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Potent in vitro antileishmanial activity of a nanoformulation of cisplatin with carbon nanotubes against *Leishmania major*

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ABSTRACT

Objectives: The aim of this study was to evaluate the cytotoxicity and antileishmanial activity of cisplatin-bonded carbon nanotubes both against promastigotes and amastigotes of *Leishmania major* in vitro.
Methods: Cisplatin-bonded single-walled carbon nanotubes (CP-SWCNT) and cisplatin-bonded multi-walled carbon nanotubes (CP-MWCNT) were considered as test compounds. In addition, SWCNT, MWCNT, free cisplatin and meglumine antimoniate (Glucantime®) were considered as controls. The effect of each compound was evaluated both on promastigote and amastigote stages of *L. major* and the results were compared.
Results: There was a statistically significant difference between the half-maximal inhibitory concentration (IC_{50}) of CP-SWCNT and each of the controls, including SWCNT, cisplatin and Glucantime® ($P < 0.05$). In addition, IC_{50} values of CP-MWCNT and each of the controls, including MWCNT, cisplatin and Glucantime®, were significantly different both for promastigotes and amastigotes ($P < 0.05$). However, the selectivity index (SI) of CP-SWCNT was <10 (5.23), indicating that this compound is not completely safe. Moreover, the SI values of CP-MWCNT (12.54) and Glucantime® (16.28) were >10, indicating the selective effect of these two compounds on the parasite. Moreover, the IC_{50} of CP-MWCNT (0.11 ± 0.09 μM) for amastigotes was 41-fold lower than that of Glucantime® (4.52 ± 1.31 μM), suggesting that a lower dose of CP-MWCNT in comparison with Glucantime® is required to kill 50% of amastigotes.
Conclusions: According to the potent in vitro antileishmanial activity of CP-MWCNT at low concentration against *L. major*, we suggest that they are evaluated in an animal model.

1. Introduction

Cutaneous leishmaniasis (CL) is a vector-borne parasitic disease caused by *Leishmania* spp. that is transmitted to humans through the bite of infected female sandflies [1]. The disease is caused by several species of *Leishmania* including *Leishmania major* and *Leishmania tropica* [2]. The prevalence of the disease is estimated to be 0.7–1.3 million cases annually worldwide, although the actual number of infected cases is estimated to be 6- to 10-fold higher than that reported [3,4].

Treatment of CL is currently based on intralesional injection or oral medication with cryotherapy, topical controlled heat, CO_2 laser and photodynamic therapy [5]. Antimonial drugs, including meglumine antimonate (Glucantime®), have been widely used for the treatment of CL [6]. In some cases, intralesional injection of antimonials is not effective and therefore systemic treatment is required. However, these systemic treatments are not completely safe; some cases of heart and kidney involvement have been reported, and in rare cases it may lead to more serious problems [7,8].

Several cases of CL treatment failure associated with different factors, including the drug delivery system, have been reported [9]. Nanocarriers are approved as an effective drug delivery system and

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facilitate the intracellular uptake of a drug into infected cells and effectively reduce the drug dosage [10].

Carbon nanostructures such as single- and multi-walled carbon nanotubes (CNT) are proposed as promising nanocarriers with different properties such as high physical, chemical, thermal and optical potency in pharmacological studies [11,12]. Due to shape of the carbon bonds in this nanoparticle, different types of materials can be loaded on the surface of this nanostructure [13].

Moreover, the lack of a proper vaccine and difficulties associated with treatment, such as drug cost, the emergence of new species, drug resistance and toxicity of existing drugs, have led researchers to search for new drugs for CL [14]. In recent years, some anticancer drugs have been reported to have antileishmanial activity [15,16]. Cisplatin is an anticancer drug with an effect on various types of cancers including sarcomas and soft tissue cancers affecting bones, muscles and blood vessels, and in recent years its antileishmanial activity has been reported [17,18].

The antileishmanial effects of cisplatin have recently been proven, and CNT with increased cell penetration are postulated to increase the therapeutic effect of this drug against leishmaniasis.

In this study, the cytotoxicity and antileishmanial activity of cisplatin-bonded CNT on promastigotes and amastigotes of *L. major* were evaluated.

