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Treatment of respiratory damage in mice by aerosols of drug-encapsulating targeted lipid-based particles

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ABSTRACT

The purpose of this study was to develop a treatment for respiratory damage caused by exposure to toxic industrial chemicals (TICs), including mass casualty events, by aerosols of dexamethasone and/or N-acetyl cysteine formulated in targeted lipid-based particles. Good encapsulation, performance as slow-release drug depots, conservation of matter, and retention of biological activity were obtained for the three drug-carrier formulations, pre- and post-aerosolization. Weight changes over a 2 week period were applied, deliberately, as a non-invasive clinical parameter. Control mice gained weight continuously, whereas a non-lethal 30 minute exposure of mice to 300 ppm Cl_2 in air showed a two-trend response. Weight loss over the first two days, reversing thereafter to weight gain, but at a rate and level significantly slower and smaller than those of the control mice, indicating the chlorine damage was long-term. The weight changes of Cl_2-exposed mice given the inhalational treatments also showed the two-trend response, but the weight gain rates and levels were similar to those of the control mice, reaching the weight-gain range of the control mice. Following this proof of concept, studies are now extended to include additional TICs, and biochemical markers of injury and recovery.

1. Introduction

Respiratory damage is among the major injuries caused by exposure, whether accidental or deliberate, to toxic industrial chemicals (TICs), such as chlorine, ammonia, hydrogen chlorine, and many others [1–3]. Treatment of such damage – addressing the surge of reactive oxygen species, the pulmonary inflammation and pulmonary edema – is still in the category of an unmet therapeutic need, despite the availability of effective drugs [1–3].

The problem is not with the drugs themselves, but with their delivery. In the available formulations the drugs are free, hence prone to the well-known deficiencies of treatment with free drugs that too often result in poor therapeutic responses, treatment failure and safety limitations [4]. The approach we devised, to turn the situation – including in cases of mass casualty events – from the current unmet, to a met, therapeutic need is composed of three linked elements: (1) to replace treatment with free drugs by drugs formulated in a targeted lipid-based carrier that can, furthermore, perform as a slow-release drug depot (2) to deliver the drug-carrier formulations in an aerosol form, directly to the airways and lungs, using a clinical portable inhalation device, and (3) to have both formulations and devices in the vehicles of the first responders so that treatment can be initiated at the field and continued thereafter in the patient's home or in a medical facility.

The carrier we selected for the task is a specialized multilamellar liposome, surface-modified by hyaluronan anchored covalently to its surface (denoted HA-L). These liposomes were shown to have active targeting to macrophages and can also bind with high affinity to the extra cellular matrix (ECM) [5–6]. The drugs selected were the antioxidant N-Acetyl-cysteine (NAC) and the anti-inflammatory corticosteroid dexamethasone (Dex). A portable nebulizer was the inhalation device.

We first formulated the drugs in the liposomes, pursued and optimized physicochemical properties, verified that the drug-carrier formulations were stable to the nebulization process and simulated the deposition of the aerosol in human airways and lungs using an Anderson Cascade Impactor (ACI).

We next conducted the first step in feasibility studies, in mice exposed to a chlorine–air mixture and treated with an aerosol of our novel drug-carrier formulations. The respiratory damage in mice, exposed to chlorine–air mixtures either by whole body or nose only, has been well established [7–13]. Similar respiratory damage in mice,

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caused by a host of other agents, was also reported [17–21]. Both invasive and non-invasive approaches have been applied to evaluate the respiratory damage and the impact of therapy. In most of those cases, where invasive approaches were applied, the experiments were for short durations, typically 1–3 days from insult. The non-invasive approach, following mice weight changes, has been applied quite extensively, mostly for periods of 3–21 days [18–21]. Defining, moreover, weight loss as a traditional indicator of an acute pulmonary bleomycin insult, Limjunyawong et al. followed mice weight changes even up to 200 days [17].

Given that our objective was to follow-up each animal for a relatively prolonged period (2–3 weeks) from exposure with/without treatment we opted for this first step in feasibility studies, to apply a non-invasive approach, such as continuous monitoring of animal weight [11,13–21]. Our goal was to have the weight changes of the chlorine-exposed and treated mice on a par with those of control mice (exposed to air alone).

2. Materials and methods

2.1. Materials

Phospholipon 90G (high purity Soybean phosphatidylcholine (SPC)) was a kind gift from Nattermann Phospholipid GmbH (Cologne Germany). Dexamethasone, NAC, dipalmitoylphosphatidylethanolamine (DPPE), cholesterol (CH) and EDC (ethyl-dimethyl-aminopropyl-carbodiimide) were from Sigma Chemical Co. (St. Louis, USA). Hyaluronan (HA) 1.5 MDa used for the liposomes was a kind gift from Genzyme (Cambridge, MA, USA). The chlorine gas was in a mini-cylinder (Portacyl®, Specialty Gases of America Inc., The American Gas Group, purchased in Israel from Maxima Company) 300 ppm chlorine balance air gas mixture. Total gas quantity per cylinder was 58 l, and the rate of gas flow was 0.5 l/min supplied via series fixed flow regulator 70 (Ashcroft®, inlet: 1000 PSIG, flow: 0.5 SLPM, purchased in Israel from Maxima Company). Liquid Scintillation cocktail, Ultima Gold™, was from PerkinElmer Life and Analytical Sciences Inc. (USA). Dialysis tubing (molecular weight cut-off of 12,000–14,000) was from Spectrum Medical Industries (Los Angeles, CA). All other reagents were of analytical grade. Ultracentrifugation was performed with a Sorval Discovery M120 SE micro ultracentrifuge (TN, USA). Lyophilization was performed with a HETO Drywinner 3 (Alleraod, Denmark). The nebulizer was DeVILBISS's PulmoAide Compact Compressor 3655. The Anderson Cascade Impactor was from ThermoFisher Scientific (Franklin, MA, USA).

