Published in final edited form as:

J Pharm Sci. 2020 June ; 109(6): 1867–1882. doi:10.1016/j.xphs.2020.01.010.

The Role of Self-Nanoemulsifying Drug Delivery Systems of CDODA-Me in Sensitizing Erlotinib-Resistant Non–Small Cell Lung Cancer

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Abstract

We have investigated the effects of combination treatment involving ERL (erlotinib) with a glycyrrhetic acid analog, CDODA-Me in overcoming ERL resistance, providing efforts to improve the oral bioavailability of this treatment using self-nanoemulsifying drug delivery systems (SNEDDS). A Qbd (quality-by-design) approach was used to prepare CDMS (CDODA-SNEDDS, 2 μ M), which was characterized using surface response methodology to optimize drug content, particle size, and drug release. CDMS/ERL combinations showed synergism in wild-type and resistant H1975 and HCC827 cell lines with combination index values less than 1. Increased apoptosis, mitochondrial membrane potential depletion, and enhanced intracellular ROS levels were also observed in combination therapy. Western blot analysis showed that combination therapy inhibited phosphorylation of epidermal growth factor receptor (EGFR) ($p < 0.01$ in all cell lines) and Met receptor tyrosine kinase (MET) ($p < 0.01$ in all cell lines). *In vivo*, the relative bioavailability of CDMS increased significantly from 22.13 to 151.76 μ g/mL compared to the dosing of oral suspension (dose equivalent). Our results demonstrate that combination therapy involving ERL and CDODA-Me overcomes resistance through dual inhibition of p-EGFR and p-MET leading to the induction of apoptosis, intracellular ROS accumulation, and decreased mitochondrial potential. Furthermore, CDMS improved the oral bioavailability of CDODA-Me.

Keywords

non-small cell lung cancer; drug resistance; erlotinib; epidermal growth factor receptor; self nano-emulsifying drug delivery system; combination therapy

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Conflict of interest: The authors declare that they have no conflict of interest.

This article contains supplementary material available from the authors by request or via the Internet at <https://doi.org/10.1016/j.xphs.2020.01.010>.

Background and Introduction

Lung cancer is the leading cancer killer accounting for 30% of all cancer deaths in the United States. Eighty-five percent of diagnosed lung cancer cases are classified as non-small cell lung cancer (NSCLC), that in most cases go undetected until reaching advanced stages limiting effective treatment options and producing low survival rates (16%).¹ Epidermal growth factor receptor (EGFR) has been found to be overexpressed in 85% of non-small cell lung cancer patients.² Targeting specific mutations, such as an exon 19 deletion and exon 21 mutation, that have resulted in increased EGFR activation driving cancer cell activity has proven to be beneficial to cancer treatment.³ EGFR is a transmembrane receptor that when bound to a substrate on its extracellular domain activates its intracellular kinase domain leading to the activation of pathways involved in cellular proliferation and survival.

ERL is an EGFR targeting tyrosine kinase inhibitor that reversibly binds to the kinase domain preventing receptor activation. This treatment has been shown to induce decreased tumor volume in 70% of patients harboring the previously stated EGFR mutations.⁴ Unfortunately, there is a high incidence of resistance developing 9–12 months after the initiation of treatment.^{5,6} The most commonly known mechanisms of ERL resistance include secondary-site EGFR mutations or amplification of the Met receptor tyrosine kinase (MET)-oncogene. The EGFR mutation T790M is the result of the methionine substitution of threonine 790 and has been found to increase the receptor's affinity for ATP overcoming the effects of the EGFR inhibitor. This has been found to be mostly responsible for the resistance to ERL treatment.^{7,8} ERL resistance can also occur through a method known as “kinase switching.” This occurs when cells switch their dependency from the targeted kinase to another that is implicated in downstream pathways allowing them to bypass the induced signaling inhibition. In the case of ERL resistance, cells switch their dependence from EGFR to MET that activates downstream AKT signals to overcome losses caused by inhibiting EGFR.^{9,10}

Methyl 2-cyano-3, 11-dioxo-18b-olean-1, 12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid methyl ester derived from glycyrrhetic acid that has been shown to be highly cytotoxic in pancreatic cancer cell lines inhibiting angiogenesis and cell growth. CDODA-Me has been shown to have antiproliferative, antiangiogenic, anti-inflammatory, and proapoptotic properties through its evaluation as a potential anticancer agent. This activity has been largely attributed to its regulation of specificity protein (Sp) transcription factors^{11,12} that in turn lead to decreased expression of Sp-regulated genes such as EGFR.

CDODA-Me is classified as having a high permeability and low solubility which makes it difficult to formulate.¹³ Self-nanoemulsified drug delivery vehicles (SNEDDS) have been used to increase drug solubility while also increasing drug absorption in the gastrointestinal (GI) tract for many poorly soluble drugs.¹⁴ SNEDDS are anhydrous, isotropic mixtures of oil(s), surfactant(s), lipophilic drug, and cosurfactant(s) or cosolvent(s), which spontaneously form oil-in-water emulsions upon aqueous dilution with gentle agitation. Micro-emulsions such as SNEDDS have become a hot topic in the field of drug delivery because of their ability to protect loads from enzymatic degradation as well as their small particle size. Their most important advantages are the ability to increase oral bioavailability

of many insoluble drugs and their high drug-loading capacity. SNEDDS have been used to reduce dose frequency, avoid first-pass metabolism, and also bypass p-glycoprotein-mediated efflux.¹⁵ SNEDDS are classified as type III lipid formulations due to their inclusion of water-soluble components and relatively low oil content. These formulations have shown to improve bioavailability through improved solubility caused by increased drug partitioning between the oil and gastrointestinal fluid as well as bypassing presystemic metabolism.^{14,16} They have been shown to increase drug distribution, increase intracellular tumor concentrations, and reduce the dose and dose-related toxicity.

Important factors involved in the formulation of SNEDDS include the type of oil, surfactant, and cosurfactant used. The oils used in SNEDDS must have the ability to solubilize hydrophobic drugs which in turn increases the potential of the drug to be transported through the intestinal lymphatic system.¹⁶ Surfactants involved in SNEDDS formulations are nonionic on account of the toxicity issues experienced with ionic surfactants and a range of 30%–60% w/w is required to form stable emulsions. This range is important because higher surfactant percentages have been shown to cause gastrointestinal irritation.¹⁵ Furthermore, particle size of emulsions is directly proportional to surfactant concentrations. Cosurfactants are used to increase drug release rate by dissolving the excess amounts of drug in the surfactant or lipid base phases.¹⁷

SNEDDS can be prepared by empirical way of “trial-error” method altering the components’ ratio or by the application of ternary phase diagram¹⁴ which can lead to many experiments and batches to make. However, recently, using hydrophobic lipophilic balance principles and response surface methodology approach was developed by Bahloul et al., which reduced the number of trials required to develop and optimize the SNEDDS with desired drug release and satisfactory pharmacokinetic parameters.^{18,19} We predict that the use of surface response methodology measuring the effects of various oil to surfactant ratios on particle size, drug entrapment, and drug release would allow for the optimization of stable SNEDDS with the desirability index providing increased entrapment and release of CDODA-Me.

