3-Hydroxykynurenine, a Tryptophan Metabolite Generated During the Infection, Is Active Against *Trypanosoma cruzi*

Carolina P. Knubel[1], Constanza Insfran[1], Fernando F. Martinez[1], Cintia Diaz Lujan[2], Ricardo E. Fretes[2], Martin G. Theumer[1], Laura Cervi[1] and Claudia C. Motran.[1, *]

[1]Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), CONICET, Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba. Haya de la Torre y Medina Allende. Ciudad Universitaria. Córdoba, Argentina., [2]Instituto de Biología Celular, Facultad de Medicina, Universidad Nacional de Córdoba, Córdoba, 5000, Argentina.

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ABSTRACT: The antiparasitic activity of 3-hydroxykynurenine (3-HK), one of the major tryptophan catabolites of the kynurenine pathway, against both *Trypanosoma cruzi* evolutive forms that are important for human infection, trypomastigotes (Tps) and amastigotes (Am), possible targets in the parasite and the drug toxicity to mammalian cells have been investigated. 3-HK showed a potent activity against Am with IC50 values in the μM concentration range while the IC50 values to cause Tps death was ~6000-times higher, indicating that the replicative form present in the vertebrate hosts is much more susceptible to 3-HK than bloodstream Tps. In addition, 3-HK showed activity against Tps and Am, at concentrations which did not exhibit toxicity to mammalian cells. Ultrastructural analysis and flow cytometry studies indicated that Am and Tps mitochondrion and nuclei contain 3-HK targets. The potency and selectivity of 3-HK, which is generated during *T. cruzi* infection in human and mice, suggest that 3-HK may be a suitable candidate for drug research and development for Chagas disease.

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, represents a major cause of heart disease and cardiovascular-related deaths in endemic areas, and causes a significant economic burden on the affected countries. Approximately 8 million people are infected with *T. cruzi* in Central and South America, and at least 120 million are at risk of infection.[1, 2]

T. cruzi infection is initiated by the entry of trypomastigotes (Tps) into the mammalian host and then the infection of different host cell types. Within host cells, *T. cruzi* Tps becomes into amastigotes (Am) that replicate in the host cell cytoplasm.[3] As immune control of the infection is established and the infection progresses into the chronic phase, the parasites are limited predominantly to heart and the skeletal muscle. The onset of human pathology may be extremely diverse and depends on the parasite biology as well as its relationship with the host.[4]

Although *T. cruzi* infection is an important cause of mortality and morbidity, no vaccines or safe chemotherapeutic agents are currently available. At present, Chagas' disease needs others treatment options since benznidazole and nifurtimox, the only existing current options, were introduced more than forty years ago and are not ideal, given that are not well tolerated, and effective only during short periods of the acute phase.[5, 6]

On exploring the innate mechanisms able to control the experimental *T. cruzi* infection *in vivo*, we found that the activity of indoleamine 2, 3-dioxygenase (IDO), an interferon-inducible enzyme that catalyzes the initial and rate-limiting step of tryptophan (Trp) catabolism through the L-kynurenine (L-Kyn) pathway (Figure S1), is critical for host resistance against this parasite infection.[7] IDO is up-regulated after *T. cruzi* infection in mice and the blocking of IDO activity *in vivo* impaired mice resistance to infection, and exacerbated both the tissue and blood parasite load, and also the infection associated pathology.[7] In addition, in contrast to the observations for other intracellular pathogens which are sensitive to Trp starvation, *T. cruzi* Am and Tps are sensitive to 3-hydroxykynurenine (3-HK) (a L-Kyn downstream metabolite), and the therapeutic administration of 3-HK during the acute phase of the infection decreases parasite load in blood and target tissues and improve the survival of lethally infected mice.[7, 8]

Based on the above considerations we thought that the activity of 3-HK on both *T. cruzi* evolutive forms relevant for mammalian infection justify further studies to evaluate the relative susceptibility of Am and Tps to 3-HK, the possible targets in the parasite and drug toxicity to different mammalian cell types.

