Published in final edited form as:

Surgery. 2016 January ; 159(1): 284–295. doi:10.1016/j.surg.2015.08.023.

sHDL Nanoparticles: A Novel Therapeutic Strategy for Adrenocortical Carcinomas

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Abstract

Background—Chemotherapeutic strategies for adrenocortical carcinoma (ACC) carry significant toxicities. Cholesterol is critical for ACC cell growth and steroidogenesis and ACC cells over-express scavenger receptor BI (SR-BI) that uptakes cholesterol from circulating high-density lipoprotein (HDL). We hypothesize that cholesterol-free synthetic-HDL nanoparticles (sHDL) will deplete cholesterol and synergize with chemotherapeutics to achieve enhanced anticancer effects at lower (less toxic) drug levels.

Methods—Anti proliferative efficacy of ACC cells for the combinations of sHDL with chemotherapeutics was tested by cell-Titer Glo. Cortisol levels were measured from the culture media. Effect on steroidogenesis was measured by RT-PCR. Induction of apoptosis was evaluated by flow cytometry.

Results—Combination-Index (CI) for sHDL and either etoposide(E), cisplatin(P) or mitotane(M) demonstrated synergy (CI<1) for anti-proliferation. sHDL alone or in combination with chemo drugs was able to reduce cortisol production by 70-90% compared to cisplatin alone or controls (p<0.01). RT-PCR indicated significant inhibition of steroidogenic enzymes for sHDL (p<0.01 vs. no sHDL). Combination therapy with sHDL increased apoptosis by 30-50% compared to drug or sHDL alone (p<0.03) confirmed by mitochondrial potential decrease.

Conclusion—sHDL can act synergistically and lower the amount of M/E/P needed for anticancer efficacy in ACC in part due to cholesterol starvation. This novel treatment strategy warrants further investigation translationally.

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Introduction

Adrenocortical carcinoma is a rare endocrine malignancy (approximately 500 new cases per year in the US) that carries a poor prognosis with advanced disease (1). Unfortunately, a majority of patients will present with advanced disease at the time of diagnosis and once metastatic, the disease has a low 10-20% five-year survival (2). For patients with metastatic disease, the only current FDA approved therapeutic is the adrenolytic agent mitotane, with initial response rates of 20-30% in advanced ACC patients and an improvement in survival rate from 14-50 months (3). Recent years have evaluated mitotane in combination with cytotoxic chemotherapeutics as in the Italian protocol, (etoposide, doxorubicin, cisplatin; EDP) or with streptozotocin (4, 5). EDPM has been shown to carry a higher response rate (23.2% vs. 9.2%) and progression free survival (5.0 months vs. 2.1 months) compared to mitotane with streptozotocin (6-8). Dose-limiting toxicities such as adrenal insufficiency, dizziness, vertigo, central nervous disturbances, hyperlipidemia, and gastrointestinal disorders remain a significant issue with both mitotane and cytotoxic agents given in combination (4). Given this toxicity in combination, development of novel drugs that have the ability to synergize with these agents could allow lower concentrations needed to achieve the same therapeutic effect and potentially mitigate some toxicity.

Normal adrenal and ACC cells require cholesterol for steroidogenesis and are known to express the scavenger receptor class B type I (SR-BI) on their surface to obtain cholesterol esters from circulating HDL (9). This SR-BI receptor is highly over-expressed in ACC cells and several other cancers (breast, prostate, ovarian, lymphoma, nasopharyngeal carcinoma) compared to normal tissues. SR-BI receptors act as bidirectional cholesterol transporters that facilitate uptake of cholesterol into cells and efflux the cholesterol out of cells (10). Since cholesterol transport is an important biologic function of cells including cancer cells, mimetic sHDL nanoparticles that bind to SR-BI recently have come under focus as a novel approach for targeting cancer (11, 12). There are a number of cholesterol-free sHDL products that have been clinically tested for the treatment of atherosclerosis by facilitation of reverse cholesterol transport (RCT) and found to be safe at high doses of 20-40 mg/kg per infusion (13). Many advanced ACC patients will develop steroid over secretion (14). Since these steroids require cholesterol (15), an agent that effluxes cholesterol from cells may have therapeutic benefit in reducing this over secretion functionality. In the current study, we use cholesterol free sHDL nanoparticles and hypothesize that they may be able to generate anti-cancer properties by depleting cholesterol from ACC cells, which could synergize with chemotherapy drugs from the Italian protocol and thereby create novel combination strategies that may lower doses of these cytotoxic drugs needed to achieve the same anticancer benefit.