Efforts to improve treatment response in resistant tumors have grown in importance as understanding the mechanisms behind them could improve the quality of life. Developing oral formulations of these treatments is equally important as it reduces cost and increases patient compliance. Our hypothesis for this study is that combination treatment of EGFR-targeted resistant cell lines with ERL and CDODA-Me will not only improve the efficacy of ERL but also overcome resistance as well. To improve the oral bioavailability of CDODA-Me, we have selected to employ the use of self-nanoemulsifying drug delivery systems (SNEDDS).

Materials and Methods

Chemicals

ERL was purchased from MedChem Express (Princeton, NJ), and CDODA-Me was graciously donated by Dr. Stephen Safe (Texas A&M University, College Station, TX). Fetal bovine serum, penicillin-streptomycin-neomycin, RPMI medium, Dulbecco’s phosphate-

buffered saline (DPBS), and crystal violet were all purchased from Sigma-Aldrich (St. Louis, MO). The primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA), and β -actin from Santa Cruz (Dallas, TX).

HPLC Analysis

Two separate HPLC methods were developed and validated for the quantification of CDODA-Me and ERL in aqueous and in biometric samples using HPLC system (Waters WAT053502) consisting of a pump (WAT054275) with integrated system controller auto sampler (e2695 separations module) and variable wavelength UV detector diode array detector (2996, Waters). Data acquisition and analysis was performed in postrun analysis using Empower software (Waters Corporation). We employed C18 Luna® (Phenomenex®, 250 × 4.6 mm) column with 5 μ m sphere-shaped particles, 100Å pore size, 400 m²g surface area, 13.5% carbon load, and calculated bonded phase coverage of 5.50 μ mole m². The spectrum analysis was carried out between 200 and 400 nm and the fixed wavelength measurement was recorded at 246 nm.

HPLC Method for CDODA-Me Estimation

A mixture of 10 mM ammonium acetate buffer (pH 4.2) and acetonitrile (90:10, v/v) was used as mobile phase at 1 mL/min flow rate for the separation of CDODA-Me. The typical best fit linear regression equations of the developed HPLC method was peak area (mV s) = $586.5 \times \text{conc. (ng/mL)} + 4176$ with $r^2 = 0.996$ in the concentration range of 30–1200 ng/mL of CDODA-Me in plasma. The system suitability parameters, k, T, HETP, Neff, HEFF, and h showed that the developed 3D view method has good reproducibility. The limits of detection (LOD) and limits of quantification (LOQ) of the developed HPLC method were 25 and 30 ng/mL, respectively. The accuracy study result of the developed HPLC method demonstrated that the % relative standard deviation (% R.S.D.) was less than 2.34 with mean % recovery range from 97.27 to 104.34. The intraday precision (% R.S.D.) study results of the developed HPLC method was in the range of 0.23 to 1.84. The repeatability of the precision samples on 3 different occasions within a day was always less than 1.10 with least % R.S.D of 0.13. The plasma concentration of CDODA-Me versus time was plotted for pure drug suspension and CDMS formulations.

Formulation-Response Surface-Central Composite QbD Approach

The solubility of CDODA-Me was determined in different oils, surfactants, and cosurfactants to select the proper excipients to be used in the formulation, and it was determined that the mixture of oils (1:1 v/v ratio of Captex 300 and Capmul MCM C8 EP), surfactants (Labrasol), cosurfactants (1:1 v/v ratio of Gelucir 44/14 & Gelucir 48/16), and cosolvent (Transcutol P) provided optimal solubilization of both compounds and was further analyzed using Design Experiment analysis. The drug-loaded CDMS was prepared by first dissolving CDODA-Me into the oil phase and mixing the other components drop wise at 50°C under magnetic stirring until a clear solution was obtained. CDMS was kept at room temperature overnight and examined for stability parameters such as turbidity and phase separation.

Response surface methodology was used to systemically investigate the effect of a wide range of independent and dependent variables. The formulation variables and their levels as shown in Supplemental Table 1 were chosen after optimizing. A-oil phase, B-surfactant, C-cosurfactant, D-cosolvent were 4 independent variables (factors) considered in the preparation of CDMS, whereas the particle size distribution, polydispersity index (PDI, emulsification time (ET), and % release were dependent variables (response). Data were analyzed using Design Expert software 7.3 (State-Ease).

To optimize and study influences of amount/concentration of formulation components on response factors such as particle size, polydispersion index, emulsification time, encapsulation efficiency, and % drug release, Central Composite Design (central composite circumscribed) was used to optimize formulation parameters and responses were analyzed using the quadratic model. Experimental batches produced are summarized in Table 1. Optimum formulations were chosen based on the desirability index. A Design of Experiments concept helped select ideal formulation composition. By using “Response Surface Central Composite Quadratic QbD Model,” different drug-loaded CDMS with varying quantity of formulation variables were prepared and evaluated to determine the best combinations. A visual representation of formulated CDMS is shown in Figure 1.

Particle Size Distribution

Five milligrams of CDMS formulation was brought to 10 mL using deionized water. The particle size distribution of the resulting dispersion was determined by laser diffraction analysis using a particle size analyzer (Nicom Zetasizer).

Determination of Drug Content

CDODA-Me was extracted from CDMS in acetonitrile using the sonication technique. Samples were sonicated for 30 min in 10 mL acetonitrile and the extract was analyzed using HPLC with previously established method (Waters).

In Vitro Drug Release

In vitro drug release studies from CDMS formulations and drug powder (2 mg/mL CDODA-Me) were performed using the dialysis bag method. Buffer solutions included 0.1 NHCl, pH adjusted to 1.2 using NaOH, and pH 6.8. Briefly, 1 mL samples were taken at various time points over a 2-h period while continually replacing it with the designated buffer. Samples were then diluted 1:1 with mobile phase, vortexed for 1 min, and centrifuged for 2 min at 10,000 rpm. Samples were analyzed using the previously mentioned HPLC methods.

Permeability Studies

Caco-2 cells were cultured in DMEM media supplemented with FBS (10% V/V), PSN (2%, v/v), and nonessential amino acid solution (1.2% v/v), and HEPES (10mM). After reaching 80% confluency, CaCO₂ cells were trypsinized and washed with PBS. The cell insert support was coated with collagen solution and single 0.5 mL of 10⁵ cells/mL cell suspension was seeded in the apical compartment of a Costar® *Trans* well® permeable support with a polycarbonate membrane (0.4 µm pore size, 12 mm diameter inserts) in a 12-well plate format. CaCO₂ cells were maintained in the *Trans*-well plate at standard culture conditions

for 21 days with media changes every 2 days. The CaCO₂ monolayer tight junction formation and integrity was monitored by the transepithelial electrical resistance (teer) using milliceller-2 v-ohm meter (Millipore, Billerica, MA). Once the *Trans*-well plate was completely confluent, the apical compartments were washed with PBS. The contents of the basolateral compartment were disposed and refilled using HBSS-HEPES buffer (pH 7.4). The plates were allowed to equilibrate for 10 min at 37°C. The apical compartment buffer was then replaced with respective drug suspension/formulation (10µg/mL CDMS and suspension) and the basolateral compartment was refilled with HBSS/HEPES buffer (pH 7.4) for absorptive permeability study. The plates were incubated at 37°C and CO₂ (5%) under constant shaking (50 rpm) for 2 h. Samples (0.2 mL) were collected at 15, 30, 45, 60, 90, and 120 min time points from the basolateral compartment and replaced with fresh buffer. The drug amount in the samples was estimated using the in-house-developed HPLC method previously mentioned. The apparent permeability was calculated using the following formula

$$PAPP \text{ (cm/s)} = [Q / (C_i \times T \times A)]$$

where Q = amount of drug in receiver (µg), C_i = the initial concentration of drug (g/cm³), T = time (sec), and A = area of insert (cm²).

