Trichomonas vaginalis

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In *Trichomonas*, the hydrogenosome, a double membrane-bounded organelle that produces ATP, also can be a good target. Other structures include mitosomes, ribosomes, and proteasomes. Metronidazole is the most frequent compound used to kill many anaerobic organisms, including *Giardia* and *Trichomonas*. It enters the cell by passive diffusion and needs to find a highly reductive environment to be reduced to the nitro radicals to be active. However, it provokes several side effects, and some strains present metronidazole resistance. Therefore, to improve the quality of the chemotherapy against parasitic protozoa is important to invest in the development of highly specific compounds that interfere with key steps of essential metabolic pathways or in the functional macromolecular complexes which are most often associated with cell structures and organelles.

anaerobic parasites chemotherapy Trichomonas vaginalis

1. Introduction

T. vaginalis and *G. intestinalis* are protist parasites causative of urogenital and intestinal infections, respectively. Trichomoniasis, caused by *T. vaginalis*, is the most common non-viral sexually transmitted infection (STI) in the world, whereas *G. intestinalis* (also named *Giardia lamblia*, *Giardia duodenalis)* is the etiologic agent of Giardiasis. This chronic condition causes one of the most common waterborne diseases, and mainly affects children. *G. intestinalis* is an extracellular flagellated protozoan parasite, class Fornicata, belonging to the order Diplomonadida, which is part of the Hexamitidae family [1].

2. Chemotherapy against Parasitic Protozoa

It is well-recognized that chemotherapy against diseases caused by parasitic protozoa is still based on the use of compounds developed many years ago. Although they have played a significant role during all these years of use, it is important to remember that they show variable toxicities. In addition, drug resistance to these compounds is increasing. Therefore, it is highly relevant to identify new active compounds against the pathogenic protozoa. Furthermore, repurposing drugs already used to treat unrelated diseases is an interesting alternative that needs to be stimulated.

There are several approaches to developing new chemotherapeutic agents against a parasitic protozoon. The first one, which corresponds to the most traditional way of identifying new compounds to be tested, is based on extracts and even molecules obtained from natural products of diverse origins. This approach allows for the identification of important molecules with anti-parasitic activities that are subsequently used as lead molecules to be improved by

chemical synthesis, allowing drug banks to establish thousands of molecules. In addition, the test of thousands of compounds in a short time was significantly facilitated by the use of high throughput techniques. A second approach corresponds to the repurposing of already-known compounds used to treat several diseases, and whose use in humans has been approved by national authorities, as is the case with the Food and Drug Administration of the United States of America. Finally, a third approach is the design of drugs that may interfere with previously established targets. In this case, it is now possible to make theoretical previsions of their actions and subsequently proceed with the chemical synthesis of those molecules considered of high potential use. Some structures and organelles, such as hydrogenosomes in trichomonads and the ventral disc in *Giardia*, are fundamental for the parasite's survival. Thus, drugs that can interfere with the functions played by these structures and organelles may constitute new alternatives for treating the diseases caused by this pathogenic protist. Furthermore, concerning compounds targeting specific metabolic pathways, it is necessary to consider that the microaerophilic/anaerobic parasitic protozoa present many metabolic enzymes involved in important processes, such as the triosephosphate isomerase in *Trichomonas vaginalis* encoded by two functional genes, TvTIM1 and TvTIM2. One of these is localized on the parasite surface, involves interaction with extracellular matrix and basement membrane proteins, such as laminin and fibronectin, and is involved in pathogenesis [2]. This fact opens the possibility of obtaining drugs that inhibit only this property. For example, recently, Ref. [3] identified one compound that interfered only with the non-glycolytic function of TvTIM and showed anti-*T. vaginalis* activity.

ATP generation in protozoa such as *T. vaginalis* and *Giardia intestinalis*, parasites that lack mitochondria, occurs exclusively through substrate-level phosphorylation. Sulfur-containing-amino-acid metabolism is a divergent metabolic pathway that occurs in both organisms and may constitute drug targets [4]. Fe-S-clusters play an important metabolic role in some protozoa, and there are three independent systems for their biosynthesis. In the case of *T. vaginalis* and *G. intestinalis*, only the so-called ISC system, localized in the hydrogenosomes and mitosomes, respectively, exist. This system may also constitute an important drug target, given Fe-S clusters' key role in these organisms.

