Mechanistic Studies of Pentamidine Analogs on *Leishmania donovani* Promastigotes

Undergraduate Honors Thesis

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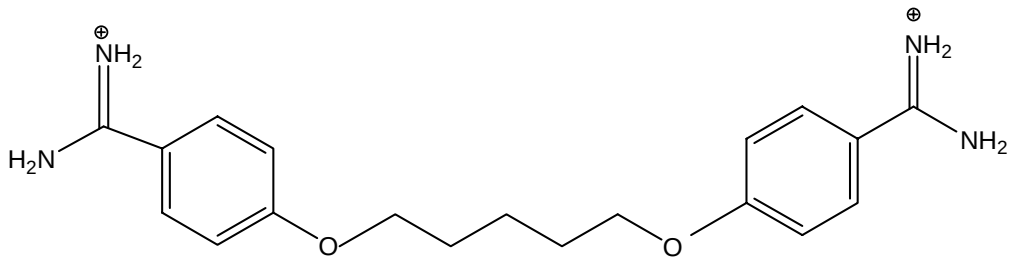
1. Introduction

Leishmaniasis is a parasitic disease caused by the genus *Leishmania*; these protozoa are transmitted by sandflies of genus *Phlebotomus* or *Lutzomyia*, which are commonly found in tropical and subtropical regions of the planet (1,2). Different *Leishmania* species are responsible for the three distinct clinical manifestations of leishmaniasis: cutaneous leishmaniasis (where self-healing skin lesions and often disfugurement of the skin occurs), mucocutaneous leishmaniasis (where the parasites affect the mucous membranes of the mouth and nose) and the most serious of the three diseases, visceral leishmaniasis (where the parasites infect the liver and spleen). Of all three variations, visceral leishmaniasis has the most debilitating effect because it affects the immune system and is often fatal if left untreated. Leishmaniasis currently affects 12 million people in 88 countries in which 80% are developing countries, making treatment a severe health problem (3). Because of the effect leishmaniasis has on humans and society, it is necessary to develop drugs that adequately treat and prevent the disease.

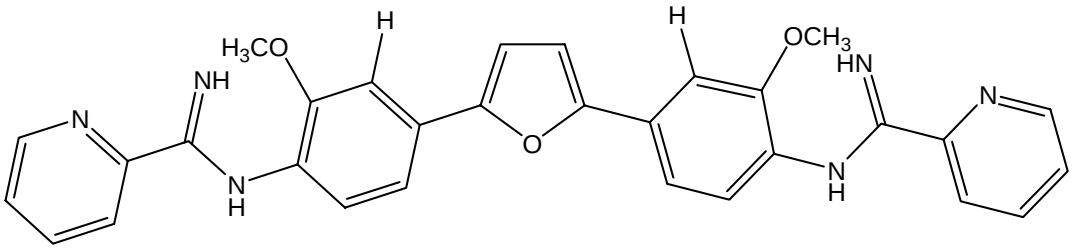
The current antileishmanial drugs have several limitations such as their route of administration (injection), toxicity and cost (4). There has also been a growing resistance to the first-line antimony-containing drugs currently available against leishmaniasis, making it necessary to develop new drugs that are orally available, inexpensive, and safe with short-term treatment. Pentamidine (Figure 1) is one of the few antileishmanial drugs currently available. It belongs to the diamidine class of drugs, which has been suggested to exert antiparasitic activity by binding to DNA, interfering with polyamine metabolism

and disrupting of mitochondrial membrane potential (5). Previous work concerning pentamidine suggests that the drug is taken up by saturable transport process in Leishmania, but the function of the transporter is still unknown (6). It also suggests that pentamidine causes damage to the mitochondrion of *Leishmania* as observed using electron microscopy (8,9). The clinical use of pentamidine is limited by its toxicity, administration by injection and development of resistance (7). Our laboratory is involved with the evaluation of pentamidine analogs as new antileishmanial drug candidates. Collaborators at Georgia State University synthesized two classes of compounds based on pentamidine: the diamidines DB111, DB1213, DB1282 and DB1288 and the reversed diamidines DB702, DB709 and DB746 (see Figure 1 for the structures of pentamidine, DB1288, and DB746). These compounds show excellent in vitro antileishmanial activity, but their mechanism of antiparasitic activity is unknown. If the mechanism of action of these agents could be determined, we may be able to design more active compounds that work in the same way and avoid toxic effects in the host.

