Methotrexate Conjugate with Branched Polypeptide Influences *Leishmania donovani* Infection in Vitro and in Experimental Animals[†]

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Methotrexate (MTX) has been coupled to various structurally related, polycationic (poly[Lys(DL-Ala$_m$)] (AK), poly[Lys(Ser$_i$-DL-Ala$_m$)] (SAK), poly[Lys(DL-Ala$_m$-Leu$_i$)] (ALK)), or amphoteric (poly[Lys(Glu$_i$-DL-Ala$_m$)] (EAK)) synthetic branched polypeptides containing poly[L-Lys] backbone by the aid of BOP reagent. The average degree of MTX incorporation was found to be dependent on the charge properties of the polymer. Under the experimental conditions used, the molar substitution ratio achieved was higher for polycations (25%) than for the amphoteric polypeptide (10%). We have studied the effect of polycationic polypeptides on *Leishmania donovani* infection. Results demonstrated that MTX conjugates in which the drug is covalently attached to carrier have pronounced leishmanicid activity. In this communication we showed that (a) a branched polypeptide−methotrexate conjugate with a polycationic carrier (ALK) increases the effect of MTX against *Leishmania donovani* infection in mice; (b) the covalent bond between the carrier and methotrexate is essential for both in vivo and in vitro activity; and (c) the number of *Leishmania donovani* parasites in infected macrophages are markedly reduced in conjugate treated animals. In vitro observation might also indicate that the MTX conjugate exhibits an effect through an uptake by macrophages which is different from that of the free drug.

INTRODUCTON

Methotrexate (MTX,[1] L-4-amino-N^{10}-methylpteroylglutamic acid), a folate antimetabolite, has been in clinical use for more than 35 years. It is a potent anticancer agent of proven benefit in the treatment of acute leukemia, osteogenic sarcoma (*1*), and of rheumatological disorders (*2*−*4*). Recently its inhibitory potential has been demonstrated against a group of intracellular parasites (*Leishmania*) of macrophages (*5*). Visceral leishmaniasis or kala-azar in man is caused by the protozoan parasite *Leishmania donovani*, which proliferates intracellularly within the mononuclear phagocytes of the infected host (*6*).

Methotrexate has been linked to various macromolecules such as BSA, mannosylated-, galactosylated-, or glucosylated-BSA (*7, 8*), mannosylated-HSA (*9*), or maleylated-BSA (*10*), for delivery into cells containing *L. donovani* parasites or to polylysine for studying the mechanism of action of cellular uptake by pinocytosis in mice and human tumor cell lines (*11*−*14*).

The methods of conjugation of methotrexate to these carriers have involved predominantly the glutamic acid moiety. Activation of α- and γ-carboxyl groups has been achieved in situ in the presence of the carrier using water soluble carbodiimide like EDC (*15*) or by ester formation with *N*-hydroxysuccinimide (*11*). (None of the above methods reported have been used for selective derivatization of α- or γ-carboxyl groups of MTX (*15*).)

MTX was also one of the first antitumor drug attached covalently to high molecular weight carriers to improve the therapeutic index by site-specific targeting and/or by changing the pharmacological properties of MTX to provide controlled release. It has been demonstrated that the biodistribution of branched polypeptide attached drugs (e.g., MTX, daunomycin, amylorid) can be strongly modified by the charge and side chain structure of the carrier resulting in elevated and more prolonged blood levels, slower drug excretion, and a longer half-life (*16, 17*).

For the investigation of the conjugate's leishmanicid activity we have prepared a new set of methotrexate-branched polypeptide conjugates (*18*) (Figure 1). In these compounds MTX molecules are attached to polycationic poly[Lys(DL-Ala$_m$)] (AK), poly[Lys(Ser$_i$-DL-Ala$_m$)] (SAK), poly[Lys(DL-Ala$_m$-Leu$_i$)] (ALK) or amphoteric poly[Lys-(Glu$_i$-DL-Ala$_m$)] (EAK) polypeptide carrier by amide bonds. Branched polypeptides with the general formula poly-[Lys(X$_i$-DL-Ala$_m$)] (XAK) or poly[Lys(DL-Ala$_m$-X$_i$)] (AXK), where $i < 1$ and $m \sim 3$, have been used as biodegradable (*19*) macromolecular carriers for targeting antitumor

