Peptide-targeted liposomal delivery of dexamethasone for arthritis therapy

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Aim: Rheumatoid arthritis is an autoimmune disease affecting the joints. Antiarthritic drugs are given systemically, thereby exposing various healthy organs to these drugs, resulting in adverse reactions. Accordingly, there is an urgent need for targeted drug delivery methods for inflamed joints. **Materials & methods:** We developed a liposomal drug delivery system using a novel peptide ligand (CKPFDRLC) named ART-2, which homes to the inflamed joints when injected intravenously to rats with adjuvant-induced arthritis. **Results:** The ART-2-coated liposomes encapsulating an antiarthritic drug, dexamethasone (DEX), were more effective in inhibiting arthritis progression than control-DEX liposomes or free DEX, despite a comparable safety profile. **Conclusion:** Peptide-targeted therapy has advantages over conventional drug delivery and can be adapted for rheumatoid arthritis therapy.

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Rheumatoid arthritis (RA) is a debilitating autoimmune disease involving chronic inflammation of the joints, which ultimately results in joint damage and deformity. This can impose a heavy socioeconomic burden on the society [1,2]. Nonsteroidal anti-inflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs), including biologics (e.g., anti-TNF- α [TNF- α] and anti-IL-6 receptor [IL-6R] antibodies), represent the mainstream antiarthritic drugs currently in use for RA therapy [3–7]. These are potent drugs, but their long-term use may lead to severe adverse reactions, such as infections in the case of biologics [7,8]. The above-mentioned drugs are administered to patients either orally or by subcutaneous/intramuscular injections, and thereby these drugs are distributed widely to several tissues in the body besides the diseased site, the inflamed joints. For this reason, prolonged arthritis therapy is frequently associated with damage to organs such as the liver and kidney. Thus, there is a need for targeting the drugs to the diseased joints in RA to enhance their efficacy in controlling arthritis.

Targeted drug delivery is a rapidly emerging area of biotechnology. Most of the effort in this regard has been invested in the treatment of cancer. In comparison, there are relatively fewer studies for the application of nanotechnology to the treatment of autoimmune diseases such as RA. Nanoparticles of diverse types (e.g., liposomes, micelles, dendrimers, etc.) [9–13] and conjugates of a drug with a polymer or folate [13–16] are being developed and tested for this purpose in different diseases, including RA. Liposomes are well-optimized nanoparticles for drug delivery [9,12]. While a large proportion of studies on liposomes used plain liposomes encapsulating a therapeutic agent, studies in tumor therapy by Ruoslahti *et al.* [11,17,18] and other investigators [19,20] have used peptide ligands to direct liposomes to specific tumors/tissues. Similar ligand-targeted nanoparticle therapy approaches are also being developed for RA [10,21–23], systemic lupus erythematosus (SLE) [24] and other autoimmune diseases.

In this study based on the rat adjuvant-induced arthritis (AA) model of human RA, we describe the development of a peptide-directed liposomal drug delivery system for arthritis therapy. In our previous studies, we identified a panel of phage-encoded peptides that home to inflamed joints of arthritic rats following intravenous injection

and systemic circulation of a phage library. In the process of bioinformatics analysis of these peptides, including random sequence variations and scrambling of potential motifs present in these peptides, we identified a peptide sequence CKPFDRALC (which we named as ART-2) that showed not only preferential homing to rat arthritic joints but also avid binding to endothelial cells (Supplementary Figure 1). Endothelial cells lining the blood vessels of the synovial tissue in the joints not only control the migration of leukocytes from blood circulation into the joint but also are vital for new blood vessel formation (angiogenesis), which is a characteristic feature of RA [25–27]. Proinflammatory cytokines and other mediators of inflammation produced in the course of arthritis cause the activation and proliferation of endothelial cells, which in turn affect the processes of cellular migration and angiogenesis [25,27]. Therefore, ligands that bind to endothelial cells are sought for modulation of their activity as well as drug delivery approaches targeting these cells. In this regard, ART-2 represents a promising ligand for endothelial cells.

