Combinatory Delivery of Etoposide and siCD47 in a Lipid Polymer Hybrid Delays Lung Tumor Growth in an Experimental Melanoma Lung Metastatic Model

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This study investigated the feasibility of lipid polymer hybrid nanoparticles (LPH) as a platform for the combinatorial delivery of small interfering RNA (siRNA) and etoposide (Eto). Different Eto loaded LPH formulations (LPH_Eto) are prepared. The optimized cationic LPH_Eto with a particle size of 109.66 ± 5.17 nm and Eto entrapment efficiency (EE%) of 80.33 ± 2.55 is used to incorporate siRNA targeting CD47 (siCD47), a do not eat me marker on the surface of cancer cells. The siRNA-encapsulating LPH (LPH_sNEG-Eto) has a particle size of 115.9 ± 4.11 nm and siRNA EE% of 63.54 ± 4.36 %. LPHs improved the cellular uptake of siRNA in a dose- and concentration-dependent manner. Enhanced cytotoxicity (3.8-fold higher than Eto solution) and high siRNA transfection efficiency (≈50 %) are obtained. An in vivo biodistribution study showed a preferential uptake of the nanosystem into lung, liver, and spleen. In an experimental pseudo-metastatic B16F10 lung tumor model, a superior therapeutic outcome can be observed in mice treated with combinatorial therapy. Immunological studies revealed elevated CD4+, CD8+ cells, and macrophages in the lung following combinatorial treatment. The study suggests the potential of the current system for combinatorial chemotherapy and immunotherapy for the treatment of lung cancer or lung metastasis.

1. Introduction

Under normal physiologic conditions, cellular homeostasis is governed by the balance between pro-phagocytic signals and anti-phagocytic signals.[1] In general, tumors express high levels of CD47 as a strategy to evade the clearance by the mononuclear phagocytic system (MPS). The interaction between CD47 and its signal regulatory protein α (SIRPα) on the macrophages delivers a “don’t eat me signal” which hinders their recognition by the immune system.[2] Blocking CD47 can be achieved by using either monoclonal antibodies[2] or small interfering RNA (siRNA).[3] Interestingly, exploiting siRNA has many advantages over antibodies including ease of manufacturing and simple incorporation into different nanocarriers with possible tumor accumulation by the enhanced permeation and retention (EPR) effect.

Combination therapy targeting multiple pharmacological mechanisms is found to improve therapeutic outcomes in cancer.[4] Etoposide (Eto) is a potent topoisomerase II inhibitor affecting mainly S and G2 phases of the cell cycle.[5] It has been explored in the treatment of many types of tumors such as lung cancer, leukemia, non-Hodgkin’s lymphoma, neuroblastoma, and gastric cancer.[6] Although Eto is broadly considered to be immunosuppressive,[7] a previous study has reported its combination with an immunotherapeutic agent can lead to complete tumor regression in immunocompetent hosts.[8] A clinical study has demonstrated improved survival in lung cancer patients receiving atezolizumab, a monoclonal antibody against PD-L1, programmed death-ligand 1, with Eto and carboplatin.[9] In a preclinical study, Eto therapeutic efficacy was also augmented when combined with a blocker of CTLA-4, a T-cell inhibitory receptor.[10] Thus, the combined efficacy of an anti-proliferative chemotherapeutic drug and immunogenic activation resulting from the co-delivery of Eto and siRNA against CD47 (siCD47) is speculated to result in a potent anti-tumor activity. There is however a need to design a delivery system capable of simultaneous delivery of the chemotherapeutic agent Eto, a hydrophobic low molecular weight drug, and the hydrophilic polyanionic siRNA.

Lipid polymer hybrid nanoparticles (LPH) are nanocarriers composed of an internal polymeric core enclosed by an outer lipid shell composed of one or more layers.[11] By virtue of its unique structure, LPH has versatile competence to encapsulate different types of payloads such as hydrophilic and hydrophobic...
The two LPH formulations prepared. Two types of LPH are prepared in the study. A) I-LPH (I refers to in situ) is prepared by dissolving a lipid mixture of lecithin: tristearin (1:1) (1 mg mL\(^{-1}\)), DSPE-PEG 2000 (1 mg mL\(^{-1}\)), Tween 80 (10 mg mL\(^{-1}\)), stearyl amine (10 mg mL\(^{-1}\)) with/or without siRNA (0.133 mg mL\(^{-1}\)) in 4% hydroalcoholic solution (0.9 mL) and heated at 70 °C. PLGA (5 mg mL\(^{-1}\)) and Eto (3 mg mL\(^{-1}\)) are dissolved in DMF (0.1 mL). The hydroalcoholic lipid phase is titrated by the drug-polymer organic solution dropwise with magnetic stirring at room temperature for 2 h.

B) P-LPH (P refers to post) are prepared by omission of siRNA from the aqueous phase and the formed LPH containing Eto are subsequently mixed with siRNA solution (at a final concentration of 0.133 mg mL\(^{-1}\)) for 2 h at room temperature. For both LPHs, the organic to aqueous phase is kept at 1:9 v/v volume ratio and the fabricated LPH is obtained by ultrafiltration using Amicon tubes (MWCO 100 K, 12 000 rpm for 30 min at 4 °C) to separate the unentrapped Eto and/or siRNA. The collected pellets (LPH) on the membrane are re-dispersed in PBS (pH 7.4, RNAse free water) to a final volume of 1 mL for further analysis.

The aim of this study was to design an LPH system suitable for the co-delivery of Eto and siCD47 for in vivo delivery and anticancer therapy in a murine tumor model. For this purpose, various formulations and process parameters were optimized using Box-Behnken design (BBD). The optimized formulae were those with the maximum entrapment efficiency (EE %) and minimum particle size (<150 nm). The ability of the optimized LPH formulations to improve siRNA uptake and gene silencing was investigated in mouse melanoma B16F10 cells. The therapeutic efficacy of the LPH co-loaded with siCD47 and Eto was investigated in an experimental mouse melanoma lung metastasis model.