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[1] Abbreviations (in order of appearance in the text): MTX, methotrexate; EAK, poly[Lys(Glu$_i$-DL-Ala$_m$)]; SAK, poly[Lys(Ser$_i$-DL-Ala$_m$)]; ALK, poly[Lys(DL-Ala$_m$-Leu$_i$)]; AK, poly[Lys(DL-Ala$_m$)]; $\overline{DP}_n$, number average of the degree of polymerization; PBS, phosphate-buffered saline, pH 7.3; $\overline{DS}$, average degree of substitution; MTX-SAK, poly[Lys(MTX$_j$-Ser$_i$-DL-Ala$_m$)]; MTX-ALK, poly[Lys(MTX$_j$-DL-Ala$_m$-Leu$_i$)]; MTX-AK, poly[Lys(MTX$_j$-DL-Ala$_m$)]; MTX-EAK, poly[Lys(MTX$_j$-Glu$_i$-DL-Ala$_m$)]; BOP benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HOBt, 1-hydroxybenzotriazole.

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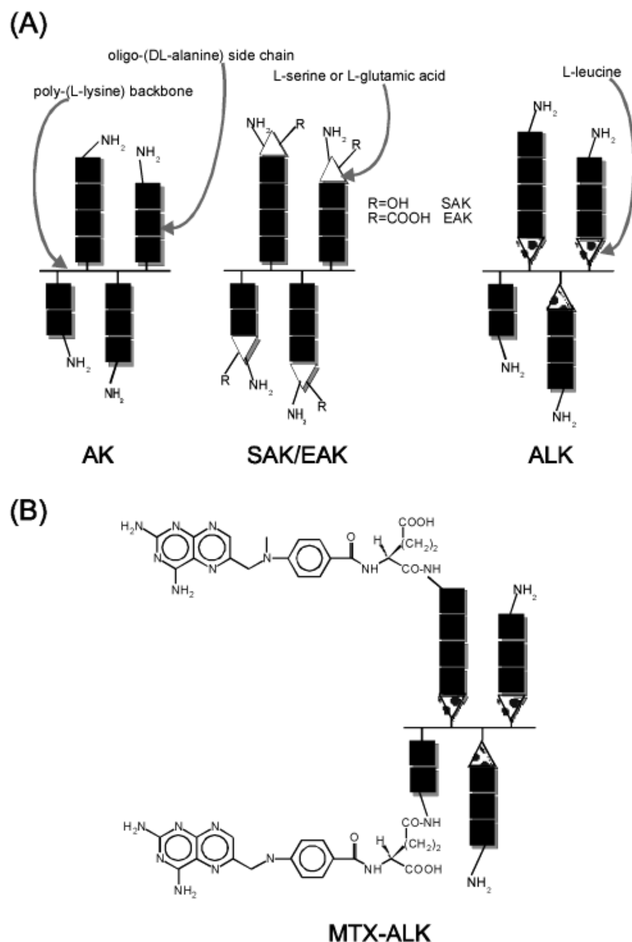

Figure 1. Schematic representation of branched polypeptide−MTX conjugates: poly[Lys(MTX$_j$-DL-Ala$_m$-Leu$_i$)] (MTX−ALK).

agents (*16−25*), radioligands (*26, 27*), immunomodulatory peptides (*28*), or constructing synthetic antigens with T or B cell epitopes (*29−31*).

In this paper we describe the improved synthesis of MTX conjugates with selected polycationic and amphoteric polypeptides and the results of comparative studies against *Leishmania donovani* infection using these compounds. These conjugates have been tested and compared with that of the free drug and of free carrier in the control of *L. donovani* infection in BALB/c mice. We have also investigated the mechanism of leishmanicid activity in mouse macrophages in vitro following their treatment with MTX, MTX + branched polypeptide mixture, and MTX-branched polypeptide conjugates.

EXPERIMENTAL PROCEDURES

Abbreviations used in this paper follow the rules of the IUPAC/IUB Commission of Biochemical Nomenclature (*32*) in accord with the recommended nomenclature of graft polymers (*33*).

Materials. Methotrexate was obtained from Lederle Laboratories (Gosport, U.K.); *N,N*-diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), and trifluoroacetic acid (TFA) were Fluka products (Buchs, Switzerland), while benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) was purchased from Bachem (Bubendorf, Switzerland). Hydroxylamin, glacial acetic acid, diethyl ether, and dimethylformamide (DMF) were purchased from REANAL (Budapest, Hungary). DMF was stored over molecular sieve. Branched polypeptides used in these studies were prepared in our