We describe below these attributes of ART-2 peptide, along with its ability to direct liposomes encapsulating dexamethasone (DEX), a potent antiarthritic drug, preferentially into inflamed joints. DEX is a glucocorticoid, which has anti-inflammatory and immunosuppressive properties, and is used in the clinic for the treatment of RA and other immune-mediated diseases, including allergies [28]. The effects of a glucocorticoid are mediated upon its binding to a specific receptor in the cytoplasm, following which the complex is transported into the nucleus, where it causes transrepression of various genes involved in the inflammatory processes. Nuclear factor-kappa B and activator protein (AP-1) are representative transcription factors inhibited in this process. Prolonged use of high dose of DEX can cause a variety of adverse effects involving the gastrointestinal system (e.g., gastritis); cardiovascular system (e.g., hypertension); musculoskeletal system (e.g., osteoporosis and muscle weakness); and metabolic disease (e.g., diabetes) [28]. Mechanisms of action of DEX in attenuating arthritic inflammation include suppression of a variety of mediators of inflammation such as cytokines, chemokines and adhesion molecules, which in turn lead to inhibition of leukocyte infiltration into the site of inflammation [29]. Also reported is DEX-induced suppression of fibroblast activation by activated memory T cells [30].

Our results show that targeted delivery of DEX using ART-2 liposomes was superior to untargeted (control) liposomal delivery of DEX or free DEX in inhibiting arthritis progression in rats.

Materials & methods

Preparation & characterization of liposomes

Materials

Cholesterol, DEX, DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and FITC (fluorescein isothiocyanate) were obtained from Sigma–Aldrich (MO, USA), whereas DSPE-PEG2000 amine (ammonium salt of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino (polyethylene glycol)-2000]) was from Avanti Polar Lipids (AL, USA). Cy7 (Cyanine 7 azide) was from Lumiprobe (MD, USA). ART-2 peptide (CKPFDRALC; Supplementary Figure 1) labeled with FITC or Cy7, and ART-2-lipopeptide (CKPFDRALC-NH-C₁₈H₃₇) (Figure 1A) were synthesized by GenScript/Lifetein (NJ, USA). The conjugation of peptide with octadecylamine was confirmed by ESI-MS (*m/z* calculated 1303.76 [for CKPFDRALC-NH-C₁₈H₃₇], observed 1303.70 [M+H]⁺ and 652.75 [M+2H]²⁺; Supplementary Figure 2A), whereas the purity of the ART-2-lipopeptide was confirmed by reverse-phase analytical HPLC (Supplementary Figure 2B).

Liposomes containing a dye (FITC/Cy7) or drug (DEX)

For preparing liposomes, various lipids and a fluorescent dye were dissolved in chloroform/methanol in a glass vial in the following molar ratio: DOPC, 1; DOPE, 0.6; cholesterol 0.4; DSPE-(PEG)₄₅-NH₂, 0.05; and FITC/Cy7, 0.05. For control liposomes, only the lipids were used. For peptide-displaying liposomes, ART-2-lipopeptide was added to the lipids at 1 molar ratio. The solvent was removed using nitrogen gas and the dried lipid film was reconstituted with 1 ml of phosphate-buffered saline (PBS) or sterile deionized water and the mixture then set overnight at room temperature (RT) to swell. Vortexing the vial for 3 min at RT yielded multilamellar vesicles. Thereafter, sequential sonication of multilamellar vesicles using an ultrasonic water bath and a probe sonifier produced small unilamellar vesicles. Subsequent centrifugation using an Amicon filter for 20 min at 5000 r.p.m removed unencapsulated dye.

