Synthetic high-density lipoprotein nanoparticles: A novel therapeutic strategy for adrenocortical carcinomas

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

Methods. *The antiproliferative 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. Effects on steroidogenesis was measured by real-time polymerase chain reaction (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 antiproliferation. Alone or in combination with the chemotherapy drugs, sHDL was able to decrease cortisol production by 70–90% compared with P alone or controls ($P < .01$). RT-PCR indicated inhibition of steroidogenic enzymes for sHDL ($P < .01$ vs no sHDL). Combination therapy with sHDL increased apoptosis by 30–50% compared with drug or sHDL alone ($P < .03$), confirmed by a decrease in the mitochondrial potential.*

Conclusion. *sHDL can act synergistically and lessen the amount of M/E/P needed for anticancer efficacy in ACC in part owing to cholesterol starvation. This novel treatment strategy warrants further investigation translationally. (Surgery 2016;159:284-95.)*

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Adrenocortical carcinoma (ACC) is a rare, endocrine malignancy (approximately 500 new cases per year in the United States) that carries a poor prognosis with advanced disease.[1] Unfortunately, a majority of patients present with advanced disease at the time of diagnosis and, once metastatic, the disease has a very low 10–20% 5-year survival.[2] For patients with metastatic disease, the only current therapeutic approved by the US Food and Drug Administration is the adrenolytic agent mitotane (M), with initial response rates of 20–30% in advanced ACC and an improvement in survival rate from 14 to 50 months.[3] Recent studies have evaluated M in combination with cytotoxic chemotherapeutics as in the Italian protocol (etoposide [E], doxorubicin [D], cisplatin [P]; EDP) or with streptozotocin.[4,5] EDPM has been shown to offer a greater response rate (23.2% vs 9.2%) and progression-free survival (5.0 vs 2.1 months) compared with M with streptozotocin.[6-8] Dose-limiting toxicities, such as adrenal insufficiency, dizziness, vertigo, central nervous disturbances, hyperlipidemia, and gastrointestinal disorders, remain a clinically relevant issue with both M 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 lesser concentrations of these noxious agents needed to achieve the same therapeutic effect and potentially mitigate some toxicity.

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Table I. Primer sequences used for real-time polymerase chain reaction analysis of steroidogenesis pathway enzymes

Gene	*Sense*	*Antisense*
StAR (NM_000349)	TTGCTTTATGGGCTCAAGAATG	GGAGACCCTCTGAGATTCTGCTT
CYP11A1 (NM_000781)	CTTCTTCGACCCGGAAAATTT	CCGGAAGTAGGTGATGTTCTTGT
HSD3B2 (NM_000198.1)	GCGGCTAATGGGTGGAATCTA	CCTCATTTATACTGGCAGAAAGGAAT
CYP11B1 (NM_000497)	TCCCGAGGGCCTCTAGGA	GGGACAAGGTCAGCAAGATCTT
CYP11B2 (NM_000498)	TTGTTCAAGCAGCGAGTGTTG	GCATCCTCGGGACCTTCTC
CYP17A1 (NM_000102)	GCTGACTCTGGCGCACACT	CCATCCTTGAACAGGGCAAA
CYP21A2 (NM_000500)	TCCCAGCACTCAACCAACCT	CAGCTCAGAATTAAGCCTCAATCC
CYP19A1 (NM_000103)	ACCAGCATCGTGCCTGAAG	CCAAGAGAAAAAGGCCAGTGA

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, which allow the cell to obtain cholesterol esters from circulating high-density lipoprotein (HDL) cholesterol.[9] This SR-BI receptor is highly overexpressed in ACC cells, and several other cancers (lymphoma and breast, prostate, ovarian, and nasopharyngeal carcinomas) compared with normal tissues. SR-BI receptors act as bidirectional cholesterol transporters that facilitate both the uptake into cells and efflux the cholesterol out of cells.[10] Because cholesterol transport is an important biologic function of cells, including cancer cells, mimetic synthetic HDL (sHDL) nanoparticles that bind to SR-BI have come under focus recently as a novel approach for targeting cancer.[11,12] A number of cholesterol-free sHDL products have been tested clinically for the treatment of atherosclerosis by facilitation of reverse cholesterol transport and found to be safe at high doses of 20–40 mg/kg per infusion.[13] Many patients with advanced ACC will develop steroid oversecretion.[14] Because these steroids require cholesterol,[15] an agent that effluxes cholesterol from cells may have therapeutic benefit in decreasing this oversecretion functionality. In the current study, we used cholesterol-free sHDL nanoparticles and hypothesized that these sHDL nanoparticles may be able to generate anticancer 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 lessen the 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 (nonsteroid secretor), were grown in 2-dimenstional culture in humidified atmosphere of 5% CO_2 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). NCI-H295R cells were grown in DMEM-Ham's F12 nutrient medium (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), 1% insulin/transferrin/selenium, and 1% penicillin/streptomycin (Life Technologies).

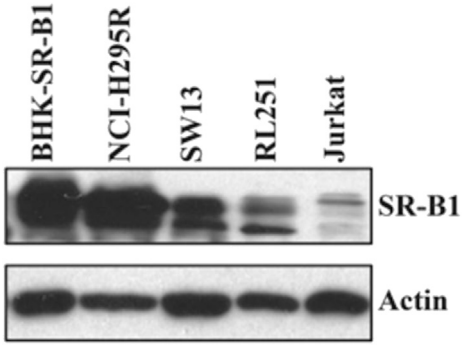

Fig 1. Expression of scavenger receptor class B type I (SR-B1) by Western blot analysis. Adrenocortical carcinoma cell lines NCI-H295R, SW13 and RL251, and BHK (baby hamster kidney) cell lines over expressing SR-B1 (positive control) as well as Jurkat cell lines (negative control) were grown in culture in appropriate medium. The cells were lysed and immunoblotted for SR-B1using the method previously described.[20] The same blot was reprobed for actin for loading control.