2. Materials and Methods

2.1. Materials

Co-polymer of DL-lactide/glycolide (50/50) conjugate and acid terminated with an inherent viscosity midpoint of 0.2 dl g\(^{-1}\) (PURASORB PLGA) was purchased from Corbion (Netherlands). Lecithin soybean (3-sn-Phosphatidylcholine ≥99 % (TLC) lyophilized powder), etoposide, tristearin, dialysis tubing (MWCO 12 kDa), absolute ethanol, dimethylsulphoxide (DMSO), Triton X-100, and 10 % neutral buffered formalin were supplied from Sigma-Aldrich, UK. DSPC-PEG 2000 was purchased from Avanti Polar Lipids (USA). Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine Iodide (DiR) and GelRed were purchased from Biotium Inc. Gibco RPMI-1640 media, fetal bovine serum (FBS), penicillin/streptomycin, GlutaMAX, Trypsin/EDTA, and phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and UltraPure Agarose were obtained from ThermoFisher Scientific (UK). RNase was obtained from New England Biolabs (UK). Isoflurane (IsoFlo) was purchased from Abbott Laboratories Ltd (UK). All reagents were used without further purification. Fluorescently labeled non-coding siRNA (siNEG), Atto 655-siNEG, was obtained from Eurogentec (Belgium). CD47 siRNA (SMARTpool) was purchased from Horizon Discovery (UK). Anti-mouse CD47-APC clone miap301 and all other antibodies were purchased from Biolegend. Falcon 70 µm cell strainers were purchased from BD Biosciences (UK).

2.2. Preparation of LPH

The single-step nanoprecipitation self-assembly method was adopted to fabricate different types of LPH systems.\(^{16}\) The preparation schemes are illustrated in Scheme 1. Two strategies were attempted to introduce siRNA into the formulation. In one method, siRNA was dissolved in the aqueous phase prior to mixing with the organic phase. The LPH prepared by this method
was referred to as I-LPH (I refers to in situ). In the second approach, referred to as P-LPH (P stands for post-incorporation), siRNA was complexed with the previously formed LPH.

To prepare I-LPH, the organic phase (0.1 mL) was prepared by dissolving PLGA (5 mg mL\(^{-1}\)) and Eto (3 mg mL\(^{-1}\)) in DMF. A 4 % v/v hydroalcoholic solution (0.9 mL) containing lecithin-tristearin (1 mg mL\(^{-1}\)). DSPE-PEG 2000 (1 mg mL\(^{-1}\)), Tween 80 (10 mg mL\(^{-1}\)), stearyl amine (10 mg mL\(^{-1}\)), and siRNA (0.133 mg mL\(^{-1}\)) was prepared and heated at 70 °C for 15 min. The organic phase was added slowly to the aqueous solution under 900 rpm magnetic stirring for 2 h at room temperature. The organic to aqueous phase was kept at a 1:9 v/v volume ratio. The PLGA, lecithin-tristearin and Eto concentrations stated above were verified to yield optimal formulation characteristics by BBD design described in the next section. To prepare P-LPH, LPH were firstly prepared using the same formula as I-LPH but without the siRNA. The formed LPH containing Eto (1 mL) was subsequently mixed with siRNA solution (0.1 mL) to a final concentration of 0.133 mg mL\(^{-1}\) for 2 h at room temperature. The unentrapped Eto, siRNA, and traces of organic solvent were removed by ultrafiltration using Amicon Ultra filters (MWCO 100 K, 12 000 rpm for 30 min at 4 °C). The recovered concentrated LPH was re-dispersed in PBS (pH 7.4, RNase free water) to a final volume of 1 mL for further analysis. Eto and siRNA containing LPH were referred to as LPH_{Eto} and LPH_{siNEG}, respectively. LPH_{siNEG,Eto} contains both siRNA and Eto. siNEG was replaced with siCD47 for therapeutic studies, that is, LPH_{siCD47}. For the biodistribution study, DiR-labeled LPH_{siNEG} was prepared by dissolving DiR in the alcoholic aqueous phase at 1 % w/w of the total lipid.

2.3. Experimental Design and Construction of BBD

Design-Expert software (Design-Expert 9.0.5.2, State-Ease Inc., USA) was used to construct a matrix and to explore the response surfaces and the statistical models to optimize LPH_{Eto} formulation. The selected critical process parameters (CPPs) were divided into formulation variables namely PLGA (A), lecithin-tristearin (B), and Eto (C) concentrations; processing variable namely the stirring speed (D). The independent variables A–D were tested at three levels: low (−1), medium (0), and high (+1). The particle size (Y1) and EE % (Y2) were the critical quality attributes (CQAs). The LPH was designed to deliver quality target product profile (QTPP) of minimum particle size and maximum EE %. The defined CPPs and CQAs, as well as the desired QTPP, are listed in Table S1, Supporting Information. The design matrix constructed by the software involved 29 different formulae (Table S2, Supporting Information).

The statistical validation of the polynomial equations generated by Design-Expert software was performed by ANOVA. Various statistical indices such as P-values, F-values, \(R^2\) (adjusted \(R^2\) and predicted \(R^2\)), and predicted residual error sum of squares (PRESS) were used to assess the statistical significance of the models; either linear two-factor interactions (2FI) or quadratic models. The 3D response surface plots were constructed by the software and the polynomial equations were authenticated. Different feasibilities were conducted over the experimental domain to find the compositions of the optimized LPH. Based on the highest desirability, the design space was generated to define the optimal CPPs to prepare LPH with the desired QTPP.\(^{[37]}\) One optimum LPH_{Eto} checkpoint was elected to confirm the chosen domain and equations. The experimental values of the responses were quantitatively compared with the predicted values and the prediction errors (\%) were calculated.

2.4. Determination of Particle Size, Size Distribution, and Zeta Potential

The particle size (z-average), size distribution expressed as polydispersity index (PDI), and surface charge (zeta potential) of the prepared LPH_{Eto} with or without siRNA (n = 3) were assessed by dynamic light scattering (DLS) with a Zetasizer Nano ZS 90 (Malvern Instruments, Southborough, MA). The as-prepared LPH dispersions were diluted with deionized water (1:10 v/v) and transferred to disposable plain folded capillary Zeta cells. All the measurements represent the average of 20 runs; each run was completed in triplicate at 25 °C.

2.5. Determination of Encapsulation Efficiency (EE \%) and Loading Efficiency (LE \%)

The EE % was measured directly by quantifying the amount of Eto encapsulated into the LPH (n = 3). Briefly, Eto LPH was centrifuged at 12 000 rpm for 30 min at 4 °C and the recovered pellets were dissolved in DMF (10 mL). Eto amount was quantified using a previously reported HPLC method.\(^{[38]}\) The HPLC system was composed of an Agilent 1100 system, equipped with a quaternary pump and a UV detector (WVD-G1314 A). A reverse-phase C8 column (Thermo BDS, 250 × 4.6 mm, 5 μ) was used for Eto separation at 25 °C at 210 nm detection wavelength. The mobile phase consisted of acetonitrile and 10 mM phosphate buffer pH 3.5 at 70:30 ratio, pH was adjusted to 4.0 ± 0.1 with formic acid at 1 mL min\(^{-1}\) flow rate.

