Parasitol Res (2012) 110:1113–1117
DOI 10.1007/s00436-011-2599-6

ORIGINAL PAPER

The effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of *Leishmania tropica* to meglumine antimoniate

Azar Shokri · Iraj Sharifi · Ali Khamesipour · Nozar Nakhaee · Majid Fasihi Harandi · Jafar Nosratabadi · Maryam Hakimi Parizi · Mohammad Barati

Received: 11 June 2011 / Accepted: 3 August 2011 / Published online: 17 August 2011

Abstract Pentavalent antimonials are the standard treatment for cutaneous leishmaniasis (CL) with low efficacy and resistance is emerging. CL is increased significantly in respect to incidence rate and expanding to new foci. In the present study, the effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of *Leishmania tropica* to meglumine antimoniate (MA, Glucantime) was evaluated using colorimetric assay (MTT) and in a macrophage model, respectively. Verapamil, as a calcium channel blocker, affects drug uptake by preventing of drug efflux from the cells. In promastigote form, several concentrations of MA with or without verapamil showed significant decrease ($P<0.05$) in optical density. The overall mean IC_{50} value with combination of MA plus verapamil (IC50=116.03 μg/ml) was significantly less than MA (IC50=225.14 μg/ml) alone ($P<0.05$) for promastigote stage. Similarly, the amastigote stage was more susceptible to treatment with MA plus verapamil to that of MA alone ($P<0.05$). Analysis of overall effect of different concentrations of MA alone, compared with combination of MA plus verapamil by mean infection rate of amastigotes in each macrophage showed a significant difference ($P<0.05$).These findings indicated some degree of synergistic effects between MA and verapamil on in vitro susceptibility of *L. tropica* to MA. Further works are required to evaluate this synergistic effect on animal model or volunteer human subjects.

A. Shokri
School of Medicine, Hormozgan University of Medical Sciences,
761-6666367, Bandarabbas, Iran

I. Sharifi (✉) · M. Fasihi Harandi · J. Nosratabadi · M. Hakimi Parizi · M. Barati
Leishmaniasis Research Center,
Kerman University of Medical Sciences,
76169-14115, Kerman, Iran
e-mail: iraj.sharifi@yahoo.com

A. Khamesipour
Center for Research and Training in Skin Diseases and Leprosy,
Tehran University of Medical Sciences,
Tehran, Iran

N. Nakhaee
Department of Community Medicine, School of Medicine,
Kerman University of Medical Sciences,
76169-14115, Kerman, Iran

Introduction

Leishmaniasis is still a significant cause of morbidity and mortality in tropical and sub-tropical countries. It is endemic in 88 countries, notably those of Southwest Asia, and is an important public health problem in some endemic regions including Iran. Two epidemiological forms of cutaneous leishmaniasis (CL) are present in Iran; anthroponotic CL, the dry, urban form, which is caused by *Leishmania tropica* and zoontic CL, the wet, rural form caused by *Leishmania major*. Ninety percent of CL cases occur in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia, and Syria (Desjeux 2004). Control measures are insufficient and complicated by the diverse ecology of different species of sand fly vectors and animal reservoirs. Existing treatments are associated with adverse effects and of fairly low efficacy (Croft et al. 2006; Firooz et al. 2006; Khatami et al. 2007; Modabber et al. 2007). In fact, the variety of epidemiological and clinical forms of leishmaniasis in the world

makes it difficult to apply a single measure, universally (Sharifi et al. 2010).

The first-line drugs, antimonials, are less effective against various forms of leishmaniasis and resistance is emerging (Jha 2006; Moreno et al. 2011; Pour et al. 2011). For clinical and persistent cases of CL, the choice of second-line drugs is very limited due to prolonged length of therapy and adverse reactions (Croft et al. 2006), even though there are promising new compounds used alone or in combination (Moreno et al. 2011; Prasad et al. 2000; Shakya et al. 2011; Tafaghodi et al. 2011). Treatment failure is common in many endemic areas (Firooz et al. 2006; Khatami et al. 2007; Modabber et al. 2007). In Iran, CL is increased significantly and resistance is reported (Hadighi et al. 2006, 2007; Pour et al. 2011). There is urgent need for development of new treatment. In *Leishmania* species, drug resistance is frequently associated with decreased cellular accumulation of the drug. However, other factors such as dose and duration of therapy, host immune status, pharmacological deficiencies, under-treatment, also lead to resistance (Croft et al. 2006).