Liposomes containing DEX were prepared using the procedure described above but with the following modifications: the dried lipid film was hydrated with DEX (150 µg) dissolved in 1 ml PBS; and to remove untrapped

Figure 1. Characteristics of liposomes. (A) ART-2-lipopeptide structure; **(B–E)** The size and transmission electron microscope image of DEX-containing ART-2-displaying **(B & D)** and Control **(C & E)** liposomes. In **D & E**, bar is 100 nm. DEX: Dexamethasone.

DEX, liposomes thus obtained were centrifuged for 20 min at 10,000 r.p.m employing an Amicon filter. After removing unencapsulated DEX, then liposomes were reconstituted with PBS [31].

Imaging of liposomes using a transmission electron microscope FEI tecnai T12

Liposomes (5 μ l) were placed on a copper grid (200-mesh size). Any excess sample was removed and 5 μ l of a 2% solution (w/v) of uranyl acetate was added. Five minutes later, the sample was air dried at RT. Thereafter, transmission electron microscope (TEM) imaging of liposomes was performed [32].

Zetasizer-based determination of liposomal size, potential & stability

Using a Zetasizer (Malvern Zetasizer Nano), the size of liposomes (in 50 μ l sample) was measured in 1-ml deionized water. The particle size was measured ten-times in triplicate using dynamic light scattering and applying zero-field correction. Similarly, zeta potential was measured ten-times by assessing the viscosity, 0.89 cP; dielectric constant, 79; and temperature, 25°C, and then Smoluchowski approximation was used to derive the average value [33]. To assess the stability of liposomes, FITC-/DEX-containing liposomes were prepared and then stored at RT. The size and polydispersity index (PDI) of liposomes were measured on day 0 and then 14 days later.

HPLC measurement of loading of DEX into liposomes

After centrifugation of the DEX-containing liposomes, the untrapped DEX was collected and analyzed by reverse-phase HPLC (Waters Inc.) and a photodiode detector. (For HPLC, the ratio of water:acetonitrile:methanol was 50:25:25 (v:v:v) ratio and the flow rate was 0.1 ml/min at 25°C.) After subtracting the amount of untrapped DEX from total DEX used, the amount of entrapped DEX was derived and presented as a percent of total.

Testing the endothelial cell-binding of liposomes in vitro

Human umbilical vein endothelial cell (HUVEC; ATCC, VA, USA) line was grown in a special medium, endothelial cell basal medium (Lonza, MD, USA) supplemented with fetal bovine serum (10%). The cells were cultured at 37°C in a 12-well plate at a density of 2×10^5 cells/well in 5% CO₂ and 95% air. After 18–24 h, cells were stained for 2 h with different concentrations (0.1, 0.25, 0.5 and 1 μ M) of liposomes, and then washed with PBS. The cells were then fixed for 15 min using 1% paraformaldehyde, and analyzed by flow cytometry. For the time kinetics study, the same procedure was followed using a single concentration (0.5 μ M) of liposomes but incubated with cells for different time points (5, 30, 60 and 120 min). Data analysis was done using FCS Express 6.

Assessing the cytotoxicity of the drug delivery vehicle

The cytotoxicity of ART-2-/Control liposomes for HUVEC was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based reduction assay [34]. The assay was performed in triplicates in a 96-well plate at a density of 10,000 cells per well. After 12 h of seeding the wells with HUVEC, the cells were treated with different concentrations of liposomes (0.1–10 μ M) and incubated for 4–48 h. After that, MTT (0.05 mg/100 μ l/well) was added to the cells and incubated for another 4 h at 37°C. Thereafter, the culture medium was replaced with 100 μ l/well of DMSO:MeOH (50:50) solution. The absorbance was measured at 570 nm using a BioRad microplate reader (CA, USA). Results were expressed as percent of cell viability = $[A570 \text{ (treated cells)} - \text{background} / A570 \text{ (untreated cells)} - \text{background}] \times 100$.