Preparation and characterization of sHDL. First, 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 (pH = 7.4) was added to the freeze-dried

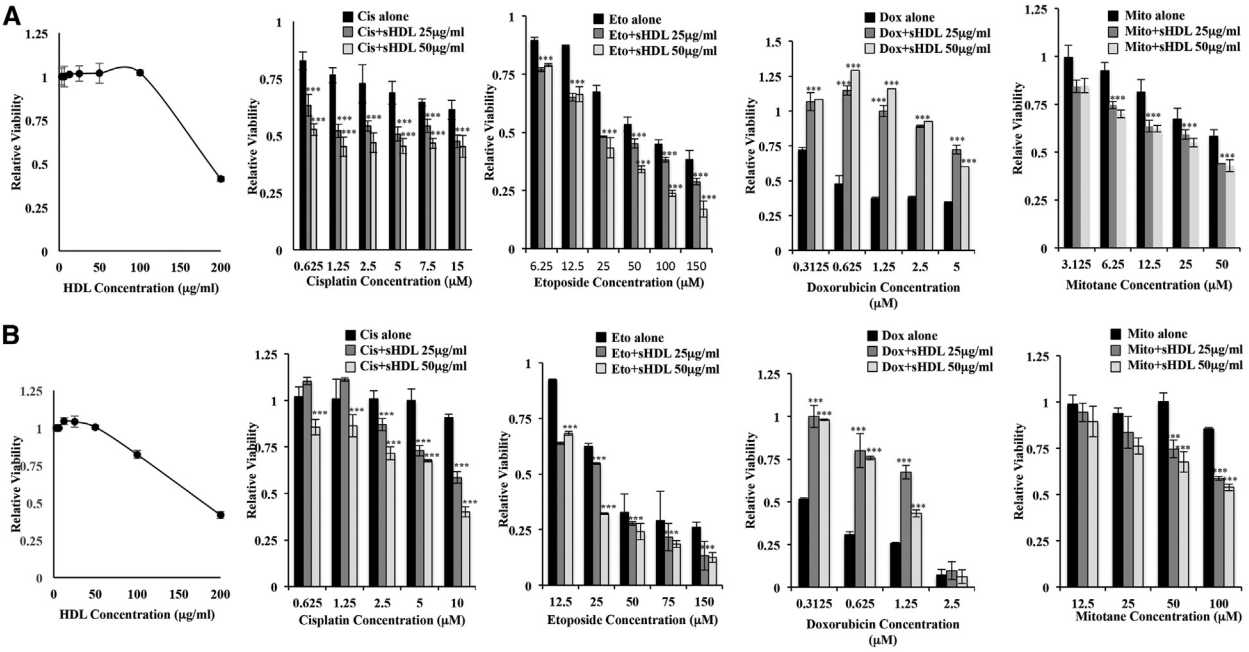

Fig 2. Effect of synthetic high-density lipoprotein (sHDL) nanoparticles in combination with cisplatin (P), etoposide (E), doxorubicin (D), or mitotane (M) on adrenocortical carcinoma (ACC) cell viability in NCI-H295R (*A*) and SW13 (*B*) cell lines. (*Left*) IC50 curves for sHDL alone showing inhibition of tumor cell viability at 100–200 μg/mL. Because the IC50s of drugs in SW13 were often greater than in NCI-H295R cells (data not shown), we often used greater 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 hours. 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 versus drug concentration on the *x*-axis. For each drug except for D, addition of sHDL significantly enhanced inhibition of cell viability compared with drug alone or sHDL alone suggesting a potential synergistic effect. The relative viability of cells compared with untreated control was plotted for all the combinations. Each experiment was done in triplicate, with the mean value and standard deviation plotted (***$P < .05$).

powder, which then was cycled 3 times between 50°C (3 minutes) and 20°C (3 minutes) with gentle shaking to obtain the sHDL. Purity of the sHDL was analyzed by gel permeation chromatography. The sHDL was further characterized by transmission electron microscopy. All images were acquired on JEM 1200EX electron microscope (JEOL USA, Peabody, MA) equipped with an AMT XR-60 digital camera (Advanced Microscopy Techniques Corp, Woburn, MA).

Cell viability assay and calculation of combination index. SW13 and NCI-H295R cells were seeded into 96-well plates in triplicate and treated with varying concentrations of either the chemotherapeutic drugs P, D, E, M (EDPM) either in combination or each alone, in simultaneous combinations with sHDL or sHDL alone for 72 hours. A large dose range was used initially to define a more selective dose range for median inhibition concentration (IC50) experiments. Serial dilutions were made from the starting concentrations, and viability of cells was then measured based on quantification of the adenosine triphosphate levels after treatment with Cell Titer-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, NJ). The CI values of <1, 1, and >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 compared with combination regimens), unless otherwise stated.