In Vivo Pharmacokinetics

In vivo pharmacokinetic studies of CDODA-Me in solution and CDMS were performed using Sprague-Dawley rats. After a 1-week acclimation period, animals were divided into 2 groups containing 6 animals (group 1, oral CDODA-Me suspension; group 2, oral CDMS). Animals were dosed 80 mg/kg CDODA-Me (3 mL drug suspension and CDMS) and blood samples were taken at various time points over the course of 4 h from the tail vein. Samples were examined using no compartmental analysis with Kinetical 5® (Thermo Fisher). Animals used in experiments were housed according to regulations set by the American Association for Accreditation of Laboratory Animal Care at 37°C and 60% humidity and procedures were modeled after methods approved by the Institutional Animal Care and Use Committee.

Cell Culture

NSCLC cell lines: HCC827, HCC827-4 µM (Acquired 4 µM ERL Resistant), HCC827-Cl4 (T790M EGFR ERL Resistant), H1975 (T790M EGFR ERL Resistant), H1975-CL1 (EGFR G796D mutation rociletinib resistant), H1299 (p53 mutated), and H460 (KRAS mutated) were cultured in RPMI supplemented with 10% FBS, 100 units per mL penicillin, and 100 mg/mL streptomycin. Conditions were maintained at 37°C in an atmosphere of 5% CO₂ and 95% relative humidity. Media was replaced every 2 days. Cells were subcultured every 3 days or upon reaching 70% confluency.

Cytotoxicity Assay

Cytotoxicity of CDODA-Me, ERL alone, and CDODA-Me-ERL combination was determined at various concentrations in wild-type and resistant cell lines. Cells were seeded in 96-well plates (approximately 10,000 cells per well) and incubated overnight. Cells were

treated for 48 h with ERL alone and in combination with CDODA-Me (2 μ M). ERL concentrations ranged from 0 to 100 μ M. Drug concentrations used were the results of diluting 50 mM erlotinib and 40 mM CDODA-Me stock solutions in cell culture grade DMSO resulting in negligible concentrations of DMSO in working drug solutions. Preliminary studies included cytotoxicity assays with CDODA-Me ranging from 0 to 40 μ M. Cytotoxicity was measured at the end of treatment by crystal violet assay after fixing cells with 2.5% glutaraldehyde (100 μ L/well) for 30 min. Absorbance was measured at 620 nm wavelength using a microplate reader (Tecan, Tecan Austria GmbH, Austria). IC₅₀ values were calculated and synergism was analyzed through the calculation of the combination index using the isobolographic analysis feature of the Compusyn software.

Determining Optimal Drug Combination Concentrations

Cells were treated for 48 h with ERL concentrations ranging between 0 and 100 μ M in combination with constant concentrations of 2 μ M, 1 μ M, and 0.5 μ M CDODA-Me. Degrees of synergism for each combination were measured using the isobolographic analysis feature of the Compusyn software (version 1.0). The concentration of CDODA-Me selected to be used in combination treatment was chosen based on the fold decrease of IC₅₀ value (indicates increased response) as well as lower combination index value at the IC₅₀ concentration (indicates higher synergism).

Apoptosis Assay

Cells were plated in 24-well plates at a cell density of 37,000 cells per well and incubated for 24 h at 37°C. Cells were treated with 12 μ M ERL, 2 μ M CDODA-Me alone, and in combination for 48 h. Each treatment group contained 6 replicates. At the completion of treatment, cells were rinsed with 500 μ L DPBS per well and fixed with 500 μ L 4% formaldehyde for 30 min at 37°C. After fixing, cells were rinsed twice with 1 mL DPBS, permeabilized for 15 min at room temperature with 0.2% Triton X 100, and stained with NucBlue fixed cell ready probes (Life technologies) according to manufacturer's protocol. The stained cells were observed using a fluorescence microscope (Olympus IX71, Tokyo, Japan) to assess cellular apoptosis.

Mitochondrial Membrane Potential

Mitochondrial membrane potential depletion was observed using rhodamine 123 staining in treated cells. Cells were seeded in a 6-well plate at a density of 1.8×10^6 cells/well. Cells were treated with 12 μ M ERL, 2 μ M CDODA-Me alone and in combination for 48 h. Cells were then fixed with 4% formaldehyde for 15 min at 37°C, permeabilized with 0.2% triton $\times$ 100, and stained with 2 mL of a 1 μ M working solution of rhodamine 123 for 30 min at 37°C. Cells were imaged with the previously mentioned fluorescence microscope. Fluorescence intensities were measured using ImageJ.

Intracellular ROS Generation

Intracellular ROS generation was observed by DCFDA staining in treated cells. Briefly, cells were seeded in a 6-well plate at a density of 1.8×10^6 cells/well. Cells were treated with 12 μ M ERL, 2 μ M CDODA-Me alone, and in combination for 48 h before addition of 20 μ M

DCFDA for 30 min at 37°C. Cells were rinsed twice with DPBS and imaged with a fluorescence microscope (Olympus IX71, U-RFL-Tlamp).

Clonogenic Assay

Cell proliferation after treatment was observed using the clonogenic assay. Briefly, cells were seeded in 6-well plates at a density of 1.8×10^6 cells/well. Cells were then treated with 12 μ M ERL, 2 μ M CDODA-Me alone and in combination for 48 h. After treatment media was removed, cells were rinsed, and cells were collected using normal cell culture procedures. Cells were reseeded in fresh 6-well plates at a density of 500 cells/well and incubated for 7 days changing media on alternate days. Cells were then fixed with 4% formaldehyde for 15 min at 37°C and stained with crystal violet for imaging. Colonies were visually counted using ImageJ.

Western Blot Analysis

Cells were seeded in 75 cm² flasks at a density of 1.5×10^6 cells and allowed to grow for 24 h at 37°C before treatment with 12 μ M ERL, 2 μ M CDODA-Me, and the combination of the 2. Cell pellets were collected through trypsinization at the completion of treatment and transferred to microcentrifuge tubes. Whole cell protein lysates were prepared using RIPA buffer (Cell Signaling, Danvers, MA) which involved the use of cell lysis cocktail composed of PMSF (phenylmethylsulfonyl fluoride), protease inhibitor, and RIPA Buffer at a ratio of 1:14:84. Samples were then centrifuged at 14,000 rpm for 20 min. After completing centrifugation, supernatant was collected and stored at -20°C until further used. Protein estimation was completed using the bicinchoninic acid assay. Briefly, 50 μ g protein was loaded into each well of a 10% SDS-PAGE gel (Mini-PROTEAN® TGX™ Precast Gels). Protein was transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) after electrophoresis. After transfer, membranes were blocked overnight using 5% bovine serum albumin in PBST. Blots were then exposed to primary (1:1000) and secondary antibodies (1:1000) for 4 h each consecutively. β -actin was used as a housekeeping protein. Imaging was performed using Clarity™ Western ECL Substrate and ChemiDoc XRS+. Protein expression was quantified by comparing sample band densities to the standard beta-actin band densities using the Image Lab software.