Induction & evaluation of AA in Lewis rats

Five to six weeks old male Lewis (LEW/SsNHsd) rats obtained from Envigo (IN, USA) were used in this study. The handling of rats and experimental procedures were performed as per institutional guidelines for the care and use of animals. Water and food were accessible to rats *ad libitum*. The rats were kept at 21–23°C at a light-dark cycle of 12 h.

For arthritis induction, heat killed *Mycobacterium tuberculosis* H37Ra (Mtb; Difco, MI, USA) suspended in mineral oil was injected subcutaneously into Lewis rats at the base of the tail (1.5 mg/rat) as described elsewhere [35,36]. The rats were then observed regularly for signs of arthritis. The disease severity was evaluated on the basis of erythema and swelling of the paws. A grade from 0–4 per paw was assigned. After completing disease scoring, the hind paws were collected, decalcified and processed for histological analysis [37–39]. Microscopic examination of the tissue sections was then performed to assess pannus growth, damage to cartilage and bone erosion [37–39].

Distribution of systemically administered liposomes *in vivo* in rats

ART-2-Cy7 liposomes and control-Cy7 liposomes (200 μ M each) were used to study the biodistribution of liposomes. The two types of liposomes of comparable fluorescence intensity were injected intravenously into arthritic or naive (control) rats. Thereafter, real-time near-infrared fluorescence imaging was done employing the IVIS[®] Spectrum system (PerkinElmer) at 2-hourly intervals up to 6 h after injection. Rats were euthanized after 6 h time point, and different organs such as the heart, lung, spleen, liver, kidney and hind paws were harvested and subjected to *ex vivo* imaging [40].

DEX (liposomal/free) treatment of rats

Arthritis was induced in Lewis rats by injection of Mtb subcutaneously as described above. At the time of disease onset, these rats were randomized and assigned to four subgroups ($n = 5$ per group) for intravenous injection (200 μ l/rat) of ART-2-DEX liposomes, control-DEX liposomes lacking ART-2, free DEX or PBS (vehicle). These injections were performed on days 10, 12, 14 and 16 after Mtb injection. The dose of DEX was 0.1 mg/kg bodyweight. Thereafter, rats were examined regularly and graded for arthritic severity as described above. After recording the arthritic scores, hind paws of rats were harvested and processed for histology.

The profiles of systemic toxicity of different treatment modalities

Blood samples ($n = 6$ per group) were collected from different groups of rats, including the above-mentioned rats on day 20 following Mtb injection and serum was separated. These samples were then analyzed for serum chemistry (Charles River, MA, USA) for the following: AST, ALT and total bilirubin for liver and biliary system toxicity; blood urea nitrogen, creatinine and ratio of blood urea nitrogen versus creatinine (B/C) for renal toxicity; lipase and amylase for pancreatic toxicity; and lactate dehydrogenase for acute/chronic tissue damage.

Statistical analysis

GraphPad Prism 6.0 was used to analyze and graph data of experiments. Analysis of variance, and where applicable, Wilcoxon rank-sum test were used for comparisons among multiple groups. Results were considered to be significant at p -value below 0.05.

Results

Characteristics of liposomes

The liposomes were 95–106 nm in size, with DEX-containing liposomes ranging from 96 to 105 nm (Figures 1B & C, 2C & D) compared with FITC-containing liposomes that measured 70–95 nm (Figure 2E & F). The size of Cy7-containing liposomes was comparable to that of FITC-containing liposomes (data not shown.) For the two types of DEX liposomes, TEM imaging validated the morphology as well as size (Figure 1D & E), whereas Zetasizer assessed their charge (Figure 2A & B). The efficiency of entrapment for the drug (DEX) was 73–78%, whereas that for the dye (FITC/Cy7) was about 100%. Furthermore, when stored at room temperature, the liposomes (DEX, Figure 2C & D; FITC, Figure 2E & F) were found to be stable for at least 2 weeks. Additional information is provided in Supplementary Table 1.

