1989) and belongs to the Phylum Zoomastigophora, Class Zoomastigophorea and Order Diplomonadida (Sogin et al., 1989; Ortega and Adam, 1997). The Genus *Giardia* includes six species. Between *G. agilis* from amphibians, *G. muris* from rodents, reptiles and birds, and *G. intestinalis* from humans and other mammals noticeable differences can be observed already by light microscopy (Ortega and Adam, 1997). Another two species, *G. ardeae* from herons and *G. psittaci* from psittacine birds such as parrots, show morphological differences that can be detected by electron microscopy. In this way they can be distinguished from one another and from *G. intestinalis* (Erlandsen and Bemrick, 1987, 1990; Ortega and Adam, 1997). *G. microti* from voles and muskrats differs by a comparison of 18S rRNA sequence from *G. intestinalis* (van Keulen et al., 1998; Adam, 2001).

G. intestinalis has a simple life cycle, which consists of two stages: non-motile cyst and motile trophozoite. Once ingested (Fig. 5 ②), cysts travel to the stomach and after being exposed to the acidic environment, they excyst in the small intestine (Fig. 5 ③). Here motile trophozoites replicate by binary fission (Fig. 5 ④), and cause symptoms of diarrhea and malabsorption. In the jejunum they encyst (Fig. 5 ⑤), under exposure to the biliary fluid and through the feces the cysts are passed further in the environment (Fig. 5 ①) and to the next host (Adam, 2001).

The cysts are 5 by 7 to 10 μm in diameter and covered by a cyst wall, whose predominant sugar component is an N-acetylgalactosamine homopolymer (GalNAc) (Jarroll et al., 1989; Adam, 2001). Trophozoites are teardrop-shaped, 12 to 15 μm long and 5 to 9 μm wide with a characteristic ventral adhesive disc, four pairs of flagella and a median body (Elmendorf, 2003; Adam, 2001).



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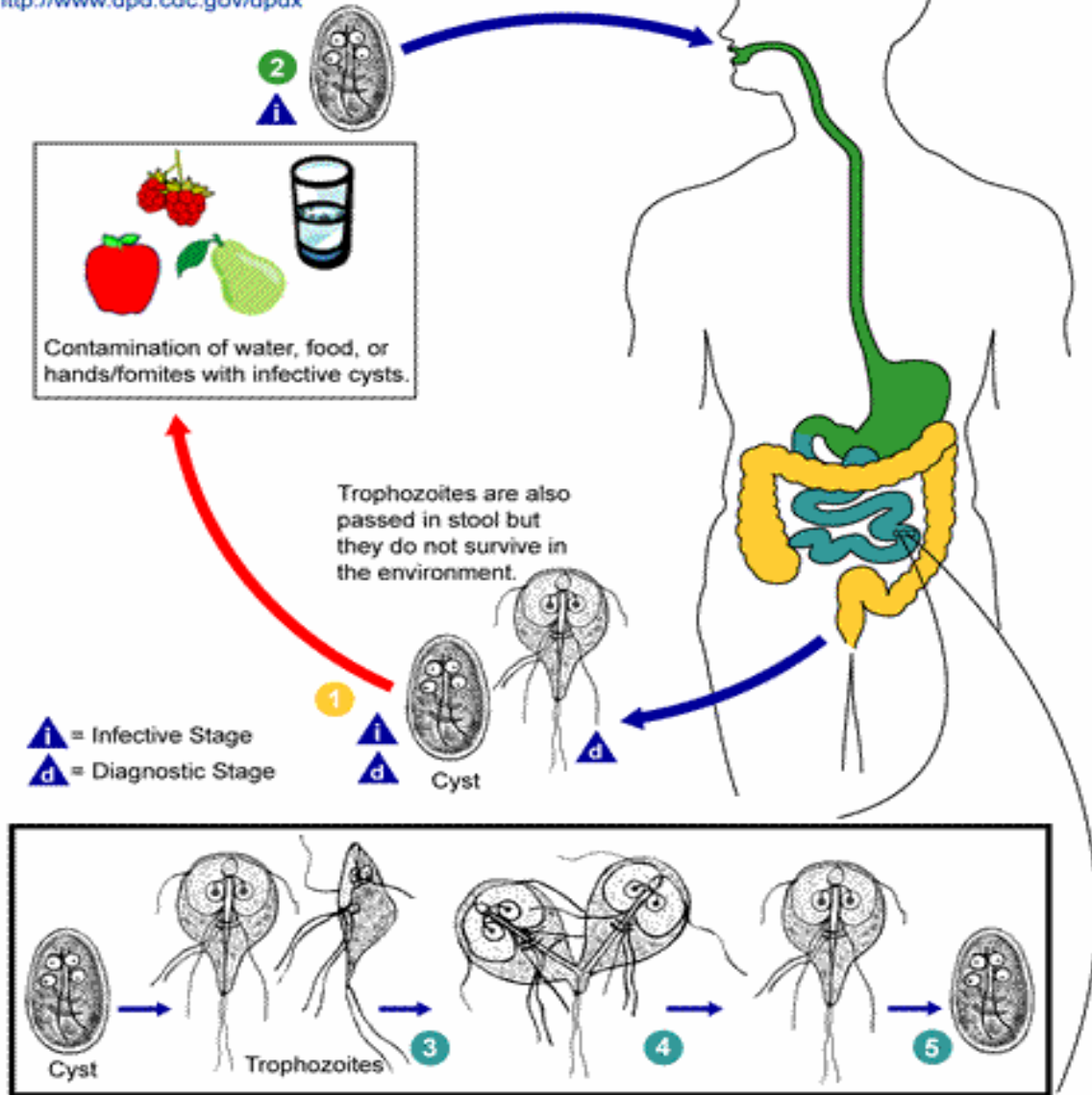


Figure 6: Life cycle of *G. intestinalis*. From CDC (Centers for Disease Control and Prevention, Atlanta, GA, USA; www.dpd.cdc.gov/dpdx)

Genotypes of *G. intestinalis*

Giardia intestinalis can be divided in seven genotypes (assemblages), of which only two (A and B) can infect humans, as well as other mammals and the rest of which are causative agents of the disease in dogs (C and D), livestock (E), cats (F) and rats (G). However, recent findings and studies of the WB isolate (assemblage A) and the

GS isolate (assemblage B) have shown such large genetic differences between the two isolates, that there is ongoing debate whether the two assemblages should be reclassified into two different *Giardia* species (Caccio and Ryan, 2008; Monis et al., 2009; Franzen et al., 2009).

1. 3. 4. Disease and diagnostics

In most cases *Giardia* infections are asymptomatic. However, if symptomatic, infections can be acute or chronic. In acute giardiasis, symptoms occur after approximately one to three weeks and include diarrhea with foul-smelling stool, malabsorption, abdominal cramps, bloating, belching, nausea and vomiting, as well as weight loss, if other symptoms persist (Ortega and Adam, 1997; Fauci et al., 2008). In chronic giardiasis, symptoms are not so severe and occur in episodes. Many patients seek professional help later in the course of the disease, so some serious consequences, such as growth retardation and dehydration may occur (Fauci et al., 2008).

Diagnosis of the disease relies on the identification of the cysts in the stool specimens, which have previously been stained with iron-hematoxylin or trichrome (Ortega and Adam, 1997). Due to a sporadic cyst passage, the stool samples should be examined at least three or even up to six times or should be concentrated by zinc sulfate or formalin-ethyl acetate methods (Ortega and Adam, 1997). There are also highly sensitive and specific enzyme immunoassay (EIA) kits for detection of *Giardia* spp. antigen in the stool (Marshall et al., 1997; Ortega and Adam, 1997), as well as direct fluorescence antibody (DFA) tests, which are used for detection of intact organisms in the stool (Ali and Hill, 2003). If there is a strong suspicion of *Giardia* infection that cannot be proven, a string test can be used for a direct examination of the intestine. The string test consists of a capsule attached to a string, which is swallowed by a patient and withdrawn and examined for trophozoites on the next day. Other methods, that are not used so often, are biopsy and gastroendoscopy with duodenal aspiration (Beal et al., 1970; Ortega and Adam, 1997).

1. 3. 5. Metabolism

Carbohydrate metabolism

Carbohydrate metabolism of *G. intestinalis* is just like in *E. histolytica* relying on substrate level phosphorylation and due to activities of pyruvate:ferredoxin oxidoreductase and 2-ketoacid oxidoreductase, among other enzymes, glucose is degraded and metabolized to acetate, ethanol, alanine and carbon dioxide. Under strictly anaerobic conditions the main product of glucose breakdown is alanine, in the presence of small concentrations of oxygen it is ethanol and under aerobic conditions the main products are acetate and carbon dioxide (Brown et al., 1998; Adam, 2001).

Amino acid metabolism

Amino acids play a much bigger role in energy production by *Giardia* than by *Entamoeba*. *Giardia* is able to take up alanine, arginine and aspartate from the medium (Mendis et al., 1992; Schofield et al., 1990, 1991; Adam 2001). The arginine dihydrolase pathway, although more common in prokaryotes, also occurs in *Giardia*, in order to convert arginine to ornithine and ammonia, generating one ATP molecule from ADP (Schofield et al., 1990, 1992; Adam, 2001).

Lipid metabolism

Giardia does not have the ability of *de novo* synthesis of fatty acids, so the required cholesterol and phosphatidylcholine are obtained from the environment (Jaroll, 1981; Farthing et al., 1985; Gillin et al., 1986; Lujan et al., 1996; Adam, 2001), where bile salts are important not only as a source, but also as transporters of lipids to the parasite surface (Lujan et al., 1996; Adam, 2001).