To evaluate the activity of 3-HK against Tps, *T. cruzi* Tps were incubated with medium alone or increasing doses of 3-HK for different times, and the percentage of mortality was evaluated by trypan blue exclusion assay. Fig. 1, A-C shows that 3-HK has a dose- and time-dependent trypanocidal effect on *T. cruzi* Tps with the dose of 0.2 μM inducing trypanocidal activity at all studied times (p<0.05). Moreover, the 50% of parasite mortality was reached with doses around of 1,200 μM, 200 μM, or between 20 and 200 μM after 24 h, 48 h or 72 h of treatment respectively. These results are in agreement with our previous ones reporting that Tps exposed to 20 μM of 3-HK suffer morphological changes.[7] As the natural milieu of Tps is mammalian blood, we assayed the effect of 3-HK on Tps in the presence of whole blood. Interestingly, when the assay was performed in 100% of whole blood at 37°C, 20 μM of 3-HK was able to decrease the number of viable Tps over the time, with only 50% of the viable Tps being observed after 96 h of exposure to 3-HK (Figure 1D). Inactivation by blood has already been reported for other trypanocidal drugs, such as naphthoquinones or lysophospholipids which interact with the serum proteins and thus reduce the amount of free compound.[9-11]

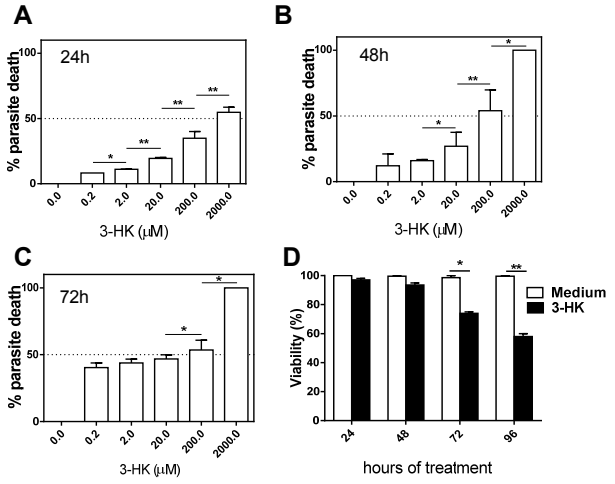
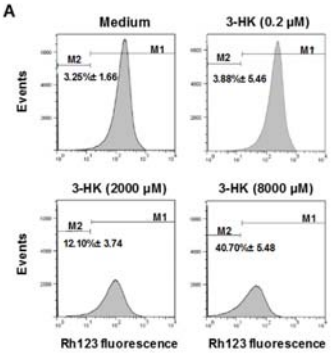


Figure 1. *In vitro* effect of 3-HK on Tps of *T. cruzi*. A-C) Effect of different doses of 3-HK against Tps at 37°C in culture medium. The percentage of parasite death was evaluated by trypan blue assay after 24 h, 48 h and 72 h of treatment. D) Trypanocidal effect of 20 μM of 3-HK on Tps in 100% of whole blood. The percentage of viable parasites was evaluated after 24 h, 48 h, 72 h and 96 h of treatment. Bars represent the means ± SD of three independent experiments. *P<0.05, **P<0.01.

In order to identify target organelles of 3-HK in the parasite and considering that previous ultrastructural studies done in our laboratory have demonstrated that 20 μM of 3-HK is able to induce striking changes in the mitochondrion of Tps [7], we used rhodamine 123 (Rh123) to monitor the mitochondria membrane potential (MMP).[9] Figure 2 shows that the treatment of *T. cruzi* Tps with 3-HK for 24 h caused a dose-dependent increase in the percentage of live parasites that displayed interference in the proton electrochemical potential gradient of the mitochondrion membrane, showing the strong effect at high 3-HK doses (8-10 mM). Only 8 to 12% of the live Tps reduced the MMP when they were treated with 3-HK doses from 0.2 to 4,000.0 μM for 24 h, while ~40% of the live Tps showed reduced MMP when doses of 8.0 and 10.0 mM were used. In contrast, only 3-5% of the untreated live Tps displayed an MMP reduction (Figure 2A). The index of variation (IV) values obtained by treating Tps with increasing concentrations of 3-HK are shown in Figure 2B. It can be noted that doses of 3-HK from 0.2 μM were able to induce mitochondrion depolarization. Therefore, although the IC50 of 3-HK to lyse the Tps is ~6000-times higher, concentrations of 3-HK as low as 0.2 μM are also able to cause organelle damage in the Tps stage. Interestingly, the type of damage observed in 3-HK-treated parasites has been reported with other compounds such as diamidines, reversed amidines and naphtoquinones derivatives.[9, 12, 13]



