ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Layered Double Hydroxide - Cyclophosphamide Composite as Vehicle in Treatment of Mice With L5178Y Lymphoma.

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ABSTRACT

Advances in nanotechnology currently have allowed us to understand and design nanoparticles as drug vehicles to enhance stability and bioavailability. In this study, cyclophosphamide (a non-ionic anti-cancer pro-drug) was loaded into layered double hydroxide nanoparticles (LDH-CPh) and characterized by solid state techniques. Subsequently, the anti-tumor effect of these particles was evaluated in a mouse model BALB/c with L5178Y lymphoblast. The number of tumor cells in the L5178Y control group decreased from $800X10^6$ to $7X10^6$ when treated with cyclophosphamide (140 mg kg^{-1}) as a positive control; and this value was reduced to ($26X10^6$) with the LDH-CPh nanoparticles at concentration of 112 mg kg^{-1}. Both treatments were statistically different ($p \leq 0.05$) when compared to the control group. The nanoparticles inhibited the lymphoma growth in a single dosage, and with a slight content of drug (112 *vs*. 140 mg kg^{-1}). The LDH nanoparticles were recovered from the mice and the solid state analysis indicated that the layered structure was retained during the 14 days within the peritoneal medium.

Keywords: Layered double hydroxide, Cyclophosphamide, Composite, L5178Y Lymphoma, Toxicity

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ISSN: 0975-8585

INTRODUCTION

Increased development of molecular biology has precisely elucidated and revealed molecular mechanisms of several cancer types where specific molecular processes have been detailed, subsequently a search of drugs to target those process has emerged.

Drug release technology is currently a tool to study in vitro cancer therapies and here, nanoparticles have been showed applications as drug matrices transport with the ability to release a high drug concentration in target tissue. Another desired behavior in these nanoparticles is sustained release along the whole medical treatment. In order to achieve a suitable performance of nanoparticles related to all these functions, materials science joins the knowledge of biochemical, interface, and nanoparticles sciences [1].

Nano systems for drug release act as drugs vehicles transport through an organism once it provides chemical and physical stability, thus improving solubility, bio distribution, and diffusion through biological barriers intended to target desired site [2]. Such vehicles must also satisfy biocompatibility and avoid immunological responses, therefore, all the physico-chemical aspects involved in the syntheses or performance must be taken into account.

Synthetic or biological polymers are the most studied nano systems as drug vehicles, and currently there is a broad range of knowledge related to its performance. Among nanoparticles composed by inorganic matter, layered double hydroxides (LDH) are promising structures to design nano systems for life sciences [3]. These structures are able tocarry drugs [4,5] and their inorganic composition also allow them to couple cations or oxide nanoparticles for advanced diagnoses and treatment purposes like magnetism, luminescence and thermotherapy [6–8].

The crystalline structure of LDH is composed by layers from divalent and trivalent cations coordinated by hydroxyl groups resulting in layers with fully covered surfaces by hydroxyl groups. The content of trivalent cations is controlled during the synthesis and this generates an excess of positive charge in the layers, which must be neutralized by interlayer ions [9]. These compounds are represented by the general formula: $[M^{2+}_{1-x}M^{3+}_{x}(OH)_2]^{x+}(A^{n-})_{x/n}\bullet mH_2O$, where M^{2+} and $M^{3+}$are divalent and trivalent cations respectively, and A is the interlayer anion with n charge [9]. The interlayer anion –in biomedicine- is commonly replaced by drugs resulting in hybrid systems to improve the dissolution mechanism of hydrophobic drugs [10], to regulate the drug release kinetics [11,12] and to improve mechanical stability [4,5,13].

The drug release from LDHs is influenced by the nanoparticles structure and this can be tailored by changing the synthesis parameters like pH, temperature and reagents concentration and by adjusting the synthesis method (ion exchange, coprecipitation or reconstruction) [14]. Another factor affecting the drug release mechanism is the ionic strength [1].

The main feature of LDHs in life science is biocompatibility, low toxicity and drug protection or genetic material transport [15]. Additionally, these nanoparticles facilitate the access of drugs to cancer cell and reduce accumulation in healthy cells, thus reducing the side effects of anti-cancer drugs.

