Published in final edited form as:

J Control Release. 2015 September 10; 213: 18–26. doi:10.1016/j.jconrel.2015.06.014.

Evaluation of self-emulsified DIM-14 in dogs for oral bioavailability and in Nu/nu mice bearing stem cell lung tumor models for anticancer activity

Apurva R. Patel^{a,1}, Ravi Doddapaneni^{a,1}, Terrick Andey^a, Heather Wilson^b, Stephen Safe^{b,c}, and Mandip Singh^{a,*}

^aCollege of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307, USA

^bInstitutes of Biosciences and Technology, Houston, TX 77843, USA

^cTexas A & M Health Sciences Center, Houston, TX 77030, USA

Abstract

3, 3-Diindolylmethane-14 (DIM-14), a novel lipophilic derivative of DIM, has demonstrated anticancer activity in different types of cancers. However, poor solubility and low oral bioavailability of DIM-14 limit its translational benefits in vivo. This study was carried out to improve the oral bioavailability of DIM-14 via self-emulsifying drug (SED) delivery system in dogs and to evaluate pharmacodynamic characteristics of SED against H1650 stem cell tumor models. DIM-14 was incorporated into an oil, surfactant, and co-surfactant mixture using labrafil and tween-80 to obtain SED. SED were characterized by droplet size, polydispersity index (PDI), zeta potential, entrapment efficiency (EE), in vitro permeability and drug release (investigated with Caco-2 monolayers and dissolution apparatus respectively). Pharmacokinetic parameters in dogs were evaluated and analyzed using Winonlin. Anti-tumor activity was carried out in H1650 lung tumor model. Particle size of SED was between 230 and 246 nm and surface charge was negative and ranged from 26.50 to 28.69 mV. Entrapment efficiency of SED was 85%. Pharmacokinetic evaluation in dogs showed increased C_{max} (39.18 ± 7.34 vs 21.68 ± 6.3 $\mu\text{g}\cdot\text{dL}^{-1}$), higher AUC_{0–t} ($34,481.34 \pm 1125.46$ vs $14,159.53 \pm 702.20$ $\mu\text{g}\cdot\text{min}\cdot\text{dL}^{-1}$) and improved absorption with 3 times more bioavailability of SED compared to DIM-14 solution. SED showed ~30–59% tumor volume/weight reduction in H1650 tumor model compared to DIM-P solution. Our studies demonstrate the potential application of self-emulsifying drug delivery system (SEDDS), that enhances oral absorption of DIM-14 and increased anti-tumor activity against lung tumor models.

Keywords

Self-emulsifying drug delivery; DIM-14; H1650 stem cell; Anti-cancer activity

*Corresponding author. mandip.sachdeva@gmail.com (M. Singh).

¹Equal Contribution.

1. Introduction

Lung cancer is one of the leading causes of cancer-related deaths worldwide and most of lung cancer cases (85%) belong to the non-small-cell lung cancer (NSCLC) type [1,40]. Despite significant advances in our knowledge about lung cancer, effective therapies to combat lung cancer are still in infancy. Although the use of targeted therapies for lung cancer has been reported, patients are yet to benefit from them. Recent reports have demonstrated that tumors contain a small subpopulation of cells, termed cancer stem cells (CSCs), which exhibit protumorigenic characteristics including high proliferation capacity, multipotent differentiation, drug resistance and long life span relative to other cells. The survival rate of lung cancer patients is very low due to the acquisition of resistance to systemic treatment regimens, which in turn may be due to the presence of cancer stem cells within the primary tumor. One new therapeutic avenue that is currently being tested in preclinical experiments for a variety of solid tumors is targeting CSCs which are thought to be responsible for tumor initiation and recurrence after chemotherapy [2]. A major challenge for treating this type of cancer due to CSCs is that, these are naturally resistant to the cytotoxic effect of radio-chemotherapy because of slow cell cycling, lower proliferation, and increased expression of DNA repair and anti-apoptosis genes [3,4]. This has led to hypothesis that targeting CSC populations is essential for an effective treatment, but this issue has not yet been experimentally tested.

New therapies using novel mechanisms to induce tumor cell death are required and natural products due to their potential anticancer compounds play a crucial role in envisaging new therapies. 3,3-Diindolylmethane (DIM) is a natural product derived from indole-3-carbinol (I3C) which is present in cruciferous vegetables such as Brussels sprouts, broccoli and cauliflower. DIM has generated much interest in cancer research because of its low toxicity and cytotoxic effects on cancer cells in vitro and inhibition of tumor growth in vivo [5]. A series of novel synthetic 1,1-bis(3-indolyl)-1-(p-substituted phenyl) methane analogs (C-DIMs) induced expression of cell cycle inhibitors such as p21 and p27, downregulated-cyclin proteins including cyclin D1, decreased expression of survival and anti-apoptotic proteins including survivin, bcl-2, bax and induced poly (ADP-Ribose) polymerase (PARP) cleavage, mitochondrial cytochrome c release and procaspase cleavage [6–8,41–43]. C-DIMs, are also potent anticancer agents and their activities are structure-dependent [9–11]. Considering that CSCs may be key mediators of metastasis, we hypothesized that targeting CSCs with DIM-14 would increase the anti-tumor potential.

Approximately 40% of new active pharmaceutical agents have poor aqueous solubility and their oral delivery is often associated with low bioavailability. Various formulation strategies which can be explored to develop oral delivery of poorly soluble drugs include utilization of surfactants, lipids, permeation enhancers, micronization, salt formation, β -cyclodextrins complexes, nanocarriers, solid dispersions, spray drying and self-emulsifying drug delivery systems (SED DS). SED DS are mixtures of oils, surfactants, solvents and co-solvents that create fine oil-in-water emulsions upon mild stirring in aqueous media. SED DS offer several advantages which includes their spontaneous formation, thermodynamic stability, improved bioavailability [12] and ease of manufacturing. Following their oral administration, these systems rapidly disperse in gastrointestinal fluids to yield micro or nano emulsions and are

rapidly absorbed through the lymphatic pathway [13]. Various bioavailability studies have reported that lipophilic compounds, such as, simvastatin and halofantrine, are more efficiently taken up from gastrointestinal tract when administered in SEDDS [14–16].

DIM-14 has showed anticancer activity in lung tumor cell lines in vitro and in vivo. Further, even though DIM-14 is a desirable anticancer agent, its activity as a single agent is limited due to its poor oral bioavailability. To address this hypothesis, we investigated the anti-tumor efficacy of the DIM-14 and SED formulations in the lung tumor models. The objective of this study was designed to evaluate SED (self-emulsifying DIM-14) in dogs for pharmacokinetic analysis and assess their anticancer activity in H1650 mice lung cancer model.