Colony formation assay. The NCI-H295R and SW13 cells were plated in 6-well plates and allowed to attach. Treatment commenced for 24 hours with drug alone or in combination with sHDL (50 μg 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

Table II. Combination index evaluations by drug concentrations

HDL (mg/mL)	*Etoposide (mmol/L)*	*CI for NCI-H295R*	*CI for SW13*
50	25	0.694	0.158
50	12.5	0.602	0.392
25	25	0.641	0.172
25	12.5	0.521	0.196
HDL (mg/mL)	*Mitotane (mmol/L)*	*CI for NCI-H295R*	*CI for SW13*
50	50	0.75	0.852
50	25	0.709	0.652
25	50	0.812	0.627
25	25	0.809	0.928
HDL (mg/mL)	*Cisplatin (mmol/L)*	*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	0.39	0.583
HDL (mg/mL)	*Doxorubicin (mmol/L)*	*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	1.19

Human adrenocortical carcinoma cell lines NCI-H295R and SW13 were treated at 25 and 50% IC50 levels of drug (etoposide [E; VP16] at 12.5 or 25 μmol/L, mitotane [M] at 25 or 50 μmol/L, cisplatin [P] at 1.25 or 2.5 μmol/L, and doxorubicin at 1.25 or 2.5 μmol/L) in combination with either 25 or 50 μg/mL synthetic high-density lipoprotein (sHDL) nanoparticle for 72 hours. 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. Whereas 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 μmol/L dose of P. Each experiment was repeated in triplicate and the viability as a percentage of untreated control was calculated using Graphpad prism.

to grow colonies of ≥50 cells for 2 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, or M alone or in combination with sHDL for 24 hours. After treatment, cells were washed, resuspended in Annexin-binding buffer, and stained using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide as described previously.[17] Induction of apoptosis was measured using the CyAn ADP Analyzer (Beckman Coulter, Inc, Indianapolis, IN) at the University of Michigan Flow Cytometry Core.

Membrane potential of mitochondria. SW13 and NCI-H295R were seeded in a 96-well black wall plate. Once attached, the cells were treated with the drugs as described. Then, 24 hour after drug treatment, 500 nmol/L tetramethylrhodamine, ethyl ester (TMRE) was added, the cells were incubated for 20 minutes at 37°C, and the fluorescent signal was measured after washing using a microplate reader (excitation = 549; emission = 575). 100 nmol/L carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added to cells 10 minutes 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 clonogic assay) for 24 hour per the manufacturer's instructions. Briefly, culture supernatant after treatment was added to the antibody-coated plates containing assay buffer (45 minutes at 25°C). After washing, tetra methyl benzidine substrate was incubated at room temperature, and the absorbance was measured using a Synergy Neo reader (BioTek).

Messenger RNA isolation and real-time polymerase chain reaction. RNA from the NCI-H295 R cells after drug treatment for 24 hour 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. Quantitative polymerase chain reaction (PCR) was performed in a step-1 real-time PCR (RT-PCR) machine using the gene-specific primer sets (Table I) 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 (Fig 1).

Treatment of 3-dimensional multicellular aggregates (MCAs). 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, Corning, NY) to generate MCAs. The MCAs generated were treated with drugs (concentrations specified in our colony formation assay) with or without 50 μg/mL sHDL nanoparticle for 24 hours. Untreated and sHDL alone treated cells served as controls. The MCAs were photographed before and

