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Novel Non-viral Vectors Target Cellular Signaling Pathways: Regulated Gene Expression and Reduced Toxicity

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Running title: Designing New Nonviral Vector with Additional Functions

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ABBREVIATIONS: NLS: nuclear localization signal; MAPK: mitogen activated protein kinase; CDCA: chenodeoxycholic acid; FXR: farnesoid X receptor; FXRRE: farnesoid X receptor response element; SM: small molecule; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DNA: deoxyribonucleic acid; CMV: cytomegalovirus.

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ABSTRACT

Advances in cell biology over the last several decades have allowed for a much greater understanding of the regulation of cellular processes. Many of these revelations have provided substantial details regarding the key players in cellular pathways and the role small molecule ligands may play in controlling their function. While much progress has been made in these areas, optimization of nonviral gene delivery vectors has not met with similar success. Many of the issues which have plagued the field, such as limited transgene activity, difficulty with specific cell targeting, inflammatory responses, and degradation of the vector among others, continue to limit the efficacy of these delivery systems. In this study, we investigate several cellular pathways in an effort to develop more efficient nonviral vectors. To increase nuclear uptake of the transgene, we explore the use of nuclear localization sequences (NLS) incorporated into our plasmid. The results indicate that the NLS did significantly increase gene expression under several circumstances in the presence of small molecule ligands, as indicated by both *in vitro* and *in vivo* studies. Furthermore, to decrease inflammatory response to the vectors, additional studies were performed to demonstrate that the incorporation of free anti-inflammatory ligands into liposome formulations does not affect transgene activity, but are able to significantly decrease the inflammatory response. Overall, these examples provide hope that free ligand can be used to effectively mediate cellular processes to overcome some of the obstacles limiting the success of gene therapy.

In recent years, many advances have been made toward understanding the mechanisms by which cells recognize other molecules as well as respond to external stimuli. Such advances have also revealed the role of small molecules in many of these regulatory pathways (Schulman and Heyman, 2004). These developments allow for the exploitation of cellular pathways to overcome some of the barriers limiting the success of gene delivery, specifically cell targeting and uptake, and inflammatory response to the delivery vehicles. Viral vectors have been shown to have superior abilities to transfect cells, however specific immune recognition has always been a concern when considering these systems for clinical trials. Explorations of nonviral systems now focus on both improvement of cell targeting and transfection to achieve levels of transgene activity that meet if not exceed levels observed in viral vectors. Furthermore, formulations of lipid and polymer have been manipulated to minimize non-specific inflammatory responses induced by the vectors (Niidome and Huang, 2002; Guo and Szoka, 2003). Currently, these approaches have only had limited success. With emergent details of cellular pathways, it is possible to utilize these advances to improve the efficacy of nonviral vectors for gene delivery.

To increase the efficiency of nonviral vectors, various realms of vector modifications have been investigated. One approach involves specifically altering the structure of the polymers and cationic lipids or addition of other “helper molecules” like polyethylene glycol (Ogris et al., 1999; Wheeler et al., 1999). Alternatively, vectors have been developed to incorporate a receptor-specific molecules (Dauty et al., 2002; Maruyama, 2002; Xu et al., 2002; Zuber et al., 2003). In some instances, the targeting molecule is specific for an external receptor, like the incorporation of folate and transferrin molecules into cancer-targeting vectors, since cancer cells tend to have substantially higher numbers of these receptors on their surfaces (Dauty et al., 2002;

Maruyama, 2002; Xu et al., 2002; Zuber et al., 2003). These previous studies involving the incorporation of helper molecules have greatly enhanced the efficiency of the vectors by targeting to receptors on the cell surface, however, there are many other possible applications for small molecule incorporation that have not been explored.

Significant advances in cell signaling have recently revealed a large number of previously unknown nuclear localization sequences (i.e. specific protein sequences that allow passage through the nuclear pore complex) (Nagy and Schwabe, 2004). Nuclear receptors belong to an evolutionary ancient superfamily, which function as ligand-activated transcription factors to assist in regulating processes such as cell growth and differentiation, inflammatory responses, and metabolism. Many studies have shown that small molecules (e.g. retinoids, steroid hormones, fatty acids, cholesterol metabolites) are involved in the regulation of numerous physiological processes often by binding to small molecules to regulate the activity of proteins (Escriou et al., 2003; Schulman and Heyman, 2004). The ability to vary nuclear receptor-dependent gene expression with small molecules has made nuclear receptors a favored target for gene and drug delivery systems (Hebert, 2003). The additions of these sequences into nonviral vectors are expected to greatly improve gene activity by increasing the internal targeting of the therapeutic gene.

Other small molecule ligands are also being investigated, specifically for their role in inhibiting inflammatory responses. Although nonviral vectors generally do not elicit a specific humoral immune response, an inflammatory response is frequently observed increasing levels of TNF-α, IL-6 and IL-12 cytokines. These nonspecific responses increase the lethality of such systems,

severely limiting their therapeutic potential as gene delivery vectors. Gaining understanding of inflammatory pathways has allowed for the discovery of many natural and synthetic small molecules that are able to inhibit the pathways leading to cytokine production. Recently, we reported a novel nonviral vector, *safeplex*, which incorporated various anti-inflammatory drugs, generally inhibiting the NF-κB inflammatory pathway, into the cationic liposomes (Liu et al., 2004). The significantly reduced immune activity has led to further investigation of other small molecules affecting cytokine inducing pathways.

The current study exploits advances in cell biology by investigating small molecules directly involved in specific cell signaling pathways. First, we incorporated a nuclear localization sequence (NLS) into the vectors. In the presence of this sequence and a small molecule ligand, a marked improvement in transgene activity was observed. Furthermore, we investigated two small molecules known to specifically inhibit either the NF-κB or the MAPK inflammatory pathway. Both vector systems were found to significantly decrease the cytokine levels in the presence of the small molecules. We overcome several of the obstacles affecting nonviral gene delivery through the incorporation of small molecule ligands.