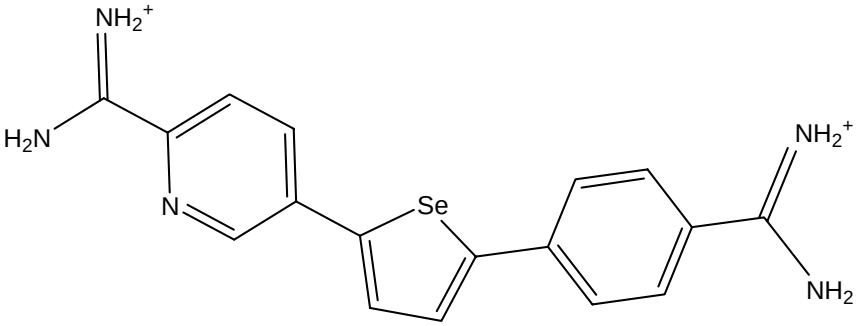
The purpose of this work is to test the hypothesis that these two classes of compounds are toxic to *Leishmania* by interfering with the parasite mitochondrion. To test this hypothesis, I planned to determine the IC_{50} values of the seven compounds against *Leishmania donovani* promastigotes, conduct mechanism of action studies employing flow cytometry and electron microscopy, and generate parasites resistant to one or more of these compounds in an effort to test this hypothesis.



Pentamidine



DB746



DB1288

Figure 1. Structures of the named compounds

2. Methods

2.1 Determining IC_{50} values against *L. donovani* axenic promastigotes.

L. donovani promastigotes were grown in vitro at 25 °C in modified RPMI 1640 medium with 25 mM HEPES and supplemented with 10 % heated inactivated fetal bovine serum (FBS), 0.2 mM glutamine, 0.1 mM adenosine, 1 µg/mL of folate, 50 units/mL of penicillin, 50 µg/mL of streptomycin and 1 x RPMI 1640 vitamins. *L. donovani* promastigotes at 10^6 parasites/mL were plated in a 96-well plate with 2-fold serial dilutions of the seven compounds mentioned above and pentamidine. Plates were incubated for 3 days at 25 °C, and then the CellTiter reagent, a tetrazolium indicator dye, was added for 3-4 hours to measure the cell viability. The drug concentration at which 50% of the parasites were inhibited compared to control (the IC_{50} value) was determined using spectrophotometric detection at 490 nm using the four parameter curve equation available in the plate reader software.

2.2 Flow Cytometry

L. donovani promastigotes at a cell density of $5x10^6$cells/mL were incubated with pentamidine, DB1288 and DB746 at their IC_{50} concentrations and 10x their IC_{50} concentrations for 24 h at 25 °C. 1 x 10^7 parasites were collected and centrifuged at 1200 x g for 10 min at RT. The supernatant was removed from samples and the pellet was resuspended in 100 µL of 0.1 mM MitoTracker Green FM (MTG) solution for 3-4 h at 25 °C. The parasites were then fixed with 400 µL of 4% formaldehyde in promastigote medium for 15 min at 25 °C. The cells were then

centrifuged as before and resuspended in 1 mL PBS and analyzed by flow cytometry.

2.3 Transmission Electron Microscopy

L. donovani promastigotes at $5x10^6$cells/mL were incubated with pentamidine at 10 µM, DB1288 at 2.5 µM and DB746 at 0.2 µM, all at 5x the IC_{50} concentrations, for 24 h at 25 °C. Samples were centrifuged for 10 min at 3200 x g at 4 °C and resuspended with 1 mL of fixative (4% paraformaldehyde, 0.1M sucrose in 0.1 M phosphate buffer) for 3 h at room temperature. The cells were rinsed 4x with 0.1 M phosphate buffer containing 0.1 M sucrose then incubated for 1 h with 1% osmium tetroxide in buffer, rinsed twice and resuspended in 2% agarose and chilled in an ice bath for 10 min to set the agarose. The agarose was cut into 1 mm blocks and stained with 2% aqueous uranyl acetate for 1 h, and then rinsed 3x with distilled water, then dehydrated in graded percentages of ethanol for 10 min at 50% - 100%. The cells were then treated with propylene oxide alone for 10 min, then propylene oxide with spur resin (1:1 for 1 h, and then 1:2 overnight) and then embedded in spur resin and polymerized overnight at 60 °C. Ultrastructural analysis was performed on a Philips CM 12 transmission electron microscope (STEM).