On the other hand, cyclophosphamide (CPh) is one of the most successful anticancer agents ever synthesized with immunosuppressive properties and thus is still widely used as a chemotherapeutic agent and in the mobilization and conditioning regimens for blood and bone marrow transplantations (BMT) [16]. CPh is an inactive prodrug that requires enzimatic and chemical activation to release active phosphoramide mustard, it belongs to the alkylant agents family [17]. It produces the inter strand and intra strand DNA cross links which impairs cell division and is responsible for the cytotoxic properties of this drug [2].

The aim of this work was to explore the viability to load CPh in LDH nanoparticles and evaluate the product as a less invasive therapeutic agent in mice BALB/c with L5178Y lymphoma.

ISSN: 0975-8585

METHODOLOGY

Synthesis of LDH:

The LDH loaded with cyclophosphamide (LDH-CPh) was attempted using the reconstruction method. A nitrate-containing LDH structure composed of zinc and aluminium cations in a molar ratio of 2.5:1 was prepared as described elsewhere [18] and the dried powder was calcined at 600 °C for 1 h. Then, 0.1142 g of the produced oxides were mixed with 0.2206 g of CPh in 25 mL of water. The suspension was stirred at 16 rpm for 15 days at room temperature. Thereafter, the powder was recovered by decantation and washed three times with de-ionized water, dried at 60 °C and stored in sealed bags.

LDH Characterization:

X-ray diffraction (XRD) profiles were acquired in a STOE diffractometer model SEIFERT Analytical X-Ray configured with 2θ geometry, using a step of 0.02 degrees and a collection time of 1 second per step. The powder samples were pressed onto a glass slide and then inserted in the sample holder. Fourier-transform infrared (FTIR) spectra for molecular analysis were collected with a Perkin-Elmer spectrometer model Spectrum One using a resolution of 4 cm^{-1} and 32 scans in the transmission mode. Morphology and structure of the particles were analyzed by transmission electron microscopy (TEM) and high resolution transmission electron microscopy (HRTEM) in a JEOL JEM2010 microscope operated with an electron beam energy of 200 keV. The samples for TEM analysis were prepared by sonicating the powder sample in distilled water and transferring one drop to the copper grid.

Bioassays:

Adult male BALB/c mice (24-32 g) were provided by the Western Biomedical Research Center of the Mexican Institute of Social Security (CIBO-IMSS). Animals were housed under standard laboratory conditions with food and water ad libitum. Each mice group used in experiment consisted of 5 animals. They were inoculated i.p. with 0.1 mL of suspension ascites fluid, containing L5178Y lymphoma 2 x 10^4 cells per mouse, on day 0. This tumor line was derived from murine thymic lymphoma (H-2d) haplotype [19]. After 24 h of inoculation, two individual groups were treated doses of 112 mg kg^{-1} i.p. with cyclophosphamide-loaded LDH nanoparticles (LDH-CPh) previously sterilizated by UV light or 140 mg kg^{-1} of cyclophosphamide (CPh), respectively. A control group was maintained for lymphoma evolution over 14 days (identified as L5178Y). For this study, all procedures involving animals were performed according to technical specifications for production, care and use of laboratory animalsapproved by Mexican official NOM-062-ZOO-1999. Mice groups were observed daily to evaluate their general conditions.

On day 14, the mice were euthanized by chloroform inhalation, and ascites fluid and tissues were removed. Antitumor activity was measured according to the following parameters: 1) Body weights recorded at day 0 and once per week for 2 consecutive weeks; 2) ascitic fluid was collected from the peritoneal cavity, along with volume measured in a graduated centrifuge tube and packed cell recovered by centrifugation at 2500 rpm for 10 min; and 3) tumor cell count was determined by viable lymphoblast cells counted (Trypan Blue exclusion) on the Neubauer counting chamber (lymphoblast cells/mL ascites fluid) and indicative of residual disease. As an alternative measure of disease in the mice, the spleen, liver and kidney weights were also recorded to evaluate macroscopic damage by treatment with nanomaterials. Statistical analyses were carry out by one way ANOVA and pos-hoc Bonferroni analysis to determine significant differences ($p \leq 0.05$).

RESULTS AND DISCUSSION

LDH:

The LDH-CPh powder analyzed by X-ray diffraction and infrared spectroscopy revealed information exclusively related to inorganic fraction. The sharp peaks presented in the diffraction pattern (pristine sample in Figure 1A) indicate that a highly crystalline structure was formed by reconstruction process after 15 days.