2. Materials and methods

2.1. Materials

Cisplatin (Sigma, St Louis, MO; purity ≥98%) and Glucantime® (Rhône-Poulenc, Paris, France) were purchased commercially and were used at different concentrations as mentioned below. Purified (≥99%) single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) were purchased from Nano-Integris Inc. (Skokie, IL), with a length of 100–1000 nm and diameter of 0.8–1.2 nm. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene glycol)2000 (DSPE-PEG2000) was purchased from Avanti® Polar Lipids Inc. (Alabaster, AL). All other chemicals were purchased from Sigma and were used without further treatment.

2.2. Parasite

Promastigotes of the reference strain *L. major* (MRHO/IR/75/ER) were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/mL streptomycin and 100 IU/mL penicillin (Gibco, Eggenstein, Germany) at 24 °C with frequent passage every 3 days.

2.3. Drug supply

2.3.1. Drug binding and encapsulation

Cisplatin was loaded onto oxidised SWCNT by two methods: first, covalent binding of the drug was carried out as explained previously [11]. Second, drug encapsulation was applied. For encapsulation, 1 mg/mL cisplatin in chloroform was prepared in the dark and the nanoplatform was gently added to the solution. The suspension was sonicated using an ultrasonic bath sonicator (Bandelin, Berlin, Germany) with a sonication cycle of 1 h sonication and 1 h rest for 2 days. Then, 10 mg of DSPE-PEG2000 was added to the prepared sample and was sonicated for an extra day. The temperature was increased to 30–70 °C and all of the chloroform was evaporated. Then, 10 mL of double-distilled water was added slowly to the target materials and was sonicated for 1 h and 15 min followed by rest for 2 h. Finally, a dialysis procedure was performed (molecular weight cut-off of 4–6 kDa) and free cisplatin was separated from the final complex pegylated SWCNT (P-SWCNT). The same procedure was used for the second complex that contained MWCNT as nanoplatform to produce pegylated MWCNT (P-MWCNT).

2.4. Characterisation of carbon nanotubes (CNT) and cisplatin-bonded CNT

2.4.1. Particle size and zeta potential

Particle size and zeta potential were determined by dynamic light-scattering analysis using a Malvern Zetasizer Nano ZS (Malvern, UK). Samples (1 mL) were loaded into a cuvette and the particle size and zeta potential were measured.

2.4.2. Fourier-transform infrared spectroscopy (FTIR) spectroscopy and thermogravimetric analysis

FTIR spectra were recorded on a Nicolet FTIR-5DX spectrometer (Nicolet Instruments, Madison, WI) using KBr pellets. Thermogravimetric analysis was performed using a Shimadzu Model TGA-50 thermogravimetric analyser (Shimadzu, Kyoto, Japan) in air with a heating rate of 10 °C/min.

2.4.3. Transmission electron microscopy (TEM)

TEM images were obtained using a Leo 912 AB electron microscope (LEO, Oberkochen, Germany) operated at 120 kV. A drop of complex solution was placed on a Formvar carbon-coated 300-mesh copper grid. All of the samples were sonicated before this step.

2.4.4. Drug loading efficiency

The drug loading percentage was calculated using the following equation:

$$\text{drug loading \%} = (\text{cisplatin detected after dialysis})/[\text{initial cisplatin amount (before dialysis)}] \times 100$$

2.5. Assessment of anti-promastigote activity

Assessment of the anti-promastigote activity was performed in 96-well plates. Initially, 1×10^5 promastigotes in 100 μL of RPMI-1640 medium were added to each well. Then, two-fold serial dilutions in ten concentrations were prepared for each compound in order to produce a logarithmic dose–response curve and to calculate the half-maximal inhibitory concentration (IC_{50}) using GraphPad Prism v.6 (GraphPad Software Inc., La Jolla, CA). The concentration ranges of the compounds used for serial dilution were as follows: cisplatin-bonded SWCNT (CP-SWCNT), 0.028–14.4 μM; cisplatin-bonded MWCNT (CP-MWCNT), 0.021–11.2 μM; SWCNT, 0.034–17.6 μM; MWCNT, 0.028–14.4 μM; free cisplatin, 0.031–16 μM; and Glucantime®, 0.21–108.8 μM. SWCNT and MWCNT were considered as control for CP-SWCNT and CP-MWCNT, respectively, and cisplatin as a controls for both. All of the experiments were performed in triplicate. In addition, three wells with the parasite and without any drug were taken as the negative control. The plates were incubated at 24 °C for 72 h and the rate of promastigote death was then calculated by mixing 20 μL of 2% formaldehyde solution in phosphate-buffered saline (PBS) and 20 μL of each well content and examining the promastigotes using a haemocytometer under a light microscope. The death rate (DR) of the promastigotes was calculated using the following formula:

$$\text{DR}(\%) = [(\text{NC} - \text{DT})/\text{NC}] \times 100$$

where NC is the number of promastigotes in the negative control and DT is the number of promastigotes in the drug-treated samples.