Methods

Cell lines

Two human ACC cell lines authenticated using genetic finger printing, NCI-H295R (cortisol secretor) and SW13 (non-steroid secretor), were grown in 2D culture in humidified atmosphere of 5% CO₂ in air at 37°C. SW13 cells were grown in Dulbecco Modified Eagle's Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal

bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY). NCI-H295R cells were grown in DMEM-Ham's F12 nutrient medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO), 1% insulin/transferrin/selenium (ITS) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY).

Preparation and characterization of sHDL

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 22A peptide (16) (weight ratio = 1:1:1) were dissolved in glacial acetic acid, which was removed by freeze-drying. Phosphate buffered saline (PBS pH = 7.4) was added to the freeze-dried powder, which then was cycled 3 times between 50°C (3min) and 20°C (3min) with gentle shaking to obtain the sHDL. sHDL purity was analyzed by gel permeation chromatography (GPC). The sHDL was further characterized by transmission electron microscopy (TEM). All images were acquired on JEM 1200EX electron microscope (JEOL USA) equipped with an AMT XR-60 digital camera (Advanced Microscopy Techniques Corp).

Cell viability assay and calculation of combination index

SW13 and NCI-H295R cells were seeded into 96 well plates in triplicate and were treated with varying concentrations of either chemotherapeutic drugs (cisplatin, doxorubicin, etoposide, mitotane or EDPM combination) alone, in simultaneous combinations with sHDL, or sHDL alone for 72 h. A large dose range was initially used to define a more selective dose range for IC₅₀ experiments. Serial dilutions were made from the starting concentrations and viability of cells was then measured based on quantification of the ATP levels after treatment with CellTiter-Glo luminescent assay reagent as per the manufacturer's instruction (Promega, Madison, WI) with luminescence quantified using a BioTek Synergy Neo plate reader (BioTek, Winooski, VT). Cell viability ratios were calculated using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) and the combination index (CI) was calculated using Chou-Talalay equation (16) using CompuSyn software (ComboSyn Inc., Paramus, USA). The CI values of less than 1, equal to 1 and greater than 1 represent synergistic, additive and antagonistic effects, respectively. For all cell-based experiments, the experimental control group was either untreated cells or cells treated with single drug alone (when comparing to combination regimens), unless otherwise stated.

Colony formation assay

NCI-H295R and SW13 cells were plated in 6-well plates and allowed to attach. Treatment commenced for 24 h with drug alone or in combination with sHDL (50µg of sHDL in terms of 22A peptide/ml). Untreated or sHDL alone-treated cells were controls. The medium was changed and surviving cells were allowed to grow colonies of 50 cells or more for two weeks, washed, fixed, and stained with coomassie blue and counted. Total colony numbers were normalized to untreated controls.

Analysis of apoptosis by flow cytometry

To analyze combination effect on apoptosis, SW13 and NCI-H295R cells grown in 60 mm plates were treated with either E, P, M alone or in combination with sHDL for 24 h. Following treatment, cells were washed, re-suspended in annexin binding buffer and stained using annexin V-FITC/Propidium iodide as previously described (17). Induction of apoptosis was measured using the CyAn ADP Analyzer (Beckman Coulter, Inc., Indianapolis, IN) at the University of Michigan Flow Cytometry Core.

Mitochondrial membrane potential

SW13 and NCI-H295R were seeded in a 96 well black wall plate. Once attached, they were treated with the drugs as described above. 24 h post drug treatment, 500 nM tetramethylrhodamine, ethyl ester (TMRE) was added, the cells were incubated for 20min at 37°C, and the fluorescent signal was measured after washing using a microplate reader (excitation=549, emission=575). 100nM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) was added to cells 10min before the addition of TMRE as a negative control.

