Published in final edited form as:

J Biomed Nanotechnol. 2015 September ; 11(9): 1608–1627.

Identification and Testing of Novel CARP-1 Functional Mimetic Compounds as Inhibitors of Non-Small Cell Lung and Triple Negative Breast Cancers

Magesh Muthu^{1,3,†}, Jaganmohan Somagoni^{9,†}, Vino T. Cheriyan^{1,3}, Sara Munie^{1,3}, Edi Levi^{1,4}, Abdelkader E. Ashour⁵, Alaa Eldeen B. Yassin⁶, Ahmed M. Alafeefy⁷, Paula Sochacki¹, Lisa A. Polin^{2,3}, Kaladhar B. Reddy⁴, Scott D. Larsen⁸, Mandip Singh^{9,*}, and Arun K. Rishi^{1,2,3,*}

¹John D. Dingell VA Medical Center, Wayne State University, Detroit, MI 48201, USA

²Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, USA

³Department of Oncology, Wayne State University, Detroit, MI 48201, USA

⁴Department of Pathology, Wayne State University, Detroit, MI 48201, USA

⁵Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

⁶Pharmaceutical Sciences Department, College of Pharmacy-3163, King Saud bin Abdulaziz University for Health Sciences, and King Abdullah International Medical Research Center, Ministry of National Guard, Health Affairs, Riyadh 11451, Saudi Arabia

⁷Department of Pharmaceutical Chemistry, College of Pharmacy, Salman Bin Abdulaziz University, Alkharj 11942, Saudi Arabia

⁸Vahlteich Medicinal Chemistry Core and College of Pharmacy, University of Michigan, Ann Arbor, MI 48109, USA

⁹College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, FL 32307, USA

Abstract

The triple negative breast cancer (TNBCs) and non-small cell lung cancers (NSCLCs) often acquire mutations that contribute to failure of drugs in clinic and poor prognosis, thus presenting an urgent need to develop new and improved therapeutic modalities. Here we report that CARP-1 functional mimetic (CFMs) compounds 4 and 5, and 4.6, a structurally related analog of CFM-4, are potent inhibitors of TNBC and NSCLC cells *in vitro*. Cell growth suppression by CFM-4 and

*Authors to whom correspondence should be addressed. Mandip.sachdeva@fam.u.edu, Rishia@Karmanos.org.

†These two authors contributed equally to this work.

Author's Contributions

Conceived and designed the experiments: Arun K. Rishi, Mandip Singh. Performed the experiments: Magesh Muthu, Jaganmohan Somagoni, Vino T. Cheriyan, Sara Munie, Edi Levi, Abdelkader E. Ashour, Ahmed M. Alafeefy, Paula Sochacki, Lisa A. Polin. Contributed reagents/materials/analysis tools: Alaa Eldeen B. Yassin, Kaladhar B. Reddy, Scott D. Larsen. Contributed to the writing of manuscript: Magesh Muthu, Jaganmohan Somagoni, Mandip Singh, Arun K. Rishi.

-4.6 involved interaction and elevated expression of CARP-1/CCAR1 and Death Effector Domain (DED) containing DNA binding (DEDD)2 proteins. Apoptosis by these compounds also involved activation of pro-apoptotic stress-activated kinases p38 and JNK1/2, cleavage of PARP and loss of mitotic cyclin B1. Both the CFMs inhibited abilities of NSCLC and TNBC cells to migrate, invade, and form colonies in suspension, while disrupting tubule formation by the human umbilical vein endothelial cells (HUVECs). Nano-lipid formulation of CFM-4 (CFM-4 NLF) enhanced its serum bioavailability when compared with the free CFM-4. Oral administration of CFM-4 NLF reduced weights and volume of the xenografted tumors derived from A549 NSCLC and MDA-MB-231 TNBC cells. Although no gross tissue or histological toxicities were noticed, the immuno-histochemical analysis revealed increased CARP-1 and DNA fragmentation in tumors of the CFM-4 NLF-treated animals. In conclusion, while stimulation of pro-apoptotic CARP-1 and DEDD2 expression and their binding underscore a novel mechanism of apoptosis transduction by CFM compounds, our proof-of-concept xenograft studies demonstrate therapeutic potential of CFM-4 for TNBC and NSCLC.