The EE% was calculated using the following equation :

\[
\text{EE\%} = \frac{\text{Amount of } Eto \text{ determined into the pellets}}{\text{Total amount of } Eto \text{ added}} \times 100 \tag{1}
\]

siRNA EE % was calculated by measuring the fluorescence intensity after staining with GelRed (excitation at 300 nm and emission at 590 nm) using a calibration curve in PBS (pH 7.4) in the range of 0.1–1 nM with coefficient of determination (\(R^2\)) 0.9971 (Model UV-1601 PC, Shimadzu, Kyoto, Japan).

LE of both Eto and siRNA was calculated using the following equation

\[
\text{LE\%} = \frac{\text{Amount of } Eto \text{ or } siRNA \text{ determined into the nanoparticles}}{\text{Total weight of nanoparticles}} \times 100 \tag{2}
\]

2.6. Transmission Electron Microscopy (TEM)

The optimized LPH_{Eto} was visualized using transmission electron microscope (TEM, Jeol, JEM-1230, Japan). A drop of the LPH...
dispersion was deposited on a copper 300-mesh grid, coated with carbon, and allowed to stand for 10 min after which, any excess fluid was absorbed with a filter paper. The sample was negatively stained with one drop of 1% phosphotungstic acid, applied, and allowed to dry for 5 min.

### 2.7. In vitro release study

Eto release from the optimized LPH<sub>Eto</sub> formulation was studied using the dialysis membrane method with slight modifications.<sup>[19]</sup> An aliquot volume of the prepared LPH (1 mL equivalent to 2.3 mg Eto) (n = 3) was placed in the pre-soaked dialysis membrane (10 K Da MWCO) and diluted with 1 mL of PBS (pH 7.4), mixed with FBS (at 50% v/v concentration). The sealed membranes were dialyzed against 50 mL PBS (pH 7.4) containing 1% Tween 80 in a thermostatically controlled shaking water bath at 250 strokes min<sup>−1</sup> ± 0.1 at 37 ± 0.5 °C.<sup>[20]</sup> To exclude the possible nonspecific binding of Eto to the dialysis membrane, the in vitro release of Eto solution in PBS (containing 10% DMSO) was also conducted under the same conditions. At predetermined time intervals, an aliquot of 1 mL of the release media was withdrawn and immediately replenished with an equivalent of preheated release media. Eto concentration was quantified by measuring the absorbance at 285 nm using a UV spectrophotometer (Perkin-Elmer Lambda 35) using dialysate from drug-free LPH as a blank.

### 2.8. In Vitro Hemolytic Assay

In vitro hemolytic activity of the optimized LPH<sub>Eto</sub> was assessed using fresh BALB/c mouse red blood cells (RBCs) (n = 3). In brief, blood was withdrawn from BALB/c mice by cardiac puncture in heparinized tubes and the RBCs were collected by centrifugation at 4000 rpm for 10 min. Consequently, the obtained RBCs were incubated with different concentrations of Eto, free or encapsulated into LPH, for 2 h at 37 °C. The samples were centrifuged at 4000 rpm for 5 min at 4 °C. The amount of the liberated hemoglobin in the supernatant was quantified spectrophotometrically at 545 nm. The respective negative and positive control were prepared by incubating RBCs with PBS (pH 7.4) and Triton X-100 (0.5% w/v), respectively.<sup>[21]</sup> Percentage (%) hemolysis was calculated using the following equation:

\[
\text{% Hemolysis} = \frac{\text{absorbance sample} - \text{absorbance negative control}}{\text{absorbance positive control} - \text{absorbance negative control}}\times 100
\]  

### 2.9. Cell Culture

B16F10 melanoma cells were cultured in RPMI media supplemented with 10% v/v FBS, 50 U mL<sup>−1</sup> penicillin, 50 µg mL<sup>−1</sup> streptomycin, and 1% v/v L-GlutaMAX. Cells were incubated in 5% CO<sub>2</sub> at 37 °C.

### 2.10. Cytotoxicity Study

In vitro cytotoxicity of Eto formulations was assessed using MTT assay. In brief, B16F10 cells were seeded in 96-well plates (n = 5) at a density of 7 k cells per well in RPMI media for 24 h. Subsequently, cells were incubated with serial concentrations of Eto in the range of 0.01–100 µM or the blank formulation at equivalent concentrations for 48 h. The incubation media was then replaced with MTT solution (120 µL) and cells were kept at 37 °C and 5% CO<sub>2</sub> for 4 h. The obtained formazan crystals were dissolved in 200 µL of DMSO then the plate was read at 570 nm using FLU-Ostar Omega plate reader (BMG Labtech). Cytotoxicity was expressed as the percentage cell survival which was calculated using the following equation:

\[
\text{% Cell survival} = \frac{\text{absorbance of treated cells at 570 nm}}{\text{absorbance of untreated cells at 570 nm}} \times 100
\]  

### 2.11. Cellular Uptake Study

Cellular uptake of siRNA was assessed by flow cytometry by incubating B16F10 cells with 30 nM or 90 nM of the fluorescently labeled siRNA, Atto655-siRNA, in free or LPH form with/without Eto (0.1 µM), for 4 and 24 h (n = 3 for each condition). Cells were washed twice with PBS, harvested and centrifuged at 1750 rpm for 3 min at 4 °C. Uptake was quantified by measuring the fluorescence of 10 000 gated cells using FL4 detector (BD FACSCalibur flow cytometer, BD Biosciences). Analysis was performed using Flowjo software (Treestar).

### 2.12. In Vitro Gene Silencing

The gene silencing ability of different LPH<sub>siCD47</sub> formulations (or their LPH<sub>siNEG</sub> counterparts) on B16F10 cells was investigated by incubation with 10, 30, and 90 nM siRNA for 48 h with/without Eto (0.1 µM) (n = 3). Cells were washed twice with PBS, trypsinized, and then stained with anti-mouse CD47-APC monoclonal antibody. CD47 expression was quantified by measuring the fluorescence of 10 000 gated cells using FL4 detector (BD FACS Calibur flow cytometer, BD Biosciences). Analysis was performed using Flowjo software (Treestar). Transfection efficiency was calculated as percentage reduction in mean fluorescence intensity (MFI) relative to control.

### 2.13. In Vitro Serum Stability

The effect of incubation with serum (50% v/v FBS) on different LPH<sub>siNEG-Eto</sub> particle size, PDI, and zeta potential after 4, 24, and 48 h incubation at 37 °C were studied (n = 3).