Immunoassay for cortisol measurement

The cortisol immunoassay (Alpco, Salem, NH) was used to quantitate cortisol levels in the culture supernatant of steroid producing NCI-H295R cells after treatment with drug combinations (same as in clonogenic assay) for 24 h per the manufacturer instructions. Briefly, culture supernatant after treatment was added to the antibody coated-plates containing assay buffer (45min at 25°C). After washing, tetra methyl benzidine (TMB) substrate was incubated at RT and absorbance was measured using a Synergy Neo reader (BioTek, Winooski, VT).

mRNA isolation and real-time (RT)-PCR

RNA from the NCI-H295R cells after drug treatment for 24 h was prepared using Qiagen RNA isolation kit (Qiagen Sciences, Valencia, CA). Approximately 500 ng of RNA was reverse transcribed using superscript RT kit from Life Technologies (Grand Island, NY). qPCR was performed in a step-one RT PCR machine using the gene specific primer sets (Table 1A) as published (18). Relative gene expression levels were calculated after normalization with internal controls. SR-BI expression level in several cancers was confirmed by Western-Blot Analysis (Table 1B).

Three-dimensional Multicellular aggregates (MCAs) treatment

To evaluate the translational potential of the combination therapy, MCAs were developed to mimic the *in vivo* tumor model as described by Jain *et al.* (19). Approximately 50,000 SW13 or 100,000 NCI-H295R cells were plated in 24-well ultralow attachment plates (Corning, NY, USA) to generate MCAs. Once MCAs were generated, they were treated with drugs (concentrations specified in our colony formation assay) with or without 50µg/ml sHDL nanoparticle for 24 h. Untreated and sHDL alone treated cells served as controls. The MCAs were photographed before and after treatment and the MCAs were quantified by Image J software (NIH) (20) as described by Jain *et al.* (19).

Statistical Analysis

All experiments were done in triplicate and the values are presented as mean $\pm$ SEM. Comparisons of differences between 2 or more means were determined by the Student unpaired *t* test (2 means) and the Fisher exact test. More than 2 means were analyzed by 2-way analysis of variance followed by the Duncan multiple range test (2 + means) and Bonferroni post hoc testing via a SPSS version 17.0 (SPSS, Inc, Chicago, IL). Significance was defined as a *P* value < 0.05.

Results

Our experimental design was to first examine the viability of cells after treatment with combination therapy to determine if synergy of sHDL with EDPM is possible. As such, we first wanted to see if

sHDL nanoparticles enhance the antiproliferative effect of E, P and M in ACC cells

Cell-Titer-GLO viability results showed inhibition of cell proliferation in a dose dependent manner for each of the chemotherapeutics drugs as expected (Fig.1 A [NCI-H295R] and B [SW13]). However, sHDL nanoparticles alone did not induce significant cell death at normal concentrations but only at high concentrations of 100-200 μ g/ml of the 22A peptide (Fig.1 A and B **left**). To determine if combining sHDL nanoparticles and the chemotherapeutic drugs results in synergy or an additive effect, we then calculated the combination index after treating the cells at different combination dosages using the method of Chou-Talalay (21). Doses were chosen based on the higher IC50 value of mitotane in SW13 cells compared to NCI-H295R cells. As indicated in Table 2 and Fig.1A and B, a true synergistic effect (combination index < 1) was observed at multiple dose ranges by combining very low concentrations of sHDL nanoparticles (25 and 50 μ g/ml) with cisplatin, etoposide, or mitotane but not with doxorubicin (CI > 1). In each combination, there was a significant reduction in viability compared to either untreated cells or single drug treated cells. We then confirmed this antiproliferative effect by clonogenic assay by testing the combination of sHDL nanoparticles with each chemotherapeutic drug (Fig 2A and B). Combination treatments had a higher reduction in viability for NCI-H295R (**A**) and SW13 (**B**) cell lines by 11.8% and 20.4% respectively for cisplatin, 44.6% and 39.52% for etoposide, 39.1% and 22.3% for mitotane compared to single drug alone (*p*<0.05 for each). HDL treatment alone had minimal effect. Representative images are shown on the right of Fig 2A and B.