ISSN: 0975-8585

The respective IR spectrum of this sample presented typical bands of LDH from –OH, NO_3^- and M-O fragments and also from molecular vibrations in the CPh reagent identified with dashed lines.

On the other hand, the LDH-CPh XRD profile obtained shows narrow reflection lines corresponding to high quality crystals with a layered structure (Figure 1B) once the profile is similar to that of a LDH [20]. The reflection close to 11 degrees (2theta) is the basal reflection associated to separation of two contiguous layers of LDH particles, which corresponds to 7.6 Å according to Bragg equation conversion.

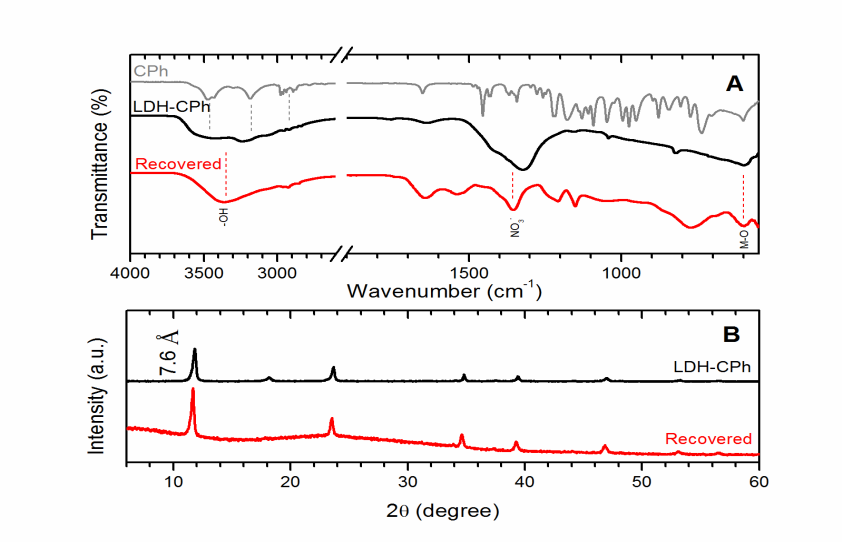

Figure 1: (A) Infrared spectra of CPh, LDH-CPh, and the LDH-CPh powder removed from mice at the end of bioassays (Recovered). (B) XRD patterns of CPh, LDH-CPh, and the LDH-CPh powder removed from mice at the end of bioassays (Recovered).

Thermal analysis:

Release of OH occurs at ca. 200 °C [21–23]. This event is endothermic [23]. Decomposition of drugs produces an exothermic peak in DSC analysis as we observed in our DSC profile [23]. The temperature where this peak occurs (200-256 °C) matches with the temperature decomposition of organic matter in Zn/Al LDH reported in the literature [22,23]. Clear evidence is that OH decomposition occurs near 200 °C and the next event is degradation of organic matter producing and exothermic peak which has been already reported in the literature [24].

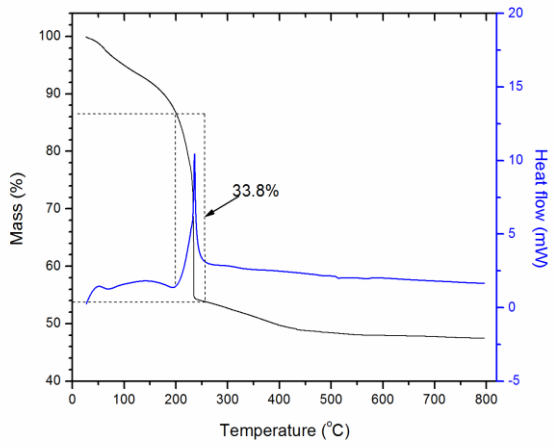

Figure 2: Thermal decomposition profile and DSC curve from LDH loaded with

Taking into account the nominal composition of synthesized LDH Zn/Al 2.5:1 the release of water due to hydroxyl decomposition is 16.9%. In our decomposition profile, the OH decomposition is probably overlapped with the CPh decomposition (once the exothermic peak is clearly present in this range) where 33.8% of mass is lost, then by discounting the percentage of OH mass, the CPh content estimated by this technique is close to 16.9% (Figure 2).