B

3-HK (μM)	Median	IV
0	114.84	0.000
0.2	81.431	-0.291
2	80.846	-0.296
20	79.112	-0.311
200	76.867	-0.331
2000	73.040*	-0.364
4000	61.5*	-0.461
8000	20.862*	-0.818
10000	19.479*	-0.830

Figure 2. Flow cytometry analysis of *T. cruzi* Tps treated with 3-HK and labeled with Rh123. Tps were treated for 24 h at 37°C with different 3-HK concentrations and then incubated for with PI plus Rh123. The alterations in the Rh123 fluorescence were quantified in the PI negative (live) population by calculating the mean percentages of parasite populations that displayed depolarization of the mitochondrion membrane (M2). A) Histograms show fluorescence intensities of untreated (medium) and 3-HK-treated PI negative Tps. M2: low-fluorescence-intensity peaks. One representative experiment out of three performed is shown. B) Data table shows the index of variation: IV= (MT -MC)/MC, where MT corresponds to the median of fluorescence for treated parasites and MC to that of control parasites. *P<0.05 *vs* untreated parasites.

Next, we evaluated the effects of 3-HK on the replication of the intracellular stage of *T. cruzi*. For that, bone marrow-derived macrophages (Mos) were infected with *T. cruzi* and treated with 3-HK for 24 h. 72 h after the infection the intracellular amastigotes were counted by immunofluorescence (IF) assay, with the results shown in Figure 3. We exposed the infected cells to 3-HK for 24 h to compare the relative susceptibility of both, Am and Tps stages of the parasite, to 3-HK. The treatment significantly reduced the intracellular parasite growth as well as the number of infected Mos (Figure 3 A-C) at all used doses. In addition, a dose-dependent effect on the number of intracellular parasites was observed with doses from 0.2 to 200 μM, while doses higher than 200 μM did not have effects in proportion to the dose (Figure 3). The intracellular anti-parasitic activity of 3-HK evaluated as endocytic index (EI) is shown in Table 1.

Table 1. Effects of 3-HK against *T. cruzi* intracellular amastigotes.

3-HK (μM)	Infected Mos (%)	Endocytic Index
0	26.84 ± 7.81	213.66 ± 58.79
0.2	10.85 ± 7.10***	67.61 ± 60.09**
2	12.40 ± 5.14***	60.59 ± 39.06***
20	8.63 ± 3.57***	51.74 ± 28.17***
200	7.76 ± 1.89***	51.43 ± 17.42***
400	14.66 ± 0.58***	89.81 ± 3.06 *
800	15.10 ± 1.60**	99.39 ± 6.54*
1,200	14.83 ± 0.76**	106.06 ± 2.46*
1,500	18.07 ±0.81**	124.36 ± 33.75*
1,800	14.32 ± 2.27**	110.69 ± 10.10*
2,000	16.31 ± 5.76**	104.34 ± 45.79*

Mos were infected with *T. cruzi* Tps for 24 h. After washing, the cultures were treated with different 3-HK concentrations or left untreated for 24 h. Cells were washed and incubated for another 48 h, fixed, and the number of infected cells and the number of intracellular parasites were counted using an IF assay. The endocytic index (EI) was calculated by multiplying the percentage of infected Mos by the average number of intracellular Am. All values correspond to the means ± SD from four independent experiments. ***P<0.0001; **P<0.001; *P<0.05 by ANOVA 3-HK-treated *vs* untreated cells.

Doses of 3-HK from 0.2 μM affected significantly both the percentage of infected cells and the EI, with this dose reducing the percentage of infected cells by 59% and the EI by 68% compared with infected untreated Mos. Once again, 3-HK concentrations higher than 200 μM did not show any significant reduction in the percentage of infected Mos or EI in proportion to the dose. The IC50 of 3-HK to reduce the number of intracellular Am after 24 h of treatment was around 0.2 μM, while the IC50 of 3-HK necessary to cause the lysis of Tps was ~6000-times higher (~1,200), indicating that the replicative form present in the vertebrate hosts is much more susceptible to 3-HK than bloodstream Tps. Interestingly, the currently available treatments for Chagas disease, Benznidazole or Nifurtimox, are more effective against the blood flagellates than the intracellular Am forms.[14, 15] The fact that 3-HK is more effective against intracellular dividing parasites is an important issue to take in account for the development of drugs against the chronic stage of Chagas disease where parasites are found intracellular in an quiescent stage.[16]