2.4 Generation of parasites resistant to DB1288

Wild-type cells were cultured in the presence of 0.4 µM DB1288 with an increase of 0.2 µM in drug pressure every four weeks to 1 µM and then a final increase in drug pressure to a concentration of 2 µM, at which the culture was stabilized for 9 months at one passage per week.

2.5 Studies with parasites resistant to DB1288

Wild-type and DB1288 resistant *L. donovani* promastigotes were plated at a density of 10^6 parasites/mL on a 96-well plate with 2-fold serial dilutions of DB1288, DB746 and pentamidine. Plates were incubated for 3 days at 25 °C, and then the CellTiter reagent was added for 3-4 h to measure the cell viability. IC_{50} values were determined by addition of the Cell Titer reagent followed by spectrophotometric detection at 490 nm as described earlier.

3. Results

3.1 Activity of Compounds against *L. donovani* promastigotes

Table 1 shows the results of the activity of the seven compounds plus pentamidine against *L. donovani* promastigotes. All seven compounds had excellent antileishmanial activity and some of the reversed diamidines showed over 10-fold greater activity against the parasites compared to the diamidines. Subsequent experiments were restricted to one agent from each class of compounds: the diamidine DB1288 and the reversed diamidine DB746, which was the most potent of the seven compounds. The IC_{50} values for DB1288 and DB746 were 0.56 μM ± 0.12 and 0.030 μM ± 0.01 respectively (values given as the mean ± standard deviation of three independent measurements).

3.2 Measurement of MitoTracker Green Fluorescence

MitoTracker Green FM (MTG), a fluorescent molecular probe that localizes in the parasite mitochondrion, was employed to determine any mitochondrial membrane potential alterations caused by the compounds. Previous work on pentamidine has shown a collapse in mitochondrial potential when *Leishmania* parasites were

treated with this compound (5). From the results in Table 2, pentamidine at a concentration of 2 µM for 24 h didn't show any significant decrease in fluorescence but at 10x the IC_{50} value, 20 µM for 24 h, there was a decrease in fluorescence but not as pronouced as in previous reports. DB1288 at 10x the IC_{50} concentration, 5 µM for 24 h, showed a decrease in fluorescent of roughly 47%, suggesting that this diamidine affects the mitochondrial potential. DB746 at the IC_{50} concentration and 10x the IC_{50} concentration for 24 h didn't show a significant decrease in MTG fluorescence, suggesting a different route of toxicity on the parasites.

3.3 Transmission Electron Microscopy (TEM)

Morphological changes in the parasites after treatment with the named compounds were observed using TEM (Figure 2). Previous work on the antileishmanial effect of pentamidine with TEM has revealed disintegration of the kinetoplast and mitochondrion (8,9). In Figure 2, DB746 at 5x the IC_{50} concentration, 0.2 µM for 24 h, did not show significant morphological alterations to the nucleus. What was observed in roughly 50% of the sample was disintegration of the cytoplasm, an intact cellular membrane and nucleus and in some cases an intact mitochondrion. In DB1288 at 5x the IC_{50} concentration, 2.5 µM for 24 h, a significant effect on the mitochondrial membrane was observed. In roughly 80% of the sample, the kinetoplast DNA was less intense compared to the control, and significant disintegration and dilation of the mitochondrion was observed.

3.4 Cross resistance studies

We measured the IC_{50} values of wild-type parasites and organism resistant to DB1288 at 2 μM (4x IC_{50} value), see Table 3 for results. We observed a 7-fold increase in the IC_{50} value of pentamidine, a 28-fold increase in IC_{50} value of DB1288 and 3.6-fold increase in IC_{50} value of DB746.