### 2.14. RNA Protection Test

The ability of LPH to protect the entrapped siRNA from FBS or RNase was investigated using agarose gel retardation assay.
LPH formulations and naked siRNA, used as a positive control, were incubated with FBS (50 % v/v) or RNase (100 µg mL⁻¹) for 4, 24, or 48 h. At the end of the incubation period, RNase was deactivated with EDTA at 50 mM final concentration and the complex was challenged with heparin (100 IU mL⁻¹, 10 % v/v) to dissociate siRNA from the complex. Untreated siRNA was used as a negative control. Samples were mixed with 6X loading dye and resolved on 2 % w/v agarose gel in sodium borate buffer at 225 V for 20 min. Bands were visualized under UV light (ChemiDoc MP system, BioRad, UK) after counterstaining with GelRed.

2.15. Storage Stability Study

The shelf-life stability of I-LPHsiNEG-Eto was studied at 4 and −20 °C for one month. The particle size, surface charge, and EE % of Eto and siNEG were determined as described above.

2.16. In Vivo Imaging and Organ Biodistribution

Animal experiments were conducted with project and personal licenses granted by the UK Home Office and in accordance with the UK Animals (Scientific Procedures) Act 1986 and UK Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (Home Office 1989). In vivo experimentation was adhered to the project licence approved by the King’s College London animal welfare and ethical review body (AWERB). In imaging studies, three female BALB/c mice aged 4–6 weeks (Envigo, UK) were anesthetized using isoflurane and intravenously injected with 200 µL DiR-labeled LPH formulations and naked siRNA, used as a positive control. Animals were sacrificed using Flowjo software. Cells were first gated using forward/side scatter profile prior to the relevant marker being assessed, using isotype staining as background. For myeloid populations, cells were gated as described previously and relative levels of CD11c and F480 were analyzed in accordance with [22].

Using this strategy three cell populations could be broadly identified, and accordingly labeled: CD11c⁺, F480⁻ dendritic cells, DC; CD11c⁻, F480⁺ alveolar macrophages, AM; CD11c⁻, F480⁺ interstitial macrophages, IM. The absolute number of each cell type was calculated by normalization of cell count to bead numbers and the cell numbers per lung weight were obtained by dividing the cell number by lung weight in mg. Data are presented as total cell number per mg of lung tissue.

2.19. Histological Examination

The collected organs including heart, lung, liver, spleen, and kidney from different treatments were fixed in 10 % neutral buffered formalin. Tissues were embedded in paraffin and sectioned for hematoxylin and eosin staining (H&E) according to the standard histological protocols at the Royal Veterinary College, UK. All stained sections were imaged using a Leica DM 1000 LED microscope (Leica Microsystems, UK) coupled with a digital camera (QImaging, UK).

2.20. Statistical Analysis

Except for the therapy study, each experiment was conducted in triplicate in the same experiment and is a representation of two independent experiments, data were presented as mean ± SD (standard deviation). For therapy experiments, experiments were done once (n = 7), data were presented as mean ± SE (standard error of the mean). Student’s t-test was applied to compare two variables while ANOVA test followed by Tukey HSD test was used for comparing different parameters between groups. For lung nodules measurements, statistical analysis was carried out using student’s T-test followed by Mann Whitney post-test. Differences were considered statistically significant at probability (p) value less than 0.05.

3. Results

3.1. LPHEto Formulation Optimization using BBD

Response surface methodology by using BBD was implemented to study the effect and the interactions of the different
investigated CPPs on CQAs which were the particle size (Y1) and EE % (Y2). Table S2, Supporting Information, shows the composition of the fabricated 29 formulations and the obtained responses. The relation between different CPPs and each of the CQAs was expressed using a polynomial equation. The statistical models were selected based on the highest $R^2$ values (adjusted and predicted) with difference below 0.2 and the least PRESS value after removing the non-significant factors. The signal to noise ratio, expressed as adequate precision, was greater than 4 proving the capability of models to navigate the design space. The quadratic model was selected as the best fit statistical model (Tables S3 and S4, Supporting Information). The positive signs of the regression coefficient indicate a direct relationship between CPPs and CQAs, while the negative sign indicates an inverse relationship between them.

3.2. Effect of Different CPPs on Particle Size (Y1) and EE % (Y2)

Table S2, Supporting Information, shows that all the prepared LPH formulae had a particle size ranging from 106.25 ± 5.41 to 263.5 ± 3.74 nm with Eto EE % in the range between 31.25 ± 2.85 and 78.11 ± 3.12%. The PDI values of the fabricated LPH were ≤0.2. The effect of different CPPs on particle size (PS) and EE % could be defined from the following equations after omitting the non-significant terms

$$PS = +137.68 + 15.30A + 11.19B + 16.43C - 54.98D + 11.67AC - 12.75BC + 47.99D^2$$

(5)

$$EE\% = +66.67 + 2.40A + 1.91B + 15.99C + 6.17D - 10.75AB - 5.66A^2 - 8.66C^2$$

(6)

Table S5 and S6, Supporting Information, listed the ANOVA of the effect of the investigated CPPs on particle size and Eto EE %, respectively, where P-values < 0.5 were considered significant. By inspecting Equation (5) and Figure S1, Supporting Information, a positive correlation between polymer, lipid, and drug concentration and PS could be observed. On the contrary, stirring speed is inversely proportional to particle size. The concomitant increase in both the polymer and drug had a positive effect on the particle size confirmed by the positive sign for AC in Equation (5) which is shown visually in Figure 1A. The interaction between lipid and drug however had an antagonistic effect on size demonstrated by the negative sign in Equation (5) and shown visually in Figure 1B. Equation (6) and Figure S2, Supporting Information, demonstrated that all the investigated parameters showed a positive influence on Eto EE %. Figure 1C showed a negative influence of the interaction between the polymer and the lipid on EE %. Design space is multidimensional CPPs combinations and their interactions, identified through design of experiments data and had been verified to afford QTPP. The design space is plotted by overlapping different CPPs’ influences on CQAs contour plots to obtain QTPP. The yellow area represents the values of CPPs when optimized to fulfill QTPP criteria; minimum particle size and maximum EE % (Figure S3, Supporting Information).

