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In vitro* effectivity of three approved drugs and their synergistic interaction against *Leishmania infantum

Leishmanicidal and synergetic effect of three drugs

Efectividad *in vitro* de tres fármacos aprobados y su interacción sinérgica contra *Leishmania infantum*

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Iman Fathy Abou-El-Naga: conceived and designed the experiments.

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All authors analyzed the data and wrote the paper

Introduction: Leishmaniasis remains one of the neglected tropical diseases. Repurposing existing drugs has proved to be successful for treating neglected tropical diseases and combination therapy is a strategic alternative for treatment of infectious diseases. Auranofin, aluvia and sorafenib are FDA approved drugs used in treatment of diverse diseases by acting on different essential biological enzymes.

Objective: In the current work mono- and combined therapeutic effects of the three drugs have been evaluated against *Leishmania infantum*.

Material and methods: The leishmanicidal effects of the three drugs on promastigotes were compared *in vitro* as regards the parasite count, the concentration of a drug that gives half-maximal response and the ultrastructural changes of the parasite. Determination of fraction inhibitory concentration index of combined drugs in a two way manner and the activity of the three drugs together was calculated to determine the synergetic effect.

Results: The three drugs as monotherapy were effective but auranofin had the best anti-leishmanial effect , its EC50 was 1.5 µM, whereas sorafinib reduced parasite growth at EC50 =2.5 µM. Scanning electron microscopy of promastigotes from all treated media showed distortion in the shape with loss of flagella and bleb formation. Acidocalcinosis was evident by transmission electron microscopy with all treatments, suggesting apoptosis. Treatment with aluvia showed signs of autophagy. The two-way combination of the drugs led to additive interactions while combination of the three drugs showed synergistic action.

Conclusion: Each drug when used as monotherapy against *Leishmania* is effective, whereas combination therapy is more effective and superior than individual drugs due to additive or synergistic effects.

Key words: *Leishmania infantum*; drug synergism; apoptosis; autophagy.

Introducción. La leishmaniasis sigue siendo una de las enfermedades tropicales desatendidas. La reutilización de medicamentos existentes ha demostrado ser exitosa para tratar enfermedades tropicales desatendidas y la terapia combinada es una alternativa estratégica para el tratamiento de enfermedades infecciosas. Auranofin, aluvia y sorafenib son medicamentos aprobados por la FDA utilizados en el tratamiento de diversas enfermedades al actuar sobre diferentes enzimas biológicas esenciales.

Objetivo. En el trabajo actual, se han evaluado los efectos terapéuticos mono y combinados de los tres fármacos contra *Leishmania infantum*.

Material y métodos. Los efectos leishmanicidas de los tres fármacos sobre los promastigotos se compararon in vitro en cuanto al recuento de parásitos, la concentración de un fármaco que proporciona una respuesta semimáxima y los cambios ultraestructurales del parásito. La determinación del índice de concentración inhibitoria de fracciones de fármacos combinados de dos maneras y la actividad de los tres fármacos juntos se calculó para determinar el efecto sinérgico.

Resultados. Los tres medicamentos como monoterapia fueron efectivos, pero la auranofina tuvo el mejor efecto antileishmanial, su CE50 fue de 1,5 µM, mientras que el sorafinib redujo el crecimiento del parásito a la CE50 = 2,5 µM. La microscopía electrónica de barrido de promastigotes de todos los medios tratados mostró distorsión en la forma con pérdida de flagelos y formación de ampollas. La acidocalcinosis fue evidente por microscopía electrónica de transmisión con todos los tratamientos, lo que sugiere apoptosis. El tratamiento con aluvia mostró signos de autofagia. La combinación bidireccional de las drogas condujo a interacciones aditivas, mientras que la combinación de las tres drogas mostró una acción sinérgica.

Conclusión. Cada medicamento cuando se usa como monoterapia contra Leishmania es efectivo, mientras que la terapia de combinación es más efectiva y superior que los medicamentos individuales debido a los efectos aditivos o sinérgicos.

Palabras clave: *Leishmania infantum*; sinergismo farmacológico; apoptosis; autofagia.