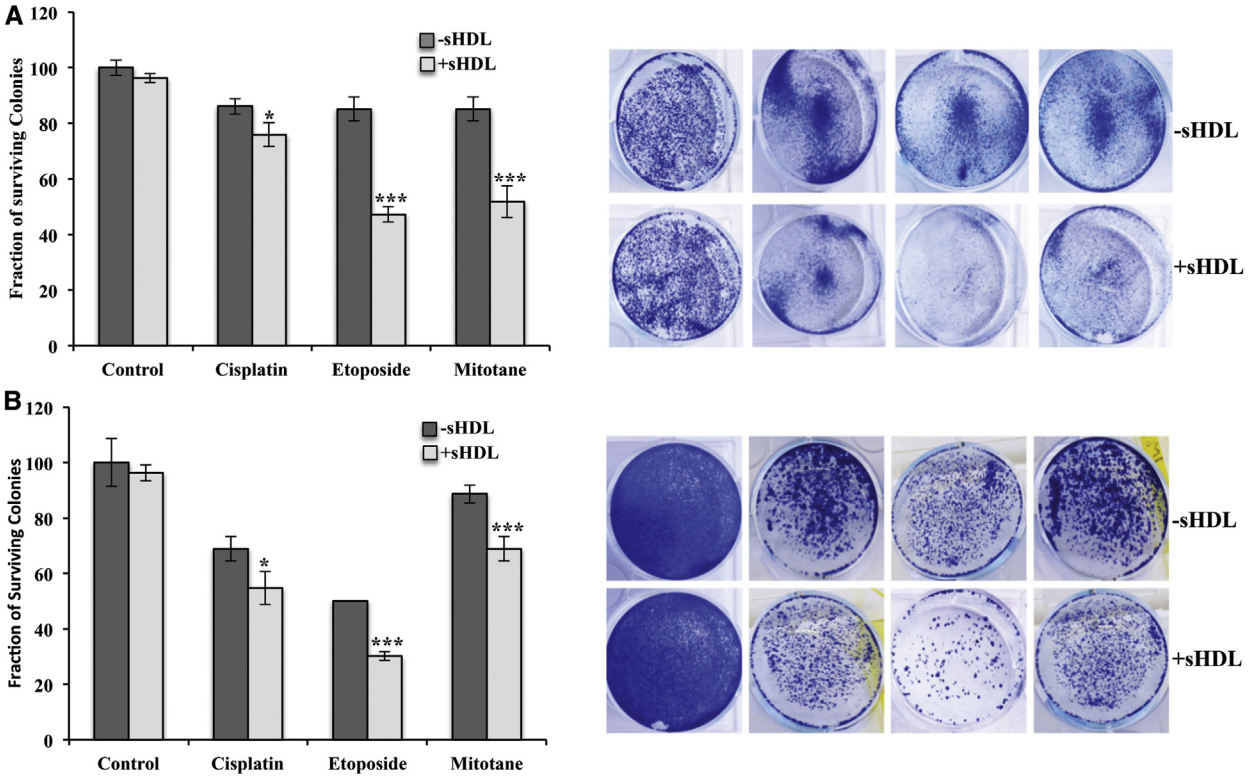

Fig 3. Clonogenic analysis of NCI-H295R (*A*) and SW13 (*B*) cell lines after etoposide (E), cisplatin (P), or mitotane (M) treatment either alone or in combination with synthetic high-density lipoprotein (sHDL). For this experiment, dosing included P at 10 μmol/L, etoposide at 150 μmol/L, or M at 50 μmol/L for NCI-H295R and 100 μmol/L for SW13 or in combination with 50 μg/mL sHDL nanoparticles. Colonies were allowed to grow for 2 weeks; those containing ≥50 cells were counted, and the results were plotted as a fraction of surviving cells compared with untreated control cells. Each experiment was done in triplicate with the mean ± standard error of the mean presented. Representative photograph images are shown on the *right* with quantitative data represented in bar graphs on the *left*. All 3 drugs showed significantly enhanced inhibition of colony formation in combination with sHDL compared with drug alone (*$P < .05$; ***$P < .01$).

after treatment and 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 values ± standard error of the mean. Comparisons of differences between ≥2 means were determined by Student's 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).

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 2). However, sHDL nanoparticles alone did not induce significant cell death at normal concentrations, only high concentrations of 100-200 μg/mL of the 22A peptide (Fig 2, *left*). To determine whether combining sHDL nanoparticles and the chemotherapeutic drugs results in synergy or an additive effect, we then calculated the CI after treating the cells at different combination dosages using the method of Chou-Talalay.[21] Doses were chosen based on the greater IC50 value of M in SW13 cells compared with NCI-H295R cells. As indicated in Table II and Fig 2, a true synergistic effect (CI < 1) was observed at multiple dose ranges by combining very low concentrations of sHDL nanoparticles (25 and 50 μg/mL) with P, E, or M but not with D (CI > 1). In each combination, there was a significant decrease in viability