2.6. Assessment of anti-amastigote activity

Murine peritoneal macrophages were extracted from Swiss Webster mice according to a protocol described previously [19]. The experiment was performed in 96-well plates. Initially, 5×10^4 macrophage cells in 100 μL of RPMI-1640 medium were added to each well. After incubating the plates at 37 °C in 5% CO_2 for 24 h, 5×10^5 stationary-phase promastigotes in 100 μL of RPMI-1640 medium were added to each well (promastigote:cell ratio, 10:1) and the plates were incubated in the same conditions. The total solution of each well was discarded to remove free promastigotes. Then, 100 μL of RPMI-1640 medium was added to each well and CP-SWCNT, CP- MWCNT, SWCNT, MWCNT, cisplatin or Glucantime® were separately added in triplicate to the wells at the same range of concentrations mentioned above to produce a logarithmic dose–response curve and to calculate the IC_{50} using GraphPad Prism software. The negative control included three wells of amastigote-infected macrophages without any treatment. Three wells each with 100 μL of RPMI-1640 medium were also considered as blanks. The plates were incubated at 37 °C in 5% CO_2 for 72 h. Subsequently, 10 μL of MTT solution (5 mg/mL stock solution in PBS) (Sigma) was added to each well (0.5 mg MTT per mL) and the plates were incubated at 37 °C in 5% CO_2 for 4 h. Then, 100 μL of dimethyl sulphoxide (DMSO) was added to each well and after mild rotation for 30 min the plates were read using a scanning multiwell spectrophotometer (Synergy™ LX ELISA reader; BioTek Inc., Winooski, VT) at a wavelength of 570 nm. The death rate (DR) was determined by the following formula:

$$DR(\%) = 1 - [(AT - AB)/(AC - AB)] \times 100$$

where AT is the mean optical density (OD) of the treated wells for each drug, AC is the mean OD of the negative control and AB is the mean OD of the blank wells.

2.7. Cytotoxicity assess and selectivity index (SI)

The effects of the compounds CP-SWCNT, CP-MWCNT, SWCNT, MWCNT, cisplatin and Glucantime® were measured on macrophages alone at the same range of concentrations mentioned previously to produce a logarithmic dose–response curve and to calculate the 50% cytotoxic concentration (CC_{50}) using GraphPad Prism software. The SI was calculated by dividing the CC_{50} by the IC_{50} of amastigotes [20]. A SI of >10 indicates the safety of a compound [21].

2.8. Data analysis

GraphPad Prism v.6 was used to calculate both absolute and relative (or maximal) IC_{50} values. However, the absolute IC_{50} was considered in this study for statistical analysis. Statistical analysis was performed by two-tailed *t*-test using IBMSPSS Statistics v.20 (IBM Corp., Armonk, NY).

3. Results

3.1. Transmission electron microscopy and infrared spectrum

TEM images of CP-SWCNT and CP-MWCNT are shown in Fig. 1, clearly showing the pegylation of carbon nanotubes (arrows) and the cutting section of acid treatment on the carbon nanostructure is visible. The IR spectrum was also utilised to identify the nature of the functional groups on the CNT. This experiment demonstrated the carboxyl and hydroxyl groups on the surface and ends of the CNT. The hydroxyl broad peak as well as the C=O bonds, C—H and C—O stretching vibrations are observed in this spectrum (Fig. 2).