2.2. Preparation of drug-free and of drug-encapsulating HA-L

2.2.1. Drug-free HA-L

The lipid composition was SPC:DPPE:CH 75:5:20 (mole ratios) and the total lipid concentration was 100 mg/ml. Liposome preparation was essentially as described under [21,23] (and the references within). The first step was preparation of regular multilamellar liposomes (RL). The lipids were dissolved in ethanol, and evaporated to obtain a dry lipid film in a rotary evaporator under reduced pressure. The swelling solution (0.1 M borate buffer at pH 9) was added to the lipid film and the system was incubated (in a shaker bath) for 2 h at 65 °C. To obtain HA-L, HA was dissolved in acetate buffer (0.1 M, pH 4.5) at the concentration of 2 mg/ml. It was pre-activated by incubation with EDC (20 mg per 1 mg HA) for 2 h at 37 °C and added to the RL suspension, at the ratio of 1:1 (v/v). This reaction mixture was incubated in a shaker bath for 24 h at 37 °C. The HA-L were freed from excess materials and by-products by centrifugation for 30 min at 4 °C and a *g* force of 160,850 followed by several successive washes and re-centrifugations in 0.1 M NH_4HCO_3, suspending the final pellets in this salt solution. Aliquots of 1 ml of these liposomes were frozen for 2 h at −80 °C, followed by lyophilization. The resultant liposome powders were stored at −18 °C until further use.

2.2.2. Drug encapsulation

2.2.2.1. N-acetyl cysteine (NAC). Buffer-free, salt-free lyophilized HA-L powders (see Section 2.2.1) were brought to room temperature and rehydrated back to original pre-lyophilization volume by the NAC stock solution (20 mg NAC/ml in PBS containing 12.5 mM EDTA at the final pH of 7.5) incubating the system at 37 °C for 24 h.

2.2.2.2. Dexamethasone (Dex). Dex-encapsulating HA-L were prepared essentially as described in Section 2.2.1 above, except the drug was added to the ethanolic lipid solution at the concentration range of 5–10 mg Dex/ml.

2.2.2.3. Co-encapsulation of Dex and NAC in the same liposome. Dex-encapsulating HA-L were prepared as in Section 2.2.2.2, and lyophilized from an aqueous suspension that was buffer-free and salt-free. These lyophilized liposomes were rehydrated in the NAC solution, as described in Section 2.2.2.1, above.

2.3. Kinetics of drug efflux and determination of encapsulation efficiency

2.3.1. NAC/HA-L

Kinetics of drug efflux from the liposomes was studied according to our previously-developed experimental set up and data processing [21–24] (and the references within). Briefly, a suspension of drug-encapsulating liposomes (see Section 2.2.2.1 above) was placed in a dialysis sac that was immersed in a continuously-stirred receiver vessel containing drug-free buffer (PBS, 12.5 mM EDTA, pH 7.5), receiver to liposome volume ratio was 15:1. At designated time points, the dialysis sac was transferred from one receiver vessel to another containing fresh drug-free buffer. NAC concentration was determined in each dialysate and in the sac (at the beginning and end of the run). The data were analyzed according to a previously derived multi-pool kinetic model, expressed in Eq. (1) below.

$$f_t = \sum_{i=1}^{n} f_i\left(1-\exp^{-k_i t}\right) \quad (1)$$

where f_t is the fraction of the total drug in the liposomal system that diffused out of the sac at time t, n is the number of independent drug pools in the liposomal system, f_i is the fraction of the total drug in the system that occupied the ith pool at time = 0, k_i is the rate constant for drug efflux from the ith pool, and t is time, the free variable.

Data processing was done by computer-aided non-linear regression analysis using the KaleidaGraph software. These kinetic experiments also yield the efficiency of drug encapsulation (f_i, for the encapsulated-drug pool, at time = 0) which is defined as the ratio of liposome-encapsulated drug to the total drug in the system.

2.3.2. Dex/HA-L

All methods were essentially as listed above for NAC, with the following exceptions. The lyophilized dry powder of Dex/HA-L (see Section 2.2.2.2 above) was rehydrated back to original volume with drug-free water or buffer. The dialysis and data processing were performed as in Section 2.3.1 above.

2.3.3. NAC + DEX/H-L

The dialysis and data processing were performed as in Section 2.3.1 above, except that the liposomal formulation in the dialysis sac was as detailed in Section 2.2.2.3 above.

2.4. Drug assays in solution and in liposomal samples

Dex was assayed in free and in liposomal formulations as previously described, by inclusion of trace ^{3}H-dexamethasone in all Dex-containing systems [23]. NAC was assayed by a colorimetric assay