Bioassays:

Weight changes were recorded for all groups (Table 1). While healthy mice did not present changes after 14 days, the untreated group (Lymphoma L5178Y) underwent a 9.0% gain weight, indicating progression of lymphoma [25]. The group administrated with CPh presented a slight increase of 0.4% in weight as expected due to standardized control of this lymphoma by CPh [19,25]. Regarding the group treated with a single dose of the LDH-CPh, significant weight decreases were observed (-22.7 %), likely as a consequence of side toxic effects from nanoparticles, this inference is based on a single assay at low dosage (14 mg/Kg) of LDH-CPh, which resulted in non-toxic effects (data not shown), however, that low dosage did not present a pharmacological effect, therefore, further attempts to load the LDH nanoparticles with a higher amount of CPh is required for future assays.

The effect of LDH-CPh in different organs of unhealthy mice (carrying lymphoblast cells L5178Y) is correlated to organs weight (Table 2). LDH treatment shows macroscopic damage (colorless and texture -easy breaking up-) in kidney and liver tissues by superficial accumulation of nanomaterial.

On the other hand, peritoneal adhesions were observed in animals treated with LDH-CPh (Figure 3). The aggregates formed are stringy bands, which are abnormal in organs from abdominal cavity. These bands occur due to peritoneal injury like incisions, cauterization, sutures, or traumatism. Adhesions in mice caused bowel obstruction and abdominal pain as observed elsewhere [26]. This data correlates with weight reduction in unhealthy mice.

The anti-tumor effect was evaluated by comparing the tumor mass after 14 days in the L5178Y and CPh control groups, against the LDH-CPh group (Figure 4).

While the L5178Y group presented an average number of tumor cells equal to $800x10^6$, the treated groups with CPh and LDH-CPh reduced the number of cells to $7x10^6$ and $26x10^6$, respectively. Both treatments displayed a significant decrease ($P \leq 0.5$) in tumor cells compared with the lymphoma L5178Y group, and there was a not significant difference between both groups treated.

Figure 3: Observation macroscopically of organs after 14 days of treatment with Cph or LDH-Cph i.p. respectivelly. Organs Accumulation of LDH-CPh nanoparticles is indicated by arrow.

ISSN: 0975-8585

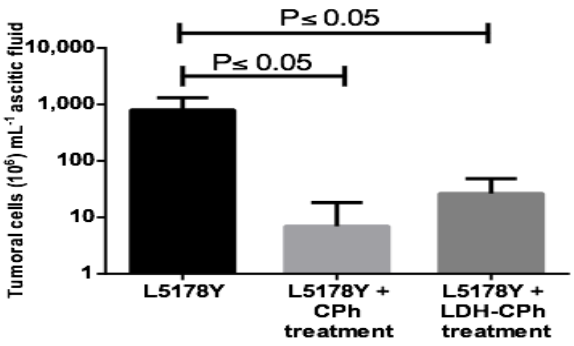

Figure 4: L5178Y lymphoblast cell in the different groups after 14 days of evolution.

Table 1: Overweight of BALB/c mice after 14 days of inoculation with 2 x10^4 cells of L5178Y lymphoma by i.p. route.

Mice group	N	Δ weight(%)
Healthy	5	0
L5178Y	5	9
L5178Y + CPh 140 mg/kg	5	0.4
L5178Y + LDH-CPh 112 mg/kg	5	-22.7

Table 2: Weight of organs in BALB/c mice after 14 days of inoculation with 2 x10^4 cells of L5178Y by i.p. route.

Group	N	Liver	Kidney (g)	Spleen
Healthy	5	1.5 ± 0.2	0.4 ± 0.2	0.1 ± 0.0
Healthy with LDH	5	1.3 ± 0.3	0.5 ± 0.1	0.1 ± 0.0
L5178Y	5	1.4 ± 0.4	0.5 ± 0.2	0.1 ± 0.0
L5178YCPh	5	1.6 ± 0.1	0.4 ± 0.2	0.1 ± 0.0
L5178Y LDH-CPh	5	1.2 ± 0.4	0.3 ± 0.3	0.2 ± 0.1

LDH after bioassays:

