Leishmania braziliensis: Cytotoxic, cytostatic and chemotactic effects of poly-lysine–methotrexate-conjugates

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HIGHLIGHTS

- Chemotaxis plays a role in Leishmania differentiation and in parasite–host–cell interaction.
- The two-chamber capillary assay evaluates MTX-conjugates chemotactic properties in *Leishmania*.
- The N-terminal amino acid (Ser or Glu) in the branches affect MTX-conjugates chemotactic activity.
- Attachment sites in MTX-conjugates (α- or γ-groups) determine their chemotactic activity.
- Cytotoxic and cytostatic effects suggest that MTX-conjugates are selective for *Leishmania*.

GRAPHICAL ABSTRACT
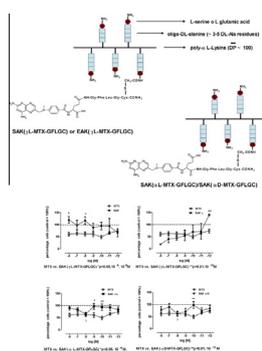
ABSTRACT

Chemotactic responses play a significant role during *Leishmania* differentiation, as well as in the course of parasite–host–cell interaction, a process that precedes a successful infection. The present study uses the modified "two-chamber capillary assay" to quantitatively evaluate the chemotactic properties and the toxic activities of methotrexate containing branched chain polymeric polypeptide conjugates in *Leishmania*. Our results demonstrate that this methodology quantitatively determines the taxis of *Leishmania* towards/against gradients of compounds. They also demonstrate that chemotaxis produced by the polypeptide–methotrexate conjugates depends on specific chemical characteristics. For example, the N-terminal amino acid (Ser or Glu) location at the branch significantly influences the elicited chemotaxis. Furthermore, the use of different attachment sites in the methotrexate conjugates (α- or γ-carboxylic groups) affect their chemotactic activity. Specific cytotoxic activities and cytostatic effects of the conjugates on parasites and on murine and human cells of the macrophage/monocyte system respectively, suggest that these ligands may be used as a group of anti-*Leishmania* substances acting selectively on *Leishmania* and different hosts.

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1. Introduction

Protozoan parasites of the genus *Leishmania* cause a disease with symptoms ranging from self-healing cutaneous lesions to non-healing mucocutaneous and visceral ailments that affect approximately 30 million people worldwide (Pozzo et al., 2009; World Health Organziation, 2010). Of the two morphologically distinct forms of *Leishmania*, the slender, spindle shaped, flagellated promastigote resides in the intestinal tract of the sand fly vector, while the small, non-flagellated, oval-shaped amastigote reside in mammalian host macrophages and other mononuclear phagocytes.

During the final phase of their development in phlebotomine female sand flies, *Leishmania* promastigotes migrate and accumulate in the prosboscis, located at the anterior region of the digestive tube of the insect. The parasites are regurgitated by the sand flies during blood feeding. Once in the skin, promastigotes are engulfed by diverse cell types, where they become amastigotes within the acidic phagolysosome (Molineux and Killick-Kendrick, 1987).

The single flagellum is a fundamental organelle that promotes promastigote attachment to insect host epithelia, plays a key role during cell translation, is crucial for parasite survival in the mammalian bloodstream and guides the final stages of cell division (Gadelha et al., 2007; Rotureau et al., 2009; Forestier et al., 2011).

Chemotaxis is an essential physiological phenomena present in all phyla, which promotes survival of microorganisms and unicellular eukaryotic cells, including *Leishmania*. For example, by means of chemotactic responses, microbes actively search the environment to guarantee their nourishment, proliferation and survival. Therefore, germs evaluate the surroundings and travel towards the attractive environment, and away from repellent, toxic compounds. However, chemotaxis is not the only migratory response at hand in microorganisms; they also react to changes in hydrostatic pressure, light, magnetic fields, osmotic pressure, temperature, etc. (Devreotes and Janetopoulus, 2003).

Small prokaryotic cells (*i.e.*, bacteria) function as "point sensors" when immersed in a chemical gradient. These microorganisms do not distinguish differences in chemical concentrations between their two axial poles, and their migratory behavior is composed of linear swimming paths and tumbling. In contrast, eukaryotic cells are larger and differentiate chemical concentrations along their axial poles (Devreotes and Janetopoulus, 2003). Surface membrane receptors allow all cell types to detect chemical gradients even in quiescent phases. However, signaling mechanisms, such as the network of Che-proteins or G-protein coupled receptors, as well as the effector's organelles (prokaryotic flagella vs. cilia, flagellar vs. ameboid movement), differ between prokaryotic and eukaryotic cells (Van Haastert and Devreotes, 2004; Wang, 2010).

All this means that understanding chemotaxis is essential for the comprehension of the behavior and physiology of motile cells, including *Leishmania*. Chemotaxis is also a fundamental step in the successful interaction between host and parasite, as chemical signals are involved in their mutual recognition, and in the migratory responses that determine infection (Pozzo et al., 2009).

