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Research paper

Nanoencapsulation of usnic acid: An attempt to improve antitumour activity and reduce hepatotoxicity

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Abstract

Despite the recognised antiproliferative and antitumour properties of usnic acid, its therapeutic application has yet to be introduced. In fact, the high hepatotoxicity and low water solubility of usnic acid have somewhat restricted its practical use in anticancer therapy. The aim of this study was therefore to investigate the antitumour activity of usnic acid encapsulated into nanocapsules prepared with lactic co-glycolic acid polymer. Usnic acid-loaded nanocapsules were obtained using the interfacial deposition of a preformed polymer. The antitumour activity was confirmed on an ascitic tumour (Sarcoma-180) implanted in Swiss mice and estimated by means of the tumour inhibition. The results of antitumour activity confirmed that the encapsulation of usnic acid into PLGA-nanocapsules produced a 26.4% increase in tumour inhibition as compared with the standard free usnic acid treatment. Vacuolization of hepatocytes and a mild lymphocytic infiltration in portal spaces were observed in animals treated with free usnic acid. However, this hepatotoxicity was substantially reduced when animals were treated with usnic acid-loaded nanocapsules. No histological changes were noticed in the kidneys or spleen of animals treated either with usnic acid or usnic acid-loaded nanocapsules. These results suggest that nanoencapsulation may be a way of enabling usnic acid to be used in chemotherapy.

Keywords: Usnic acid; *Cladonia substellata*; Nanocapsules; Antitumour activity; Toxicity

1. Introduction

The antitumour activity of usnic acid was first reported more than two decades ago. In 1975, the inhibition activity of usnic acid against Lewis lung carcinoma was reported [1]. Among several lichen constituents, (+)-usnic acid enantiomer exhibited the highest activity in an assay for evaluating the inhibition of tumours induced by the

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Epstein–Barr virus, and its inhibitory activity appeared to be rather stronger than that of (−)-enantiomer [2].

The fact that usnic acid is a non-genotoxic antineoplastic agent that works in a p53-independent manner [3] makes it a potential candidate for novel cancer therapies. Nevertheless, applications of usnic acid have been to a certain extent limited by its poor solubility in water [4] and its hepatotoxicity [5–7], which has also limited its use in anticancer therapy.

Nanotechnology is particularly valuable for formulating new therapeutic dosage forms containing drugs derived from biotechnology (e.g. peptides, proteins, genes and anticancer drugs), because it can protect drugs from degradation in biological fluids and improve their penetration into cells. It can also be important with respect to small hydrophobic molecules, as it can provide ultra-dispersed pharmaceutical dosage forms, which allow rapid drug dissolution. Nanosystems thus may be able to improve the efficacy of existing drugs, and systems involving new active molecules are expected to be available soon [8].

In accordance with the above mentioned findings the usnic acid was encapsulated into nanocapsules [9], providing a suitable dosage form allowing further *in vivo* studies. In the present study, the antitumour activity of usnic acid-loaded nanocapsules was assessed in Sarcoma 180-bearing mice. Additionally, histopathological, haematological and biochemical analyses were carried out to verify the renal and hepatotoxicity of free and encapsulated usnic acid on animals in a subchronic toxicity study.

2. Materials and methods

Poly (D,L-lactic-acid-co-glycolic acid) polymer (PLGA 50/50, inherent viscosity 0.57 dl/g, 30 °C) was purchased from Birmingham Polymers (Alabama, USA); soya phosphatidylcholine (Epikuron® 200) was obtained from Lucas Meyer (Germany); poloxamer 188 was generously supplied by ICI (France); purified soybean oil, usnic acid used as a reference in standard calibration curves, and trehalose were all purchased from Sigma–Aldrich (St. Louis, USA); HPLC grade methanol, analytical grade solvents and reagents were obtained from Merck (Darmstadt, Germany). The Tween 80 and standard usnic acid (UA) were obtained from Sigma–Aldrich (St. Louis, USA). Usnic acid was extracted and purified from *Cladonia substellata* (Vainio). The lichen material was collected on sandy soils of *tabuleiro* (savannah-like vegetation in the Mamanguape region of Paraíba, northeast Brazil) and identified through morphological and chemical talus characterization [10].

