Gene expression modulation and the molecular mechanisms involved in Nelfinavir resistance in Leishmania donovani axenic amastigotes

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Summary

Drug resistance is a major public health challenge in leishmaniasis chemotherapy, particularly in the case of emerging Leishmania/HIV-1 co-infections. We have delineated the mechanism of cell death induced by the HIV-1 protease inhibitor, Nelfinavir, in the Leishmania parasite. In order to further study Nelfinavir–Leishmania interactions, we selected Nelfinavir-resistant axenic amastigotes in vitro and characterized them. RNA expression profiling analyses and comparative genomic hybridizations of closely related Leishmania species were used as a screening tool to compare Nelfinavir-resistant and -sensitive parasites in order to identify candidate genes involved in drug resistance. Microarray analyses of Nelfinavir-resistant and -sensitive Leishmania amastigotes suggest that parasites regulate mRNA levels either by modulating gene copy numbers through chromosome aneuploidy, or gene deletion/duplication by homologous recombination. Interestingly, supernumerary chromosomes 6 and 11 in the resistant parasites lead to upregulation of the ABC class of transporters. Transporter assays using radiolabelled Nelfinavir suggest a greater drug accumulation in the resistant parasites and in a time-dependent manner. Furthermore, high-resolution electron microscopy and measurements of intracellular polyphosphate levels showed an increased number of cytoplasmic vesicular compartments known as acidocalcisomes in Nelfinavir-resistant parasites. Together these results suggest that Nelfinavir is rapidly and dramatically sequestered in drug-induced intracellular vesicles.

Introduction

Leishmania is a trypanosomatid protozoan responsible for a diverse spectrum of human diseases termed leishmaniasis. These parasites exhibit a dimorphic life cycle, consisting of extracellular promastigotes that reside within the midgut of the sandfly vector and intracellular amastigotes that reside within the phagolysosome of host macrophages (Alexander and Russell, 1992). After establishing infection in the human host, amastigotes multiply in an intracellular vacuolar compartment, leading to macrophage lysis and serial infection of other surrounding macrophages. Depending on the species involved, the disease occurs in several forms ranging from simple cutaneous to more serious and potentially fatal visceral form. Visceral leishmaniasis (VL), which is primarily caused by Leishmania infantum (L. infantum) and L. donovani, is now also emerging as an important opportunistic disease found in patients infected with human immunodeficiency virus type-1 (HIV-1) (Alvar et al., 2008). Leishmania and HIV-1 both infect the cells of monocyte lineage (i.e. macrophages and dendritic cells) (Carter and Ehrlich, 2008; Garg et al., 2009). In this regard, it has been reported that HIV-1 infection promotes higher growth of L. infantum in macrophages (Zhao et al., 2006) and, on the other hand, the parasite is able to potentiate and upregulate virus replication, at least in vitro (Zhao et al., 2004). Interestingly, we have recently shown that, in culture, uninfected bystander cells, not macrophages productively infected with HIV-1, account for enhanced phagocytosis and higher multiplication of Leishmania parasites (Lodge et al., 2012).

No effective vaccines are yet available against Leishmania and treatment relies primarily on chemotherapy. Furthermore, interaction of Leishmania with HIV-1 worsens disease progression and complicates treatment. The recent development of highly active antiretroviral therapy
(HAART) has significantly improved the prognosis of patients infected with HIV-1. This therapeutic strategy consists of at least three anti-retroviral drugs, typically two nucleoside or nucleotide reverse transcriptase inhibitors used in combination with a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor (PI). In patients co-infected with *Leishmania* and HIV-1, HAART is initiated to partially restore immune functions but it has also been found to prevent VL in dually infected individuals (de la Rosa et al., 2001), as reflected by the sharp decrease in the incidence of VL in Europe following the widespread use of HAART (Rosenthal et al., 2001). Recently, our group and others have reported that HIV-1 PIs exhibit antileishmanial activity in a dose-dependent manner *in vitro* (Savoia et al., 2005; Trudel et al., 2008; Santos et al., 2009). Furthermore, it has been demonstrated that HIV-1 PIs interfere with the cellular proliferation and ultrastructure of *L. amazonensis*, and its capacity to macrophages (Santos et al., 2009). We have also shown that Nelfinavir (NFV), an HIV-1 PI, acts as a powerful inhibitor of the intracellular growth of *Leishmania* in both monocyteid THP-1 cells and primary human monocyte-derived macrophages (Trudel et al., 2008). However, little is known about the precise mode of action of NFV in *Leishmania*. PIs block the active site of aspartyl protease, a HIV-1 enzyme known to be essential for the maturation of viral proteins, by mimicking target peptides (Deeks et al., 1997). Recently, it has been shown that a Ddi1-like protein from *L. major* is an active aspartyl proteinase and the biochemical analysis of the recombinant *L. major* Ddi1-like protein shows that it hydrolyses specific substrates in common with the A2 aspartyl proteinase family, of which the HIV-1 protease is a member (Perteguer et al., 2012). In this regard, it has been shown that an orthologue of the yeast Ddi1-like protein in *Leishmania* is a potential target for the HIV-1 PIs including NFV (White et al., 2011). Furthermore, we have previously reported that NFV induces DNA fragmentation in *Leishmania* axenic amastigotes, and that a NFV-resistant parasite treated with subcytotoxic concentrations of NFV undergoes less apoptosis than its NFV-sensitive counterparts (Kumar et al., 2010). Indeed, NFV induces programmed cell death in *Leishmania* axenic amastigotes by a caspase-independent mechanism (Kumar et al., 2010). Finally, ultrastructure studies of NFV-treated *L. amazonensis* showed cytoplasm shrinking and an increased number of intracellular vesicles in comparison to untreated parasites (Santos et al., 2009).

In the case of *Leishmania/HIV-1* co-infections, interaction of drug resistant parasites with HIV-1 may greatly complicate the disease. In this regard, it has been reported that antimony (Sb) resistant *L. donovani* induces upregulation of ATP-binding cassette (ABC) transporters in the host's macrophages and are responsible for the intracellular loss of Sb (Mookerjee Basu et al., 2008). Furthermore, HIV-1 PIs are effluxed by ABC transporters potentially making host cells sanctuary sites for HIV-1 replication (Jones et al., 2001). Drug resistance is a complex multi-gene phenomenon and different types of mutations are responsible for resistance in a single parasite. Indeed, several studies dealing with drug resistance in *Leishmania* emphasized the plasticity of its genome (Ubeda et al., 2008; Leprohon et al., 2009; Downing et al., 2011). Genome wide expression profiles provide useful information about the mechanisms of drug resistance in the *Leishmania* parasite. For example, DNA microarrays have been useful for investigating the mode of action of drugs (Wilson et al., 1999) and their mechanisms of resistance (Brazas and Hancock, 2005; Ouellette et al., 2007).