Methotrexate (MTX) is a folate anti-metabolite used since more than 40 years as a potent anticancer agent in cases of leukemia, sarcoma and rheumatic disorders (Kóczán et al., 2002). *Leishmania* parasites are also affected by this compound (Kóczán et al., 2002). With the aim of increasing cell targeting and delivery, MTX has been conjugated to macromolecules including BSA, poly-lysine complexes, high molecular weight carriers like oligotuftsin (Mező et al., 2006; Bai et al., 2008) and polycationic or amphoteric branched chain polymeric polypeptide carriers (Kóczán et al., 2002; Szabó et al., 2008). These MTX conjugates, may also improve drug-targeting and effectiveness of the drug against *Leishmania*

and therefore, could increase selectivity towards the microorganism, reduce the selection of resistant parasites by drug pressure and minimize adverse effects of the treatment. In fact, one MTX-conjugate (ALK) that is known to accumulate selectively in the spleen (Clegg et al., 1990) reduces *Leishmania donovani* infection *in vitro* and in experimental animals (Kóczán et al., 2002).

Various techniques have been described to measure chemotaxis in *Leishmania*. Bray (1983) was the first to propose that *Leishmania* could actively respond to a chemical gradient. Oliveira et al. (2000) and later on Leslie et al. (2002) improved the sensitivity of the Bray's method. Additional sophistications were introduced by Barros et al. (2006), to differentiate, chemotactic from osmotactic responses in *Leishmania*, and the mechanisms involved in this physiological response. Finally, the use of optical tweezers permitted the evaluation of the strength and directionality of the flagellar force in *Leishmania amazonensis* (Pozzo et al., 2009).

Peptide-drug conjugates acting as anti-*Leishmania* agents may target parasites in different stages of their life cycle, including the intracellular amastigote form. Additionally they may affect the viability of host cells. Therefore, to evaluate the activity of the conjugates on the host cells, we determined the cytotoxic effects of conjugates against *Leishmania* promastigotes, their chemotactic potential and their cytostatic effects on murine and human monocytic/macrophages used as reference cells.

To study chemotaxis in *Leishmania* promastigotes we employed the "two-chamber capillary assay", originally developed to measure chemotaxis in the protozoan ciliate *Tetrahymena pyriformis* (Kőhidai et al., 1995). Our results suggest that this improved methodology quantitatively evaluates the taxis of *Leishmania* towards/against gradients of the conjugates. In fact, our data indicate that chemotaxis induced by the MTX-branched chain polymeric polypeptide conjugates depends on specific chemical properties. Their specific cytotoxic activities (on parasites) and cytostatic effects (on cells of the macrophage/monocyte system) suggest that these polypeptide conjugates constitute a group of substances acting selectively on *Leishmania*. Finally, on the basis of our herein presented data we conclude that the evaluation of chemotaxis in *Leishmania* by the "two-chamber capillary assay" is a rapid and reliable screening method that may be helpful also in diagnosis and drug development.

2. Materials and methods

2.1. Materials

The materials used for the synthesis of the conjugates, as well as those used to grow the cells and perform the different biological experiments were of analytical grade and were purchased to Sigma–Aldrich Co (St. Louis, MS, USA).

2.2. Synthesis of poly-lysine-methotrexate conjugates

2.2.1. Chloroacetylated branched chain polypeptide poly[Lys(ClAc$_{0.5}$-X-DL-Ala$_{3.0}$)]

The branched chain polymeric polypeptide, poly[Lys(X-DL-Ala$_{3.0}$)] (where X = Ser (SAK) or Glu (EAK) with a poly-lysine (poly[Lys]) backbone was synthesized as described earlier (Mező et al., 1997). Briefly, poly[Lys] was synthesized by the polymerization of N$^{\alpha}$-carboxy-N$^{\varepsilon}$-benzyloxycarbonyl-lysine anhydride, with an average degree of polymerization of 100 units. After cleavage of the protecting groups, poly[Lys(DL-Ala$_{3.0}$)] (AK) was prepared by grafting short oligomeric DL-Ala side chains; that is, the respective N$^{\alpha}$-carboxyalanine anhydride onto the ε-amino groups of poly[Lys]. The benzyloxycarbonyl-protected amino acid derivative (Z-Ser-OPcp or Z-Glu(OBzl)-OPcp) was coupled to the end of the

side chains of AK by the 1-hydroxybenzotriazole (HOBt)-catalyzed active ester method. The blocking groups were removed completely by the use of hydrogen bromide (HBr) 35% in glacial acetic acid. The composition of the branched chain polypeptides was determined by amino acid analysis. The size and molecular weight of the polymer was calculated from the amino acid composition and the sedimentation analysis of poly[Lys] (Hudecz et al., 1984). Chloroacetylation of SAK and EAK was carried out as follows: 100 mg SAK or EAK were dissolved in 1 mL water; the solution was further diluted with 4 mL of N,N-dimethylformamide (DMF). Chloroacetyl (ClAc) groups were attached at the N^{α}-amino group of Ser or Glu residues by 70 mg chloroacetic acid pentachlorophenyl ester (ClAc-OPcp) dissolved in 5 mL DMF (Mező et al., 2003). The reaction mixture was stirred overnight at room temperature (RT). The solution was dialyzed for 2 days in Visking tubes (cutoff 8000–12,000 Da) against acetic acid 0.1%, and freeze-dried. According to the elemental analysis of chlorine ca. 50% of the side chains of the polypeptides were blocked by ClAc groups.