With synergy observed in combination with several of the drug compounds in inhibiting cell viability, we next wanted to evaluate if this effect was due to induction of apoptosis or merely a toxic effect of the drug leading to cell necrosis.

sHDL synergizes with chemotherapeutic drugs to induce apoptosis

Next combination dosing was evaluated by flow cytometry for a synergistic effect on apoptotic cell death in both ACC cell lines as determined by analysis of DNA fragmentation using sub-toxic concentrations of E, P, or M alone or in combination with sHDL for 24 h. Given the antagonistic effect of doxorubicin with sHDL on proliferation we did not test this

combination here. Cells undergoing early as well as late apoptosis and necrosis were differentiated based on phosphatidylserine staining on the outer leaflet of the apoptotic cells by Annexin V-FITC / PI staining. Combination treatments with sHDL resulted in a significantly greater increase in apoptotic or necrotic cells compared to each drug alone with negligible cell death noted with sHDL alone or untreated cells. The sHDL nanoparticles in combination with chemo drugs resulted in an increase in the percentage of apoptotic cells (early and late) by 6.16%, 36.46%, and 36.01% for P, E and M, respectively ($p < 0.05$ vs. drug alone) with minimal changes in necrosis compared to single drug alone for the NCI-H295R cells (Fig. 3A). In the case of non-cortisol secreting SW13 cells, the necrotic cell death increased by 14.71% for P and 21.33% for E ($p < 0.01$) while the apoptotic cell death increased by 11.9% ($p < 0.05$) for M when combined with sHDL vs. drug alone (Fig. 3B).

Given this synergistic effect on cell growth and induction of apoptosis, and since apoptosis and necrosis are mitochondrial dependent pathways, mitochondrial membrane potential was assessed.

Mitochondrial membrane potential is altered by combination therapy with sHDL

To elucidate the role of mitochondrial function in inducing apoptosis, we evaluated the mitochondrial potential (Ψ) using TMRE staining after treatment of cells with sHDL and E, P, or M for 24 h. As a negative control the cells were pretreated with an ionophore FCCP to eliminate mitochondrial membrane potential changes. Treatment of NCI-H295R and SW13 cells with sHDL in combination with each of the chemo drugs resulted in a significant reduction in Ψ by 13.09% and 6.5% for P, 9.2% and 31.54% for E, and 14.49% and 19.8% for M ($p < 0.05$ vs. chemo drug alone), respectively (Fig. 4A (NCI-H295R) and B (SW13)). This effect was blocked in the presence of mitochondrial depolarizer FCCP.

The effect of combination therapy with sHDL on Cortisol levels

To verify how changes in the steroidogenic pathway are influenced by combination therapy, we measured the concentration of Cortisol in the culture supernatant of hormone-producing NCI-H295R cells after E, P or M treatment alone or in combination with sHDL for 24 h. Treatment of cells with drug alone decreased Cortisol production levels by 89.6% for sHDL, 84.7% for M, and 82.1% for E ($p < 0.01$ each vs. controls while P decreased it only by 8.39% ($p = \text{NS}$) (Fig. 5). In combination with sHDL, this effect was not significantly different for E, or M but decreased 82% with P similar to that seen for the HDL alone.

Next, since Cortisol levels were significantly decreased with sHDL, we wanted to look more at its mechanistic effect on steroidogenesis in these ACC cells. To explore the effect of combination treatment on steroidogenesis, we evaluated the expression of genes involved in steroidogenesis by quantitative RT-PCR after 24 h treatment of hormone producing NCI-H295R cells with either drug alone or in combination with sHDL (primers listed in Table 1A). Relative expression levels of factors of Cortisol biosynthesis by RT-PCR including steroidogenic acute regulatory protein (StAR), the intra mitochondrial cholesterol transporter, CYP11A1 and others were examined. During combination of sHDL with either cisplatin, etoposide, or mitotane, the levels of StAR (0.18-1.88), CYP21A2 (0.05-2.6) and CYP19A1 (0.22-4.9) increased (in terms of fold changes); whereas the levels of CYP11A1

(0.1-0.5), CYP11B1 (0.1-0.64), CYP11B2 (0.04-0.52), CYP17A1 (0.1-0.58) and HSD3B2 (0.1-0.2 for P and M respectively; but increased by 2.1 for E) decreased as fold change. Representative fold changes compared to monotherapy ($p<0.05$) are shown graphically for StAR, CYP17A1, CYP21A2, CYP11B1, and CYP11B2 (Fig. 6A-E).