In order to study gene expression modulations associated with NFV resistance on a full genomic scale, we have analysed the RNA expression profile as well as performed a comparative genomic hybridization of both NFV-resistant and -susceptible *L. donovani* amastigotes using 60-mer oligonucleotide microarrays. These experiments pinpointed specific mechanisms and novel changes in aneuploidy of chromosomes that correlated with NFV resistance. Interestingly, our genomic study identified upregulation of a particular class of ABC transporters, which are involved in vesicular trafficking. Using electron microscopy imaging, we observed an increased number of vacuoles in the NFV-resistant parasites when compared with their drug-sensitive counterparts.

**Results**

**Characterization of *L. donovani* amastigotes selected for NFV resistance**

In order to further delineate the mode of action of NFV in *Leishmania*, we generated NFV-resistant parasites by increasing the drug concentration in the growth media at each passage, as previously described (Kumar et al., 2010). The NFV IC$_{50}$ value of the drug-sensitive parental Ld9518 amastigotes was 8.4 μM, whereas an IC$_{50}$ of 22.9 μM was observed for drug-resistant parasites, thus displaying a ~ 3-fold increase in resistance when compared with the NFV-sensitive strains (Fig. 1A). The stability of the resistant phenotype of Ld9518 axenic amastigotes was tested by growing the cells in absence of NFV. Revertants obtained after up to 25 passages lost most of their resistance to NFV with an IC$_{50}$ of 12.7 μM. Given that NFV-resistant parasites were selected in a stepwise manner, it is possible that multiple NFV resistance mechanisms may exist in these cells. In another set of experiments, the effect of NFV was monitored in luciferase-expressing parasites (NFV-sensitive and -resistant) following their infection of primary human monocyte-derived macrophages (MDMs). We observed a significant increase in the growth of NFV-resistant parasites when...
compared with the NFV-sensitive parental strain, in cultures treated with different concentrations of NFV (Fig 1B). For example, NFV inhibited the intracellular growth of drug-resistant parasites by 32.7% and 44.3%, at 12.5 μM and 25 μM, respectively, as compared with 60.1% and 70.5% for the drug-sensitive parental strain. Thus, NFV-resistant parasites maintained this important feature even when growing in macrophage cultures.

**Gene expression profiles of *L. donovani* amastigotes**

RNA expression profiling was compared between NFV-resistant and -sensitive axenic amastigotes using full genome 60-mer oligonucleotide microarrays as described previously (Ubeda et al., 2008; Leprohon et al., 2009). The differential hybridization data using RNA probes derived from sensitive and resistant parasites are represented for each chromosome (Fig. 2A). We observed that most genes were expressed at similar levels in both the NFV-resistant and -sensitive strains. Indeed, the bulk of the gene expression ratios when comparing NFV-resistant and -sensitive strains were close to 1. However, we identified notable differences in the expression of some genes. We observed that the RNA of 158 genes was upregulated whereas that of 157 genes was downregulated (cut-off 1.5, *P* < 0.05) in the resistant strain as compared with the sensitive type (Table S1).

We further investigated the genomic background of NFV-resistant and -sensitive strains by comparative genomic hybridization (CGH) (Fig. 2B). When compared with the previously obtained RNA expression profiles, DNA genomic hybridization was found to be similar for each strain, which was expected given that trypanosomatids lack transcriptional gene regulation. Indeed, the probe hybridization of 201 genes was enhanced whereas that of 110 genes was decreased (cut-off 1.5, *P* < 0.05) in the NFV-resistant strain as compared with that of the sensitive strain (Table S2). Furthermore, we observed notable downregulation for both RNA gene expression and DNA probe hybridization on chromosome 10 in the NFV-resistant amastigotes when compared with the drug-sensitive strain (Fig. 2A and B).

Moreover, both the CGH and RNA expression analyses suggested that several candidate genes are putatively correlated to resistance. Among these modulated genes, we observed overexpression of a putative ABC transporter-encoding gene (i.e. *LinJ06_V3.0080*) linked to chromosome 6, as well as genes encoding cyclophilin (i.e. *LinJ06_V3.0120*), DHFR-TS (i.e. *LinJ06_V3.0890*) and terbinafine resistance locus protein (i.e. *LinJ23_V3.0280*). Interestingly, histone-encoding genes (i.e. *LinJ27_V3.1070* and *LinJ29_V3.2950*) were also overexpressed in the resistant strain in comparison to the sensitive strain. However, genes encoding for metallopeptidase and cysteine peptidase (i.e. *LinJ26_V3.2450* and *LinJ31_V3.0450*) were downregulated. The *lmgt2* gene encoding for a glucose transporter (i.e. *LinJ36_V3.6550*) was also downregulated in the NFV-resistant strain in comparison to the sensitive parasites. The microarray data were supported by selected quantitative real-time reverse transcription PCR (qRT-PCR) assays (Fig. 3).

**A deletion in chromosome 10 in NFV-resistant amastigotes**

Gene deletion and formation of extra-chromosomal DNA are present in *Leishmania* due to the homologous recombination events taking place at repeated sequences
Fig. 2. Gene expression profile of *L. donovani* axenic NFV-resistant amastigotes. DNA microarray data were analysed with GeneSpring GX3.1 to illustrate the NFV-resistant/NFV-sensitive amastigote gene expression ratio on a chromosome by chromosome basis (1 to 36).