4. Discussion

The results obtained with DB1288 support the hypothesis that this diamidine interferes with the parasite mitochondrion. Disintegration and dilation of the mitochondrion occurs in *Leishmania* treated with DB1288, also causing degradation of the kinetoplast DNA compared to the control in TEM micrographs and a possible dilation of the mitochondrion, which winds throughout the cell in Figure 2H and G. We also observed a significant decrease in MitoTracker Green fluorescence in parasites exposed to DB1288, indicating that DB1288 alters the mitochondrial membrane potential in *Leishmania*. We also discovered that DB1288 localized in the nucleus and kinetoplast of *Leishmania*, although we could not obtain high quality micrographs to demonstrate this. Previous work with the fluorescent diamidine DAPI on *Leishmania* has revealed its localization in the parasite nucleus and kinetoplast DNA (11). The results from the studies in Table 3 show that the DB1288 resistant parasites are cross-resistant to pentamidine, consistent with a common mechanism of action for the two diamidines. This result is similar to a published paper in which pentamidine resistant *L. donovani* expressed cross-resistance to other diamidines (10).

Effects of DB746 were more difficult to demonstrate. From the flow cytometry experiments in measuring MitoTracker Green fluorescence, it is questionable to suggest that DB746 interferes with mitochondrial membrane potential as was observed from the results in Table 2. Also, the micrographs from TEM suggest that DB746 exerts its antileishmanial activity by altering the cytoplasm of the parasites and in a few cases by dilating of the parasite mitochondrion membrane as observed in Figure 2C. We also observed from the results in Table 3, that DB746 showed only a 3.6-fold increase in the IC_{50} value compared to DB1288 (28-fold increase) and pentamidine (7-fold increase). Taken together these results suggest an additional mechanism of antileishmanial activity for DB746.

Further studies into the mechanism of action of these agents are needed to adequately design more active compounds against *Leishmania* parasites that have non-toxic effects in the host. Such studies include examining MTG fluorescence in resistant and control parasites as a measure of membrane potential and carrying out electron microscopy and MTG experiments on *L. donovani* exposed to lower concentrations of DB746

Acknowledgements. I would like to thank Dr. Werbovetz for giving me the opportunity to be involved in drug discovery and development research. I would also like to thank Dawn Delfin for assisting me through all my experiments.

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TABLE 1 Activity of compounds against wild-type *L. donovani* promastigotes

Compound	Average IC_{50} ± error (μM)*
Pentamidine	1.5 ± 0.56
DB1312	0.89 ± 0.31
DB1111	2.3 ± 0.60
DB1282	0.16 ± 0.04
DB1288	0.56 ± 0.12
DB709	0.32 ± 0.07
DB702	0.036 ± 0.01
DB746	0.030 ± 0.01

*IC_{50} values represent the mean ± standard error of at least three independent measurements

TABLE 2 Analysis of MitoTracker green fluorescence in *L. donovani* promastigotes

Compounds	% Decrease in Fluorescence Intensity compared to control
Pentamidine, 2μM[b]	3.4
Pentamidine, 20μM[a]	22 ± 12
DB1288, 0.5μM[a]	12 ± 5
DB1288, 5μM[a]	47 ± 7
DB746, 0.04μM[a]	7.9 ± 5.9
DB746, 0.4μM[a]	16 ± 11

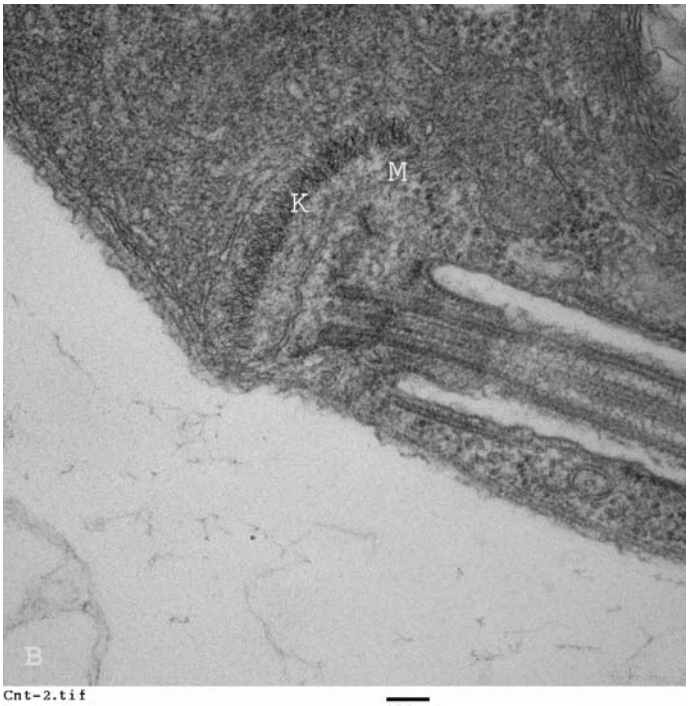
[a]Flow cytometry results represent the mean ± standard error of at least three independent measurements
[b]Flow cytometry result represents one independent experiment