Combination therapy with sHDL is effective in targeting *in vivo* mimicking MCAs

To confirm whether the cytotoxic effect of combination therapy in targeting cells can be translated to tumors *in vivo*, we have used three-dimensional MCAs as a mimic for tumor model. First, MCAs were developed by seeding the cells in ultralow attachment plates and then treating them with either drug alone or in combination with sHDL. As evident from the results shown in Fig. 7A, we observed approximately 20%, 50% and 30% reduction in NCI-H295R MCAs for cisplatin, etoposide and mitotane, and approximately 50%, 25% and 50% reduction in SW13 MCAs (Fig.7B), respectively, when used in combination with sHDL. These results indicate that sHDL combination is effective in targeting even three-dimensional MCAs.

Combination therapy enhances the efficacy of EDPM

Given the antagonistic effect for doxorubicin, we examined if the complete EDPM regimen with sHDL would still be synergistic in inhibiting ACC cell viability. The viability of the cells was determined as before by CellTiter-Glo after treating both NCI-H295R and SW13 cells with either EDPM (25%, 50%, 75%, or 100% MTD levels) alone or in combination with 25 μ g/ml or 50 μ g/ml of sHDL. Untreated cells or sHDL alone treated cells served as controls. Despite the antagonistic effect of doxorubicin, we observed an enhanced dose dependent decrease in viability for both NCI-H295R (Fig.8A) and SW13 (Fig.8B) for sHDL combinations compared to EDPM alone. These results clearly demonstrate that combination therapy with sHDL nanoparticles effectively target ACC cells at lower doses of EDPM, which may lower toxicity profiles.

Discussion

Despite recent insights into the molecular mechanisms underlying the carcinogenesis of ACC and the development of novel targeted therapies for other cancers, advanced ACC remains a deadly disease (22). Patients with ACC rarely present with classic symptoms related to their tumor with only 40-60% of the patients present with symptoms characteristic of hormone excess (22). Hypercortisolism is the most common presentation in 50-80% of the patients with hormone excess and ACC patients diagnosed with hypercortisolism commonly have hypokalemia and hypertension (22). Recently, it was noted that mitotane induces CYP3A4 (23). Since many anti-neoplastic drugs are metabolized by CYP3A4, drug-drug interactions including toxicities could result from such combinations. Therefore, novel treatment options that avoid such drug interactions and have the potential for mitotane dose/toxicity reduction would be a real advance to the field.

Synthetic HDL nanoparticles have good safety in clinical trials thus far and demonstrate a unique antineoplastic effect in part due to their ability to efflux cholesterol selectively from cancer cells. As such, we have investigated the effect of combination therapy of sHDL with

commonly used chemotherapeutic drugs for ACC. Building on our preliminary findings (Table 1B) indicating that ACC cells express the highest level of SR-BI compared to several other cancers and normal cells, we hypothesized that sHDL nanoparticles can target and inhibit ACC cell viability effectively in combination with suboptimal concentrations of chemotherapeutic drugs.

We first performed a simple cell viability study to determine if combination dosing of sHDL and E, D, P or M would result in a synergistic, additive, or antagonistic effect by calculating combination indices for each combination. Our results clearly demonstrate that cisplatin, etoposide and mitotane act synergistically with sHDL at multiple concentrations whereas doxorubicin acts as an antagonist. This effect was then confirmed using a gold-standard clonogenic assay to demonstrate that combinations of sHDL and low-doses of E, P, or M resulted in improved inhibition of cell viability compared to E, P or M alone at normal therapeutic concentrations. We then demonstrated that combination treatment of E, P or M with sHDL lead to significant increases in ACC apoptosis and decreases in mitochondrial membrane potential compared to monotherapy. As mitochondrial depolarization can be due to either oxidative stress or apoptosis, further studies are needed to fully understand how this combination modulates the mitochondrial respiratory chain activity. Synergistic increases in apoptosis compared to necrosis suggest a mechanism-based pathway synergy, supporting the benefit of combination therapy on tumor biology as opposed to a merely non-targeted toxicity effect observed with necrosis.