A. RNA expression profile.

B. CGH. Green features indicate genes overexpressed in NFV-resistant amastigotes, whereas red features indicate genes down regulated in NFV-resistant amastigotes. Yellow features indicate genes equally expressed in both parasites. Vertical bars represent individual genes on each chromosome and their location above or below the strand represent the transcribed strand. Gene modulation is represented on a chromosome basis, some chromosomes are entirely overexpressed or down regulated at the DNA and RNA levels. These include chromosomes 4, 6, 11, 26 and 31. The plot represents the average values of three independent hybridizations.
In the NFV-resistant parasites, we observed a region of chromosome 10, containing 6 genes encoding hypothetical proteins, the expression of which was decreased (Fig. 4A). Close examination of the sequences flanking the region revealed the presence of repeated sequences. We therefore verified if this sequence was deleted through homologous recombination, using PCR (Fig. 4A and B). We found a DNA amplification product in both NFV-sensitive and -resistant strains (Fig. 4C), suggesting that a subpopulation of parental NFV-sensitive parasites harboured the deletion in chromosome 10 and thus that these cultured Leishmania parasites are heterogeneous in nature. However, this region was found to be completely deleted from the entire population of the resistant strain as shown from PCR amplification of one of the deleted region genes (Fig. 4D). In order to confirm that this region is entirely deleted from the NFV-resistant strain, we performed Southern blot hybridization (Fig. 4E). PCR amplification and Southern blot hybridization both displayed a band in the sensitive strain; however, it was absent in the NFV-resistant strain (Fig. 4D and E). Sequencing of a PCR generated amplicon derived from NFV-resistant parasites (GenBank: JX683526) confirmed the scenario of homologous recombination between the repeated sequences (Fig. S1).

**Chromosome aneuploidy and gene copy number variation in NFV-resistant L. donovani amastigotes**

The genomes of L. donovani and L. infantum each consist of 36 chromosomes, and are very similar (Downing et al., 2011). This genomic similarity allowed us to compare the variation of chromosome and gene copy numbers in NFV-resistant L. donovani using a whole genome array based on L. infantum genome sequences. Most of the genes modulated in NFV-resistant parasites were linked to chromosome aneuploidy as shown from both RNA expression and CGH profiles (Fig. 2A and B). Indeed, the majority of genes on chromosomes 4, 6 and 11 appeared upregulated (predominantly coloured in green) while the expression of genes located on chromosomes 26 and 31 was.

Fig. 3. Validation of DNA microarray expression data by qRT-PCR. The mean log2 ratios of selected genes from the microarray expression data (grey bars) are compared with qRT-PCR data (black bars). The microarray expression data are the average of three biological replicates (with one dye swap), while the qRT-PCR data are an average of three biological replicates and normalized with the LinJ18_V3.0630 gene encoding a putative 60S ribosomal protein.
Fig. 4. Gene deletion mechanism on chromosome 10.
A. A portion of the *Leishmania* chromosome 10, with grey boxes representing genes. Forward primer 1a and reverse primer 1b are represented by arrows, and direct repeats are depicted by black boxes. The microarray data (mean log₂ ratio) obtained from comparative genomic hybridization (DNA) and RNA expression profiling (RNA) are shown below.
B. Model for gene deletion on chromosome 10 mediated by homologous recombination between direct repeats.
C. PCR amplification of a 2.3 kb DNA fragment with primers 1a and 1b to support the model shown in (B).
D. PCR with primers 1c and 1d of gene *LinJ10_V3.1410* to support the gene deletion event in the entire population of NFV-sensitive amastigotes.
E. Southern blot hybridization with the probe generated from primers 1c and 1d to further confirm the deletion event as shown in the model (B).
Lane M, molecular mass markers; Lane 1, *L. donovani* NFV-sensitive amastigotes; lane 2, *L. donovani* NFV-resistant amastigotes.
appeared mostly downregulated (predominantly coloured in red). Plotting the gene expression data as a function of microarray probes for selected chromosomes confirmed the variation of chromosome numbers (aneuploidy) in the NFV-resistant strain (Fig. 5). An analysis of the relative gene expression ratios of NFV-resistant and -sensitive parasites also showed that the expression of most genes was either upregulated or downregulated by 0.8- to 1.5-fold (log2 ratio) in the resistant strain, except for chromosome 4, on which genes were upregulated by 0.8- to 2.2-fold (log2 ratio) (Fig. 5). This was further supported by quantitative Southern blot hybridization, which showed that the DNA content of chromosomes 4, 6, 11 and 31 was increased by 1.8-, 1.4- and 1.4-fold, respectively, in NFV-resistant parasites (Fig. 5A–C, lanes 1 and 2), while that of chromosomes 26 and 31 decreased by ~ 2-fold in the NFV-resistant strain (Fig. 5D and E). LinJ18_V3.0630, which expression was unchanged in the microarray data, was used as a control to normalize the fold change for Southern blot quantitative analysis.

As Leishmania lack RNA Polymerase II-directed transcription (Clayton, 2002), the NFV-resistant strain mRNA profile is due to changes in chromosome copy numbers, as illustrated by comparing the DNA and RNA profiles, which displayed similar changes in gene expression along the whole chromosome length (Fig. 6). In order to further verify that aneuploidy is associated with NFV resistance in axenic amastigotes, we generated a revertant strain by growing the NFV-resistant axenic amastigotes in the absence of drug for up to 25 passages. The NFV resistance decreased in the revertant line, close to the levels of the parental NFV-sensitive parasites (Fig. 1). The decrease in resistance following 25 passages in the absence of NFV correlated with the change in aneuploidy of chromosomes 4, 6, 11 and 31, which suggested that the status of these chromosomes was similar to that of a NFV-sensitive strain (Fig. 5A–C and E, lane 3). Chromosome 26 which was haploid in the resistant mutant remained haploid in the revertant line (Fig. 5D, lane 3).

Our previous observations on chromosome aneuploidy in NFV-resistant axenic amastigotes do not exclude the existence of such a phenomena in the parental, drug-sensitive strain. We performed real-time PCR of selected genes of which copy numbers were varying in the NFV-resistant strain (Fig. S2A). Interestingly, the real-time PCR cycle threshold (Ct) values confirmed that the selected genes, each present as a single copy on either chromosome 4, 6, 11 or 26, were predominantly disomic in the NFV-sensitive axenic amastigotes, with a minor presence of monosomic and trisomic forms in the population. This confirmed that these chromosomes were naturally aneuploid. Furthermore, the Ct value of LinJ31_V3.0030 on chromosome 31 indicated that this chromosome exists in a tetrasomic form, as has been reported previously (Downing et al., 2011). Similarly, the Ct values of these genes on chromosomes 4, 6, 11, 26 and 31 in the NFV-resistant strain showed that the aneuploidy of these chromosomes was modulated according to the CGH and RNA expression profiles (Fig. S2B).