TABLE 3 Activity of compounds against resistant DB1288 at 2 μM *L. donovani* promastigotes

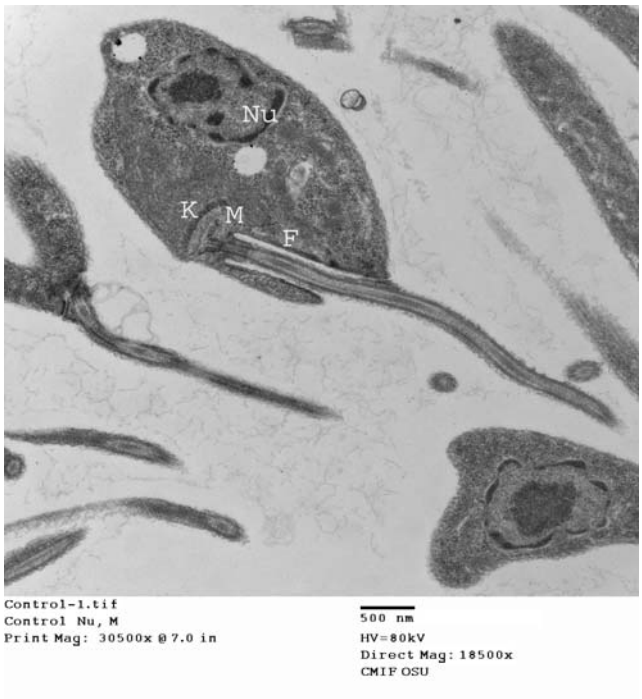
Compounds	Average IC_{50} ± error (μM)*
Pentamidine	14 ± 3
DB1288	14 ± 4
DB746	0.11 ± 0.05

*IC_{50} values represent the mean ± standard error of at least five independent measurements