To investigate the morphological changes at the ultrastructural level induced by 3-HK on the Am form of *T. cruzi*, infected Mos treated with 20μM of 3-HK or left untreated were fixed and processed for transmission electron microscopy (TEM). Ultrastructural images of untreated intracellular Am showed typical structures, such as the endoplasmic reticulum, nucleus, mitochondrion, and flagellum (Figure 4A). As was already reported for 3-HK-treated Tps [7], the alterations related to nuclei and mitochondria were the most common and frequent effects induced by 3-HK on intracellular Am. Am treated for 24 h displayed alterations mainly in the mitochondrion which are swollen (Figure 4B, D), and the presence of low electrodense structures in the cytoplasm of the parasite (Figure 4B). In addition, 3-HK induced different level of kinetoplast structure disorganization like as an open and dispersed mesh (Figure 4 C, D). Moreover, most of the treated parasite showed the subpelicullar microtubules, flagellum and flagellar pocket with normal structure (Figure 4 B-D). The treatment of infected cells with 3-HK for 24 h inhibited *T. cruzi* Am replication within host cells, resulting in Mos containing only a few Am, and we did not observe a marked Am lysis (only few Am digested), suggesting that this compound interferes with the proliferation rather than the integrity of the Am and, therefore, the effect of 3-HK on Am stage might be more trypanostatic than trypanocidal. Taking into account that although no sterilizing effect was observed after 3-HK treatment of *T. cruzi*-infected mice, this treatment is effective in reducing both the incidence and severity of the electrocardiogram alterations and also the inflammatory infiltrates and fibrosis.[7] Therefore, the induction of lysis might not be essential for the development of drugs against the chronic stage of Chagas disease.

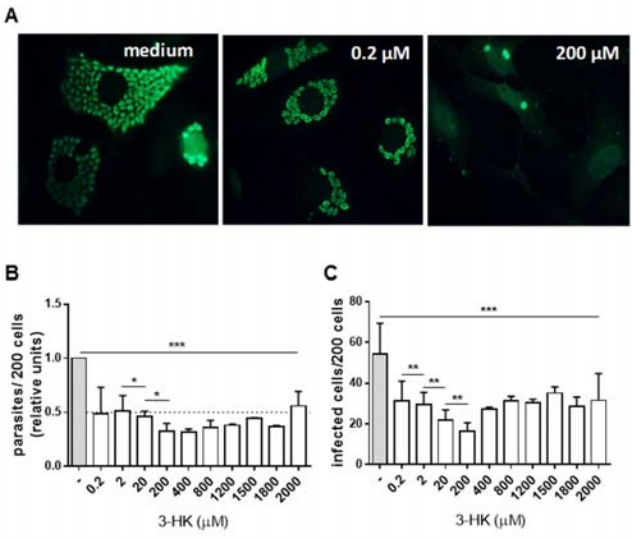


Figure 3. Effect of 3-HK on *T. cruzi* intracellular Am replication. Mos were infected with *T. cruzi* Tps, treated with different 3-HK concentrations for 24 h or left untreated. The number of infected cells and the intracellular parasites were counted by IF after 72 h post-infection. A) Mos were observed at X 1000. Representative fields for 0.2 μM- and 200 μM-treated Tps are shown. B) Bars represent relative units calculated by dividing the number of intracellular parasites per 200 cells in differentially treated cultures by the number of intracellular parasites per 200 cells in medium-treated cultures. Values are averages ± SD from 4 independent experiments. ***P<0.001 vs. medium. * P<0.05. C) Number of infected cells counted in 200 Mos. Values correspond to the averages ± SD from 4 independent experiments. **P< 0.01 and *P<0.05 *vs*. medium.