Here just a few examples of new compounds will be mentioned. For *G. intestinalis*, interesting results have been obtained with (a) the antibiotic fumagillin isolated from *Aspergillus fumigatus* and showed to be active even in metronidazole-resistant isolates, (b) proton pump inhibitors such as omeprazole which also inhibit triosephosphate isomerase, an important enzyme involved in the glucose and glycogen metabolism, (c) auranofin, a gold(I) complex already approved by the FDA to be used to treat rheumatoid arthritis and gold(I) phosphine derivatives, that inhibit thioredoxin reductase [5]. Another potential drug target is related to mechanisms involved in controlling gene expression, such as transcription factors [6][7] and enzymes involved in modifying histones. Examples include recent studies showing that some sirtuin inhibitors interfere with *G. intestinalis* growth, induction of multinucleated cells, and even cell death [8]. Nicotinamide also arrests *Giardia* trophozoites in the G2 phase [9].

3. *Trichomonas vaginalis* Features

T. vaginalis is an extracellular, microaerophilic protozoan that colonizes humans' genital or urinary tracts. Trichomoniasis is a sexually transmitted infection affecting approximately 400 million people worldwide [10]. Women

are more symptomatic, with vaginal odor, discharge, and frequent miscarriages, which can lead to infertility [11]. Men are usually asymptomatic, although it can lead to urethritis and prostate cancer [12]. A greater predisposition to HIV (human immunodeficiency vírus) [13] and HPV (human papillomavirus) is also related to trichomoniasis [14].

T. vaginalis is 9 to 23 in length and 7 µm in width and can display different morphologies. This variation will depend on its virulence level, the strain, and if there is proximity to other cells, such as epithelial cells or bacteria. Another important change in morphology occurs when the parasites are under intense stress due to nutrient deprivation or drug treatment. When *T. vaginalis* are grown in an axenic medium (TYM) [15], the cells are free-swimming and pyriform. However, in vivo or when interacting with host cells, *T. vaginalis* changes its shape, displaying a variable amoeboid form with various projections to increase contact with the host cell.

Trichomonads do not present the cyst form, only the trophozoite, where the flagella are externalized. However, endoflagellar forms are observed in several situations, either in samples recently obtained from infected people or by stressful induction in laboratory experiments. In this case, starvation, removal of iron from the culture medium, and addition of anti-mitotic drugs induce the internalization of flagella, which are kept in cell vacuoles, and the parasite takes a rounded shape. The flagella continue beating inside intracellular vacuoles [16]. This form, named pseudocyst, or endoflagellar form, is observed in large numbers and is generally reversible when the stressful situation is eliminated.

Trichomonads present one anterior nucleus with a random distribution and six chromosomes which are conservated in size and number among isolates [17]. Its mitosis occurs without nuclear envelope breakdown (closed mitosis) and with an extranuclear spindle (paradesmosis) [18].

T. vaginalis has five flagella, four anterior, and one recurrent flagellum forming the undulating membrane. The basal bodies are in the most parasite anterior region, where the flagella emerge through the flagellar canal. The recurrent flagellum projects toward the posterior cell region, contacting the cell's plasma membrane and forming the undulating membrane. It has been observed by deep etching that there are filamentous bridges connecting the cell surface with the recurrent flagellum [19]. All flagella participate in the cell's movement.

The axonemes of these flagella are typically eukaryotic with a 9 + 2 array of microtubules. By freeze-fracture techniques, the anterior flagella display an impressive array of rosettes formed by 9–12 intramembranous particles [20]. In contrast, the base of the recurrent flagellum exhibits a flagellar necklace with a distinct array of particles [21].

The basal bodies in trichomonads are atypical structures since they are associated with several filaments and striated roots in hook-shaped lamellae. These fibers can be contractile and noncontractile, such as the costa [22].

Trichomonads contain organelles that are common to all eukaryotic cells, such as the Golgi complex, endoplasmic reticulum, one nucleus, lysosomes, and a complex cytoskeleton forming the mastigont system. Several unique structures form the mastigont system, such as the pelta and the axostyle, which form the pelta axostylar complex, the parabasal filaments, and the costa.