MATERIALS and METHODS

Plasmid DNA preparation. Plasmid pNGVL3-Luc, containing the complementary DNA of firefly luciferase driven by the cytomegalovirus promoter (CMV), was custom prepared by Bayou Biolabs (Harahan, LA). To examine nuclear receptor targeting, the following plasmids were examined. Plasmid, pFXRRE-Luc, contains a farnesoid X receptor response element (FXRRE), and a thymidine kinase promoter in front of the firefly luciferase gene. A third plasmid, pCMV-FXR, contains cytomegalovirus promoter and the farnesoid X receptor gene. Both were a gift from Dr. Xie's lab (University of Pittsburgh). The FXR-related plasmids were amplified in DH5α strain of *Escherichia coli*, isolated by alkaline lysis, and purified by ion exchange column chromatography (Qiagen Inc., Valencia, CA).

Preparation of the vectors. DOTAP liposomes were prepared from chloroform solution to a concentration of 10 mg/ml. Briefly, the lipid solution was placed under a stream of nitrogen to evaporate the solvent until a thin lipid film formed at the bottom of a glass tube. It was further vacuum desiccated for 1 h and then rehydrated in 5% dextrose solution. The lipid suspension was briefly sonicated and then extruded through a polycarbonate membrane with pore size of 0.2 μm. For novel formulations, small molecules (SM) including chenodeoxycholic acid (CDCA, Sigma, St. Louis, MO), capsaicin (Sigma) and PD-98059 (EMD Biosciences, Inc. San Diego, CA) were dissolved in organic solvents at a concentration of 5 mg/ml and mixed with DOTAP before solvent evaporation. The DOTAP liposomes, DOTAP:SM liposomes, and plasmid DNA were diluted with 5% dextrose solution prior to mixing. Lipoplexes were formed by adding the DNA

solution dropwise into the freshly prepared DOTAP or DOTAP:SM liposome solution in a glass tube while the tube was gently swirled**.**

Turbidity studies. Fifty μl of each sample, CDCA alone, DOTAP liposomes and DOTAP:CDCA liposomes, was diluted to 500 μl with H_2O. Turbidity was measured over 60 min by watching for the appearance of a peak at an absorbance of 600nm. Absorbances were measured on a DU-800 UV-Vis spectrophotometer (Beckman-Coulter, Fullerton, CA). Stability experiments for DOTAP-capsaicin and DOTAP-PD98059 liposomes were performed under the same conditions.

Encapsulation Efficiency. Two hundred μl of DOTAP liposome (10 mg/ml) with 5 mg/ml of drug was applied to a Microcon YM-3 spin column (Millipore, Billerica, MA). After a 30 min centrifugation at 14,000 x *g*, 10 μl of filtrate was combined with 90 μl of 50% alcohol solution. Capsaicin was detected at a wavelength of 281 nm, and PD98059 at 238 nm.

***In vitro* gene transfer.** Murine melanoma BL-6 cells (9×10^4 cells/well) were seeded in a 24-well plate and cultured in RPMI medium with 10% FBS for 24 hours before transfection. The cells were transfected with 20 μl of the vector containing DOTAP/CDCA and plasmid DNA (2 μg/well) with a charge ratio of 2 to 1. Luciferase expression was measured 24 h after the transfection using a kit purchased from Promega (Madison, WI, USA) and a luminometer (Autolumat LB953, EG & G, Berthhold, Germany).

***In vivo* gene transfer.** Hydrodynamic injection of CD1 female mice was performed by injecting via the tail vein a large volume (8% of body weight) of solution containing 10 μg of DNA with or without the small molecule CDCA (0.6 mg, ip). For inflammatory inhibitor studies by *in vivo* gene transfer, CD1 female (18−20 g) mice were injected intravenously with the vector containing 25 μg plasmid DNA with a charge ratio of 12 to1 (±). Serum was isolated from blood taken by tail vein at 2 h following injection. Mice were sacrificed 6 h after the injection to monitor gene expression. Lungs were collected and placed in 1 ml of ice-cold lysis buffer and homogenized with a tissue tearor (BioSpec Products, Bartlesville, OK) for 20 s at the highest speed. The homogenates were then centrifuged at 14,000 x *g* for 5 min at 4°C. Ten microliters of the supernatant was analyzed with the luciferase assay system (Promega, Madison, WI).

Analysis of cytokine activity. Two hours after the *in vivo* gene transfer, blood was collected and allowed to clot on ice for at least 4 hours. The sample was then centrifuged at 3000 x *g* for 20 min at 4°C and serum was collected for the cytokine assay. The cytokine concentration (TNF-α) was determined with mouse cytokine immunoassay kits (R&D Systems, Minneapolis, MN).

Imaging of liposome particles. Liposomes were prepared with CDCA as described above. 5 μl of the liposomes were placed on a formvar coated copper grid (Ted Pella, Redding,CA). The liposomes were visualized using uranyl acetate negative staining, then imaged on a JEOL 100CX at the University of Pittsburgh Center for Biological Imaging. Images were taken at 100,000 X magnification.

RESULTS

Overcoming limitations to nonviral gene delivery is a task that has been confronted in various ways by researchers in the field. The most recognized issues with gene delivery include: delivery to target tissues, cellular and nuclear uptake, release from the delivery vehicle, and induction of an immune response, among others. The vector systems described below address two of these obstacles through the incorporation of small molecules in the nonviral vector formulation. The first system improves nuclear targeting by the inclusion of a gene for the nuclear receptor FXR within the plasmid DNA as well as an associated small molecule ligand. Secondly, the non-specific inflammatory response is significantly reduced by a lipoplex containing small molecules known for their inhibition of signaling pathways in specific cytokine induction.