Verapamil, as a calcium channel blocker, affects drug uptake by preventing drug efflux from the cells and has been widely used for treatment of hypertension, cardiac arrhythmia, angina pectoris, and most recently, cluster headaches. Verapamil is also used as a vasodilator during cryopreservation of blood vessels and as an inhibitor of drug efflux pump proteins such as P-glycoprotein (Bellamy 1996).

In the present study, the effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of *L. tropica* to meglumine antimoniate was evaluated using colorimetric assay (MTT) and in a macrophage model, respectively.

Materials and methods

Drug preparation

Meglumine antimoniate (MA, Glucantime Rhône–Poulenc, France) and verapamil (V, Alborz Pharmacy, Iran) were obtained from commercial sources. Verapamil was dissolved in sterile distilled water according to the manufacturer's instructions and aliquots stored at −20°C until use. Verapamil at constant concentration of 8 μM was used along with different concentrations of MA in either stage according to the method described (Ranjini Valiathan et al. 2006). The stock solutions were defrosted and diluted in medium just prior to assay. MA solution was stored at room temperature and diluted in medium to prepare final concentrations of 5.85, 11.71, 23.43, 46.87, 93.75, 187.5, 375, and 750 μg/ml (Carrio et al. 2000).

Parasite culture

L. tropica standard strain MHOM/IR/02/Mash2 was cultured in NNN medium, incubated at 25°C±1°C and subcultured in RPMI-1640 (Gibco, UK) supplemented with 15% heat-inactivated FCS, penicillin (200 IU/ml) and streptomycin (200 μg/ml).

Promastigote assay

The susceptibility of promastigotes was carried out according to the method described by Carrio et al. (2000). Serial dilutions of MA in RPMI-1640 (PH, 7.2) were prepared in 96-well microtiter plate. Promastigotes (10^5) were harvested at log phase, and 100 μl of medium was added to each well and incubated at 25°C±1°C for 72 h. Promastigotes were cultured in medium with no drug and used as positive control, and medium with no organism was used as blank. All experiments were performed in triplicate. MTT assay was performed by preparing MTT (Sigma Aldrich, USA) in sterile PBS and 10 μl of MTT solution was added in each well, incubated at 25°C±1°C for 3 h. The reaction was stopped by using isopropanol alcohol and read by ELIZA reader (ELX800) at 492 nm. IC50 was determined using the following formula:

$$\text{Log IC50} = \log x_1 + \left[\left(y_1 - y_{0/2}\right)/\left(y_1 - y_2\right)\right] \times \left[\log(x_2) - \log(x_1)\right]$$

Amastigote assay

Macrophages were collected from Balb/c mouse by injecting 5 ml of cold RPMI-1640 medium into mouse peritoneal cavity; aspirated macrophages were washed twice and resuspended in RPMI-1640. The amastigote viability test was carried out according to the method described elsewhere (Carrio et al. 2000). Every experiment was done in triplicate similar to promastigote assay. Viability test was performed by adding 90 μl of trypan blue solution (0.2%) in saline containing 0.01% sodium aside to 10 μl of cell suspension (10^6cells per milliliter). After 2 min, cells were counted under light microscope, and viability was calculated as follows:

$$\%\text{Viability} = (\%\text{ of live cells } / \text{all counted cells}) \times 100$$

Then, 200 μl of the cells (10^6cells per milliliter) was added into 8-chamber slide (Lab-Tek, Nalge Nunc International NY, USA), incubated at 37°C with 5% CO_2 for 2 h. Promastigotes were added to macrophages and incubated at 37°C with 5% CO_2 for 24 h. Then MA concentrations (10 μl) in medium as added to the slides and incubated at 37°C for 72 h. Dried slides were fixed with methanol, stained by Giemsa and studied under light microscope. Macrophages containing amastigotes

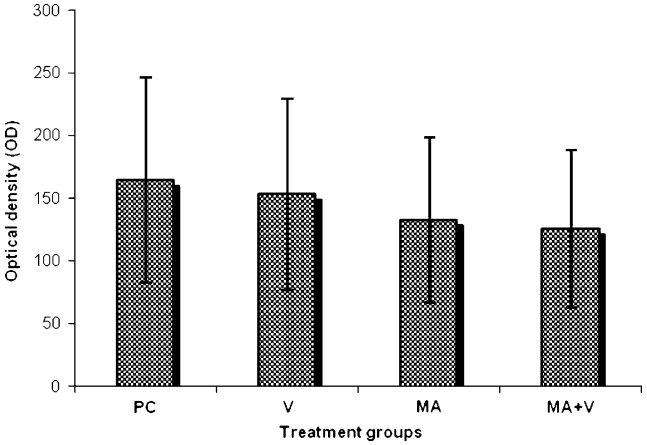

Fig. 1 Comparison of optical density (*OD*) between positive control (*PC*), verapamil (*V*), meglumine antimoniate (*MA*), and meglumine antimoniate plus verapamil (MA + V) on the susceptibility of promastigotes by colorimetric assay

with no drugs and macrophages alone were considered as positive and negative controls, respectively. Drug activity was evaluated by counting the number of amastigotes in the macrophages by examining 100 macrophages.