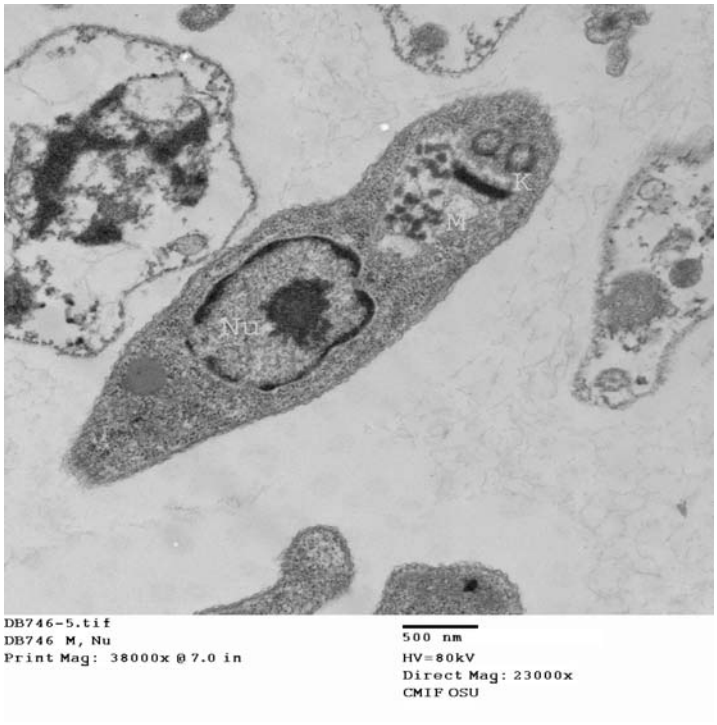
A. Control-1



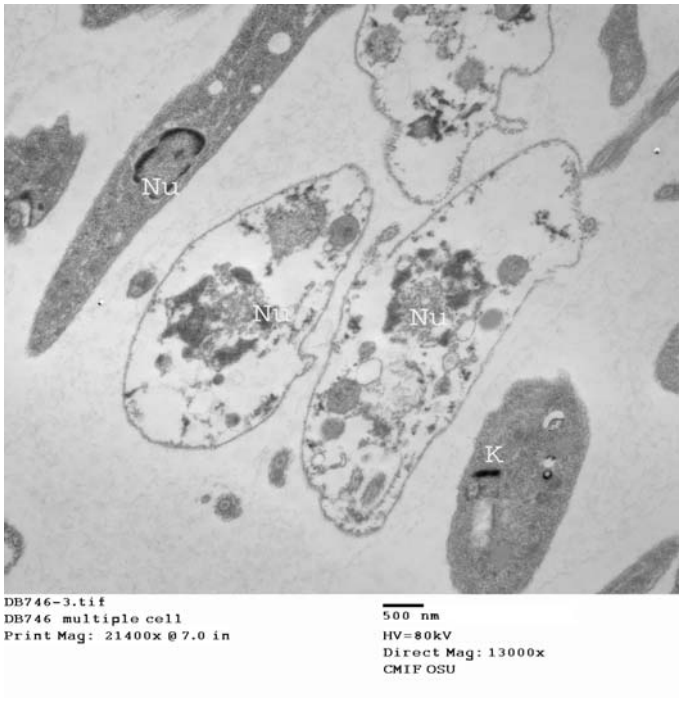
B. Control-2



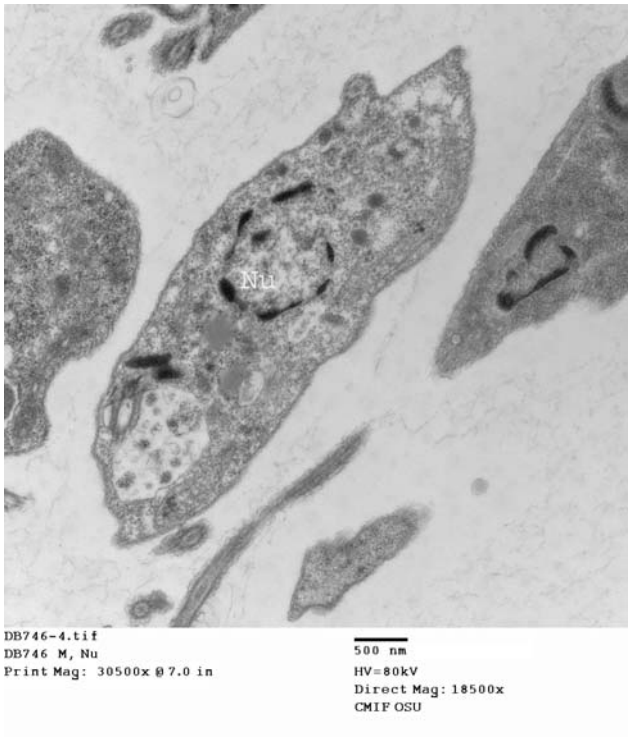
C. DB746-1



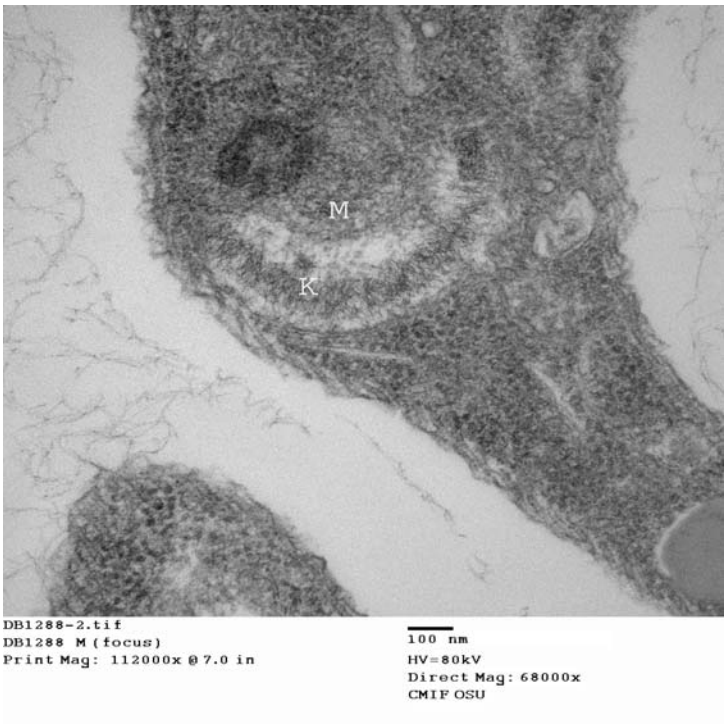