Leishmaniasis is one of the neglected tropical diseases. It affects as many as 12 million people living in endemic areas in 98 countries. About 350 million people are considered to be at risk, most of them in developing countries (1-3). *Leishmania* species cause a wide clinical spectra that includes cutaneous, mucocutaneous and visceral leishmaniasis. The most common is the cutaneous form which causes disfiguring and stigmatizing skin lesions whereas mucocutaneous leishmaniasis is significantly less common. Visceral leishmaniasis is fatal if not treated (4).

Currently, limited choices of drugs are used for treatment of leishmaniasis. There are no approved vaccines nor prophylactic drugs. Pentavalent antimonial compounds, sodium stibogluconate, pentamidine, various amphotericin B (AmB) formulations, miltefosine and paromomycin are the approved therapeutics now. Imiquimod and sitamaquine are under clinical assessment (5). However, the available drugs have limitations which include toxicity, long courses, high costs, undesirable route of administration, teratogenicity and drug resistance. Therefore, so far no safe and effective anti-leishmania drug is present in the market (6). Recent research funded by various organizations is only directed towards clinical trials and diagnostic studies of leishmaniasis in endemic countries. Consequently, there is still an urgent need to develop new therapeutics for leishmaniasis.

New drug trials aim at interfering with vital biochemical and metabolic pathways of the parasite and in this rationale enzymes are the most important focus. The target enzymes in the parasite should have major structural and functional differences from the mammalian host ones to achieve selective inhibition of the target sites (7). Repurposing existing drugs has been proved to be successful for treating neglected tropical diseases. The new uses of Food and Drug Administration (FDA) approved drugs are a short cut between the preclinical testing and clinical trials. This strategy

reduces the funds needed for the preclinical researches as well as the study of the safety profiles and the pharmacological characteristics (8,9).

In the current study three FDA approved drugs, namely auranofin (Ridura), lopinavir/ritonavir (Aluvia) and sorafenib (Nexavar), acting as inhibitors of different protease enzymes, have been chosen to study their effect as monotherapy and combination therapy on *Leishmania infantum* (*L. infantum*). *Leishmania* proteases are very important virulence factors as they are involved in host tissue invasion, survival inside macrophages and host immune response modulation. Hence, these enzymes are considered good targets in the parasite biology (10). The efficacy of the drugs was compared to that of AmB. AmB is a polyene antibiotic which acts on the membrane sterols of *Leishmania* promastigote resulting in a loss of the permeability barrier to small metabolites (11). Although, AmB is widely used in treatment of leishmaniasis, its toxicity is considerable (12).

Auranofin (Ridura) is a gold containing drug used in treatment of rheumatoid arthritis. It emerged as a strong inhibitor of mammalian thioredoxin reductases (13). Recently the drug showed a remarkable antiparasitic activity as a result of inhibition of parasitic enzymes involved in the control of the reduction/oxidation (redox) process. These enzymes are essential for maintaining intracellular levels of reactive oxygen species. *Leishmania* and other Trypanosomatids contain trypanothione reductase, a key enzyme of redox metabolism (14). Trypanothione reductase and mammalian glutathione reductase show notable differences in the structure validating specific inhibitors designed against trypanothione reductase to be an ideal drug against *Leishmania* parasite without changing the mammalian glutathione reductase activity (7).

Aluvia is a highly active anti-retroviral therapy (HAART) used against Human Immune Deficiency Virus (HIV) (15). The drug is an aspartyl peptidases inhibitor and

composed of two anti-retroviral drugs, lopinavir and ritonavir in a ratio of 4:1(16). Peptidases enzymes are essential in a wide range of biological functions (17). They are recognized as therapeutic targets for important diseases and many micro-organisms including *Leishmania* (18-20). These enzymes are classified into five distinct clans (AA, AC, AD, AE and AF) and 16 families according to MEROPS database. Clan AA/ the classical aspartic peptidases is further subdivided into eight families of which family A2 includes the HIV peptidase. In Trypanosomidae, the aspartic peptidases belong to two clans; clan AA and clan AD (21).

In recent decades, co-infection of *Leishmania* and HIV is increasingly reported in endemic areas of leishmaniasis (4). The introduction of HAART has shown a recognizable decrease in *Leishmania*/HIV co-infection as regards incidence, pathology and clinical presentation of the disease (22). Experimental studies on different *Leishmania* species using HIV peptidases inhibitors (HIV-PIs) have enforced the epidemiological results documenting decrease incidence of *Leishmania*/HIV co-infection after treatment with these drugs (21-25).