In contrast to the majority of NFV-regulated genes, we found that some differentially expressed genes in the NFV-resistant parasites were not linked to changes in chromosome aneuploidy, and were present in different copy numbers on their chromosome. In this regard, we observed that the expression ratio of the gene encoding Histone H2A (i.e. LinJ29_V3.1850) was ~ 5-fold (according to the RNA expression profile) higher in the NFV-resistant strain (Table S1); however, chromosome 29 was not modulated in these parasites. We also determined that the glucose transporter gene imgt2 (i.e. LinJ36_V3.6550), which was greatly downregulated (RNA expression profile, Table S1) in the resistant strain, was also suppressed independently of NFV-induced chromosome aneuploidy. In order to verify that both the genes were modulated at the genomic level, we performed quantitative Southern blot hybridization (Fig. 7). LinJ18_V3.0630 was used as a control to normalize the fold change for quantitative analysis. We observed that LinJ29_V3.1850 and LinJ36_V3.6550 were modulated 1.2-fold and 0.7-fold, respectively, in the NFV-resistant strain, which confirmed our previous data using RNA expression profiling (Fig. 7 and Table S1).

**NFV accumulation and change in ultrastructure in NFV-resistant parasites**

Ploidy changes in chromosomes 6, 11, 26 and 31 of resistant parasites lead to an increase in the number of genes encoding for Leishmania ABC transporters (Fig. 8); this prompted us to evaluate the role of ABC transporters in parasite NFV resistance. Multiple protein sequence alignment was performed on ABC transporters coded by these chromosomes modulated in NFV resistant parasites. The resulting alignment was subjected to phylogenetic analyses by neighbour-joining algorithms. Interestingly, we determined that both the ABCG and ABCA classes of ABC transporters were upregulated, while other classes of ABC transporters were downregulated, in the resistant strain (Fig. 8). In mammalian cells, ABCG2 transporters have been linked to the formation of extracellular vesicles which are responsible for the sequestration of the anticancer drug Mitoxantrone (Ifergan et al., 2005). Furthermore, the ABCA class of transporters is involved in vesicular trafficking in Trypanosoma cruzi (Torres et al., 2004). The vesicular sequestration of drugs is a well-known mechanism of drug resistance in mammalian cells (Sognier et al., 1994; Kramer et al., 1998) and has also been described in Leishmania (Légardé et al., 2001). We thus investigated the
ultrastructure of the NFV-resistant and -sensitive *L. donovani* axenic amastigotes by transmission electron microscopy. Interestingly, though we observed some augmentation of intracellular vacuoles in drug-treated parasites, they were more abundant in the drug-resistant parasites, (Fig. 9, panels A–D). Furthermore, the observation of ultra-thin sections of axenic amastigotes by electron microscopy suggests that these vesicles might be acidocalcisomes, according to their electron-density (Corrêa et al., 2002). Given that acidocalcisomes contain particularly high levels of polyphosphate (polyp) (Docampo and Moreno, 1999), we measured the polyP levels in NFV-sensitive and -resistant parasites using DAPI fluorescence staining (Aschar-Sobbi et al., 2008). We observed higher levels of polyP in NFV-resistant parasites in comparison to NFV-sensitive ones (Fig. 9, panels E and F). Interestingly, there is a good correlation between electron microscopy and polyp levels in NFV-sensitive and -resistant parasites.

Given that such vacuoles may sequester NFV, acting as cytoplasmic drug disposition chambers, we performed drug-accumulation assays using [3H]-labelled NFV. We determined that NFV accumulates in greater amounts in the drug-resistant parasites, and in a time-dependent manner (Fig. 9G). Together, these observations suggest that NFV is sequestered in *Leishmania* intracellular vesicles, the formation of which may contribute to the establishment of NFV resistance in axenic amastigotes.

**Discussion**

Leishmaniasis is now emerging as a significant opportunistic infection in HIV-1-infected patients, and the global

**Fig. 5.** Chromosome aneuploidy in *L. donovani* amastigotes selected for NFV resistance. The mean log₂ expression ratio of each individual gene was plotted as a function of the location of microarray probes for the upregulated chromosomes 4 (A), 6 (B) and 11 (C) and of the downregulated chromosomes 26 (D) and 31 (E). For each plot, the log₂ expression ratios of chromosome 18, which genes were equally expressed in NFV-resistant and -sensitive amastigotes, are shown as a control (black line). Quantitative Southern blot hybridization was performed to correlate gene expression modulation of entire chromosomes with the chromosome DNA copy number. Two to three distant probes per chromosome were hybridized to Sacl digested DNA either from NFV-sensitive amastigotes (lane 1, SEN), NFV-resistant amastigotes (lane 2, RES), or the revertant amastigotes (lane 3, REV). The hybridization signal of gene LinJ 18_V3.0630 was used for normalization. The hybridization signals were quantified using NIH ImageJ and the fold differences in DNA copy number of NFV-resistant (bar 2, RES) and revertant (bar 3, REV) amastigotes are compared with NFV-sensitive (bar 1, SEN) parasites, as represented by the bar graph.

**Fig. 6.** Correlation between CGH and RNA expression profile microarray hybridization data. Genomic DNA- or RNA-derived probes prepared from *L. donovani* NFV-resistant amastigotes or their drug-sensitive parental strain were hybridized to DNA microarrays. The correlation between DNA and RNA gene expression data is represented by a subset of whole chromosomes. Examples shown are: genes from chromosomes 4, 6 and 11 showing increased DNA and RNA, from chromosomes 26 and 31 showing decreased DNA and RNA and chromosome 18, where either DNA or RNA remain unchanged. The expression ratio of each gene is represented by green for overexpressed genes, red for downregulated genes and yellow for non-modulated genes in NFV-resistant parasites.
distribution of Leishmania/HIV-1 co-infection is expanding. Furthermore, due to the emergence of both antileishmanial and antiviral drug resistance, the treatment of co-infected patients is increasingly difficult, particularly in developing countries. NFV mimics the peptide linkage(s) of newly expressed virus precursor polyproteins, which is cleaved by aspartyl protease of HIV-1 into mature capsid proteins and enzymes (Deeks et al., 1997). NFV, along with other HIV-1 PIs used in HAART, have been shown to inhibit the growth of Leishmania parasites in vitro (Savoina et al., 2005; Trudel et al., 2008; Santos et al., 2009). For example, we and others have shown that NFV inhibits the growth of Leishmania parasites in culture and in virus-infected human primary monocyte-derived macrophages, and that it was the most effective drug against the parasite when compared with other PIs (Trudel et al., 2008; Santos et al., 2009). We have also shown that NFV induces oxidative stress and caspase-independent, endonuclease G-mediated apoptosis in L. donovani axenic amastigotes (Kumar et al., 2010).