2. Materials and methods

2.1. Chemicals

DIM-14 was prepared as described [5]. All culture media contained antibiotic-antimycotic solution PSN mix by Gibco-Invitrogrn (Grand Island, NY, USA). Laminin, accutase, poly-D-lysine, epidermal growth factor, and fibroblast growth factor were purchased from Sigma Aldrich (St. Louis, MO); labrafil M1944 and labrasol from Gattefosse (Paramus, NJ). Kolliphor™ TPGS (pharma grade) was purchased from BASF fine chemicals. All other chemicals used were of analytical grade.

2.2. Source of cells

H1650 parent cells and side population cells (SP)/cancer stem cells (CSCs) were generously donated by Dr. Srikumar Chellappan of the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL) and Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). H1650 CSCs were cultured in DMEM:F12 base medium enriched with fibroblast and epidermal growth factors (10 µg/ml) and 2% PSN cocktail. Cells were maintained at 37 °C under an atmosphere of 95% air and 5% CO₂. H1650 CSCs were cultured on a basement membrane-coated matrix consisting of immobilized laminin on a poly-D-lysine layer.

2.3. Animals

Nu/nu mice (20–30 g) were used for the current studies. The protocols were approved by the Institutional Animal Care and Use Committee, Florida A & M University. Animals were maintained on standard animal diet, in a controlled room (22 ± 1 °C @ 35–50% RH) for a week prior to experiments. Eighteen month old female intact Labrador retriever dogs were acquired from an internal canine breeding colony maintained at Texas A&M, College of Veterinary Medicine. All canine protocols were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Dogs were housed in large runs and allowed outdoor play time and toys for enrichment. They were fed standard dog chow and water ad libitum for the duration of the study.

2.4. H1650 SP cell viability

H1650 SP cells were seeded in a 96-well format (1×10^4 per well) and incubated for 16–18 h. Treatment was carried out for 72 h with different concentrations of DIM-14. The cells were washed with PBS 2× and fixed in 0.1 ml glutaraldehyde solution (0.025% w/v) and incubated at 37 °C for 30 min. Glutaraldehyde was aspirated and 0.1 ml crystal violet solution (0.01% w/v) added and incubated at room temperature for 15 min. Crystal violet solution was aspirated followed by 2 washes with PBS; the plates were air-dried and disodium hydrogen phosphate solution added to dissolve the crystal violet. The absorbance of crystal violet was read at 540 nm and the cell viability calculated as a percentage of the control. Determinations of cell viability were made at least 3× and the data presented as mean $\pm$ SD.

2.5. Preparation of liquid self-emulsifying system and SB DIM-P

The liquid self-emulsified (SE) formulations were prepared as previously reported [17]. Initially, solubility of DIM-14 was determined in different oils and surfactants to select the suitable oil to be used for the formulation. The mixture of oils, surfactants and co-surfactants was optimized by DOE analysis. Briefly, DIM-14 was dissolved into the mixture of oil, surfactant, and co-surfactant with help of heating at 50 °C in a water bath and vortexed until a clear solution was obtained. Then it was kept at room temperature for 24 h and examined for stability parameters such as turbidity/phase separation. Optimized concentrations of Enova oil, TPGS, surfactant (tween 80) and co-surfactant (labarafil) were determined from QbD design. The optimized composition is showed in Table 1.

2.6. Characterization of self-emulsified spray dried formulations

2.6.1. Emulsification time—The emulsification time (the time for a pre-concentrate to form a homogeneous mixture upon dilution) was monitored by visually observing the disappearance of SED and the final appearance of the emulsion in triplicate. A dissolution apparatus (Dissolution Tester USP, Type-II) was used at a paddle speed of 50 rpm with 200 ml buffer medium at 37 °C. The SED (1 g) was added slowly to the medium and time required for the disappearance of SED was recorded.

2.6.2. Droplet size and zeta potential determination—Five milligrams of the SED formulation was diluted with water to 10 ml in a flask and gently mixed. The droplet size distribution and zeta potential of the resultant emulsion was determined by laser diffraction analysis using a particle size analyzer (Nicomp Zetasizer, US). The sizing of the emulsion droplet was determined in a small volume module. Particle size was calculated from the volume size distribution. All studies were replicated ($n = 3$) and statistically analyzed ($P < 0.05$).

2.6.3. Dilution studies/robustness on dilution—A dilution study was done to access the effect of dilution on the SED pre-concentrate. In this study, the optimized formulation was subjected to various dilutions (i.e., 1:50, 1:100 and 1:500) with various diluents (i.e., water, 0.1 N HCl, phosphate buffer pH 7.5) and the droplet size was recorded.

2.6.4. Determination of drug content—DIM-14 from an SED formulation was extracted in acetonitrile using the sonication technique. The extract was analyzed for DIM-14 content by HPLC method already established in our laboratory [18]. Entrapment efficiency was estimated using the formula:

$$\text{Entrapment efficiency, EE(\%)} = [(W_n - W_r) / W_n] \times 100$$

where, W_n = weight of DIM-14 in SED, and W_r = weight of DIM-14 in filtrate.

2.6.5. In vitro release studies—DIM-14 release from SED formulations were performed using USP XXIII, dissolution apparatus II with 200 ml of distilled water as dissolution medium at $37 \pm 5^\circ\text{C}$ with paddle speed at 50 rpm. SED formulation equivalent to 2 mg of DIM-14 was introduced into the dissolution tester. At predetermined time intervals, an aliquot of 1 ml was collected, filtered, and analyzed for the content of DIM-14 by HPLC. An equivalent volume (1 ml) of fresh dissolution medium was replaced to compensate the loss due to sampling.