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and Microsoft excel. All values were expressed as mean $\pm$ SD. Comparisons were carried out using one-way ANOVA–Bonferroni test and Student t-test. A $p < 0.05$ was considered to indicate statistically significant differences.

Results

Characterization of SNEDDS

Particle Size Distribution—The model F-value of 4.64 implies the model is significant. There is only a 0.28% chance that a “model F-value” this large could occur due to noise. The “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. The particle size distribution ratio of 9.903 indicates an adequate signal. This model can be used

to navigate the design space. It is shown that increasing oil:surfactant ratios lead to a smaller particle size (Fig. 2). ANOVA analysis values are shown in Supplemental Table 1. The calculated equation for particle size distribution is as follows.

$$\begin{aligned} \text{Particle size distribution} = & +202.00 + 49.33 * A + 11.83 * B - 18.08 \\ & * C - 2.92 * D - 3.87 * A * B - 18.00 * A * C + 0.25 * A * D + 4.88 * B \\ & * C - 5.12 * B * D - 6.25 * C * D + 57.65 * A^2 + 4.77 \\ & * B^2 + 2.02 * C^2 + 5.77 * D^2 \end{aligned}$$

Polydispersity Index—The model F-value of 3.23 implies the model is significant. There is only a 1.57% chance that a “model F-value” this large could occur due to noise. The “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. The PDI ratio of 7.562 indicates an adequate signal. This model can be used to navigate the design space.

$$\begin{aligned} \text{PDI} = & +0.45 + 0.089 * A - 0.010 * B - 0.027 * C - 0.014 * D - 0.042 * A \\ & * B + 6.438\text{E-}003 * A * C + 0.027 * A * D + 0.014 * B * C - 0.032 * B \\ & * D - 8.062\text{E-}003 * C * D + 0.071 * A^2 - 1.802\text{E-}003 * B^2 + 0.039 * C^2 + 9.448\text{E-}003 * D^2 \end{aligned}$$

Emulsification Time—The Model F-value of 9.15 implies the model is significant. There is only a 0.01% chance that a “Model F-Value” this large could occur due to noise. “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. This shows that as the oil:surfactant ratio increases the time required to obtain a clear suspension increases as well. The ET ratio of 12.608 indicates an adequate signal. This model can be used to navigate the design space.

$$\begin{aligned} \text{Emulsification time} = & +95.00 + 20.58 * A + 1.92 * B - 2.83 \\ & * C - 2.33 * D - 1.62 * A * B - 1.62 * A * C + 0.25 * A * D - 0.25 * B * C - 1.12 \\ & * B * D + 1.38 * C * D + 0.50 * A^2 - 1.62 * B^2 - 2.25 * C^2 - 0.37 * D^2 \end{aligned}$$

Percent Release—The Model F-value of 4.39 implies the model is significant. There is only a 0.36% chance that a “Model F-Value” this large could occur due to noise. “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. This shows the amount of drug released. The release ratio of 8.381 indicates an adequate signal. This model can be used to navigate the design space.

$$\begin{aligned} \% \text{ Release} = & +76.85 - 12.56 * A + 0.37 * B + 1.13 * C + 0.88 * D + 4.41 * \\ & A * B - 0.16 * A * C - 0.29 * A * D - 0.78 * B * C + 0.34 * B * D - 1.250\text{E-}003 \\ & * C * D - 2.38 * A^2 - 0.43 * B^2 - 1.76 * C^2 - 1.75 * D^2 \end{aligned}$$

CDMS Formulation Improved CDODA-Me Intestinal Permeability

CaCo-2 cells were used to model intestinal permeability *in vitro* due to their ability to form a high degree of tight junctions and expression of drug transporters and enzymes that

drastically limit their permeability. When compared to the drug suspension, CDMS provided increased permeability with rates of 0.211 ± 0.021 cm/s versus 0.131 ± 0.043 cm/s. Both rates showed strong linearity with r^2 values > 0.95 (0.9933 and 0.9589 for suspension and CDMS, respectively).

CDMS Formulations Improve CDODA-Me Oral Bioavailability

In vitro drug release showed (Fig. 3a) that CDMS steadily increased CDODA-Me release with 95% percent of the drug being released within 2 h. The suspension showed little membrane permeability with only 10% ($\pm 5.7\%$) of drug being released in the same time period. Pharmacokinetic parameters of CDODA-Me suspensions and CDMS are shown in Figure 4b and Table 2 after oral administration. CDMS formulations improved plasma drug concentrations for CDODA-Me when compared to the suspension. CDMS increased maximum plasma concentrations from 0.33 to 1.02 $\mu\text{g/mL}$. This shows an approximate 3-fold increase in absorption. The half-life was increased from 32 min to 175 min and the mean residency times from 69.87 to 246.14 min. This shows that CDMS not only showed an improvement in drug absorption but improved circulating times as well.

Combination Therapy Shows Synergistic Response Leading to Decreased Cell Viability

Resistant cells showed little response to ERL treatment. IC_{50} values for ERL treatment were 6.4 ± 2.03 μM , 18.7 ± 2.1 μM , 43.7 ± 3.1 μM , 36.7 ± 1.9 μM , 44.5 ± 2.3 μM , 16.3 ± 1.2 μM , and 46.68 ± 1.24 μM ERL for HCC827, HCC827-4 μM , HCC827-CL4, H1975, H1975-CL1, H460, and H1299, respectively compared to combination treatment IC_{50} values of 3.1 ± 1.4 μM , 6.9 ± 2.9 μM , 13.7 ± 3.1 μM , 18.8 ± 2.4 μM , and 19.2 ± 1.8 μM , 8.8 ± 2.3 μM , and 42.44 ± 1.2 μM ERL. All cell lines showed similar dose responses to CDODA-Me with IC_{50} values of 4.5 ± 1.6 μM , 4.4 ± 1.3 μM , 5.7 ± 2.04 μM , 6.2 ± 1.2 μM , 6.6 ± 1.1 μM , 5.5 ± 0.8 μM , and 5.4 ± 2.4 μM respectively (cell viability is shown in Figs. 5 and 6). Although resistant cell lines showed improved response, it is important to note that combination index values indicated strong synergism. The IC_{50} and combination index data are summarized in Table 3. After treating cells with drug-loaded CDMS, we observed that the formulation showed no effect on cell viability with IC_{50} values similar to the suspension (Fig. 7).