2.1. Preparation and characterization of usnic acid-loaded nanocapsules

Usnic acid-loaded nanocapsules prepared with 50/50 poly (lactic-glycolic acid) copolymer (PLGA) were obtained as previously described [9]. The polymer, the soybean oil, the usnic acid and the soya phospholipid were each dissolved in acetone. All these organic solutions were then mixed and maintained at 40 °C under magnetic stirring at 150 rpm for 10 min. The aqueous phase consisted of the poloxamer®188 and 10% trehalose dissolved in 50 ml of a phosphate buffer solution (pH 7.4) at 0.2 M. Next, the organic solution was gradually poured into the aqueous phase under magnetic stirring at 150 rpm for 30 min. Nanocapsules were produced as the acetone was removed under reduced pressure at 40 °C. The colloidal suspension was concentrated to a 10 ml final volume by removing the water in similar conditions. Finally, the suspension of nanocapsules was conditioned in sealed vials and stored at 4 °C.

The particle size and surface charge potential of usnic acid-loaded nanocapsules were determined using a Zetasizer® (Nano-ZS90, Malvern, United Kingdom). Samples of nanocapsules were diluted in water as required for a satisfactory particle count. The distribution and the mean diameter of particles were evaluated as well as their standard deviation and polydispersity index (PDI). The surface charge of usnic acid-loaded nanocapsules was determined by measuring the zeta potential (ξ) through an electrophoresis technique. Results are presented as the average of at least three measurements of different samples for a same batch of nanocapsules.

2.2. Antitumour activity of usnic acid-loaded nanocapsules

In the present investigation five groups of six animals each were randomised into positive and negative controls and treated groups. A negative control group with healthy animals was added. The ascitic tumour (5×10^6 cells/ml/animal) was subcutaneously inoculated in the inguinal area of male Swiss mice (32–41 g body weight, 45–60 days old). Treatment of the animals was started 24 h after tumour inoculation with daily i.p. injections of usnic acid suspension (0.5% Tween 80) or usnic acid-loaded nanocapsules at a dose of 15 mg/kg/day for 7 days. The positive and negative control groups were treated daily with phosphate-buffered saline (pH 7.4) for 7 days. After a week of treatment, the animals were anaesthetised with ketamine and xylazine and sacrificed by decapitation. Blood samples were collected in CBC bottles containing 3% EDTA and were analyzed for haematology within 20 min. Tumours and organs (liver, kidneys and spleen) were excised, and their weights were measured prior to histopathological analysis. The antitumour activity was calculated as % tumour inhibition $= (C - T)/C \times 100$, where C is the average tumour weight of the control group and T is the average tumour weight of the usnic acid-treated groups [11].

The animal experiments were performed according to the protocol recommended by the National Cancer Institute (NCI) [12] with the approval of the Ethics Committee for Experiments on Animals of the Federal University of Pernambuco (Recife, Brazil).

2.3. Histopathological analysis

Sample tissues of tumour, liver, kidneys and spleen were fixed in 10% neutral buffered-formalin solution, trimmed,

embedded in paraffin, sectioned at 4–6 μm, and stained with haematoxylin and eosin. Tissue sections were examined by light microscopy (Olympus BH-2, Japan).

2.4. Haematology

Blood samples were analyzed for red blood cells (RBC), white blood cells (WBC), and haematocrit (Ht) determination. For the cell count, blood smears were prepared, stained with Panotic dye (Instant Prov, São Paulo, Brazil) and observed by light microscopy (Olympus BH-2, Japan).

2.5. Survival of tumour-bearing mice

Male Swiss mice (32–41 g body weight, 45–60 days old) were randomly divided into three groups with 10 animals each. Sarcoma 180 cells (5×10^6 cells/ml/animal) were subcutaneously inoculated in the inguinal area of the animals. Twenty-four hours after tumour inoculation the mice began to receive daily i.p. injections of usnic acid suspension or usnic acid-loaded nanocapsules at a dose of 15 mg/kg/body weight/day for 7 days. The control group received phosphate-buffered saline (pH 7.4) daily for 7 days. The behaviour of the animals was monitored during the treatment and the length of survival recorded.