2.2.2. Mtx-GFLGC-NH₂

The MTX-containing GFLGC peptide was synthesized on Rink Amide 4-methylbenzhydrylamine (MBHA) resin using fluorenylmethyloxycarbonyl chemistry as described previously (Mező et al., 2006). To block the thiol function of Cys a trytyl (Trt) group was used. Additionally, the 9-fluorenylmethoxycarbonyl (Fmoc) group was used to temporary protect the α-amino group of amino acids. MTX was attached to the peptide resin by the use of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and N-Hydroxybenzotriazole (HOBt) reagents (2 equiv each) in the presence of 4 equiv Diisopropylethylamine (DIEA). The peptide was cleaved from the resin with trifuloroacetic acid (TFA) in the presence of an appropriate scavenger mixture at RT for 1.5 h. Three main peaks were observed in the crude mixture. Two peaks in the HPLC chromatogram were identified as the compounds in which the MTX was attached to the pentapeptide through its © γ- or ⟨-α-carboxylic group (γL-MTX-GFLGC and αL-MTX-GFLGC). The third peak proved to be the D-isomer, derived by the racemization of glutamic acid present in the MTX moiety. The compounds were separated by RP-HPLC and characterized by analytical HPLC and ESI-MS.

2.2.3. Conjugation of Mtx-GFLGC-NH₂ to chloroacetylated branched chain polypeptides

All three isomers of MTX-GFLGC-NH₂ were attached to the chloroacetylated SAK polypeptide (ClAc-SAK) by selective ligation through a thio-ether linkage. In the case of the chloroacetylated EAK polymer (ClAc-EAK) only the γ-isomer was conjugated. ClAc-SAK and ClAc-EAK were dissolved in TRIS buffer 0.1 M (pH 8.1) at a concentration of 1 mg mL⁻¹. MTX-GFLGC-NH₂ (1.5 equiv, calculated according to the chlorine content of the polymers) was added to the reaction mixture. These reaction mixtures were stirred for 24 h at RT, afterwards free cysteine was added -in excess- to the solution, to block the unreacted chloroacetyl groups. After 3 h the solution was transferred to Visking tubes (cut off 8000–12,000 Da) and intensive dialysis was performed for 2 days. The conjugates were then isolated by freeze-drying. In all cases 20–25% side chains of the branched chain polypeptides were substituted with the MTX containing spacer according to the amino acid analysis of the conjugates.

2.3. Strains and culture conditions

The reference strain Leishmania (V.) braziliensis (MHOM/BR/LTB300) was kindly provided by Dra. Noris Rodríguez (Instituto de Biomedicina, Universidad Central de Venezuela). Leishmania promastigotes were grown at 26 °C in semisolid blood agar supplemented with glucose-NaCl medium (glucose 1.5%, NaCl 0.85%, weight:vol) until used.

Promastigotes at late log growth phase were collected by centrifugation at 1500 g for 10 min at RT. The medium was decanted and the cells were suspended in buffer A:Hepes 10 mM pH 7.3; NaCl 132 mM; KCl 3.5 mM; CaCl₂ 1 mM and MgCl₂ 0.5 mM. The cells were centrifuged again and the buffer was discarded; the cells were suspended in buffer A up to the desired cell density. Buffer A (osmolality = 288 mOsm/kg) was used as the control solution to test parasite's chemotaxis.

J774.1 murine macrophages and the MonoMac6 (MM6) human monocyte cell line (Wright et al., 1996) were cultured in RPMI-1640 medium, containing 10% FCS, 2 mM L-glutamine, and streptomycin 0.16 mg mL⁻¹ (complete medium) at 37 °C, 5% CO₂ atmosphere. Cells were harvested at confluence.

2.4. Solutions

D-glucose and D-fructose stock solutions were prepared in buffer A and kept frozen at −20 °C until use. MTX and MTX-branched chain polymeric polypeptide conjugate stock solutions were prepared in water and kept frozen at −20 °C until use. Dilutions of carbohydrates (D-glucose 4–100 mM and D-fructose 100–225 mM) and MTX-branched chain polymeric polypeptide conjugates (10⁻¹²–10⁻⁵ M) were freshly prepared for each experiment in buffer A. The solution's osmolality (mOsm/kg) was measured with a single sampler osmometer (The Advanced™ Osmometer Model 3D3; Advanced Instruments, INC. USA), with a straight line reproducibility between 0 and 400 mOsm/kg less than 1 SD. All solutions used in the study were evaluated by this method.

2.5. Standardization of chemotaxic assays

To guarantee the reproducibility and validity of the data to be obtained by the two-chamber capillary assay both, incubation time and osmolality of solutions used were standardized.

The cell density used herein (4×10^7 cell mL⁻¹), is within the range used in the literature (Bray, 1983; Oliveira et al., 2000; Leslie et al., 2002). Since our aim was directed towards the evaluation of chemotactic rather than chemokinetic responses, the maintenance of the concentration gradients was a fundamental characteristic of the method. Therefore the incubation time was selected after analyzing data obtained from experiments done at 15, 30, 45 and 60 min incubation times. The best results were obtained at 30 min; fifteen min incubations were not sufficient to induce a chemotactic response. On the contrary, 45 and 60 min incubations resulted in a significant migration of cells into the inner chamber ($p = 0.001$); however, at these incubation times the gradients tend to disappear and equilibrate. For these reasons, the 30 min ($p = 0.0045$) incubation time was considered as the optimal one.