2.7. Caco-2 permeability studies of DIM-14

Single cell suspensions (10^5 cells per ml) were seeded in the apical compartment of a Costar® Transwell® permeable support with a polycarbonate membrane (0.4 μm pore size) in a 12-well plate format in a 0.5 ml volume. The cells were maintained for 21 days with media changes done on alternate days for 14 days and daily subsequently. Formation and integrity of Caco-2 monolayer and tight junctions were monitored by the transepithelial electrical resistance (TEER). Permeation studies were done by adding buffer (HBSS-HEPES buffer) to the donor (pH 6.5) and acceptor (pH 7.4) compartments for 5 min at 37°C . An absorptive permeability study was done by adding sample solution to the donor compartment (pH 6.5) and free buffer to the acceptor compartment (pH 7.4). Sampling was carried out from the acceptor compartment at 15, 30, 45, 60, 90, and 120 min under sink condition. Secretory studies were carried out by adding the sample to the acceptor chamber (pH 7.4) and sampling from the donor chamber (pH 6.5). Samples were eluted by HPLC and amount of DIM-14 was obtained by interpolating the peak area from a standard plot of area versus amount of DIM-14. The apparent permeability (P_{app}) of DIM-14 was computed using the formula:

$$P_{app}(\text{cm/s}) = [Q / (C_i * T * A)],$$

where Q = amount of DIM-14 in receiver (μg); C_i = initial concentration of DIM-14 (g/cm^3), T = time (sec), and A = Area of insert (cm^2).

2.8. Bioavailability of DIM-14 and SED

2.8.1. Bioavailability in dogs—Pharmacokinetic profile of DIM-14 in Dogs was determined following IV and oral administration. Animals were randomly distributed into three experimental groups ($n=3$). DIM-14 was formulated as described earlier for intravenous administration and for oral solution; DIM-14 was dissolved in corn oil. The oral

treatment groups were given 3.33 mg/kg of DIM-14 solution and SED equivalent to 3.33 mg/kg of DIM-P was administered orally by syringe. Each dog had a central venous catheter (long saphenous) placed on the day of the study. The third group was given DIM-14 (0.5 mg/kg) intravenously. Dogs were fasted overnight before the start of pharmacokinetic studies. Blood samples were collected from the venous catheters into heparinized tubes. Samples were collected at baseline, and at 15, 30, 60, 120, 180, 240, 360, 480, 600, 720, 1440 min after administration of a single dose of DIM-14 solution and SED. Blood samples were immediately centrifuged and plasma was collected and stored at -80°C until analysis. At the end of the study, major organs were collected for further evaluation.

2.8.2. Data analysis—Pharmacokinetic parameters were determined using non-compartmental techniques with WinNonlin® 5.0 software (Pharsight Corporation, Mountain View, CA, USA). SHAM analysis (i.e., Slope, Height, Area, and Moment) [19] utilized plasma concentration-time data to estimate the area under the curve (AUC), terminal elimination half-life ($t_{1/2}$), and the area under the first moment of the plasma concentration-time curve (AUMC). The AUC was calculated for each animal using the piecewise log trapezoidal areas. The non-compartmental parameters were calculated for each rat before averaging dose groups.

2.9. In vivo anticancer evaluation in lung cancer models

2.9.1. H1650 cancer stem cell xenograft tumor model—H1650 CSCs at 80–90% confluency were harvested using accutase (Sigma-Aldrich, St. Louis, MO) (Bajpai, Lesperance et al. 2008) and washed $2\times$ with PBS. Cells were counted (1×10^6) and suspended as single cells in 50 μl serum-free DMEM:F12 media and diluted in matrigel (3 mg/ml) to a final volume of 300 μl on ice. Cell suspension in matrigel (300 μl) was injected on the right flank of mice using a pre-chilled 24-gauge needle and syringe. Animals were maintained under standard husbandry for xenografts to develop.

2.9.2. H1650 cancer stem cell orthotopic lung tumor model—Mice were placed under isoflurane-induced anesthesia under aseptic conditions. The left lateral chest was doused with iodine and cleaned with an alcohol swab. A small lateral incision ($\sim 5\text{ mm}$) was made to the left chest in plane of the left fore-limb just below the scapula. A cell suspension-filled B-D® 1 ml latex free syringe connected to a 27-gauge Surflo® winged infusion set was used to deliver an inoculum of 1×10^4 cells (in a 0.1 ml volume of serum-free DMEM:F12 base media) through the sixth intercostal space into the left lung. Incisions were closed with surgical skin clips and animals observed for full motor and cognitive recovery. Mice were maintained for 30 days for development of lung tumor verified by dissection of a random mouse for anatomical observation.

2.10. Treatment of animals

Mice were randomly divided into the following groups ($n = 12$) to receive DIM-14 formulations by oral gavage. The control group received vehicle (No DIM-14); the second group received DIM-14 (20 mg/kg) solution every other day; the third group received SED (20 mg/kg). To check for evidence of toxicity, the animals were weighed twice weekly. At the end of study, all animals were sacrificed by exposure to a lethal dose of carbon dioxide.

After dissection and removal of the lungs, the lungs and tumor mass were washed in sterile PBS and weighed. The lung weights and tumor volume were used for assessment of the therapeutic activity of the treatments.

2.11. Western blot analysis

Protein was extracted from tumor nodules collected from control-untreated and treated tumors using RIPA buffer (50 mM Tris-HCL, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxychlorate, and 0.1% sodium dodecyl sulfate) with protease inhibitor. The lysate from normal lung tissues was also prepared in a similar manner as described above. Protein content was measured using BCA Protein Assay Reagent Kit (PIERCE, Rockford, IL). Equal amounts of supernatant protein (50 μ g) from the control and different treatment groups were denatured by boiling for 5 min in sample buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 and probed with antibodies against ER stress markers and β -actin (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Proteins were visualized using enhanced chemiluminescent solution (Pierce, Rockford, IL) and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

2.12. Statistical analysis

Results obtained were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Statistical significance in differences in cell viability and migration were determined by unpaired t-test (***P < 0.0001); and one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (***P < 0.05); tumor weight and lung weight (*P < 0.05) by one-way ANOVA followed by Tukey's post-test (***P < 0.0001; **P < 0.05). Results are presented for at least three determinations and presented as mean $\pm$ SD.

3. Results

3.1. H6150 SP cells are resistant to chemotherapy

The IC₅₀ of DIM-14 in H1650 and H1650 SP cells were 8.70 ± 0.13 nM and 33.75 ± 3.65 nM respectively. The concentration of drug treatment resulting in 50% cell death (IC₅₀) was estimated from cell viability data using linear regression analysis.

3.2. Characterization of SED DIM-14

3.2.1. Droplet size, zeta potential determination and drug content—The formulation composition ratio in Table 1 gave the particle size (240 ± 24.23 nm, mean $\pm$ SD, n = 3) than other SED formulations. The PDI (polydispersity index) was 0.11 ± 0.02 . Also, the coefficient of variance (COV) for the measurement set was less than 5%. The charge of oil droplets in SED was negative due to the presence of free fatty acids and the zeta potential of the formulation was -28.9 ± 0.42 (mean $\pm$ SD, n = 3). We have measured the particle size and zeta potential aging after 24 of dispersion and it was found that there was no statistical differences compare to time zero readings. Drug content of the formulation was found to be $85.34 \pm 0.42\%$ (mean $\pm$ SD, n = 3).

