ACTIVITY OF D-CARNITINE AND ITS DERIVATIVES ON *TRYPANOSOMA* INFECTIONS IN RATS AND MICE

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Summary:

Little progress has been made in the treatment of African trypanosomiasis over the past decades. L-carnitine has a major role in glycolysis-based energy supply of blood trypanosomes for it stimulates constant ATP production. To investigate whether administration of the isomer D-carnitine could exert a competitive inhibition on the metabolic pathway of the L-form, possibly resulting in parasite replication inhibition, several formulations of this compound were tested on *Trypanosoma lewisi* and *T. brucei rhodesiense* in rodent models. High oral dosages of D-carnitine inner salt and propionyl-D-carnitine were not toxic to animals and induced about 50 % parasite growth inhibition in reversible, i.e. competitive, fashion. A putative mechanism could be an interference in pyruvate kinase activity and hence ATP production. Considering both, lack of toxicity and inhibitory activity, D-carnitine may have a role in the treatment of African trypanosomiasis, in association with available trypanocidal drugs.

KEY WORDS : *Trypanosoma lewisi*, *Trypanosoma brucei rhodesiense*, D-carnitine, rat, mouse.

Résumé : ACTIVITÉ DE LA D-CARNITINE ET DE SES DERIVÉS SUR LES INFECTIONS À *TRYPANOSOMA* CHEZ LE RAT ET LA SOURIS

Dans les dernières décennies, il y a eu très peu de progrès dans le traitement de la trypanosomiase africaine. La L-carnitine joue un rôle important dans la production d'énergie basée sur la glycolyse chez les trypanosomes sanguins, car elle stimule la production constante d'ATP. Pour vérifier si l'administration de l'isomère D-carnitine pourrait exercer une inhibition compétitive vis-à-vis de la forme L, en provoquant si possible une inhibition de la réplication du parasite, plusieurs formulations de ce composé ont été testées sur *Trypanosoma lewisi* chez le rat et *T. brucei rhodesiense* chez la souris. Des dosages élevés par voie orale de D-carnitine et de propionyl-D-carnitine se sont avérés non toxiques pour les animaux et ont entraîné une inhibition de la réplication du parasite de 50 % de manière réversible, c'est-à-dire compétitive. On pourrait supposer un mécanisme d'interférence dans l'activité de la pyruvate kinase et donc dans la production d'ATP. En considérant les deux, le manque de toxicité et l'activité d'inhibition, la D-carnitine peut jouer un rôle dans le traitement de la trypanosomiase africaine, en association avec les autres médicaments trypanocides.

MOTS CLÉS : *Trypanosoma lewisi*, *Trypanosoma brucei rhodesiense*, D-carnitine, rat, souris.

INTRODUCTION

It has been recently estimated that some 60 million people in 36 sub-Saharan African countries are at risk of sleeping sickness (human African trypanosomiasis) caused by *Trypanosoma brucei gambiense* in Central and West Africa, and by *T. b. rhodesiense* in Southern and East Africa (World Health Organization, 2001). The estimated prevalence in 1999 was between 300,000 and 500,000, but only 45,000 cases have been officially reported. In the veterinary field, the tse-tse transmitted animal trypanosomiasis due to *T. congolense*, *T. vivax* and *T. b. brucei* are considered the most important diseases of livestock, interesting about 44 million cattle. It has been calculated that, owing to the pre-

sence of tse-tse fly, 7 millions km² are unsuitable for livestock and that the estimated annual cost of this situation is about 1,340 million USD (Peregrine, 1994; Gu *et al.*, 1999).

Chemotherapy of African trypanosomiasis remains unsatisfactory because of low efficacy and high toxicity of available drugs (Gradoni, 1996). They consist in old compounds such as suramin and pentamidine for the treatment of the initial phase of the disease, or the highly toxic arsenical derivative melarsoprol for the neurological phase treatment. More recently, studies on specific metabolic pathways of *T. brucei* s.l. have led to the discovery of eflornitine, an inhibitor of polyamine biosynthesis registered in 1990, which however is effective only against *T. b. gambiense* (Bacchi *et al.*, 1990). In this situation, any observation concerning compounds involved in metabolic pathways of *Trypanosoma* could be useful for a better targeting of new drugs (Fairlamb, 1989).

