International Journal of Pharmaceutics 376 (2009) 84–91

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Liposomal dexamethasone–diclofenac combinations for local osteoarthritis treatment

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ARTICLE INFO

Article history:
Received 5 February 2009
Received in revised form 14 April 2009
Accepted 20 April 2009
Available online 3 May 2009

Keywords:
Liposome
Osteoarthritis
Diclofenac
Dexamethasone
Cyclooxygenase
MRI

ABSTRACT

Conventional chronic and acute treatments for osteoarthritis (OA) are by oral NSAIDs (such as diclofenac) and intra-articular injected glucocorticosteroids (such as dexamethasone). In free form, diclofenac and dexamethasone generate severe adverse effects with risks of toxicity. To reduce these drawbacks, we investigated local injections of liposomal formulations for diclofenac and dexamethasone (each alone, and their combination). Bioadhesive liposomes carrying hyaluronan (HA-BAL) or collagen (COL-BAL) as their surface-anchored ligand were used for the task. Each drug alone or their combination showed high efficiency encapsulations (≥80%) and performance as slow-release depots (half-lives in the range of 1–3 days under the fastest conditions). Employing RIA and immunoblot assay techniques, it was verified that the encapsulated drugs retained their biological activities: inhibitions of Cyclooxygenases enzyme-activity (diclofenac) and of Cyclooxygenases protein-expression (dexamethasone). Using live-animal MRI, a single intra-articular injection of each liposome-drug(s) formulation sufficed to reduce knee joint inflammation in OA rats over a time span of 17 days, HA-BAL better than COL-BAL. The most effective treatment was by the combination of both drugs in HA-BAL, a single dose reducing the inflammation volume down to 12.9% from initial over that time span. We find all three HA-BAL formulations worthy of further studies.

1. Introduction

Osteoarthritis (OA) is a multifactorial pathology leading to a range of structural and functional disorders of the synovial joints (Ge et al., 2006; Hunter and Felson, 2006; Sarzi-Puttini et al., 2005, Braunwald, 2001). To date, there are neither OA preventive measures nor cure and the goal of contemporary treatment is to control pain, minimize disability and improve joint function (Ge et al., 2006; Sarzi-Puttini et al., 2005; Braunwald, 2001; Felson et al., 2000). A major strategy to control inflammation and alleviate pain is by reducing the levels of prostaglandins (PGs)—the paracrinc and autocrinc mediators of inflammation (Smith et al., 2000; Vane et al., 1998). This makes the Cyclooxygenase (COX1 and COX2) – key enzymes in PG production – drug targets for OA treatment (Chen et al., 2008; Ge et al., 2006; Hardy et al., 2002; Felson et al., 2000; Smith et al., 2000). Two classes of drugs can reduce COX activity: NSAIDs that are COX inhibitors and corticosteroids that inhibit COX synthesis (Ge et al., 2006; Towheed, 2006; Schumacher and Chen, 2005; Saravanan et al., 2004; Kim et al., 2003; Stove et al., 2002; Smith et al., 2000; Vane et al., 1998). Drugs from both classes are administered in their free form, and treatments with drugs of each class are riddled with problems. Chronic OA treatment, by oral administration of NSAIDs, is accompanied by sever adverse effects that include gastrointestinal (GI) toxicity, gastric ulcers and anaphylaxis (Ge et al., 2006; Sarzi-Puttini et al., 2005; Braunwald, 2001; Felson et al., 2000; Vane et al., 1998). Corticosteroids are given by intra-articular injection and their repetitive administration causes cartilage damage, joint breakdown and other adverse effects that lead to limitation in treatment duration (Butoescu et al., 2009; Schumacher and Chen, 2005; Kim et al., 2003; Stove et al., 2002). The approach we propose, to improve OA therapy while reducing the adverse effects is: (i) to formulate such drugs alone or combined in a suitable carrier and (ii) to administer the resultant drug(s)-carrier formulations locally, via intra-articular injection to the inflamed joint (Butoescu et al., 2009, 2008; Elron-Gross et al., 2009, 2008; Cevc et al., 2008; Larsen et al., 2008; Gerwin et al., 2006; Kim and Martin, 2006; Thakkar et al., 2005; Kallonteri et al., 2002; Tuncay et al., 2000). This local route maintains the conventional treatment by free corticosteroids and switches the conventional route of free NSAID from oral to local. The carriers selected for this task are our previously developed bioadhesive liposomes, in particular the multilamellar liposomes (MLV) that have hyaluronan (denoted HA-BAL) or collagen (denoted COL-BAL) anchored covalently to their surface (Elron-Gross et al., 2008; Yerushalmi et al., 1994; Yerushalmi and Margalit, 1994). These bioadhesive liposomes were found to maintain the safety features of conventional liposomes, as well as the abilities to encapsulate hydrophilic and hydrophobic drugs and to act as slow-release

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doi:10.1016/j.ijpharm.2009.04.025

depots (Elron-Gross et al., 2008; Yerushalmi and Margalit, 1998, 1994; Margalit, 1995, 1991). An added and critical advantage of these bioadhesive liposomes is their high-affinity binding to recognition sites present in the target area such as extracellular matrix, cartilage, and membrane-embedded receptors (Elron-Gross et al., 2008; Yerushalmi and Margalit, 1998, 1994; Margalit, 1995, 1991; Yerushalmi et al., 1994).