1. 3. 6. The genome

Giardia intestinalis has two nuclei, which have the same size, DNA amount and transcriptional activity (Kabnick and Peattie, 1990; Yu et al., 2002; Ankarklev et al.,

2010). The genome consists of 12 Mbp with 46% of G+C content. Each nucleus has five chromosomes (Adam, 2000), although there are findings, which suggest that in some *Giardia* isolates nuclei show aneuploidy (Tumova et al., 2007). The *Giardia* genome project revealed that the parasite has simplified metabolic pathways in comparison to other eukaryotic organisms as well as simplified machinery for DNA replication, transcription and RNA processing (Morrison et al., 2007).

1. 3. 7. Pathogenicity

The question why some patients develop symptoms and others do not, although they are infected with *Giardia*, still remains to be answered (Fauci, et al., 2008). Trophozoites adhere to the epithelium, however, no tissue invasion is observed. There are several theories, why do symptoms occur: villous blunting, lymphocyte infiltration, lactose intolerance, as well as malabsorption and diarrhea. One of the theories is that the symptoms are an answer to diffuse shortening of the microvillus brush border and linked with it loss of enzyme activities (Farthing, 1997; Ortega and Adam, 1997; Ali and Hill, 2003; Scott et al., 2004; Buret, 2007; Fauci, 2008; Hodges and Gill, 2010). Another theory maintains that it is a reaction by the host immune response, that is causing mucosal inflammation after infiltration of lymphocytes and mast cells or symptoms could be caused by a reaction to a bile content alteration (Ortega and Adam, 1997; Ali and Hill, 2003; Fauci, 2008; Hodges and Gill, 2010; Ankarklev et al., 2010).

Virulence factors

The first requirement for developing infection is attachment to the epithelial cells and colonization of the small intestine, so the ventral adhesive disc as well as the four pairs of flagella, are very important virulence factors (Palm, 2005; Weiland et al., 2003; Ankarklev et al., 2010).

Antigenic variation is the ability of *G. intestinalis* to switch on or switch off the gene expression of the cysteine-rich variant-specific surface proteins (VSP) (Prucca and Lujan, 2009; Ankarklev et al., 2010). There are approximately 200 genes coding for the proteins of each VSP family in assemblages A and B and no identical VSP were detected in two assemblages (Morrison et al., 2007; Franzen et al., 2009;

Ankarklev et al., 2010). The size of the proteins varies between 20 and 200 kDa and they are the parasites' main tool for immune evasion. The giardial surface is completely covered with them, but their expression is mutually exclusive. Several proteins are expressed at the same time only during switching and differentiation, which occurs spontaneously every 6 to 13 generations (Nash et al., 1990; Svärd et al., 1998; Prucca and Lujan, 2009; Ankarklev et al., 2010).

1. 3. 8. Host immune response

Since the majority of *Giardia* infections are self-limiting and asymptomatic, it can be concluded that host defense is in most cases very effective (Faubert, 2000). There are natural barriers to parasite colonization of the human duodenum and jejunum. It is a very hostile environment presented with mucus layer and digestive enzymes, which prevent immediate access to the epithelial cells (Roskens and Erlandsen, 2002; Roxström-Lindquist et al., 2006). Furthermore, peristalsis of the intestine is also a natural way for eliminating the parasite. That is the reason why *Giardia* is constantly reattaching itself (Roxström-Lindquist et al., 2006). Mouse experiments have shown that probiotic bacteria have an inhibitory effect on *Giardia* by increasing the production of parasite-specific intestinal IgA and serum IgG (Benyacoub et al., 2005; Roxström-Lindquist et al., 2006). There are other proteins, such as defensin and lactoferrin, which as a part of innate immune system have shown *in vitro* anti-giardial activity (Eckmann, 2003).

1. 4. Treatment of amoebiasis and giardiasis - a new drug is needed

There are two groups of drugs used for treatment of *E. histolytica* infection. One group includes poorly absorbed luminal amoebicides, paromomycin, iodoquinol and diloxanide furoate, which are able to eradicate trophozoites only from the intestinal lumen of a patient, but they are able to prevent spread of infection by eradicating cysts (Ravdin and Stauffer, 2005; Pritt and Clark, 2008). This group, however, is totally ineffective in the case of amebic colitis and liver abscess, which are treated with well-absorbed tissue amoebicides. These drugs are presented as 5-nitroimidazole family, most of all metronidazole and tinidazole (Powell et al., 1966; WHO, 1997; Pritt and Clark, 2008; Orozco et al., 2009). Usual therapy is a 5 to 10-day course of metronidazole and additional treatment with luminal amoebicides for cyst eradication (Pritt and Clark, 2008; Orozco et al., 2009).

The 5-nitroimidazole drugs, metronidazole, tinidazole, secnidazole, ornidazole and nimorazole are, as well, drugs of choice for the treatment of giardiasis (Ortega and Adam, 1997; Orozco et al., 2009). Other drugs, furazolidone, paromomycin and the first anti-giardial drug quinacrine are either less effective or like quinacrine have more severe side effects (Ortega and Adam, 1997). Albendazole is, however, as effective as metronidazole, and some data indicate that its toxicity profile is also more favorable (Solaymani-Mohammadi et al., 2010).

As stated above, therapy for both protozoans is based on metronidazole and its derivatives. However, metronidazole is known to cause frequent adverse reactions, such as metallic taste, headache, nausea, vomiting, epigastric discomfort and diarrhea (Solaymani-Mohammadi et al., 2010). Furthermore, central nervous system toxicity, as well as pancreatitis have been reported when metronidazole is administered in high doses (Hill et al., 1984; Roe, 1985; Gardner and Hill, 2001). The biggest concern in metronidazole use is the evidence that the drug may be carcinogenic in mice and rats (Lindmark and Muller, 1976; Bost RG, 1977; Voogd CE, 1981; Gardner and Hill, 2001). The International Agency for Research on Cancer (IARC) has listed metronidazole as animal carcinogen and the European Directorate for the Quality of Medicine and HealthCare in its Safety Data Sheet has issued a warning that metronidazole may cause cancer (IARC, 1987; EDQM, 2010). Nevertheless, there is not enough evidence to consider metronidazole a human carcinogen. To confirm or dismiss such a statement a more comprehensive study

with large number of patients treated with metronidazole and a follow up period of more than 20 years would need to be conducted (Bendesky et al., 2002). Considering that metronidazole can cross the placenta, a possible teratogenicity and the influence on the embryo in the first trimenon has also been debated over the years (Cudmore et al., 2004).

Another concern is growing resistance to metronidazole, especially of *Giardia* with 20% prevalence for clinical resistance and easily acquired laboratory resistance by gradual exposure to increased drug concentrations (Upcroft, 2001; Boreham et al., 1988; Orozco et al., 2009). Furthermore, there have been reports that *Giardia* after failing treatment with metronidazol showed resistance to other known anti giardial drugs as well (Brasseur et al., 1995; Orozco et al., 2009). *Entamoeba* until now did not show any capacity to develop metronidazol resistance. However, in laboratory conditions by stepwise exposure to elevated drug concentrations partial resistance has been induced (Wassmann et al., 1999; Upcroft, 2001; Orozco et al., 2009). The only clinical resistance that has been reported was in patients with liver abscesses, however those patients have been successfully cured after the therapeutic drainage of the abscess (Hanna et al., 2000).

2. Materials and Methods

2. 1. Parasite strains and media

The strains of parasites used in this study were *Entamoeba histolytica* HM-1:IMSS (ATCC 30459) and *Giardia intestinalis* WB clone 6 (ATCC 50803). The trophozoites were cultivated axenically in 40 ml tissue culture flasks (Becton Dickinson Labware, NJ, USA), at 36.5°C in TYI-S-33 media (Diamond et al., 1978), modified by Kollaritsch for *E. histolytica* and by Keister's for *G. intestinalis* (Keister, 1983). With current modifications in order to prepare 1 l of medium, 20 g of casein digest peptone, 20 g (10 g for *G. intestinalis* medium) of yeast extract, 10 g of glucose, 2 g of sodium chloride, 1 g (2 g) of cysteine hydrochloride, 1 g of dibasic potassium phosphate, 0.6 g of monobasic potassium phosphate, 0.2 g of ascorbic acid and 22.8 mg of ferric ammonium citrate (brown form) are dissolved in 870 ml (900 ml) of purified H₂O (Milli-Q Integral 5, Millipore, Vienna). The *G. intestinalis* medium is supplemented with 0.75 g of bovine bile (Sigma-Aldrich). The pH is adjusted to 6.85 for *Entamoeba* and to 7.0–7.2 for *Giardia* medium. After the medium has been autoclaved for 15 min at 120°C and cooled down, both media are supplemented with 10% (v/v) complement-inactivated bovine serum, 10 ml/l penicillin/streptomycin antibiotic mixture (Invitrogen) and *Entamoeba* medium with 30 ml of Diamond Vitamin Tween 80 Solution (40x) (Sigma-Aldrich) as well. *E. histolytica* trophozoites are subcultured twice and *G. intestinalis* trophozoites three times a week.

2. 2. Compounds

Leaves, stem or root barks of tropical plants from Rutaceae, Meliaceae and Asteraceae families were collected on several rainforest locations in Thailand and Costa Rica and brought to the Institute of Botany in Vienna, where the extracts were prepared and the compounds were isolated by preparative column and thin layer chromatography (TLC). The purity was evaluated by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) (Greger et al., 1996; Hofer et al., 2000). All fourteen compounds were dissolved in 100% dimethylsulphoxide (DMSO, Sigma–Aldrich) to a stock concentration of 10 mg/ml.

2. 3. Susceptibility assays

The parasites were tested in 96-well microtiter plates under anaerobic conditions which were established in 1.1 litre plastic air-tight boxes (EMSA GmbH Emsdetten, Germany) by use of Anaerocult A (Merck Darmstadt, Germany). The establishment of anaerobic conditions in the boxes was tested using the Anaerotest strips (Merck).