The rough endoplasmic reticulum in trichomonads is around the nucleus, in the outer nuclear membrane, and dispersed in the cell's cytosol. The ER participates in the autophagic process, enlarging when the parasite is submitted to drug treatment [23]. *T. vaginalis* present a large Golgi complex and has been named Parabasal Apparatus, which includes the parabasal filaments [24].

T. vaginalis shows intense phagocytic activity, incorporating bacteria and various particles, forming large vacuoles. The endocytosed material travels to lysosomes, where it is digested.

The pelta is made of stable microtubules and involves the flagellar canal from which the flagella emerge; it overlaps with the axostyle microtubules forming the pelta-axostylar system [25][26]. The axostyle is formed by a well-organized array of stable microtubules extending across the length of the cell and is a supportive entity. In addition, it participates in trichomonads mitosis, providing constriction of the nucleus during karyokinesis.

4. *Trichomonas* and Its Unusual Structures

Trichomonads' cytoskeleton presents other unusual proteinaceous structures such as some filaments and lamellae as the sigmoidal filaments, the supra- and infra-kinetosomal bodies, rootlets fibers, and striated fibers as the costa and parabasal filaments [24].

The costa is a proteinaceous, periodic structure placed along the cell, dissipating the stress caused by the beating of the recurrent flagellum [26][27][28]. Fine fibers connect the undulating membrane to the cytoplasmic side, where the costa is found (Benchimol et al., 1993). The costa size reaches a length of about 14.38 µm and 36.5 nm wide with alternating bands of 13.8 nm and 241.3 nm in width [29].

The costa is a non-motile structure and presents many proteins, some of which are uncharacterized. In addition, a costa accessory structure has been demonstrated [29]. Further analyses of the costa fraction identified 54 hypothetical proteins, with fourteen proteins as the fraction's major components. Thus, the costa structure presents a new class of proteins not described in other cells. More recently, one major protein (*T. foetus* ARM 19800.1 protein) was characterized and localized in the costa and designated as costain 1 [30].

Hydrogenosomes

T. vaginalis does not have mitochondria but contains the hydrogenosome, an unusual organelle surrounded by two closely apposed membranes; it has this name because it produces molecular hydrogen.

Hydrogenosomes are considered divergent forms of mitochondria adapted to anaerobic life. In trichomonads, the hydrogenosome has 0.5 µm in diameter, is usually spherical, and may contain a peripheral, flattened, membrane-bounded compartment that contains high calcium levels, magnesium, and phosphorus, possibly functioning in the regulation of intracellular calcium [31][32][33]. Thus, all evidence points to the organelle as the primary calcium storage in *Trichomonas*.

The hydrogenosome participates in the pyruvate metabolism and produces ATP and molecular hydrogen [34]. Since two membranes coat the hydrogenosome, it divides like mitochondria [35], imports proteins post-translationally [36], and produces ATP [37], it has been considered a modified mitochondrion. However, it does not have a genome, Krebs cycle, or the typical membranous respiratory chain. In addition, the hydrogenosome also lacks the F0–F1 ATPase, cytochromes, and oxidative phosphorylation [38]. Hydrogenosomes use the pyruvate or malate to acetate for ATP production and produce molecular hydrogen using substrate-level phosphorylation [39]. One important characteristic of hydrogenosomes is the presence of cardiolipin (de [40], a bacterial and mitochondria membrane phospholipid suggesting its endosymbiotic origin.

Previous work [41] demonstrated that hydrogenosomes and mitochondria present common core membrane components, which are important for protein import and metabolite exchange. In addition, many proteins have been localized in the matrix of hydrogenosomes, and enzymes responsible for iron–sulfur (Fe–S) cluster assembly have been localized in the *T. vaginalis* hydrogenosome [42][43].

Previous studies provided a detailed proteomic analysis of the *T. vaginalis* hydrogenosome and showed that it contains 569 proteins [44]. In addition, the authors found many proteins that function in energy and amino acid metabolism, flavin-mediated catalysis, Fe-S cluster formation, membrane translocation, oxygen stress response, proteolytic processing, chaperonin activities, and ATP hydrolysis, which are responsible for ~30% of the hydrogenosome proteome.