Combination Therapy Induces Apoptosis in Resistant Cell Lines

In Hoechst33342 staining, apoptotic cells exhibited bright fluorescence due to shrunken nucleus and fragmented chromatin, whereas normal and healthy cells showed lower fluorescence. The microscopic images in Figure 8 showed that the control cells have lower fluorescence, but the number of brightly stained cells was increased in all the treated groups. In both nonresistant and resistant cells, the combination of CDODA-Me and ERL treatment groups showed higher number of brightly stained cells than the control group. Histogram showed that approximately $21.68 \pm 1.8\%$, $64.23 \pm 1.7\%$, and $67.66 \pm 4.4\%$ apoptotic cells were present in CDODA, ERL, and combination, respectively, in HCC827 cells. Moreover, combination therapy percentages when compared to the ERL treatment with values of $54 \pm 7.7\%$ versus $11 \pm 2.9\%$, $39 \pm 5.6\%$ versus $8 \pm 2.5\%$, $43 \pm 4.5\%$ versus $15 \pm 3.7\%$, $44 \pm 3.3\%$ versus $9 \pm 2.7\%$, $62 \pm 2.4\%$ versus $5 \pm 4.3\%$ for HCC827-4 μM , HCC827-CL4, H1975, H1975-CL1, and H460, respectively.

Mitochondrial Membrane Potential Is Reduced as a Response to Combination Therapy

Rhodamine 123 staining was used to identify the changes in mitochondrial membrane potential in both treated and untreated cells. Decrease in green fluorescence intensity indicates decreased mitochondrial membrane potential. Figure 9 showed that in control groups, the fluorescence intensity was higher, which indicates healthy normal cells. The decrease in mitochondrial membrane potential was found highest in combination group than in both CDODA-Me and ERL single treatment for all resistant and nonresistant cells. Combination relative fluorescent intensities were found to be 0.044 ± 0.04 , 0.04 ± 0.03 , 0.13 ± 0.08 , 0.08 ± 0.09 , 0.07 ± 0.06 , 0.09 ± 0.01 for HCC827, HCC827-4 μ M, HCC827-CL4, H1975, H1975-CL1, and H460, respectively, compared to ERL single treatment values of 0.062 ± 0.06 , 1.03 ± 0.08 , 1.15 ± 0.1 , 1.03 ± 0.09 , 0.84 ± 0.22 , and 0.843 ± 0.643 also, respectively. $p < 0.001$ was obtained for all cell lines excluding HCC827. Histogram shows (Fig. 10) the relative decrease in fluorescence intensity levels in different treatment groups in both resistant and nonresistant HCC827 cells.

Intracellular ROS Accumulation Increases in Cells Treated With Combination

DCFDA staining identifies intracellular ROS production in the treated cells (Fig. 11). Microscopic images and relative fluorescence intensity histogram showed that combination therapy increased fluorescence when compared to ERL treatment with values of $61 \pm 6.2\%$, $8 \pm 5.8\%$, $11 \pm 9.15\%$, $6 \pm 4.9\%$, $73 \pm 6.22\%$, and $18 \pm 8.4\%$ for HCC827, HCC827-4 μ M, HCC827-CL4, H1975, H1975-CL1, and H460, respectively, compared to combination treatment values of $82 \pm 7.04\%$, $84 \pm 8.03\%$, $81 \pm 11.08\%$, $83 \pm 9.09\%$, $74 \pm 10.07\%$, and $70 \pm 7.10\%$ also, respectively.

Colony-Forming Ability of Cells After Treatment Is Greatly Reduced After Combination Treatment

Re-seeding cells after treatment allowed us to model the long-term effects treatment had on surviving cells. Clonogenic assays showed that combination treatment decreased the colony number (Fig. 12). Each cell line showed a decrease in colony formation compared to the single treated cells when treated with the combination. ERL treatment decreased colony formation in HCC827 cells ($p < 0.001$), HCC827-4 μ M ($p < 0.001$), and H460 ($p < 0.001$) cells, whereas only combination treatment showed these effects in HCC827-CL4 ($p < 0.001$), H1975 ($p < 0.001$), and H1975-CL1 ($p < 0.001$).

Combination Therapy Decreases Kinase Activation Inhibiting Growth Signaling

Western blot analysis showed that treatment had no effects on total EGFR in all cell lines. P-EGFR expression was significantly decreased in ERL ($p < 0.01$) and combination treatment ($p < 0.05$) groups in HCC827. In HCC827-4 μ M cells, only combination therapy induced significant effects decreasing p-EGFR expression as well ($p < 0.001$). The same trend was observed in HCC827-CL4, H1975, and H460 cell lines. Although both ERL and combination treatment appeared to decrease p-EGFR expression in H1975-CL1 cells, this change was not significant. Treatment also showed no effects on total MET expression in all cell lines. Combination treatment decreased expression of p-MET in HCC827 ($p < 0.001$), HCC827-4 μ M ($p < 0.001$), HCC827-CL4 ($p < 0.01$), H1975 ($p < 0.01$), and H1975-CL1 (p

< 0.001). ERL also decreased p-MET expression in H1975 ($p < 0.01$), H1975-CL1 ($p < 0.001$), and HCC827-CL4 ($p < 0.01$). This is summarized in Figure 13.

Discussion

Targeted therapy is one of the profound NSCLC treatment modalities which elicits initial tumor responses in patients but EGFR mutations in ~50% NSCLC patient limits the success of this therapy.²⁰ The high degree of molecular mutations makes it possible for cancer cells to evade apoptosis as well as adapt too many clinically effective treatments. The development of ERL was a major break-through in the treatment of non-small cell lung cancer with over 80% of patients carrying the EGFR mutation that it targets showing decreased tumor growth, but unfortunately, the nature of the cells led to the development of resistance.³ We have for the first time not only shown that combining an ERL treatment regimen with a nontoxic concentration of CDODA-Me could overcome resistance but also that employing the use of CDMS could improve the oral bioavailability of CDODA-Me.

The oral route of administration is usually preferred on account of its high degree of patient compliance as well as its elimination of expenses associated with physician-assisted dosing commonly coupled with many chemotherapeutic agents. We have focused on the development of an oral dosage form of CDODA-Me that could be prescribed alongside the commercially available Erlotinib oral dosage form (Tarceva). Because CDODA-Me is very lipophilic in nature, this makes it difficult to formulate it for oral administration while maintaining bioavailable concentrations to provide effective treatment. For such compounds, the dissolution of the drug is the limiting step for absorption of the drug from the GI tract. Self-nanoemulsifying drug delivery systems have gained much interest in recent years in efforts to reduce dose frequency and maximum drug absorption.

Surface response methodology showed that particle size was inversely related to the surfactant percentages contained in the formulation while it was directly related to oil phase percentages. Previously published literature relates a smaller particle size to increased SNEDDS stability and higher drug release percentages.^{13,15,17} It has also been reported that when formulating SNEDDS, it is important to limit the surfactant concentrations to reduce the incidence of GI irritation while remaining within the 30%–70% w/w range required to form a stable nano-emulsion.²¹ This study also showed that drug release decreased in response to decreased oil:surfactant ratios. Modeling for the effect of oil and surfactant phases on particle size, polydispersity, ionization, percent drug released, and percent drug entrapped all showed F-values greater than 4.0 indicating that the models are accurate and not influenced by the presence of noise. Longer emulsification times slightly increased drug loading. *In vitro* permeability studies showed that CDMS formulations doubled permeability for CDODA-Me when compared to drug suspensions. This trend was also observed *in vivo* with pharmacokinetic studies showing that CDMS both improved plasma drug concentrations and doubled the half-life of CDODA-Me.