Sorafenib is a multi-kinase inhibitor used for treatment of advanced hepatocellular and renal cell carcinoma. Recently, it was identified as active agent against *L. donovani* and different species of *Leishmania* causing cutaneous leishmaniasis (26). Large number of kinases especially cyclin-dependent and mitogen-activated kinases are responsible for cell-cycle control in *Leishmania*. Although kinases are recognized as targets for many diseases, they are poorly studied as targets for *Leishmania* (27). The anti-leishmanial potency of sorafenib is due to a non-specific inhibition of many diverse protein kinases rather than that of the mammalian kinases (28).

Combination therapy is a strategic alternative for treatment of infectious diseases. It is currently considered as one of the most rational alternatives to increase drug

activity, reduce treatment duration and dosage, reduce toxicity and delay or prevent drug resistance. It has been efficiently used for treatment of malaria, tuberculosis and AIDS (29). However, it is uncommon to treat leishmaniasis by combined drugs (30-32). Recently, the need for combination therapy for leishmaniasis has emerged (33).

In the current study the anti-leishmanial effect of auranofin, aluvia and sorafenib drugs against *L. infantum* promastigotes was evaluated compared to the gold standard drug for leishmaniasis AmB. The synergistic, additive or antagonistic effects of combined therapy were also investigated. The morphological changes of the parasite treated with the aforementioned drugs at the ultrastructural level were also analyzed.

Material and methods

Leishmania *strain and its maintenance*

L. infantum MON1 is the visceral leishmaniasis strain used in this study. It was kindly provided by Professor Jean Dupouy Camet, president of the European Federation of Parasitologists. It was further maintained in the Laboratory of Medical Parasitology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt. *L. infantum* promastigotes were maintained under standard culture conditions in Novey-MacNeal-Nicoll (NNN) media. Parasites were sub-cultured every 7 days (34).

Tested drugs

Three commercially available FDA-approved drugs were used in this study; auranofin, purchased from Abbott; alluvia, purchased from Astellas pharma SPA and sorafenib, purchased from Bayer. AmB was used as a gold standard drug.

Determination of the* in vitro *anti-leishmanial activity

Ten µM stock solutions were prepared from each drug in 1% DMSO. Negative control was prepared from 1% DMSO whereas positive control was 10 µM of AmB. 1×10^6 *Leishmania* promastigotes suspended in 100 µL of culture media were incubated for three hours before adding the test drugs. After 48 hours of incubation with each drug preparation at 25° C, an aliquot of each tube was added to an equal amount of a solution containing 0.2% formalin, to stop parasite movement in order to facilitate parasite counting using Neubauer chamber (35). Auranofin, aluvia and sorafenib were tested in ascending concentrations range from 0.1 to 20 µM. AmB, was tested in a dilution range from 0.01 to 17 µM.

Determination of fraction inhibitory concentration (FIC) index, isobologram construction, and classification of the nature of interaction

Fraction inhibitory concentration (F-IC) is the inhibitory concentration that caused a 50% decrease in growth (EC50) of promastigotes. It was calculated for each tested drug and for each concentration. All tests were performed in triplicate (9).

To test for synergy, drugs were evaluated in quadruplicate individually to determine EC25, each individual compound in a pair was required to inhibit 25±10% of growth in untreated media.

Drug combinations observed to have possible synergism were subjected to formal isobologram analysis using the fixed ratio method (36).

Serial two-fold dilutions were performed in triplicate. For each ratio, an EC50 was calculated for each of the drugs. The fractional inhibitory concentrations (FIC) were calculated as the following:

EC50 when in combination / EC50 of drug alone

The sum of the FIC was calculated as follows: Σ FICs = FIC drug A + FIC drug B. The mean sum of the FIC ($\overline{X}$ Σ FIC) was calculated as the average of SFIC from the three different fixed ratios. The interactions were considered synergistic for $\overline{X}$ Σ FIC ≤ 0.5, additive for $\overline{X}$ Σ FIC between 0.5 and 4, and antagonistic for $\overline{X}$ Σ FIC >4 (9).