Based on the high desirability, one formula was selected and prepared as a checkpoint to validate the developed models. Scheme 1 and Table S7, Supporting Information, illustrate its composition and the corresponding predicted and experimental particle sizes and EE % values respectively. The percentage error was <4 % thus the developed model was judged suitable for studying and predicting CPPs for the preparation of LPH with the desired QTPP. This formula was used for the incorporation of siRNA using either preparation methods, in situ (I pre-fix) or post-incorporation of siRNA (P pre-fix), with or without Eto. Table 1 shows that all P-LPH formulations had a significantly higher particle size than their respective I-LPH type (P < 0.05). All the prepared LPH had PDI < 0.2 confirming the monodispersity of the system. All the proposed formulae had a positive surface charge which significantly diminished upon siRNA incorporation (P < 0.05). Eto EE % ranged from 74.67 ± 5.35 to 81.59 ± 2.55 % which was not affected by the presence of siRNA. The LE % of Eto and siRNA ranged between 6–8 % and 0.1–0.3 nmole siRNA/mg LPH, respectively. The obtained LE results are in accordance with previously reported studies. The presence of siNEG had insignificant effect on Eto LE%. On the other hand, Eto significantly reduced siNEG LE % (P < 0.05). I-LPH with or without Eto had significantly higher LE than P-LPH (P < 0.05). The LPH retained their starting size, surface charge, drug, or siRNA EE % (Table S8, Supporting Information). Transmission electron microscope (TEM) images of I-LPH with siNEG revealed the presence of spherical-shaped, non-aggregated nanoparticles in the size range of 100–110 nm, consistent with the DLS measurements (Figure 2A). The core-shell structure could be visualized in the photomicrographs; the white core and dark zone represent the polymeric matrix and the lipid coat, respectively, confirming the core-shell structure of LPH.

3.3. LPH Exhibits Controlled Drug Release Characteristics and Reduces Blood Hemolysis of Eto

The optimized LPH formulation demonstrated a biphasic release profile which began to plateau from 12–24h. The release rate was approximately 6.3 % h⁻¹ in the first 8 h followed by a more controlled rate of approximately 2.2 % h⁻¹ in the second phase (from 8–24 h). A total of 20 %, 50 %, and 80 % releas was measured at 2, 8, and 24 h, respectively, in the presence of 50 % FBS at pH 7.4 compared to a 100 % release achieved within 3 h in the free form of the drug (Figure 2B).

The hemolytic activity of LPH was compared to that of the free drug (Figure 2C). Eto solution showed a dose-dependent hemolytic activity with hemoglobin release ranging from 12.6 to 62 %, in agreement with previous reports. On the contrary, less than 5 % of hemoglobin release was noted for LPH at equivalent Eto concentrations at a maximum of 100 µg mL⁻¹, which is within the acceptable range of the new consensus ASTM E2524-08-Standard test method for analysis of hemolytic properties of nanoparticles.
Figure 1. Response 3D plot for the significant parameters interaction on LPH PS (Y1) and EE% (Y2). A) Effect of PLGA and Eto concentration interaction on PS. B) Effect of lipid and Eto concentration interaction on PS. C) The effect of PLGA and lipid concentration interaction on EE%. PLGA–Eto and lipid–Eto interactions increased and decreased the PS, respectively. C) PLGA–lipid interaction had a negative influence on EE%.

3.4. LPH Formulation Shows Increased Anti-Cancer Activity Compared to Free Drug and can be Used to Deliver siRNA

A dose-dependent reduction in B16F10 cell viability was observed after incubation with Eto solution or LPH$_{\text{Eto}}$ at a concentration range of 0.01–100 µM after 48 h incubation (Figure 2D). The $IC_{50}$ for Eto solution or LPH$_{\text{Eto}}$ was 0.6611 and 0.1723 µM, respectively. In addition, the calculated $IC_{50}$ of I-LPH$_{\text{siNEG,Eto}}$ and P-LPH$_{\text{siNEG,Eto}}$ was 0.1791 and 0.1893 µM respectively (Figure S4A, Supporting Information). Drug-free LPH with or without siNEG exhibited > 80 % cell viability up to 10 µM equivalent drug concentration (Figure S4B, Supporting Information). A significant reduction in cell viability at higher concentrations could be attributed to the presence of stearyl amine. Altogether the results cooperated that the enhanced toxicity of Eto in an LPH form is not due to an additive toxicity of the nanocarrier.

The ability of I-LPH and P-LPH to improve the cellular internalization of fluorescently labeled siRNA was assessed in B16F10 cells at two concentrations (30 and 90 nM) and two incubation periods (4 and 24 h) with/without Eto (0.1 µM). The extent of cellular uptake was expressed as MFI. Figure 3A shows a representative histogram of Atto655-siRNA cellular uptake. Both I-LPH and P-LPH ($p > 0.05$) improved siRNA uptake in a time-dependent fashion (Figure 3B). A significant reduction in CD47 expression by 50–60 % was observed independent of LPH types and doses (Figure 3C–D). No gene silencing was
Table 1. Characterization of selected LPH formulation.

| Formulation               | siRNA | Eto | Size [d.nm]a),f) | PDIa),f) | Charge [mV]b),f) | siRNA EE [%]c),f) | Eto EE [%]d),f) | siRNA LE [nmole mg⁻¹]e),f) | Eto LE [%]e),f) |
|--------------------------|-------|-----|------------------|---------|-----------------|-----------------|----------------|----------------------------|----------------|
| LPHEto                   | –     | +   | 109.66 ± 5.17    | 0.12 ± 0.01 | 27.25 ± 2.34    | –               | 81.59 ± 2.55    | –                          | 8.12 ± 0.13    |
| I-LPHsiNEGg),h)          | +     | –   | 118.39 ± 4.98    | 0.15 ± 0.01 | 15.69 ± 2.11    | 66.58 ± 3.98    | –              | 0.272 ± 0.016              | –              |
| I-LPHsiNEG-Eto g),h)    | +     | –   | 115.9 ± 4.11     | 0.18 ± 0.02 | 17.47 ± 2.4     | 63.54 ± 4.36    | 74.67 ± 5.35    | 0.184 ± 0.012              | 7.43 ± 0.32    |
| P-LPHsiNEGg),i)          | +     | –   | 138.91 ± 3.92    | 0.17 ± 0.01 | 10.15 ± 2.24    | 32.58 ± 3.14    | –              | 0.14 ± 0.015               | –              |
| P-LPHsiNEG-Eto g),i)     | +     | –   | 142.1 ± 7.24     | 0.20 ± 0.03 | 8.92 ± 1.02     | 31.11 ± 5.87    | 81.24 ± 3.95    | 0.1 ± 0.01               | 7.81 ± 0.14    |

a) Measured by dynamic light scattering  
b) Surface charge measured by electrophoresis  
c) Calculated as percentage of initial siRNA added, determined by gel red method  
d) Calculated as percentage of initial etoposide added, determined by HPLC  
e) Calculated as percentage of entrapped etoposide or siRNA weight to the LPH weight  
f) Expressed as mean ± SD (n = 3)  
g) LPH composed of PLGA (5 mg mL⁻¹), lecithin:tristearin (1:1) (1 mg mL⁻¹), DSPE-PEG 2000 (1 mg mL⁻¹), Tween 80 (10 mg mL⁻¹), stearyl amine (10 mg mL⁻¹) with or without siRNA (0.133 mg mL⁻¹), and Eto (3 mg mL⁻¹).  
h) I stands for in situ incorporation of siRNA  
i) P stands for post-incorporation of siRNA.