Small molecule ligand activates a nuclear receptor response element to increase gene expression in naked DNA delivery. The farnesoid X receptor (FXR) is member of the nuclear receptor (NR) superfamily, specifically associated with the bile salt circulation pathway and cholesterol homeostasis (Maglich et al., 2003; Redinger, 2003). Pharmaceutical models of FXR regulation are being investigated in hopes of gaining a better understanding of the cholesterol/bile acid metabolism for the development of novel drugs to control this pathway. FXR is expressed only in specific tissue types, including liver, gut, adrenal gland and kidney. In the system described below, FXR is exploited for its ability to target to the nucleus via NLS as well as for its relevance in a major metabolic pathway.

FXR must form a functional heterodimer with the retinoid X receptor in order to be active in bile acid synthesis (Fig. 1). This dimer will bind with the FXR response element (FXRRE) in the presence of select ligand molecules to activate transcription factors and initiate synthesis. These ligands are now known to be largely responsible for the regulation of metabolic pathways (Redinger, 2003; Sonoda et al., 2003). The following experiment was designed to specifically employ the aforementioned characteristics of FXR.

A plasmid containing the FXRRE upstream from a firefly luciferase gene (referred to as pFXRRE-Luc) was delivered to the livers of female CD-1 mice by hydrodynamic delivery (Liu et al., 1999; Zhang et al., 1999). Luciferase levels were measured to determine the efficiency of gene delivery. When the FXRRE-luc plasmid was delivered, as shown in Fig. 2, luciferase levels were approximately 3 x 10^8 RLU/liver due to endogenous FXR. The FXRRE-luc plasmid was then co-delivered with a second plasmid containing a strong promoter (CMV) in front of the FXR gene, to enhance the production of FXR. No significant increase in luciferase expression was observed. However, when the FXRRE-luc plasmid was injected in the presence of an activator ligand specific to FXR, chenodeoxycholate (CDCA), luciferase expression was enhanced by approximately 3 fold. Furthermore, when the pFXRRE-Luc and pCMV-FXR plasmids were co-delivered with the ligand, expression was enhanced nearly 6 fold over the levels observed when pFXRRE-Luc plasmid was delivered alone.

Delivery of a stable lipid-based vector containing small molecule ligands increases transgene activity both *in vitro* and *in vivo*. Gene delivery in the presence of a nuclear receptor was further investigated in lipid-based vectors. The cationic lipid, 1,2-Dioleoyl-3-

Trimethylammonium-Propane (DOTAP) has been frequently used in gene delivery systems. A novel liposome formulation was prepared containing DOTAP and the ligand, CDCA, at 10 mg/ml and 5 mg/ml, respectively. The DOTAP and DOTAP/CDCA liposomes were examined by transmission electron microscopy (TEM) to determine if the presence of the ligand introduced any obvious physical defects into the liposomes. The DOTAP/CDCA liposomes (Fig. 3A) were well dispersed and relatively uniform in size at approximately 100 nm, similar to liposomes of DOTAP alone (data not shown). To ensure that the CDCA did not leak from the liposomes, turbidity experiments were performed on the various liposomes and CDCA alone (Fig. 3B). DOTAP/CDCA liposomes were not observed to have any change in the solution turbidity over 50 minutes, indicating stability of the novel liposome formulation.

After verifying the stability of the liposomes, the two formulations were compared for activity *in vitro*. Lipoplexes, complexes of liposomes and plasmid DNA, were transfected into the mouse melanoma cell line, BL6. DOTAP/pFXRRE-Luc lipoplexes were not observed to have significant levels of luciferase expression (Fig. 4, n=6). DOTAP/CDCA/pFXRRE-Luc lipoplexes also did not show significant levels of luciferase expression. Minimal gene activity was anticipated in both instances as the BL6 cell line was not expected to contain endogenous FXR or other related stimulatory molecules (i.e. CDCA). When the pFXRRE-Luc plasmid was co-delivered with the plasmid containing pCMV-FXR in DOTAP liposomes, the luciferase expression increased nearly 3 fold over transfection with the lipoplexes containing only pFXRRE-Luc. Moreover, co-delivery of the plasmids in the ligand-containing liposomes enhanced gene expression more than 10 fold over the controls.

Incorporation of anti-inflammatory molecules can decrease the immunogenicity of non-viral vectors. A second factor that largely limits the effectiveness of non-viral gene delivery systems is the inflammatory response, which results from the complexation of the liposomes and plasmid DNA. Although the immune response triggered by nonviral delivery systems generally do not involve specific recognition, a substantial inflammatory response is frequently observed, which can diminish the therapeutic value of the vector (Zhou et al., 2004). While naked DNA has been found to be non-immunostimulatory when used alone, complexation with lipids or polymers draws unwanted recognition of the CpG motifs found in plasmid DNA (Krieg et al., 1995; Krieg, 2002). Many approaches have been taken to minimize this effect, including the creation of novel formulations of lipid, pegylation of the molecules (Ogris et al., 1999; Xu et al., 2002; Zuber et al., 2003), and the incorporation of anti-inflammatory molecules conjugated into the vector (Tan et al., 1999; Liu et al., 2004). We have previously described a nonviral vector system that incorporated several general molecules including nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal compounds such as dexamethasone and prednisone into the formulation (Liu et al., 2004). In this study, we incorporated a small molecule ligand to block cytokine formation by specifically inhibiting the NF-κB and the MAPK pathways (Fig. 5). First, capsaicin, commonly used in over-the-counter anti-inflammatory treatments, was incorporated into the vectors to directly inhibit the NF-κB pathway (Singh et al., 1996). A second small molecule, PD-98059, a selective non-competitive inhibitor of the MAPK pathway, was incorporated into the lipid-based vector and also investigated for effectiveness of inhibiting TNF-α production (Yoshizumi et al., 2003).