2.6. Subchronic toxicity of usnic acid

Healthy male Swiss mice (32–41 g body weight, 45–60 days old) were acclimatized for approximately 1 week and randomly assigned to three groups of six animals each. The two treated groups received daily i.p. injections of usnic acid suspension (0.5% Tween 80) or usnic acid-loaded nanocapsules (15 mg/kg/day) for 15 days. The control group was treated with pH 7.4 phosphate-buffered saline. Throughout the study period, the behaviour of the animals was observed and body weights measured on a daily basis. Clinical signs of toxicity such as tremors, morbidity and ataxia were monitored. After treatment, the animals were anaesthetised with ketamine and xylazine and sacrificed by decapitation. Blood was recovered and submitted to biochemical analysis. Blood samples were centrifuged at 3000 rpm for 10 min within 1 h after collection. The serum samples were stored at −80 °C prior to analysis. Blood urea nitrogen (BUN), creatinine (CRT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a biochemistry analysis kit (Katal Biotecnologica, Brazil). Immediately after the animals were sacrificed, liver, kidneys and spleen were removed and histopathological analysis performed as previously described.

2.7. Statistics

The mean value and standard deviations were calculated from tumour and organ weights, haematology and biochemistry parameters of the treated and untreated groups of animals. The statistical significance of data from the treated groups compared with the control group was analyzed using a one-way analysis of variance (ANOVA) and matched-pair comparisons were also performed using the Tukey test. As usual, the level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Usnic acid-loaded nanocapsules

Nanocapsules of PLGA containing acid usnic presented a mean particle size of 214 ± 75 nm with a narrow polydispersity index (PDI = 0.26). It was observed that the drug encapsulation did not affect the mean size of particles. In fact, unloaded-PLGA-nanocapsules exhibited a mean diameter of 167 ± 55 nm (PDI = 0.19). Moreover, the particle size evolution of the usnic acid-loaded nanocapsule suspension was negligible for 120 days (285 ± 116 nm; PDI = 0.43) when stored at 4 °C. The size of usnic acid-loaded nanocapsules in the range of 200 nm can be considered suitable for *in vivo* administration through the intraperitoneal route.

The surface charge of usnic acid-loaded nanocapsules was determined by measuring the zeta potential (ξ), which was -28.4 ± 8 mV.

3.2. Antitumour activity of usnic acid-loaded nanocapsules

Recent investigations have demonstrated that the distribution profile of anticancer drugs can be partially controlled by their encapsulation in colloidal nanosystems. These nanocarriers are thus able to increase antitumour effectiveness, thereby reducing drug side effects [8].

Studies have been conducted to investigate the use of usnic acid as an anticancer agent. To this end, this drug was encapsulated into PLGA-nanocapsules and its cytotoxicity evaluated in NCI H-299 and Hep2 cells as previously reported [9]. In the present investigation, the antitumour activity of usnic acid-loaded nanocapsules was evaluated in Sarcoma 180-bearing mice treated with 15 mg/kg daily for 7 days. Furthermore, subchronic toxicity was evaluated in healthy animals treated with the same dose for 15 days. Each evaluation was performed by comparing the nanoencapsulated form with an usnic acid suspension in 0.5% Tween 80, chosen on account of its low water solubility.

All animals survived to the end of the treatment and no clinical or behavioural abnormalities were observed. A delay in the development and a reduction in the size of the tumours, as well as increased infiltration by lymphoid cells, granulation tissue, and fibrosis surrounding the tumour, were detected with the usnic acid treatment. After treatment of the animals with usnic acid suspension or usnic acid-loaded nanocapsules, a variation in the tumour mass was found. Untreated animals presented a progressive increase in tumour growth, achieving a weight of 1.07 ± 0.15 g on average at 7 days after inoculation with