It is widely accepted that L-carnitine is found in all biologic systems where it plays a key role in fatty acid metabolism (Wieland *et al.*, 1969; Cederblad & Lind-

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stedt, 1976; Rebouche, 1977; Bremer, 1983). It has long been observed that L-carnitine has a major role also in the glycolysis-based energy supply of blood trypanosomes, in which fatty acid oxidation is very scarce, for it stimulates constant ATP production (Gilbert & Klein, 1982, 1984; Gilbert *et al.*, 1983). In *T. b. brucei*, L-carnitine is found at concentrations of 1-5 mM, comparable to the highest values detected in any biological systems (Klein *et al.*, 1982). These data, together with the fact that trypanosomes assume L-carnitine very actively, also against gradient (Keilman & Dusanic, 1971), led us to suppose that the administration of the isomer D-carnitine could exert a competitive inhibition of the metabolic pathway of the L-form, possibly resulting in energy metabolism alteration which could induce, in turn, inhibition of parasite replication.

Following promising observations on antitrypanosomal activity of D-carnitine *in vitro* (Manganaro *et al.*, 1991), new studies have been carried out to confirm such activity by using two *in vivo Trypanosoma* models: *T. lewisi* in rats, and *T. b. rhodesiense* in mice.

MATERIAL AND METHODS

ANIMALS

Male Fisher rats weighing 150-200 g, and male Balb/c mice weighing 16-18 g (Charles River, Calco, Lecco) were used. Upon arrival at our animal facilities, the animals were acclimatized for three days before being used and kept at constant room temperature of 21°C. Pellet diet and water were administered *ad libitum*.

PARASITES

The strain ISST1 of *T. lewisi*, maintained as cryostabulate since 1980, was used in the rat model. This parasite is not pathogenic for man, has no extravasal growth phase and is considered useful for a preliminary screening of substances for trypanosomiasis treatment. In laboratory rats the maximum peak of *T. lewisi* parasitaemia is usually detected on day 5 post infection (p.i.). During the study period the strain was maintained in rats with i.p. injections every five days of 1 ml of whole blood containing about 1×10^7 trypanosomes/ml.

The strain IBADAN 73 of *T. b. rhodesiense*, supplied to Istituto Superiore di Sanità by the London School of Tropical Medicine and Hygiene, London, and kept as cryostabulate since 1982, was used in the mouse model. In mice, this strain induces a sub-acute chronic infection characterized by a first peak of parasitaemia on day 5, and by a second peak on days 14-15 p.i. During the study, trypanosomes were maintained in Balb/c mice by i.p. injections every 5-10 days.

In rats, the infected blood was collected with an heparinized syringe from the abdominal vena cava from anesthetized animals, whereas in mice it was withdrawn from tail veins. Parasites were counted with the aid of an haemocytometer after dilution of blood in Turk's solution. Counts were performed in triplicate at 400 ×.

DRUGS AND TREATMENT SCHEDULES

For a preliminary screening in the *T. lewisi*-rat model, L-carnitine, D-carnitine inner salt (IS), D-carnitine hydrochloride (HC), and the derivatives isovaleryl (IV)-, isobutiryl (IB)- and propionyl (PP)-D-carnitine (kindly supplied by Sigma Tau Pharmaceutical Inc, Pomezia, Rome) were dissolved in saline and administered orally via cannula, always at the same hour, to groups of 25 rats at doses ranging from 75 to 300 mg/kg/day, from day 1 to day 5 p.i. Untreated infected animals were used as control. On day 5, parasites were counted from both treated and untreated animals.

Basing upon activity results on *T. lewisi*, D-carnitine IS and PP-D-carnitine were selected for further experiments, and given orally to groups of 25 *T. b. rhodesiense*-infected mice at doses from 75 to 300 mg/kg/day from day 1 to day 15. Parasite counts were performed on days 5 and 15 p.i. and compared to those of untreated controls.

HISTOPATHOLOGY

In mice infected with *T. b. rhodesiense*, liver and spleen were collected for weight measurements and histological examinations. Moreover, a further experiment included three groups of 14 animals, which were respectively infected, or infected and treated with D-carnitine IS at the dose of 300 mg/kg/day for 15 days, or only treated with D-carnitine IS at the same dose. On day 2, and every other day from day 5 to day 15 p.i., two animals of each group were sacrificed and liver, spleen and brain were collected and fixed in 10 % formaldehyde for histological examination. Sections were stained with hematoxylin-eosin.

STATISTICAL ANALYSIS

Data from each animal group were expressed as mean $\pm$ SD. Student's paired T test was used for significance.