In previous studies we focused on the NSAID diclofenac (SD) alone, formulated in two types of carriers: in the bioadhesive liposomes defined above, investigating molecular and *in vitro* properties; in non-liposomal microspheres (denoted collagomers) where we also ventured into *in vivo* studies (Elron-Gross et al., 2009, 2008).

In the present study we have taken a new direction that, at the same time, also completed the previous liposomal SD studies (Elron-Gross et al., 2008). We added a second drug used for the treatment of OA: The corticosteroid dexamethasone (DEXA). Moreover, a key element of the novel direction was to pursue not only each drug alone, but the potential of their co-encapsulation in the same liposome. Both drugs reduce COX activity, but in two different mechanisms, expected to have no mutual interference. SD is a reversible inhibitor of both iso-enzymes while DEXA inhibits their synthesis, especially the inducible isomer COX2 (Elron-Gross et al., 2008; Smith et al., 2000; Vane et al., 1998). In addition to these mechanistic differences, these drugs also differ in solubility, leading to the postulation that both drugs can be accommodated in the same liposome, DEXA in the lipid regions and SD in the aqueous core.

Six drug-liposome formulations were investigated: the two liposome species (i.e., HA-BAL and COL-BAL) and for each liposome species we tested SD alone (extending the previously reported molecular and *in vitro* studies), DEXA alone and both drugs in the same liposome.

The molecular and *in vitro* studies indicated that all six formulations are suitable for *in vivo* testing, which was performed, and is also reported here, in an OA rat model using live animal MRI. Favorable therapeutic responses were generated, HA-BAL emerging as the better carrier and treatment with the drug combination better than with a single drug. These results affirm the working hypothesis, including the new direction of co-encapsulating two drugs – NSAID and corticosteroid – in the same liposome.

2. Materials and methods

2.1. Materials

Phospholipon 90G (high purity Soybean phosphatidylcholine (SPC)) was a kind gift from Nattermann Phospholipid GmbH (Cologne Germany). Dipalmitoyl phosphatidylethanolamine (DPPE), collagen, glutaraldehyde, EDC (ethyl-dimethyl-aminopropyl-carbodiimide) diclofenac, dexamethasone, arachidonic acid (AA), indomethacin, 2,2,2-tribromoethanol, and monosodium iodoacetate were from Sigma Chemical Co. (St. Louis, USA.) Hyaluronan (HA) 1.5 MDa was a kind gift from Genzyme (Cambridge MA. USA). Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), heat inactivated fetal bovine serum (FBS), MEM-eagle non-essential amino acids (X100), L-glutamine, penicillin (10,000 U/ml) + streptomycin (10 mg/ml) + nystatin (1250 U/ml) solution, and 0.25% trypsin–EDTA solution were from Biological Industries (Beit Haemek, Israel). [^{14}C]diclofenac, [^{3}H]PGE$_2$, [^{3}H]dexamethasone, and horseradish peroxidase conjugated goat anti-rabbit antibody were from Amersham Pharmacia Biotech (Buckinghamshire, UK). COX-2 (murine) polyclonal antibody was from Cayman Chemical (MI, USA). Pico chemilumunescent substrate was from Thermo Fisher Scientific-Pierce (IL, USA). Male Wistar rats were from Harlan (Rehovot, Israel). Costar tissue culture flasks and plates were from Corning (Corning, USA). Dialysis tubing (molecular weight cutoff of 12,000–14,000) was from Spectrum Medical Industries (Los Angeles, USA). Ultracentrifugation was performed with a Sorval Discovery M120 SE micro ultracentrifuge (TN, USA). Lyophilization was performed with a HETO Drywinner 3 (Alleraod, Denmark). Sonication was performed using Bath sonicator (Fisher Scientific 550). Densitometry was performed using ImageMaster 1D, Pharmacia (Stockholm, Sweden). MRI experiments were performed on 7 T BioSpec Magnet 70/30 USR system (Bruker, Germany), Medical Image Analysis (MIA version 2.4), MATLAB image processing toolbox.

2.2. Bioadhesive liposomes

Multilamellar liposomes (MLV) were composed of soybean phosphatidylcholine (SPC) and dipalmitoyl phosphatidylethanolamine (DPPE) at the mole ratio of SPC:DPPE 95:5, total lipid concentration was 100 mg lipid/ml.

2.2.1. SD-encapsulating bioadhesive liposomes

The liposomes were prepared essentially as previously described (Elron-Gross et al., 2008). Drug-free MLV suspended in the swelling solution (0.1 M borate buffer at pH = 9) were taken to surface modification, binding hyaluronan or collagen, according to the previously reported processes (Elron-Gross et al., 2008; Yerushalmi and Margalit, 1998, 1994; Yerushalmi et al., 1994). Briefly, to obtain HA-BAL, 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 for 2 h at 37 °C and added to the MLV suspension, at the ratio of 1:1 (v/v). To obtain COL-BAL, collagen was dissolved in 0.01% acetic acid to the final concentration of 2 mg/ml, allowing 24 h at 4 °C for complete dissolution. The collagen was added to the MLV suspension at a ratio of 1:1 (v/v), followed by the addition of glutaraldehyde to a final concentration of 1%. Each reaction mixture was incubated for 24 h under shaking or stirring. Incubation temperatures were 37 °C and 4 °C, for the HA-BAL and for COL-BAL, respectively. The bioadhesive liposomes 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 PBS pH = 7.6, suspending the final pellets in this buffer. Aliquots of 1 ml of these liposome types – HA-BAL and COL-BAL – were frozen for 2 h at −80 °C, followed by lyophilization. The resultant liposome powders were stored at −18 °C until further use. For SD encapsulation the lyophilized liposomes, brought to room temperature, were rehydrated with an aqueous SD solution, incubating the systems for 2 h at 37 °C.