3.2.2. Release studies—At initial 12 h, SED formulation released 99% of DIM-14. Fig. 1 reveals the in vitro drug release profiles of DIM-14 from free drug and solution with ~10% and ~40% of drug release in 24 h. The release profile of optimized formulation was found to release more than 50% of DIM-14 within 4 h from the SED formulation, indicating a highly desirable release profile.

3.2.3. Caco-2 permeability studies of DIM-14—The TEER values of $>400 \Omega \cdot \text{cm}^2$ and mean permeability values of paracellular control Lucifer Yellow of $< 0.15 \times 10^{-6} \text{ cm/s}$ were within normal limits, thus confirming paracellular integrity of monolayers. The average Peff, A–B assay for DIM-14 was approximately $0.26 \pm 0.05 \times 10^{-6} \text{ cm/s}$, and B–A was $0.41 \pm 0.09 \times 10^{-6} \text{ cm/s}$. The TEER values of monolayers were indicative of tight junction integrity, and TEER values did not significantly change throughout the experiment. The A–B apparent permeability (Papp) values using the Caco-2 assay for SED were $9.45 \pm 0.07 \times 10^{-6} \text{ cm/s}$ and $10.95 \pm 0.08 \times 10^{-6} \text{ cm/s}$ respectively for unstirred and stirred water layers respectively.

3.3. Optimization of SED using desirability function

The contour plots and the prediction profiler of interactions between independent variables are shown in Fig. 2. Using the desirability function, all measured responses were combined into a single response. The optimized batch was identified with a desirability value of 0.757 for SED. Individual and overall desirability for all measured responses were evaluated.

3.4. Bioavailability studies of SED

3.4.1. Bioavailability in dogs—The plasma concentration-time profiles of DIM-14 formulations in dogs are shown in Fig. 3. The concentration-time plot of DIM-14 in dog plasma followed a single i.v. bolus injection which was estimated from peak area of HPLC chromatograms of injected plasma samples (Fig. 3A). Non-compartmental pharmacokinetic parameters were measured. Curve fitting into a two-compartment model was done using the WinNonlin software. Following intravenous bolus injection, there was a rapid distribution of drug into peripheral tissues ($K_{12} = 0.39 \text{ l/h}$) and a comparatively slow distribution from the peripheral compartment to the central compartment ($K_{21} = 0.15 \text{ l/h}$), and a slower elimination from the central compartment ($K_{10} = 0.003 \text{ l/h}$). Volume of distribution (V_d) of DIM-14 was 0.321 l/kg and remained unchanged at steady state ($V_{dss} = 0.319 \text{ l/kg}$).

AUC was calculated by trapezoidal methods ($P < 0.05$), and secondary parameters were calculated. Oral delivery of DIM-14 (3.33 mg/kg) showed poor bioavailability ($< 10\%$) and a shorter plasma half-life compared to that of SED. However, the half-life for SED was increased by ~3 h with increase in bioavailability by ~25%. Pharmacokinetic evaluation in dogs showed improved absorption of SED formulations compared to solution; increased C_{max} (39.18 ± 7.34 vs $21.68 \pm 6.3 \mu\text{g dL}^{-1}$) and higher AUC_{0-t} ($34,481.34 \pm 1125.46$ vs $14,159.53 \pm 702.20 \mu\text{g min dL}^{-1}$). The relative oral bioavailability of SED calculated on the basis of AUC_{0-t} was about 3 fold more as compared to solution.

3.5. In vivo anticancer activity of oral DIM-P

3.5.1. H1650 cancer stem cell xenograft tumor model—The results in Fig. 4 showed that lung tumor weights were significantly (*, $P < 0.001$) decreased by 30 and 64% after treatment with DIM-14 and SED respectively compared to vehicle control. In mice treated with the DIM-14 and SED, lung tumor volumes were decreased by 29 and 61% respectively. A non-significant ($P > 0.05$) change in average number of tumor nodules was observed among central, mid and peripheral regions of harvested lungs from each of the treated groups (Fig. 4). DIM-14 and SED treatment showed significant (*, $P < 0.001$) decrease in average number of tumor nodules by 29 and 56% respectively compared to control groups. We did not observe any weight loss or other signs of toxicity in mice treated with DIM-14.

3.5.2. H1650 cancer stem cell orthotopic lung tumor model—Treatment was started ten days after tumor implantation and continued for a total of 30 days. Ten days after inoculation with tumor cells, the average lung weights and tumor volumes were 198 ± 17.25 mg and 87 ± 18.87 mm³, respectively (Based on pilot study). The results (Fig. 5) showed that lung tumor weights were significantly (*, $P < 0.001$) decreased by 24 and 59% after treatment with DIM-14 and SED respectively compared to vehicle control. In mice treated with the SED& DIM-14, lung tumor volumes were decreased by 67 and 34% respectively. A non-significant ($P > 0.05$) change in average number of tumor nodules was observed among central, mid and peripheral regions of harvested lungs from each of the treated groups (Fig. 5). DIM-14 and SED treatment showed significant (*, $P < 0.001$) decrease in average number of tumor nodules by 22 and 49% respectively compared to control groups. We did not observe any weight loss or other signs of toxicity in mice treated with DIM-14.

3.6. Hematoxylin and eosin staining

The lung tumor histology was evaluated by H & E staining of lung tumor tissue. DIM-14 and SED treated tumors exhibited only occasional, isolated microvessels, while tumors from untreated mice had well-formed capillaries surrounding nests of tumor cells. Histological examination of the lungs and tracheobronchial epithelium showed no signs of inflammation or edema among all groups which suggests a safer toxicity profile for both DIM-14 and SED therapy.