RESULTS

T. LEWISI-RAT MODEL

In this model, all formulations and derivatives of D-carnitine induced a certain degree of parasite inhibition as compared to untreated controls and to ani-

Compound	Parasite inhibition (%)
L-carnitine	7.3 ± 4.1
D-carnitine inner salt	42.9 ± 12.4*
D-carnitine hydrochloride	27.6 ± 13.2*
Isovaleryl-D-carnitine	36.0 ± 9.7*
Isobutiryl-D-carnitine	38.1 ± 13.5*
Propionyl-D-carnitine	54.2 ± 14.5*

* Significantly different ($P < 0.05$) from untreated controls.

Table I. – Percent parasite inhibition in rats infected with *T. lewisi* and treated with L-carnitine or different formulations and derivatives of D-carnitine at the dose of 300 mg/kg/day for five days.

mals treated with L-carnitine, with no evidence of toxicity. Data reported in Table I, which shows results at the highest dose tested (300 mg/kg/day), indicate that the IS formulation and the PP derivative of D-carnitine were the most active.

In a dose-response assay of L-carnitine, D-carnitine IS and D-carnitine HC (Fig. 1), results have shown that both formulations of D-carnitine display a dose-related activity, which was significant from concentrations higher than 150 mg/kg/day.

Parasites recovered from drug-treated rats were as infectious as those from untreated controls when sub-injected into healthy rats (data not shown).

Fig. 1. – Dose-response assay of L-carnitine, D-carnitine inner salt (IS) and D-carnitine hydrochloride (HC) in rats infected with *Trypanosoma lewisi*.

* Significantly different ($P < 0.05$) from L-carnitine at the same dosages.

Days from infection	D-carnitine IS (mg/kg/day)			PP-D-carnitine (mg/kg/day)		
	75	150	300	75	150	300
5	49.9 ± 17.7	48.8 ± 12.5	48.2 ± 17.6	21.7 ± 4.6	38.6 ± 9.4	40.2 ± 20.1
15	30.3 ± 13.3	44.0 ± 19.6	54.2 ± 14.2	27.3 ± 13.5	21.9 ± 9.4	22.4 ± 10.1

Table II. – Percent parasite inhibition in mice infected with *Trypanosoma brucei rhodesiense* and treated for 15 days with different doses of D-carnitine inner salt (IS) or propionyl (PP)-D-carnitine.

T. B. RHODESIENSE-MOUSE MODEL

At the doses tested, both D-carnitine IS and PP-D-carnitine induced a significant ($P < 0.05$) decrease in *T. b. rhodesiense* replication, as detected on days 5 and 15 p.i. (Table II). After five days of treatment, D-carnitine IS did not display any dose-related activity, whereas this was significantly shown at the assessments performed on day 15 p.i. (the highest inhibition induced being 54.2 %). The contrary situation was observed for PP-D-carnitine, which inhibited parasite replication in dose dependent manner only in the early phase of infection (for a maximum of 40.2 % inhibition), but not in the late one. Parasites recovered from drug-treated mice showed same infectiousness than those from untreated controls when sub-injected in healthy mice (data not shown).

To investigate on organ pathology features, liver and spleen were weighted from PP-D-carnitine treated mice and compared with those from untreated controls. Liver enlargement in mice treated with the drug doses of 150 or 300 mg/kg/day was significantly lower ($P < 0.01$) than in controls, when comparing 15 day p.i. with 5 day p.i. specimens (Fig. 2). Similarly, spleen enlargement was significantly less evident in mice

Fig. 2. – Percent increase of liver weight in *Trypanosoma brucei rhodesiense*-infected mice untreated or treated with different doses of PP-D-carnitine for 15 days.

* Significantly different ($P < 0.01$) from untreated controls.

treated with higher dosages of PP-D-carnitine, although this was less marked (data not shown).

Brain, liver and spleen histology did not reveal any pathological feature in healthy mice treated with D-carnitine IS. The same was observed in brain specimens from infected mice, both untreated and treated, suggesting that CNS was not involved at this stage of infection. Liver specimens from infected untreated mice showed lymphomononuclear infiltrates frequently associated with hepatocyte necrosis; this finding was progressively increasing from day 2 to day 5 p.i. These features were less marked in infected animals treated with D-carnitine IS; furthermore, from day 9 onwards regeneration features were evidenced by the increase of mitotic processes. In spleen specimens from both groups of infected mice, early white pulp hyperplasia was observed together with some extramedullary erythropoiesis foci; in some of the D-carnitine-treated animals, macrophage and plasma cell counts increased from day 11 onwards, this being probably correlated to early immune response.