Table 1. Characteristics of Branched Polypeptides

polypeptide	code[a]	amino acid composition[b] Lys	Ala (m)	Ser/Leu/Glu (i)	$\overline{M_w}$[c] (±5%)
poly[Lys(DL-Ala$_m$)]	AK	1.00	2.06	−	16 700
poly[Lys(Ser$_i$-DL-Ala$_m$)]	SAK	1.00	4.08	0.92	28 800
poly[Lys(Glu$_i$-DL-Ala$_m$)]	EAK	1.00	5.00	0.90	41 500*
poly[Lys(DL-Ala$_m$ -Leu$_i$)]	ALK	1.00	3.00	0.97	34 800

[a] Code of branched polypeptides is based on one letter symbol of amino acids. [b] Determined by amino acid analysis after hydrolysis in 6 M HCl at 105 °C for 24 h. [c] Calculated from the number average degree of polymerization of polylysine ($\overline{DP}_n$ =60 ± 2 or $\overline{DP}_n$ = 90 ± 2*) and from the amino acid composition of branched polypeptides.

laboratories as described earlier (*19−21*). The characteristics of these compounds are summarized in Table 1. Polylysine with $\overline{DP}_n$ = 60 ± 2 or $\overline{DP}_n$ =90 ± 2 (EAK) and $\overline{M_w}/\overline{M_n}$ =1.65 ± 0.15 was used.

Animals. Inbred mice (BALB/c strain) were obtained from the National Institute of Nutrition, Hyderabad, India. Female mice, 6−8 weeks old and of 20−25 g body weight were used in these experiments.

Parasites. The *Leishmania donovani* strain (BI2302) was isolated from the bone marrow material of a kala-azar patient. The strain was maintained in vivo through serial passages in hamsters (*35*).

METHODS

Coupling of Methotrexate to Branched Polypeptides. Coupling was performed with BOP in the presence of 1-hydroxybenzotriazole (HOBt) and DIEA in water−DMF (1:9, v/v) mixture. Briefly: Branched polypeptide ALK (10 mg, 0.24 μmol) was dissolved in 0.5 mL of deionized water, and the clean and viscous solution was diluted with 4.5 mL of DMF (c = 2 mg/mL). Dried methotrexate (5.62 mg, 11.0 μmol) (0.5 mol calculated for amino group of side chains of the polymer), 4.88 mg (11.0 μmol) of BOP reagent, 1.7 mg (11.0 μmol) of HOBt, and 2.75 μL (22 μmol) of *N,N*-diisopropylethylamine (DIEA) were dissolved in 2 mL of DMF and the reaction proceeded for 10 min at 4 °C. The solution with preactivated MTX was added to the solution of branched polypeptide. The mixture was stirred at room temperature and allowed to react for 12 h at RT in the dark. The final concentration of polypeptide was 1.53 mg/mL. The input molar ratios of MTX to polypeptide were 0.1:1, 0.3:1, and 0.5:1. Since the relative amounts of α- and γ-coupled material are unknown, the error introduced by assuming no effect of coupling, although probably small, is not known accurately. After conjugation, the solvent was removed in vacuo, and the resulting oil was triturated with 5 mL of diethyl ether three times. The product was dissolved in 1 mL of glacial acetic acid and diluted with 3 mL of water. The solution was placed in Visking tubing (molecular mass cutoff 12000−14000 Da, Fisons, Loughborough, UK) and dialyzed extensively against 1.0% acetic acid for 48 h. The average degree of molar substitution ($\overline{DS}$) was estimated by amino acid analysis and spectrophotometrically at λ = 373 nm assuming that the extinction coefficient of methotrexate at λ = 373 nm is 7800 M^{-1} cm^{-1}. Analytical data on the conjugates are presented in Table 2.

Analysis of Ester Bound Methotrexate to Poly-[Lys(Ser$_i$-DL-Ala$_m$)]. Ser-containing conjugate poly[Lys-(MTX$_j$-Ser$_i$-DL-Ala$_m$)] (MTX-SAK) was treated with 1.1 M hydroxylamine (0.1 M final concentration) in 0.1 M carbonate buffer (pH 9.0) at 37 °C overnight, similarly

Table 2. Chemical Characterization of MTX-Branched Polypeptide Conjugates

conjugate	code[a]	$\overline{DS}_j$[b] (%)	$\overline{M}_w$[c] (±5%)
poly[Lys(MTX$_j$-DL-Ala$_m$)]	MTX-AK	26.3	24 800
poly[Lys(MTX$_j$-Ser$_i$-DL-Ala$_m$)]	MTX-SAK	25.1	36 200
poly[Lys(MTX$_j$-Glu$_i$-DL-Ala$_m$)]	MTX-EAK	10.2	53 200*
poly[Lys(MTX$_j$ -DL-Ala$_m$-Leu$_i$)]	MTX-ALK	25.0	37 800

[a] Code of conjugates is composed of the abbreviation of branched polypeptide and of methotrexate attached. [b] Average degree of substitution determined from the amino acid analysis after hydrolysis in 6 M HCl at 105 °C for 24 h. [c] Calculated from the number average degree of polymerization of polylysine ($\overline{DP}_n$ = 60 ± 2 or $\overline{DP}_n$ = 90 ± 2*) and from the amino acid composition of conjugates.

to the method of Endo et al. (*34*) with some modifications (*39*). The samples were run on RP-HPLC as described below.