The quick tests with end concentrations of 2.5 µg/ml and 10 µg/ml of each compound and with 40,000 cells/ml as a starting concentration of the protozoans were performed in triplicates in 300 µl volumes of media. As controls, untreated cells, cells treated with 10 µM of metronidazole and cells treated with DMSO were used. The plates were incubated for 24 h and 48 h at 37°C. The experiments were repeated twice with both protozoans. Evaluation was done by transferring cells to Eppendorf tubes, concentrating them to 50 µl volumes by centrifugation in a microcentrifuge (Galaxy-Mini, VWR), and staining the cells with the same volume of a 0.4% solution of trypan blue (Sigma-Aldrich) and counting living and dead cells in a Bürker-Türk hemocytometer. The cell number per milliliter was determined by counting the cells in all 4 x 16 fields in the counting chamber, to get more accurate values. This number was then divided by four and multiplied with 10,000.

Assays with the compound that had shown activity in the quick tests were performed in the same manner, just in more concentrations of the compound, 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml and 20 µg/ml, respectively. EC50 and EC90 values of the substance were calculated *via* log probit analysis available in the SPSS 16.0 statistics software suite (SPSS Inc., now part of IBM, Chicago, IL).

Assays with metronidazole, as a control drug, were also conducted under the same defined conditions; EC50 and EC90 were calculated, so that efficacy of two drugs could be compared.

Furthermore, trophozoites of *E. histolytica* and *G. intestinalis* were cultivated under anaerobic conditions in media containing different cysteine concentrations: 0 g/l, 0.125 g/l, 0.25 g/l, 0.5 g/l and 1 g/l for *Entamoeba* and 0 g/l, 0.25 g/l, 0.5 g/l, 1 g/l and 2 g/l for *Giardia*. Then the susceptibility of the parasites to the active compound at concentrations of 0 µg/ml, 1 µg/ml, 5 µg/ml, and 20 µg/ml, respectively, was examined as described above in each of the media.

2. 4. *E. histolytica* proteome analysis

Two-dimensional gel electrophoresis (2DE) was performed by an adapted protocol for *E. histolytica* cell extracts (Leitsch et al., 2005).

Sample preparation

The cultivated cells were transferred from the 40 ml flasks in 50 ml Falcon tubes, centrifuged (700 x g, 5 min), the supernatant was discarded and the pellet was washed twice with 20 ml 1x PBS (700 x g, 5 min) and then suspended in 500 µl ddH₂O, transferred in 2 ml Eppendorf tubes and filled up with 1.5 ml 12% (w/v) trichloroacetic acid (TCA) in acetone (at -20°C). TCA precipitation is very important because it destroys protease activity and keeps the salt concentration as low as possible, so it will not interfere with isoelectric focusing. The precipitation step should last for at least 60 min at -20°C. The proteins were sedimented at 14,000 rpm for 20 min at 4°C (Sigma centrifuge 1-15 PK) and washed twice with 1 ml ice-cold 90% acetone (v/v) / 10% ddH₂O in order to remove residual TCA, which would disturb isoelectric focusing as well. The pellets were air-dried for 30 min and resuspended in 500 µl of 2DE solubilization buffer (7 M urea, 2 M thiourea, 1% (w/v) DTT, 0.5% (w/v) ampholytes 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)). Resolubilization was carried out overnight at 23°C under mild shaking on a thermomixer. After 30 min centrifugation at 14,000 rpm (4°C) in order to remove any residual cell debris, the protein concentration was measured with the Bradford-Assay (Bio-Rad Laboratories, Vienna, Austria) at 595 nm according to the instructions of the provider.

For the 17 cm isoelectric focusing (IEF) strips with a pH range between 5-8, 500 µg/ml of proteins were diluted with 2DE solubilization buffer to a total volume of 400 µl. Prepared samples were loaded on an IEF tray and covered with at least 2 ml of mineral oil in order to prevent evaporation during first the 12 h of rehydration.

Isoelectric focusing (IEF)

The PROTEAN IEF System (Bio-Rad Laboratories, Vienna, Austria) was used for isoelectric focusing. The program consists of the following steps:

1. Rehydration 12 h (50 mV)
2. 250 V, 1 h, rapid slope

3. 500 V, 1 h, rapid slope
4. 2000 V, 2 h, linear slope
5. 5000 V, 2 h, linear slope
6. 10000 V, 5 h, rapid slope or 8000 V, 6 h, rapid slope

Strip equilibration

After IEF the strips were transferred to the plastic tray, washed with ddH₂O in order to remove mineral oil and immediately equilibrated, although at this point, they can be stored at -80°C as well. Equilibration is preformed in two steps:

1. reduction of the cysteine groups with 1% dithiothreitol (DTT)
2. alkylation of the reduced cysteines with 4% iodoacetamide

The equilibration buffer consists of: 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris/Cl pH 8.8. Both steps should not take more than 15 to 20 min in order to prevent protein loss due to diffusion.

SDS-PAGE

After the large gels for the second dimension were prepared, each equilibrated strip was placed on the top of one gel and overlaid with proteinase-free agarose solution. The gels were run at 4°C overnight on 15–25 mA.

Visualization

Staining was done with Coomassie staining (0.87g Coomassie Brilliant Blue R250 (Bio-Rad), 45% (v/v) ddH₂O, 45% (v/v) methanol, 10% (v/v) glacial acetic acid) for 20 min on room temperature. The gels were destained in 20% (v/v) methanol and 10% (v/v) glacial acetic acid in ddH₂O for 2 h, and afterwards washed and kept in ddH₂O.

The staining and destaining steps were carried out on a Belly Dancer shaker (Stovall Greensboro, NC USA).

Identification of proteins

Coomassie-stained protein spots were excised from the gels, treated with trypsin and the resulting peptides were analyzed by liquid chromatography and tandem mass spectrometry by Dr. Alain Guillot from the PAPPSO (Plateforme d'Analyse Protéomique de Paris Sud-Ouest) core facility, Paris, France.

Buffers

Lysis buffer:

7 M urea

2 M thiourea

1% (w/v) DTT

0.5% (w/v) ampholytes

4% (w/v) CHAPS

4x Resolution buffer for 2nd dimension:

1.5 M Tris/Cl pH 8.8

0.4% (w/v) SDS

Equilibration buffer:

6 M urea

30% (v/v) glycerol

2% (w/v) SDS

50 mM Tris/Cl pH 8.6 or pH 8.8 (use resolution buffer as stock)

Acrylamide stock 30% (300 ml)

90 g acrylamide

2.4 g bisacrylamide

Take up in 300 ml ddH₂O and filter

Second dimension gels (100 ml = 2 large gels)

40 ml acrylamide 30% (w/v)

25 ml 4x resolution buffer

25 ml water

10 ml glycerol (87%)

Running buffer for second dimension (1litre):

10 ml of 10% (w/v) SDS

3 g Tris

14 g glycine

3. Results

3. 1. Establishment of the susceptibility assay

In order to conduct the susceptibility assays, it was necessary at first to establish a system in which the parasites would grow in 96-well microtiter plates, which had to be used because of limited available amounts of extracted compounds (1 mg or less). Trophozoites showed normal growth in 24- and 48-well microtiter plates, however they could not survive in 96-well plates, incubated at 37°C for 24 h in a normal aerated incubator. Several approaches were tried out: I – the microtiter plate covered with parafilm and the lid, II – the candle-box, which is the gold standard in malaria field research (Trager and Jensen, 1976), finally III – and IV – two assays which we established and in which plates were kept in air-tight plastic boxes under microaerophilic and anaerobic conditions.

The microaerophilic and anaerobic assays consisted of one airtight plastic box (EMSA GmbH Emsdetten Germany) with a volume of 1.1 litres for one microtiter plate (Fig. 7). In the box, one half of an Anaerocult A sachet (Merck, Darmstadt, Germany) is added to establish anaerobic conditions, or one Anaerocult C sachet (Merck) for microaerophilic conditions. Anaerobic conditions were controlled with Anaerotest strips (Merck). Different concentrations of the cells: 10,000, 20,000, 30,000, 40,000 and 50,000 cells/ml were incubated for 24 h at 37°C. The cells were counted and based on results we decided for the best method (Fig. 8).



Figure 7: Test box for anaerobic and microaerophilic conditions

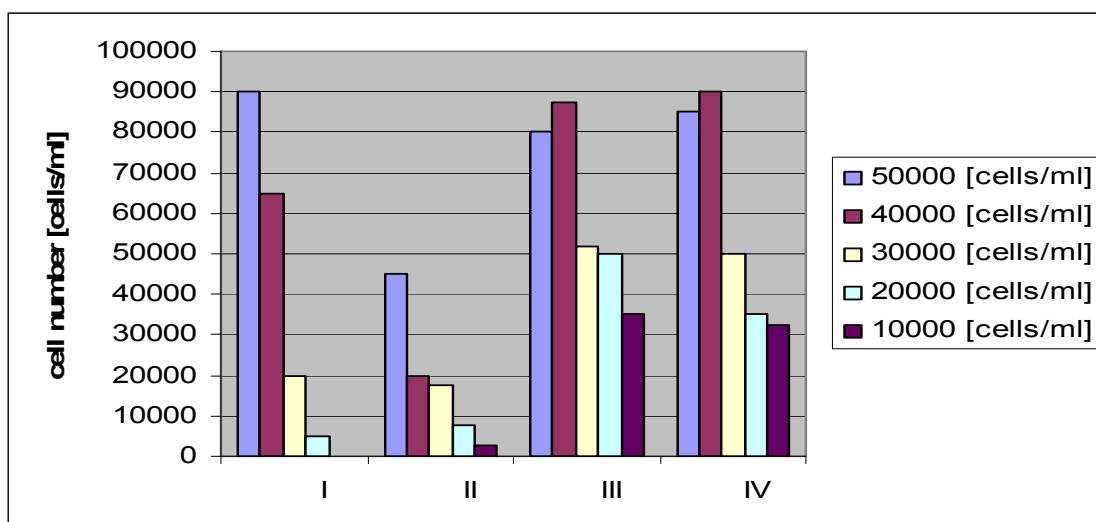


Figure 8: Results of the microtiter plate cultivation experiment

- I – plate covered with parafilm and the lid
- II – plate in a candle box
- III – plate under microaerophilic conditions
- IV – plate under anaerobic conditions

It was decided to use the anaerobic assay to conduct the screenings, because we found the cell numbers were slightly superior to the microaerophilic assay, and with the use of the Anaerotest strips we could test easily that anaerobic conditions were indeed established.