2.2.2. DEXA-encapsulating bioadhesive liposomes

The liposomes were prepared essentially as previously described using the veteran film method (Yerushalmi and Margalit, 1998, 1994; Schumacher and Margalit, 1997; Yerushalmi et al., 1994), except ethanol replaced the chloroform:methanol mixtures for lipid dissolution, the swelling solution was 0.1 M borate buffer at pH 9, and incubation of the dry lipid film was for 2 h at 65 °C, in a shaker bath. For DEXA encapsulation, the drug was added to the initial lipid-ethanol solution. The surface modification and the rest of the production were conducted as listed in Section 2.2.1.

2.2.3. DEXA-SD co-encapsulating bioadhesive liposomes

Lyophilized DEXA-encapsulating bioadhesive liposomes were rehydrated in an aqueous solution of SD as described previously, incubating the systems for 2 h at 37 °C.

2.3. Drug encapsulation efficiency and kinetics of drug release

Kinetic of drug release were studied according to our previously developed experimental set-up and data processing (Elron-Gross et al., 2008; Peer et al., 2003; Margalit, 1991). Briefly, a suspension of drug-encapsulating liposomes was placed in a dialysis sac that was immersed in a continuously stirred receiver vessel containing drug-free buffer (PBS pH 7.6), receiver to liposomes 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. Drug 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, processing the data by computer-aided non-linear regression using the KaleidaGraph software (Elron-Gross et al., 2009, 2008; Peer and Margalit, 2000; Yerushalmi and Margalit, 1994; Margalit, 1991). These kinetic experiments also yield the efficiency of drug encapsulation, which is defined as the ratio of liposome-encapsulated drug to the total drug in the system. In all studies drug concentrations were assayed using trace radioisotopes, [^{14}C] diclofenac and [^{3}H] dexamethasone.

2.4. Cell culture growth and maintenance

Monolayers of CT-26 cells (mouse colon carcinoma) that express intracellular COX1 and COX2, were grown in T75 flasks in Dulbecco's modified eagle's medium (DMEM) containing 10% heat inactivated foetal bovine serum (FBS), 1% MEM-eagle non essential amino acids (X100), 1% L-glutamine, 1% penicillin (10,000 unit/ml) + streptomycin (10 mg/ml) + nystatin (1250 unit/ml) solution. Cultures were maintained at 37 °C in 5% CO_2. For all experiments cells were harvested from subconfluent cultures using 0.25% trypsin–EDTA solution and were resuspended in fresh full serum-supplemented growth medium before plating.

2.4.1. COX inhibition by free and by liposomal SD–DEXA combinations in intact cells

Forty-eight to seventy-two hours prior to an experiment CT-26 cells were seeded onto 24-multiwell plates and the experiment was initiated upon 70% confluence. The cell-growth media was replaced with 500 μl of media containing a desired combination of SD–DEXA (free or liposomal), drug concentrations ranging from 1 to 100 nM. Wells receiving drug-free medium or "empty" (i.e. drug-free) liposomes served as controls. Incubations were for 16 h at 37 °C, followed by addition of the substrate arachidonic acid (AA), at a final concentration of 30 mM. The reaction was arrested after an additional incubation of 20 min at 37 °C, by the addition of indomethacin at a final concentration of 5 μM. The medium of each well was collected and subjected to a radioimmunoassay (RIA) (Elron-Gross et al., 2008; Levin et al., 2000) using [^{3}H]PGE_2, to determine the PGE_2 concentration. Total protein determination was by the Bradford method.

2.4.2. Evaluation of COX expression by immunoblotting (Western blotting)

Seventy-two hours prior to an experiment CT-26 cells were seeded onto T-75 tissue culture flasks and the experiment was initiated upon 70% confluence. The cell-growth media was replaced with 10 ml of media containing free or liposomal formulations of the following drugs (at 100 nM): SD, DEXA or a combination of SD–DEXA (100 nM each drug). Cells were incubated with the chosen treatment for 16 h at 37 °C, 5% CO_2, after which the cells were harvested using 0.25% trypsin–EDTA solution. The cells were resuspended in an ice cold lysis buffer (100 mM Tris, pH = 8, 150 mM NaCl, 6.25 μM DETC, 0.1% NP-40, 80 μM PMSF), sonicated for 1 min in an ice cold bath sonicator, and centrifuged for 10 min at 4 °C, 10,000 × *g*. The supernatants were collected and stored at −80 °C for further use. Protein concentrations in the supernatants were determined using Bradford method. The proteins were boiled in sample buffer (×2), separated by chromatography on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with 5% milk proteins in Tris buffered saline–Tween (TBST; 190 mM NaCl, 0.05% Tween 20, 25 mM Tris, pH = 7.6), and incubated with COX-2 (murine) Polyclonal antibody (1:2000 dilution), for 18 h at 4 °C. Membranes were washed four times; each wash was 15 min in TBST, followed by 1 h incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000 dilution). After additional four washes with TBST the membranes were developed using super signal west pico chemilumunescent substrate and densitometry was performed using ImageMaster 1D (Gallagher et al., 2008; Holla et al., 2005).