The control of leishmaniasis and HIV-1 infection rely mainly on chemotherapy and emerging drug resistance is one of the major clinical problems. Thus, the identification of molecular mechanisms involved in drug resistance in the Leishmania parasite is essential for the design of new therapeutic strategies to control leishmaniasis as well as treatment in Leishmania/HIV-1 co-infections. Currently, work on drug resistance has been limited to in vitro selected resistant Leishmania promastigotes, and little is known of the drug resistance mechanisms in the amastigote stage of the parasites. This is surprising, given that amastigotes are the circulating form of Leishmania in the human host. In this regard, we developed in vitro NFV-resistant axenic amastigotes by stepwise increase of drug pressure. Although it has been reported that axenic amastigotes are not equal to lesion-derived amastigotes and promastigote-specific genes might be expressed in the axenic amastigotes (Holzer et al., 2006), we observed that in vitro selected NFV-resistant axenic amastigotes maintained their resistant phenotype in macrophage cultures. It should further be noted that the NFV concentrations used in this study were in the micromolar range, which are the therapeutic doses of NFV reached in the blood plasma (Skinner-Adams et al., 2004). Thus, these are also relevant when used directly in media cultured parasites. Furthermore, in the case of co-infections, HAART does not prevent leishmaniasis relapse despite increasing CD4+ T cell counts and obtaining undetectable HIV-1 loads (Lopez-Velez, 2003). In such cases, Leishmania might develop PI-resistance following HAART, complicating the treatment. We developed an in vitro resistant strain keeping in mind such possible cases of resistance might occur under physiological conditions. In this regard, Santos and colleagues have recently reported that a Leishmania clinical isolate from a HIV-1 coinfected patients under HAART is resistant to Nelfinavir (Santos et al., 2012).

DNA microarray technology has been shown to be useful for understanding both the mode of action of drugs as well as the mechanisms involved in drug resistance (Wilson et al., 1999; Brazas and Hancock, 2005; Ouellette et al., 2007). In order to identify the underlying molecular events responsible for NFV resistance in Leishmania, we performed CGH and RNA expression profile analyses using whole Leishmania genome DNA microarray chips (Ubeda et al., 2008; Leprohon et al., 2009; do Monte-Neto et al., 2011). We found a qualitative correlation between both the RNA expression and CGH profiles (Figs 2 and 6). This observation is explained by the lack of genetic transcriptional control in trypanosomatid parasites (Clayton,
Thus, these parasites modulate their RNA transcript levels by modulating genomic DNA. However, we also observed differences in RNA for some genes in the absence of copy number modulation at the DNA level, which is an infrequent event in \textit{Leishmania} drug resistance. This difference may arise due to increased stability of mRNAs in the \textit{Leishmania} parasite (Haimeur et al., 1999).

We further observed that most of the modulated genes were associated with chromosome aneuploidy. Indeed, analyses of CGH and RNA expression profiles showed that gene expression modulation of entire chromosomes (i.e. chromosomes 4, 6, 11, 26 and 31) was associated with change in ploidy in the NFV-resistant \textit{L. donovani} axenic amastigotes. Furthermore, we observed a reversal of chromosomal ploidy to NFV-sensitive levels in the revertant parasites (Figs 2 and 5). Aneuploidy appears to arise frequently in drug pressure and it is well tolerated in the amastigote stage of the NFV-resistant parasites. Indeed, chromosome aneuploidy was previously observed in \textit{methotrexate-resistant} \textit{L. major} (Ubeda et al., 2008) and \textit{antimony-resistant} \textit{L. infantum} (Leprohon et al., 2009). Chromosome aneuploidy is also associated with drug resistance in the yeast \textit{Candida glabrata} (Polakova et al., 2009) and is associated with a strong selective advantage to environmental stresses in \textit{Saccharomyces cerevisiae} (Rancati et al., 2008).

Advancements in whole genome sequencing technology enabling that of multiple \textit{L. donovani} clinical isolates pinpointed extensive variation in chromosome and gene copy number (Downing et al., 2011). However, despite the well-established biology of this parasite, the exact mechanism of chromosome aneuploidy is still enigmatic. Recently FISH analysis showed that each chromosome exists in different ploidy states in the parasite population and this variation in chromosome copy number is due to asymmetric chromosome allotment during mitosis (Sterkers et al., 2010). Accordingly, we observed that chromosomes 4, 6, 11 and 26 were mostly disomic; however, we found that some monosomic and trisomic forms also exist within the population of the wild-type, NFV-sensitive strain (Fig. S2A). Surprisingly, we observed that chromosome 31 is in a tetrasomic form in these parasites (Fig. S2A), although this has been previously reported (Akopyants et al., 2009; Rogers et al., 2011). In the NFV-resistant strain, the copy numbers of these chromosomes varied greatly, providing the genetic basis of selection under drug pressure (Fig. S2B).

ABC transporters have been associated with drug resistance and trafficking in various cells. For example, the ABCG2 transporter is involved in drug sequestration in extracellular vesicles of cancer cells (Ifergan et al., 2005) and ABCA transporters are involved in vesicular trafficking in the protozoan parasite \textit{Trypanosoma cruzi} (Torres et al., 2002). Thus, these parasites modulate their RNA transcript levels by modulating genomic DNA. However, we also observed differences in RNA for some genes in the absence of copy number modulation at the DNA level, which is an infrequent event in \textit{Leishmania} drug resistance. This difference may arise due to increased stability of mRNAs in the \textit{Leishmania} parasite (Haimeur et al., 1999).

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Advancements in whole genome sequencing technology enabling that of multiple \textit{L. donovani} clinical isolates pinpointed extensive variation in chromosome and gene copy number (Downing et al., 2011). However, despite the well-established biology of this parasite, the exact mechanism of chromosome aneuploidy is still enigmatic. Recently FISH analysis showed that each chromosome exists in different ploidy states in the parasite population and this variation in chromosome copy number is due to asymmetric chromosome allotment during mitosis (Sterkers et al., 2010). Accordingly, we observed that chromosomes 4, 6, 11 and 26 were mostly disomic; however, we found that some monosomic and trisomic forms also exist within the population of the wild-type, NFV-sensitive strain (Fig. S2A). Surprisingly, we observed that chromosome 31 is in a tetrasomic form in these parasites (Fig. S2A), although this has been previously reported (Akopyants et al., 2009; Rogers et al., 2011). In the NFV-resistant strain, the copy numbers of these chromosomes varied greatly, providing the genetic basis of selection under drug pressure (Fig. S2B).