The small anti-inflammatory molecules were incorporated into DOTAP liposomes as described in the Materials and Methods. The novel formulations were characterized for the stability of the liposomes over time. Data indicates that these liposomes are only stable at ratios below 10:1 (DOTAP:small molecule) based on turbidity studies (data not shown). Optimized complexes were determined to be stable over 1 h, with turbidity curves similar to that of DOTAP: CDCA liposomes (Fig. 3). Encapsulation efficiency of the small molecule-containing liposomes was examined by centrifugation studies. The complexes were briefly run over a spin column, where any free drug passing into the column eluate was detected by UV-spectroscopy (Materials and Methods). Both capsaicin and PD-98059 liposomes were found to maintain the drug with greater than 98% efficiency. Incorporation of DNA to form lipoplex was not found to significantly increase leaking of the drug from the complexes (Table 1). It should be noted that ratios of lipid: small molecule are substantially lower than those used for the nuclear receptor targeting experiments (Fig. 3B) and in our previous studies (Liu et al., 2004). This is largely because the anti-inflammatory molecules were effective at lower concentrations. Concentration studies revealed that the incorporation of higher quantities of drug reduced the stability of the complexes as well as induced toxicity *in vivo* (data not shown).

Transgene activity was examined by measuring luciferase expression in the lungs of mice after tail vein injection of the respective vectors. Luciferase levels for lipoplexes containing capsaicin and PD-98059 or DOTAP alone were found to be the same within error for all three formulations (Fig. 6A). Cytokine activity was analyzed by measuring TNF-α levels in the serum. DOTAP lipoplexes had TNF-α levels at approximately 1000 pg/ml. Lipoplexes containing the anti-

inflammatory inhibitors were significantly lower at 225 pg/ml and 525 pg/ml for capsaicin and PD-98059, respectively (Fig. 6B; $p < 0.05$).

DISCUSSION

The above results describe two highly effective approaches for improving the efficacy of nonviral gene delivery. Both approaches incorporate small molecule ligands into the formulation and act to overcome multiple limitations currently affecting the progress of gene delivery, specifically increased/regulated transgene expression and the non-specific inflammatory response.

Increased expression of the transgene was examined by expression of firefly luciferase protein. The aforementioned experiments systematically investigated the role of the FXR nuclear hormone receptor and the associated ligand for activating this promoter region (Fig. 2 and 4). This nuclear receptor (FXR) was chosen to serve as a model for proof of concept. These experiments demonstrate that to some extent all 3 essential components of the system were still present after cellular uptake, as transfection with any 2 components did not demonstrate optimal transgene activity. Additionally, FXR is only expressed in limited tissue types. It was clearly demonstrated that the presence of this nuclear receptor alone does not make significant improvements in transgene activity, however, in the presence of the activating ligand, activity was improved up to 10-fold over the controls. Since several essential elements are required for nuclear receptor activation, this system becomes tunable to specific cell types. Delivery of engineered plasmids containing a hormone receptor response element, without both the receptor

and activating ligand would be ineffective in tissues that do not already contain both the receptor and the small molecule. Therefore, it is possible to regulate both location and level of transfection in any cell type by incorporating both a nuclear receptor and the ligand into a nonviral formulation. Moreover, this stepwise investigation of the nuclear receptor and ligand stresses the significant role these small molecules play in regulating cellular pathways.

Other small molecules (i.e. capsaicin and PD-98059) incorporated into lipid formulations successfully minimized the anti-inflammatory response commonly observed in nonviral vectors. These molecules, selected for their specificity toward inflammatory pathways, targeted the NF-κB and MAPK pathways. Incorporation of the small molecules into the liposomes did not alter the efficiency of gene delivery as compared to the DOTAP lipoplexes (Fig. 6A). Since the encapsulation efficiency and turbidity experiments did not indicate any structural defects in the liposomes at these concentrations (Table 1), we presume that no function-altering changes occurred in liposomes containing the small anti-inflammatory molecules. The results suggest that the modified lipoplex were successfully taken up by cells, but also released the free drug after injection, thereby reducing systemic activation of inflammatory cytokines.

The cytokine inhibitors chosen for these experiments are known to inhibit two specific pathways leading towards TNF-α production. The results clearly show that TNF-α levels are significantly reduced when either the MAPK or the NF-κB pathway was inhibited (Fig. 6B). However, it is important to recognize that the level of cytokine reduction was two-fold lower for the capsaicin-inhibited pathway. This may indicate the inhibition of the NF-κB pathway is a more effective method for reducing TNF-α levels. Based on these results, it may be possible to inhibit the

cytokine levels by affecting one or more activation pathways by the incorporation of small molecules. Thus, manipulation of small molecules to most efficiently down-regulate specific anti-inflammatory pathways may allow vectors to be modified toward limiting specific cytokine production.

Recent advances in cell signaling and trafficking have introduced a much greater understanding into many intra- and intercellular pathways. Gaining perspective on the roles of small molecules to up- or down-regulate these pathways and functions has allowed us to exploit this information to improve gene delivery vectors. The above examples used several small molecules to overcome two main limitations to nonviral gene delivery (i.e. nuclear uptake and inflammation). Incorporation of these small molecules was met with significant success, and provides evidence that small molecule ligands may greatly improve the efficacy of nonviral gene delivery vectors. The nature of the small molecule ligands and their stability in liposome formulations may also allow for the incorporation of multiple small molecules into liposomes, or possibly for use in conjunction with other advances in the field, such as the inclusion of specific cell targeting molecules (e.g. transferrin or EGF). Thus, by combining the breakthroughs in cell biology with advances in gene delivery, it may truly be possible to optimize vectors and to overcome many of the obstacles currently limiting the success of nonviral gene delivery.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Activation pathway of the farnesoid X receptor response element (FXRRE). The activation is dependent upon the dimerization of the retinoic X receptor (RXR) and the farnesoid X receptor (FXR), which only occurs in the presence of the ligand, chenodeoxycholic acid (CDCA). In this case, the FXRRE is located upstream from the firefly luciferase reporter gene. Upon dimerization and activation of the FXRRE, transcription would be initiated and luciferase expression observed.