3. 2. Results of the susceptibility assays

After conducting quick tests for all of the substances with *E. histolytica* as well as *G. intestinalis* from the number of living and dead cells for each concentration and the control, the growth inhibition percentages (GI), after 24 h and 48 h, were calculated, using the formula (Rolòn et al., 2006):

$$GI (\%) = [(Gc - Gp) / Gc] \times 100$$

Gc – the mean number of living cells per milliliter in control

Gp – the mean number of living cells per milliliter at the different drug concentrations

Substance	<i>Entamoeba histolytica</i>			
	GI [%] 24 h		GI [%] 48 h	
	10 µg/ml	2.5 µg/ml	10 µg/ml	2.5 µg/ml
Arborinin	15.27	32.42	-33.17	-57.29
Arborin	22.92	36.64	-51.53	-34.68
Methyldambullin	27.40	13.95	-1.02	-24.39
Sakambullin	25.86	8.14	-13.57	-56.81
Yukocitrin	11.08	15.79	-41.91	-21.86
5-Hydroxynoracronycine	31.09	26.62	26.41	-41.79
Zanthobungeanin	28.72	9.71	2.29	-7.05
Kokusaginin	8.80	21.43	0	-25.85
Methylacarol	-13.59	2.83	-16.13	-18.93
Iso-gamma-fagarine	5.9	27.45	16.07	-28.75
Dictamnin	6.2	-5.37	15.71	-6.45
Aglafolin	16.30	11.35	7.84	6.73
Microminutin	26.50	14.50	-6.59	13.38
Methylgerambullin	96.50	48.27	96.78	30.95

Table 1: Results of the susceptibility assays (quick tests) for *Entamoeba histolytica*

Substance	<i>Giardia intestinalis</i>			
	GI [%] 24 h		GI [%] 48 h	
	10 µg/ml	2.5 µg/ml	10 µg/ml	2.5 µg/ml
Arborinin	17.04	5.73	12.16	13.19
Arborin	27.76	18.59	9.10	7.52
Methyldambullin	54.79	21.24	26.37	3.62
Sakambullin	7.21	7.07	10.77	0.65
Yukocitrin	7.53	14.90	7.71	3.70
5-Hydroxynoracronycine	-2.13	-0.11	18.24	16.05
Zanthobungeanin	28.22	0.04	41.11	40.06
Kokusaginin	18.36	24.65	36.09	28.87
Methylacarol	27.90	15.84	19.22	26.76
Iso-gamma-fagarine	36.25	0.06	2.65	-1.77
Dictamnin	24.02	11.77	1.86	0.54
Aglafolin	62.58	63.74	73.92	75.35
Microminutin	21.48	10.39	-2.98	-5.63
Methylgerambullin	96.99	54.14	99.48	36.85

Table 2: Results of the susceptibility assays (quick tests) for *Giardia intestinalis*

The results of the susceptibility assays showed that only one of the fourteen substances, namely methylgerambullin (Fig. 9), had an effect against both of the parasites, by killing 96.78% of *E. histolytica* and 99.48% of *G. intestinalis* cells at the highest concentration after 48 h.

As methylgerambullin had shown quite promising results not only against *E. histolytica* and *G. intestinalis*, but also against trypanosomatids and *Plasmodium falciparum*, a contract was given to Selvita Life Sciences Solutions (Krakow, Poland) for the chemical synthesis of methylgerambullin. This synthesis was performed according to Ikegami et al. (1989) by the following scheme (Fig. 9). The availability of sufficient amounts of methylgerambullin allowed further experiments, in particular the proteomic studies described below.

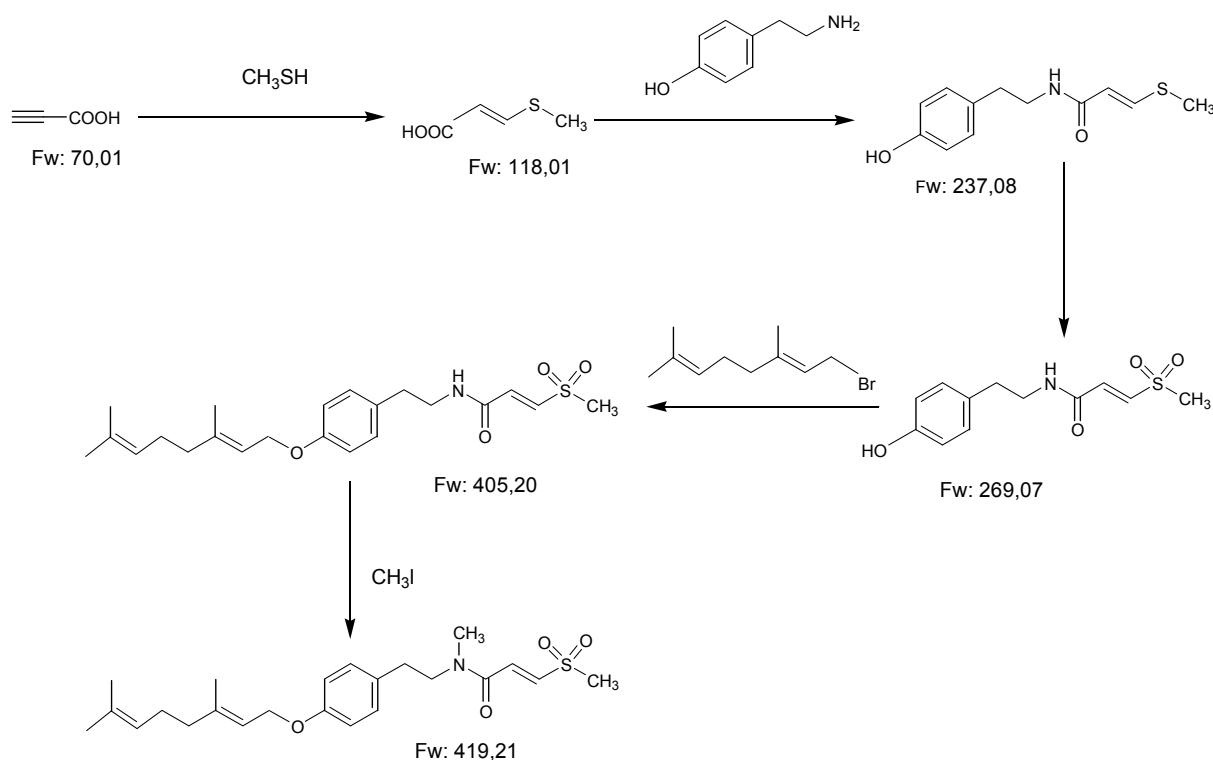


Figure 9: Chemical synthesis of methylgerambullin

Assays with both protozoans and different concentrations of methylgerambullin, 1 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$, 7.5 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$, respectively were performed in order to calculate EC50 and EC90 values of the compound after 24 h and 48 h of the exposure to the compound. The assays for each parasite were repeated three times. EC50 and EC90 with the results of each assay were calculated and out of those results the geometric mean (G) was calculated as the final result.

***Entamoeba histolytica* – methylgerambullin**

EC50 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	Exp. 3	G
24 h	4.31	9.11	5.72	6.08
48 h	4.59	13.77	6.23	7.33

EC90 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	Exp. 3	G
24 h	16.77	88.23	20.86	31.37
48 h	16.29	258.55	43.53	56.81

Table 3: The EC50 and EC90 values of three experiments and calculated geometric mean (G) for *E. histolytica* treated with methylgerambullin

***Giardia intestinalis* – methylgerambullin**

EC50 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	Exp. 3	G
24 h	27.06	2.94	2.91	6.14
48 h	196.06	4.94	3.73	15.34

EC90 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	Exp. 3	G
24 h	48.53	9.45	6.99	14.74
48 h	3452.68	14.03	9.80	78.00

Table 4: The EC50 and EC90 values of three experiments and calculated geometric mean (G) for *G. intestinalis* treated with methylgerambullin

In order to compare EC50 and EC90 of methylgerambullin with those values of metronidazole, the drug which is used for the treatment of *E. histolytica* and *G. intestinalis* infections, the assays with both parasites and metronidazole were conducted under same conditions and EC50 and EC90 were calculated.

***Entamoeba histolytica* – metronidazole (control)**

EC50 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	G
24 h	0.32	0.52	0.41
48 h	0.15	0.39	0.24

EC90 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	G
24 h	0.87	1.24	1.04
48 h	0.56	0.87	0.70

Table 5: The EC50 and EC90 values of three experiments and calculated geometric mean (G) for *E. histolytica* treated with metronidazole

***Giardia intestinalis* – metronidazole (control)**

EC50 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	G
24 h	0.59	0.49	0.54
48 h	0.31	0.36	0.33

EC90 [$\mu\text{g ml}^{-1}$]	Exp. 1	Exp. 2	G
24 h	1.83	1.21	1.49
48 h	1.85	1.01	1.37

Table 6: The EC50 and EC90 values of three experiments and calculated geometric mean (G) for *G. intestinalis* treated with metronidazole

3. 3. The activity of methylgerambullin depends on the cysteine concentration in the medium

The experiments described above had shown a rather high variability, in general the effect of methylgerambullin was weaker in freshly-prepared media. In order to test if the activity of methylgerambullin depends on the concentration of cysteine in the medium, five assays with defined concentrations of cysteine and different concentrations of methylgerambullin for each parasite were conducted. The results indeed show an inverse relation between concentration of the cysteine in the medium and the efficacy of the methylgerambullin (Figs 10, 11).