Ultrastructural study

Scanning electron microscope (SEM) (JEOL-JSM-25 SII) and transmission electron microscope (TEM) (JEOL 100 CX), were used to examine *L. infantum* promastigotes after their treatment with auranofin, aluvia and sorafenib for 48 h at 26° C in comparison to positive and negative controls. The specimens were processed for SEM and TEM (37,38).

Statistical analysis

All parasite burden data were expressed as the mean ± standard deviation. Abnormally distributed data was expressed using Median (Min. – Max.) and was compared using Kruskal Wallis test. Significance between groups was done using Mann Whitney test. A p-value of $p < 0.05$ was considered statistically significant (39).

Results

In vitro *anti-leishmanial activity*

It was clear that all used individual drugs, auranofin, aluvia, sorafenib and AmB limit *in vitro* parasite growth after 48 hours of parasite replication. Whereas DMSO had no significant effect. Lopinavir/ Ritonavir (aluvia) reduced parasite growth at 1.7 µM. As regards to auranofin, the lower drug concentration that was able to limit parasite growth by 50% (EC50) was 1.5 µM. Sorafenib and AmB showed the highest EC50 concentrations (2.5 µM and 2 µM respectively) (table 1 and 2).

Synergy testing and isobologram analysis

EC25 values were measured for each of the used drugs in every possible combination (table 3). Four combinations were tested in formal isobologram analyses in order to quantify the interactions by this standard method.

Fraction inhibitory concentration (FIC)

Sum of FICs of combination A (Auranofin+ Lopinavir/Ritonavir) = 1.53+0.45 = 1.98 = additive**.**

Sum of FICs of combination B (Sorafenib + Lopinavir/ Ritonavir) = 0.59+ 0.8 = 1.39 = additive**.**

Sum of FICs of combination C (Auranofin + sorafenib) = 1.2+0.48 =1.68 = additive**.**

Sum of FICs of combination D (Auranofin+Lopinavir/Ritonavir+sorafenib) = 0.17+0.12+0.2=0.49 = synergism**.**

When these results were statistically studied, aluvia showed no significant difference from other drugs either when used alone or in combination form. The growth inhibition in auranofin treated media was significantly greater than that treated with sorafenib and AmB, but no statistically significant difference was found between it and any combination. In spite of reduction in parasite growth, sorafenib was the least in potency in comparison to auranofin and combination B, C and D. Combination B, C and D showed a significant reduction in the growth of promastigotes compared to AmB and combination A (table 4).

Ultrastructural study

SEM of the parasites from 48 hours' culture that were inoculated in fresh media in absence of any drug showed normal morphology (figure 1a and b). Promastigotes from all treated media showed distortion in the parasite shape with loss of flagella and bleb formation. Auranofin treated promastigotes exhibited sever distortion in the

parasite shape and some of them showed rounded form (figure 1c). Irregularities in the cell membrane of the parasite were highly evident in aluvia treated promastigotes (figure 1d), while dimple like structures on cell surface of promastigotes were observed well in sorafenib treated parasites (figure 1d).

Normal ultrastructure contents were detected using TEM for examination of the parasites from 48 hours' culture that were inoculated in fresh media in absence of any drug (figure 2a and b). Acidocalcinosis was evident in the parasites from all treated media suggesting apoptosis. Auranofin treated promastigotes showed clearly evident acidocalcinosis (figure 2c). Aluvia treated promastigotes showed well evident acidocalcinosis and degenerated nuclear membrane and chromatin granules suggesting apoptosis. Vacuoles with different densities and autophagy vesicles with double membrane were also present (figure 2d). Sorafenib induced apoptosis with shrinkage of the cytoplasm (figure 2e).

Discussion

Despite several trials, there are no effective vaccines against *Leishmania* up to now and chemotherapy remains the mainstay for the control of leishmaniasis. The currently used drugs are unsafe, expensive, and lead to resistance (40). Moreover, treatment of leishmaniasis with protease inhibitors has been tried before in several research studies. Accordingly, in this study, we evaluated the effect of three different, commercially available, enzyme inhibitors against *L. infantum* spp. We compared the mono and combination therapy of auranofin, lopinavir / ritonavir (aluvia) and sorafenib. The drugs were chosen for their well-known history of safe clinical profile and for their inhibition of essential enzymes (41).