Figure 2. Hydrodynamic injection of naked DNA into mice livers. Naked DNA was delivered to the mice using hydrodynamic injection of 8% of body weight of solution. Injection of plasmid containing FXRRE-luc and injection of FXRRE-luc with plasmid containing the CMV-luc combination were found to have nearly ten-fold less expression than delivery of the same combinations in the presence of CDCA. Quantities of FXRRE-luc injected were 10 μg/mouse; CMV-FXR was 4 μg/mouse; CDCA were 0.6 mg/mouse via intraperitoneal injection.

Figure 3. Incorporation of CDCA into DOTAP liposome for gene delivery. A) Transmission electron micrograph (TEM) of DOTAP:CDCA liposomes viewed by negative staining of the liposomes. The w/w ratio of the components is 10:2. B) Turbidity study of CDCA alone, DOTAP liposomes and DOTAP:CDCA liposomes over 60 min. Based on the turbidity of the solution, it is evident that DOTAP:CDCA liposomes exhibit similar stability to DOTAP liposomes. Turbidity was examined at an absorbance of 600nm.

Figure 4. Luciferase expression in BL6 cells of DOTAP:CDCA liposomes. The DOTAP:CDCA liposomes were mixed with plasmid DNA in a 2 to 1 ratio. CDCA concentration was 5mg/ml. Luciferase expression was measured 24 h after transfection. Since BL6 cells are not known to endogenously express FXR, observed expression is a result of the co-delivery of the plasmids ($p< 0.01$). ***p* values were calculated using the student's t-test.

Figure 5. Inflammatory Signaling pathways. This diagram describes the steps leading to the activation of the cytokine, TNF-α. Initially, the pathways are the same but then split off to lead to the production of NF-κB or MAPK and AP-1. The inhibitors used in the anti-inflammatory experiments, capsaicin and PD98059, were chosen because they are specific for the NF- κB and the MAPK pathways, respectively. Structures of these inhibitor molecules are shown within the figure. Areas of inhibition are denoted with arrows.

Figure 6. *In vivo* gene expression and cytotoxicity results for novel vector formulations. A) Bioactivity of DOTAP liposomes in mouse lungs. Mice were injected with lipoplexes containing 20 μg of DNA at a 12:1 ratio with the cationic lipid DOTAP. DOTAP: capsaicin and DOTAP:PD98059 lipoplexes were examined for luciferase activity in mouse lungs as compared to DOTAP lipoplexes. The resulting gene expression levels were found to be the same for all lipoplexes within experimental error. B) Examination of TNF-α response to lipoplex delivery. DOTAP lipoplex induced 4-fold and 2-fold greater levels of TNF- α than the lipoplexes containing capsaicin and PD98059, respectively (n=5). The DOTAP:capsaicin and DOTAP:PD98059 lipoplexes were 10:0.125 (w/w) and 10:0.25 (w/w), respectively.

Table 1. Encapsulation efficiency of lipoplexes for anti-inflammatory drugs.

After formation, lipoplexes were applied to a size exclusion column (MW 3000) and were separated from molecules not contained within the complexes. Excess of the anti-inflammatory drugs, capsaicin and PD98059 were quantified by UV spectroscopy to give the resulting concentrations. In both cases, only 1-2% of the total drug was found to be excluded from the lipoplex.

	Encapsulation Efficiency		p value
	liposome/inhibitor	liposome/inhibitor/DNA	
Capsaicin	98.2 ± 1.4	98.9 ± 1.9	0.47
PD98059	99.6 ± 1.6	98.7 ± 1.3	0.56