Entamoeba histolytica:

Methylgerambullin:		0 µg/ml		1 µg/ml		5 µg/ml		20 µg/ml	
		alive	dead	alive	dead	alive	dead	alive	dead
Cysteine:	0 g/l	50,000	21,250	23,750	26,250	/	71,250	/	71,250
	0.125 g/l	62,500	31,250	36,250	20,000	5,000	61,250	/	70,000
	0.25 g/l	80,000	27,500	55,000	23,750	28,750	63,750	/	55,000
	0.5 g/l	100,000	26,250	36,250	11,250	35,000	36,250	/	72,500
	1 g/l	146,250	33,750	126,250	27,500	53,750	25,000	2,500	103,750

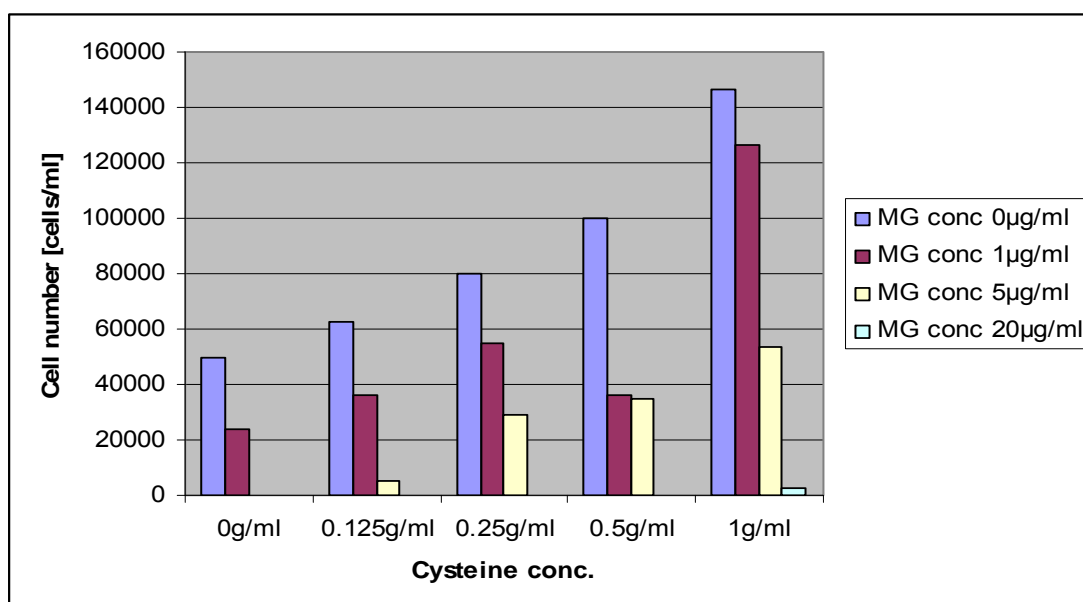


Figure 10: *Entamoeba histolytica* cultivated in medium with different cysteine concentrations and treated with different concentrations of methylgerambullin

***Giardia intestinalis*:**

Methylgerambullin:		0 µg/ml		1 µg/ml		5 µg/ml		20 µg/ml	
		alive	dead	alive	dead	alive	dead	alive	dead
Cysteine:	0 g/l	95,000	13,750	36,250	16,250	/	70,000	/	52,500
	0.25 g/l	88,750	12,500	63,750	23,750	2,500	70,000	/	73,750
	0.5 g/l	146,250	12,500	60,000	15,000	13,750	35,000	/	81,250
	1 g/l	226,250	15,000	145,000	13,750	89,100	14,100	19,100	51,600
	2 g/l	320,000	22,500	271,250	26,250	243,750	23,750	191,250	25,000

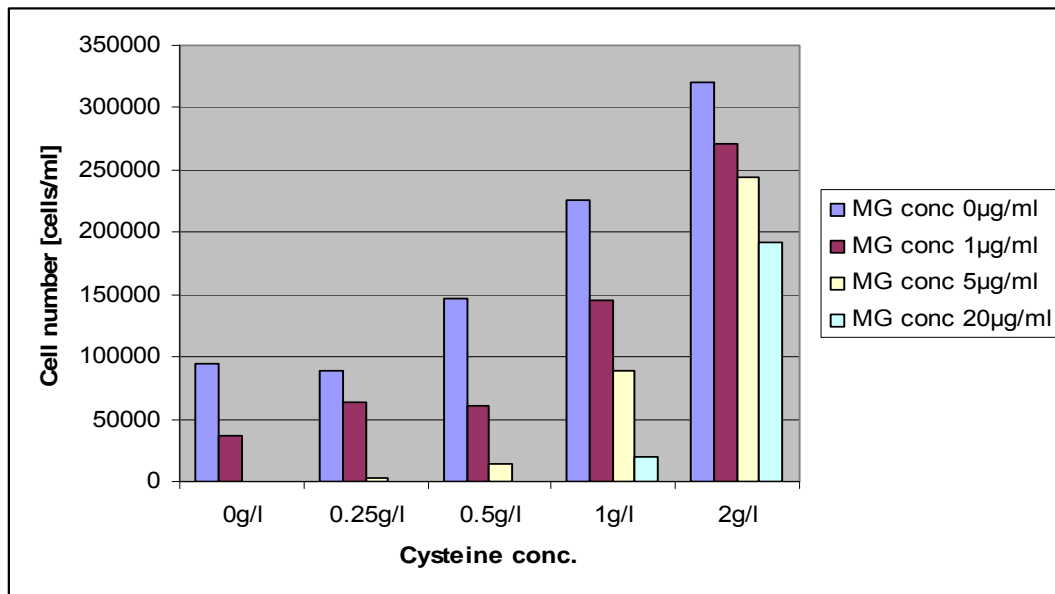


Figure 11: *Giardia intestinalis* cultivated in medium with different cysteine concentrations and treated with different concentrations of methylgerambullin

3. 4. Comparison of methylgerambullin-treated and untreated *E. histolytica* by two-dimensional gel electrophoresis

In order to observe whether methylgerambullin has any effect on the *E. histolytica* proteome, two-dimensional gel electrophoresis was performed by an adapted protocol for *E. histolytica* cell extracts (Leitsch et al., 2005). Four 40 ml flasks of the culture were incubated at 37°C for four days, until the culture reached the required density. The cells were centrifuged at 700 x *g* for 5 min, the supernatant was discarded and the cells were resuspended in TYI-S-33 medium without cysteine and split in two 40 ml flasks. The cells in one flask were treated with 40 µg/ml of methylgerambullin and the other ones were used as a control. After 3 h and 30 min, when the treated cells were rounded and clearly affected by the compound, both of the cell cultures were harvested by centrifugation on 700 x *g* for 5 min. Extracts from the cells were prepared and two-dimensional gel electrophoresis was then performed as described above, and the gels were Coomassie-stained.

The stained gels (Figs. 12, 13) showed that there were four newly induced protein signals in *E. histolytica* treated with methylgerambullin. Tandem mass spectrometry (MS) carried out by Dr. Alain Guillot at the PAPPSO core facility identified these proteins as isoenzymes of alcohol dehydrogenase (ADH) and isoenzymes of pyruvate:ferredoxin oxidoreductase (PFOR).