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et al., 2004). Our phylogenetic analyses of these ABC transporters showed an upregulation of both the ABCG and ABCA classes of these transporters in the NFV-resistant parasite (Fig. 8). Both RNA expression and CGH profiles of the NFV-resistant parasite revealed that the overexpression of the ABCG and ABCA classes of ABC transporters is due to an increase in the copy numbers of chromosomes 6 and 11 respectively (Fig. 2). Upregulation of transporters involved in vesicular trafficking in the NFV-resistant parasite suggests a possible sequestration of the drug in a cytoplasmic vesicular compartment. Electron microscopy of the ultrastructure of NFV-resistant parasites revealed the presence of an increased number of such vacuoles in comparison to the parental NFV-sensitive strain (Fig. 9A). Recently, it was reported that similar concentrations of NFV in L. amazonensis promastigotes also resulted in the formation of vacuoles, which were identified, based on their electron-density, as possible acidocalcisomes (Santos et al., 2009). Moreover, a greater accumulation of [3H]labelled-NFV in the resistant parasites also suggests sequestration of NFV in these cells (Fig. 9G). Therefore, the ABCG or ABCA classes of transporters might be involved in such sequestration. For instance, vesicular sequestration of paromomycin has recently been reported in Leishmania (Chawla et al., 2011), and it has been proposed that the ABCA3 transporter-mediated sequestration of thiol-Sb (III) is involved in Leishmania antimony-resistance (Leprohon et al., 2006). However, attempts to overexpress ABCA3 or ABCH1 in a L. infantum WT background did not result in antimony resistance (Leprohon et al., 2006), suggesting that these genes are not sufficient to trigger resistance, which likely constitutes a multifactorial process. In another study, Dodge and colleagues showed that upregulation of the multidrug resistance protein 1 (mdr1) confers resistance to the hydrophobic drug vinblastine by pumping the drug into various compartments of the endomembrane system (Dodge et al., 2004). Furthermore, it has been reported that Sitamaquine, a lipophilic weak base, rapidly accumulates in intracellular compartments of Leishmania parasites called acidocalcisomes (López-Martín et al., 2008). NFV also being a lipophilic weak base (Ford et al., 2004), we postulated it might accumulate also in acidocalcisomes that are known to contain particularly high levels of polyP. Our electron microscopy study and polyP level measurements (Fig. 9E and F) indeed suggest that the increased number of vesicles in NFV-resistant parasite might be acidocalcisomes.

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Modulation of transcripts through gene amplification and gene deletion by homologous recombination is a common mechanism of drug resistance in Leishmania (Beverley, 1991; Ouellette et al., 1991; Chow et al., 1993; Grondin et al., 1993) and this is probably due to the lack of transcription control in the trypanosomatid parasites (Clayton, 2002). Homologous recombination takes place when the repeat sequences of two genes with homologous sequences recombine, leading to the modulation of the expression of other genes in the vicinity. An example of this is the deletion of the folate transporter-encoding gene in the methotrexate-resistant parasite by homologous recombination between two members of the folate biotin transport gene family (Ubeda et al., 2008). We also observed entire groups of gene loci deleted on chromosome 10 by homologous recombination and repeat sequences bordering the deleted gene were involved in these events (Fig. 4). However, this region was not found to be involved in NFV-resistance, given that this region was also deleted from the entire population of the revertant line (data not shown). Indeed, such events are common and repeat sequences are involved in the gross rearrangement of the Leishmania genome. Recently, it has been reported that BRCA2 and RAD51 promote homologous recombination in L. infantum (Genois et al., 2012). Several key genes which have been previously reported as involved in drug resistance were also found to be modulated in NFV resistance. For example, we observed that the gene encoding the glucose transporter (i.e. LinJ36_V3.6550) is downregulated in the NFV-resistant strain (Fig. 3), and Southern blot hybridization confirmed the downregulation of this gene at the genomic level (Fig. 7). Decreased glucose uptake provides metabolic adaptation in glibenclamide-resistant L. amazonensis (Machuca et al., 2006). Furthermore, it has been suggested that antimony-resistant L. amazonensis minimizes the generation of reactive oxygen species (ROS) by downregulating the glucose transporter (do Monte-Neto et al., 2011). NFV generates oxidative stress and caspase-independent apoptosis in L. donovani (Kumar et al., 2010). Therefore, the downregulation of the glucose transporter in the NFV-resistant parasite might be responsible for less glucose uptake and minimization of ROS, which provides for a selective advantage under drug pressure.

Among other genes that are differentially regulated in the NFV-resistant parasite, the genes encoding for Histone H1 (i.e. LinJ27_V3.1070) and Histone H2A (i.e. LinJ29_V3.1850) are upregulated (Fig. 3); such upregulation of histones has been previously associated to drug resistance (Singh et al., 2010). The gene encoding for Heat-shock protein 70 (HSP70) has also been reported to be upregulated in antimony- and arsenite-resistant parasites (Brochu et al., 2004), and we observed its upregulation in the NFV-resistant L. donovani amastigotes. HSP70 acts as a molecular chaperone and protects the cell from a variety of stresses including heat radiation, chemical and biochemical shocks (Hartl and Hayer-Hartl, 2002). HSP70 also protects the cell from oxidative stress (Miller et al., 2000). Given that NFV induces oxidative stress in the sensitive strain (Kumar et al., 2010), upregulation of HSP70 in the NFV-resistant line would be involved in protecting the parasite from greater oxidative damage. Taken together, the modulation of these genes (glucose transporters, histones and HSP70) might be used for the selection of biomarkers in the identification of drug resistant Leishmania phenotypes.

Recently, White and co-workers reported that Leishmania harbours an aspartyl proteinase activity, and that such activity can be blocked by HIV PIs (White et al., 2011). Furthermore, considerable reduction in aspartic peptidase activity was observed when a NFV-resistant clinical isolate of L. chagasi was incubated with the drug (Santos et al., 2012). Thus, it is possible that the mechanism of NFV resistance in the Leishmania parasite is related to the downregulation of the aspartyl peptidase. However, we did not observe modulation of an aspartic peptidase gene. It is possible that the host cell environment poses challenges to Leishmania and that gene modulation events associated with in vivo resistance might differ from resistance ex vivo. It is noteworthy that we observed a diminution of some cysteine peptidase and metallopeptidase genes in the NFV-resistant parasites (Table S1). However, enhanced activity of both cysteine peptidase and metallopeptidase has been previously reported in parasites incubated with HIV-1 PIs (Santos et al., 2009), and we also observed upregulation of other cysteine and metallopeptidase genes in the NFV-resistance parasite (Table S1). Thus, it is possible that NFV-resistant parasites differentially and specifically modulate peptidase expression in order to compensate for NFV action on these enzymes. However, the exact involvement of these different classes of peptidases in the mechanism of NFV resistance in Leishmania remains an open question.