2.5. Animals

The Tel Aviv University Institutional Animal Care and Use Committee approved all animal procedures according to the guidelines of the Office of Laboratory Animal Welfare (L-06-045). The experiments were done with male Wistar rats weighing 120–150 g at the beginning of the experiments. Animals were group-housed (3 per cage) in solid-bottomed plastic cages designed to allow easy access to standard laboratory food and water ad lib. The rats were kept in a 12:12 light–dark cycle in a controlled temperature chamber (24 ± 1 °C).

2.5.1. Induction of OA

On day 1 of induction, animals were sedated with 2,2,2-tribromoethanol, at the dose of 1% animal weight. One milligram of monosodium iodoacetate (MIA) dissolved in 30 μl PBS (pH 7.6) was injected to the infrapatellar ligament of the right knee using a 30-gauge needle. Sterile saline was injected similarly to the left knee of the same animal. These processes were repeated on days 2 and 3 (Elron-Gross et al., 2009; Pomonis et al., 2005; Bove et al., 2003; Guzman et al., 2003; Guingamp et al., 1997). At the following day the animals were subjected to visual observation, after which they were taken to MRI (see Section 2.5.3, below), to assess the formation and the level of inflammation.

2.5.2. Treatment

Treatment was applied after it was verified (by MRI) that the desired disease state was achieved. Day of treatment is defined as day zero. The animals were treated with a single intra-articular (IA) injection of the desired formulation to the OA knee, and each animal received a PBS injection to the control knee. There were four animal groups for each type of liposome: (i) No treatment, but the OA knee was pricked by the needle (ii) Liposomal SD (iii) Liposomal DEXA (iv) Liposomal DEXA–SD. All injected volumes were 30 μl (Elron-Gross et al., 2009; Pomonis et al., 2005; Bove et al., 2003; Guzman et al., 2003; Guingamp et al., 1997). Each SD dose and each DEXA dose, whether single or together, was 1 mg/kg body weight. The injection process was similar to that described above for generating the disease state, including the sedation.

2.5.3. Determination of inflammation volume

The MRI procedure was applied as follows: Each animal was anesthetized with isoflurane (5% for induction, 1–3% for maintenance) using a flow rate of 1 l/min oxygen, and was installed in a head-holder to assure reproducible positioning inside the probe. Rate of respiration was monitored and was 60–80 breaths min^{-1} throughout the imaging period. The MRI device was equipped with gradient coil system capable of producing pulse gradient of up to 40 G/cm in each of the three directions. The MRI protocol included transverse T_1- and T_2-weighted MR images. The

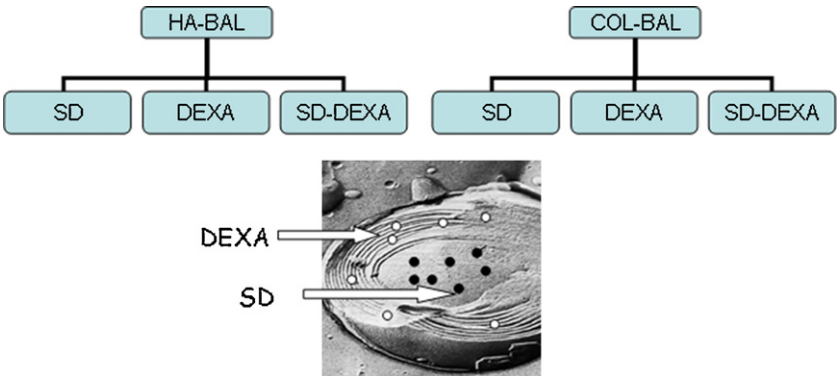

Fig. 1. (A) A scheme representing the six drug-liposome formulations. (B) An electron micrograph of an open MLV illustrating the localizations proposed for DEXA and for SD.

T_1-weighted images were acquired for the right and left knees using the FLASH sequence with a repetition delay (TR) of 263 ms, an echo delay (TE) of 6 ms, matrix dimension of 128 × 128 and one average, corresponding to an image acquisition time of 34 s. The T_2-weighted images were acquired for the right and left knees using the RARE sequence with a TR of 3500 ms, TE effective of 50 ms, 256 × 128 matrix (interpolated to 256 × 256), RARE factor of 8, and four averages, corresponding to an image acquisition time of 3 min 44 s. Twenty-four continuous slices with slice thickness of 1 mm were acquired with a field of view (FOV) of 4.5 cm × 4.5 cm (Jiang et al., 2000, Loeuille et al., 1997). The volume was calculated from the T2-weighted MR images using the Medical Image Analysis (MIA version 2.4), MATLAB image processing toolbox. Each animal was subjected to 3–4 MRI sessions spaced over 17 days. The first session was conducted before treatment, the others at intervals post-treatment. MRI experiments were performed on 7 T BioSpec Magnet 70/30 USR systems.