RP-HPLC Analysis of MTX-Branched Polypeptide Conjugates. The HPLC system consisted of one Model 600 ternary gradient pump and controller, a Model 490E programmable multi-wavelength UV−visible detector, a Model 717 autosampler, and an in-line degasser (all from Waters, Milford, MA). All injections were made by the autosampler. Data were recorded and processed with Millenium manager software. A 15 cm × 4.6 mm column with a spherical 5 μm silica (300 Å pore size) with a C18 hydrophobic bonded phase were used (Ultrasphere, Beckman, CA). The mobile phase consisted of 0.1% (v/v) TFA in HPLC grade water (eluent A), and 0.1% (v/v) TFA in 80% (v/v) acetonitrile−water mixture (eluent B). Gradient elution was used at 1 mL/min flow rate; the B content of the eluent was increased from 5% to 60% between 5 and 35 min. All samples were dissolved in distilled water and were filtered before analysis using 0.45 μm Spartan 13 (Schleicher and Schuell, Dassel, Germany) filters. UV absorbance was monitored at λ = 373 nm for MTX containing samples and at λ = 214 nm for free polypeptide samples. The injected volume was selected so that the absorbance value at the peak maximum was below 1.0 absorbance unit. Quantitative analysis of conjugates was performed using peak-area measurement calibrated with appropriate standards. All analyses were carried out at ambient temperature.

Amino Acid Analysis. The amino acid composition of branched polypeptides and their MTX conjugates was determined by amino acid analysis using a Beckman (Fullerton, CA) model 6300 amino acid analyzer. Prior to the analysis, samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

In Vivo Treatment of *L. donovani* Infected BALB/c Mice with MTX Alone or in Combination with MTX Conjugates. Groups of four to five mice were infected with *L. donovani* amastigotes (about 2 × 10^7 parasites/0.1 mL/animal) via the intracardial (ic) route. Different groups of animals received different types of treatment as per the following protocol. For initial experiments groups of animals were treated with MTX (50, 100, or 200 μg/injection/animal) by the intraperitoneal (ip) route on every alternate day starting from day 10 following the infection. Five such injections were given and animals were sacrificed on day 28 following the infection, i.e., 10 days after the last injection. The degree of parasitaemia in the sacrificed animals was determined by microscopic counting of the number of amastigotes in their liver impression smears stained with Giemsa. At least 500 nucleated cells were screened for the purpose and results are expressed as the number of amastigotes/100 cells (*36*).

Other groups of infected animals were similarly treated with five doses of MTX covalently coupled to different branched polypeptides (poly[Lys(MTX$_j$-DL-Ala$_m$)] (MTX-AK), MTX-SAK and poly[Lys(MTX$_j$-DL-Ala$_m$-Leu$_i$)] (MTX-ALK)) so that the administered compounds contained equivalent amounts of MTX (100 μg/injection/animal). Whenever required, groups of infected mice were also treated with unconjugated branched polypeptides or with a mixture of MTX and branched polypeptides using a similar protocol. The control group of infected mice received only normal saline (0.9% NaCl) injections. All mice (treated or control) were sacrificed on day 28, and parasitemia in their liver was determined microscopically as described above.