Finally, we wanted to ensure that 3-HK effects were produced only in the different stages of the parasite and did not involve the host cells. To evaluate 3-HK selectivity, mammalian cell from different origins were treated with different concentrations of the compound for 24 h or 48 h and the

relative toxicity of 3-HK determined by trypan blue exclusion assay. Treatment of PBMC, Swan 71 cell line and Jurkat cell line, all cells from human origin representing different tissue types, with doses of 3-HK from 0.2 to 2000 µM for 24 h did not result in any significant loss of cellular viability (not shown). A similar behavior was observed when Mos or J774 cell line were evaluated (not shown). However, weak but significant effects on cell viability were observed in PBMC and Jurkat cells (lymphocytes) after 48 h of treatment with concentrations of 3-HK from 1,200 µM (Figure 5A). Interestingly, IC50/48 h of 3-HK for bloodstream Tps death was ~6-times lower (200 µM). Next, we treated Mos for 24 h or 48 h with doses of 3-HK from 0.2 to 10,000 µM and analyzed for apoptosis (hypodiploid DNA using PI) by FACS and observed that none of the concentrations used modified the percentages of cells carrying hypodiploid DNA observed in untreated cells (Figure 5B). Therefore, 3-HK exhibited considerable activity against intracellular Am at doses that did not cause host cell damage. After 24 or 48 h of treatment, no damage was observed in Mos by using doses up to 2000 µM, but only 0.2 µM of 3-HK for 24 h (a 10000-fold lower dose) was enough to reduce host cell infection by ~60%.

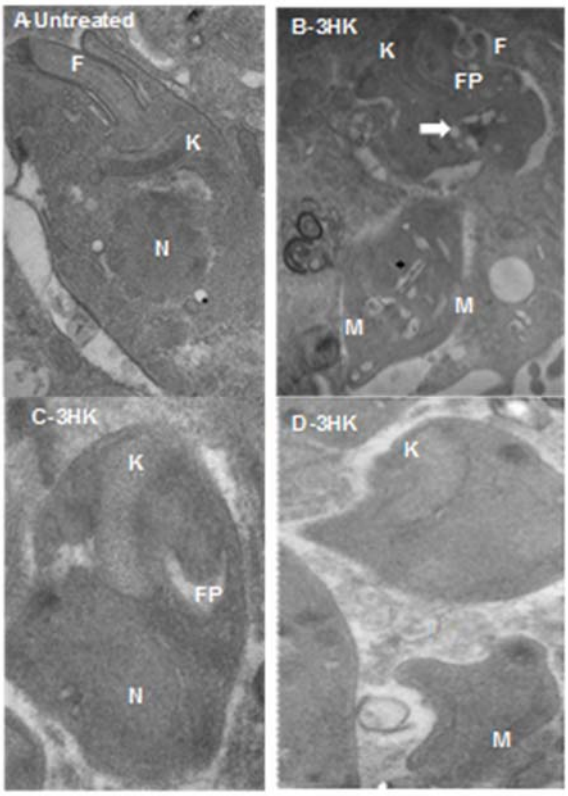


Figure 4. Ultrastructural examination of the intracellular Am into *T. cruzi* infected Mos treated with 3-HK. Mos were infected with *T. cruzi* for 24 h and treated with 3-HK (20µM) for 24 h or left untreated. A) Am showing normal structures. B-D) 3-HK-treated Mos. M, mitochondrion; N, nucleus; F, flagellum; FP, flagellar pocket; K, kinetoplast; low electrodense structures (arrow) Original magnification: A and C 16700X; B, 13000X, D, 13999X.

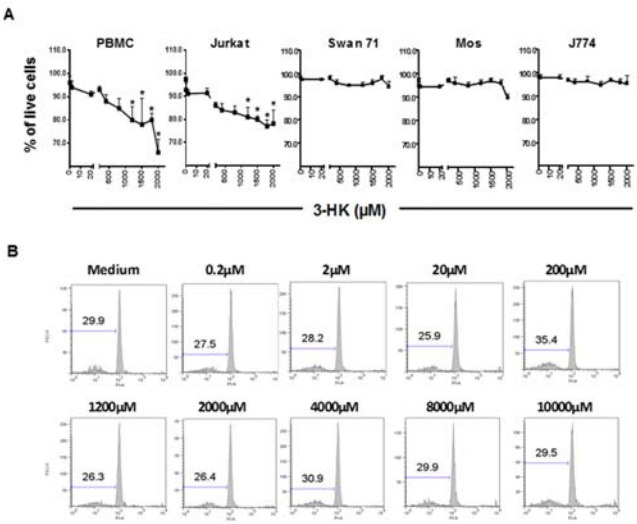


Figure 5. Effect of the 3-HK on mammalian cells. A) Mammalian cells representing different tissue types were treated for 48 h with different 3-HK concentrations or left untreated. Percentage of viable cells was evaluated by light microscopy using the trypan blue exclusion assay after 48 h of treatment. Values represents the means ± SD from 4 independent experiments. **P*<0.05 vs untreated. B) Mos treated with medium alone or different 3-HK concentrations for 24 h were first labeled with FITC-labeled F4/80 antibody and then stained with PI and the cells subjected to hypodiploid DNA content by FACS. The numbers indicate the percentage of cells with hypodiploid DNA content. A representative histogram for each 3-HK concentration is shown.