The current results showed that auranofin had the most effective anti-leishmanial activity among the tested drugs. It is the only drug that when used individually led to

a significant inhibition in the parasite count compared to AmB. At 10 µM auranofin also significantly reduced the parasite count more than sorafenib but the results were insignificant compared to that of aluvia. Furthermore, auranofin gave the lowest growth percentage of the parasite and had the lowest LC50 in comparison to aluvia, sorafenib and AmB. The effect of auranofin against *L. infantum* is due to inhibition of trypothione reductase enzyme; one of the top targets in drug discovery for leishmaniasis as it protects the parasite from oxidative damage and toxic heavy metals and allows the delivery of the reducing equivalents for DNA synthesis (14,42). Aluvia was found to be higher in efficacy than of AmB. The proteolytic activity of the HIV PIs had been demonstrated by other studies of different *Leishmania* species (21,24,25,43). Another mode of action was explained by Alves et al (44), through modulation of innate defense mechanisms via different cellular pathways. They also showed that although HIV protease inhibitors are highly efficient to control HIV, these drugs might also influence the course of leishmaniasis in HIV-*Leishmania*-co-infected individuals.

In the present study, sorafenib showed the least leishmanicidal activity among the studied drugs. It didn't significantly reduce the promastigotes than AmB and had a higher LC50. The drug is a multi-kinase inhibitor and was found to be active against *L. donovani* in culture identifying cycline dependent kinase and mitogen activated kinase as targets for anti-leishmanial treatment (26,27). Recently, it was found that sorafenib utilizes a non-apoptotic form of cell death (ferroptosis) to perform its effect on tumor cells. Ferroptosis is a regulated form of cell death results from iron-dependent lipid peroxide accumulation as shown by Yu *et al* (45).

Combination therapy between commercially available drugs aims at reducing cost, toxicity and duration of treatment and represents a promising alternative rational

(46). Therefore, we tried the combination of these drugs in a two-way and in a three-way combinations. The results showed that the interactions between the drugs in the two-way combination were found to be additive. More importantly, the combination of the three drugs showed synergetic effect. Although the present results showed that neither aluvia nor sorafenib gave significant inhibition in the parasite when used individually, significant reduction was achieved by the combination of both drugs (combination B). Butcher attributed this unexpected result to the combination between two drugs with two different defined biomolecular targets (47). Furthermore, the combination between auranofin and aluvia (combination A) led to significant reduction in the parasite count than AmB and this could be due to the strong anti-leishmanial effect of auranofin added to that, the different mechanism of action of aluvia in modulating the immune system. Lewis et al supports our findings in animal model where a combination of auranofin and antiretroviral drug was able to significantly reduce the post-therapy viremia (48).

The use of drugs with synergistic or additive activity in combination therapy delays or prevents the development of resistance and may shorten the treatment regimen which in turn decreases the undesirable effects made by each drug (49,50). Moreover, this alternative strategy leads to reduction of cost and time (41). The search for synergism by combination of approved drugs can rapidly move the results into preclinical and clinical phases (51).

In an attempt to explore the effect of each drug on the promastigotes, ultrastructural studies of treated parasites were done. SEM of the promastigotes treated by auranofin, aluvia and sorafenib for 48 hours showed sever distortion in the shape, loss of flagella with irregularities on the cell surface. Some promastigotes treated with auranofin exhibited a rounded appearance. Sharlow et al found the same

morphology of the promastigotes of *L. amazonensis* treated by auranofin (52). Rigobello et al (13) and Ilari et al (14) attributed this rounded swelling to the inhibition of trypanothione reductase and to the membrane permeability transition. This morphological distortion had not been observed with any known leishmanicidal drugs (52).

In the current work, the TEM showed evidences suggested that auranofin, aluvia and sorafenib exerted their anti-leishmanial effect on *L. infantum* promastigotes by inducing apoptosis. There were some changes in the essential organelles including the nucleus, the mitochondria and the cell membrane in addition to changes in the cytoplasmic contents. There were also irregularities of the cell membrane. The most striking ultrastructure change was the presence of large number of acidocalcisomes in the cytoplasm which is an important evidence of apoptosis (53). Only the promastigotes that were treated with aluvia showed autophagy in addition to apoptosis. The increased number of the vesicles with different densities in the cytoplasm, rupture of the nuclear envelope and the presence of dense chromatic granules represent signs of autophagy (24,54). The two major forms of programmed cell death, the apoptosis and autophagy were also verified in the ultrastructure study of *L. amazonensis* treated by HIV PIs (24).