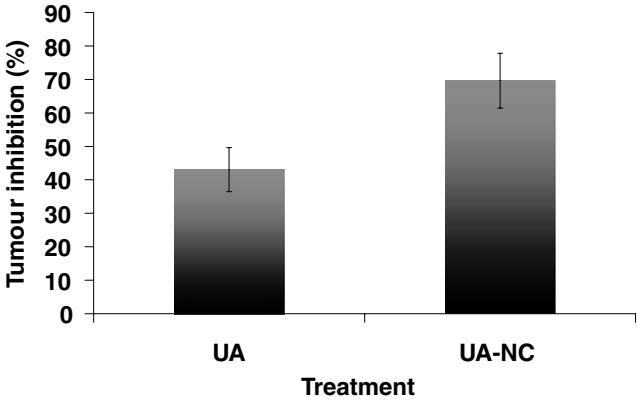

Fig. 1. Evaluation of the antitumour activity of free and encapsulated usnic acid against Sarcoma 180-bearing mice: UA, usnic acid suspension and UA-NC, usnic acid-loaded nanocapsules.

tumour cells. In contrast, the treatment with encapsulated usnic acid produced a decrease in tumour mass (0.281 ± 0.013 g) as compared with the drug suspension (0.995 ± 0.050 g). These results show that the encapsulated usnic acid dosage form presents a higher tumour regressive effect than the usnic acid suspension one.

In view of such a tumour inhibition, it can be seen that the group treated with usnic acid-loaded nanocapsules presented a $69.7 \pm 6\%$ inhibition as compared with the control group. Nanoencapsulation improved the activity of usnic acid by 26.4% in contrast with its suspension form, which presented an antitumour activity of $43.3 \pm 4\%$ as compared with to the control group (Fig. 1).

These results are corroborated by a recent investigation of the antitumour activity of usnic acid encapsulated into PLGA-microspheres, which showed an improvement of 21% in tumour inhibition in comparison with usnic acid suspension on Sarcoma 180-bearing mice [13]. Taking into account the fact that the same experimental protocols were adopted for both studies, nanoencapsulation seems to be more effective than microencapsulation in improving the antitumour activity of usnic acid. These findings could be explained by the faster penetration of nanocapsules into the tumour tissue than microspheres, which can accumulate in the peritoneal cavity, making the uptake of microparticles by the tumour cells more difficult. These results corroborate the widespread hypothesis that the size of particles of nanocarrier systems is an important parameter to be considered in drug bioavailability studies.

3.3. Histopathological analysis

The histopathological analyses of tumour and liver of animals treated with free and encapsulated usnic acid are illustrated in Figs. 2 and 3, respectively. Microphotographs of tumour revealed the presence of both typical and atypical cells in constant mitosis after treatment with usnic acid (Figs. 2a and b). However, more extensive necrotic areas with uncharacterized pyknotic nuclear cells were observed

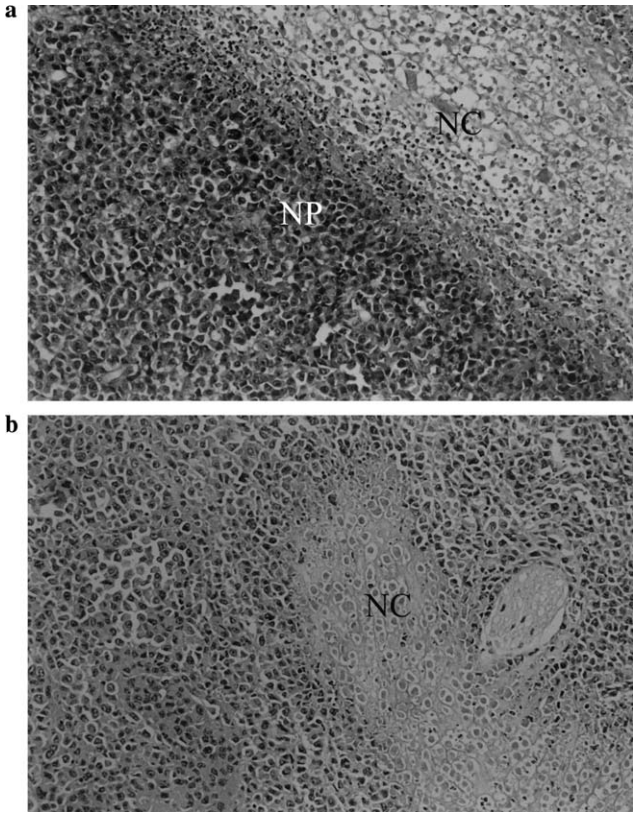

Fig. 2. Histopathological analysis of tumour-bearing mice (H & E, 50×): usnic acid suspension (a), and usnic acid-loaded nanocapsules (b). NC, necrosis; NP, pyknotic nucleus.

on the tumour tissue of animals treated with usnic acid-loaded nanocapsules (Fig. 2b). In fact, a few residual neoplastic cells inserted into a large central area of necrosis were observed in the tumour.