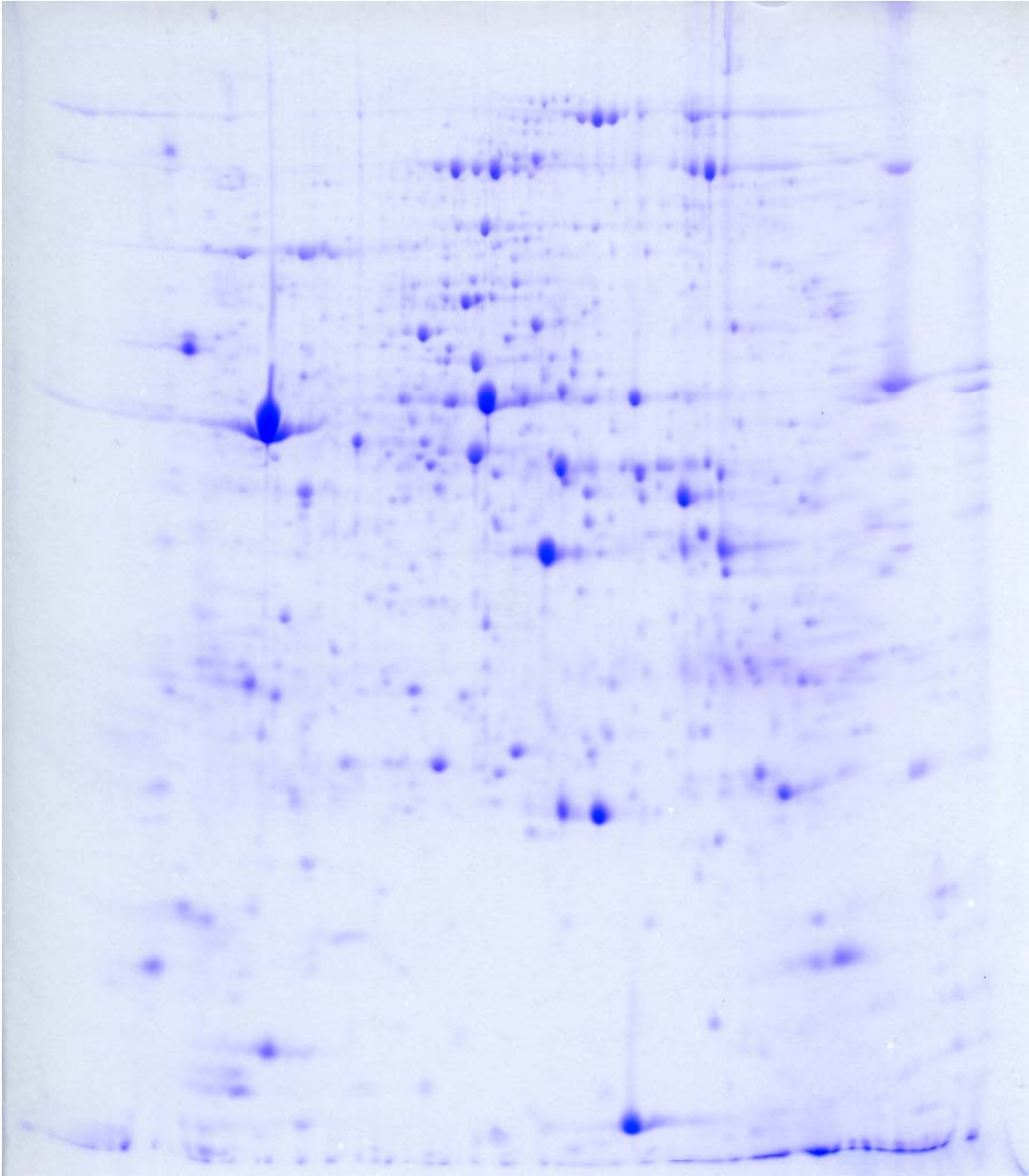


Figure 12: Coomassie-stained gel of untreated *E. histolytica* cell extract

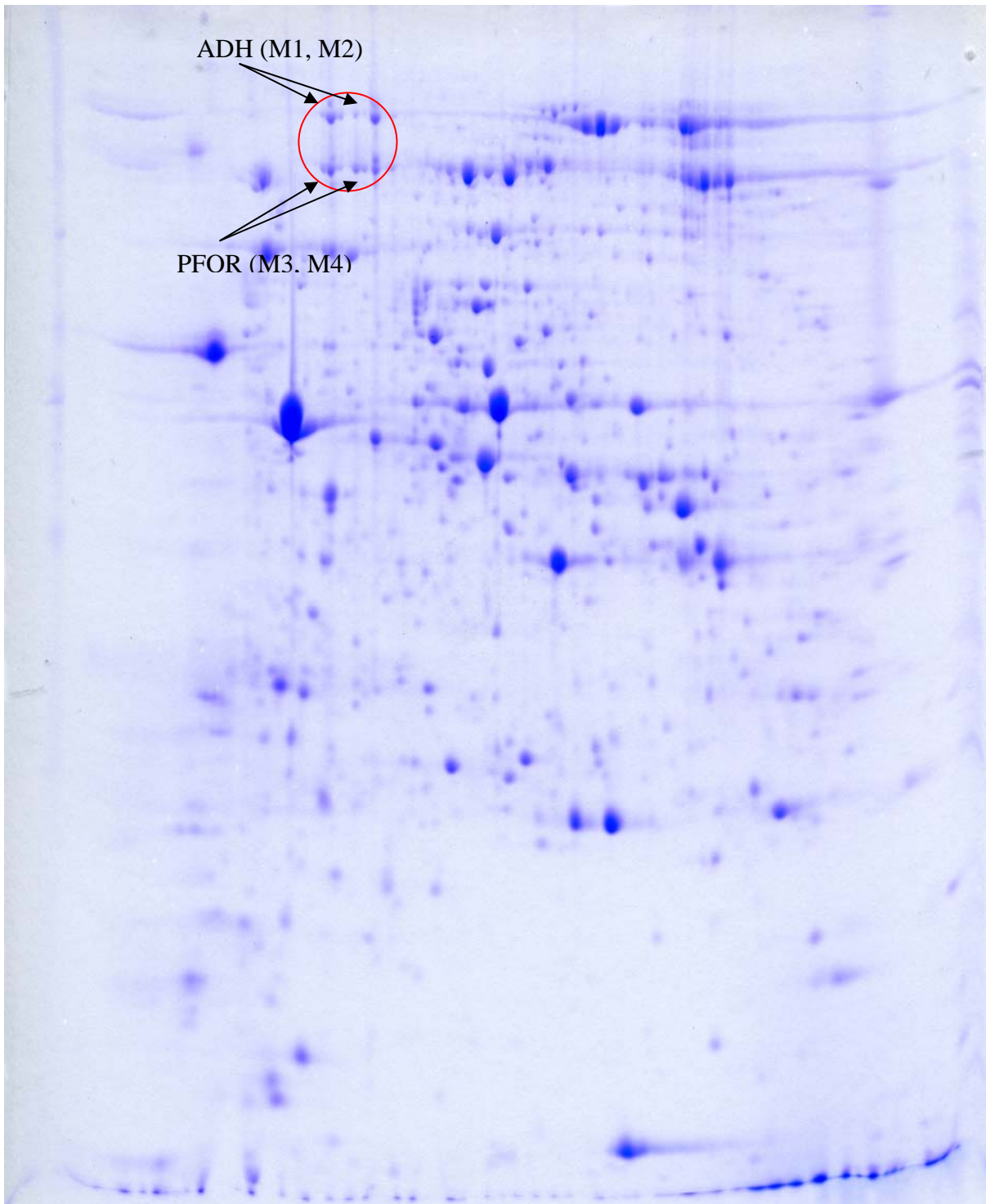


Figure 13: Coomassie- stained gel of *E. histolytica* treated with 40 µg/ml of methylgerambullin. Circled in red are four induced proteins, which cannot be seen on the gel of untreated *E. histolytica*: M1 and M2 spots were identified as isomers of alcohol dehydrogenase (ADH) and M3 and M4 spots as isomers of pyruvate:ferredoxin oxidoreductase (PFOR).

4. Discussion

4. 1. Natural products against tropical diseases

Protozoa can cause severe diseases, including malaria, leishmaniosis, Chagas disease, sleeping sickness, amoebiasis and giardiasis. All these protozoan infections are responsible for misery and death, particularly in tropical countries. In spite of various efforts, there are still no protective vaccines available against any of the protozoan diseases, and parasite resistance to existing drugs has become a serious problem. Furthermore many of the available drugs are old or cause serious adverse reactions. Due to lack of financial returns, research in this field is of limited interest to pharmaceutical companies. As a consequence, only 13 of 1393 new molecular entities authorised between 1975 and 1999 were designated for tropical diseases (Trouiller et al., 2002). As mentioned in the Introduction, many pharmaceutical companies have dismissed their departments for natural product research, because the drug discovery from medicinal plants was seen as more time and money consuming than other drug discovery methods (Butler, 2004; Koehn and Carter, 2005; Balunas and Kinghorn, 2005).

Focus of pharmaceutical research and drug development in the 1980s shifted from natural products to high-throughput synthesis and combinatorial chemistry, which provide a larger amount of produced substances. They are also time- and cost-effective and do not raise a question of intellectual property rights, as the use of natural products does (Harvey, 2008). However, although there are libraries with dozen thousands of combinatorial new chemical entities available, only one of those molecules, namely sorafenib, a kinase inhibitor, has been approved for treatment of a human disease, a renal carcinoma (Newman, 2007). To compare, in only two years period, from 2005 to 2007, thirteen natural product-related drugs were approved for treatment and five of them were newly discovered chemical classes of drugs (Butler, 2008; Harvey, 2008). Furthermore, according to the *Pharmaprojects* database (www.pharmaprojects.com) from March 2008 there are in total 225 natural product related drugs at different stages of development, of which 108 are of plant origin (Harvey, 2008). In a recent review Harvey also highlights that in a period from 1994 to 2010 almost 50% of newly introduced drugs were natural product-related drugs (Harvey et al., 2010).

Advances in technology of purification and identification of chemical substances derived from natural products made this kind of drug research and development again more relevant and interesting not only for the scientist, but for pharmaceutical companies as well. One of the greatest improvement happened in the field of separation technology, where high performance chromatography methods (Foucault, 1995; McChesney et al., 2007), as well as more recently, the multi-channel counter-current chromatography (CCC) method (Wu et al., 2008) made separation and fractionation faster and more efficient. Assigning the chemical structure to the isolated compound is the next step in the process, which was especially advanced with the development of high field NMR spectrometry (Crosmun et al., 1994; Claridge, 1999; McChesney et al., 2007), in particular the two-dimensional NMR technique. Mass spectrometry techniques alone and coupled with liquid chromatography are broadly used as well. Together with the available data bases of mass spectra they add the necessary speed and accuracy to the structure elucidation process.

All these data suggest that in the field of drug research and development one should rely more on natural products screening and possibly combine it with combinatorial chemistry. An isolated active compound can be a lead to the chemists, which could then through different processes, using newest technology and accumulated knowledge, further improve it. Such improvements in efficacy, selectivity, solubility, better absorption or easier excretion from the organism, could make a newly isolated active compound a perfect drug candidate. Some of the scientists argue that nature has been combining and choosing the substances with specific biological advantages for millions of years and that people should try to learn from its wisdom (McChesney et al., 2007).

4. 2. Methylgerambullin against *Entamoeba histolytica* and *Giardia intestinalis*

In the present study fourteen plant-derived substances were tested for their activity against the microaerophilic protozoan parasites, *Entamoeba histolytica* and *Giardia intestinalis*. Only the sulphur-containing amide methylgerambullin showed promising, high anti-amoebic and anti-giardial activity. The obtained EC₅₀s in *E. histolytica* were 6.08 µg/ml after 24 h and 7.33 µg/ml after 48 h in comparison to the standard drug metronidazole, which showed EC₅₀s of 0.41 µg/ml after 24 h and 0.24 µg/ml after

48 h of treatment. Metronidazole also had lower EC90s with 1.04 µg/ml after 24 h and 0.70 µg/ml after 48 h, respectively. The obtained EC90s of methylgerambullin against *E. histolytica* were 31.37 µg/ml after 24 h and 56.81 µg/ml after 48 h of treatment. So interestingly, the EC50 and EC90 of methylgerambullin are lower after 24 h than after 48 h. This could mean that after 48 h at this drug concentration *E. histolytica* trophozoites are able to slightly recuperate.