Next, we evaluated the role of combination treatment on ACC steroidogenesis and cholesterol production using NCI-H295R human ACC cells that secrete steroid. Drugs like mitotane are known to inhibit CYP11A1, CYP11B1 and others (23). Although studies have demonstrated the role of LDL in increasing steroidogenesis (24, 25) very little is known about the role of sHDL in steroidogenesis. Because elevated cortisol levels are a known negative prognostic factor for ACC, we used RT-PCR to examine how our combination treatment modulates key factors involved in the steroidogenesis pathway. Our results indicated that compared to monotherapy, combination therapy with sHDL increased expression levels of StAR, the intra mitochondrial transporter; CYP21A2, the enzyme metabolizing 17-hydroxyprogesterone into 11-deoxycortisol, and CYP19A1 whereas the levels of CYP11B1, CYP11B2, CYP11A1 and CYP21A2 decreased. These results suggest that our combination has multiple effects and blocks both the upstream and the downstream regulators of cortisol. To evaluate if this effect on steroidogenesis leads to down regulation of cortisol production, we measured cortisol production levels in response to treatment. Interestingly, sHDL alone or in combination with P (which does not alter cortisol levels) decreased cortisol production >80%, similar to mitotane. We then showed that the EDPM + sHDL combination treatment retained its synergy despite the antagonism of doxorubicin and that this combination effect is translatable to 3-dimensional cell growth in aggregates. Given the statistically significant reduction in MCAs and viability after EDPM and sHDL treatment, further *in vivo* evaluation is warranted to confirm if this combinational synergy translates to NCI-H295R xenografts.

In conclusion, our results demonstrate for the first time that sHDL nanoparticles act synergistically with chemotherapy agents used in ACC, allowing lower doses in

combination to generate *in vitro* efficacy. This synergy may be due in part to targeting of the steroidogenic pathway, similar to mitotane, for potentiating enhanced apoptosis. Since these sHDL nanoparticles have already demonstrated some safety in clinical trials, this combination strategy may be a novel, less toxic approach to improve treatment in combination and avoid dose-limiting toxicities while maintaining the therapeutic benefits of mitotane and the Italian protocol. Further translational evaluation of this combination treatment *in vivo* will provide additional preclinical evidence to support new treatment strategies for patients with advanced ACC.

Acknowledgments

This work was partly funded by the National Institutes of Health (T32 CA009672, R01 CA173292), The University of Michigan M-TRAC Program, the University of Michigan Comprehensive Cancer Center Support Grant, and the University Of Michigan Department Of Surgery.

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Figure 1. Effect of sHDL nanoparticles in combination with cisplatin, etoposide, doxorubicin, or mitotane on ACC cell viability in NCI-H295R (A) and SW13 (B) cell lines

Panels on the left are IC₅₀ curves for sHDL alone showing inhibition of tumor cell viability at 100-200 µg/ml. Since IC₅₀s of drugs in SW13 were often higher than in NCI-H295R cells (data not shown), we often used higher doses of E, D, P, or M in these cells. Cells were treated with serial dilutions of drugs alone or with sHDL nanoparticles for 72 h. Cell viability was calculated by Cell-Titer Glo assay. The 8 graphs at the right are plotted as a function of cell viability on the y-axis vs. drug concentration on the x-axis. For each drug except for doxorubicin, addition of sHDL significantly enhanced inhibition of cell viability compared to drug alone or sHDL alone suggesting a potential synergistic effect. The relative viability of cells compared to untreated control was plotted for all the combinations. Each experiment was done in triplicate and the mean and standard deviation are plotted (***p<0.05).

Figure 2. Clonogenic analysis of NCI-H295R (A) and SW13 (B) cell lines after E, P, or M treatment either alone or in combination with sHDL

For this experiment, dosing included cisplatin at 10 μ M, etoposide at 150 μ M, or mitotane at 50 μ M for NCI-H295R and 100 μ M for SW13 or in combination with 50 μ g/ml sHDL nanoparticles. Colonies were allowed to grow for 2 weeks and those containing 50 or more cells were counted and the results were plotted as a fraction of surviving cells compared to untreated control cells. Each experiment was done in triplicate and the mean $\pm$ SEM were presented. Representative photograph images are shown on the right with quantitative data represented in bar graphs on the left. All three drugs showed significantly enhanced inhibition of colony formation in combination with sHDL compared to drug alone (* p <0.05; *** p <0.01).