We validated the chemotactic response of Leishmania braziliensis promastigotes with increasing concentrations of D-glucose and D-fructose. These ligands are good chemotactic agents (Oliveira et al., 2000). The results demonstrated that D-glucose promotes Leishmania migration (25 mM) compared to cells not exposed to carbohydrates. Likewise, D-fructose induced chemo-attractant responses at concentrations in the range of 200–225 mM. Finally, we determined the influence of osmolality in cell migration. Therefore, we analyzed the chemotactic activity of carbohydrate treated cells compared to non-treated cells versus solutions osmolality, at various carbohydrate concentrations (Díaz et al., 2011). Our results suggest that L. braziliensis promastigotes react to osmotic gradients. The response differs between D-glucose and D-fructose. Indeed, in the presence of D-glucose, cell migration was noteworthy when solutions with an osmolality at least 22 mOsm/kg higher than the control solution (288 mOsm/kg)

was used; when D-fructose was used, migration only occurred in solutions where the osmolality was at least 212 mOsm/kg higher than in the control solution (Díaz et al., 2011).

2.6. Chemotactic effect of MTX-branched chain polymeric polypeptide conjugates on L. braziliensis

The chemotactic response of Leishmania promastigotes was determined by a modification of the vertical two chamber capillary assay (Kőhidai et al., 1995; Díaz et al., 2011). Briefly, the tips of an 8-channel-micropipette were used as the inner chamber and the wells of a 96-well plate were used as outer compartments of the two chamber system. The tips were filled with the experimental substance (100 µL control, or increasing concentrations of MTX-branched chain polymeric polypeptide conjugates, 10^{-12}–10^{-6} M). The wells were filled with the L. braziliensis suspension (200 µL, 4×10^7 cells mL^{-1}). The cells were incubated for 30 min. At the end of the incubation time the cells that migrated into the inner chamber were fixed in formaldehyde (2%) diluted in PBS (phosphate buffer 0.05 M, pH 7.2; NaCl 0.9 M). The cells were then counted in a Neubauer haemocytometer. Of note, the changes in osmolality produced by the MTX-branched chain polymeric polypeptide conjugates represented maximal 0.003% of the of the overall solution's osmolality, small enough to guarantee that the assay measures chemotactic responses instead of osmotactic responses.

2.7. Promastigote susceptibility to MTX-branched chain polymeric polypeptide conjugates

Promastigotes (1×10^7 cells mL^{-1}) were seeded in 1 mL blood agar–glucose medium, in the absence of MTX-branched chain polymeric polypeptide conjugates. The cells were incubated for 24 h at 26 °C and further exposed to increasing concentrations of the MTX-branched chain polymeric polypeptide conjugates. The tubes were incubated for three additional days. Each day cell density was evaluated by the use of a Neubauer haemocytometer. MTX was used as a reference compound and positive control.

2.8. Cytostatic effect of MTX-branched chain polymeric polypeptide conjugates

Monocytic cells (J774.1 and MM6) were seeded into 96 well tissue-culture plates in complete medium, at an initial cell density of 5×10^3 cells mL^{-1}, in 100 µL/well final volumes. After 24 h 100 µL either of compounds (dissolved in serum free medium), or serum free medium (for untreated wells) were added to the wells. After a 3–6 h of incubation the cells were washed twice with serum free medium. Afterwards 100 µL fresh complete medium were added to each well. Cells were maintained at 37 °C, 5% CO$_2$ for further 72 h. Survival of cells was then determined by adding 0.36 mg mL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to each well (Mosmann, 1983; Vannier-Santos et al., 2008). After an incubation of 3.5 h the absorbance (at 540 nm) was measured by the use of an ELISA-reader (Labsystems MS Reader). The absorbance at 620 nm was used as reference wavelength. Differences in absorbances (A_{540}–A_{620}) were calculated, and the cytostatic effect was expressed as percentage of impairment of growth compared to that of untreated control cells. The 50 percent inhibitory concentration (IC$_{50}$) was determined by fitting a sigmoid curve to the data points and the calculating X values at Y = 50 using Origin Pro 8.0 software.

2.9. Data analysis

Data on drug susceptibility is expressed as mean values ± standard error of the mean (SEM) of at least three experiments. To determine the concentration that decreases the growth of parasites by 50% (IC$_{50}$) the Graph Pad Prism-5© software was used to fit the data by non-linear regression analysis. Data on chemotaxis is expressed as mean ± SEM of the number of cells that migrated to the inner chamber (n = at least 5 experiments). The statistical significance of differences in migrating cells between cells exposed to MTX-branched chain polymeric polypeptide conjugates and control cells was determined with the Student t test. Statistical analysis of cytostatic assays was performed by Student's t test using the Origin Pro 8.0 at the 95% confidence level (n = at least 5 experiments).

3. Results

Fig. 1 illustrates the two dimensional representations of the MTX-conjugates emphasizing the sterical organization of the different amino acids present in the peptides. It is important to emphasize that the images shows only simple lines; therefore the steric configurations (D, L), although present, are not visible.