3.7. Anti-tumor activity of DIM-14 and SED formulations

Western blot analysis of Bcl-2 expression suggested that the anti-apoptotic marker, Bcl-2, was significantly down regulated in SED group compared to untreated control and DIM-14 treated groups (Fig. 6). The densitometric analysis of western blot bands revealed that β -actin relative Bcl-2 expression was reduced 1.8 and 4.2 fold, respectively in DIM-14 and SED groups compared to untreated control tumors. The SED produced 2.4 fold higher repression in the Bcl-2 expression compared to DIM-14, suggesting the superior anticancer effects of SED in lung cancer stem cell model. Similarly, the expression of cell survival marker survivin was also significantly down regulated (0.9 and 2.1 fold) in DIM-14 and SED groups compared to control groups (Fig. 6). Typical properties of CSCs include their capacities for self-renewal and differentiation, in vivo tumorigenic potential and resistance to

chemotherapy. The stem cell self-renewal marker SOX2 expressions was found to be higher in control tumor lysates. Treatment with SED formulation resulted in significant down regulation of SOX2. The relative expressions were found to be reduced 1.9 and 4.8 fold significantly in DIM-14 and SED formulations respectively compared to untreated control tumor lysates (Fig. 6). Also, the stem cell marker Nanog indicated the promising anticancer effects of both DIM-14 and SED formulations. Compared to untreated control, the relative expression of Nanog was decreased (2.4 and 5.5 fold) in DIM-14 and SED groups respectively; SED produced 2.8 fold decreased Nanog expression compared to DIM-14 (Fig. 6). The expression of another stem cell marker, Oct4, was significantly decreased in SED formulation compared to untreated control and DIM-14 treated groups, with 2.16 and 3.50 fold reduction respectively (Fig. 6). The expression of NF-KB was also significantly down regulated in both DIM-14 and SED formulation treated tumors (Fig. 6). All these results suggest that SED formulation showed superior anticancer effects than DIM-14 treated groups, suggesting its superior anticancer activity.

4. Discussion

DIM-14, in its native state exhibits poor aqueous solubility and less oral bioavailability with no detectable toxicity. Different analogs of DIM have been synthesized in order to enhance its clinical usefulness [10,11,20–23]. However, low bioavailability properties of these DIMs preclude their translational utility outside of cultured cells. Various drug delivery approaches using carriers aimed at increasing oral bioavailability have shown some promise [24–27]. In the current study, first time we developed DIM-14 into a self-emulsified drug delivery system and explored its ability to overcome its solubility and bioavailability related problems [18,44].

For development of SED formulations, the selection of suitable oil is crucial because it solubilizes the lipophilic drug and increases its transport via the intestinal lymphatic system, thus enhancing its absorption from GIT. Based on our solubility screening, since both sesame and corn oil had the maximum solubility for DIM-14, Enova oil was selected as oil phase because it contains higher amount of triglycerides with medium chain fatty acids; which have lower interfacial tension, better water solubility and partitioning ability as an emulsifier than triglycerides with long chain fatty acids [28]. We also screened various nonionic surfactants with HLB values >10 for their efficiency as emulsifier and Tween 20 (HLB 16) was selected as the surfactant for preparation of the binary mixture. In SED, the primary means of self-emulsification assessment were visual estimation and rate of emulsification (index for the assessment of the efficiency of emulsification). The emulsification time study showed that the optimized formulation employed could emulsify within 55 s (rapidity of the formulation). The SED formulation was optimized using desirability functions such as droplet size and drug release. It was found that the theoretical (predicted) and observed (experimental) values were in close agreement. Higher values of correlation coefficient (R²) for the dependent variables indicated a good fit for SED experimental model.

In-vivo pharmacokinetic analysis with the desired formulation in dogs showed significantly ($P < 0.05$) higher absolute oral bioavailability of ~25% compared to DIM-14 solution. The

increased AUC and $C_{\max}$ values with SED formulation demonstrated the superior oral performance of our formulation compared to drug in free form. The relative bioavailability of the SED was 3 fold higher in dogs compared to DIM-14 alone. These support the hypothesis that SED is effective in improving the oral bioavailability of DIM-14.

The plasma concentration of the DIM-14 solution reached $C_{\max}$ within 360 min after oral administration. Compared with the plasma concentration profile of the DIM-14 solution, the plasma concentration profile of the SED was a little faster during the first 120 min, and was stabilized in sustained fashion over 720 min, followed by rapid decline in DIM-14 plasma concentration, resulting in sustained release effect of SED formulation. Since this is the first study of DIM-14 in dogs, we do not have any previous information on bio-distribution of DIM-14 but we observed three compartment distribution for DIM-P previously [18]. Oral bioavailability of DIM-5, DIM-7, DIM-8 and DIM-12 was found to be 39, 30, 6 and 42% respectively by De Miranda et al. [29]. Pharmacokinetic profile for all of them was found to be similar expect DIM-8 having the high first pass metabolism. The time for each DIMs to reach $C_{\max}$ ($T_{\max}$) after oral gavage administration was about 60 min for DIM-8 and 120 min for all others, which is similar to DIM-14. Similarly, Clearance as a function of bioavailability, extrapolated volume of distribution, and mean residence time were dependent on DIMs structure and route of administration.

In the present case, an increase in the AUC of DIM-14 was observed with SED and results revealed that the SED significantly promoted and sustained DIM-14 absorption. One unique property of SED is that it has superior absorption in the gastrointestinal tract when loaded with lipophilic drugs. The small droplet size (in the range of 500 nm) of the microemulsions may penetrate the absorption site via the transcellular pathway, and could protect the drug from enzyme degradation. Therefore, the higher bioavailability of DIM-14 through SED may be due to the enhanced absorption through the lymphatic pathway, as previously reported [30,31]. A relatively high ratio of emulsifier in SED may also contribute to the increased permeability by disturbing the cell membrane [32].

For the first time in the current study, we evaluated the effect of DIM-14 against H650 lung cancer stem cells. This evaluation of DIM-14 SED as potential anticancer agent in H1650 lung cancer mice model highlighted the significant increase in anticancer activity in SED than that of the DIM-14 solution. This is expected because of the improved oral absorption and increased half-life of SED. The improved oral bioavailability of DIM-14 in SED is further complemented by the increased apoptosis compared to DIM-14 solution at the same dose of 20 mg/kg.

An effective therapeutic approach against cancers should focus on elimination of pool of CSCs which are quiescent or slowly replicating and thus more resistant to apoptosis induced by current cytotoxic regimens [2]. So far, very few drugs have been reported to exhibit CSCs inhibitory activity. One such drug, salinomycin, a potassium ionophore used as an agricultural antibiotic, reduced the proportion of CSCs by more than 100-fold relative to paclitaxel [3]. In the same study, nigericin, structural similarity to salinomycin, also exhibited selective toxicity on breast CSCs, suggesting that salinomycin killing CSCs may be due to its action as a potassium ionophore [3]. Metformin, a standard agent for diabetes,

selectively killed cancer stem cells in several breast cancer cell lines in vitro and in vivo [4]. Unfortunately, salinomycin is very toxic to humans that prevent its clinical use [33]. Metformin, on the other hand, is not very potent; against CSCs it works only at high concentrations of 100–300 μ M, which is beyond physiologically achievable limit. This served as the basis for our study to look for new agents that could selectively target CSC.