Figure 2. Morphological and in vitro studies. Morphological characterization of the optimized LPHEto by Transmission Electron Micrography (A). LPHEto appeared as core-shell nanostructure with particle size in consistency with DLS technique. In vitro release profile of Eto from the optimized LPH in PBS (pH 7.4) containing 1% Tween 80 in the presence of FBS (50% v/v) at 37 °C (B). Drug release from LPH is measured by dialyzing LPHEto in the presence of 50% FBS against PBS (pH 7.4). Drug concentration in the dialysate is assessed by UV spectroscopy at λmax 285 nm. Datapoint represents mean and SD (n = 3). The in vitro hemolysis assay of LPHEto (C). BALB/c mice RBCs are incubated with different Eto concentrations either solution or encapsulated in LPH (5–100 µg mL⁻¹) for 2 h at 37 °C. Positive and negative controls are 0.5 w/v% Triton X-100 and PBS (pH 7.4), respectively. Samples are centrifuged at 4000 rpm for 5 min at 4 °C and the absorbance of the released hemoglobin is determined at 545 nm. Results are expressed as mean ± SD (n = 3). All the tested LPHEto had acceptable hemolysis range (<5%). The encapsulation of Eto into LPH significantly reduced the drug-associated hemolysis at tested concentrations (*p < 0.05). Cytotoxicity of Eto on B16F10 cells by MTT assay (D). Cells are incubated with either Eto or LPHEto for 48 h at increasing drug concentrations (0.01–100 µM). Cell viability is determined by MTT assay and data is presented as viable cells as a percentage of non-treated cells (n = 5). Statistical analysis is done by student’s t-test and *p < 0.05 is considered significant.

obtained with LPHsiNeg (Figure S5, Supporting Information) suggesting that silencing is specific and is not a toxicity artefact. Additionally, Eto had an insignificant effect on siRNA internalization and siCD47 silencing (p > 0.05) (Figure S6, Supporting Information).

3.5. LPH are Stable in the Presence of Serum

Changes in size, PDI, and zeta potential of LPH were assessed following incubation with 50% serum for 4, 24, and 48 h (Figure 4A–C). In contrast to P-LPH, I-LPH maintained the size, PDI,
Figure 3. The encapsulation of siRNA into LPH improves cellular uptake and consequently gene silencing efficiency. Intracellular delivery of LPH_{siAtto655} at concentrations 30 and 90 nM after 4 and 24 h is assessed using flow cytometry. Representative flow cytometry histograms obtained at the 24 h time point after incubation with 30 nM I-LPH_{siAtto655} (blue) and P-LPH_{siAtto655} (black) in comparison to isotype stained control cells (red) are shown in (A). Cellular uptake is quantified by MFI using flow cytometry and FL-4 detector. Quantitative uptake of siRNA, expressed as MFI, is shown in (B). siRNA uptake significantly improved after encapsulation in LPH (*p < 0.05). The gene silencing efficiency of LPH is assessed on B16F10 cells at three different concentrations (0, 10, 30, and 90 nM) at 48 h. Representative flow cytometry histograms obtained after incubating B16 cells with 30 nM I-LPH_{siCD47} (blue), P-LPH_{siCD47} (black), or untreated control (red) are shown in (C). The knock-down efficiency of CD47, relative to untreated control calculated as reduction in MFI relative to control, is shown in (D). Statistical analysis is carried out using student’s t-test *p < 0.05. Data points represent mean and SD (n = 3).

and zeta potential up to 24 h incubation. A significant increase in size and PDI and a reduction in the zeta potential of both types were observed after a 48-h incubation (p < 0.05). The ability of LPH to protect the entrapped siRNA from serum and RNAse degradation was tested (Figure 4D). Expectedly, naked siRNA suffered from extensive degradation. I-LPH offered better siRNA protection from FBS and RNAse than P-LPH, evident by the comparable band intensities to untreated siRNA. Due to the overall better stability and higher siRNA EE % of I-LPH compared to P-LPH, the former was selected for further in vivo evaluation.

3.6. DiR-LPH Accumulates in MPS Organs of Mice after Intravenous Injection

The biodistribution profile of DiR-labeled I-LPH_{siNEG} was studied over 24 h after a single intravenous injection. Figure 5A shows the whole-body images of mice taken at 0, 1, 4, and 24 h post-injection. High accumulation of LPH in liver was captured up to 24 h, indicating the retention of LPH in liver. Uptake in spleen was also shown by whole-body imaging. No signs of clearance were observed at 24 h, the latest studied time point in this study. Ex vivo images of major organs confirmed the level of accumulation of LPH in the major MPS organs in the order of liver, spleen, and lung (Figure 5B,C). This imaging study suggested the candidacy of LPH to deliver Eto/siRNA to metastatic cancer to lung or the liver.

3.7. Dual siCD47 and Eto Therapy Resulted in Reduced Tumor Nodules in the Lungs Compared to Monotherapy

The therapeutic efficacy of I-LPH_{siCD47,Eto} was evaluated in the experimental lung metastatic B16F10 tumor model after two
intravenous injections. Monotherapies with siCD47 and Eto in LPH were tested for comparison. Mice in both PBS and I-LPHsiNEG groups showed significant weight loss (p < 0.05) compared to monotherapy (I-LPHsiNEG-Eto and I-LPHsiCD47) and dual therapy group (I-LPHsiCD47-Eto) suggesting increased disease severity (Figure 6A). Post-mortem macroscopic analysis (day 13) demonstrated apparent reduction in melanoma nodules in the following order: I-LPHsiCD47-Eto > I-LPHsiNEG-Eto > I-LPHsiCD47 > I-LPHsiNEG and PBS groups (Figure 6B and Figure S7, Supporting Information). Significantly lower lung weights were observed in both Eto treated groups (I-LPHsiNEG-Eto and I-LPHsiCD47-Eto) (p < 0.05) in comparison with the other groups (Figure 6C). Lung weight values for I-LPHsiNEG-Eto, I-LPHsiNEG-Eto, I-LPHsiCD47, I-LPHsiNEG, and PBS were 0.237 ± 0.087, 0.326 ± 0.099, 0.65 ± 0.14, 1.024 ± 0.063, and 0.832 ± 0.143 g, respectively. In addition to lung weight measurements, we employed another method to quantify tumor burden by counting the tumor nodules in the lungs (Figure 6D). Nodule counts confirmed that combinatorial therapy is significantly more effective than drug monotherapy in reducing tumor burden, which agreed with macroscopic analysis (Figure 6B) and histopathological analysis (Figure S8, Supporting Information).