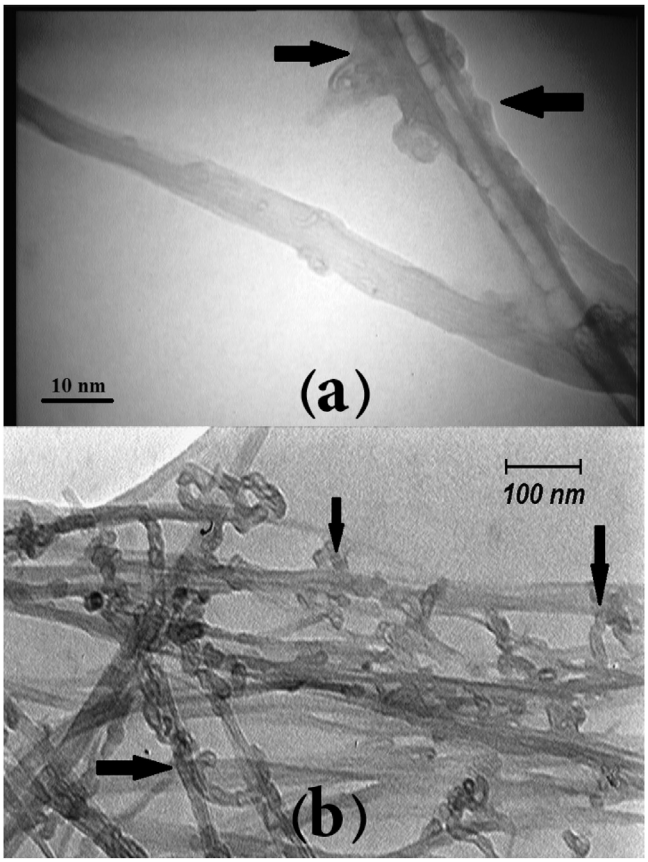

Fig. 1. Transmission electron microscopy images of (a) cisplatin-bonded single-walled carbon nanotubes (CP-SWCNT) and (b) cisplatin-bonded multi-walled carbon nanotubes (CP-MWCNT). Arrows indicate the pegylated parts.

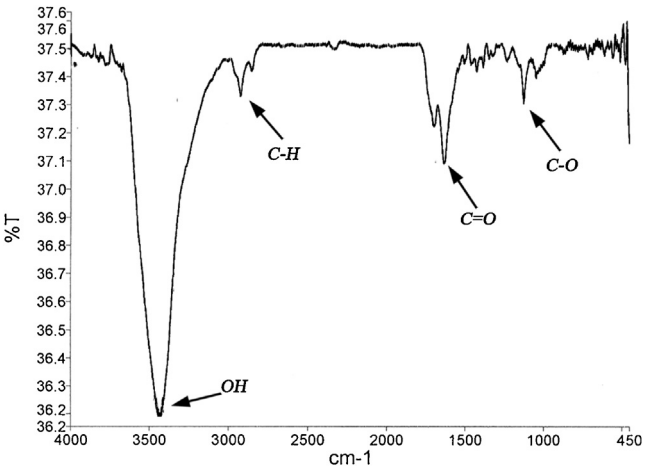

Fig. 2. Infrared spectrum of carbon nanotubes following acid treatment.

The TEM images show that the particle size of the P-SWCNT and P-MWCNT was 100–1000 nm in the longitudinal direction. The diameter of synthesised complexes was <10 nm and <30 nm for P-SWCNT and P-MWCNT, respectively. The zeta potential of both complexes was ca. −23 mV.

3.2. Drug-treated promastigotes

The IC_{50} values obtained for CP-SWCNT and CP-MWCNT were 0.39 μM and 0.24 μM, respectively ($P > 0.05$) (Fig. 3). There was a

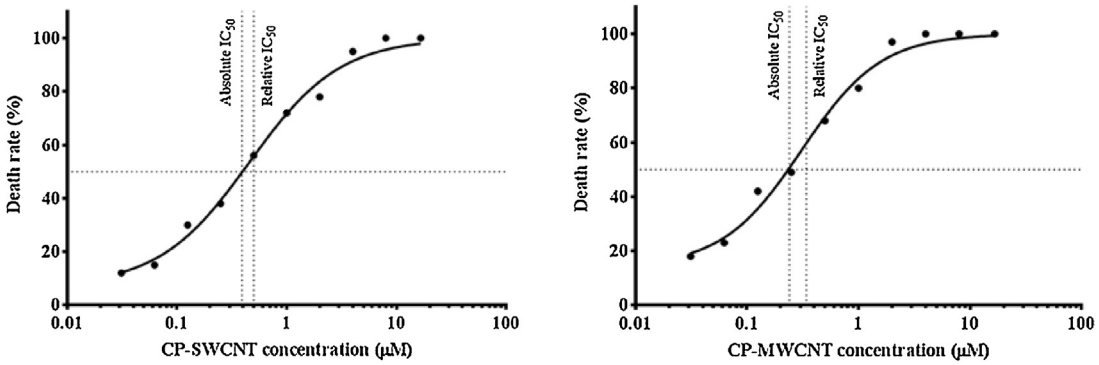

Fig. 3. Effect of cisplatin-bonded single-walled carbon nanotubes (CP-SWCNT; left) and cisplatin-bonded multi-walled carbon nanotubes (CP-MWCNT; right) on promastigotes of *Leishmania major*. For CP-SWCNT, absolute IC_{50} = 0.39 μM and relative IC_{50} = 0.51 μM; and for CP-MWCNT, absolute IC_{50} = 0.24 μM and relative IC_{50} = 0.34 μM. IC_{50}, half-maximal inhibitory concentration.