The chemotactic response of Leishmania promastigotes was determined by a modification of the vertical two-chamber capillary assay (Kőhidai et al., 1995; Díaz et al., 2011). The setup of the capillary chemotaxis assay used is thoroughly described in Fig. 2. Our results on chemotactic response of Leishmania promastigotes are summarized in Fig. 3. In each panel the results compare the percentage of migrating parasites incubated in the absence (control, expressed by the value 100%) of polypeptide-MTX conjugates, in the presence of MTX and, in the presence of increasing concentrations of a polypeptide-MTX conjugate. The results demonstrate that within the concentration range tested and compared to control conditions, (a) MTX elicited an inhibitory chemotactic response ca. 30–40%, even at concentrations far lower its IC$_{50}$; (b) that EAK (γL-MTX-GFLGC) did not influence significantly the migration of Leishmania parasites within the concentration range herein tested. On the other hand SAK (αL-MTX-GFLGC), elicited a significant negative chemotactic response compared to control conditions, at 10^{-8} M, a concentration far lower than its IC$_{50}$ (as discussed afterwards). Interestingly, the conjugate SAK (αD-MTX-GFLGC) did not affect the viability of the cells at concentrations up to 10^{-6} M (as discussed afterwards) and produced a significant negative chemotactic response at 10^{-6}, 10^{-8}, 10^{-9}, 10^{-11} M. Finally, SAK (γL-MTX-GFLGC) elicited a dual effect compared to control conditions, being chemotactic positive at concentrations lower than 10^{-9} M and chemotactic negative at concentrations higher than 10^{-8} M, near the IC$_{50}$ for this compound (as discussed afterwards). In conclusion, our results indicate that chemotaxis produced by the MTX-branched chain polymeric polypeptide conjugates in Leishmania depends on specific chemical properties and suggest that the terminal amino acid significantly influence the response, as the presence of serine instead of glutamic acid elicited negative chemotaxis, and in the serine conjugates the α-isomers produced a strong negative chemotactic response while γ-isomer exhibited a dual response.

The previous results encouraged us to further test the cytotoxic activity of the MTX-branched chain polymeric polypeptide conjugates in comparison with the cytotoxic activity of MTX in vitro against L. braziliensis. Table 1 summarizes the obtained results. The concentrations of EAK (γL-MTX-GFLGC), SAK (αL-MTX-GFLGC) and SAK (γL-MTX-GFLGC) that produced a 50% reduction in promastigotes growth were one order of magnitude higher than the concentrations of MTX needed, and concentrations up to 5 µM of SAK (αD-MTX-GFLGC) did not decrease the growth of L. braziliensis. The data indicate that L. braziliensis is less susceptible to the MTX-branched chain polymeric polypeptide conjugates than to MTX.
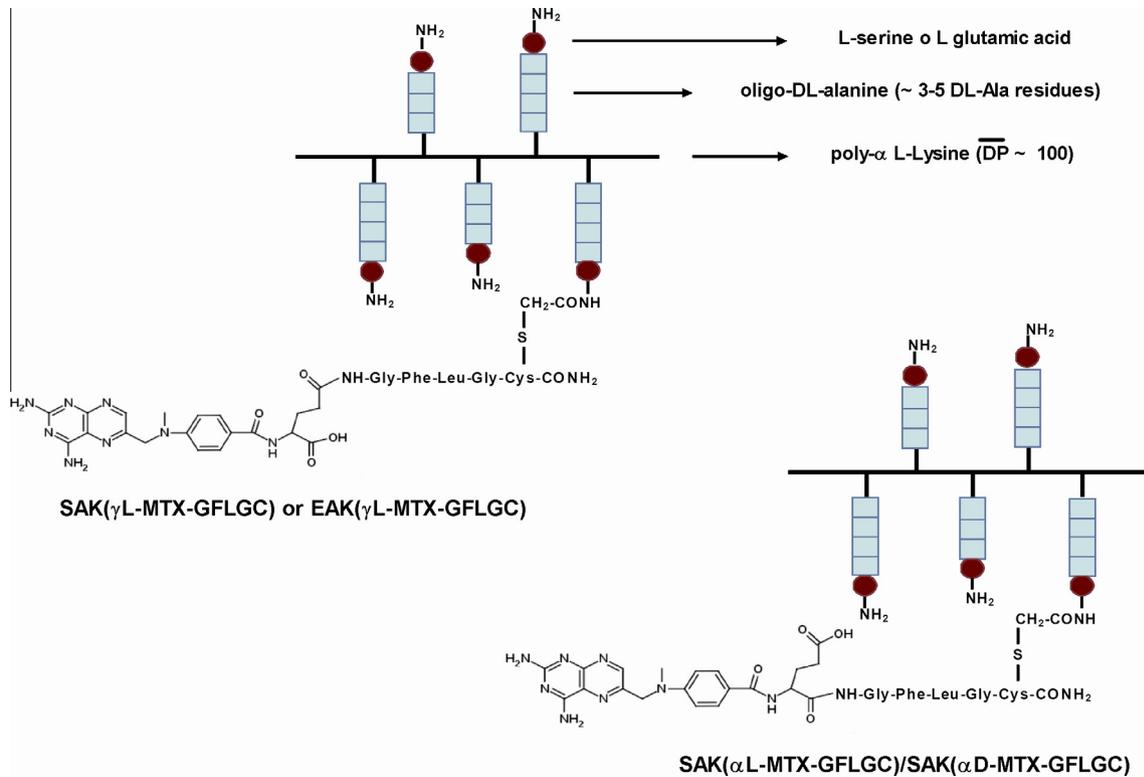
Fig. 1. Two dimensional representations of the MTX-conjugates showing the sterical organization of the different amino acids present in the peptides. The steric configurations (D, L) are not visible.
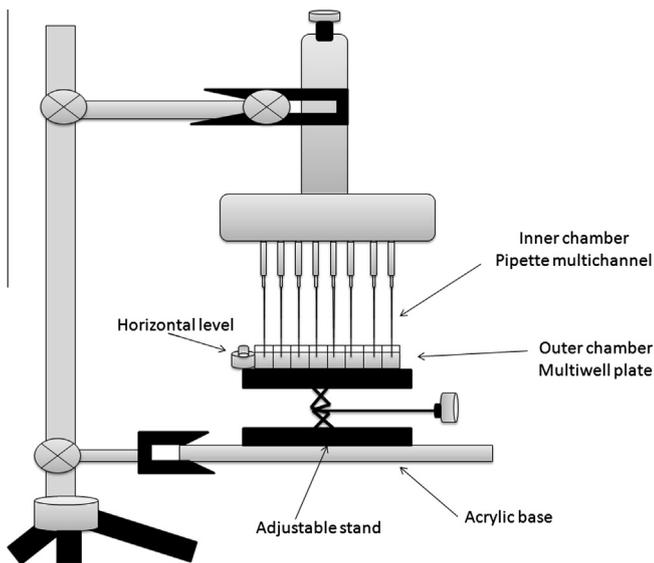
Fig. 2. Setup of capillary chemotaxis assay with multichannel pipette. The two chambers were connected only via capillary entries of the tips containing test substances. The tips of an 8-channel-micropipette were used as the inner chamber and the wells of a 96-well plate were used as outer compartments of the two chamber system. The tips were filled with the experimental substance (100 μL; control, or increasing concentrations of MTX conjugates). The wells were filled with the *L. braziliensis* suspension (200 μL). The cells were incubated for 30 min at room temperature. At the end of the incubation the cells that migrated into the inner chamber were fixed in formaldehyde (2%) diluted in PBS. The cells were then enumerated in a Neubauer haemocytometer.