3.8. Dual siCD47 and Eto Therapy Resulted in Altered Lung Leukocyte Population Density in the Lungs

To establish if there was a correlation between the anti-tumor activity observed and immune responses, we performed immunological assessments in tumor-bearing lung tissues. It was observed that all mice receiving Eto containing I-LPH had significantly elevated numbers of CD8+ cells when compared to mice receiving the I-LPHsiNEG (Figure 7A). This increase was most pronounced in the case of mice receiving I-LPHsiCD47-Eto, however, significance between I-LPHsiCD47-Eto and I-LPHsiNEG-Eto could not be obtained, possibly due to variation between mice. This general trend was conserved in the CD4+ population however, in contrast, levels in the I-LPHsiCD47-Eto group were significantly higher.
Figure 5. Fluorescently-labeled LPH shows preferential accumulation in the highly perfused MPS organs following intravenous injection. A) Representative whole-body images up to 24 h post intravenous injection. B) Ex vivo imaging of organs excised at 24 h post-injection. C) Quantitative analysis of organ biodistribution. Mice are injected intravenously with 200 µL of DiR labeled (1 w/w% of total lipid) I-LPHsiNeg in PBS. Animals are imaged immediately after injection (0 h) and up to 24 h using IVIS Lumina III in vivo imaging system (excitation at 740 nm and emission at 790 nm). Data are expressed as mean ± standard deviation (n = 3).

than the I-LPHsiNEG-Eto group (Figure 7B) Due to the proposed mechanism of siCD47, we were particularly interested in the lung myeloid cells, especially antigen-presenting cells. To address this, we utilized a relatively simple staining strategy to identify three putative cell populations, these being: IM, AM, and DC (Figure 7C, 7D, and 7F respectively). A representative flow cytometry dot plot is shown in Figure 7E. Using this analysis, it was observed that there were the highest number of IMs present in mice receiving I-LPHsiCD47-Eto, this being the only group significantly different from I-LPHsiNEG (Figure 7C). Interestingly, there were elevated levels of AMs in mice treated with I-LPHsiCD47 and I-LPHsiNEG-Eto. These differences were significant compared to I-LPHsiNEG but not to I-LPHsiNEG-Eto. I-LPHsiCD47-Eto, in turn, was not significantly different from I-LPHsiNEG (Figure 7D). The DC numbers followed no discernible trend and there were no significant differences observed between any of the treatment groups (Figure 7F).

3.9. Histological Analysis

Microscopic examination on lung tissues further confirmed the therapeutic effectiveness of LPH containing Eto, siCD47, or the combination (Figure S8, Supporting Information). Large areas of tumor invasion were observed in lung tissues of the PBS and I-LPHsiNEG groups, whilst more areas of alveoli and bronchioles were present in the lungs of mice treated with I-LPHsiNeg-Eto or I-LPHsiCD47. In the I-LPHsiCD47-Eto group, most of the lung tissues showed normal histological features and only a few small tumor nodules were observed. Histological examination was also carried out on major organs including heart, liver, spleen, and kidney. As shown in Figure S9, Supporting Information, no obvious histologic changes were observed in these organs in the treatment groups compared to the PBS group.

4. Discussion

Many tumors suppress the host immune system allowing for immune escape and leading to tumor growth and progression. Targeting CD47 using monoclonal antibodies or siRNA has been suggested previously in preclinical and clinical studies. Indeed, there is data to suggest this is a viable approach both preclinically and clinically. However, one future drawback of the use of CD47-based therapeutics may be that it is relatively widely distributed, including on RBCs. Administration of anti CD47 monoclonal antibodies intravenously may result in rapid dilution and toxicity through off-target effects.
Figure 6. The combination of siCD47 and Eto in LPH dual therapy shows an improved antitumor activity over monotherapy after two intravenous injections. Mouse melanoma lung metastasis tumor model is developed by injecting C57BL/6 with B16F10 cells (5 * 10^5 cells suspended into 100 µl PBS) intravenously. On days 7 and 12, mice are i.v. injected with either PBS, I-LPHsiNEG, I-LPHsiNEG-Eto, I-LPHsiCD47, or I-LPHsiCD47-Eto (n = 7 per group). Changes in mouse weight relative to starting weight throughout the time course are reported in (A). Representative lung images of different groups are shown in (B). At the end of the study on day 23, mice are culled and individual organ weight is recorded (C). Lung tumor nodule counts of I-LPHsiNEG-Eto and LPHsiCD47-Eto are shown in (D). In (A–C), statistical analysis is performed using one-way ANOVA followed by Tukey post-test *p < 0.05. Bars represent mean ± SEM. In (D), statistical analysis is carried out using student’s t-test followed by Mann Whitney post-test *p < 0.05.

siCD47 formulated in a particulate system could possibly negate or minimize these issues by blocking the CD47 axis at a more local level. As CD47 is proposed to exert its effects through the phagocytosis activity of macrophage, it may be speculated that CD47 therapy is most suited to tumors or organs with high levels of macrophages, either tumor-associated or tissue-resident. To this end, we designed an LPH for delivery to the lung. As there is an abundance of macrophage present in the lung, targeting them, via the CD47 axis, rather than T cells via the PD-1, CTLA4 axis may have a logical foundation. To compliment lung delivery of siCD47 etoposide was selected. Eto is currently approved in clinic as part of the lung cancer chemotherapeutic regimen and is effective against the B16F10 cell line used in the pseudo metastatic mouse model and therefore a viable candidate.