The anti-giardial EC50 of methylgerambullin after 24 h was 6.14 µg/ml and the EC90 was 14.74 µg/ml. After 48 h methylgerambullin also showed a higher anti-giardial EC50 (15.34 µg/ml) and EC90 (78.00 µg/ml) than after 24 h of treatment. Metronidazole was more efficient in *G. intestinalis* with an EC50 of 0.54 µg/ml and an EC90 of 1.49 µg/ml after 24 h as well as EC50 of 0.33 µg/ml and EC90 of 1.37 µg/ml after 48 h. All other newly tested compounds did not show any or just marginal activity against *E. histolytica* and *G. intestinalis*.

When we compared the EC50 and EC90 values from three consecutive experiments with each parasite, we noted a high variation of EC50 between the experiments with *G. intestinalis*, 2.94 µg/ml, 2.91 µg/ml, and third 27.06 µg/ml. This led us to the idea that these variations could be in correlation with freshness of the used cultivation medium. After a new bottle of the medium has been opened, it is kept in the refrigerator for a few days, until is used up. There is a noticeable difference as the *Giardia* medium changed colour from light to dark yellow and the *Entamoeba* medium from light to dark brown. This colour change could be caused by the depletion of cysteine.

In order to test whether cysteine is important for methylgerambullin activity additional experiments with both parasites, cultivated in medium with different cysteine concentrations and treated with methylgerambullin, were conducted. The results, indeed, showed an inverse relation between methylgerambullin activity and concentration of cysteine. We looked at the cysteine concentrations of 0.5 µg/ml for *Giardia* and 0.25 µg/ml for *Entamoeba*, which are both four times lower than concentrations normally used for preparing the medium. At these concentrations trophozoites have enough cysteine to grow and divide, so during the *Giardia* experiment the cell concentration tripled and it doubled in the *Entamoeba* experiment. For both parasites the highest concentration of 20 µg/ml of methylgerambullin was effective, by killing all the cells, and even lower concentrations of 5 and 1 µg/ml showed efficacy. However, in the experiments with

the highest concentrations of cysteine in the medium, methylgerambullin even in the highest concentration of 20 µg/ml was not effective. This led us to believe that cysteine reacts with methylgerambullin (Fig. 14) and in this way blocks it, so the cells are protected, when there is enough cysteine in the medium. Furthermore, we presume that using the same mechanism, methylgerambullin could interact with the thiols of the parasite, what could be potentially important for its mode of action and the way it destroys the cells.

Proposed reaction:

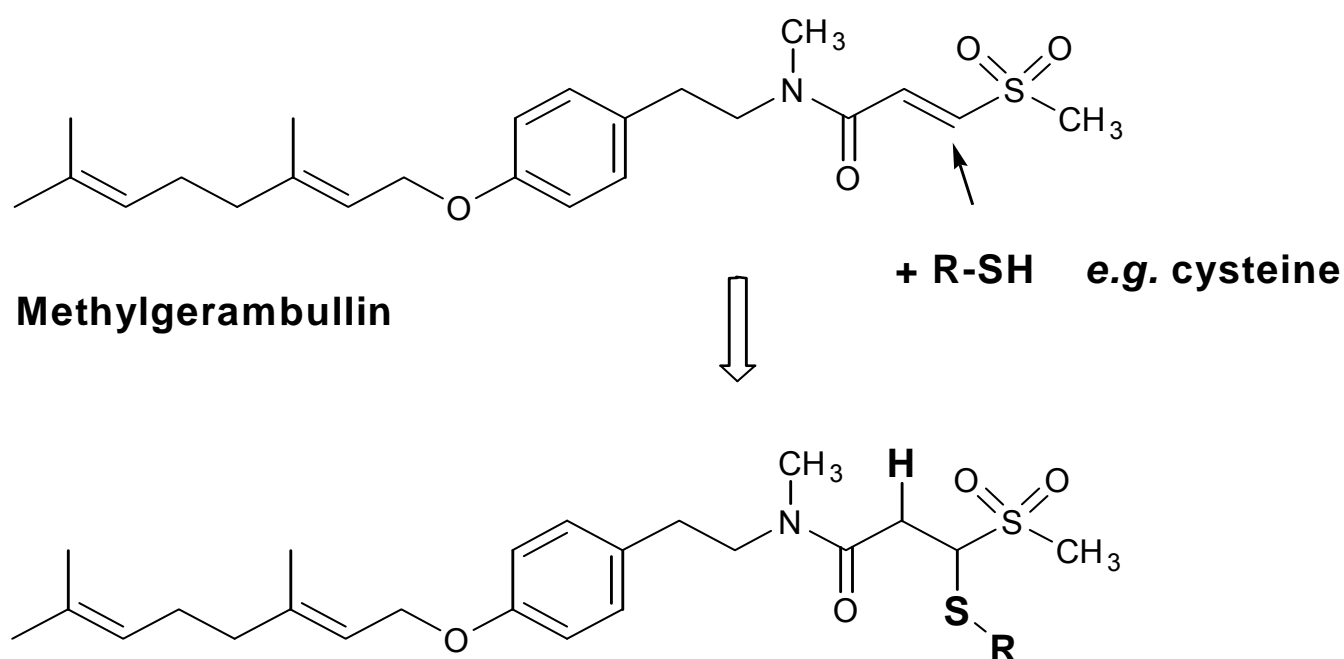


Figure 14: Proposed reaction scheme for the reaction of methylgerambullin with cysteine

Cysteine (Cys) is a non-essential amino acid, however, it is very important for the cell function of prokaryotes as well as the eukaryotes, where it plays an essential role in maintenance of the redox state of the plasma proteins (Jones et al., 2000; Nkabyo et al., 2006). In the mammalian gut lumen glutathione (GSH), which is generated from cysteine, has a protective role. Its antioxidant character diminishes the damage caused by electrophiles (Martensen et al., 1990; Aw et al., 1992; Dahm and Jones, 1994; Nkabyo et al., 2006). The total concentration of cysteine, homocysteine and glutathione was determined as 35.4 nmol/mg protein in proximal colonic mucosa and 32 nmol/mg protein in distal colonic mucosa (Morgenstern et al., 2003). Although we

definitively expect methylgerambullin to be active in an environment like the human gut, this activity could be diminished by the thiols in this environment.

When we compared the protein patterns of untreated *Entamoeba* and *Entamoeba* treated with methylgerambullin by two-dimensional gel electrophoresis, the analysis revealed specific differences. As it can be seen in Fig. 13, circled in red, there are four additional spots on the gel of the treated cells. Mass spectrometry revealed that spots M1 and M2 are isoenzymes of alcohol dehydrogenase (ADH) and spots M3 and M4 isoenzymes of pyruvate:ferredoxin oxidoreductase (PFOR). These proteins are normally to be seen at a more basic pI. The reason why they could have shifted to more acidic pI remains to be discovered. One of the theories is that the enzymes, through treatment of *Entamoeba* with methylgerambullin, were phosphorylated and due to this modification the position of the enzymes in the gel changed.

A possible phosphorylation could also change the activity of the enzymes. ADH and PFOR are both part of the glucose catabolism pathway and every change in their conformation, which changes their activity, could also influence levels of ATP in the cell. Drastical decrease of ATP levels could lead to the cell death.

According to the *Entamoeba* genome project there are more than 150 kinases as well as more than 100 putative phosphatases in *Entamoeba* so protein phosphorylation could be important for many activities in the cell (Clark, 2007).

4. 3. Properties of methylgerambullin

So far, methylgerambullin could only be extracted from the genus *Glycosmis*, family Rutaceae, in contrast to other sulphur-containing amides, which also occur in the genus *Raphanus*, family Brassicaceae (Moon et al., 2010). A specimen of glycosmis, also called orangeberry, is shown in Fig. 15. To obtain more of the compound, an efficient, high-yielding method to chemically synthesize methylgerambullin was found (Obwaller et al., unpublished). Chemical synthesis is of high interest and has big advantages, like lower costs, batch to batch consistency and purity. Further advantages are that chemical synthesis is less time consuming than extraction and purification and a constant yield can be achieved, therefore it is easier to establish GMP standards for synthetic compounds than for extracted compounds.



Figure 15: *Glycosmis* sp. (source: Botanical garden, Vienna)

What is also of great importance is that methylgerambullin can easily be dissolved in the non-toxic polyethylene glycol (PEG) in a concentration as high as 10 mg/ml, overnight. Methylgerambullin, as well as all the other tested compounds, obeys the famous Lipinski's "Rule of Five", which predicts passive oral absorption of the drug in regard to its molecular weight, H-bond donors and acceptors and log-P. These are important requirements for further *in vivo* and other pharmacological studies.

The sulphur-containing acid moieties of the structure are most likely derived from the amino acid cysteine, whereas the amine parts are mostly characterized by phenethyl or phenylethenyl groups that additionally can be linked to various prenyloxy structures, e.g. in methyldambullin and sakambullin, or geranyloxy groups, e.g. in methylgerambullin. These prenylated amides are mostly oxidized to sulfones or sulfoxides (Hofer et al., 2000).

Several sulphur-containing amides are reported to have anti-fungal or insecticidal activity (Greger et al., 1996). Methylgerambullin showed cytostatic activity in CEM-SS (T-lymphoblastic leukaemia), KU812F (chronic myelogenous leukaemia), HT29 (colon cancer) and UACC-62 (melanoma) cell lines, however, methylgerambullin has

been shown to be non-toxic in peripheral blood mononuclear cells (Mohamed et al., 2004).