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Figure 1

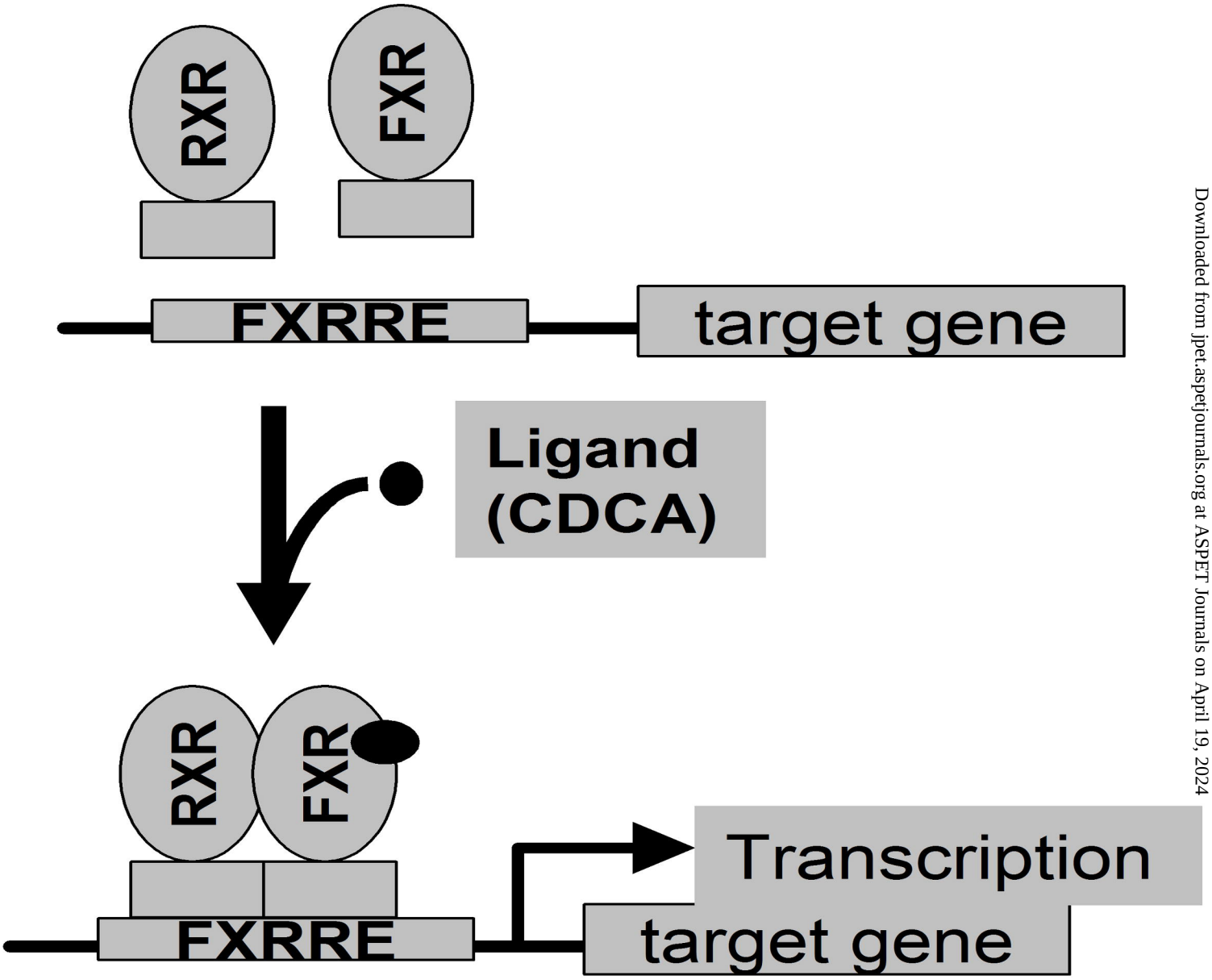

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Figure 2

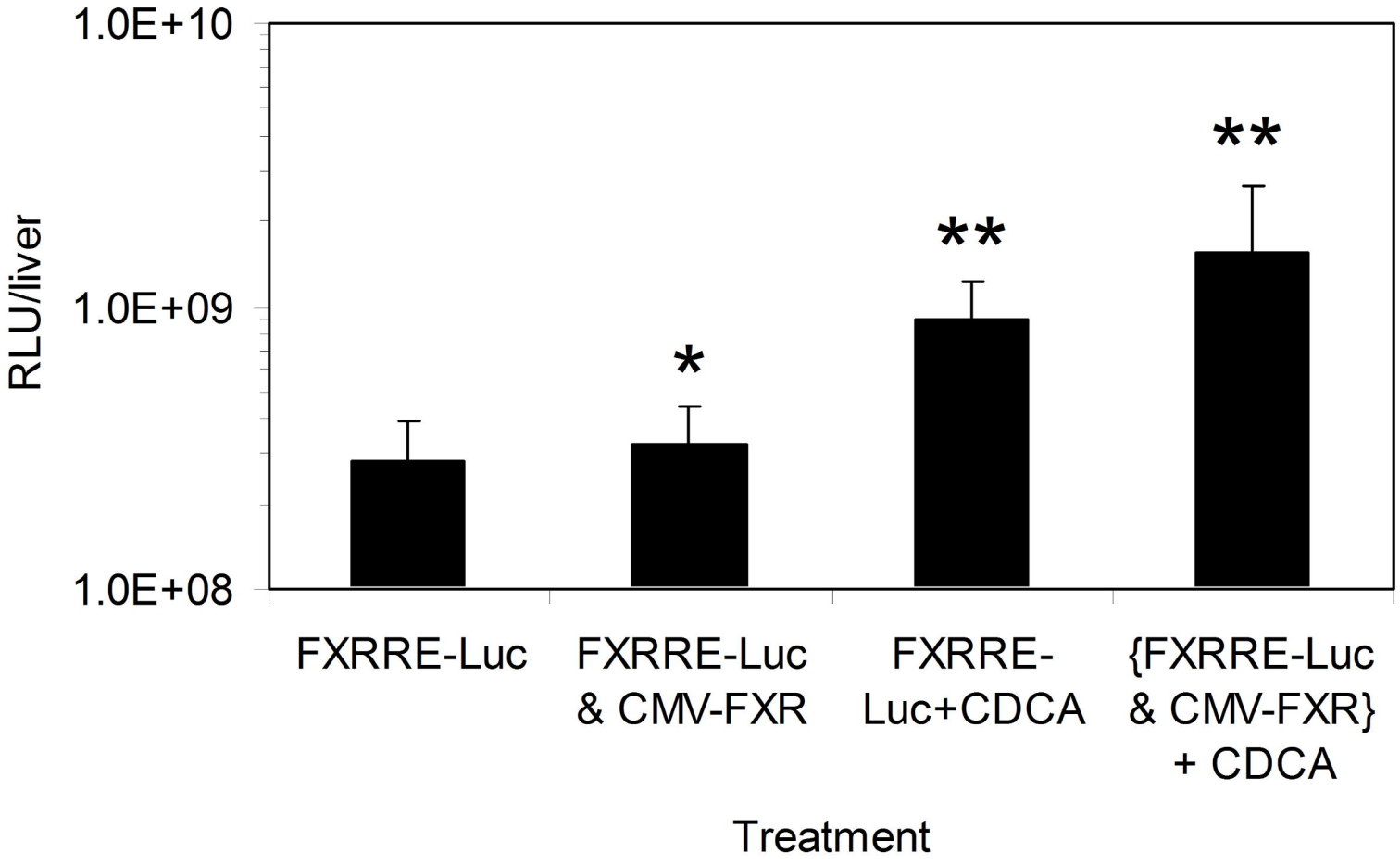

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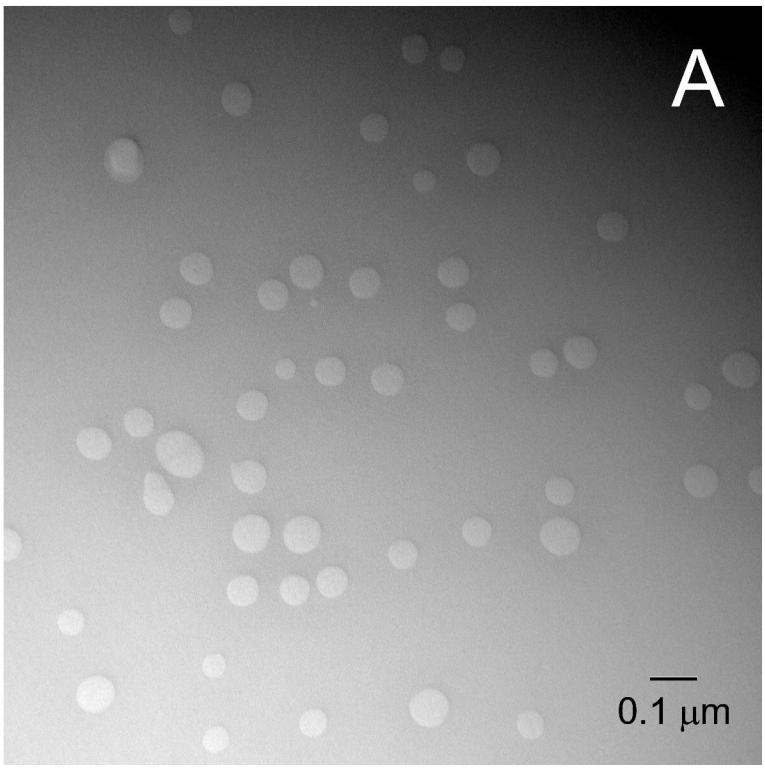
Figure 3