D. DB746-2



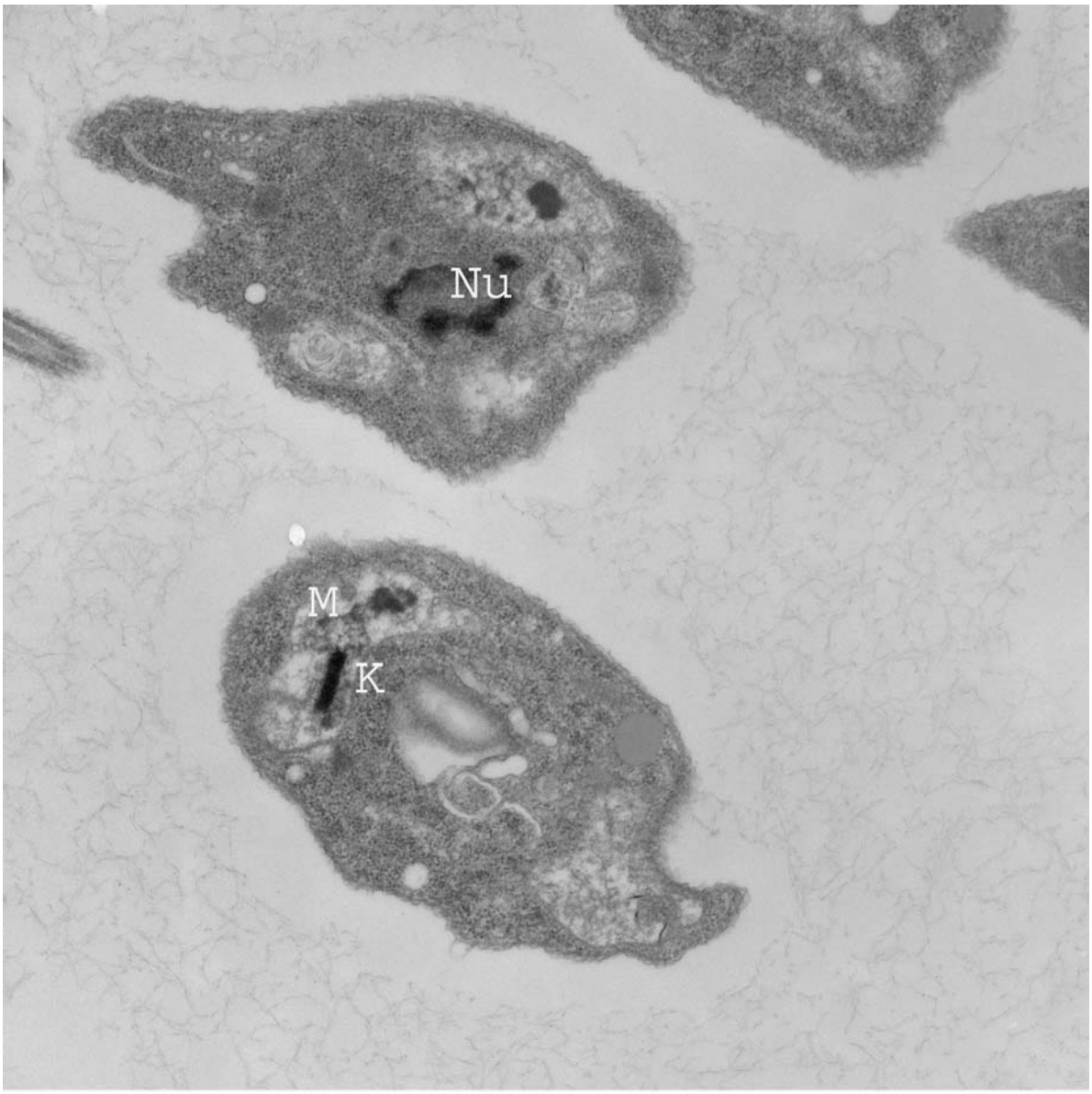
E. DB746-3



F. DB1288-1



G. DB1288-2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H. DB1288-3