Cell viability studies showed that CDODA-Me at nontoxic doses showed -synergism when used in combination with ERL reducing the viability in both resistant and nonresistant cells. Killing of ERL-resistant cells by combination of ERL and CDODA-Me suggested the

possibility of using CDODA-Me to overcome ERL resistance in NSCLC. Usually, higher concentrations of drugs are needed to kill drug-resistant cells than nonresistant cells. Our data confirm higher IC₅₀ values for both ERL and ERL-CDODA-Me combination in all the resistant cell lines than nonresistant cells. Killing of both resistant and nonresistant cells was further supported by fluorescence imaging which showed an increase in apoptosis in combination treatment than ERL and CDODA-Me alone. Based on our cytotoxicity and apoptosis assay, 12µM ERL and 2µM CDODA-Me were selected for further studies.

Cell viability studies were performed on a number of NSCLC cell lines. HCC827 cells were either sensitized to erlotinib (HCC827 harboring a mutation within EGFR tyrosine kinase domain E746 - A750 deletion) or resistant (HCC827-4µM ERL [erlotinib acquired resistant] and HCC827-CL4 [EGFR T790M mutation harboring]). When comparing the response of ERL, single treatment resistant cells showed decreased response with a 7-fold increase in IC₅₀ concentration for HCC827-CL4 compared to HCC827. Combination treatment was able to improve treatment response in each cell line showing a 2-fold decrease in IC₅₀ values of HCC827. Combination treatment also improved response giving HCC827-4µM an IC₅₀ value comparable to that of HCC827 thereby overcoming resistance as well as showing a 3-fold decrease in IC₅₀ value of HCC827-CL4 showing cells to be more sensitive.

H1975 cells each were resistant to ERL on account of the presence of the T790M EGFR mutation, but H1975-CL1 cells were previously cultured to have acquired resistance to the irreversible EGFR TKI rociletinib. Both cell lines showed similar responses to ERL single treatment with high IC₅₀ values but the combination treatment showed a 2-fold increase in response. H1299 and H460 cell lines were used as controls because they did not have EGFR mutations (ERL sensitizing/resistant). H1299 has a p53 deletion and H460 has a KRAS mutations that caused it to be over activated. ERL treatment showed varying responses across all cell lines with H1975 cells showing the highest degree of resistance. This was to be expected as they have the T790M EGFR mutation that inhibits ERL activity. This response was comparable to the EGFR T790M mutated HCC827-CL4 as well. H1299, a cell line that was not confirmed to have ERL-sensitizing EGFR mutations, behaved similarly as well. It is also interesting to note that all cell lines showed similar responses to CDODA-Me single treatment. Furthermore, 2 µM CDODA was found to show <20% cell viability in all cell lines. This concentration was chosen because it was the IC₂₀ in the resistant HCC827 cells. Studies were previously performed with 0.5 µM and 1 µM, but those concentrations showed no significant differences when compared to ERL single treatment. The lowest concentrations of the combination showed cell viability less than one because although the concentration of ERL is decreasing the concentration of CDODA-Me remained at a constant 2µM even as ERL became zero.

The combination of ERL and CDODA-Me to improved response showed varied effects sensitizing all cells with EGFR mutations, whether they are ERL-sensitizing or ERL-resistant mutations. The combination treatment also showed improved response in KRAS-mutated H460 cells. Previously reported literature has identified KRAS upregulation as a key contributor to EGFR targeting TKI resistance, but little work has been done on studying the effects of this upregulation in inducing response in cells that have not been shown to have sensitizing EGFR mutations. A recent study conducted by Zou et al.²² showed that

H460 cells with wild-type EGFR showed increased response to ERL when combined with low doses of chloroquine through the inhibition of cytoprotective autophagy pathways. Eberlein and associates²³ found that H1975 cells resistant to gefitinib treatment had a KRAS copy number gain when compared to wild-type and the combination of MEK inhibition sensitized cells to treatment.

Most research on CDODA-Me has been focused on its molecular influences on cell death and angiogenesis through its effect on Sp proteins.²⁴ It has previously been shown that Sp protein regulation of CDODA-Me is influenced by the induction of intracellular ROS accumulation²⁵ in bladder cancer. Previous literature has reported that long-term treatment with EGFR targeting tyrosine kinase inhibitors leads to increased ROS that drives the epithelial to mesenchymal cellular transition producing more aggressive cancer types.²⁶ Resistant cells maintained higher levels of ROS under normal untreated conditions and CDODA-Me alone increased the ROS levels in the resistant cells as well. Resistant cells also displayed higher migratory ability indicative of EMT which was also decreased upon exposure to combination therapy. This increase in ROS is maintained and stabilized through a process referred to as a prooxidant shift which leads to re-programming cellular activity to better tolerate prooxidant conditions. Overcoming this transformation was achieved by increasing oxidative stress above the toxicity threshold, inducing the intended cell death.^{27,28} Thus, our data have shown that combination therapy effects on migration could possibly be dependent on the accumulation of ROS.

In the present experiment, combination of CDODA-Me and ERL showed higher ROS levels than individual dose in both resistant and nonresistant cells. Present data showed a decrease in mitochondrial membrane potential by the combination of ERL and CDODA-Me in both resistant and nonresistant cell lines. Increased mitochondrial activity drives the increased cellular metabolism that drives the invasive and aggressive nature of cancer cells. It is also a hallmark pathway for cells to overcome apoptotic signaling leading to increased proliferation. A decrease in mitochondrial membrane potential leads to decreased mitochondrial activity as well as an increase in the release of cytochrome-c which is one of the major driving forces behind apoptotic signaling.²⁹

Janmaat et al. (2003)³⁰ observed that increased colony-forming ability is indicative of ERL resistance and it can thus be suggested that inhibiting this increased colony formation could also be characteristic of reversing resistance. When measuring the colony-forming ability in resistant cells, they initially formed a higher number of colonies than the nonresistant cells, but after combination treatment, the number of colonies not only decreased but also was comparable to those of the nonresistant cells.

In recent years, combination therapy has gained interest as a way to illicit higher tumor response increasing survival rates for a number of cancer types. Our lab has focused on NSCLC combination treatments in the past as well.^{31–34} This trend remains evident in the case of EGFR mutated NSCLC as well. The combination of cetuximab (also an EGFR inhibitor) and with nontoxic doses of STAT inhibitor CYT387 showed synergistic effects in resistant NSCLC as well.³⁵ Statistical analysis on clinical results has shown that patients suffering from EGFR point mutations eliciting erlotinib sensitivity or resistance could

particularly benefit from combination therapy showing increased remission periods.³⁶ These results served as a driving force to test the effects of CDODA-Me and erlotinib combination therapy in resistant NSCLC.

CDODA-Me has been previously shown to decrease Sp protein expression which is involved in the regulation of transcription factors responsible for many of the growth signaling pathways cancer cells take advantage of. Because of Sp proteins role in growth and maturation, they have been found to be overexpressed in many cancer types. Through silencing Sp transcription factors, it was found that VEGFR and EGFR signaling decreased causing a decrease in angiogenesis.³⁷ Because of this, we predicted that CDODA-Me when combined with ERL would prove to be a promising treatment regimen.