Therefore, it can be concluded that administration of the combination of drugs is more effective and superior than individual drugs. The combined administration of the drugs in two way combinations led to additive interactions. Furthermore, the combination of the three drugs had shown synergistic action. The synergism shown with the combined drugs brings us to the concept of structure-function approach in fighting leishmaniasis. The electron microscopic study revealed that the three drugs exerted their anti-leishmanial action by inducing apoptosis, in addition to autophagy

in case of aluvia. It is possible that the effectivity of this drug combination is attributed to their similar mechanisms of action. However, further experimental design to establish the curative combination ratio and toxicity parameters of these compounds is needed. Also, further studies to test drug effectivity on amastigotes are to be done in the future.

Conflicts of interest

The author declares absence of any conflict of interest.

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Table 1. Effect of different drugs on in vitro proliferation of *L. infantum* promastigotes

Drug	**Growth percentage**
Auranofin (Ridaura 3mg)	4.27%
Lopinavir/ Ritonavir (Aluvia 200mg)	6.8%
Sorafenib (Nexavar 200mg)	9.84%
Amphotericin B	11%
Auranofin+ lopinavir/ ritonavir (Combination A)	3.75%
Lopinavir/ ritonavir+ sorafenib(Combination B)	5.1%
Auranofin+ sorafenib (Combination C)	8.25%
Auranofin+ sorafenib+ lopinavir/ ritonavir (Combination D)	4.75%

Leishmanicidal effect of the drugs is tested after 48h of incubation with the parasite

Table 2. EC50 of different drugs (EC50)

Drug	**EC50 (µM)**
Auranofin (Ridaura 3mg)	1.5 µM
Lopinavir/ Ritonavir (Aluvia 200mg)	1.7 µM
Sorafenib (Nexavar 200mg)	2.5 µM
Amphotericin B	2 µM

EC50 values are means of triplicate assays

Table 3. EC25 of different drugs in every combination

Drug	**Lopinavir/Ritonavir**	**Auranofin**	**Sorafenib**
Auranofin+ Lopinavir/ Ritonavir (combination A)	0.77 µM	2.3 µM	
Lopinavir/ Ritonavir+ sorafenib(combination B)	1 µM		2 µM
Auranofin+ sorafenib(combination C)		1.8 µM	1.2 µM
Auranofin+ sorafenib+Lopinavir/ Ritonavir(combination D)	0.3 µM	0.3 µM	0.3 µM

EC25 values are means of triplicate assays

Table 4. *In vitro* activity of different drugs and combinations against promastigotes