Tumour histopathological studies thus confirmed a great reduction in tumour size after treatment with usnic acid-loaded nanocapsules as compared with the unloaded drug. Moreover, histological observation indicated an increase in the number of lymphocytes on the peritumoural tissue. These findings suggest that the immune system was stimulated, providing an increased host response to the tumour.

Our *in vivo* results bear out the mechanism of the antitumour activity of usnic acid in Sarcoma 180-bearing mice via necrosis without apoptosis involvement as postulated from previous *in vitro* studies. In fact, the adverse effects of usnic acid were investigated in cultured murine hepatocytes [5], suggesting that the hepatotoxicity induced by usnic acid was directly related to the inhibition of the mitochondrial function by disruption of the electron transport chain. An increase in the production of reactive oxygen species was observed, which ultimately leads to cell death. A corresponding drop in cellular ATP levels was also detected after usnic acid treatment, confirming a disruption in mitochondrial bioenergetics. From that study it can be inferred that usnic acid acts as both an inhibitor and uncoupler of mitochondria. Usnic acid causes ATP deple-

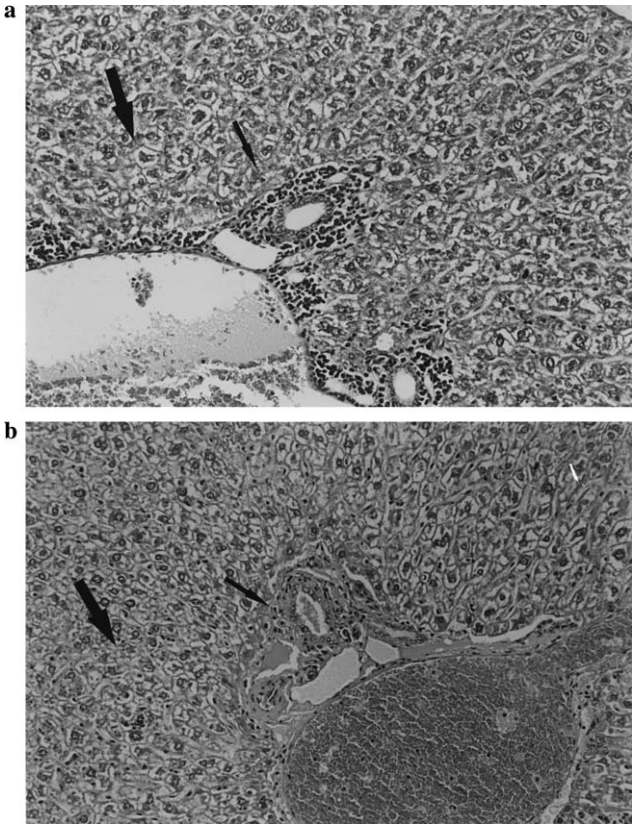

Fig. 3. Histopathological analysis of liver in Sarcoma 180-bearing mice (H & E, 50×): usnic acid suspension (a), and usnic acid-loaded nanocapsules (b). Thin arrows indicate periportal leukocyte infiltration and thick arrows show uncharacterized hepatocytes presenting karyorexis.

tion in hepatocytes and induces necrosis. Oxidative stress and mitochondria inhibition play an important role in mediating usnic acid-induced hepatotoxicity.

Moreover, a recent study on the relationship between the antineoplastic activity of usnic acid and p53 activation [3] revealed that usnic acid has antiproliferative activity against the wild-type p53 (MCF7), as well as the nonfunctional p53 (MDA-MB-231) breast cancer cell lines, and the lung cancer cell line H1299 (null for p53), acting as a non-genotoxic anticancer agent, which works in a p53-independent manner.