NSCLC develop acquired resistance to ERL by 2 different mechanisms which includes mutation of so-called “gatekeeper” T790M residue and amplification of MET. Previous studies reported that mutation in T790M residue prevents hydrogen bonding with the inhibitor and allows the retention of ATP binding with the kinase which results T790M mutation mediated resistance.² In addition, amplification of MET in EGFR mutant lung cancer reactivates downstream PI3K/AKT signaling pathways which drives resistance to kinase inhibitor.⁹ Hence, despite continuous inhibition of EGFR by ERL, downstream pathway reactivation emerges resistance to ERL. Therefore, current research and clinical studies are focused on dual inhibition of EGFR and MET. To identify and confirm the pathways involved in overcoming drug resistance by CDODA-Me and ERL combination treatment, the expression of p-EGFR, p-Met were studied in treated cells. Our western blot data showed that in ERL resistant cells, the expression of p-Met was higher in ERL-alone group than CDODA-Me and significant downregulation of p-MET was observed in the combination group. Moreover, downregulation of p-EGFR was observed in combination treatment than CDODA-Me or ERL alone. Downregulation of p-Met and p-EGFR expression in combination treatment indicates the inhibition of both Met and EGFR signaling by CDODA-Me and ERL combination treatment which concurred with the previous reports and suggested a possible mechanism to overcome ERL resistance in NSCLC. As stated earlier, 2 known mechanisms of ERL resistance include increased affinity of the receptor for ATP and upregulation of MET. The receptors increased affinity for ATP allows the receptor to reduce interactions with ERL thereby preventing its inhibitory effects. Upregulation of MET allows the cell to decrease its dependence on the EGFR receptor while still activating necessary pathways due to the overlapping nature of the 2 receptors. CDODA-Me alone showed no effect on MET or EGFR expression levels but decreased the levels of those proteins in their phosphorylated forms when treated with the combination. The same activity trend was observed by Pang et al.³⁸ when they discovered that CDODA-Me inhibits angiogenesis through decreased activity rather than decreased expression of VEGFR, another Sp regulated tyrosine kinase receptor. They also reported that CDODA-Me showed greater growth inhibition of endothelial cells at angiogenic phases compared to those grown in normal culture treated with the same dose. This was also observed in our resistant NSCLC which showed a higher fold increase in response at the same dose compared to nonresistant cells.

Protein kinase inhibitors such as erlotinib have elicited significant response in patients suffering from oncogene addiction caused by uncontrolled activation of proteins such as the epidermal growth factor receptor. Uncontrolled activation of other kinase proteins such as MET, BCR-ABL, and platelet-derived growth factor receptor has also been shown to lead to oncogene addiction that has led to the development of tyrosine kinase inhibitors, tivantinib, dasatinib, and sorafenib to target them, respectively. Mechanisms of resistance to these inhibitors have also been shown to be driven by the cancers ability to switch dependence to a number of overlapping kinase pathways. We believe that not only can CDODA-Me serve as an erlotinib sensitizer but shows potential to aid in overcoming the resistance of these molecules as well.

Conclusions

We have shown that combining glychyrrhetic acid derivative, CDODA-Me, with ERL could improve cell response in both resistant and nonresistant NSCLC inducing apoptosis and inhibiting the phosphorylation of Met and EGFR preliminarily indicating a synergism between these 2 pathways. Although we previously predicted that this synergistic effect would contribute to CDODA-Me's known regulation of EGFR and MET expression, our results show that this compound prevents the activation of these kinase receptors showing no effect on actual expression levels. This leaves the exact mechanism of induced response in resistance cell lines requiring more experimentation to be further understood. We have also found that combination treatment had the ability to sensitize NSCLC cell lines that did not have EGFR sensitizing mutations. Although this has been previously shown in the literature the mechanism behind this sensitization remains unconfirmed as well. On the other hand, cell viability assays have shown that 48 h treatment with erlotinib in combination with CDODA-Me at nontoxic doses showed response in resistant cell lines comparable to erlotinib single treatment in responsive NSCLC cells. This study only focused on Met and EGFR activity as a mode of resistance leading the investigation to other known mechanisms imperative to completely understand the molecular basis of this improved response. We have also shown that self-nanoemulsifying delivery systems could improve the oral bioavailability of this proposed treatment regimen increasing both absorption and circulation of CDODA, but the effect of this formulation on the treatment of tumors *in vivo* has yet to be quantified. In conclusion, this study shows that CDODA-Me/ERL SNEDDS could prove to be a promising cancer treatment through the induction of apoptosis mediated by inhibiting the phosphorylation of EGFR and MET in resistant and nonresistant NSCLC.

Acknowledgments

This project was supported by the Research Center in Minority Institute (RCMI) 2454MD007582-34A1 U54 grant and FAMU CREST Center for Complex Designs for Multidimensional Printing (Award number 1735968).

Abbreviations used:

AKT	protein kinase b
ANOVA	analysis of variance
CDMS	CDODA-Me-loaded SNEDDS

CDODA-Me	methyl 2-cyano-3, 11-dioxo-18b-olean-1, 12-dien-30-oate
DC	drug content
DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
DPBS	Dulbecco's phosphate-buffered saline
ECL	enhanced chemiluminescence
EGFR	epidermal growth factor receptor
ERL	erlotinib
ET	emulsification time
HBSS	hanks buffered saline solution
HLB	hydrophobic lipophilic balance
HPLC	high-performance liquid chromatography
IC₅₀	half the maximum inhibitory concentration
LOD	limit of detection
LOQ	limit of quantification
MET	met receptor tyrosine kinase
NSCLC	non-small cell lung cancer
PDI	polydispersion index
PSD	particle size distribution
PVDF	polyvinylidene fluoride
QbD	quality by design
R.S.D	relative standard deviation
RFI	relative fluorescent intensity
ROS	reactive oxygen species
RSM	response surface methodology
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNEDDS	self-nanoemulsifying drug delivery system
Sp	specificity protein

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Figure 1.

Visual schematic representing the SNEDDS formulation process. Drug is solubilized in the oil phase followed by the addition of surfactants at 50°C under mild agitation. Upon the addition of aqueous phase (water) components naturally assembled into a stable emulsion.

Figure 2.

3D response surface plots for the influence of oil phase to surfactant ratios on (a) particle size distribution, (b) drug loading, (c) emulsification time, and (d) %drug released. This ratio was found to be directly proportional indicating increased oil phase allows for higher drug loading and require higher emulsification time while decreased oil phase also allows for smaller particle size.

Figure 3.

Plasma concentration curves (a) as well as drug release profiles (b) comparing CDODA-SNEDDS to CDODA-Me drug suspensions. SNEDD formulations both increased drug release *in vitro* as well as drug absorption *in vivo* as shown above. Animals were dosed 80 mg/kg CDODA-Me (3 mL drug suspension and SNEDDS). A dose of 2 mg/mL CDODA-Me was tested for drug release this is correlated to be an 8.06 mM dose of CDODA-Me. Animals were dosed 80 mg/kg CDODA-Me (3 mL drug suspension and SNEDDS) for PK studies; this correlates to a dose range of 2.6–2.8 mM CDODA-Me.

Figure 4.

Overlay plot produced as a result of optimizing formulation parameters such as minimizing surfactant content to provide increased drug loading, decreased particle size, and increased drug release. Highlighted regions show the optimal range of SNEDD components.

Figure 5.