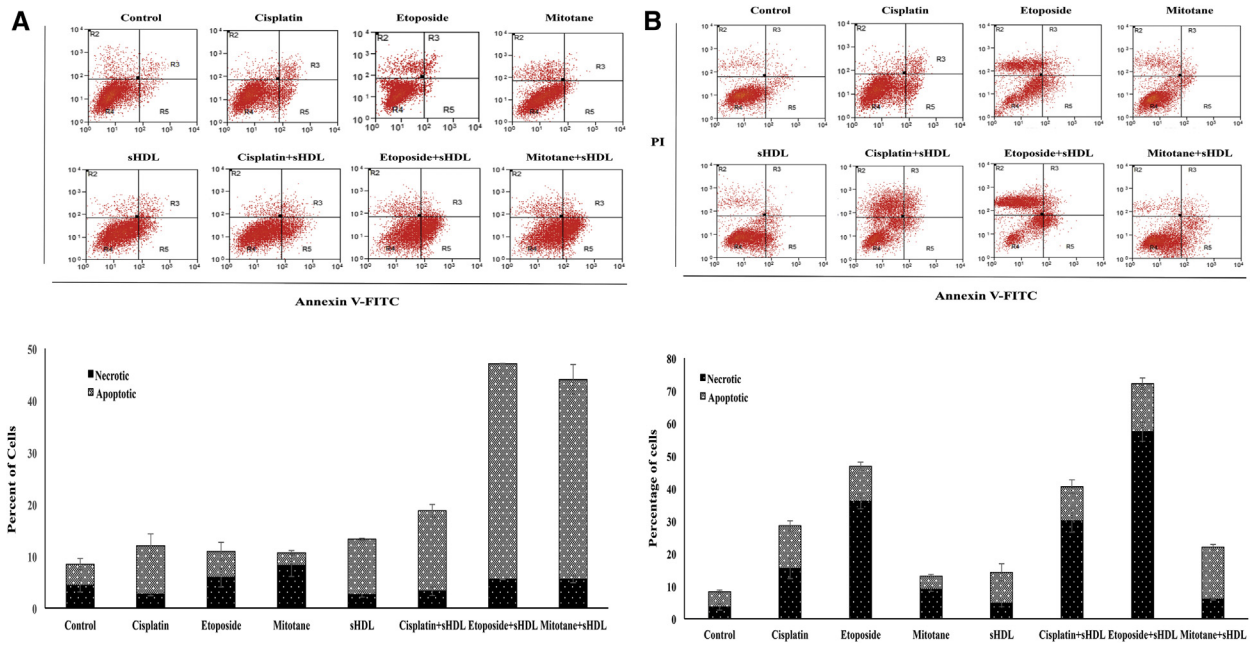

Fig 4. Analysis of apoptosis by flow cytometry after treatment of NCI-H295R (*A*) and SW13 (*B*) cells with either etoposide (E), cisplatin (P), or mitotane (M) alone or in combination with synthetic high-density lipoprotein (sHDL) nanoparticles. *Top row* of flow cytometry plots show adrenocortical carcinoma cells treated with either P, E, or M alone or in combination with 50 μg/mL sHDL nanoparticles as describe in Fig 3 in the clonogenic assay for 24 hours. Cells were stained with Annexin V fluorescein isothiocyanate and propidium iodide and apoptosis analyzed by flow cytometry (*top row* of flow cytometry plots). For each drug, the addition of sHDL in combination enhanced the amount of cells gated toward apoptosis on flow, which is depicted more quantitatively in the *bottom bar graphs*. Although 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 with the mean ± standard error of the mean presented.

compared with 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 3). Combination treatments had a greater decrease in viability for NCI-H295R (Fig 3, *A*) and SW13 (Fig 3, *B*) cell lines by 12% and 20%, respectively, for P, 45% and 40% for E, and 39% and 22% for M compared with single drug alone ($P < .05$ each). HDL treatment alone had minimal effect. Representative images are shown on the right of Fig 3.

With synergy observed in combination with several of the drug compounds in inhibiting cell viability, we next wanted to evaluate if this effect was owing 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 subtoxic concentrations of E, P, or M alone or in combination with sHDL for 24 hours. Given the antagonistic effect of D with sHDL on proliferation, we did not test this combination. 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. Combination treatments with sHDL resulted in a significantly greater increase in apoptotic or necrotic cells compared with each drug alone with negligible cell death noted with sHDL alone or untreated cells. The sHDL nanoparticles in combination with the chemotherapy drugs resulted in an increase in the percentage of apoptotic cells (early and late) by 6%, 36%, and 36% for P, E, and M, respectively ($P < .05$ vs drug alone) with minimal changes in necrosis compared with single drug alone for the NCI-H295R cells (Fig 4). In the case of non–cortisol-secreting SW13 cells, the necrotic cell death increased by 15% for P and 21% for E ($P < .01$), whereas the apoptotic cell death increased by 12% ($P < .05$) for M when combined with sHDL versus drug

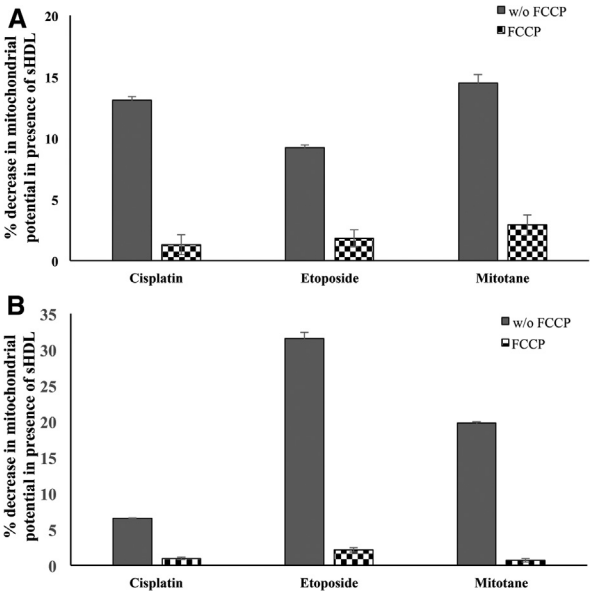

Fig 5. Combination therapy with synthetic high-density lipoprotein (sHDL) nanoparticles increases the mitochondrial membrane potential. After 24 hours of treatment of NCI-H295R (*A*) and SW13 (*B*) cells with cisplatin (P), etoposide (E), or M either alone (similar concentrations as that in Fig 3) or along with 50 μg/mL sHDL nanoparticles, cells were stained with tetramethylrhodamine, ethyl ester (TMRE) for 20 minutes, and the changes in mitochondrial potential were evaluated by measuring the fluorescence intensity in a microplate reader. Cells pretreated with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone for 10 minutes 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%, 10%, or 15% for P, E, and M, respectively. All experiments were done in triplicate and reported as mean ± standard error of the mean.

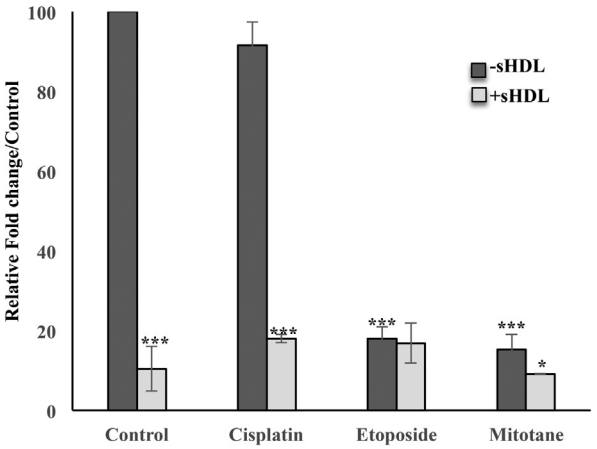

Fig 6. Evaluation of cortisol production with drug treatment. The cortisol levels in the culture medium were estimated by radioimmunoassay after treatment of cortisol-producing NCI-H295R cells with synthetic high-density lipoprotein (sHDL) nanoparticles along with either cisplatin (P) or etoposide (E) or mitotane (M) at concentrations used in Fig 3 for 24 hours. E, M, and sHDL each decreased cortisol production (***$P < .05$) with treatment, whereas P alone did not. The addition of sHDL to P decreased levels to that of sHDL alone and addition of sHDL to M enhanced this effect (*$P < .05$).

alone (Fig 4). Given this synergistic effect on cell growth and induction of apoptosis, and because apoptosis and necrosis are mitochondrial-dependent pathways, the membrane potential of mitochondria was assessed.