Statistical analysis

SPSS was used to analyze the data. ANOVA test was used to define a possible relation between MA alone and MA plus verapamil. The IC50 values of MA alone and with verapamil for both promastigote and amastigote stages were compared using t-test, and $P<0.05$ was considered as a significant difference.

Results

Promastigote assay

In promastigote form, several concentrations of MA with or without verapamil showed significant decrease ($P<0.05$) in optical density (OD) as measured by MTT method. The mean OD for MA was 132.5±11.8, for MA plus verapamil was 125±23.2, and for verapamil alone was 153.1±20.5 (Fig. 1). The overall growth rate of promastigotes treated with various concentrations of MA plus V (IC_{50}=116.03 μg/ml) was significantly lower ($P<0.05$) than growth rate in MA alone (IC_{50}=225.14 μg/ml).

Amastigote assay

The effect of MA alone and with verapamil was evaluated by the mean infection rate (MIR) of each macrophage and also by the mean number of amastigotes in each macrophage. Comparison of the MIR showed that different concentrations of MA in absence of verapamil significantly inhibited growth of macrophages by 187.5 μg/ml ($P<0.05$), whereas there was no effect, thereafter, as compared with positive control (Table 1). However, the effect of MA alone on the mean number of amastigotes in each macrophage was significant by 46.87 μg/ml ($P<0.05$), when compared with positive control ($P<0.05$).

The effect of various concentrations of MA plus verapamil or MA alone on the MIR reduced infection rate by at least 23.43 μg/ml (Table 2), although, infected macrophages failed to survive at 5.85 μg/ml of MA plus verapamil. But, when the mean number of amastigotes is being considered a significant difference was observed at various concentrations of MA along verapamil.

Analysis of overall effect of different concentrations of MA alone, compared with combination of MA plus verapamil by mean infection rate and mean number of amastigotes in each macrophage is shown in Table 3. T-test analysis indicated MA plus verapamil showed a synergistic effect at concentrations of 375, 118.5, and 93.75 μg/ml as compared with the respective concentrations of MA alone when the MIR is considered.

Table 1 The effect of various concentrations of meglumine antimoniate (MA) alone on the mean infection rate and number of amastigotes per macrophage in comparison with infected macrophages with no treatment as positive control

	Mean infection rate		Mean number of amastigote in each macrophage	
Concentration	Mean±SD	*P* value	Mean±SD	*P* value
0 μg MA(control)	66.00±2.00	Not significant	3.54±0.24	Not significant
750 μg MA	50.66±6.65	$p<0.001$	2.05±0.10	$p<0.001$
375 μg MA	55.33±4.16	$p<0.001$	2.31±0.31	$p<0.001$
187.5 μg MA	59.00±2.64	$p<0.05$	2.74±0.20	$p<0.01$
93.75 μg MA	61.33±3.51	Not significant	2.84±0.21	$p<0.01$
46.87 μg MA	63.33±4.50	Not significant	2.99±0.29	$p<0.05$
23.43 μg MA	64.33±3.51	Not significant	3.15±0.31	Not significant
11.71 μg MA	66.00±3.60	Not significant	3.26±0.35	Not significant
5.85 μg MA	67.33±4.04	Not significant	3.43±0.44	Not significant

Table 2 The effect of various concentrations of meglumine antimoniate (MA) plus verapamil (V) or V alone on the mean infection rate and number of amastigotes per macrophage in comparison with infected macrophages with no treatment as positive control