2.6. Statistics

Data were expressed as mean ± SD (Fig. 2) and means ± SEM (Fig. 4). Statistical analysis of the data was performed using the two-tail unequal variance Student's t test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Physicochemical characterization of the drug-liposome formulations

A scheme representing the six drug-liposome formulations is illustrated in Fig. 1, together with an electron micrograph of an open MLV in which the two separate localizations anticipated for each of the drugs–DEXA in the lipid regions and SD in the aqueous core, are pointed out. Encapsulation efficiencies and release kinetics, were explored for each drug in all test formulations. Under unidirectional flux conditions all systems showed a multi-phase pattern whether the drug was singly encapsulated or co-encapsulated. The data analyzed according to equation (1) below, where $f_{(t)}$ is the fraction of drug that diffused from the pool at time = t, normalized to the total drug in the system at time = 0; k is the rate constant for drug diffusion from the pool. The data fit the case of a two-pool model, one assigned to the un-encapsulated drug present in the system at time = 0 (k_1 and f_1) and the other to the liposome-encapsulated drug (k_2 and f_2) (Elron-Gross et al., 2008; Peer et al., 2003; Yerushalmi and Margalit, 1994; Margalit, 1991). The parameters obtained for the liposome-encapsulated drugs are listed in Table 1.

$$f_{(t)} = f_1(1 - \exp^{-k_1 t}) + f_2(1 - \exp^{-k_2 t}) \quad (1)$$

For the two liposome systems encapsulating SD alone, the encapsulation efficiencies and release rate constants were determined anew. The respective magnitudes were found similar to those previously reported (Elron-Gross et al., 2008), namely high encapsulations (79% and 87%) and slow-release performance (half-lives of 1.3 and 1.5 days) for COL-BAL and HA-BAL, respectively. The two liposome systems encapsulating DEXA alone also showed high encapsulation-efficiencies of 83(±2)% and 92(±3)%, for HA-BAL and COL-BAL respectively. DEXA's release was slower than that of SD, with 2.2 and 2.9 days for the half-lives of DEXA release from HA-BAL and COL-BAL, respectively.

In the co-encapsulating systems, the use of two different radioisotope labels made it possible to determine the individual parameters of each drug. Co-encapsulation of SD and DEXA in the same liposome had minor effects on the encapsulation efficiencies that did not exceed 2–10% (Table 1). Compared to each of the drugs encapsulated alone, k_2 values for SD remained the same, whereas for DEXA k_2 values increased 2-fold when it was co-encapsulated with SD. As can be seen in Table 1, the rates of DEXA's release in the co-encapsulated systems are quite similar to those determined for SD, indicating (and supported by the *in vivo* results) retention of the slow-release attribute.

3.2. Biological activities of the drug-liposome formulations

The processes of carrier preparation and of drug encapsulation may harm the drug's activity. Hence it is imperative, before considering *in vivo* studies with novel drug-carrier formulations, to verify that the encapsulated drug has retained activity. To that end the CT-26 cell line, known to express the COX enzymes, was used as the test system. Free drugs at dose levels similar to those of the encapsulated drugs, were used as a control to verify that the desired activities, COX inhibition by SD and inhibition of protein expression by DEXA, were indeed obtained in this cell line.

3.2.1. Inhibition of enzyme activity

We have previously found that SD alone in each of the two types of bioadhesive liposomes does, indeed, act as a COX inhibitor (Elron-Gross et al., 2008). In this study we extended the evaluation to the co-encapsulating systems, to verify that the presence of DEXA does not interfere with SD activity. As seen for the RIA results (Fig. 2), there was a dose-dependant pattern of COX inhibition. Moreover, DEXA did not prevent SD from acting, whether the two drugs were in free form or co-encapsulated in each of the liposome systems.

3.2.2. Inhibition of protein expression

Using the immunoblot approach to assess levels of protein expression, we tested the effects of liposomal DEXA alone and with SD (free drugs were also tested as controls). DEXA alone (Fig. 3)

Table 1
Physicochemical properties of the tested formulations.

Liposome species	Drug[a]	Encapsulation (%)		k_2 (h^{-1})		$\tau_{1/2}$ (h)	
HA-BAL	SD	79(±5)		0.019(±0.002)		38	
	DEXA	83(±2)		0.013(±0.001)		53	
	SD–DEXA	76.5(±2.6)[b]	82(±2)	0.023(±0.002)	0.021(±0.001)	30	32
COL-BAL	SD	87 (±5)		0.023(±0.002)		30	
	DEXA	92(±3)		0.010(±0.001)		69	
	SD–DEXA	77 (±4)	81 (±3)	0.025(±0.002)	0.022(±0.001)	28	32

[a] The concentration of each drug in all systems was 5 mg/ml. The parameters are each an average of three independent batches and the numbers in parenthesis are the standard deviations.
[b] In each set of data for the co-encapsulated systems, the first value is for the SD and the second value is for the DEXA.