The membrane of mitochondria potential is altered by combination therapy with sHDL. To elucidate the role of mitochondrial function in inducing apoptosis, we evaluated the mitochondrial potential ($\Delta\Psi$) using TMRE staining after treatment of cells with sHDL and E, P, or M for 24 hours. As a negative control, cells were pretreated with an ionophore FCCP to eliminate potential changes in the mitochondrial membrane. Treatment of NCI-H295R and SW13 cells with sHDL in combination with each of the chemotherapy drugs resulted in a decrease in $\Delta\Psi$ by 13% and 7% for P, 9% and 32% for E, and 15% and 20% for M ($P < .05$ vs chemotherapy drug alone), respectively (Fig 5). This effect was blocked in the presence of the 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 hours. Treatment of cells with drug alone decreased cortisol production levels by 90% for sHDL, 85% for M, and 82% for E ($P < .01$ each vs controls; P decreased it only by 8%; P = NS; Fig 6). In combination with sHDL, this effect was not different for E or M, but decreased 82% with P similar to that seen for the HDL alone.

Next, because cortisol levels were decreased significantly with sHDL, we wanted to examine the 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 hours of treatment with hormone-producing NCI-H295R cells with

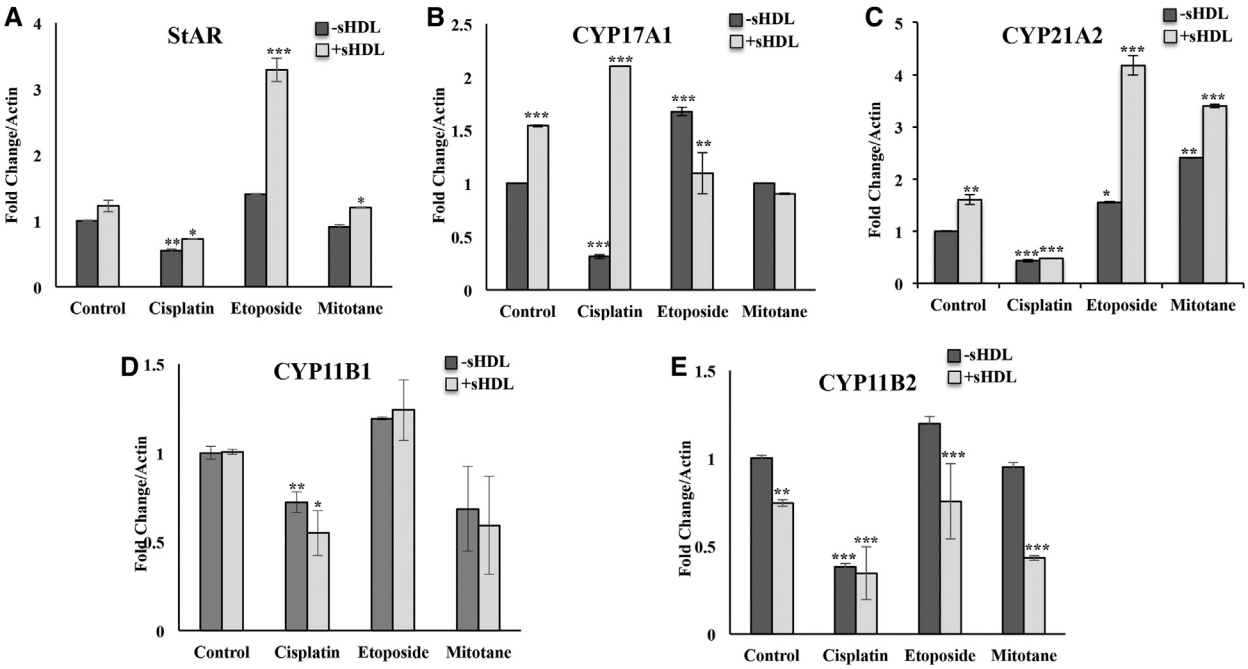

Fig 7. *A–E*, Messenger RNA level expressions of key steroidogenesis pathway enzymes in hormone-producing H295R cells after treatment with either cisplatin (P), etoposide (E), or mitotane (M) alone or in combination with synthetic high-density lipoprotein (sHDL) nanoparticles for 24 h. Clockwise from *top left*, enzymes include (*A*) steroidogenic acute regulatory protein, (*B*) CYP17A1, (*C*) CYP21A2, (*D*) CYP11B1, and (*E*) CYP11B2. Expression levels were measured by real-time polymerase chain reaction. P consistently decreased enzyme expression levels with treatment, whereas etoposide enhanced them. M only increased CYP21A2 levels, whereas the addition of sHDL to drug enhanced this effect in most of the enzymes. Data were analyzed by relative expression method with values presented as mean ± standard error of the mean. Each experiment was repeated in triplicate. ($P < .05$ each *, **, ***).

either drug alone or in combination with sHDL (primers listed in Table I). Relative expression levels of factors of cortisol biosynthesis by RT-PCR, including the steroidogenic acute regulatory protein (StAR), the intramitochondrial cholesterol transporter, CYP11A1, and others were examined. During combination of sHDL with either P, E, or M, 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 C and M, respectively; but increased by 2.1 for E) decreased as fold change. Representative fold changes compared with monotherapy ($P < .05$) are shown graphically for StAR, CYP17A1, CYP21A2, CYP11B1, and CYP11B2 (Fig 7).