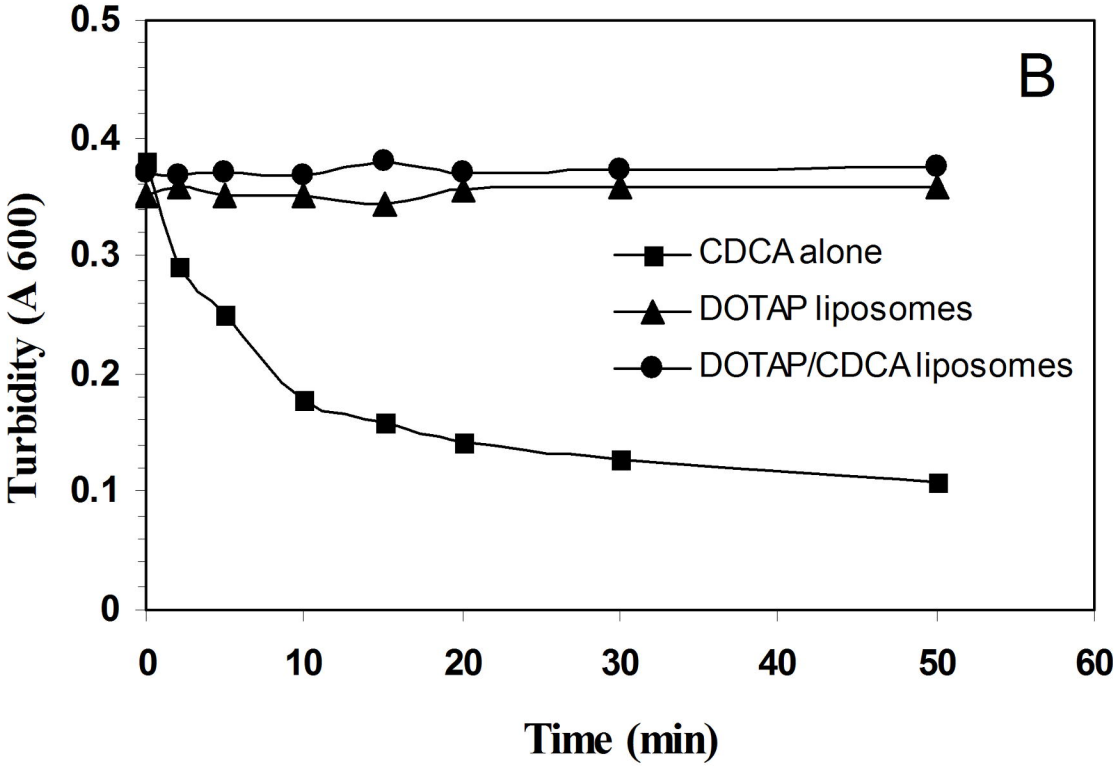


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Figure 4

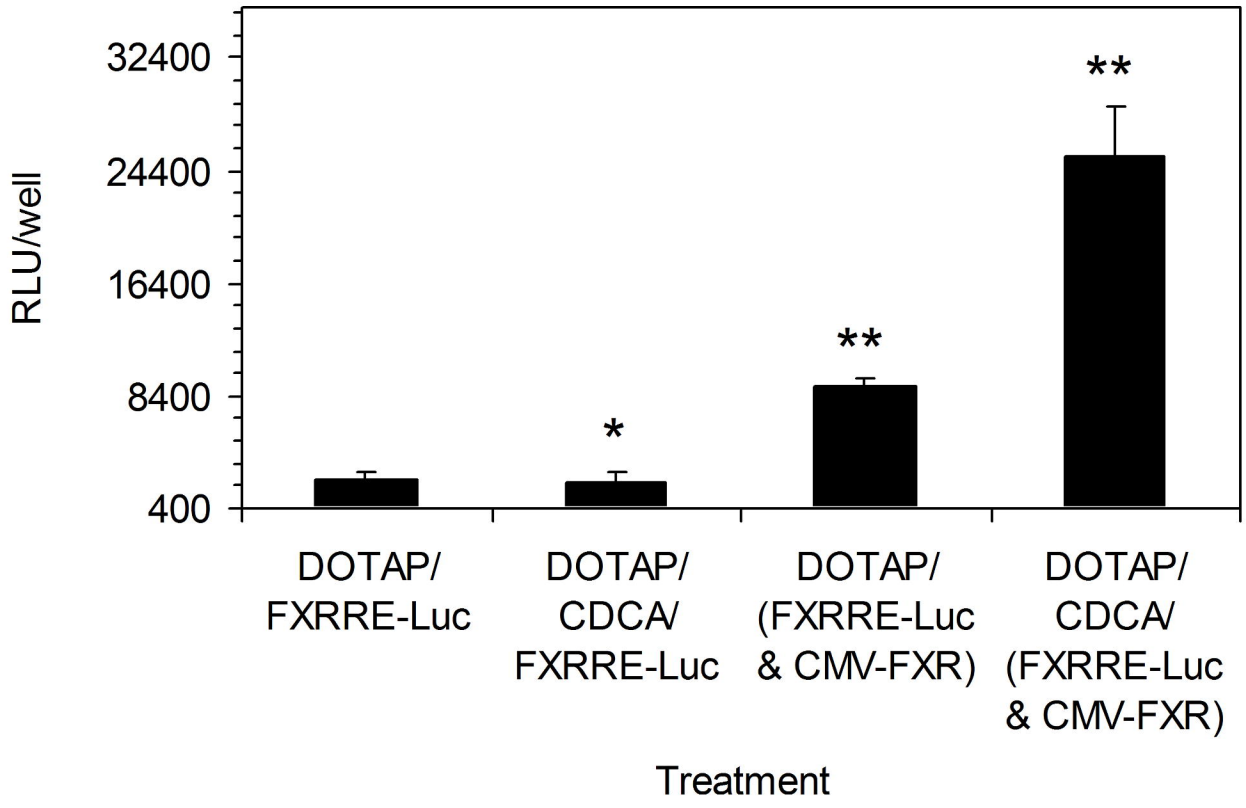