statistically significant difference between the IC_{50} of CP-SWCNT and each of the other compounds, including SWCNT, cisplatin, and Glucantime® ($P < 0.05$). Furthermore, the IC_{50} obtained for CP-MWCNT showed a statistically significant difference compared with MWCNT, cisplatin and Glucantime® ($P < 0.05$). The IC_{50} values for all compounds are presented in Table 1.

3.3. Drug-treated amastigotes

The IC_{50} values obtained for CP-SWCNT and CP-MWCNT were 0.17 μM and 0.11 μM, respectively ($P > 0.05$) (Fig. 4). Statistical analysis showed a significant difference between the IC_{50} of CP-SWCNT and each of the other compounds, including SWCNT, cisplatin and Glucantime® ($P < 0.05$). In addition, comparison of the IC_{50} obtained for CP-MWCNT with the IC_{50} values of MWCNT, cisplatin and Glucantime® showed a statistically significant difference ($P < 0.05$). Moreover, the lowest SI was for CP-SWCNT (5.23) and the highest SI was for Glucantime® (16.28). The IC_{50}, CC_{50} and SI values for all compounds are given in Table 1.

4. Discussion

Owing to drug resistance and side effects of current medications, introduction of a new drug has constantly been a goal for the treatment of CL. According to previous studies, some anticancer drugs such as 9-anilinoacridines, 8-hydroxyquinolines, phospholipid analogues, trans-platinum drugs and several registered patents have shown antileishmanial activity [17]. However, because of the side effects of these anticancer drugs, the aim has been to reduce their dosage for treatment of leishmanial infection. An effective approach to reduce the drug dosage is to improve the drug delivery system using nanoparticles. In the current study, P-SWCNT and P-MWCNT were used as nanocarriers on which the drug cisplatin was bound.

In the realm of drug delivery, CNT have gained tremendous attention as promising nanocarriers owing to their distinct characteristics such as high surface area, enhanced cellular uptake, and the possibility to be easily conjugated with many therapeutic agents including small biological molecules with superior efficacy, enhanced specificity and diminished side effects [22,23]. On the other hand, some researchers believe that the safety, efficacy, sensitivity and specificity of nanoparticles for the treatment of human diseases still need to be evaluated further [24,25].

The results of the current study showed that both CP-SWCNT and CP-MWCNT were more effective than free cisplatin on promastigotes ($P < 0.05$). Similar results were obtained regarding the effect of these two compounds on amastigotes compared with free cisplatin ($P < 0.05$). This showed that nanoformulation could improve the effect of these two compounds and reduce the dose of cisplatin. The SI of CP-SWCNT was <10 (5.23), indicating that this compound is not completely safe and affects both macrophage and amastigotes. However, the SIs of CP-MWCNT (12.54) and Glucantime® (16.28) were >10, indicating the selective effect of these two compounds on the parasite. Nevertheless, the IC_{50} of CP-MWCNT (0.11 ± 0.09 μM) for amastigotes was 41-fold lower than that of Glucantime® (4.52 ± 1.31 μM), suggesting that a lower dose of CP-MWCNT in comparison with Glucantime® is required to kill 50% of amastigotes. Thus, further studies are required to exactly determine the efficacy and safety of CP-MWCNT in vivo.

In a previous study, the efficacy of betulin and CNT-attached betulin were evaluated against *Leishmania donovani* and the results showed that CNT-attached betulin was more effective with lower toxicity than the free form of betulin [26].

Table 1
Values for half-maximal inhibitory concentration (IC_{50}), 50% cytotoxic concentration (CC_{50}) and selectivity index (SI) for promastigotes and amastigotes of *Leishmania major* treated with various compounds.