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 used 3-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 shown in Fig 8, we observed approximately 20%, 50%, and 30% decrease in NCI-H295R MCAs for P, E, and M, and approximately 50%, 25% and 50% decrease in SW13 MCAs (Fig 8), respectively, when used in combination with sHDL. These results indicate that sHDL combination is effective in targeting even 3-dimensional MCAs.

Combination therapy enhances the efficacy of EDPM. Given the antagonistic effect for D, we examined whether 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 Cell Titer-Glo after treating both NCI-H295R and SW13 cells with either EDPM (25%, 50%, 75%, or 100% maximum tolerated dose (MTD) levels) alone or in combination with 25 or 50 μg/mL of sHDL. Untreated cells or sHDL alone treated cells served as controls. Despite the antagonistic effect of D, we observed an enhanced, dose-dependent decrease in viability for both NCI-H295R (Fig 9) and SW13 (Fig 9) for

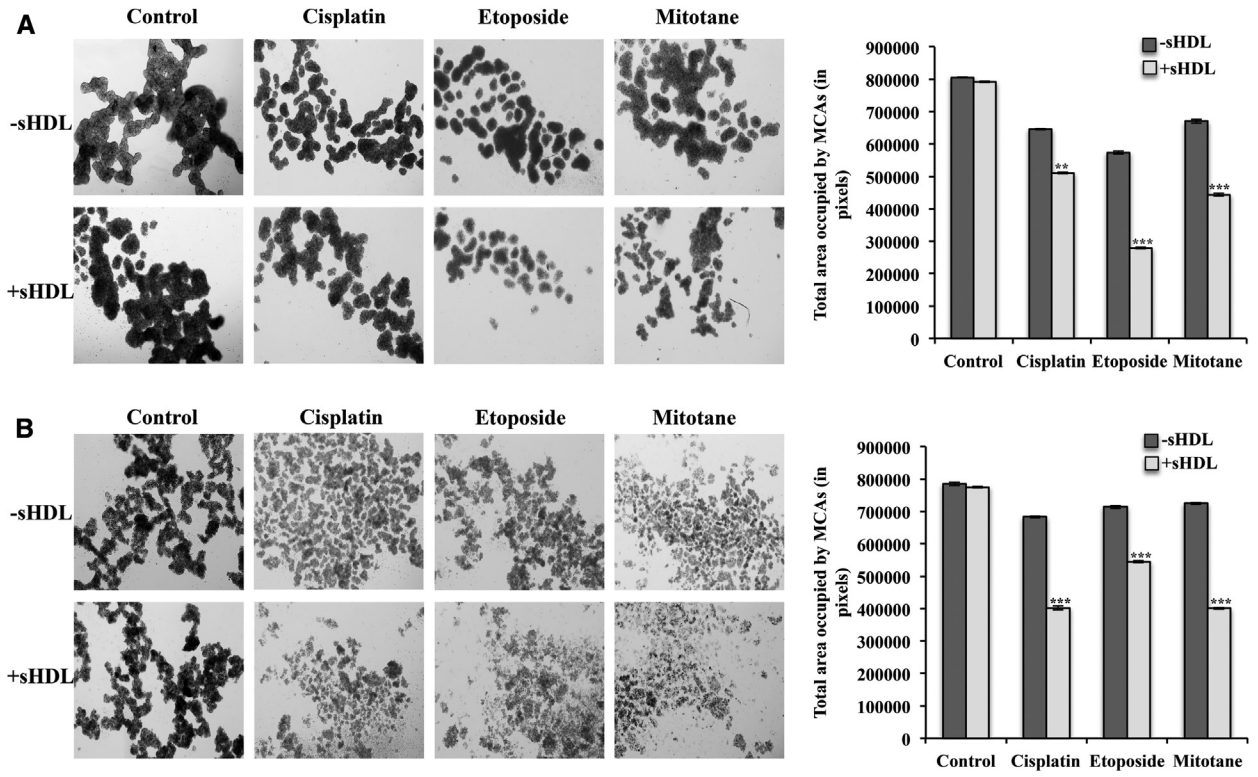

Fig 8. Effect of synthetic high-density lipoprotein (sHDL) combination with chemotherapeutics on multicellular aggregates (MCAs). Approximately 100,000 NCI-H295R (*A*) and 50,000 SW13 (*B*) cells were seeded on ultralow attachment plates and incubated at 37°C in a 5% CO_2 incubator. After the formation of MCAs, the MCAs were photographed and then treated with either drugs alone or in combination with 50 μg/mL sHDL nanoparticles. After treatment for 24 hours, the MCAs were photographed again (*left*) and quantified using Image J software from NIH (*bar graph* data shown on the *right*). In each case, the addition of sHDL to either E or P or M demonstrated inhibition of aggregate formation (***$P < .05$). All experiments were done in triplicate.

sHDL combinations compared with EDPM alone. These results demonstrate that combination therapy with sHDL nanoparticles target ACC cells effectively at lesser doses of EDPM, which may lessen the 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 and 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 have hypokalemia and hypertension commonly.[22] Recently, it was noted that M induces CYP3A4.[23] Because many antineoplastic drugs are metabolized by CYP3A4, drug–drug interactions causing additive toxicities can result from such combinations. Therefore, novel treatments that avoid such drug interactions and have the potential for a decrease in the required dosage of M would be a real advance to the field.