Our next step was to test the MTX-branched chain polymeric polypeptide conjugates and analyze their cytostatic effects on murine macrophages and human monocytes (Fig. 4). MTX exerted a cytostatic effect on murine macrophages and human monocyte

and both cell lines were susceptible to MTX at concentrations close to those that affected the viability of promastigote cells. On the contrary, the concentrations of EAK (γL-MTX-GFLGC) and SAK (γL-MTX-GFLGC) that were cytostatic to murine macrophages and human monocytes were much higher (>2000 fold) than those that affected the viability of promastigotes. As a result, the SI between murine macrophages and human monocytes, and promastigotes were extremely high, especially for the MM6 cells reaching values near 50,000. This all means that EAK (γL-MTX-GFLGC) and particularly SAK (γL-MTX-GFLGC) seem to be especially selective towards promastigotes when compared with human cell lines.

4. Discussion

Many studies in the literature analyze chemotaxis and other taxis performed by cells and micro organisms (Rao et al., 2008; Barros et al., 2006). The capillary assay, first developed by Pfeffer (1888) and later improved by Adler (1973), is a commonly used quantitative method useful for this purpose. Herein we combined the two chamber and the capillary assays, a modification initially developed by Kőhidai et al. (1995) to measure chemotaxis in *Tetrahymena pyriformis*.