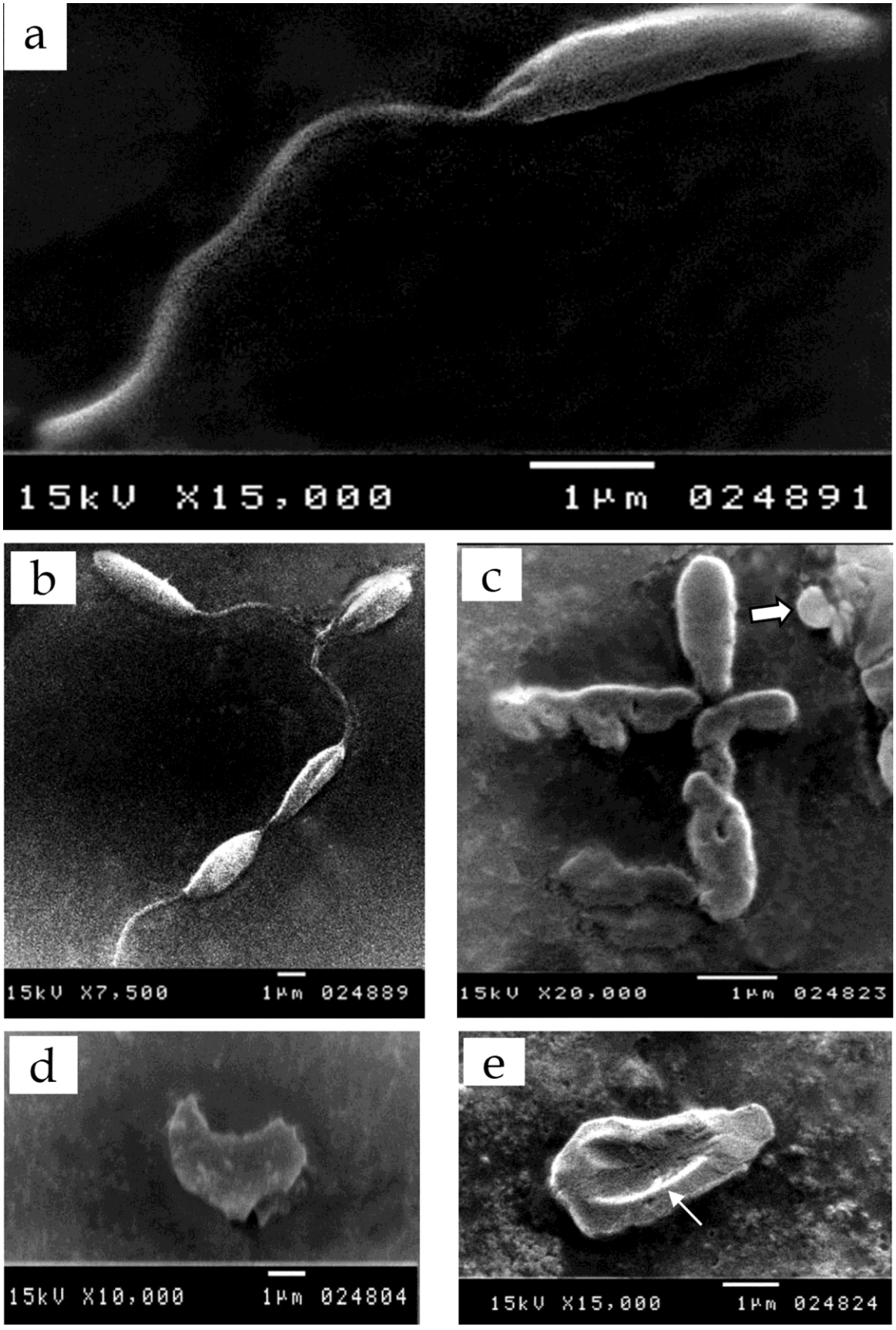
Parasite count in culture media treated with different drugs	**Median (Min. – Max.)**
Auranofin (Ridaura 300 mg)	38 (27 – 77.5)[b]
Lopinavir/ Ritonavir (Aluvia 200mg)	70 (18 – 100)[ab]
Sorafenib (Nexavar 200mg)	101 (40 – 150)[a]
Amphotericin B	107.5 (88 – 137)[a]
Auranofin+ Lopinavir/ Ritonavir (combination A)	37.5 (15 – 60)[b]
Lopinavir/ Ritonavir+ sorafenib(combination B)	51 (20 – 82)[b]
Auranofin+ sorafenib (combination C)	82.5 (45 – 120)[ab]
Auranofin+ sorafenib+Lopinavir/ Ritonavir (combination D)	40 (20 – 90)[b]

Abnormally distributed data was expressed using Median (Min. – Max.) and was compared using Kruskal Wallis test, Sig. bet. grps was done using Mann Whitney test