The display of ART-2 on liposomes enhanced their endothelial cell-binding attribute

We described above (under Introduction section) the binding of peptide ART-2 itself (not as part of liposomes) to endothelial cells (HUVEC; Supplementary Figure 1B & C). The same peptide but conjugated to a lipid (ART-2-lipo-peptide; Figure 1A) was then used for preparing the test liposomes, while control liposomes lacked this peptide. Therefore, we assessed whether conjugation of peptide ART-2 to lipids and/or the incorporation of the resulting lipo-peptide into liposomes had any significant effect on the binding of peptide ART-2 to endothelial cells. It was evident from both the dose titration (Figure 3A & B) and time kinetics (Figure 3C & D) that ART-2-liposomes showed much higher endothelial cell-binding compared with that of control liposomes. We also tested whether our drug delivery vehicle, namely the ART-2/Control liposomes, had any toxic effect on cells. For this purpose, we tested the effect of these liposomes on HUVEC using an MTT-based cell viability assay as described under Methods section. Our results (Supplementary Figure 3) showed a minimal toxic effect of the two liposomal preparations when tested on HUVEC for 4–48 h. At each of the liposomal concentrations tested, the cell viability of HUVEC ranged from about 80–100% (Supplementary Figure 3). The difference between the liposomal-treated cells and

Figure 2. The zeta potential and stability of liposomes. The liposomes described in Figure 1 were tested for their Zeta potential (A & B). For stability testing, liposomes stored at room temperature were tested on days 0 and 14 for their size (C & E) and polydispersity index (PDI) (D & F). These measurements are shown for the two types of Dexamethasone (DEX)-containing (C & D)/fluorescein isothiocyanate-containing (E & F) liposomes indicated in the figure. Additional information is depicted in Supplementary Table 1. DEX: Dexamethasone; PDI: Polydispersity index.

untreated cells was not statistically significant. Thus, our drug delivery vehicle was safe for *in vitro* and *in vivo* testing.

Figure 3. Endothelial cell-binding of liposomes *in vitro*. Human umbilical vein endothelial cells were treated with ART-2-FITC liposomes or control-FITC liposomes for 2 h at the indicated four concentrations (A & B) or for different duration of time at one concentration (0.5 μM) (C & D) and then examined by flow cytometry. For each set, the fluorescence intensity is shown as a histogram as well as a graph.

* $p < 0.01$

FITC: Fluorescein isothiocyanate.

Arthritic joints show high concentration of fluorescence signal following intravenous injection of dye-entrapping ART-2-liposomes

We compared the fluorescence intensity in hind paws of arthritic rats after injecting intravenously ART-2-Cy7 liposomes versus control-Cy7 liposomes. The fluorescence signal in hind paws was much higher with ART-2-Cy7 liposomes than that with control-Cy7 liposomes at each of the time points tested (Figure 4A). In the case of the

Figure 4. *In vivo* imaging of systemically administered liposomes. (A) ART-2-displaying or control liposomes containing Cy7 within them were injected intravenously into arthritic rats and their distribution in the body was assessed by Near-infrared fluorescence-imaging at periodic intervals spanning from 5 min to 6 h. (B) Various organs (indicated in the figure) were harvested after 6 h of injection of Cy-7-containing liposomes into arthritic or naïve rats, and *ex vivo* imaging was performed using the same equipment as that used for *in vivo* imaging above. (C) *In vivo* imaging of arthritic and naïve rats after injection of Cy-7-containing liposomes as indicated in the figure.

former, a signal in the joints was detectable as early as 5 min after injection, which peaked at 4 h, followed by slightly reduced intensity at 6 h time point. Interestingly, the fluorescence signal was higher in the hind paw with more severe arthritis than in the opposite paw with less severe disease, even in the same rat. At the termination of experiment at 6 h, fluorescence signal was detectable not only in the arthritic paws but also in the liver and kidney (Figure 4B). Apparently, the signal from these abdominal organs might be because of Cy7 clearance by them. However, under the same test conditions, hind paws of naive rats showed no fluorescence signal (Figure 4C). Our assessment of the fluorescence signal is comparative among different paws and organs, and our results show an association between higher disease severity and higher fluorescence signal in arthritic rats.