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Figure 5

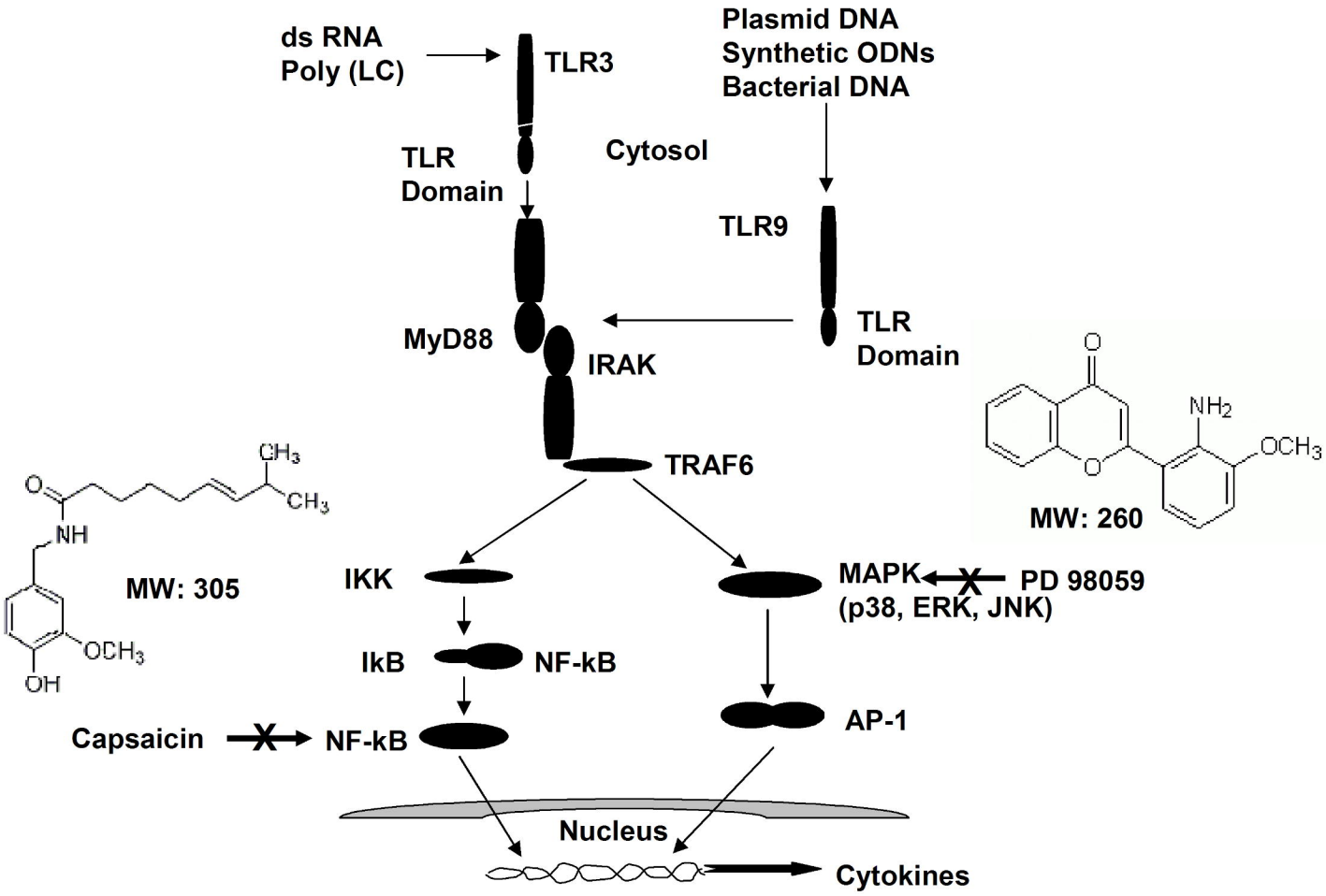

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Figure 6