DIM-14 in the current study depicted potential anticancer activities as it was able to suppress various molecular markers associated with cancer expression. Anticancer activity could be correlated with decrease in the levels of BCL-2 and survivin in lung cancer stem cell tumor animal models. There are several reports suggesting the relation between CSCs and BCL2 level. Madjd et al. [34] showed that BCL-2 was highly expressed in breast CSCs and potentially affects the chemo-resistance by inducing other signaling pathways required for CSC survival. Lagadinou et al. [35] reported that BCL-2 inhibition reduced oxidative phosphorylation and selectively eradicated quiescent LSCs (Leukemia stem cells). In addition to BCL2 suppression, inhibition of survivin — an inhibitor of apoptosis protein, could potentiate the anticancer activity of DIM-14 and SED. Survivin is generally highly expressed in most cancers and expected to play a significant role in chemotherapy resistance, increased tumor recurrence, and shorter patient survival [36].

To date, very little is known regarding how Oct4, Sox2 and Nanog contribute to CSC properties at the molecular level. For the first time we report the effect of DIM derivatives on expression of these stemness factors. DIM-14 was found to efficiently impair the growth of LCSCs by reducing the expression of pluripotent stem cell transcription factors (Oct4, Sox2, Nanog). These factors are called as stemness factor because of their ability to determine the fate to stem cells. Inappropriate time and level of expression of these transcription factors would result in cancer stem cells rather than normal pluripotent stem cells or differentiated somatic cells. Upregulation of Oct4, Sox2 and Nanog, is correlated with poor survival outcome of patients with various types of cancer [37, 38]. Interestingly, levels of inflammatory marker NF- κ B were also found to be decreased in DIM-14 and SED treated stem cell lung cancer model. This indicates that DIM-14 could have inhibited the inflammatory pathway necessary for transformation and lung cancer stem cell formation. Along with suppression or inhibition of all above mentioned CSCs markers, DIM was also found to reverse the drug resistance of CSC as well as decrease their self-renewal potential of CSC in several carcinoma and melanoma cell lines [39]. Semov et al. [39], demonstrated that DIM is a selective and potent inhibitor of CSCs and pre-treatment of tumor spheres with DIM before implantation to mice significantly retarded the growth of primary tumors compared to tumors formed by untreated tumor spheres. These factors make DIM-14 a potential candidate of choice to be implemented in lung cancer prevention and therapy.

Our study attains significance because of the new potential applications attached to DIM-14 in cancer therapy. The unique ability of DIM-14 to eliminate cancer stem cells highlights its potential to use as a medium to bring about a dual therapeutic effect when combined along with conventional cancer treatment regimen consisting chemotherapeutic agents or irradiation. While a bulk of cancer non-stem cells in tumor can be efficiently eliminated by conventional cytotoxic agent or by irradiation, DIM-14 can selectively eliminate cancer stem cells. This dual therapeutic treatment of cancers by application of DIM-14 along with

conventional treatment has the enormous potential to rule out the development of chemoresistance, metastases and most importantly, tumor recurrence. Thus our unique in vivo model successfully establishes the potential application of DIM-14 to inhibit lung cancer stem cells and paves ways for other researchers to evaluate its application in other types of cancers also.

5. Conclusion

The improved oral bioavailability and superior anticancer effect of DIM-14 in lung cancer model through the self-emulsified formulation, gives a novel opportunity and technology to deal with poorly water soluble and low oral bioavailable drugs. Based on superior pharmacokinetic and pharmacodynamic profile, DIM-14 can be a potential anticancer drug to treat/prevent different cancer types by oral administration. In addition, DIM-14 SED in the current study was shown to be effective against lung cancer stem cells as well. In conclusion, this unique approach of drug formulation has numerous industrial applications and provides an effective alternative to deal with poorly water soluble, gastric acid sensitive, first pass metabolizing drugs.

Acknowledgments

The authors acknowledge the financial assistance of this research from the National Institute on Minority Health and Health Disparities (NIMHD) P20 program [Grant # 1P20MD006738-03; to M.S.]; and the Department of Defense (DOD) Breast Cancer Program [Grant #W81XWH-11-1-0211] and the National Institutes of Health (NIH-NCI) [1R21CA175618-01A1 to M.S.]. The authors thank Dr. Srikumar Chellappan, (Professor, H. Lee Moffitt cancer center and research institute, Tampa, Florida) for the kind gift of lung cancer stem cells for our work.

References

1. Ichite N, Chougule MB, Jackson T, Fulzele SV, Safe S, Singh M. Enhancement of docetaxel anticancer activity by a novel diindolylmethane compound in human non-small cell lung cancer. *Clin. Cancer Res.* 2009; 15(2):543–552. [PubMed: 19147759]
2. Wang J, Li ZH, White J, Zhang LB. Lung cancer stem cells and implications for future therapeutics. *Cell Biochem. Biophys.* 2014; 69(3):389–398. [PubMed: 24549856]
3. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell.* 2009; 138(4):645–659. [PubMed: 19682730]
4. Hirsch HA, Iliopoulos D, Tschlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res.* 2009; 69(19):7507–7511. [PubMed: 19752085]
5. Qin C, Morrow D, Stewart J, Spencer K, Porter W, Smith R III, Phillips T, Abdelrahman M, Samudio I, Safe S. A new class of peroxisome proliferator-activated receptor gamma (PPARgamma) agonists that inhibit growth of breast cancer cells: 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes. *Mol. Cancer Ther.* 2004; 3(3):247–260. [PubMed: 15026545]
6. Chinthalapalli S, Smith R III, Samudio I, Zhang W, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl) methanes induce peroxisome proliferator-activated receptor gamma-mediated growth inhibition, transactivation, and differentiation markers in colon cancer cells. *Cancer Res.* 2004; 64(17):5994–6001. [PubMed: 15342379]
7. Su Y, Vanderlaag K, Ireland C, Ortiz J, Grage H, Safe S, Frankel AE. 1,1-Bis(3'-indolyl)-1-(p-biphenyl)methane inhibits basal-like breast cancer growth in athymic nude mice. *Breast Cancer Res.* 2007; 9(4):R56. [PubMed: 17764562]