We have previously standardized and validated the method for *Leishmania* parasites and have found that 30 min incubation time, and strict controls of the osmolality of solutions are critical parameters that should be carefully controlled (Díaz et al., 2011). There we demonstrated that (a) incubation times should be kept at 30 min to evaluate chemotactic rather than chemokinetic responses; (b) that this methodology allowed us to corroborate previous results meaning that D-glucose 25 mM is chemo-attractant to the parasites (Oliveira et al., 2000; Leslie et al., 2002) and that D-fructose increase cell migration at much higher concentrations (200 and 225 mM) with dramatic changes in osmolality.
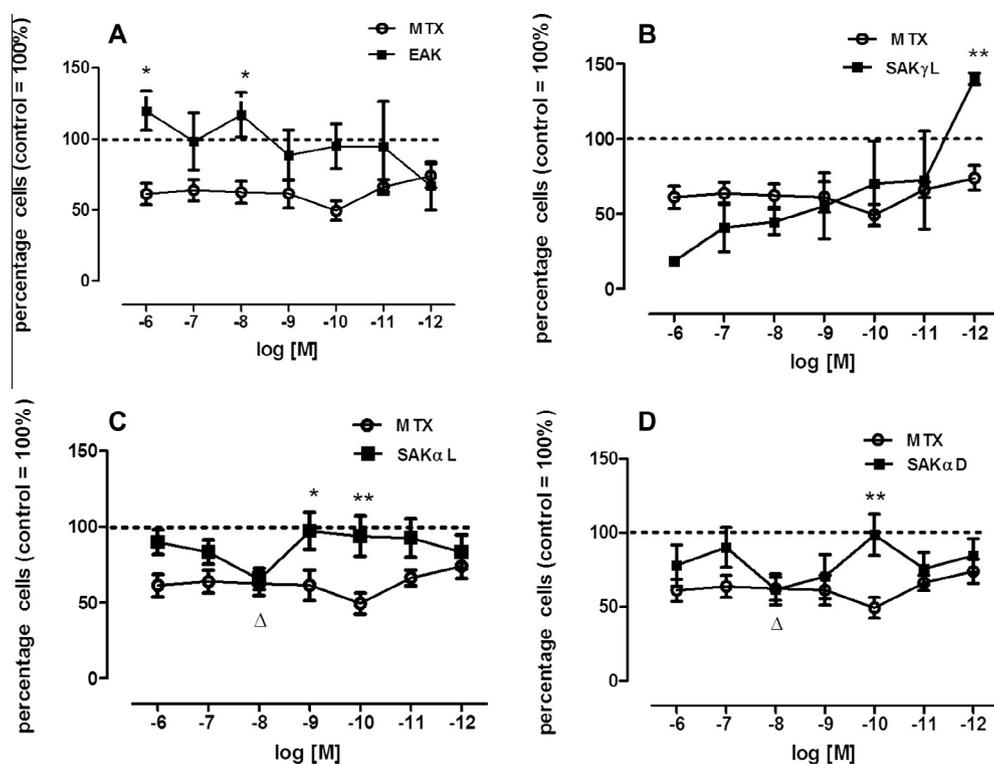
Fig. 3. Concentration dependence of chemotaxis elicited by MTX-branched chain polymeric polypeptide conjugates. *L. braziliensis* migration was affected by MTX-branched chain polymeric polypeptide conjugates: EAK (γL-MTX-GFLGC, upper panel left) did not affect parasite migration, differences were not significant between control and any concentration (MTX vs. EAK 10^{-6}, 10^{-8} M $*$ $p < 0.05$); selected concentrations of SAK (γL-MTX-GFLGC, upper panel right) significantly affected parasite migration (control vs. 10^{-6}, 10^{-8}, 10^{-12} M, $p < 0.05$) (MTX vs. SAKγL 10^{-12} M $**p < 0.01$); selected concentrations of SAK (αL-MTX-GFLGC, lower panel left) (control vs. 10^{-8} M, $p < 0.05$) (MTX vs. SAKαL 10^{-9} M $*p < 0.05$, 10^{-10} M $**p < 0.01$) affected parasite migration; and selected concentrations of SAK (αD-MTX-GFLGC) affected parasite migration (control vs. 10^{-6}, 10^{-8}, 10^{-9}, 10^{-11} M, $p < 0.05$) (MTX vs. SAK$\alpha\delta$ 10^{-10} M $**p < 0.01$). δ = not significant.

In the results presented herein we used 25 mM glucose as a positive control for our assays. The aim of cell targeting and delivery of compounds may increase drug selectivity, reduce the appearance of drug resistance and minimize drug adverse effects. In this regard, the conjugation of branched chain polymeric polypeptide as carriers for MTX may improve drug-targeting and increase MTX effectiveness against *Leishmania*, as has been demonstrated for *Leishmania donovani* infection *in vitro* and in experimental animals (Kóczán et al., 2002). Our aim in the present work has been to evaluate the effect of MTX-branched chain polymeric polypeptide conjugates on the migration of *Leishmania* promastigotes as a first step to design compounds that may interrupt the host-cell-parasite interaction. Therefore, herein we have analyzed the chemotactic capacities of some MTX-branched chain polymeric polypeptide conjugates on *L. braziliensis*. These compounds influence chemotaxis and survival of eukaryotic unicellular model organisms like *Tetrahymena pyriformis* (Szabó et al., 2008). Within the concentration range tested, MTX elicited an inhibitory chemotactic response. However, EAK (γL-MTX-GFLGC) did not influence the migration of *Leishmania* parasites, although the serine-MTX conjugate SAK (αL-MTX-GFLGC) elicited a significant negative chemotactic response at concentrations far lower than their IC_{50}. Interestingly, SAK (αD-MTX-GFLGC) did not affect the viability of the cells at concentrations up to 10^{-6} M and still elicited a significant negative chemotactic response at very low concentrations. Finally, SAK (γL-MTX-GFLGC) had a dual effect being chemotaxic positive at concentrations lower than 10^{-9} M a concentration far lower than the IC_{50} for this compound.

Our results indicate that the chemotactic responses to these conjugates depend on specific chemical properties. That is, (a) the N-terminal amino acid in the branches of the polymeric poly-

peptide -serine (SAK) instead of glutamic acid (EAK)-determines a significant chemotactic response; (b) in the serine conjugates, the αD-isomer elicited a strong chemotactic (negative) response without killing the parasites, while the γL-isomer elicited a strong chemotactic (positive) response at concentrations that did not kill the parasites. Previous results demonstrate that branched polypeptides with poly-L-backbones are essentially nontoxic and do not affect macrophage chemotaxis, the *Leishmania* host cell (Szabó et al., 2008). They also demonstrate that SAK conjugates, as well as EAK conjugates are incorporated into non-adherent and adherent cells only at very high concentrations (Szabó et al., 2008). Therefore, although studies on the internalization of the herein analyzed MTX-branched chain polymeric polypeptide conjugates have not been done, we may suggest that they can be used as non-toxic "neutral" drug carriers. This all means that they might be extremely useful to decrease host-cell parasite interaction, especially if they contain a terminal serine amino acid and are αD-isomers.