Concentration	Mean infection rate		Mean number of amastigote in each macrophage	
	Mean±SD	*P* value	Mean±SD	*P* value
0 μg MA(control)	66.00±2.00	Not significant	3.54±0.24	Not significant
V alone(8 μg)	53.33±1.52	$p<0.001$	2.83±0.10	$p<0.01$
750 μg MA+V	43.66±2.50	$p<0.001$	2.06±0.33	$p<0.001$
375 μg MA+V	47.66±2.50	$p<0.001$	2.33±0.29	$p<0.001$
187.5 μg MA+V	51.00±1.00	$p<0.001$	2.45±0.29	$p<0.001$
93.75 μg MA+V	54.33±0.50	$p<0.001$	2.57±0.23	$p<0.001$
46.87 μg MA+V	59.33±1.15	$p<0.001$	2.74±0.14	$p<0.001$
23.43 μg MA+V	62.00±1.73	$p<0.01$	2.85±0.06	$p<0.01$
11.71 μg MA+V	63.66±1.50	Not significant	2.96±0.75	$p<0.05$
5.85 μg MA+V	65.66±1.15	Not significant	3.05±0.12	$p<0.05$

Discussion

Despite effort and progress over the years, the number of drugs available for treatment of CL remains limited. MA is the treatment of leishmaniasis for nearly 65 years. In addition to the low response recently reported against various forms of leishmaniasis due to resistance (Croft et al. 2006; Hadighi et al. 2006, 2007; Pour et al. 2011) treatment with antimonials is long, painful, and associated with toxicity (Alvar et al. 2006). In Iran, ACL due to *L. tropica* is a chronic disease and at present combination of treatment such as MA with cryotherapy or along with other drugs are used (Khatami et al. 2007; Layegh et al. 2009; Modabber et al. 2007). Therefore, the need for new drug development and treatment regimens, effective against different forms, are highly recommended. Hopefully, there are promising new developments currently used in drug treatment of leishmaniasis (Moreno et al. 2011; Shakya et al. 2011).

Most of the previous pre-clinical drug studies have been concentrated on promastigotes of *Leishmania* species; in vitro drug susceptibility experiments on both promastigote and amastigote stages are fairly rare (Ranjini Valiathan et al. 2006). In the present study, the effect of verapamil as a calcium channel blocker in combination with MA in the growth of promastigote and amastigote stages of *L. tropica* have been investigated. Various concentrations of MA along with verapamil inhibited the growth of both stages of *L. tropica* and caused significant response, as compared with MA alone. It is shown that verapamil when used in combination with chloroquine, enhances the accumulation of chloroquine within a parasitic cell digestive vacuole, rendering it incapable of detoxifying itself and making it more susceptible to death (Martin et al. 1987).

The overall mean IC50 values of MA plus verapamil for promastigote stage was significantly lower than those with MA alone ($P<0.05$). Similarity, the amastigote stage was more susceptible to treatment with MA plus verapamil, compared to similar concentrations of MA alone. In general, both amastigotes and promastigotes findings are in agreement with others (Ranjini Valiathan et al. 2006). However, they used clinical isolates and standard strain of *Leishmania donovani* and showed that the amastigote stage is 2–60 folds more sensitive to sodium stibogluconate than

Table 3 Analysis of overall effect of different concentrations of meglumine antimoniate (MA) alone as compared with meglumine antimoniate plus verapamil(V) by the mean infection rate and mean number of amastigotes in each macrophage

Concentration	Mean infection rate		Mean number of amastigote in each macrophage	
	Sig (two-tailed)		Sig (two-tailed)	
MA (750 μg/ml)& MA+V(750 μg/ml)	0.164	Not significant	0.963	Not significant
MA (375 μg/ml)&MA+V(375 μg/ml)	0.050	Significant	0.940	Not significant
MA (187.5 μg/ml)&MA+V(187.5 μg/ml)	0.008	Significant	0.235	Not significant
MA (93.75 μg/ml)&MA+V(93.75 μg/ml)	0.027	Significant	0.219	Not significant
MA (46.87 μg/ml)&MA+V(46.87 μg/ml)	0.211	Not significant	0.247	Not significant
MA (23.43 μg/ml)&MA+V(23.43 μg/ml)	0.360	Not significant	0.181	Not significant
MA(11.71 μg/ml)&MA+V(11.71 μg/ml)	0.360	Not significant	0.226	Not significant
MA (5.85 μg/ml)&MA+V(5.85 μg/ml)	0.530	Not significant	0.225	Not significant

promastigote stage. There is a significant difference between promastigotes in biochemistry and sensitivity to standard and experimental drugs as indicated previously (Lira et al. 1999). The effect of verapamil has already been evaluated, as arsenite-resistant *L. donovani* promastigotes express an enhanced membrane P-type adenosine triphosphatase activity that was sensitive to verapamil treatment (Prasad et al. 2000).

Conclusion

The results of this study showed some degree of synergistic effect of verapamil on MA in inhibiting growth of promastigote and amastigote stages of *L. tropica*. Further works are required to evaluate this synergistic effect on animal model or volunteer human subjects.