In another study, the three sulphur-containing amides involved in the present study, namely methylgerambullin, methylgerambullin and sakambullin, showed high activity against *Trypanosoma cruzi* Y *in vitro* (Astelbauer et al., 2010) and all three compounds were significantly more effective, with lower EC50s and EC90s, than benznidazole, the standard drug in the treatment of Chagas disease. After 72 h treatment, all three compounds also had lower EC90s than amphotericin B, another drug that is used in the treatment of Chagas disease. As against *E. histolytica* and *G. intestinalis* in the present study, methylgerambullin was demonstrated to be the most efficient anti-trypanosomal compound with the lowest EC90, namely 1.92 µg/ml after 72 h of treatment, compared to amphotericin B with 15.54 µg/ml (Astelbauer et al., 2010). Interestingly the other two sulphur-containing amides which also showed high anti-trypanosomal activity, namely methylgerambullin and sakambullin, did not show any anti-amoebic or anti-giardial activity in the present study.

Furthermore, in the study conducted with 43 fresh isolates of *P. falciparum*, measuring the inhibition of schizont maturation *in vitro*, methylgerambullin achieved total schizont maturation inhibition with an IC50 of 14.29 ng/ml and an IC90 of 118.5 ng/ml (Astelbauer et al., unpublished).

Additionally, we demonstrated promising, high *in vitro* activity of methylgerambullin against promastigote *L. infantum* MCAN/ES/89/IPZ 229/1/89, Zymodeme MON 1 with EC50s of 0.97 µg/ml and 0.20 µg/ml and EC90s of 0.58 µg/ml and 0.45 µg/ml after 24 h and 48 h of treatment, respectively (Astelbauer et al., unpublished). Therefore, methylgerambullin was again the most efficient newly tested compound like in *E. histolytica* and *G. intestinalis*. Methylgerambullin was even more efficient than the control substance miltefosine, which showed EC50s and EC90s with concentrations of 0.73 µg/ml, and 3.85 µg/ml after 24 h and 0.52 µg/ml and 0.94 µg/ml after 48 h of treatment, respectively.

No anti-bacterial or anti-fungal activity has been observed for methylgerambullin (Abdullah et al., 2006). In further experiments, no anti-trichomonal activity of any of the plant-derived substances included in the present study was observed (Astelbauer et al., unpublished).

Further *in vitro* studies towards *Plasmodium vivax* (Astelbauer et al., in press) and fungi including yeasts, e.g. *Candida albicans* (ATCC 90028) and *C. glabrata* (ATCC

90030) as well as conidials and sporangiospores, e.g. *Aspergillus fumigatus* III/191 LP Jan/2007, *Rhizopus oryzae* III 122, *Fusarium solani* III/100 (LP) were evaluated recently (Willinger et al., unpublished).

It is interesting to observe how the exact structure of methylgerambullin plays an important role in its activity. If we compare it to metronidazole and other 5-nitroimidazole drugs which are used in the treatment of a variety of parasitic and bacterial diseases, we can see that regardless of their side chain all the drugs of this group show high efficacy. In contrast, sulphur-containing amides differ in the way that every change in their side chain changes the efficacy of the compound. So we found that all three sulphur-containing amides were effective against *T. cruzi*, two against *P. falciparum*, namely methylgerambullin and sakambullin, two against *Leishmania* spp., methylgambullin and methylgerambullin and only methylgerambullin against *G. intestinalis* and *E. histolytica*. Since it is obvious that the exact structure of methylgerambullin plays an important role in its activity, it remains an open question whether the efficacy of the compound could be further improved by modifications of the side chains that could be introduced by chemical synthesis.

Finally, we presume that the mode of action of metronidazole and methylgerambullin are different. If methylgerambullin might sometimes become available as a commercial drug, especially against *Trypanosoma* and *Leishmania*, it could also be used against metronidazole-resistant *G. intestinalis* or *E. histolytica*.

5. Abbreviations

2DE	two-dimensional gel electrophoresis
ADH	alcohol dehydrogenase
CCC	counter-current chromatography
CHAPS	3-[(3-cholamidopropyl)dimethylammonio] -1- propanesulfonate
ddH ₂ O	"doubly distilled" water - molecular biology grade water from Millipore system
DFA	direct fluorescence antibody
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDQM	European Directorate for the Quality of Medicines &HealthCare
EIA	enzyme immunoassay
GMP	Good Manufacturing Practice
GSH	glutathione
HPLC	high performance liquid chromatography
IARC	International Agency for Research on Cancer
IEF	isoelectric focusing
MS	mass spectrometry
NCEs	new chemical entities
NCI	National Cancer Institute
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PAPPSO	Plateforme d'Analyse Protéomique de Paris Sud-Ouest
PBS	phosphate buffered saline
PEG	polyethylene glycol
PFOR	pyruvate:ferredoxin oxidoreductase
SPSS	Statistical Package for the Social Sciences
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TCM	traditional Chinese medicine
TLC	thin layer chromatography
USDA	United States Department of Agriculture
VSP	variant-specific surface proteins
WHO	World Health Organization

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7. Curriculum vitae

Personal information:

Name: Mirjana Drinić
Date of birth: 09.05.1979
Place of birth: Rijeka, Croatia

Education:

1986-1992 Elementary school "Eugen Kumičić", Rijeka, Croatia
1992-1994 Elementary school "Branko Ćopić", Prijedor, BiH
1994-1998 High school "Sveti Sava", Prijedor, BiH
1998-2003 Medical University Banja Luka, BiH
2004-2005 German course on Vienna University
2005-2009 Faculty of Life Sciences on Vienna University, Vienna, Austria
Genetics and Microbiology
2009-2012 Diploma theses and parallel involvement in other projects in
Molecular Microbiology group (group-leader Michael Duchêne) at
the Institute of Specific Prophylaxis and Tropical Medicine, Center
for Pathophysiology, Infectiology and Immunology, Medical
University of Vienna

Teaching:

2012 Lab/Practical Tutor: Parasitology in Hygiene for medical students in
Molecular Parasitology group (led by Julia Walochnik)

Presentations at meetings:

1. Activity of plant-derived substances against *Entamoeba histolytica*
Mirjana Drinić, Florian Astelbauer, Adriane Raninger, David Leitsch, Walther
Wernsdorfer, Brigitte Brem, Andreas Obwaller, Julia Walochnik, Harald Greger,
Michael Duchêne (Poster)

24th Meeting of German Society for Parasitology and 29th Meeting of German Society for Protozoology, Düsseldorf, Germany, March 16–20, 2010

2. Activity of plant-derived substances against *Entamoeba histolytica* and *Giardia intestinalis*

Mirjana Drinić, Florian Astelbauer, Adriane Raninger, David Leitsch, Walther Wernsdorfer, Brigitte Brem, Andreas Obwaller, Julia Walochnik, Harald Greger, Michael Duchêne

(M. Drinić, oral presentation)

10th Congress of the Croatian Society of Biochemistry and Molecular Biology Opatija, Croatia, September 15–18, 2010

3. Anti-amoebic and anti-giardial activity of substances derived from tropical plants

Mirjana Drinić, Florian Astelbauer, Adriane Raninger, David Leitsch, Walther Wernsdorfer, Brigitte Brem, Andreas Obwaller, Julia Walochnik, Harald Greger, Michael Duchêne (M. Drinić, oral presentation)

44. Jahrestagung der Österreichischen Gesellschaft für Tropenmedizin und Parasitologie

Graz, Meerscheinschloss, November 18-20, 2010

4. *In vitro* activity of methylgerambullin from *Glycosmis mauritiana* against *Entamoeba histolytica* and *Giardia intestinalis*

Mirjana Drinić, Florian Astelbauer, Adriane Raninger, David Leitsch, Walther Wernsdorfer, Brigitte Brem, Andreas Obwaller, Julia Walochnik, Harald Greger, Michael Duchêne (M. Drinić, oral presentation)

VI European Congress of Protistology (ECOP 2011)

Berlin, July 25–29, 2011

5. Activity of methylgerambullin, isolated from the tropical plant *Glycosmis mauritiana*, against the parasites *Entamoeba histolytica*, *Giardia intestinalis*, *Trypanosoma* spp. and *Leishmania* spp.

Mirjana Drinić, Florian Astelbauer, Adriane Raninger, David Leitsch, Walther Wernsdorfer, Brigitte Brem, Andreas Obwaller, Julia Walochnik, Harald Greger and Michael Duchêne (Poster)

Retreat of the Center for Pathophysiology, Infectiology and Immunology, Vienna, September 27, 2011

6. Pentamycin shows high anti-protozoal activity *in vitro*

Mirjana Drinic, Florian Astelbauer, Hans-Peter Fuehrer, Peter Starzengruber, Michael Duchêne, Harald Noedl, Manfred Schulz, Cees Winnips, Julia Walochnik (Poster, 1st poster prize)

45. Jahrestagung der Österreichischen Gesellschaft für Tropenmedizin und Parasitologie

"From Bugs to Drugs", Wien, Gesellschaft der Ärzte, November 17–19, 2011

Publications:

1. Downregulation of flavin reductase and alcohol dehydrogenase-1 (ADH1) in metronidazole-resistant isolates of *Trichomonas vaginalis*

David Leitsch, Mirjana Drinić, Daniel Kolarich, Michael Duchêne
Molecular and Biochemical Parasitology **183**: 177-183 (2012).

2. Anti-amoebic and anti-giardial activity of methylgerambullin from *Glycosmis* spp.

Mirjana Drinić, Florian Astelbauer, Adriane Raninger, David Leitsch, Walther H. Wernsdorfer, Brigitte Brem, Andreas Obwaller, Julia Walochnik, Harald Greger, Michael Duchêne, in preparation