*: Statistically significant at $p \leq 0.05$

Different superscripts are statically significant

Figure 1. Ultrastructural changes observed in promastigotes from different studied media. **a** and **b:** Normal shape of the parasite from 48 hours' culture that were inoculated in fresh media in absence of any drug. **c:** Parasite after being treated with auranofin showed shape distortion, loss of flagella and some of them showing a round form (arrow). **d:** Severe distortion in the shape and loss of flagella with detached membrane in aluvia treated promastigote. **e:** Promastigote treated with sorafenib showed a large dimple (arrow) on body surface in addition to loss of flagella.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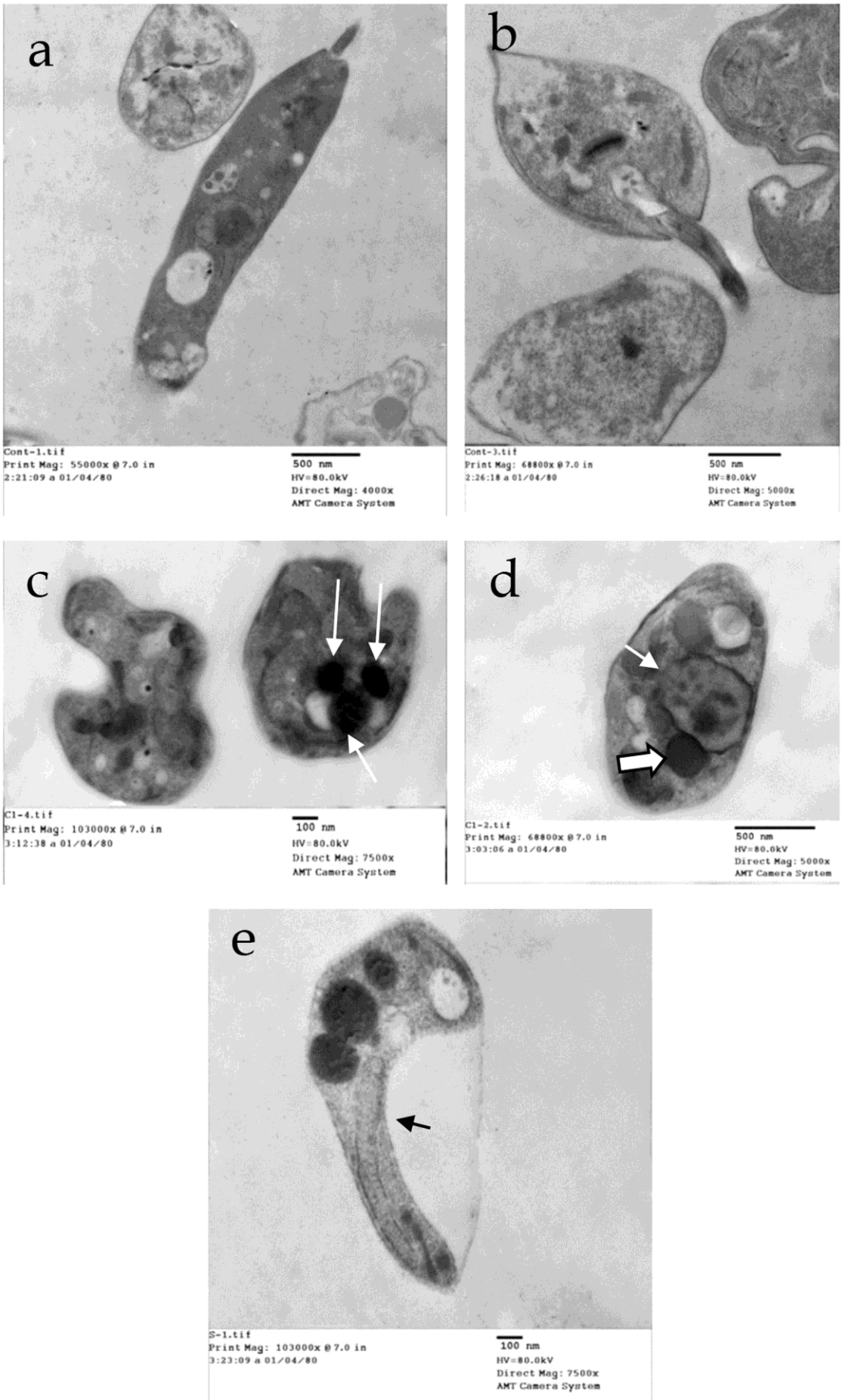
b
15kV X7,500 1µm 024889
c
15kV X20,000 1µm 024823
d
15kV X10,000 1µm 024804
e
15kV X15,000 1µm 024824

Figure 2. Ultrastructural changes in promastigotes from different media. **a** and **b:** normal parasite from 48 hours culture that were inoculated in fresh media in absence of any drug. **c:** Auranofin treated promastigotes showed well evident acidocalcinosis (arrows). **d:** Aluvia treated promastigotes showed well evident acidocalcinosis (thick arrow) and degenerated nuclear membrane (thin arrow) with condensed chromatin granules close to the nuclear membrane suggesting apoptosis. Vacuoles with different densities and autophagy vesicles with double membrane were also present. **e:** Sorafenib treated promastigotes showed acidocalcinosis and shrinkage of the cytoplasm (arrow).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d
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