Figure 3. Analysis of apoptosis by flow cytometry after treatment of NCI-H295R (A) and SW13 (B) cells with either E, P or M alone or in combination with sHDL nanoparticles

Top row of flow cytometry plots show ACC cells treated with either cisplatin, etoposide or mitotane alone or in combination with 50 μ g/ml sHDL nanoparticles as describe in Figure 2 in the clonogenic assay for 24 h. Cells were stained with annexin V-FITC and propidium iodide and then apoptosis was analyzed by flow cytometry (top row of flow cytometry plots). For each drug, addition of sHDL in combination enhanced the amount of cells gated toward apoptosis on flow. This is depicted more quantitatively in the bottom bar graphs.

While the level of necrosis remained relatively stable with treatment, the level of apoptosis significantly increased with addition of sHDL to the drug in each case. The number of both apoptotic and necrotic cells were plotted for different treatments. The experiments were carried out in triplicate and mean $\pm$ SEM were presented.

Figure 4. Combination therapy with sHDL nanoparticles increases the mitochondrial membrane potential

After 24 h treatment of NCI-H295R (**Figure 4 A**) and SW13 (**Figure 4 B**) cells with cisplatin, etoposide or mitotane either alone (similar concentrations as that in Figure 2) or along with 50 μ g/ml sHDL nanoparticles, cells were stained with TMRE for 20min and the changes in mitochondrial potential were evaluated by measuring the fluorescence intensity in a micro plate reader. Cells pretreated with FCCP for 10min before the addition of TMRE served as a negative control. The changes in mitochondrial membrane potential between monotherapy and sHDL combination therapy are plotted in the bar graphs with NCI-H295R cells having the greatest change in membrane potential at 13.1%, 9.5% or 14.5% for C, E, and M, respectively. All experiments were done in triplicate and reported as mean $\pm$ SEM.

Figure 5. Evaluation of cortisol production with drug treatment

The cortisol production levels were estimated by radioimmunoassay after treatment of cortisol producing NCI-H295R cells with sHDL nanoparticles along with either cisplatin or etoposide or mitotane (at concentrations used in Figure 2) for 24h. Etoposide, mitotane, and sHDL each significantly reduced cortisol production (***) with treatment while cisplatin alone did not. Addition of sHDL to cisplatin reduced levels to that of sHDL alone and addition of sHDL to Mitotane enhanced this effect (*).

Figure 6. A-E mRNA level expressions of key steroidogenesis pathway enzymes in hormone producing H295R cells after treatment with either cisplatin, etoposide or mitotane alone or in combination with sHDL nanoparticles for 24 h
 Clockwise from top left: enzymes include **Panel A = STAR**, **Panel B = CYP17A1**, **Panel C = CYP21A2**, **Panel D= CYP11B1**, **Panel E = CYP11B2**. Expression levels were measured by RT-PCR. Cisplatin consistently decreased enzyme expression levels with treatment, whereas etoposide enhanced them. Mitotane only increased CYP21A2 levels and addition of sHDL to drug enhanced this effect in most of the enzymes. Data were analyzed by relative expression method and the values presented as mean $\pm$ SEM. Each experiment was repeated in triplicate. ($p < 0.05$ each *, **, ***)

Figure 7.

Effect of sHDL combination with chemotherapeutics on MCAs. Approximately 100,000 NCI-H295R (A) and 50,000 SW13 (B) cells were seeded on ultralow attachment plates and incubated at 37°C in 5%CO₂ incubator. After the formation of MCAs they were photographed and then treated with either drugs alone or in combination with 50µg/ml sHDL nanoparticle. Post treatment for 24 h they were photographed again (left photos) and the MCAs were quantified using Image J software from NIH (bar graph data shown on the right). In each case, addition of sHDL to either E or P or M demonstrated a significant inhibition of aggregate formation (**p<0.05). All experiments were done in triplicate.