LDH-CPh treated mice presented accumulation of nanoparticles in kidney, liver, spleen and intestine tissues. Other researchers have observed accumulation of LDH loaded with indocianine green in the liver and spleen and they propose this carrier system to transport the drug to a specific organ [3,27]. In our results, the accumulation affected the size, color and texture of organs with the probable repercussion on their function. The accumulated powder was separated, washed with deionized water and analyzed. The IR spectrum of this compound (Fig 1A) contains additional bands with strong intensity at 1643, 1542, 1207, 115 and 780 cm^{-1}. Among these bands, water is well-known to present the band at 1642 cm^{-1}, and phosphate ions are probably producing the bands at 1207, 115 and 780 cm^{-1} [28,29]. Although these assays are not detailed enough to identify the new components adhered to the powder nanoparticles, it is valid to infer that salts of small molecular weight could be present since few vibration bands appeared. Additionally, the new components are not modifying the crystalline structure of the LDH matrix considering that the XRD pattern (Figure 1B) only shows reflections related to the layered matrix. The sharp and defined peaks at the same 2theta degrees of the administrated LDH-CPh indicates that crystal structure are maintained in the bioassay period even with the same basal space (7.6 Å). The height of the base-line in this diffraction profile rose due to the amorphous glass holder used to place the small amount of nanoparticles removed from the mouse. The TEM micrographs presented in Figure 5 show that LDH-CPh powder is formed by nanoparticles with an average size of 10 nm (SD = 3, n = 47) and during the bioassay produce aggregates of 25 nm (SD = 13, n = 43). Although the crystalline phase of those aggregates corresponds to LDH, the composition is altered by new compounds

detected from IR analysis and they could act as a matrix to concentrate the nanoparticles. Although other authors have associated the aggregation of LDH nanoparticles in specific organs to a selective response that allow to target specific organs with LDH vehicles [27], the aggregation might affect the macroscopic morphology and then the tissues function as it occurred in the mice studied in this work. Therefore, a challenge for future studies is to improve the dispersion in the peritoneal cavity to expose the large surface area of nanoparticles and improve the treatment with CPh.

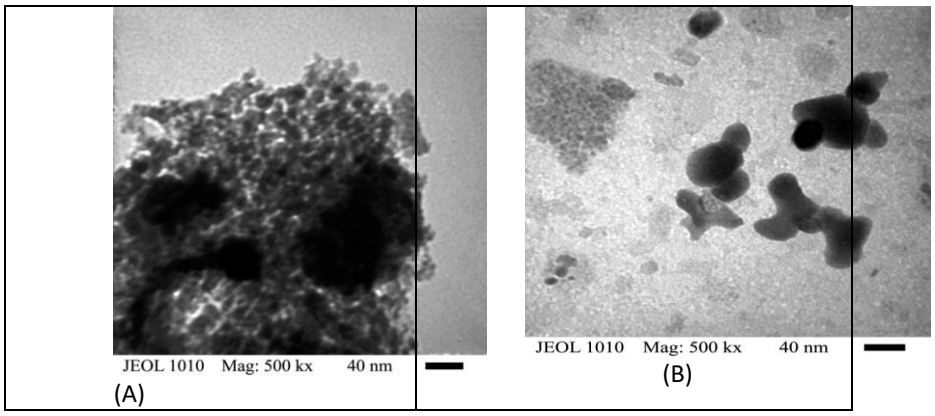

Figure 5: TEM micrographs of (A) LDH-CPh nanoparticles and (B) the powder accumulated in tissues of mice recovered after 14 days of i.p. administration.

CONCLUSIONS

LDH nanoparticles can be loaded with CPh, although intercalation into the layered space was not evidenced by XRD, the loading probably occurred onto the external surface of nanoparticles and this content presents an inhibitory effect on the L5178Y lymphoma in a single dose reaching the reduction of tumor cells as was produced by CPh control in a slightly lower CPh dosage (112 vs. 140 mg kg^{-1}). Nevertheless, the LDH-CPh nanoparticles formed aggregates in liver, kidney, spleen or intestine tissues affecting the weight and texture, and probably influencing the function of these organs producing the 22% of reduction weight in mice. Therefore, more efforts are needed to increase the CPh content in the LDH nanoparticles in order to reduce the dose of the LDH-CPh product as well as to avoid aggregation in the intra-peritoneal cavity.

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