Morphological alterations were detected in the liver of the animals treated with either free or encapsulated usnic acid treatments (Fig. 3). Vacuolization of hepatocytes and an intensive lymphocyte infiltration in portal spaces can be seen in the liver of animals treated with usnic acid suspension (Fig. 3a) in comparison with the controls. On the other hand, the liver of animals treated with usnic acid-loaded nanocapsules presented only morphologically uncharacterized hepatocytes and a mild lymphocyte infiltration in portal spaces (Fig. 3b). Moreover, a process of hepatocytic necrosis was also shown to be present. However, this hepatotoxicity was substantially reduced as animals were treated with usnic acid-loaded nanocapsules. Liver histopathological analysis shows thereby that the nanoencapsulation of usnic acid was able to reduce hepatotoxicity when compared with the usnic acid suspension in Tween 80.

No histological changes were noticed in the spleen or kidneys of any of the animals treated with either free or encapsulated usnic acid.

3.4. Haematology

No significant alterations in RBC levels in treated and untreated Sarcoma 180-bearing mice (from 5.42 to 6.57×10^6 cells/ml) were observed in comparison with the negative control group ($6.6 \pm 0.7 \times 10^6$ cells/ml) (Table 1). However, the haematological findings did not show any treatment-related effects and were not significantly different from positive control values. No significant reduction in WBC levels occurred in the animals treated with either usnic acid suspension or usnic acid-loaded nanocapsules in comparison with the positive control group. As expected, lymphocyte levels were increased in tumour-bearing animals as compared with healthy animals. Moreover, no significant differences in lymphocytes and neutrophils were observed between the treated groups and the untreated positive control group.

The results suggested that usnic acid either in suspension or nanoencapsulated caused no haematological toxicity in the treated animals. Furthermore, usnic acid, whether free or encapsulated, has no immunological effects, since the same quantity of leukocytes is observed in the untreated tumour-bearing animals. However, further investigations should be carried out to support the contention that usnic acid has no effect on the immuneresponse of the host.

3.5. Survival of tumour-bearing mice

Analysis of the survival of Sarcoma 180-bearing mice revealed that the behaviour and clinical findings were preserved in the first week of treatment of animals with free and encapsulated usnic acid. From the second week on, animals presented bristling hair, reduced reflexes, weight

Table 1
Haematological findings in mice treated with usnic acid suspension (UA) and usnic acid-loaded nanocapsules (UA-NC) at a dose of 15 mg/kg for 7 days

Haematological parameters	Treatments			
	UA	UA-NC	Control (+)	Control (−)
RBC (10^6/ml)	5.42 ± 1.38	6.57 ± 2.01	6.18 ± 1.16	6.6 ± 0.7
Ht (%)	40 ± 1	40 ± 3	39 ± 2	42 ± 1
WBC (10^4/mm^3)	1.5 ± 0.03	1.6 ± 0.07	1.6 ± 0.03	1.3 ± 0.09
Lymphocytes (%)	53.0 ± 15	41.8 ± 10	59.3 ± 1	74.9 ± 20
Neutrophils (%)	36.6 ± 13	40.0 ± 12.5	32.5 ± 7.5	19.9 ± 15

Values are presented as Means ± SD.
Control (−), untreated healthy mice; Control (+), untreated Sarcoma180-bearing mice; RBC, red blood cell count; WBC, white blood cell count; Ht, haematocrit.

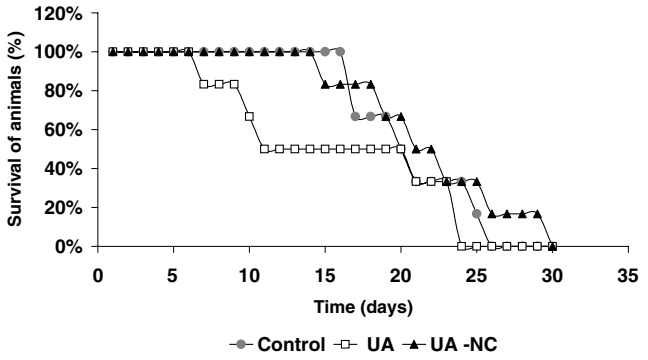

Fig. 4. Survival evaluation of Sarcoma-bearing mice treated with free and encapsulated usnic acid. UA, usnic acid suspension and UA-NC, usnic acid-loaded nanocapsules.