8. Ichite N, Chougule M, Patel AR, Jackson T, Safe S, Singh M. Inhalation delivery of a novel diindolylmethane derivative for the treatment of lung cancer. *Mol. Cancer Ther.* 2010; 9(11):3003–3014. [PubMed: 20978159]
9. Chintharlapalli S, Papineni S, Baek SJ, Liu S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl) methanes are peroxisome proliferator-activated receptor gamma agonists but decrease HCT-116 colon cancer cell survival through receptor-independent activation of early growth response-1 and nonsteroidal anti-inflammatory drug-activated gene-1. *Mol. Pharmacol.* 2005; 68(6):1782–1792. [PubMed: 16155208]
10. Chintharlapalli S, Papineni S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl) methanes inhibit growth, induce apoptosis, and decrease the androgen receptor in LNCaP prostate cancer cells through peroxisome proliferator-activated receptor gamma-independent pathways. *Mol. Pharmacol.* 2007; 71(2):558–569. [PubMed: 17093136]
11. Lei P, Abdelrahim M, Cho SD, Liu X, Safe S. Structure-dependent activation of endoplasmic reticulum stress-mediated apoptosis in pancreatic cancer by 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes. *Mol. Cancer Ther.* 2008; 7(10):3363–3372. [PubMed: 18852139]
12. Kohli K, Chopra S, Dhar D, Arora S, Khar RK. Self-emulsifying drug delivery systems: an approach to enhance oral bioavailability. *Drug Discov. Today.* 2010; 15(21–22):958–965. [PubMed: 20727418]
13. Singh B, Bandopadhyay S, Kapil R, Singh R, Katare O. Self-emulsifying drug delivery systems (SEDDS): formulation development, characterization, and applications. *Crit. Rev. Ther. Drug Carrier Syst.* 2009; 26(5):427–521. [PubMed: 20136631]
14. Zhang P, Liu Y, Feng N, Xu J. Preparation and evaluation of self-microemulsifying drug delivery system of oridonin. *Int. J. Pharm.* 2008; 355(1–2):269–276. [PubMed: 18242895]
15. Barakat NS. Enhanced oral bioavailability of etodolac by self-emulsifying systems: in-vitro and in-vivo evaluation. *J. Pharm. Pharmacol.* 2010; 62(2):173–180. [PubMed: 20487196]
16. Patil P, Patil V, Paradkar A. Formulation of a self-emulsifying system for oral delivery of simvastatin: in vitro and in vivo evaluation. *Acta Pharm.* 2007; 57(1):111–122. [PubMed: 19839411]
17. Balakrishnan P, Lee BJ, Oh DH, Kim JO, Lee YI, Kim DD, Jee JP, Lee YB, Woo JS, Yong CS, Choi HG. Enhanced oral bioavailability of Coenzyme Q10 by self-emulsifying drug delivery systems. *Int. J. Pharm.* 2009; 374(1–2):66–72. [PubMed: 19446761]
18. Patel AR, Spencer SD, Chougule MB, Safe S, Singh M. Pharmacokinetic evaluation and in vitro-in vivo correlation (IVIVC) of novel methylene-substituted 3,3' diindolylmethane (DIM). *Eur. J. Pharm. Sci.* 2012; 46(1–2):8–16. [PubMed: 22342559]
19. Jusko, WJ., Schentag, JJ., Evans, WE., Jusko, WJ. *Applied Pharmacokinetics.* Applied Therapeutics, Inc.; San Francisco: 1980.
20. Chintharlapalli S, Papineni S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes inhibit colon cancer cell and tumor growth through PPARgamma-dependent and PPARgamma-independent pathways. *Mol. Cancer Ther.* 2006; 5(5):1362–1370. [PubMed: 16731770]
21. Inamoto T, Papineni S, Chintharlapalli S, Cho SD, Safe S, Kamat AM. 1,1-Bis(3'-indolyl)-1-(p-chlorophenyl)methane activates the orphan nuclear receptor Nurr1 and inhibits bladder cancer growth. *Mol. Cancer Ther.* 2008; 7(12):3825–3833. [PubMed: 19074857]
22. Lei P, Abdelrahim M, Cho SD, Liu S, Chintharlapalli S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes inhibit colon cancer cell and tumor growth through activation of c-jun N-terminal kinase. *Carcinogenesis.* 2008; 29(6):1139–1147. [PubMed: 18460448]
23. Lei P, Abdelrahim M, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes inhibit ovarian cancer cell growth through peroxisome proliferator-activated receptor-dependent and independent pathways. *Mol. Cancer Ther.* 2006; 5(9):2324–2336. [PubMed: 16985067]
24. Moretti MD, Gavini E, Juliano C, Pirisino G, Giunchedi P. Spray-dried microspheres containing ketoprofen formulated into capsules and tablets. *J. Microencapsul.* 2001; 18(1):111–121. [PubMed: 11201334]
25. Nazzal S, Khan MA. Controlled release of a self-emulsifying formulation from a tablet dosage form: stability assessment and optimization of some processing parameters. *Int. J. Pharm.* 2006; 315(1–2):110–121. [PubMed: 16563673]