In Vitro Treatment of *L. donovani* Infected Mouse Macrophages with MTX Alone or in Combination with Branched Polypeptides. Antileishmanial activities of MTX or branched polypeptide ALK or their combinations were determined in vitro by using mouse macrophages infected with leishmania parasites (*37*). For this, peritoneal exudate cells were collected from mice pretreated with 2% (w/v) thioglycolate solution. Washed cells were resuspended in RPMI-1640 medium containing 10% heat inactivated fetal calf serum (RPMI−FCS) and overlaid on glass cover slips taken in 35 mm diameter plastic Petri dishes. Dishes were kept at 37 °C in air containing 5% CO_2 for 4 h to allow attachment of cells. Following washing, cells remaining attached to the cover slips were further incubated overnight with RPMI−FCS. Next, *L. donovani* promastigotes freshly harvested from a log phase culture of the parasite grown in a liquid culture medium (*38*) were added to the Petri dishes in a cell-to-parasite ratio of about 1:10. After incubation for 6 h at 37 °C, free parasites were removed by washing and the Petri dishes were incubated with RPMI−FCS containing appropriate doses of MTX (25 μg/mL of incubation medium) or ALK (108 μg/mL) or their mixture or poly[Lys(MTX$_j$-DL-Ala$_m$-Leu$_i$)] (MTX-ALK) conjugate. The control dish (with parasitized macrophages in cover slips) contained neither drug nor conjugates. Following incubation of the dishes for a period of 72 h, cover slips containing parasitized macrophages were taken out, fixed, stained with Giemsa, and examined microscopically to determine the number of intracellular parasites per macrophage. At least, 100 macrophages were screened in each cover slip for the purpose.

RESULTS

Synthesis and Chemical Characterization of MTX-Branched Polypeptide Conjugates. The coupling of MTX to branched polypeptide was achieved by the BOP reagent-based activation method in which one carboxyl group of the molecule was linked to the α amino group of the side chains of branched polypeptide AK, SAK, ALK, or EAK to provide covalent α-amide type bonding (Figure 1). The carboxyl groups of MTX were activated by equimolar BOP and HOBt in situ using a tertiary amine (DIEA). It should be noted that no precipitate was observed during the synthesis of the conjugate under these conditions. The MTX-XAK/AXK preparations were triturated followed by dialysis, and free MTX content was assessed by reversed-phase HPLC. The amino acid composition of purified conjugates was determined by amino acid analysis. Considering the amino acid composition of the free carrier and of the Glu content of MTX the average degree of molar substitution ($\overline{DS}$) was calculated. Characteristic values of the conjugates (MTX content, $\overline{M}_w$) are summarized in Table 2. The results presented in this table indicate that the amount of MTX

incorporation into the polypeptide conjugate depends mainly on the charge properties of the terminal amino acid residue of the side chain. The average molar substitution ratio was in the range of 10.2−26.3% expressed as % of modified side chains in the conjugates. Interestingly enough no marked differences have been observed among the respective $\overline{DS}$ values of polycationic polypeptides ($\overline{DS}$ for MTX-AK 26.0%, for MTX-SAK 25.1%, and for MTX-ALK 25.0%), while in case of MTX conjugate with amphoteric EAK only 10.2% of the side chains were substituted at 0.5:1 input MTX:EAK molar ratio. These values indicate that 10−26% of the free amino groups of branched polypeptide was modified by MTX. Consequently, all MTX conjugates studied still preserved the polycationic or amphoteric character of the branched polypeptide used.

Under these conditions the active ester derivative of MTX could react with the hydroxyl group of the Ser residue producing an ester linkage between MTX and branched polypeptide SAK. The ester bond can be cleaved by hydroxylamine (*34, 39*). To verify the absence of ester-linked MTX two sets of MTX-SAK conjugate samples were investigated. Conjugates before and after hydroxylamine treatment were analyzed by RP-HPLC using two wavelengths to access free drug in the MTX conjugate preparations. The retention time for MTX, absorbing at $\lambda = 373$ nm, was found to be 10.1 min. Free SAK was detected at $\lambda = 214$ nm at the void volume, whereas the t_R value for MTX-SAK was 14.5 min at both $\lambda = 214$ and 373 nm, indicating the presence of covalently bound MTX. Quantitative analysis, performed on a reversed-phase HPLC column (pore size of 300 Å, gradient elution), indicated that less than 0.01% of free MTX besides the total MTX content in the conjugate samples can be detected using peak-area measurement calibrated with appropriate standards. It should be noted that no free MTX was detected in the MTX-SAK samples before hydroxylamine treatment. These data are in accord with our earlier observation that conditions for the preparation of conjugates highly influence the extent of ester bound formation between OH group and active ester derivative of MTX. The lack of free MTX in MTX-SAK samples suggests that the formation of the amide linkage between MTX and branched polypeptide is favored in a DMF−water 9:1 (v/v) solvent mixture.

After analyzing the purity of freshly synthesized compounds, their stability was investigated. Typical chromatograms of free MTX and of MTX-SAK conjugate containing free MTX as a control are shown in Figure 2. The stability data of conjugate samples obtained after 4−180 days storage at 4 °C. No free MTX was detected (data not shown).

Treatment of *L. donovani* Infected Mice with MTX Alone or in Combination with Branched Polypeptides. In preliminary experiments, different groups of *L. donovani* infected mice were treated with different doses (50−200 μg/injection/animal) of MTX, and their liver parasite load was estimated. About 20%, 36%, and 47% reduction of their liver parasite load was demonstrable following treatment with 50 μg, 100 μg, and 200 μg/injection/animal, respectively (five injections administered as per the protocol described in the Materials and Methods section). A dose of 100 μg of MTX was subsequently chosen for all subsequent experiments for the determination of any additional benefit, which may be derived from the combination therapy of MTX and MTX conjugates.

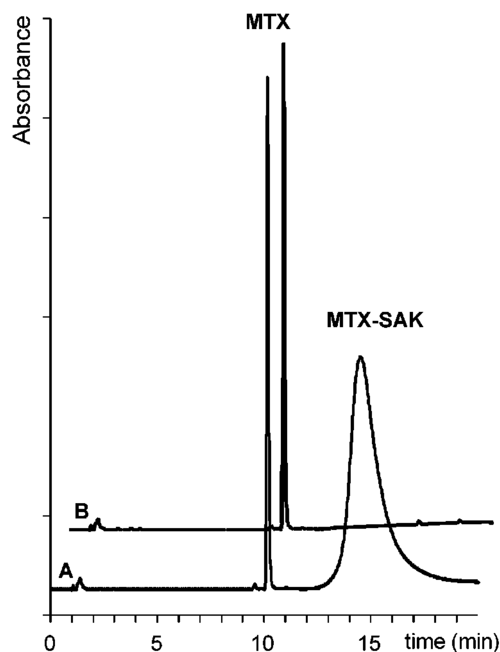

Figure 2. RP-HPLC chromatograms of a mixture of poly[Lys-(MTX_i-Ser_i-DL-Ala_m)] (MTX−SAK) conjugate and MTX (A) and of free MTX (B).

Initially, different groups of *L. donovani* infected mice were treated with MTX branched polypeptide conjugates (MTX-AK, MTX-SAK, and MTX-ALK). It should be noted that due to the high tendency of gel formation under experimental conditions MTX-EAK conjugate was not investigated. All three preparations showed significant levels of antileishmanial activity (data not shown). However, the conjugate MTX-ALK produced most encouraging data (>90% reduction in the number of parasite). Therefore, detailed studies were undertaken with this conjugate and with the corresponding branched polypeptide, ALK.

Table 3 shows the effect of treatment of leishmania infected mice with free MTX as well as in its conjugated form with ALK. About 42% reduction in the liver parasite burden was noted in the MTX treated group of animals as compared to those of untreated control group, and the difference was statistically significant ($0.01 < P < 0.05$). Treatment of animals with MTX-ALK conjugate, however, led to more marked reduction (∼95%) of their liver parasite burden as compared to those in the control group and the difference was statistically highly significant ($P < 0.001$). The MTX-ALK conjugate treated group had also significantly ($0.001 < P < 0.01$) less number of liver parasites than that of the MTX alone treated group. Further experiments were carried out to determine the antileishmanial effect of ALK treatment alone or in the form of a mixture with MTX. Results presented in Table 3 demonstrate that treatment of *L. donovani* infected mice with free ALK did not induce any significant ($P > 0.1$) level of reduction of their liver parasite burden. On the other hand, treatment of animals with the MTX+ALK mixture produced about 35% reduction in the parasitemia as compared to that of the control group and the decrease was statistically significant ($0.001 < P < 0.01$). However, the MTX+ALK mixture treated group failed to show any significant difference ($P > 0.1$) in their liver parasitemia as compared to that of the MTX alone treatment group.

Treatment of *L. donovani* Infected Mouse Macrophages in Vitro with MTX Alone or in Combination with the Branched Polypeptide ALK. The effect

Table 3. Reduction of Parasite Burden in the Liver of *L. donovani* Infected Mice Following Treatment with MTX (alone) or in Conjugation with ALK

treatment group[a,b]	no. of parasites/ 100 nucleated cells	mean ± SE	statistical significance[c]
I. control	119.9	115.2 ± 4.5	
($n = 5$)	124.3		
	123.1		
	103.9		
	104.6		
II. MTX	89.1	66.2 ± 14.6	$0.01 < P^* < 0.05$
($n = 5$)	32.6		
	110.9		
	53.0		
	45.5		
III. ALK	112.4	103.7 ± 5.5	$P^* > 0.1$
($n = 5$)	102.2		
	102.6		
	124.1		
	77.6		
IV. ALK+ MTX	84.7	75.6 ± 4.1	$0.001 < P^* < 0.01$
mixture	67.6		$P^{**} > 0.1$
($n = 5$)	92.9		
	62.3		
	51.4		
V. ALK-MTX	12.5	6.0 ± 1.9	$P^* < 0.001$
($n = 5$)	4.6		$0.001 < P^{**} < 0.01$
	7.3		
	1.5		
	3.9		

[a] n = number of animals. [b] Animals were treated with MTX (100 μg/injection/animal), with ALK (432 μg/injection/animal) or ALK+MTX (a mixture of 100 μg of MTX and 432 μg of ALK/injection/animal) or ALK−MTX (100 μg of MTX conjugated to 432 μg of ALK/injection/animal). [c] *Compared to group I. **Compared to group II.

of in vitro treatment of parasite infected mouse macrophages with MTX alone or in combination with ALK was studied, and results are presented in Table 4 and Figure 3. It may be seen that treatment of macrophages with MTX (alone) (Figure 3B) or in the form of a mixture with ALK (Figure 3C) induced only marginally significant ($0.05 < P < 0.1$) reduction in the intracellular parasite level while ALK (alone) failed to show any reduction of this kind ($P > 0.1$). On the other hand, considerable (about 54%) decrease of the parasite load was noted following MTX-ALK conjugate treatment (Figure 3D vs Figure 3A), and the reduction was significant ($0.001 < P < 0.01$) as compared to that of the control. The MTX-ALK conjugate was also found to be more efficient than MTX + ALK mixture or MTX alone ($0.01 < P < 0.05$) in reducing the number of amastigotes within the macrophages.

DISCUSSION

Synthetic effort with soluble carriers to discover alternate means to introduce MTX into cells could lead

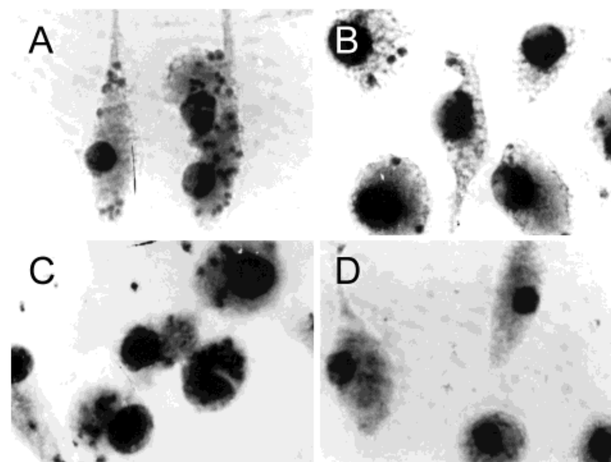

Figure 3. Effect of treatment of *L. donovani*-infected mouse macrophages in vitro with MTX (B), MTX + poly[Lys-(DL-Ala$_m$-Leu$_i$)] (ALK) mixture (C) and poly[Lys-(MTX$_j$-DL-Ala$_m$-Leu$_i$)] (MTX−ALK) conjugate (D). (A) show infected macrophages without any treatment (control). Microscopic magnification: 330×.

increased and selective uptake of the drug and thus more beneficial therapeutic effect. For the construction of macromolecule−drug conjugates it is important to provide rational basis to the selection of the proper carrier. To this end we have previously reported data on the synthesis, biodistribution and in vitro cytotoxicity of the antimetabolite drug methotrexate-branched polypeptide conjugates using osteogenic sarcoma cell line (*16*). In this contribution we extended our studies by investigation of antileishmanial effect of MTX conjugates in experimental animals. For this a new set of branched polypeptide-based conjugates was prepared by an improved synthetic procedure. After covalent coupling of MTX to the α-amino groups of AK, SAK, or ALK conjugates with a similar average degree of molar substitution the overall charge of the compounds remains positive.

It is believed that positive charge density is required for uptake of MTX−linear poly(α-amino acid) (e.g., polylysine, polyornitine) by various mouse or human tumor cells (*11, 13*). A mode of action of the conjugates involves binding to the cell-surface, subsequent endocytic internalization, localization in the lysosomal system, and degradation by lysosomal enzymes to liberate the drug (*12, 13, 40*). This hypothesis was supported by experimental results using polylysine-coupled MTX and L929 mouse fibroblast (*12*) and mouse mammary tumor MM46 cells (*40*) in vitro. MTX attached to poly(D-lysine) directly or through a Leu-Ala-Leu-Ala tetrapeptide spacer between the drug and the ε-amino groups of poly(D-lysine) was also investigated. No effect was observed with MTX-poly(D-lysine), while the conjugate containing MTX at the