ART-2-targeted liposomal DEX was more effective in suppressing arthritis in rats than untargeted liposomal DEX or free DEX

We tested three different treatment modalities based on DEX for their relative efficacy in suppressing arthritis in rats. These modalities included two liposomal forms of DEX and free DEX. An equal amount of DEX was delivered via different treatment modalities. ART-2-DEX liposomes were more efficacious in inhibiting AA than either control-DEX liposomes or free DEX (Figure 5A & B). On the other hand, control-DEX liposomes showed a similar level of antiarthritic effect as free DEX, when both were compared with vehicle-treated controls. The differences in arthritic scores of ART-2-DEX versus control rats; ART-2-DEX versus control-DEX; and ART-2-DEX versus free DEX were statistically significant. The severity of clinical arthritis of various treatment groups of rats was further validated by histological examination of hind paw sections (Figure 5C).

DEX delivered via liposomes showed a similar profile of systemic adverse effects as DEX given via plain (control) liposomes or without liposomes (free DEX)

The sera of rats treated with DEX delivered in different forms (liposomes or free) were tested for any effects on the liver and biliary system, pancreas and kidneys, as well as general tissue damage. The serum chemistry profile of the three groups of treated rats was comparable to each other, as well as to that of untreated arthritic controls (Figure 6). Taken together, the results of arthritis-inhibitory effects (Figure 5) and serum chemistry analysis, the latter serving as an indirect pointer to systemic toxicity effects (Figure 6), revealed that the highest therapeutic index of DEX was achieved via targeted delivery using ART-2-DEX liposomes than that with other drug delivery approaches tested.

Discussion

Despite the availability of potent antiarthritic drugs for the treatment of RA, there is limitation imposed by the potential risk of adverse effects involving other organs such as the liver and the kidney [3–7]. For the newer class of drugs, the biologics (e.g., anti-TNF- α), a risk of severe infections is a concern [7,8]. One of the factors contributing to some of the adverse effects is the unavoidable exposure of healthy organs to the systemically administered drugs, whose primary target otherwise is the inflamed joint. Thus, novel ways to improve the benefit/risk ratio (or therapeutic index) of these drugs are being sought. These include, but are not limited to, the use of different types of nanoparticles [9–13] and drug–polymer/folate conjugates [13–16]. The baseline parameters under consideration here are the dose of a drug and its systemic adverse effects. Therefore, therapeutic index of a drug can be improved by: increasing its efficacy at a given dose and reducing its adverse effects; increasing efficacy at the same dose but without affecting the extent of adverse effects; maintaining efficacy at the same dose but reducing adverse effects; and maintaining efficacy at a reduced dose but without affecting the extent of adverse effects. In this study, we describe a peptide-directed liposomal drug delivery method to improve the therapeutic index of DEX, which represents a widely used class of drugs for RA therapy. This outcome was achieved by increasing efficacy at the same dose but without affecting adverse effects.

Targeted drug delivery aimed at a particular organ requires a suitable ligand that can direct the nanoparticle carrying that drug to that tissue. In our system, we used a novel peptide ART-2 to achieve this objective. We used liposomes as carriers of DEX, and these liposomes displayed peptide ART-2 on their surface along with PEG. The latter reduces the clearance of liposomes by the reticuloendothelial system of the liver. Control-DEX liposomes had all the same ingredients except peptide ART-2. The test/control liposomes were within the expected range of size (nm). The ART-2-DEX liposomes had a slightly different net charge than control liposomes, and we believe that this difference is because of the ART-2 peptide displayed on the test liposomes. For an objective comparison, for *in vivo* treatment of arthritis in rats, an equal dose of DEX (amount per kg bodyweight), either in the form of liposomes or as free drug, was administered intravenously to rats. It is clear from our results that the peptide-targeted