Figure 8. Effect of multi-drug combination studies with sHDL on ACC cell viability
 NCI-H295R (A) and SW13 (B) cells were treated with EDPM (25%, 50%, 75%, or 100% MTD levels) alone or in combination with 25µg/ml or 50µg/ml of sHDL. Untreated cells or sHDL alone treated cells served as control. Despite the antagonistic effect of doxorubicin, we observed an enhanced dose dependent decrease in viability for both sHDL combinations compared to EDPM alone. Relative viability was calculated in terms of untreated controls. The mean and standard deviation from three different experiments are presented with *** representing $p < 0.05$.

Table 1**A. Primer sequences used for RT-PCR analysis of steroidogenesis pathway enzymes. B. Expression of SR-B1 by western blot analysis**

ACC cell lines NCI-H295R, SW13 and RL251, and BHK (baby hamster kidney) cell lines over expressing SR-B1 (positive control) as well as Jurkat cell lines (negative control) were grown in culture in appropriate medium. The cells were lysed and immunoblotted for SR-B1 using the method previously described (20). The same blot was reprobed for actin for loading control.

Gene	Sense	Antisense
Star (NM_000349)	TTGCTTTATGGGCTCAAGAATG	GGAGACCTCTGAGATTCTGCTT
CYP11A1 (NM_000781)	CTTCTTCGACCCGAAAATTT	CCGGAAGTAGGTGATGTTCTTGT
HSD3B2 (NM_000198.1)	GCGGCTAATGGGTGGAATCTA	CCTCATTATATACTGGCAGAAAGGAAT
CYP11B1 (NM_000497)	TCCCGAGGGCCTCTAGGA	GGGACAAGGTCAGCAAGATCTT
CYP11B2 (NM_000498)	TTGTTCAAGCAGCGAGTGTTG	GCATCCTCGGGACCTTCTC
CYP17A1 (NM_000102)	GCTGACTCTGGCGCACACT	CCATCCTTGAACAGGGCAAA
CYP21A2 (NM_000500)	TCCCGAGCACTCAACCAACCT	CAGCTCAGAATTAAGCCTCAATCC
CYP19A1 (NM_000103)	ACCAGCATCGTGCCTGAAG	CCAAGAGAAAAAGGCCAGTGA

Table 2
Combination Index evaluations by drug concentrations

Human ACC cell lines NCI-H295R and SW13 were treated at 25 and 50% IC50 levels of drug [Etoposide (VP16) at 12.5 or 25 μ M, mitotane at 25 or 50 μ M, cisplatin at 1.25 or 2.5 μ M, and doxorubicin at 1.25 or 2.5 μ M] in combination with either 25 μ g/ml or 50 μ g/ml sHDL nanoparticle for 72 h. The viability of the cells was calculated by cell-Titer Glo and the combination index (CI) was calculated using the Chou-Talalay equation (19). CI<1 is defined as a combination demonstrating synergy (with stronger synergy for CI<0.5), CI=1 means the combination is additive, not synergistic, and CI>1 means an antagonistic effect. While E, P, and M each showed synergy with sHDL in both cell lines, stronger synergy was observed with etoposide and sHDL and in the 1.25 μ M dose of cisplatin. Each experiment was repeated in triplicate and the viability as a percentage of untreated control was calculated using Graphpad prism.

sHDL (μ g/ml)	Etoposide (μ M)	CI for NCI-H295R	CI for SW13
50	25.0	0.694	0.158
50	12.5	0.602	0.392
25	25.0	0.641	0.172
25	12.5	0.521	0.196
sHDL (μ g/ml)	Mitotane (μ M)	CI for NCI-H295R	CI for SW13
50	50	0.750	0.852
50	25	0.709	0.652
25	50	0.812	0.627
25	25	0.809	0.928
sHDL (μ g/ml)	Cisplatin (μ M)	CI for NCI-H295R	CI for SW13
50	2.5	0.759	0.666
50	1.25	0.462	0.812
25	2.5	0.572	0.619
25	1.25	0.390	0.583
sHDL (μ g/ml)	Doxorubicin (μ M)	CI for NCI-H295R	CI for SW13
50	2.5	5.232	1.289
50	1.25	3.619	1.844
25	2.5	5.982	1.385
25	1.25	4.257	1.190