Compound	Promastigotes	Amastigotes		
	IC_{50} (μM) (mean ± S.D.)	IC_{50} (μM) (mean ± S.D.)	CC_{50} (μM) (mean ± S.D.)	SI[a]
CP-SWCNT	0.39 ± 0.09	0.17 ± 0.07	0.89 ± 0.22	5.23
CP-MWCNT	0.24 ± 0.11	0.11 ± 0.09	1.38 ± 0.54	12.54
SWCNT	1.22 ± 0.09	0.73 ± 0.15	7.94 ± 1.63	10.88
MWCNT	3.11 ± 0.26	1.3 ± 0.3	8.06 ± 3.08	6.20
Cisplatin	1.75 ± 0.40	0.76 ± 0.12	4.97 ± 1.07	6.53
Glucantime®	4.41 ± 0.98	4.52 ± 1.31	73.63 ± 5.56	16.28

S.D., standard deviation; CP-SWCNT, cisplatin-bonded single-walled carbon nanotubes; CP-MWCNT, cisplatin-bonded multi-walled carbon nanotubes; SWCNT, single-walled carbon nanotubes; MWCNT, multi-walled carbon nanotubes.
[a] $SI = CC_{50}/IC_{50}$.

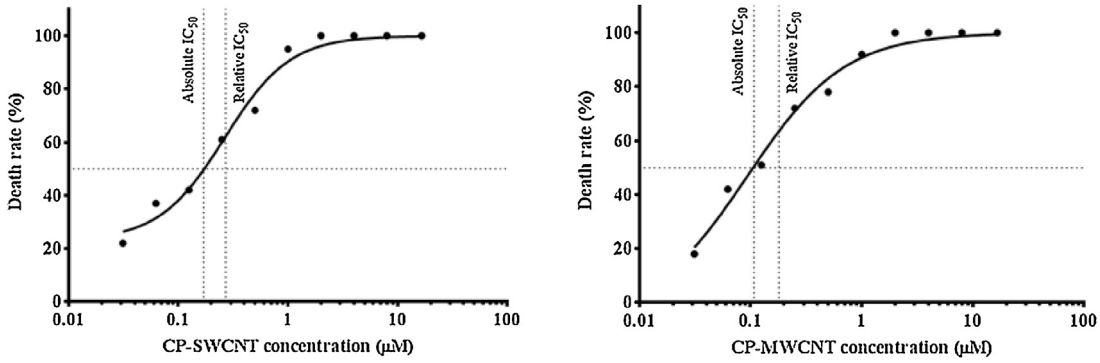

Fig. 4. Effect of cisplatin-bonded single-walled carbon nanotubes (CP-SWCNT; left) and cisplatin-bonded multi-walled carbon nanotubes (CP-MWCNT; right) on amastigotes of *Leishmania major*. For CP-SWCNT, absolute IC_{50} = 0.17 μM and relative IC_{50} = 0.26 μM; and for CP-MWCNT, absolute IC_{50} = 0.11 μM and relative IC_{50} = 0.18 μM. IC_{50}, half-maximal inhibitory concentration.

Some studies have shown that when an antileishmanial drug bonds to nanoparticles, its toxic effects are reduced. In one study, amphotericin B-loaded MWCNT showed a greater effect on intracellular amastigotes than amphotericin B alone. Furthermore, MWCNT loaded with amphotericin B showed no toxic effect on the liver and kidney of *Leishmania*-infected hamsters and significantly inhibited parasite replication in vivo [27]. In another study, researchers orally administrated the same aforementioned MWCNT loaded with amphotericin B to *Leishmania*-infected hamsters and observed that they significantly inhibited parasite growth by 99% [28]. Likewise, other studies revealed that CNT effectively increased the drug efficacy on *Leishmania*-infected cells and reduced the dosage and toxicity of the drug [29].

Despite many studies conducted on the use of nano-formulated drugs/compounds in the treatment of leishmaniasis, many factors still need to be optimised for use of these drugs in humans, of which pharmacokinetics and pharmacodynamics are two highly important categories [30]. Until now, the only nanoparticle-based antileishmanial drug approved for humans by the US Food and Drug Administration (FDA) is AmBisome® [31].

It seems that the use of nanotechnology improves the function of drugs in the treatment of leishmaniasis, leading to a reduction in the dosage and duration of treatment and likely preventing drug resistance.

In conclusion, in this study CP-MWCNT showed considerable antileishmanial activity on amastigotes of *L. major* with a selective killing effect on infected macrophages as well as greater efficacy in comparison with Glucantime®. As a whole, according to the potent in vitro antileishmanial activity of CP-MWCNT at low concentration against *L. major*, we suggest that they are evaluated in an animal model.

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Competing interests

None declared.

Ethical approval

Not required.

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