Acknowledgments This investigation was supported by the Kerman Leishmaniasis Research Center and Vice Chancellor for Research, Kerman University of Medical Sciences (Project no.86/94-k/86/31).

Conflict of interest The authors declare that they have no conflict of interest in this study.

References

Alvar J, Croft S, Olliaro P (2006) Chemotherapy in the treatment and control of leishmaniasis. Adv Parasitol 61:223–274

Bellamy WT (1996) P-glycoprotein and multidrug resistance. Annu Rev Pharmacol Toxicol 36:161–183

Carrio MC, Reiva C, Gallego M, Arboix M, Portus M (2000) *Leishmania infantum:* stage specific activity of pentavalent antimony related with the assay conditions. Exp Parasitol 95: 209–214

Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in leishmaniasis. Clin Microbiol Rev 19(1):11–26

Desjeux P (2004) Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 27:305–318

Firooz A, Khamesipour A, Ghoorchi MH, Nassiri-Kashani M, Eskandari SE, Khatami A, Hooshmand B, Gorouhi F, Rashighi-Firoozabadi M, Dowlati Y (2006) Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis. Arch Dermatol 142 (12):71575–71579

Hadighi R, Mohebali M, Boucher P, Hajjaran H, Khamesipour A, Ouellette M (2006) Unresponsiveness to glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med 3(5):659–667

Hadighi R, Boucher P, Khamesipour A, Meamar AR, Roy G, Ouellette M, Mohebali M (2007) Glucantime-resistant *Leishmania tropica* isolated from Iranian patients with cutaneous leishmaniasis are sensitive to alternative antileishmania drugs. Parasitol Res 101 (5):1319–1322

Jha TK (2006) Drug unresponsiveness and combination therapy for kala-azar. Indian J Med Res 123(3):389–398

Khatami A, Firooz A, Gorouhi F, Dowlati Y (2007) Treatment of acute Old World cutaneous leishmaniasis: a systematic review of the randomized controlled trials. J Am Acad Dermatol 57(2): 335.e1–29

Layegh P, Pezeshkipoor F, Soruri AH, Navaifar P, Moghyman T (2009) Efficacy of cryotherapy versus intralesional meglumine antimoniate (glucantime) for treatment of cutaneous leishmaniasis in children. Am J Trop Med Hyg 80(2):172–175

Lira R, Sundar S, Makharia A, Kenney R, Gam A, Saraiva E, Sakhs D (1999) Evidence that the high incidence of treatment failure in Indian Kala azar is due the emergence of antimony resistant strains of *Leishmania donovani*. J Infect Dis 180(2):564–567

Martin SK, Oduola AM, Milhous WK (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science 235:899–901

Modabber F, Buffet PA, Torreele1 E, Milon G, Croft SL (2007) Consultative meeting to develop a strategy for treatment of cutaneous leishmaniasis. Institute Pasteur, Paris 13–15June, 2006. Kinetoplastid Biol Dis 24(6):3.

Moreno D, Plano D, Baquedano Y, Jiménez-Ruiz A, Palop JA, Sanmartin C (2011) Antileishmanial activity of imidothiocarbamates and imidoselenocarbamates. Parasitol Res 108(1):233–239

Pour R, Sharifi I, Kazemi B, Zarean M (2011) Identification of nonresponsive isolates to glucantime in patients with cutaneous leishmaniasis in Bam. J Kerman Univ Med Sci 18(2):123–133

Prasad V, Kaur J, Dey CS (2000) Arsenite-resistant *Leishmania donovani* promastigotes express an enhanced membrane P-type adenosine triphosphatase activity that is sensitive to verapamil treatment. Parasitol Res 86(8):661–664

Ranjini Valiathan ML, Dubey RC et al (2006) *Leishmania donovani:* Effect of verapamil on in vitro susceptibility of promastigote and amastigote stages of Indian clinical isolates to sodium stibogluconate. Exp Parasitol 114:103–108

Shakya N, Sane SA, Gupta S (2011) Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator–picroliv. Parasitol Res 108(4):793–800

Sharifi I, Fekri AR, Aflatoonian MR, Khamesipour A, Mahboudi F, Dowlati Y, Nadim A, Modabber F (2010) Leishmaniasis recidivans among school children in Bam, South-east Iran, 1994–2006. Int J Dermatol 49:557–561

Tafaghodi M, Khamesipour A, Jaafari MR (2011) Immunization against leishmaniasis by PLGA nanospheres encapsulated with autoclaved *Leishmania major* (ALM) and CpG-ODN. Parasitol Res 108(5):1265–1273