26. Nazzal S, Nazzal M, El-Malah Y. A novel texture-probe for the simultaneous and real-time measurement of swelling and erosion rates of matrix tablets. *Int. J. Pharm.* 2007; 330(1–2):195–198. [PubMed: 17049771]
27. Nekkanti V, Karatgi P, Prabhu R, Pillai R. Solid self-microemulsifying formulation for candesartan cilexetil. *AAPS PharmSciTech.* 2010; 11(1):9–17. [PubMed: 20013081]
28. Driscoll DF, Nehne J, Peterss H, Franke R, Bistran BR, Niemann W. The influence of medium-chain triglycerides on the stability of all-in-one formulations. *Int. J. Pharm.* 2002; 240(1–2):1–10. [PubMed: 12062496]
29. De Miranda BR, Miller JA, Hansen RJ, Lunghofer PJ, Safe S, Gustafson DL, Colagiovanni D, Tjalkens RB. Neuroprotective efficacy and pharmacokinetic behavior of novel anti-inflammatory para-phenyl substituted diindolylmethanes in a mouse model of Parkinson's disease. *J. Pharmacol. Exp. Ther.* 2013; 345(1):125–138. [PubMed: 23318470]
30. Trevaskis NL, McEvoy CL, McIntosh MP, Edwards GA, Shanker RM, Charman WN, Porter CJ. The role of the intestinal lymphatics in the absorption of two highly lipophilic cholesterol ester transfer protein inhibitors (CP524,515 and CP532,623). *Pharm. Res.* 2010; 27(5):878–893. [PubMed: 20221896]
31. Zhang Z, Ma L, Jiang S, Liu Z, Huang J, Chen L, Yu H, Li Y. A self-assembled nanocarrier loading teniposide improves the oral delivery and drug concentration in tumor. *J. Control. Release.* 2013; 166(1):30–37. [PubMed: 23266449]
32. Scott Swenson E, Curatolo WJ. (C) Means to enhance penetration: (2) Intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity. *Adv. Drug Deliv. Rev.* 1992; 8(1):39–92.
33. Story P, Doube A. A case of human poisoning by salinomycin, an agricultural antibiotic. *N. Z. Med. J.* 2004; 117(1190):U799. [PubMed: 15107902]
34. Madjd Z, Mehrjerdi AZ, Sharifi AM, Molanaei S, Shahzadi SZ, Asadi-Lari M. CD44+ cancer cells express higher levels of the anti-apoptotic protein Bcl-2 in breast tumours. *Cancer Immun.* 2009; 9:4. [PubMed: 19385591]
35. Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, Ashton JM, Pei S, Grose V, O'Dwyer KM, Liesveld JL, Brookes PS, Becker MW, Jordan CT. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell.* 2013; 12(3):329–341. [PubMed: 23333149]
36. Rahman KM, Banerjee S, Ali S, Ahmad A, Wang Z, Kong D, Sakr WA. 3,3'-Diindolylmethane enhances taxotere-induced apoptosis in hormone-refractory prostate cancer cells through survivin down-regulation. *Cancer Res.* 2009; 69(10):4468–4475. [PubMed: 19435906]
37. Wang ML, Chiou SH, Wu CW. Targeting cancer stem cells: emerging role of Nanog transcription factor. *Onco Targets Ther.* 2013; 6:1207–1220. [PubMed: 24043946]
38. Liu K, Lin B, Zhao M, Yang X, Chen M, Gao A, Liu F, Que J, Lan X. The multiple roles for Sox2 in stem cell maintenance and tumorigenesis. *Cell. Signal.* 2013; 25(5):1264–1271. [PubMed: 23416461]
39. Semov A, Iourtchenko L, Liu LF, Li S, Xu Y, Su X, Muijnek E, Kiselev V, Alakhov V. Diindolylmethane (DIM) selectively inhibits cancer stem cells. *Biochem. Biophys. Res. Commun.* 2012; 424(1):45–51. [PubMed: 22727906]
40. Fulzele SV, Chatterjee A, Shaik MS, Jackson T, Ichite N, Singh M. 15-Deoxy-Delta12,14-prostaglandin J2 enhances docetaxel anti-tumor activity against A549 and H460 non-small-cell lung cancer cell lines and xenograft tumors. *Anticancer Drugs.* 2007; 18:65–78. [PubMed: 17159504]
41. Chougule M, Patel AR, Sachdeva P, Jackson T, Singh M. Anticancer activity of Noscapine, an opioid alkaloid in combination with Cisplatin in human non-small cell lung cancer. *Lung Cancer.* 2011; 71:271–282. [PubMed: 20674069]
42. Fulzele SV, Chatterjee A, Shaik MS, Jackson T, Singh M. Inhalation delivery and anti-tumor activity of celecoxib in human orthotopic non-small cell lung cancer xenograft model. *Pharm Res.* 2006; 23:2094–2106. [PubMed: 16902813]

43. Haynes A, Shaik MS, Chatterjee A, Singh M. Evaluation of an aerosolized selective COX-2 inhibitor as a potentiator of doxorubicin in a non-small-cell lung cancer cell line. *Pharm Res.* 2003; 20:1485–1495. [PubMed: 14567645]
44. Patel AR, Godugu C, Wilson H, Safe S, Singh M. Evaluation of Spray BIO-Max DIMP in Dogs for Oral Bioavailability and in Nu/nu Mice Bearing Orthotopic/Metastatic Lung Tumor Models for Anticancer Activity. *Pharm Res.* 2015; 32:2292–2300. [PubMed: 25576245]

Fig. 1.
In-vitro release study of DIM-14 free drug, DIM-14 solution and SED formulation.

Fig. 2.
Contour plots showing the effect of independent variables on desirability and dependent variables during process design SED.

Fig. 3. A) Plasma concentration (µg/dL) vs time profile (hr) following intravenous administration of DIM-14 (0.5 mg/kg), B) plasma concentration (µg/dL) vs time profile (hr) following oral administration of DIM-14 solution (3.33 mg/kg) and self-emulsified formulation of DIM-14 (3.33 mg/kg) (SED) in dogs (n = 4).

Fig. 4.

Effects of DIM-14 solution and SED on H1650 stem cell xenograft lung tumor volume (a); tumor weight (b); mice body weight (c). Lung weights and tumor volumes were determined for measurement of therapeutic activity of the treatments. One-way ANOVA followed by post Tukey test was used for statistical analysis. $P < 0.05$ (*, significantly different from untreated controls; **, significantly different from DIM-P solution). Data presented are means $\pm$ SD (n= 12).

Fig. 5. Effects of DIM-14 solution and SED on H1650 stem cell orthotopic lung tumor volume (a); tumor weight (b); lung tumor tissues (c); mice bodyweight (D). Lung weights and tumor volumes were determined for measurement of therapeutic activity of the treatments. One-way ANOVA followed by post Tukey test was used for statistical analysis. $P < 0.05$ (*, significantly different from untreated controls; **, significantly different from DIM-P solution). Data presented are means $\pm$ SD ($n = 12$).

Fig. 6.

Western blot analysis of different proteins in tumor lysates from control-untreated and treated groups. Lane 1 = control; Lane 2 = DIM-14 solution; Lane 3 = SED. Protein expression levels (relative to β -actin) were determined. Mean $\pm$ SEM for three replicate determinations. One-way ANOVA followed by post Tukey test was used for statistical analysis. $P < 0.01$ (*, significantly different from untreated controls; **, significantly different from single treatments).

Table 1

Optimize ingredients of DIM-14 as self-emulsified drug delivery system (SEDDS).

Ingredients	Composition of SEDDS (% w/w)
DIM-14	20
Enova oil	50
TPGS	10
Labrafil 1944	20