Figure 5. Comparative efficacy of liposomal dexamethasone and free dexamethasone for arthritis therapy. Arthritis was induced in Lewis rats by subcutaneous injection of *Mycobacterium tuberculosis* (Mtb). At the time of appearance of signs of arthritis (disease onset), rats were randomized ($n = 5$ per group) and given intravenously four injections every 2 days starting from day 10 (indicated by arrows) of the following preparations: ART-2-Dexamethasone (DEX) liposomes, control-DEX liposomes or free DEX. Another arthritic group of rats given only Phosphate-buffered saline (vehicle) served as a reference for comparison with other groups. All groups received the same dose of DEX, namely 0.1 mg/kg. **(A)** Disease severity is indicated by arthritic scores (mean $\pm$ SD), and $p < 0.05$ indicates statistically significance. **(B)** Representative hind paw photographs and **(C)** histological features of hind paws (bone: B; cartilage: C; joint space: J; and pannus: P) are shown. DEX: Dexamethasone; Mtb: *Mycobacterium tuberculosis* H37Ra, heat-killed.

liposomal DEX had a significantly higher inhibitory effect on arthritis progression than nontargeted liposomes or free DEX.

As discussed above, another parameter for assessment of therapeutic index is the extent of adverse reactions. In our study, we employed serum chemistry of known enzymes/markers that are associated with physiological functions and tissue pathology of different organ systems (e.g., liver function, kidney function, pancreatic function, general tissue toxicity, etc.). Our results showed that despite increasing the efficacy of the antiarthritic activity of DEX by ART-2-DEX liposomes, there was no additional increase in the enzymes/markers of tissue pathology when compared with that of control liposomes or free DEX. In view of different treatment categories mentioned above, ART-2-DEX liposomes had an improved therapeutic index via enhanced efficacy but without increasing the adverse effects.

The use of liposomes to deliver drugs for arthritis therapy is not new. However, unlike the use of peptide ART-2 in our study, many of those studies used plain liposomes that lacked a ligand to direct them to arthritic

Figure 6. Serum testing for the relative adverse effects of dexamethasone in different treatment groups. Sera were collected from rats ($n = 6$ per group) treated with DEX delivered via ART-2-displaying liposomes, Control-liposomes or as free drug. Serum collection time was day 20 after Mtb injection. Sera were tested for the indicated enzymes or other analytes to assess **(A)** liver and biliary system toxicity; **(B)** pancreatic toxicity; **(C)** kidney toxicity; and **(D)** acute/chronic tissue damage. DEX: Dexamethasone; Mtb: *Mycobacterium tuberculosis*, H37Ra, heat-killed.

joints. For example, other investigators have reported liposomal delivery of antiarthritic drugs such as prednisolone, betamethasone, DEX and methotrexate (MTX) in animal models of RA, including collagen-induced arthritis and AA [41–45]. Plain liposomes were used in these studies. Another set of studies employed a peptide ligand to direct liposomes for arthritis therapy in the AA model [10,21,22], but that ligand was different from our ART-2 peptide. For example, the following types of liposomes have been used for arthritis therapy in different studies: liposomes containing DEX and displaying a peptide with RGD motif on the surface [10]; liposomes-encapsulating prednisolone and presenting on their surface a peptide ligand that has avidity for fibroblast-like synoviocytes [21];