Using CGH and gene expression profiling of whole genome microarrays, we have established that aneuploidy is involved in the establishment of drug resistance in Leishmania axenic amastigotes. The molecular mechanisms involved in NFV resistance are a multifactorial phenomenon and several mechanisms coexist together. Our results suggest that the modulation of key genes, such as the downregulation of the gene encoding the glucose transporter and upregulation of those encoding for histones and HSP70, enable the parasite to adapt physiologically and metabolically in developing the resistance against drugs. This would ultimately lead to the sequestration of NFV in intracellular vacuoles. However, further work
is required to delineate the mechanism involved in the vesicular sequestration of NFV.

Experimental procedures

**Parasite culture and selection of NFV-resistant axenic amastigotes**

*Leishmania donovani* promastigotes field strain 9518 (Ld9518) (Lira et al., 1999; Vergnes et al., 2007) were maintained at pH 7.0 and a temperature of 25°C. Promastigotes were cultured in RPMI-1640 medium (Wisent, St Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS), 5 μg ml⁻¹ hemin (Sigma-Aldrich, St Louis, MO), 25 μmol l⁻¹ HEPES and 2 mmol l⁻¹ NaHCO₃. We obtained axenic amastigotes by switching promastigotes (25°C, pH 7) to a phagolysosomal like environment (37°C, pH 5.6 and 5% CO₂). Axenically grown amastigotes of Ld9518 were maintained at 37°C with 5% CO₂ by weekly sub-passages in MAA/20 medium (Sereno and Lemesre, 1997) at pH 5.6 in 25-cm² flasks. MAA/20 consists of modified medium 199 (Gibco, Life Technologies, Burlington, ON, Canada) with Hank’s salts, supplemented with 0.5% soybean trypto-casein (Pasteur Diagnostics, Marne la Coquette, France), 15 mM D-glucose, 5 mM L-glutamine, 4 mM NaHCO₃, 0.023 mM bovine hemin and 25 mM HEPES at a final pH of 7.0 and supplemented with 20% of FBS. These axenic amastigotes show morphological, biochemical and biological characteristics similar to those of amastigotes isolated in vivo. The anti-HIV-1 PI NFV was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, USA). This compound was resuspended to a concentration of 50 μM in dimethylsulphoxide (DMSO) for a maximum final concentration of 12.5 or 25 μM. Controls consisted of dilutions of DMSO corresponding to those used to prepare the drug solutions. Next, cells were washed 3 times with phosphate-buffered solution (PBS) and lysed, and luciferase activity in the lysates was measured using a luminometer (Varioskan, Thermo Scientific) as previously described (Roy et al., 2000). The experiments were performed in triplicate. The statistical significance of the results was defined by performing one-way analysis of variance with Bonferroni multiple comparison tests. All statistical analyses were performed using Prism software 3.03. P values less than 0.05 were considered statistically significant.

**Microarray design**

The full genome high-density oligonucleotide arrays were described previously (Ubeda et al., 2008). The microarray chip by Agilent Technologies (Mississauga, Canada), includes full genome 60-mer oligonucleotide microarrays for all protein coding genes of the protozoan parasites *L. major* and *L. infantum*. These microarray chips have been used previously in different drug resistance studies of *Leishmania* parasites (Ubeda et al., 2008; Leprohon et al., 2009; do Monte-Neto et al., 2011).

**Genomic DNA preparation and labelling**

Genomic DNA was prepared from 10⁸ amastigotes using DNAzol according to manufacturer’s instructions (Invitrogen). Genomic DNA was then sheared by passing the DNA preparation successively through 22G1 and 27G1/2 needles (Becton Dickinson, Franklin Lakes, USA). Fragmented DNA was then double digested with *Pvu*II and *Mse*I restriction enzymes. Digested and fragmented DNA was further purified using phenol-chloroform phase extraction, followed by ethanol precipitation. For each probe, 8 μg of digested genomic DNA was converted to fluorescently labelled DNA using *Cy₅*- or *Cy₀*-dCTP (Amersham, GE Healthcare, Piscataway, USA), dNTP (Roche Applied Science, Laval, QC, Canada), random hexamers (Roche Applied Science) and the exo⁺ Klenow DNA polymerase (NEB, Ipswich, MA). Fluorescent probes were then purified with ArrayIt columns (Tel-eChem International, Sunnyvale, USA).

**RNA preparation and labelling**

Total RNA was isolated from 10⁸ amastigotes during the mid-log phase using the Illustra RNA Spin Mini kit as...
instructed by the manufacturer (GE Healthcare). The RNA preparation was treated with TURBO DNase (Ambion, Austin, USA) to avoid any genomic DNA contamination. The quality and quantity of the RNA were measured using RNA 6000 Nano Assay chips on a Bioanalyzer 2100 (Agilent Technologies). For each probe, 7 μg of RNA were converted to aminooallyl-dUTP incorporated cDNA using random hexamers (Roche Applied Science) and the Superscript III RNase H Reverse Transcriptase (Invitrogen) according to the manufacturer’s recommendations. Unincorporated aminooallyl-dUTPs were removed from the labelled cDNA using ArrayIt columns. Thereafter, aminooallyl-dUTP incorporated cDNA were coupled to Alexa Fluor 555 or Alexa Fluor 647 (Invitrogen) according to the manufacturer’s recommendations. Fluorescent cDNA probes were purified with ArrayIt columns.

**Microarray hybridization**

The labelled and purified probes from *L. donovani* NFV-resistant or -sensitive parasites were mixed with 200 μg ml⁻¹ salmon sperm DNA (Agilent Technologies), 200 μg ml⁻¹ yeast tRNA (Sigma-Aldrich), 1x blocking agent buffer (Agilent Technologies) and 1x hybridization buffer (Agilent Technologies). Mixed probes were denatured for 3 min at 95°C followed by incubation at 37°C for 30 min. Combined labelled probes were applied on the array under a LifterSlip. Hybridization was performed for 24 h at 65°C, and slides were washed 5 min at room temperature in 0.5× SSC (75 mM NaCl, 7.5 mM Sodium Citrate, pH 7.0), 0.005% Triton X-102 with gentle agitation and subsequently washed 5 min in pre-warmed 0.1× SSC, 0.005% Triton X-102 at room temperature with occasional stirring.