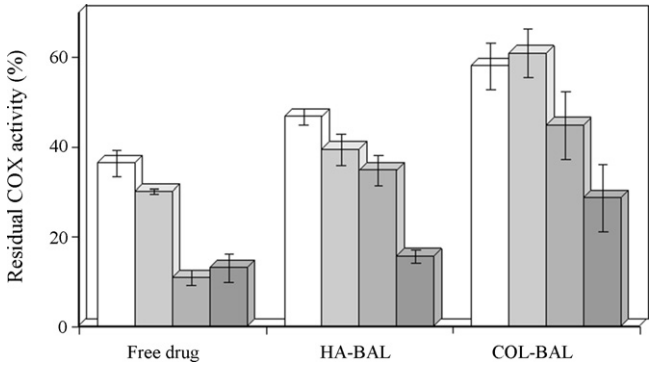

Fig. 2. COX inhibition, expressed as residual intracellular-COX activity in intact CT-26 cells, as function of formulation type, free or liposomal SD–DEXA, and drug concentration. For each test formulation, drug concentrations increase with the increase in bar shading from light-to-dark, corresponding to 1, 3, 10 and 100 nM. Enzyme activity was determined for arachidonic acid as the substrate, following the increase in the PGE_2 product. Each bar is an average of six determinations.

induced significant decreases in COX2 expression, in keeping with the natural activity of corticosteroids (Smith et al., 2000, Vane et al., 1998). The results with SD alone (Fig. 3), showed the expected modest increases in COX2 expression, for cases where enzyme inhibition may lead to denovo protein synthesis. For the SD–DEXA combination, it is clear (Fig. 3) that the presence of SD does not interfere with DEXA's activity. Moreover, in this combination the ability of DEXA to inhibit protein expression was powerful enough to also eliminated the increase in COX2 expression induced by SD (see SD–DEXA vs. SD alone).

3.3. Studies in animal model: OA rats

The animal model was set up as detailed under methods. Development of the local inflammation was verified and its volume measured for each animal, by MRI examination. In all groups animal weights increased normally, with no significant differences among the groups—an indication that the disease model itself as well as the treatments, were not toxic to the animals. All treated animals received the same liposomal (HA-BAL or COL-BAL) drug dose of

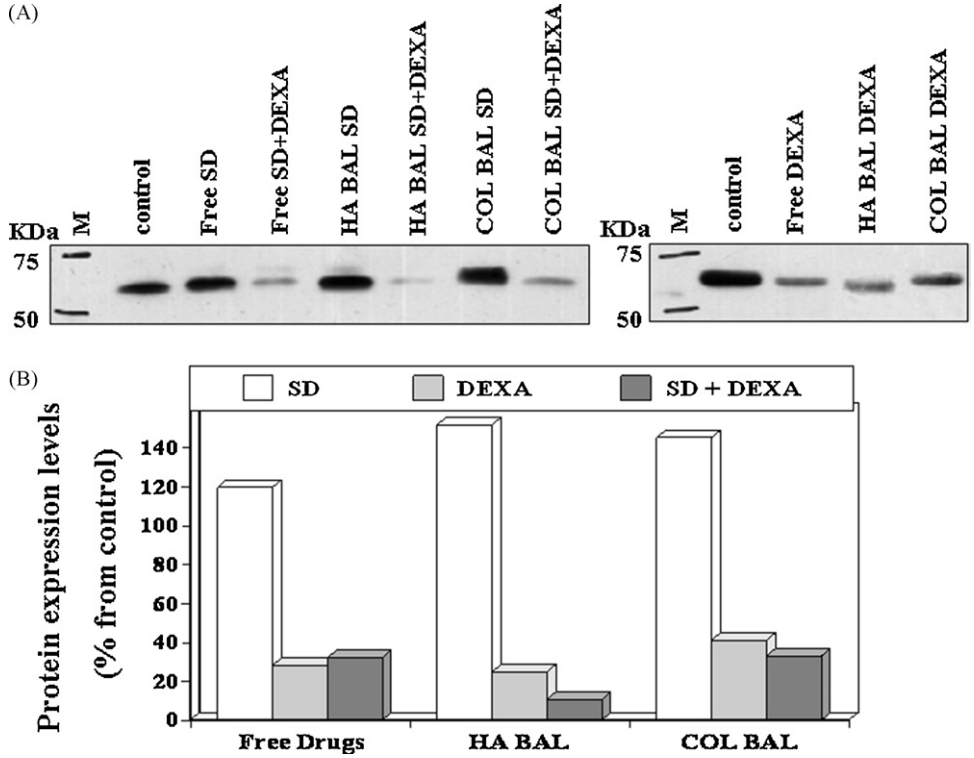

Fig. 3. Immunoblot analysis of COX expression in CT-26 cells. (A) Membranes were incubated with a COX2 (murine) Polyclonal Antibody), followed by the addition of horseradish peroxidase conjugated goat anti-rabbit antibody, then developed with the Super Signal West Pico chemilumunescent substrate. Densitometry was performed using ImageMaster 1D. (B) A Histogram showing the effects of the SD and DEXA free or liposomal, alone or co-encapsulated, on COX expression in CT-26 cells. In all formulation the drugs concentration was 100 nM.