loss, and irregularity in respiratory frequency and consequent death (Fig. 4). Fifty per cent of the animals treated with usnic acid suspension had died at 20 days, and all had succumbed 24 days after the tumour inoculation. Moreover, in the group treated with encapsulated usnic acid, a 50% death rate was recorded on the 21st day and 16.7% of the animals survived for 29 days. During this period, the mice in the aforementioned two groups presented clinical features similar to those of the group that received usnic acid suspension, although the alteration in respiratory frequency was stronger in the former group. In the control group, the death rate was recorded 2 weeks after the tumour inoculation. At 20 days, it stood at 50%, reaching 100% at 25 days. These results show that the encapsulation of usnic acid was able to achieve a 33% survival rate, when compared with the group treated with usnic acid suspension 24 days after the tumour inoculation.

3.6. Subchronic toxicity of usnic acid

There were no statistically significant differences in body weight between the control and treated animal groups. No noteworthy clinical signs were observed in any animal and no deaths occurred during treatment. The serum levels of BUN, CRT, ALT and AST of the control group were 140 mg/dl, 1.07 mg/dl, 289.4 IU/l and 241.5 IU/l, respectively. Whereas the serum levels of BUN, CRT, ALT and AST of the animals treated with usnic acid suspension were 156 mg/dl, 0.94 mg/dl, 552 IU/l, and 403.1 IU/l, the encapsulated usnic acid produced values of 138 mg/dl, 0.82 mg/dl, 406 IU/l and 322.5 IU/l, respectively (Table 2).

No alterations in serum levels of BUN and CRT were observed with the usnic acid treatment, strongly suggesting that the renal function of animals was preserved with this treatment. Therefore, the usnic acid seems not to cause any renal toxicity when administered as a long-term treatment.

Serum transaminase activity in the treated groups was significantly higher than in the control group. Serum transaminase activities were increased with the usnic acid treatment, indicating liver cell injury. The hepatotoxicity of usnic acid was thus confirmed in the subchronic toxicity study. High serum levels of transaminases suggest a chronic hepatic dysfunction caused by usnic acid. Nevertheless, the encapsulation of usnic acid was able to reduce the hepatotoxicity of usnic acid.

Table 2
Serum biochemical findings in Swiss mice treated with usnic acid suspension (UA) and usnic acid-loaded nanocapsules (UA-NC) at a daily dose of 15 mg/kg for 15 days

Biochemical parameters	Treatments		
	Control	UA	UA-NC
BUN (mg/dl)	140 ± 11.50	156 ± 5.58	138 ± 8.83
CRT(mg/dl)	1.07 ± 0.09	0.94 ± 0.10	0.82 ± 0.10
ALT (IU/l)	289.44 ± 23.30	552 ± 0.82	406 ± 9.45
AST (IU/l)	241.51 ± 14.80	403.10 ± 6.76	322.48 ± 32.36

BUN, blood urea nitrogen; CRT, creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Our results are partially in disagreement with those previously reported [6] for a toxicity study of (+)-usnic acid in rats at a dose of 50 mg/kg or 200 mg/kg for 5 days. In that investigation, no significant change in serum transaminase activity was found; however, signs of liver cell damage were detected by electron microscopy as a marked swelling of the mitochondria and endoplasmic reticulum.

Histological observation of the liver of the animals treated with usnic acid suspension and usnic acid-loaded nanocapsules for 15 days revealed extensive necrotic areas on liver tissue after treatment with usnic acid suspension (data not shown), although this abnormality was substantially reduced with the treatment using usnic acid-loaded nanocapsules. The results demonstrated that usnic acid caused hepatotoxicity in mice treated with 15 mg/kg/day for 7 days, as shown by the signs of liver cell damage.

4. Conclusions

The present investigation performed in tumour-bearing mice confirmed the antitumour activity of usnic acid and also corroborated the hepatotoxicity induced by usnic acid caused by the process of necrosis. Furthermore, the findings showed that the encapsulation of usnic acid into PLGA-nanocapsules was able to maintain and improve its antitumour activity and considerably reduce the hepatotoxicity of this drug. These results also clearly suggest the nanoencapsulation can be offered as a way of introducing usnic acid into chemotherapy.

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