DISCUSSION

In both rodent models of trypanosomiasis investigated, high dosages of D-carnitine IS and PP-D-carnitine induced about 50 % parasite growth inhibition. When treatment was suspended, or parasites from treated animals were sub-injected into healthy ones, trypanosomes quickly reached burdens similar to those of untreated controls, indicating that D-carnitine and its derivatives have a reversible, i.e. competitive, activity rather than irreversible, i.e. trypanocidal, action. On the other hand, there was no evidence of drug toxicity to animals at all dosages tested. The histological findings in mice suggest that not only D-carnitine does not induce damage in major organs, but it seems to facilitate hepatic restoration in *T. b. rhodesiense*-infected animals, as evidenced by numerous mitotic processes observed after nine days of therapy. Further confirmation of a probable protective drug effect on liver, is the finding that hepatomegaly was significantly reduced in treated animals, in a dose-dependent manner (see Fig. 2).

Our observations confirm the importance of carnitine in the metabolic functions of trypanosomatidae, and suggest the opportunity to investigate on the pathway(s) in which this aminoacid is involved. A limit of our *T. b. rhodesiense* model is that both early and late infections were caused by monomorphic slender-shaped parasites. The absence of stumpy procyclic forms is probably due to the syringe propagation of our strain, which is known to induce partial segregation with a loss of plasticity of the organism in the absence of natural fly transmission (Fairbairn & Culwick, 1946).

This observation may be relevant because there are marked metabolic differences between slender and stumpy forms (Riley, 1962).

As regards D-carnitine uptake, it has been observed that while all trypanosomal enzymes involved in carnitine metabolism are specific for the L-isomer, the transporter protein that maintains high cellular levels of this aminoacid versus plasmatic concentrations does not discriminate between L- and D-isomers (Bahl & Bressler, 1987). This observation would indicate that D-carnitine we administered, and at least some its derivatives, were absorbed by the parasites. As regards a comparison of metabolic pathways in our two models of trypanosomiasis, classical biochemical studies on mammalian blood trypanosomes have pointed out major differences in energy metabolism between the *Trypanosoma* species employed in our assays; a) a cytochrome system is present in *T. lewisi* but not in *T. b. rhodesiense* (Fulton & Spooner, 1959); b) while *T. b. rhodesiense* degrades glucose to a mixture of pyruvate and glycerol, *T. lewisi* converts glucose to a lactate, acetate and succinate mixture (Grant & Fulton, 1957; Riley, 1956, 1962). Nevertheless, the last biochemical step common to both trypanosomes is the production of pyruvate, which accounts for 83 % of metabolized glucose (Flynn & Bowman, 1973); this step could represent the common site in which L-carnitine is active, and D-carnitine may exert competitive inhibitory activity. If so, a putative mechanism could be an interference with pyruvate kinase (PK) activity and hence ATP production. Several studies have demonstrated that after ATP has been produced by PK along the way to pyruvate, the increasing levels of ATP have inhibitory activity on this enzyme, owing to decreased enzyme-substrate affinity. Analogously, acetyl-CoA synthesized following pyruvate dehydrogenation inhibits PK (Cox *et al.*, 1993). As a final result, the two metabolic steps lead to a decrease of energy production. It is well known that acetyl-CoA does not cross mitochondrial membrane. The conversion of acetyl-CoA to acetyl-carnitine by acetyl-carnitine transferase (CAT) enables acetyl group to cross this membrane; the subsequent reconversion of acetyl-carnitine to acetyl-CoA allows this molecule to entry in the tricarboxylic cycle and frees carnitine, which starts again shuttle function (Cox *et al.*, 1993). Hence, it has been hypothesized that in *Trypanosoma* carnitine and CAT system exert a buffering effect that protects the limited cellular CoA pool from the metabolic "acetyl pressure" (Klein *et al.*, 1982). In conclusion carnitine, through CAT activity, stimulate PK and consequently ATP production by removing acetyl-CoA inhibitory action. This interpretation of carnitine metabolic role is supported by the high CAT concentration observed in these parasites.

Considering both, the lack of toxicity of D-carnitine, even if administered at high dosages for long periods,

and the 50 % reduction in parasite load obtained in two *in vivo* models of *Trypanosoma* (which suggests a broad activity of this compound), it could be interesting to evaluate a therapeutic efficacy of D-carnitine in association with available antitrypanosomal drugs. This approach could lead to synergistic effects and to a substantial dose reduction of these highly toxic drugs and, consequently, fewer adverse events and an improved cost-effectiveness (Keiser *et al.*, 2001).

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