**Microarray data acquisition and analyses**

The G2565CA microarray scanner (Agilent Technologies) was used to detect the fluorescent signal from microarray slides at a resolution of 5 μm as previously described (Leprohon et al., 2009). GenePix Pro 6.0 (Axon Instruments, Union City, USA) was used to quantitate the fluorescence signal intensities of the microarray. Three different RNA or DNA preparations from Ld9518 NFV-resistant or -sensitive amastigotes were analysed including dye-swaps. Normalization and statistical analyses of microarray data were performed using R2.2.1 software with the LIMMA (Linear models for microarray data) 2.7.3 package as previously described (Smyth and Speed, 2003; Smyth, 2004; Smyth et al., 2005). Background corrections were performed using the ‘Edwards’ methods; the ‘within array normalization’ was achieved by loss and ‘between array normalization’ by the ‘quantiles of A’ methods for each array (Yang et al., 2003). Multiple testing corrections were obtained using the ‘false discovery rate’ method with a threshold P value of 0.05. Only statistically significant genes with an absolute log ratio greater than 0.58 (log1.5) were considered as differentially expressed. Gene-Spring GX 3.1 software was used for chromosome by chromosome analysis. The entire data set has been deposited in GEO under the Accession Number GSE42197. The comparative genomic hybridization data are deposited under Accession Number GSE42195 and the RNA expression profiling data under Accession Number GSE42196.

**Quantitative real-time PCR (qRT-PCR)**

Three independent RNA preparations were used for each qRT-PCR experiment. First-strand cDNA was synthesized from 2.5 μg of RNA using random primers (Roche Applied Science) and SuperScript III RNase H Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. The resulting cDNA samples were stored at −20°C until use. Control PCR was performed with GAPDH primers to evaluate the uniformity of cDNA synthesis in different samples. Equal amount of cDNAs were run in triplicate and amplified in 20 μl reactions containing 2× PerfeCta® SYBR® Green Supermix with Low ROX™ (Quanta Biosciences, Gaithersburg, MD), 40 nM forward and reverse primers and 1 μl target cDNA. qRT-PCR was carried out using a rotator thermocycler Rotor Gene (RG 3000, Corbett Research, San Francisco, USA). Initially, mixtures were incubated at 95°C for 4 min and then cycled 40 times at 95°C for 20 s, 55°C for 15 s and 72°C for 20 s. No-template controls were used as recommended. Amplification was normalized to the LinJ18_V3.0630 and LinJ36_V3.0850 genes, for which a highly stable expression was noted in several conditions by different microarray experiments. The quantities of target genes were calculated according to standard curves using the Rotor-Gene Real-Time analysis Software 6.0 (Build 24, Corbett Research). Primers were designed using primer 3 plus (http://www.primer3plus.com/). A comprehensive list of primers used in this study can be found in supporting information (Table S3).

**Southern blot hybridizations**

Genomic DNA was isolated using DNAzol (Invitrogen) according to the manufacturer’s instructions. For quantitative Southern blots, the genomic DNA was digested with appropriate restriction enzymes (i.e. *SacII, BamHI* and *EcoRV*) and migrated overnight at 30 V in a 0.8% agarose gel lacking ethidium bromide. The digested DNA was blotted by capillary transfer with 20× SSC on positively charged nylon membranes (Roche Applied Science). Digoxigenin-labelled probes were obtained using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). Digoxigenin-labelled probes were obtained using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science) according to the manufacturer’s instructions. Southern hybridization and washing were performed following standard protocols (Sambrook et al., 1989). For chromosome copy number determination, signal intensity was quantified using Image J software (http://rsbweb.nih.gov/ij).

**Electron microscopy**

Log phase amastigotes were incubated with 25 μM NFV for the indicated time periods. The cultures were then centrifuged at 2000 g for 5 min, and the cell pellets washed with HEPES-NaCl (pH 7) and fixed with cacodylate buffer (0.1 M, pH 7.4) containing 4% (v/v) -formaldehyde and glutaraldehyde for 12 h at 4°C. The cells were dehydrated with graded ethanol, and embedded in Eponate resin (Pol/Bed 812; Polysciences, Burlington, Canada). Ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 1010 transmission electron microscope.
**NFV accumulation assays**

Nelfinavir-sensitive and -resistant strains of *L. donovani* amastigotes were pelleted during the mid-log phase. For each assay, 5 x 10⁷ cells were washed twice in MAA/20 medium without FBS and re-suspended in 200 μl MAA/20 medium containing 20% FBS. NFV accumulation assays were initiated by adding 200 μl MAA/20 medium supplemented with 25 μM NFV and 250 nM of [³²P] NFV (5 Ci mmol⁻¹) (American Radiolabeled Chemicals, St Louis, MO). The mix was layered over 100 μl of dibutylphthalate (Sigma-Aldrich) in a 1.5 ml microtube and incubated at 37°C. Unincorporated NFV was removed by aspiration and cells were washed once in HEPES-NaCl buffer and the accumulation was measured in a liquid scintillation counter (Beckman LS6000TA). The quantity of incorporated radioactivity was normalized with the number of cells and the background transport was removed by subtracting the accumulation value obtained on ice.

**Measurement of intracellular polyP levels**

We measured the acidicocalcisomal polyP content in NFV-sensitive and -resistant parasite by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (Aschar-Sobbi et al., 2008). Briefly, *L. donovani* axenic amastigotes (2 x 10⁷) were resuspended in 1 ml of PBS and incubated for 10 min at room temperature with DAPI (10 μg ml⁻¹). Cell density was equilibrated in all the samples by measuring the optical density at 600 nm before the fluorescence measurement. After two washes with PBS, DAPI emission spectrum was collected between 450 and 650 nm using 415 nm excitation wavelengths. DAPI fluorescence was recorded with a Varioskan Flash Multimode Reader (Thermo Scientific). The experiments were performed in triplicate. The statistical significance of the results was defined by performing Student’s t-test. Statistical analyses were performed using Prism software 3.03. P values less than 0.05 were considered statistically significant.

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**References**


Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.