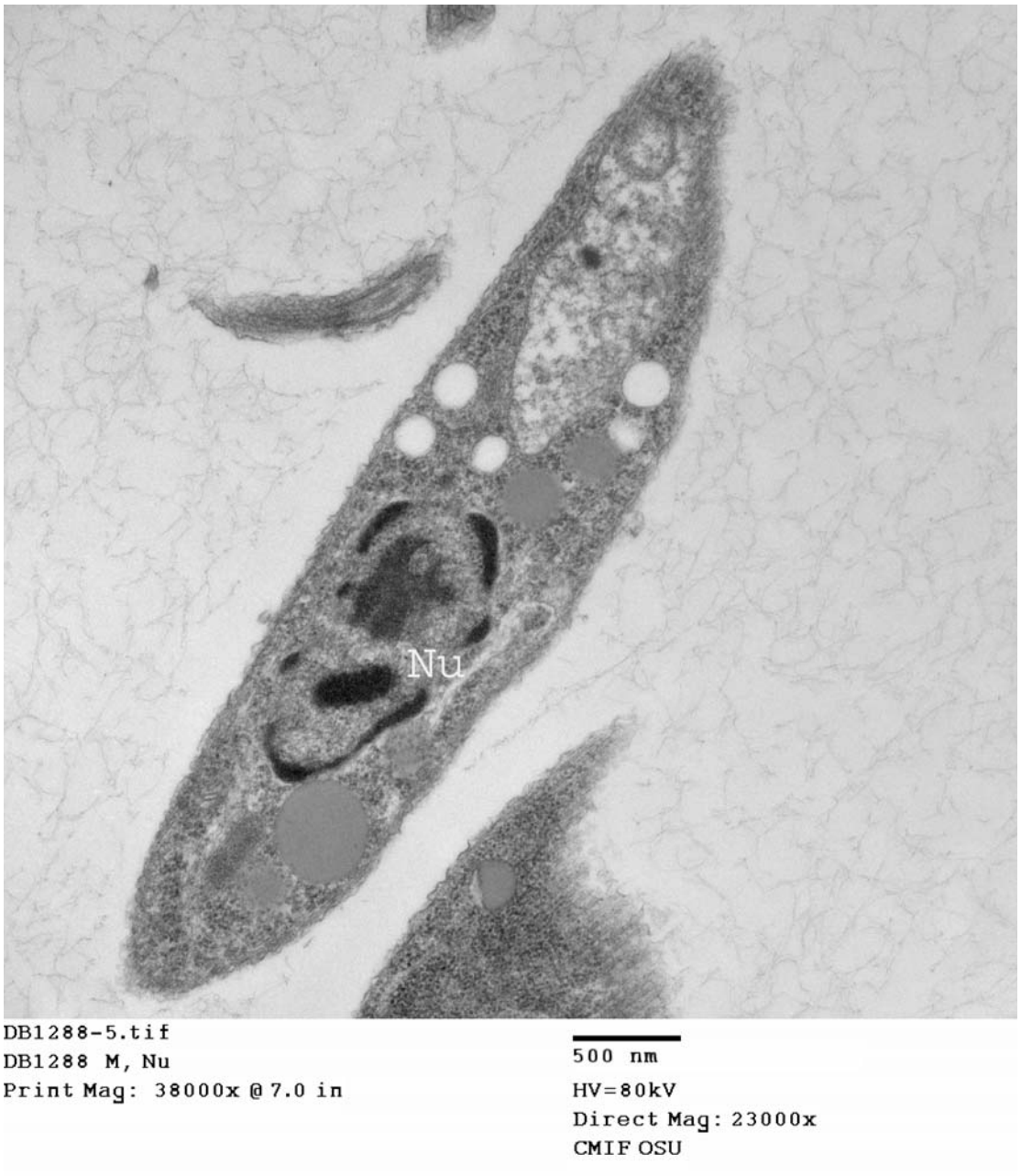

Figure 2. Transmission electron micrographs of *L. donovani* promastigotes for 24 h of Controls (2 graphs), DB1288 at 2.5 µM (3 graphs) and DB746 at 0.2 µM (3 graphs)