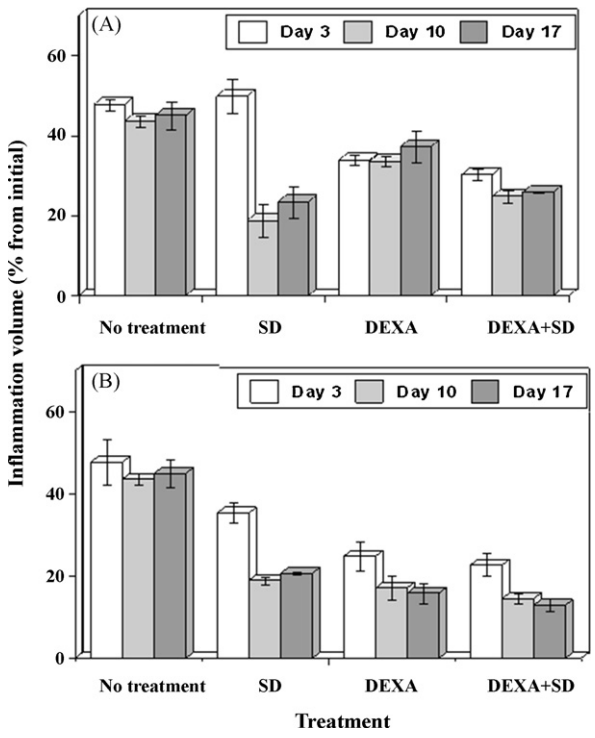

Fig. 4. Inflammation volumes (normalized to day zero) as function of time and treatment. The volume for each animal at each time point was determined on the basis of all 24 T_2-weighted images taken. (A) Drugs in COL-BAL. B: drugs in HA-BAL. The numbers of animals/group were: $n = 9$ in the untreated group, $n = 6$ in each of the treatments groups. Each bar is an average of animal/groups (n). Day zero is defined as the day of treatment.

1 mg/kg body weight, for the singly encapsulated drug (i.e. SD or DEXA) and for each drug in the co-encapsulated system.

Fig. 4 summarizes the results of the *in vivo* studies. A single dose of encapsulated drug(s) in COL-BAL reduced the volume of inflammation with time, compared to untreated animals (Fig. 4A). There was no significant difference among the tested formulations. For all treatments the therapeutic impact of the single dose was evident over the entire test period of 17 days, and was statistically significant compared to no treatment ($p < 0.001$ for DEXA, SD and SD–DEXA vs. untreated animals, day 17).

A single dose of drug(s), encapsulated singly or combined in HA-BAL, generated substantial reductions in the volume of inflammation, compared to the untreated controls (Fig. 4B). These results were, moreover, significantly better than those with COL-BAL (for all drugs $p < 0.0005$, day 17). The best results were obtained with SD–DEXA in HA-BAL where the inflammation volume (day 17) was 12.9% from initial, compared to 16% and 20.4% for DEXA and SD alone in the same liposome type. A demonstration of this best formulation is shown by the MRI data in Fig. 5. For the untreated animal (upper panels) there was little change in the level of inflammation over the entire period. For the treated animal (lower panels), even-though the level of inflammation was initially higher, the impact of the treatment is evident—the inflammation cleared by day 17.

4. Discussion

In this study we tested liposomes that encapsulated either a single drug, SD or DEXA, or their combination in the same liposome. For the single-drug systems, physicochemical properties of the DEXA-liposomes are reported here for the first time, and those of the SD-encapsulating liposomes were reported earlier (Elron-Gross et al., 2008). DEXA has been formulated in particulate carriers such as PLGA and liposomes. These systems vary from the present case in several aspects that do not allow for direct comparison: different pathologies and routes of administration (mostly systemic) (Asgeirsdottir et al., 2007; Kim and Martin, 2006; Koninh et al., 2006; Kallonteri et al., 2002); different particulate carriers and drug species such as the original hydrophobic DEXA (as we used) or soluble derivatives such as dexamethasone-phosphate and dexamethasone-acetate (Butoescu et al., 2009; Asgeirsdottir et al., 2007; Kim and Martin, 2006; Koninh et al., 2006; Kallonteri et al., 2002). Yet despite this wide scope of differences, two properties—encapsulation and retention of drug activity—can be compared to the present study. Depending in part on particle size and solubility of the drug derivatives, high encapsulation levels in the 70–90% range were reported, which is quite similar to the current case (Table 1) (Butoescu et al., 2009, 2008; Koninh et al., 2006). Where therapeutic activities, *in vitro* and/or *in vivo*, were reported encapsulation did not harm drug activity which is also similar to