Keywords

CFMs; TNBCs; NSCLC; CARP-1; SAPKs; Apoptosis; NLF

INTRODUCTION

Cancers cause one in four deaths in the US, and remain a major public health problem in the US and world over. The American Cancer Society estimates highest percent of new cases and mortality resulting from lung and breast cancers in females, while prostate and lung cancers account for highest percentage of new cases and associated mortality among men.¹ Throughout the last decade, the overall incidence rates and consequent mortality associated with the lung, breast, and colorectal cancers decreased among women, and a similar trend also occurred for lung, prostate and colorectal cancers in men.¹ The encouraging downward trends in cancer associated mortality could partly be attributed to recent advances in diagnosis and therapeutic modalities, particularly targeted therapeutics for a number of cancers. In this context, development of therapeutics that target estrogen receptor (ER) function and biosynthesis, and the human epidermal growth factor receptor (EGFR) 2 (aka Her2) have benefited a vast majority of the breast cancer patients, while EGFR targeting agents have also been utilized in treatments of non-small cell lung (NSCLC) and other cancers. However, for a significant percent of breast cancers that lack ER, progesterone receptor (PR), and Her2, often grouped as triple-negative breast cancers (TNBC), chemotherapy including an anthracycline and taxane-based regimen remain the current best standard of care. A recent study has revealed further stratification and existence of molecular subtypes among TNBCs and stresses the need for identification of better molecular-based therapies.² Coupled with occurrence of gain-of-function mutations in many cancers, including the NSCLCs, frequent relapses in clinic occur that often result in emergence of resistant, hard to treat disease. Although a number of cell growth and survival pathways are being actively pursued for targeting TNBCs,³ and many other difficult to treat cancers including NSCLCs, new therapeutic strategies are urgently needed to overcome drug resistance and improve therapeutic outcomes.

CARP-1 (Cell cycle and apoptosis regulator 1, aka CCAR1) is a peri-nuclear phosphoprotein and a regulator of cell growth and apoptosis signaling.⁴⁻⁷ CARP-1 not only functions as a transcriptional co-activator of steroid family of nuclear receptors and a regulator of adipogenesis through the glucocorticoid receptor (GR), it also regulates Adriamycin (ADR)-dependent apoptosis in part through co-activation of p53.^{4, 6} CARP-1 expression is often elevated in cells experiencing stress due to growth factor withdrawal or chemotherapy-induced cell cycle arrest and apoptosis.^{6, 7} Our recent studies revealed that apoptosis stimulation following blockage of EGFRs involved CARP-1 phosphorylation at tyrosine¹⁹², increased CARP-1 levels, activation of p38 MAPK and caspase-9.⁵ In addition, pharmacologic blockage of protein kinase A (PKA) also resulted in CARP-1 threonine⁶⁶⁷ phosphorylation, suppression of c-Myc transcription and inhibition of human breast cancer (HBC) cell growth.^{7, 8} Knockdown of CARP-1 resulted in resistance to apoptosis by ADR or EGFR tyrosine kinase inhibitors demonstrating requirement of CARP-1 in cell growth inhibitory and apoptosis signaling by these agents.^{4, 5}

To better define molecular mechanisms of signal transduction involving CARP-1, we conducted yeast-two-hybrid analyses and found that CARP-1 interacted with cell cycle regulatory anaphase promoting complex/cyclosome (APC/C) E3 ligase subunit APC2.⁹ Our investigation revealed that CARP-1 functions as a coactivator of the APC/C E3 ligase.⁹ APC/C is a multisubunit ubiquitin E3 ligase protein that plays a distinct role in cell cycle transitions, and misregulation of APC/C substrates such as securin, polo-like kinase (Plk) has been demonstrated to correlate with tumor progression.¹⁰⁻¹² We then tested whether and to the extent abrogation of CARP-1-APC/C interaction and consequent interference with APC/C functions could be detrimental to progression of cancer cell cycle and growth. A chemical biology-based high-throughput screening of a chemical library resulted in identification of a number of novel, small molecule inhibitors (SMIs) of CARP-1 binding with APC/C subunit APC2. These compounds, termed CARP-1 functional mimetics (CFMs), inhibit cell growth by inducing apoptosis in various cancer types.^{9, 13, 14} Here we provide evidence that CFMs and their analogs are potent inhibitors of TNBC and NSCLC cell growth *in vitro*, and the nano-lipid formulation of CFM-4 (CFM-4 NLF) inhibited growth of TNBC and NSCLC xenografted tumors in nude mice.

MATERIALS AND METHODS

Materials

DMEM, RPMI-1640 medium, and antibiotics (penicillin and streptomycin) were purchased from Invitrogen Co. (Carlsbad, CA). FBS was purchased from Denville Scientific Inc. (Metuchen, NJ). CFM-1 [(Z)-5-(5-bromo-7-methyl-2-oxoindolin-3-ylidene)-2-thioxothiazolidin-4-one], CFM-4 (1-(2-chlorobenzyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one), CFM-5 (5-bromo-1-phenethyl-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one), and CFM-4 analogs CFM-4.1 (1-methyl-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one), CFM-4.2 (5-bromo-1-methyl-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one), CFM-4.3 (1-methyl-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4]thiadiazole]), CFM-4.4 (5'-benzyl-1-methyl-3'H-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one), CFM-4.5 (1-(2-chlorobenzyl)-3'-

phenylspiro[indoline-3,2'-thiazolidine]-2,4'-dione), and CFM-4.6 (1-(naphthalen-1-ylmethyl)-5'-phenyl-3'H-spiro[indoline-3,2'-[1,3,4] thiadiazol]-2-one) were obtained from ChemDiv, San Diego, and Ryan Scientific, