In vitro study of Methotrexate- polypeptide effect on Leishmania donovani Replication rates

*Raheem J.M. & **Ayad Q. mahdi

Abstract

(MTX) has been supported by the polypeptide (insulin). the degree of MTX incorporation into Methotrexate polypeptide (1:0.5) the molar substitution ratio was applied in the experimental reaction . the spectrophotometers were used for characterization of MTX-polypeptide product. The in vitro coupling of MTX-polypeptide are shown to be an inhibitant of L.donavani growth. The growth rate of parasites decreased in parallel with increasing the concentration of MTX- polypeptide (0.02, 0.04 and 0.06 mg/L) & lowering as compared to the control group. The growth rate is counted during period of as long as 72 hrs. The maximum decline in growth rate was obtained in the third day at concentration of 0.06 mg/L of MTX-polypeptide. There is a significant difference ($p<0.05$) in replication rates of promastigotes among the different concentrations of MTX- polypeptide (mainly at 0.06 mg/L).

Key words: Methotrexate-polypeptide, Leishmania donovani, in vitro, insulin, replication rates

Introduction

Infection by Leishmania gives rise to a variety of clinical manifestations classically labeled as visceral, cutaneous, & mucocutaneous Leishmaniasis,cutaneous leishmaniasis is the most common form and chemotherapy is the usual therapeutic approach against this disease[1].the clinical manifestations of cutaneous leishmaniasis result from the interaction of factors including ,parasite species , site of inoculation and host immune status[2].

Drug production is an approach aimed at drug targeting and decreasing the undesired effects. Different peptide hormones were used for treatment of some cancers; also they are used as carrier molecules for different chemotherapeutic agents. Methotrexate (MTX, L-4-amino-N^{10}–methylpteroyl-glutamic acid), a folate antimetabolite has been in clinical use for more than 35 years. It is coupled to various structurally related, polycationic or amphoteric polypeptides [3]. Methotrexate 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 Methotrexate to provide controlled release [4].

In order to interfere with the normal development of an intracellular parasite, a drug must reach the compartment where the parasite lives; death of the parasite then occurs, either directly or through various cell-killing mechanisms in the host cell. The triggered by the active drug complex life cycle of Leishmania & the intracellular nature of some of its developmental stages make such a task more difficult.

Furthermore, as some species of Laishmania migrate to various tissues, they can be associated with all types of Leishmanial diseases [5]. Although pentavalent antimonials like sodium antimony gluconate (SAG) are the age-old conventional therapy for visceral Leishmaniasis increasing resistance (VL), in more recent times, to SAG has emerged as a major barrier in the treatment of VL [6] . Methotrexate is a potent anticancer agent of proven benefit in the treatment of acute leukemia, osteogenic sarcoma [7], and of rheumatological disorders [8,9]. And used in severe psoriasis and even as an abortifacient in combination with misoprostol [10]. Recently its inhibitory potential has been demonstrated against a group of intracellular parasites (Leishmania) of macrophages [3]. It has been demonstrated that the biodistribution of branched polypeptide attached drugs(e.g., Methotrexate , 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[3,4]. In other experimental studies Methotrexate has been used to induce in vitro resistance in strains of P.falciparum but has not been considered anti-malarial itself [11].

The aim of study: the current study evaluate the MTX –polypeptide Parasiticidal effect on L.donovani promastigotes in vitro.

Material and methods

Equipment and supply

All the materials are supplied from flukes company except polypeptide (insulin) is supplied from Novo Nordisk A/S.

characterization of MTX- Preparation and polypeptide resin

The one neck reaction vessel was charged with 0.01 mole (4.544 gm) of Methotrexate dissolved in 100 ml of distilled water, and 0.02 mole of insulin (100 IU/ml). The reaction mixture is left for 30 hrs at 35 $^{\circ}$C with stirring. The solvent is evaporated by rotary evaporated 40 $^{\circ}$C The UV-visible spectrophotometry (Jeho model 40) .spectra of MTX-polypeptide shows sharp intensity band at 300 nm and broad band at 375 nm. Compare with parent methotrexate as shown in Figure 1and 2

KBr disk was used in the FT.IR spectrophotometric study (shimadzu Bruker model Equino X55).The FT.IR spectra of MTX – polypeptide estimate many distinguishing bands compare with FT- IR spectrum of methotrexate . the stretching vibration band of OH group in 3384-3446 cm^{-1}, C-H band in 2900 – 3000 cm^{-1} , carbonyl group in 1600 – 1700 cm^{-1} , C=C in 1400 – 1450 cm^{-1} and C-O in 1040 – 1050 cm^{-1}.[12] as shown in figure 3 and 4.

Parasite isolation:

A cloned line of L.donovani (MHOM/IQ/2005/MRC10) was obtained from the center of medical research at Al-Nahrayn University in Baghdad; this sample is isolated from the bone marrow of a patient with Kala-azar.

Culture:

The promastigotes had been grown on NNN media which is a diphasic media contain; solid phase [13] and Liquid phase which also called lock solution [14], 2 ml of lock solution is added to the solid phase of NNN media .

Growth studies:

Growth rate experiment are conducted by inoculating parasites at a density of 1 x 10^{6} cell/ml in NNN media at 24 $^{\circ}$C after 24 hr. of inoculation[15]. Twelve inoculated vials of NNN media has been used for this study with three vials for each concentration. When growth is confirmed after 24 hrs of inoculation by counting the base line growth rate, the drug MTX – groups of polypeptide has been added to three

Vials with different Concentration (0.02 mg/L), (0.04 mg/L) and (0.06 mg/L) and the 4th group of vials is left as a control media without addition (each group contain 3 vials). The growth rate of parasites at different times (within three consecutive days) were counted in a Neubauer Hematocytometer under light microscopy by using the following equation[16]:

No. of parasites in 1 mm^3 = No. of parasites in five small squares X 800

Statistical analysis: all values were expressed as the mean ± SE ,the significant difference is determined by using analysis of variance test (Microsoft Excel program) and P value < 0.05

are considered significant.

Results

The average degree of MTX support was dependant on the charge properties of polypeptide. Under the experimental conditions used, the molar substitution ratio was achieved. The UV visible spectra of the MTX- polypeptide was estimated at the same two transition bands of the parent MTX. The transition bands of MTX-polypeptide were shown high and broad compared with spectra of parent MTX due to formation of strong hydrogen bonds (Fig.1 and 2)

The FT.IR spectra of MTX- polypeptide (Fig 4) showed several distinguishing bands but lower intensity compared with parent MTX (Fig.3)

Comparing the growth patterns of parasites in the three concentrations of MTX –polypeptide (0.02 ,0.04 and 0.06 mg /L) with the growth (Table 1) , &pattern of the control group(Fig. 5) there was a significant difference (P<0.05) .The growth rate of parasites is lower within the three concentrations of MTX –polypeptide as compared to the control group .

In the first day of the experiment, there is significant difference among the growth rates of the three concentrations of MTX –polypeptide when comparing with each other's .the maximum growth rate is shown in Concentration 0.02 mg/L whereas minimum growth rate is shown in concentration 0.04 mg/L.

In the second day of experiment in promastigote culture media, the parasites counts mg/L were mg/L & 0.04 in concentration of 0.02 approximately similar in comparism with mg/L which shows a concentration of 0.06 significant decrease in the promastigote count (P<0.05). While in the third day of experiment, mg/L exhibit attenuated concentration of 0.06 growth compared with the other concentrations mg/L .while the growth rate are similar in the 0.02 mg/L concentrations. & 0.04

mg/L concentration there is Within the 0.02 significant difference in growth rate in the second & third day as compared to the first day.

mg/L has significant The concentration of 0.04 variation in growth rate with first, second, & third days (p<0.05), where the maximum replication rate is found in the second day. (Table 1)

mg/L , a In regard to the concentration of 0.06 significant decrease in the number of promastigotes was observed in the third day (p<0.05) as compared to first & second days which exhibit no significant difference between them.

Discussion

There are many advantages for MTX- polypeptide over parent MTX,1- increase effective surface area , 2- slow release of MTX (increase duration of action) , 3- decrease side effect of MTX , 4- increase bioavailability & solubility , and 5-]. 7increase stability [1

Methotrexate is an antimetabolite drug used in treatment of many cancerous diseases mainly & also as a chemotherapy constitute the main tool] .it is a folic 8for the control of Leishmaniosis[1

acid antagonist competitively inhibits dihydrofolate reductase, preventing the synthesis of tetrahydrofolic acid which is implicated in synthetic processes in the metabolism of Leishmania ,it was also showed that absence of folic acid inhibit Leishmania growth[1].9

Regarding the control group , we observe that there is increase in growth rate from first to second day , the peak of growth rate occur in second day ,then growth rate decrease in third day .this can be explained by the fact that after period of adjustment (in the first day) cell division rapidly occur with the population doubling at constant rate ,then as nutrients depleted & toxic products accumulate , cell growth slows to a stop(stationary phase) & eventually enters a phase of decline (death)[20].

in the first day, the significant difference among the 3 concentrations is suggested to be because of suboptimal & optimal doses of MTX –polypeptide i.e. the concentration 0.02 mg/L & 0.04 mg/L were considered suboptimal so that there is a little toxic effect of MTX –polypeptide observed in these 2 media, while the concentration of 0.06 mg/L is approximately optimal dose that affect the growth rate of promastigote obviously.

In another study the promastigote had been grown on a single medium with increasing the dose of drug gradually over long period of time (5 days) which allows the drug to accumulate sufficiently within the parasite , they found that increasing the dose leads to more decrease in growth rate ,which is resemble to our study. But it differ from our study in that it increases the dose of drug on the same medium & not use different concentrations on separated media . [21]

In the second day, the concentration of 0.04 mg/L showed an increase in the growth rate in as compared to the first day, this can be explained by development of resistant strain of parasites .this suggestion is more accepted as it goes with other study which states that resistance of Leishmania against a given drug may be either natural, or may be acquired when the parasite are exposed to suboptimal dose [2]. 3

In the second day also we observed that 0.02 mg/L &0.04 mg/L were approximately alike this can be due to approximation of the 2 doses as been subeffective dose & to that the 2 media has similar baseline of parasite before addition of the dose ,&also to that both growth rates are counted after 48 hours period of time .

In the third day, the maximum decline in growth rate occur at concentration of 0.06 mg/L, this is attributed to that MTX –polypeptide dose is nearly optimal & to the fact that MTX –polypeptide needs 72 hours to eliminate the parasite effectively as been proved by study [25], although the other study prove this fact in 24, vivo while our experiment is done in vitro.

Conclusion

The binding of MTX with a polypeptide has a potent parasiticidal effect. Increasing the dose of MTX causes more decrease in growth rate of promastigotes. Suboptimal doses of MTX may lead to development of drug resistance by the parasite. The effect of MTX in killing parasites may take as long as 72 hrs to be maximized.

دراسة في خارج الجسم الحي لتاثير عقار ميثوتراكزيت المقترن ببيبولي ببتايد على معلل تكاثر طفيلي

اللشمانيا الاحشائية

Leishmania donovani

لستاذ مساعد: رحيم جميل **محسن**(فرع الكيمياء الصيدلانية - كلية الصيدلة - **جامعة البصرة**)

مدرس : اياد قاسم مهدي (فرع الادوية والعلوم المختبرية السريرية – كلية الصيدلة - جامعة البصرة)

الخلاصة

تم اقران Methotrexate الى بولي ببتايد (انسولين) وكانت نسبة الاضافة (1:05) اعتماداً على النسبة المئوية للتجربة . واستخدمت الطرق الطيفية لتشخيص المركب المحضر , وتمت دراسة تاثير المركب الناتج على نمو الطور المسوط Promastigote لطفيلي اللشمانيا الاحشائية Leishmania donovani خارج الجسم الحي in vitro المعزوله من الوسط الزرعي (NNN media) , وقد قل معدل نمو الطفيلي عكسياً مع زيادة التراكيز المستخدمة للعقار المحضر بتراكيز (0.02 , 0.04 , 0.06) ملغم /لتر بالمقارنة مع المجموعة الضابطة وحسب معدل النمو خلال فترة اقصاها 72 ساعة . واظهرت الدراسة ان اقل معدل نمو للطفيلي قد سجل في اليوم الثالث من التجربة وعند تركيز (0.06) ملغم/لتر , وقد تبين ان هنالك فروق معنوية عند مستوى ثقة (P<0.05) للتراكيز المختلفة للعقار المحضروخصوصاً عند تركيز 0.06 ملغم /لتر.

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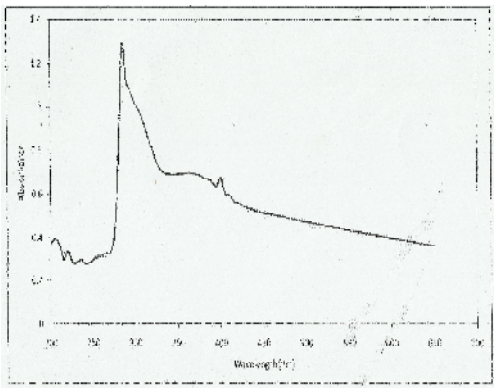

Transmissio

Figure 1: UV-visible spectrum of Methotrexate (10^{-3} M in

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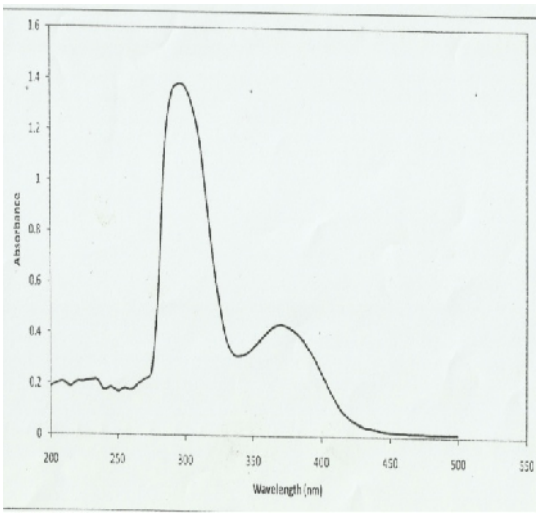
Wave number Cm $^{-1}$

Wave number Cm $^{-1}$

Figure 3: FT-IR spectrum of Methotrexat



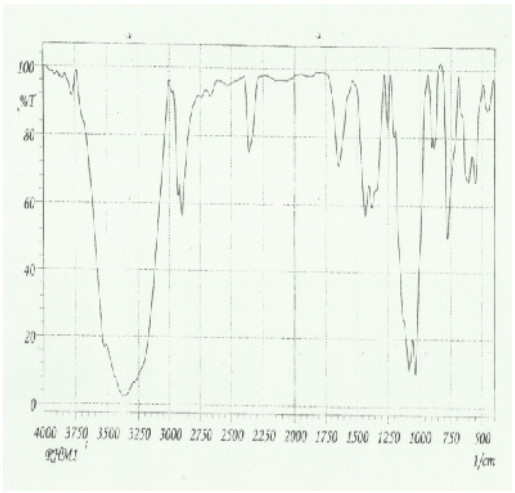
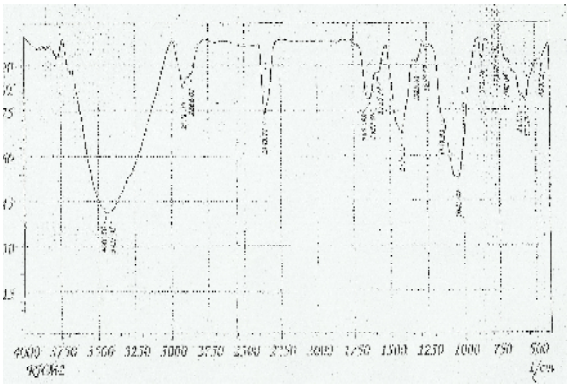


Figure 2: UV-visible spectrum of Methotrexate-peptide polymer(10^{-3} M in water)



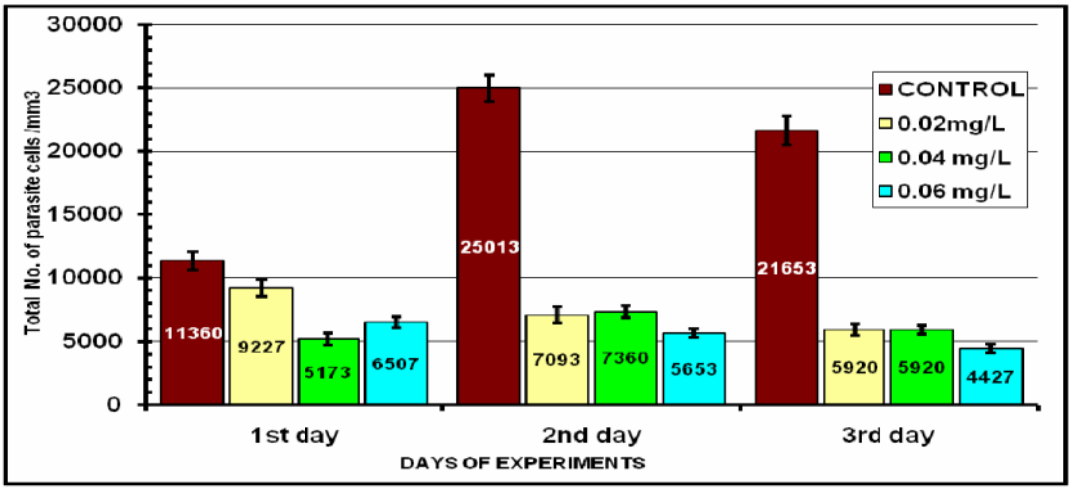


Fig (5): Illustrate the replicaton rates of L.donovani promastigotes treatred with three different Concentration of MTX –polypeptide during a period of three days

Table (1): The effects of various concentrations of MTX –polypeptide on the L.donovani Promastigotes growth rates in vitro.

	parasite cells expressed in mean ± standard of errors Count of								
	1st day			2nd day			3rd day		
	MEAN ±	SE		MEAN ±	SE		MEAN ±	SE	
CONTROL	11360.00 ±	712.13		25013.33 ±	1028.26	*	21653.33 ±	1172.29	*, a
0.02 mg/L	9226.67 ±	680.02	b	7093.33 ±	628.79	*,b	5920.00 ±	492.53	*,b
0.04 mg/L	5173.33 ±	474.04	b, c	7360.00 ±	482.53	*, b	5920.00 ±	347.40	a, b
0.06 mg/L	6506.67 ±	447.58	b, c	5653.33 ±	361.72	b, d	4426.67 ±	320.63	*,a , c, d

* Significant different at p<0.05 as compared with first day values

[a] Significant different at p<0.05 as compared with second day values

[b] Significant different at p<0.05 as compared with controls values

[c] Significant different at p<0.05 as compared with 0.02 mg/L values

[d] Significant different at p<0.05 as compared with 0.04 mg/L values