Table 4. Effect of Treatment of *L. donovani* Infected Mouse Macrophages in Vitro with MTX, ALK, and Their Combinations

treatment group[a]	number of parasites/macrophage (after 72 h of treatment) expt. 1	expt. 2	expt. 3	expt. 4	mean ± SE	statistical significance[c]
I. control	3.9	3.3	3.2	5.0	3.8 ± 0.7	
II. MTX	3.2	2.3	2.2	3.6	2.8 ± 0.3	$0.05 < P^* < 0.1$
III. ALK	3.0	2.8	n.d.[b]	3.9	3.2 ± 0.3	$P^* > 0.1$
IV. ALK+MTX (mixture)	2.8	2.4	n.d.	3.2	2.8 ± 0.2	$0.05 < P^* < 0.1$
V. ALK-MTX	2.1	1.7	1.3	2.2	1.8 ± 0.2	$0.001 < P^* < 0.01$
						$0.01 < P^{**} < 0.05$
						$0.01 < P^{***} < 0.05$

[a] Macrophages were treated with MTX (25 μg/mL of culture), or ALK (108 μg/mL) or ALK+MTX mixture (25 μg/mL of MTX and 108 μg of ALK/mL) or ALK−MTX (c) (25 μg of MTX conjugated to 108 μg of ALK/mL). [b] n.d. = not determined. [c] *Compared to Group I. **Compared to Group II. ***Compared to Group IV.

N-terminal α-amino group of the Leu-Ala-Leu-Ala unit exhibited potent cytotoxicity, indicating that the spacer is cleaved in the secondary lysosomes (*40*). In addition, the cytotoxicity of a terner conjugate in which MTX-Leu-Ala-Leu-Ala was coupled to monoclonal antibody specific for MM46 cells was not inhibited by TPP, an inhibitor of the membrane active transport system for MTX (*40*). Taken together these data suggest that the transport pathways of MTX and of MTX−carrier conjugates are different and independent (*12*). Trouet et al. (*41*) has demonstrated that a daunorubicin conjugate with Ala-Leu-Ala-Leu spacer and succinylated BSA carrier was stable in serum but was cleaved by lysosomal hydrolases. These findings could be adopted for the speculation on the mechanism of action of MTX-ALK conjugate with marked antileishmania activity observed in vivo and in vito.

It has been described that MTX attached to mannose−BSA strongly inhibits the growth of *Leishmania donovani* inside macrophages. This conjugate was 100 times more active than the free MTX. In contrast MTX conjugated to BSA or other nonspecific neoglycoproteins such as galactose−BSA and glucose−BSA have leishmanicidal effects comparable to that of the free MTX (*7*). Further results indicated that mannose−BSA based MTX conjugate binds specifically to mannose-specific receptor of macrophages and is internalized and degraded in lysosomes, releasing the active drug to act on *Leishmania* parasites (*8, 9, 42*). These data suggest that the uptake mechanism of mannose-containing conjugates is related to the presence of mannose receptor of infected macrophages. On the other hand, our findings clearly indicate that MTX conjugates without a mannose moiety could also be introduced into macrophages.

Recently it has been documented that *Leishmania* and other trypanosomatid protozoa require reduced pteridines (pterins and folates) for growth, suggesting that inhibition of these pathways could be targeted for effective chemotherapy (*43*). Findings suggest that successful antifolate chemotherapy in *Leishmania* will have to target simultaneously both dihydrofolate reductase (DHFR) and pteridine reductase 1 (PTR1). It is attractive to speculate on the mechanism of action of branched polypeptide attached MTX. On the basis of published data outlined above and our own observations reported in this communication, it is likely that MTX released from MTX-branched polypeptide conjugate might act as an inhibitor of enzymes involved in the unusual pteridine metabolism in this lower eukaryote.

CONCLUSION

These studies have indicated that synthetic branched polypeptides can be considered as potential candidates for constructing suitable conjugates for methotrexate delivery into *Leishmania donovani* infected macrophages. In this communication we showed that (a) a branched polypeptide−methotrexate conjugate with a polycationic carrier (ALK) increase the leishmanicid activity of MTX in mice; (b) the covalent bond between the carrier and methotrexate is essential for the effect; and (c) the number of *L. donovani* parasites in infected macrophages are markedly reduced in conjugate treated animals. The latter observation might indicate that the MTX conjugate exhibits its effect throught an uptake by macrophages which is different from that of the free drug. Further comparative studies are in progress to clarify the mechanism of action.

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