We have not addressed the mechanism for the trypanocidal action of 3-HK. However, some reports have described the antimicrobial effects of Trp catabolites and we have demonstrated that not only 3-HK, but also 3-HAA and quinolinic acid (QA) are active against *T. cruzi* Am in vitro.[7] 3-HK and its catabolites have been demonstrated to have ~~a strong~~ microbicidal activity against Gram negative bacilli, Gram positive cocci and *C. albicans*, with the IC50 doses to inhibit the microorganism growth ranging from 30 to 137 µM.[17] In addition, picolinic acid (the last breakdown product of the L-Kyn pathway) has direct microbicidal activity, presumably by depriving the microorganisms of the free nutrient iron essential for their growth.[17, 18] Interestingly, 3-HK and 3-HAA have the capacity of generate superoxide and hydrogen peroxide (H_2O_2)[19], such as naphthoquinones[20, 21, 9] with these last compounds inducing similar ultrastructural changes in Tps of *T. cruzi*. H_2O_2 can oxidize ferrous ions from microsomal systems to form hydroxyl radicals, which are the compounds harmful to the parasite.[22] Thus, taking into account that H_2O_2 is toxic for *T. cruzi* and other members of the Trypanosomatidae family[23], substances that increase H_2O_2 generation or decrease H_2O_2 utilization [24, 25] or that significantly accelerate the homolytic breakdown of H_2O_2[26] to form more hydroxyl radicals are potential trypanocidal drugs.[20]

The data show the *in vitro* activity of 3-HK against Tps and Am, at concentrations that exhibit low toxicity to host cells.

In addition, our data indicates that Am are much more susceptible to 3-HK than Tps and suggest that mitochondrion and nuclei from both form of the parasite contain 3-HK targets. The differential susceptibility of both forms of the parasite, the definition of dose-response and the characterization of 3-HK targets are all important data to take in account to evaluate alternative or combined therapies that will probably improve current anti-*T. cruzi* dose regimens. In addition, the potency and selectivity of 3-HK, suggest that this compound may be a suitable candidate for drug research and development for Chagas disease.

SUPPORTING INFORMATION

Figure S1 and experimental proceedings detail. The Supporting Information is available free of charge on the ACS Publications website

AUTHOR INFORMATION

Corresponding Author

*Claudia Cristina Motrán, Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), CONICET, Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba. Haya de la Torre y Medina Allende. Ciudad Universitaria. Córdoba, Argentina. Phone: 54-351-5353851, ext. 3148, Fax: 54-351-433-3048, ext. 3177. E-mail: cmotran@fcq.unc.edu.ar.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

3-HK, 3-hydroxykynurenine; 3-HAA, 3-hydroxyanthranilic acid; Am, amastigotes; EI, endocytic index; F, flagellum; FACS, flow cytometry; FITC, fluorescein isothiocyanate; FP, flagellar pocket; IC50, half maximal inhibitory concentration; IDO, indeleamine 2,3-dioxygenase; IF, immunofluorescence; IV, index of variation; K, kinetoplast; L-Kyn, L-Kynurenine; M, mitochondrion; MC, median of fluorescence for control parasites; Mos, bone marrow-derived macrophages; MT, median of fluorescence for treated parasites; MMP, mitochondria membrane potential; N, nucleus; PBMC, human peripheral blood mononuclear cells; PI, propidium iodide; Rh123, rhodamine 123; SM, subpeliculliar microtubules; TEM, transmission electron microscopy; Tps, trypomastigotes; Trp, tryptophan.

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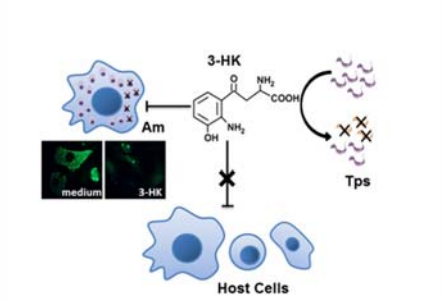
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