Recently, an overview of various aspects of this organelle, such as its biogenesis, hydrogenosomal protein import, and membrane translocases [45].

Hydrogenosomes participate in various protein synthesis, including components localized in the outer and inner membrane, and transported into the organelle using elaborated import machinery that presents some similarities to the system found in mitochondria. Several enzymes include processing peptidases, adenylate kinase, acetate: succinate CoA transferase, hydrogenase, pyruvate: ferredoxin oxidoreductase, superoxide dismutase, and several others that are involved in metabolic activity.

5. Drugs Affecting Trichomonas

5.1. Metronidazole

Metronidazole and other nitroimidazoles have been used in trichomoniasis treatment. Two important problems in trichomoniasis treatment using metronidazole are the resistance and side effects, such as metallic taste, vomiting, nausea, dizziness, and insomnia [46]. In some cases, it provokes leucopenia and neuropathies. Moreover, this drug is prohibited during pregnancy [47], and some strains exhibit 5’-nitroimidazoles resistance. In addition, several hydrogenosomal proteins are altered in drug resistance, resulting in severe organelle modifications [48]. The authors noted a marked reduction of pyruvate: ferredoxin oxidoreductase and ferredoxin levels in resistant strains. Furthermore, one group [49] described a different pathway involved in the metronidazole activation within the

hydrogenosome. They reported that trichomonads acquired a high level of metronidazole resistance when both the pathways of malate and pyruvate that activate metronidazole were eliminated.

Metronidazole enters the cell as an inactive prodrug by simple diffusion and goes to the hydrogenosome using the same way [50]. It needs to find a highly reductive environment to be reduced to the nitro radicals to be active. Oxidoreductases like pyruvate ferredoxin oxide reductases make such a reduction. Major oxygen-scavenging enzymes include Flavin reductase and NADH oxidase. For instance -N(2-hydroxyethyl) oxamic acid and acetamide may damage the DNA of replicating cells. The mode-of-action of the heterocyclic aromatic nitro-compounds is considered to be due to the radical damage caused by the reactive and toxic species that are obtained from the reduction of the nitro groups and that interact with several intracellular molecules, including DNA [51][52]. Resistance of the parasites to metronidazole reaches around 4.3% of the isolates in the USA [53]. Hydrogenosomes are the main target for activating 5-nitroimidazole drugs [54]. In the hydrogenosome, metronidazole is activated to a cytotoxic form. In addition, morphological studies provided evidence for hydrogenosome alterations in size, shape, and behavior when these drugs are used in vitro [48][55].

5.2. Effects on Trichomonas Structures by Other Drugs (Table 1)

The plasma membrane, the endoplasmic reticulum (ER), and the Golgi complex are key components of the cells. They may present unique components involved in the capacity of the protozoa to interact with host cells. For *Trichomonas* and *Giardia*, there is evidence of some proteins exposed on the protozoan surface or secreted via extracellular vesicles (exosomes, ectosomes) that are fundamental for the protozoan to exert its pathogenic side. Examples include cysteine proteases and variable surface proteins (VSP), some of which, as in the case of *G. intestinalis*, are involved in antigenic variation. The synthesis and the fate of these molecules to the cell surface involve the participation of intracellular vesicles and the cytoskeleton components used to transport them mediated by dyneins and kinesins. Interruption of these processes may interfere with parasite viability; therefore, they constitute molecular targets that can be interfered with by specific molecules. Indeed, there are several examples where parasites become inviable when the synthesis of a certain protein is blocked. Thus, this is an exciting area for further development of anti-parasitic drugs.