sHDL nanoparticles have good safety in clinical trials thus far and demonstrate a unique antineoplastic effect in part owing 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 (Fig 1) indicating that ACC cells express the highest level of SR-BI compared with several other cancers and normal cells, we hypothesized that sHDL nanoparticles would 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 whether combination dosing of sHDL and E, D, P, or M would result in a

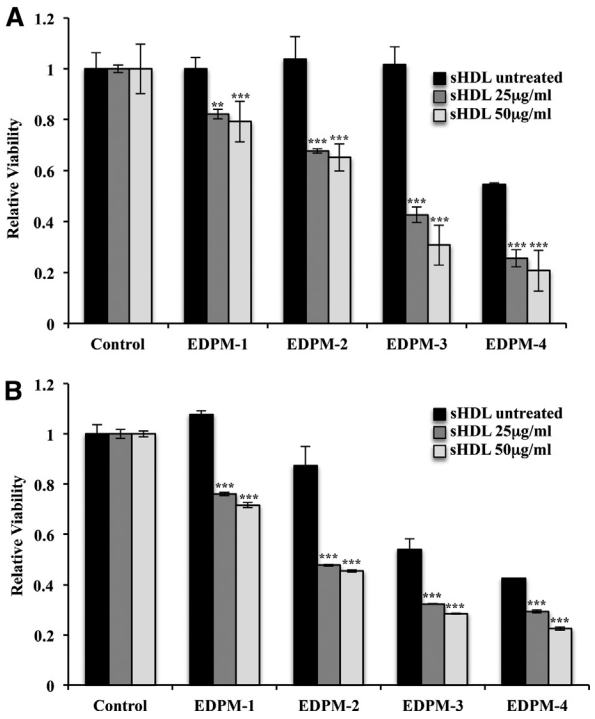

Fig 9. Effect of multidrug combination studies with synthetic high-density lipoprotein (sHDL) on adrenocortical carcinoma cell viability. NCI-H295R (*A*) and SW13 (*B*) cells were treated with EDPM (25%, 50%, 75%, or 100% MTD levels) alone or in combination with 25 or 50 μg/mL of sHDL. Untreated cells or sHDL alone treated cells served as controls. Despite the antagonistic effect of D, we observed an enhanced dose-dependent decrease in viability for both sHDL combinations compared with EDPM alone. Relative viability was calculated in terms of untreated controls. The mean value and standard deviation from three different experiments are presented with *** representing $P < .05$.

synergistic, additive, or antagonistic effect by calculating combination indices for each combination. Our results demonstrate that P, E, and M act synergistically with sHDL at multiple concentrations, whereas D acts as an antagonist. This effect was then confirmed using a clonogenic assay to demonstrate that combinations of sHDL and low doses of E, P, or M resulted in improved inhibition of cell viability compared with 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 membrane the potential of the mitochondria compared with monotherapy. Because mitochondrial depolarization can be owing to either oxidative stress or apoptosis, further studies are needed to understand fully how this combination modulates the activity of the mitochondrial respiratory chain. Synergistic increases in apoptosis compared with necrosis suggest a synergy in mechanism-based pathway, supporting the benefit of combination therapy on tumor biology as opposed to a merely nontargeted 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 M 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 increased 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 with monotherapy, combination therapy with sHDL increased expression levels of StAR, the intra mitochondrial transporter; CYP21A2, the enzyme metabolizing17-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 whether this effect on steroidogenesis leads to a downregulation 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 by >80%, similar to M. We then showed that the EDPM plus sHDL combination treatment retained its synergy despite the antagonism of D and that this combination effect is translatable to 3-dimensional cell growth in aggregates. Given the significant decrease 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 that sHDL nanoparticles act synergistically with chemotherapy agents used in ACC, allowing substantially lesser doses in combination to generate in vitro efficacy. This synergy may be owing in part to targeting of the steroidogenic pathway, similar to M, for potentiating enhanced apoptosis. 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 M and the Italian protocol.

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DISCUSSION

Dr Bradford K. Mitchell (Morgantown, WV): I have nothing to disclose. Can you tell us a little bit about the uptake of these nanoparticles? You suggest by the discussion on the sphere formation and its impact the possibility that these particles' uptake is based on receptor expression on the surface or something else on the surface that's going to dramatically change in your cells grown in monolayer. I wonder if that would impact your decision making about further testing.

Dr Mark S. Cohen (Ann Arbor, MI): We have actually looked at this in sphere formation, and we have recently completed, actually, some animal data as well. If you look at the SRB1 receptor expression levels, ACCs have about 100-fold greater expression than any other cancer and almost a thousand fold greater expression than any other cell type. It makes a very selective target for this particular HDL type of therapy. What we are seeing in the aggregate is an example of what we would expect to see in a tumor. We actually do see targeting and localization in vivo, which I have not presented in this paper, but that is part of our ongoing studies.

Dr Bradford K. Mitchell: What is the expression in the monolayer?

Dr Mark S. Cohen: We did look at monolayer expression, and we have some data on Western to show that I did not include here, but compared with tissue levels, it is pretty high. I would say it is 10- to 20-fold higher than what you would see in normal cells at least, maybe even 100-fold higher, but it is pretty dense expression.