Effect of erlotinib and CDODA-ME on cell viability. Cells were treated with the indicated concentrations of erlotinib, CDODA-Me, and erlotinib in combination with a constant concentration of 2 μ M CDODA for 48 h. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells (100%).

Figure 6.

Effect of CDODA-Me on cell viability. Cells were treated with the indicated concentrations of CDODA-Me for 48 h. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells (100%).

Figure 7.
Effects of SNEDDS combination treatment in comparison to the combination suspensions. Cells were treated with the indicated concentrations of ERL in combination with 2 μ M CDODA-Me for 48 h. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells (100%).

Figure 8.

Cell apoptosis morphologic changes were examined by Hoechst33258 staining and observed under a fluorescence microscope at 200 magnification. The apoptotic cells detected by the fluorescence microscopy displayed condensed and fragmented nuclei. Combination therapy increased the observance of apoptosis in all cell lines. Cells were treated with CDODA-Me (2 μ M), ERL (12 μ M), and the combination of these concentrations.

Figure 9.

The effects of treatment on mitochondrial membrane potential indicated by decreased fluorescent intensity after staining with rhodamine123. Cells were again observed through fluorescent microscopy. Combination shows increased depletion of membrane potential in all cell types. Cells were treated with CDODA-Me (2 μ M), ERL (12 μ M), and the combination of these concentrations.

Figure 10.

Graphical data corresponding with apoptosis staining, mitochondrial membrane potential staining, and reactive oxygen species staining. Significance shown is relative to control measurements. p values represented as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

Figure 11. CDODA-Me (2 μ M) and ERL (12 μ M) single treatment and combination-induced reactive oxygen species (ROS). Cells were treated for 48 h 2, 7-dichlorofluorescein (DCF) fluorescence was visualized by fluorescence microscopy. ROS is indicated through the presence of brightly stained cells displaying rounded morphology.

Figure 12.

Clonogenic assay results. Cells were treated for 48 h with 2 μ M CDODA-Me, 12 μ M erlotinib, and a combination of the 2; trypsinized; and reseeded in a 6-well plate at a cell density of 100 cells/well. Image was taken 5 d after reseeding. Cells were fixed in 2.5% glutaraldehyde and stained with crystal violet for imaging (a). Histogram represents the number of colonies counted for each treatment group (b). Significance shown is relative to control measurements. p values represented as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

CDODA-Me, (3) 12 μ M Erlotinib, (4) 2 μ M CDODA-Me + 12 μ M erlotinib on receptor kinase expression as well as apoptotic and survival markers. Histograms display density analysis ratios of protein expression as normalized by beta actin. Significance is shown relative to control (untreated cells). p values represented as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

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Table 1

Batches Generated Through Design Expert With Actual Particle Size Distribution (PSD), Polydispersity Index (PDI), Emulsification Time (ET), Drug Content (DC), and Drug Release (Release)

Run	Formulation Variables Levels				Response				
	Oil Phase (% w/w)	Surfactant (% w/w)	Cosurfactant (% w/w)	Cosolvent (% w/w)	PSD (nm)	PDI	ET (Sec)	DC (%)	Release (%)
1	30	25	12	12	300	0.8	120	84.45	45.5
2	30	25	4	4	352	0.74	124	80.54	47.5
3	21	40	8	8	245	0.64	105	60.45	62.2
4	12	25	12	12	160	0.32	58	98.24	96.4
5	21	40	8	8	202	0.62	95	85.58	76.8
6	30	55	12	12	348	0.71	118	74.52	55.2
7	12	55	12	12	218	0.57	72	78.54	80.5
8	21	40	8	8	202	0.62	95	85.58	76.8
9	21	40	16	16	201	0.57	85	86.54	70.5
10	21	40	8	8	202	0.62	95	85.58	76.8
11	21	10	8	8	224	0.65	90	70.54	68.5
12	12	25	12	12	150	0.31	61	97.57	95.4
13	12	25	4	4	194	0.47	70	85.47	87.8
14	3	40	8	8	452	0.74	70	56.41	74.5
15	30	55	4	4	401	0.71	124	74.52	55.2
16	12	55	4	4	200	0.51	72	80.54	86.5
17	21	40	8	8	202	0.62	95	85.58	76.8
18	21	40	8	8	202	0.62	95	85.58	76.8
19	12	25	4	4	180	0.42	60	95.41	92.5
20	39	40	8	8	456	0.81	132	74.52	55.2
21	12	55	12	12	218	0.37	68	85.54	84.5
22	21	70	8	8	261	0.62	95	85.58	76.8
23	30	55	12	12	256	0.51	95	74.52	65.2
24	30	55	4	4	372	0.61	115	78.52	61.2
25	30	25	12	12	286	0.61	98	84.52	58.2
26	30	25	4	4	365	0.71	105	74.52	54.2

Run	Formulation Variables Levels				Response			
	21	40	8	8	248	0.52	90	87.58
27	21	40	8	8	248	0.52	90	87.58
28	12	55	4	4	184	0.51	68	84.51
29	21	40	0	0	262	0.82	95	75.21
30	21	40	8	8	202	0.62	95	85.58
								72.5
								81.5
								64.2
								76.8

Table 2

Calculated Pharmacokinetic Parameters After Oral Dosage of CDODA-Me SNEDDS and Suspension Using Noncompartmental Analysis

Parameter	Unit	SNEDDS	Suspension
$t^{1/2}$	min	175.31	32.77
Tmax	min	30.00	30.00
Cmax	µg/mL	1.02	0.33
AUC _{0-t}	µg/mL *min	97.39	21.64
AUC _{0-Inf}	µg/mL *min	151.76	22.13
MRT _{0-inf}	min	246.14	69.87
V _{z/f}	(mg)/(µg/mL)	33.33	17,625.55
Cl/f	(mg)/(µg/mL)/min	0.13	37.28

SNEDD formulation showed decreased clearance as well as increased absorption. Animals were dosed 80 mg/kg CDODA-Me (3 mL drug suspension and SNEDDS).

Table 3

Summarized Cytotoxicity Data After 48 h Treatment With Erlotinib and CDODA-Me Alone and in Combination

Cell Line	ERL IC ₅₀	CDODA-Me IC ₅₀	ERL + CDODA-Me IC ₅₀	Combination Index
HCC827	6.43 ± 2.03	4.5 ± 1.6	3.1 ± 1.4	0.89 ± 0.11
HCC827-4uM	18.7 ± 2.1	4.4 ± 1.3	6.9 ± 2.9	0.64 ± 0.06
HCC827-CL4	43.7 ± 3.1	5.7 ± 2.04	13.7 ± 3.1	0.67 ± 0.04
H1975	36.7 ± 1.9	6.2 ± 1.64	18.8 ± 2.1	0.66 ± 0.03
H1975 CL1	44.5 ± 2.3	6.6 ± 1.1	19.2 ± 1.8	0.61 ± 0.06
H460	16.3 ± 1.2	5.5 ± 0.8	8.9 ± 2.3	0.92 ± 0.04
H1299	46.7 ± 1.2	5.5 ± 2.4	42.4 ± 1.2	0.95 ± 0.12

Combination index values less than 1 are indicative of synergism and values less than 0.5 indicate strong synergism. Combination therapy showed a higher degree of synergism in resistant cells although some degree of synergism is observed in all cell lines.