The lipidic component of the cell membranes is also a drug target explored for some parasitic protozoa. For example, it has been shown that the introduction of carbocyclic rings in the lipid portion of alkyl phosphocholines leads to drugs with potent activity against some protozoa. Miltefosine is an alkylphosphocholine synthetic lipid analog shown to present activity in cancer cells and parasites protozoa such as *Leishmania, T. cruzi*, and *T. vaginalis* [56]. It is nowadays used in clinics by oral via. Other ether phospholipid derivatives were synthesized and showed improved activity and lower toxicity against parasites tested for *T. vaginalis* [57][58][59]. Using miltefosine (MLT), several alterations, such as wrinkled and rounded cells, membrane blebbing, intense vacuolization, and nuclear condensation, occurred, all indicative of cell death by apoptosis [56]. In addition, cells treated with the IC50 of MLT significantly reduced the number of viable parasites. One group [60] used clotrimazole (CTZ) and zinc compounds, and CTZ complexed with zinc salts acetate [Zn(CTZ)2(Ac) and a chloride [Zn(CTZ)2Cl2] complexes against *T. vaginalis*. The incubation of the parasites with [Zn(CTZ)2(Ac)2] complex inhibited parasite growth and

provoked changes in the shape of treated parasites with cell membrane projections. In addition, hydrogenosomes, endoplasmic reticulum, and Golgi complex were altered. Therefore, the authors inferred that [Zn(CTZ)2(Ac)2] is a highly effective compound against *T. vaginalis* in vitro, indicating its potential alternative use as an agent against trichomoniasis. *T. vaginalis* treated with [Zn(CTZ)2(Ac)2] exhibited a reduction of pyruvate: ferredoxin oxidoreductase in hydrogenosomes.

It has been reported cell alterations provoked by Δ(24(25))-sterol methyltransferase inhibitors on *T. vaginalis* [46]. The authors described parasites forming cell clusters with wrinkled cells, membrane blebbing, and cell disruption. In addition, the Golgi was abnormal, the hydrogenosomes were damaged, and several autophagic vacuoles were observed [46]. When a comparative study was performed using metronidazole and BPQ-OH [61], both drugs provoked cell death, intense cell vacuolization, and membrane blebbing. However, BPQ-OH was less toxic for human cells in vitro.

One group reported that the compounds amioder and dronedarone affected *T. vaginalis* [62]. The parasites were killed by an apoptosis-like process and showed morphological changes with disturbance in the hydrogenosome structure.

5.3. Proteasomes

The proteasome is a macromolecular complex that controls exceeding protein formation and erroneous proteins, acting on its degradation. Thus, cell homeostasis can be maintained due to its proteolytic activities involving breaking the peptide bonds. It has been reported that the protist parasite *Tritrichomonas foetus*, a relative of *T. vaginalis*, presents a 20S proteasome [63]. The proteins are tagged via a single ubiquitin molecule with the enzyme ubiquitin ligase. Any interference with this system causes severe damage to the cell. Therefore, it may constitute an important drug target. For instance, drugs such as Ixazomib and carmaphycin-17, which interfere with proteasomes, are effective against *T. vaginalis* [64]. In addition, the fungal metabolite gliotoxin inhibits proteasome proteolytic activity. As a result, it induces an irreversible pseudocysts transformation (endoflagellar form (EFF) and cell death in *Tritrichomonas foetus*, a protist cattle parasite [65]). Lactacystin, a well-known specific proteasome inhibitor, interfered in transforming *T. foetus* to endoflagellar form (EFF) in a dose-dependent manner. Lactacystin treatment also resulted in an accumulation of ubiquitinated proteins. Furthermore, it caused an increase in the number of endoplasmic reticulum membranes in the parasite, thus suggesting that the ubiquitin-proteasome pathway is required for the cell cycle and EFF transformation in *T. foetus* [63].

Recently Ibánez-Escribano et al. [66] designed and synthesized several thiosemicarbazones (Schiff-based analogs) with modification of the stereoelectronic effects of the substituents on N-1 and N-4. In addition, the authors made an isosteric replacement of sulfur with selenium in some of them. Thiosemicarbazones (numbers 49, 51, and 63) showed high activity against *T. vaginalis* with IC50 of 16.39, 14.84, and 14.89 µM, respectively. No significant cytotoxic activity against Vero cells was observed (CC50 of 256 µM). On the other hand, selenoisosters 74 and 75 showed IC50 in the range of 11 µM. These compounds did not interfere with the hydrogenosome membrane

potential as analyzed by labeling with the fluorescent dye JC-1. Scanning electron microscopy of drug-treated *T. vaginalis* showed the presence of rounded cells, some resembling pseudocysts, surface invaginations, and pores.

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