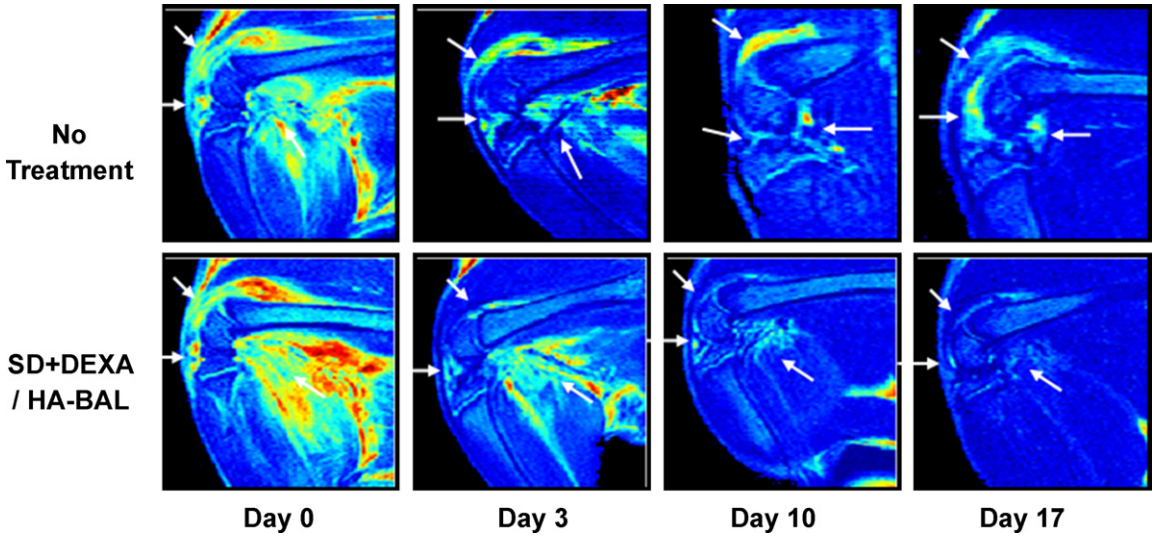

Fig. 5. MRI of osteoarthritic rat knees as function of time (days 0, 3, 10 and 17 post treatment) and treatment, each image is one of 24 taken per animal, per time point. Day zero is defined as the day of treatment administration, taking these images prior to treatment. (Upper panels) Images from an untreated animal. (Bottom panels) Images from an animal receiving a single treatment of intra-articular injection of SD–DEXA-encapsulating HA-BAL at the drug dose of 1 mg/kg body weight. The white arrows point to the inflammation, which is observed through the water accumulation that appears as red, light-yellow and pale-blue regions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the present case (Figs. 2–4) (Butoescu et al., 2009; Asgeirsdottir et al., 2007; Kim and Martin, 2006; Koninh et al., 2006).

The concept of co-encapsulating two drugs in the same carrier has been tested in the past for a variety of combination as exemplified by the following cases (Butoescu et al., 2009, 2008; Tardi et al., 2007; Gursoy et al., 2004): drug (DEXA) and nanoparticles in PLGA particles; two hydrophilic chemotherapies in liposomes; two anti-tuberculosis drugs, one hydrophilic and one hydrophobic (as in our case). These studies confirmed co-encapsulation is feasible without mutual interferences, which is also similar to the present case (Figs. 2–4).

The only quantitative difference between the single-drug and the co-encapsulating systems was with DEXA (Table 1): its efflux rate constant (k_2) increased 2-fold in the presence of SD. In multi-step processes an increase in a rate constant is attributed, in principle, to decreased energetic costs. This could be either at the original rate-limiting step or due to a shift in the location of that step. For the present case, the latter could arise from a SD-induced modulation of DEXA's localization within the liposomal membrane. Although SD is sufficiently water-soluble to localize preferentially in the internal water compartment of liposomes, it is also relatively amphiphilic (octanol/water partition coefficient is 13, in phosphate buffer pH, 7.4) (Menasse et al., 1978). The ability of SD to interact with PC may affect membrane ordering, causing the modest acceleration in DEXA's release (Ferriera et al., 2005; Lopes et al., 2004; Margalit, 1991). We wish to stress that this 2-fold decrease in half-life still keeps DEXA in the "slow-release" category, in fact at the same level of SD.

The question of whether each of the drugs retains its biological activity in the liposomal formulations (alone and co-encapsulated) was investigated *in vitro* and *in vivo*. *In vitro*, we applied two independent approaches (Figs. 2 and 3) and it is clear that all six formulations were active, and that co-encapsulation did not impair neither enzyme activity nor protein expression. These conclusions were further supported by the *in vivo* results.

Inflammation contraction is driven by two factors, host defense mechanisms and appropriate medication. The body's ability to induce inflammation contraction was no better than 50% (Figs. 4 and 5) (Elron-Gross et al., 2009). All six tested liposomal formulations defined in Fig. 1 showed (Fig. 4) reduction of inflammation volume compared to the untreated animals (day 17). Co-encapsulating SD–DEXA in HA-BAL had the most favorable therapeutic response. We credit the greater effect of HA-BAL to differences in liposome-target affinity. Binding of these liposomes to cell membranes well-exceeds that of COL-BAL (Elron-Gross et al., 2008).

5. Conclusions

Two different drug species can be encapsulated in the same liposome, without mutual interference, and with full retention of biological activities.

Several of the novel formulations merit further *in vivo* pursuit, with the potential of matching the formulation to the therapeutic needs of the OA patient. For chronic NSAID treatment we propose SD encapsulated in HA-BAL and also in the non-liposomal microspheres (Elron-Gross et al., 2009). For acute corticosteroid treatment we propose DEXA-encapsulating HA-BAL and for the most sever cases, we propose the combination of SD–DEXA in HA-BAL.

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