liposomes entrapping betamethasone and expressing folate on liposomal surface for imaging and therapeutic studies [22]; and liposomes expressing a peptide ligand for endothelial cells and containing within them IL-27, an immunomodulatory cytokine [46,47]. In the present study, although the drug (DEX) used is same as that used in a few of the previous studies by other investigators, the novelty here lies in the use of a peptide ligand not described before. In addition, we also assessed adverse effects by measuring serum enzymes/markers, which was not done in many of the above-mentioned studies that were limited to measurement of arthritis inhibition alone. Furthermore, in our study using DEX, the peptide ligand (ART-2) showed binding to endothelial cells, a property shared with RGD peptide mentioned above [10]. However, the dose of DEX used in that study [10] was 1 mg/kg injected as a single intravenous injection in comparison to four intravenous injections of 0.1 mg/kg DEX each in our study. Furthermore, in that study, although DEX delivered via RGD-targeted liposomes was more efficacious than untargeted liposomes [10], as also found in our study based on ART-2 peptide, but a direct comparison with free DEX was not performed there.

Another novel approach for drug delivery that has been described for arthritis involves conjugation of a cytokine (e.g., IL-4) [48] or a drug (e.g., MTX) [16,49] to a targeting ligand. For example, conjugation of IL-4 to a peptide ligand (e.g., a synovial endothelium-targeting peptide CKSTHDRLC) was shown to be highly effective when tested in the synovial xenograft model of RA [48]. Similarly, MTX conjugated to dextran sulfate was shown to have a marked antiarthritic effect against collagen-induced arthritis in mice [16,49]. One of these MTX-based formulations targeted the scavenger receptor on activated macrophages [49]. We have attempted a related approach by directly conjugating MTX to a joint-homing peptide ligand for treatment of arthritis in the AA model and found it to be more effective compared to the use of free drug (data not shown; manuscript in preparation).

The application of targeted drug delivery is advancing rapidly in the field of autoimmune diseases. Besides its use for arthritis therapy summarized above, liposomal drug delivery has also been used for therapy in animal models of experimental autoimmune uveoretinitis [50], experimental autoimmune encephalomyelitis [51–53]; and SLE (or lupus) [24,54]. Besides the use of peptides and folate described above, antibodies (e.g., anti- α 8 integrin or anti-CD4 antibody) [24,54] and glutathione [55] have also been employed as ligands for drug delivery in above studies.

Conclusion

We describe here a peptide-guided liposomal drug delivery approach that helped improve the therapeutic index of a known antiarthritic drug by enhancing its efficacy compared with free drug, while retaining a comparable profile of adverse effects. This methodology can be adapted for targeted delivery of a biologic or another drug for arthritis therapy in RA patients.

Future perspective

It is hoped that the application of targeted drug delivery will move fast beyond cancer to include autoimmune and other diseases. Regardless, with rapid developments in the field of nanotechnology and drug delivery, it is imperative that uniform guidelines for the preparation of different types of drug delivery vehicles are set internationally, so that results of different studies performed in different laboratories worldwide can be compared comprehensively. Furthermore, the mechanisms by which nanoparticles interact with different cell types and deliver their contents at the site of disease remain to be better elucidated. Nevertheless, the future appears bright for this growing field, and we hope to see more advances in combination therapy, with two or more therapeutic agents in the same delivery vehicle, and possibly imaging and therapeutic agents delivered either in the same vehicle or concurrently in two separate vehicles.

Summary points

- Rheumatoid arthritis (RA) is a debilitating autoimmune disease requiring long-term therapy.
- Drugs to treat RA are given systemically and thereby can be toxic to various healthy tissues.
- Drug delivery methods to target inflamed joints are needed to limit toxicity of such drugs.
- We developed a liposomal drug delivery system using a novel joint-homing peptide, ART-2.
- We tested the efficacy of liposomal versus free dexamethasone (DEX) in suppressing arthritis in rats.
- Peptide-targeted liposomal DEX was more effective than control liposomal or free DEX.
- The safety profile of liposomal DEX was comparable to that of free DEX.
- Peptide-targeted therapy can be adapted for RA.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2018-0501

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The handling of animals and the procedures performed on them were done following approval by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine, MD, USA and the Baltimore VA Medical Center, MD, USA. The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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