Generally, the physicochemical properties such as size of nanocarriers dictate their intracellular internalization and their subsequent therapeutic applications. In addition, higher drug EE % is an important factor in the clinical translation of different nanoparticles. In an attempt to produce LPH with an optimal size <150 nm and maximum Eto EE %, different formulation parameters and processes were employed and the effects on size and EE % was studied. The concomitant increase in particle size with increasing polymer, lipid, or Eto concentrations could be attributed to the increase in viscosity which inversely affects the evaporation rate of the organic solvent and opposes the effect of stirring speed to breakdown the particles into smaller ones. On the contrary, increasing the stirring speed increases the mechanical and hydraulic shear which decreases the particle size. PLGA, lecithin:tristearin, and Eto concentration, as well as, stirring speed showed a positive effect on EE %. The pronounced effect of increasing PLGA concentration on augmenting EE % was attributed to the availability of larger core material to
I-LPHsiCD47-Eto significantly alters lung leukocyte population density. At a predetermined terminal end point mice are culled and lungs removed. A single lung cell suspension is obtained through physical dissociation of lung tissue. Cells extracted are stained with Anti-CD4-FITC and Anti-CD8-PE or anti-F480-FITC and anti-CD11c-APC a fixed volume of precision count beads is also added to each sample prior to acquisition on a FACs Calibur flow cytometer. A) Total CD8+ and B) CD4+ cell counts obtained are normalized to lung weight. The myeloid subpopulations are distinguished into interstitial macrophages (IM F480+, CD11c−) alveolar macrophages (AM F480+, CD11c+) or Dendritic cells (DC, F480−, CD11c+) based on F480/CD11c staining (E). C) Total IM, D) AM, and F) DC are counted and normalized as above. Data is plotted as mean ± SEM and is analyzed with Graphpad Prism 8 using students t-test with Mann Whitney post-test ns, non-significant *p < 0.05, **p < 0.005.
pro-inflammatory signals unlike some other chemotherapeutic agents. Significantly improved antitumor efficacy obtained after the systemic administration of I-LPH siCD47-Eto is likely to be attributed to the effective delivery of LPH to the cancerous nodules. Despite delivery to both healthy lung tissues and cancerous nodules, it is known that chemotherapeutic drugs and RNAi have more pronounced effects in rapidly dividing cells (tumor) compared to non-dividing cells (healthy lung tissues) which could have led to the selective pharmacological toxicity observed. This hypothesis was supported by the normal histological appearances in lung and spleen in the mice which received I-LPH siNEG or drug-loaded LPH treatments.

High amounts of LPH were detected in the lung. The significantly improved antitumor efficacy obtained after the systemic administration of I-LPH siCD47-Eto could be attributed to the effective delivery of LPH to the lung, leading to the release of the cytotoxic effect of Eto and the successful transfection of siCD47. This observation demonstrates hypothetically synergistic or additive effects of combining both Eto and siCD47. We have demonstrated that macrophage numbers (innate activation) and T cell numbers increased (innate/adaptive activation) suggesting that treatment with siCD47 LPH activates the immune responses. It is widely reported that macrophage can phagocytose cancer cells and this effect is improved with CD47 blockade. Indeed, this has been previously shown in the B16F10 lung metastatic model using siCD47. As shown in the data the numbers of T cells remain relatively low, so it was not possible to extract enough numbers of T cells from lungs to perform ex vivo activation studies. We however speculate that the increase in T cell numbers is a direct response to macrophage activation (release of cytokines, establishment of chemokine gradients, etc.).

Eto itself has a complex interplay with the immune system depending on the model and the criteria used for assessment. It has been shown that splenocytes adoptively transferred from mice which resolved tumor following Eto treatment can protect naive mice from tumor challenge. This strongly suggests that antitumor immunity is established successfully. The authors speculate that this may be due to the drug’s effects on the tumor cells, causing the release of antigen containing apoptotic bodies to antigen-presenting cells, including macrophages. Interestingly, it has also been reported that Eto selectively ablates activated T cells, in a hemophagocytic lymphohistiocytosis model leading to immune suppression. In the same study, it was noted that following in vivo Eto treatment macrophages maintain the ability to present antigen and mature. The detailed role of Eto in cancer treatment and immune system modulation may seem elusive but studies have suggested that cancerous cells killed through Eto treatment induce macrophages to produce TNFα and other pro-inflammatory signals unlike some other chemotherapeutic agents. Although, Eto is not considered to be a classic “immunogenic cell death” inducer as it does not induce translocation of calreticulin, it can however cause the release of inflammatory mediators ATP and HMGB1. Conversely, macrophages have been shown to either enhance or reduce Eto-induced apoptosis depending on the phenotype (i.e., M1 or M2 macrophages). In our study, although the activation state of T cells was not assessed, we observed elevated numbers of T cells in the lungs with I-LPH siCD47-Eto treatment. It is possible that, as the lung has a distinctly high macrophage population, the proinflammatory activity of the macrophages on the T cells predominates. It could be speculated that following the combinatory treatment, Eto provides a source of apoptotic tumor cell fragments which can provoke an inflammatory response. These fragments can be taken up to a greater extent by antigen-presenting cells including macrophages, as a result of concomitant CD47 axis blockade.

In this study, we were able to demonstrate that the co-delivery of siCD74 and Eto in a single system could both reduce tumor growth and induce both innate and adaptive immune responses. It will be interesting to test in future studies whether using a subtherapeutic Eto dose combined with siCD47 for example will show a similar therapeutic effect to the therapeutic Eto treatment as a monotherapy. Additionally, this study showed that the co-delivery of a chemotherapeutic drug such as Eto, as a model drug, and a siRNA in the LPH system developed is therapeutically useful so future studies will focus on replacing Eto with other chemotherapeutic drugs with known immunomodulatory activities such as doxorubicin at subtherapeutic and therapeutic doses.

5. Conclusion

The current study confirmed the feasibility of the proposed LPH system as a platform for the simultaneous delivery of Eto and siCD47 for the treatment of lung’s metastasized tumor nodules. The combinatory treatment of Eto with siCD47 delivered by the preferred LPH formulation offered an improved therapeutic outcome compared to drug monotherapy in the lung metastatic model of melanoma B16-F10 cells. The anti-tumor responses correlated well with immune cell activation pattern in the tumor-bearing lungs.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

H.A. is a recipient of Newton Musharafa Fellowship. This project has received funding from the Brain Tumor Charity (GN-000398), Institutional Link- British Council (IL4337313), and Wellcome Trust (WT103913). After initial online publication, the name of the university and city in affiliation 1 was changed to University of Sadat City and Sadat City, respectively, on April 7, 2021.

Conflict of Interest

The authors declare no conflict of interest.
Author Contributions

H.M.A.B. designed the work, performed the experiments, and wrote the manuscript. A.A.W. designed and performed the immunological studies and assisted in manuscript writing. J.W. performed anti-tumor studies. K.T.A.J. supervised the work and wrote the manuscript. All authors assisted in data analysis and manuscript proof reading.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created in data analysis and manuscript proofreading. H.M.A.B. designed the work, performed the experiments, and wrote the manuscript. J.W. performed anti-tumor studies. H.M.A.B. designed the work, performed the experiments, and wrote the manuscript. J.W. performed anti-tumor studies.

Keywords

CD47, chemotherapy, immunotherapy, macrophages

Received: October 22, 2020
Revised: February 4, 2021
Published online: March 4, 2021

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