The monocyte-macrophage cell system is considered the preferential host for *Leishmania*. For this reason we have tested the MTX-branched chain polymeric polypeptide conjugates to analyze their cytostatic effects in these potential target cells. Our results demonstrate that EAK (γL-MTX-GFLGC), a MTX-branched chain polymeric polypeptide conjugate that does not influence parasite migration, and SAK (γL-MTX-GFLGC), a MTX-branched chain polymeric polypeptide conjugate that has a strong dual effect on parasite migration, need concentrations 138–50,000 fold higher to be cytostatic to vertebrate model murine and human cells. This result confirms previous data that demonstrate that branched polypeptides, especially those with poly-L-backbones are essentially nontoxic to mammalian cells (Szabó et al., 2008). Interestingly, both vertebrate cell lines express a differential susceptibility to the conjugates.

Table 1
In vitro activity of MTX-branched chain polymeric polypeptide conjugates on *L. braziliensis* and murine macrophages and human monocyte.

Compound	L. braziliensis $IC_{50} \times 10^{-6}$ [M] (Confidence intervals)	J774.1 $IC_{50} \times 10^{-6}$ [M] (Confidence intervals) (SI)	MM6 $IC_{50} \times 10^{-6}$ [Ml] (Confidence intervals) (SI)
MIX	0.2 (0.129–0.332)	0.06S (0.0167–0.277) 0.34	3.43 (1.25–943)
EAK (yL-M TX-GFLGQ)	1.15 (0.784–IM)	1181.00 (876–1630) 1027	28,000 (640–1,21,000) 24,347
SAKy (yL-M TX-GFLGQ)	107 (1.56 2.74)	138 (76.5–252) 67	44,900 (16,500–122000) 21,691
SAK aL (aL-MTX-GFLGQ)	4.64 (1.63–13.2)	ND	ND
SAK αD (αD-MTX-GFLGQ)	>5.00	ND	ND

ND = not determined, SI = selectivity index obtained by dividing the IC_{50} against mammalian cell to the against promastigotes.
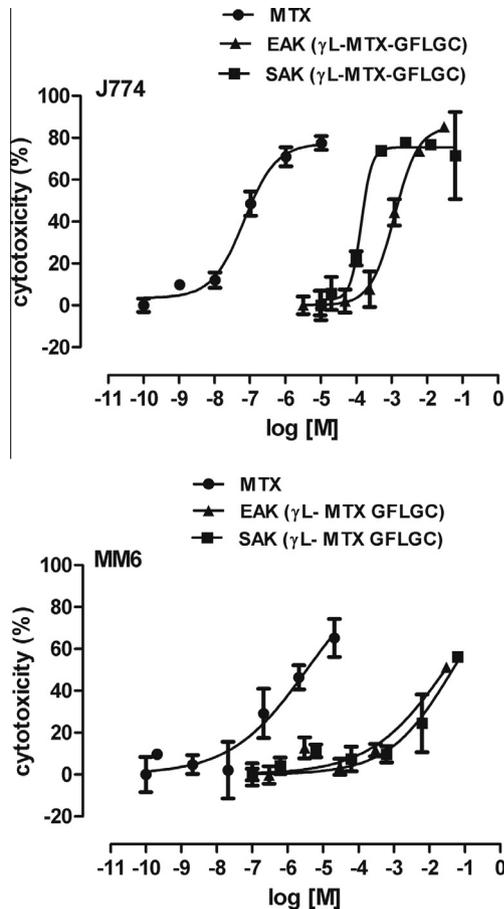
Fig. 4. Dose response curves showing the effects of the two representative substances EAK (γL-MTX –GFLGC), SAK (γL-MTX-GFLGC) and MTX in J774.1 and MM6 cells. Macrophages were seeded into 96 well tissue-culture plates. After 24 h compounds or serum free medium (for untreated wells) were added to the wells. After a 3–6 h of incubation the cells were washed twice with serum free medium and complete medium were added to each well. Survival of cells was then determined 72 h later.

Differential responsiveness of murine *versus* human cells supports the idea of a novel MTX-branched chain polymeric polypeptide conjugates with selective anti-*Leishmania* activity and species specific host specificity. The results presented above support this assumption and suggest the presence of a highly selective signaling system despite slight changes in conformational properties of the ligand (see diverse effects of SAK and EAK conjugates in monocytes).

Understanding host-parasite interactions in *Leishmania* is essential to identify fundamental steps involved in the successful interaction, mutual recognition, and migratory responses that determine infection by the parasite. On the basis of the herein presented data we thus conclude that this technique is a novel, rapid and reliable screening method to evaluate chemotaxis in *Leishmania*. The method described and standardized, guarantees cellular integrity, do not cause damage to the cells and therefore permits the comprehension of complex behaviors as is the case of chemotaxis involved in the initial steps of *Leishmania* parasite infection.

Since research in chemotaxis is essential for understanding host-parasite interactions as occurs in *Leishmania* invading its hosts, the study of this cellular response could be helpful to identify fundamental steps involved in the successful and mutual recognition, and in the migratory responses that determine infection. This identification can help to develop alternative and successful treatments for this serious disease. In fact, some of the herein presented results suggest that to decrease host-cell parasite interaction the ideal branched chain polymeric polypeptide-MTX conjugate should contain a terminal serine amino acid and should be an αD-isomer. Further studies in this direction, to understand fundamental steps involved in the successful interaction between host and parasite, in their mutual recognition and in the migratory responses that determine infection may help to develop new alternatives for control and treatment for this serious disease.

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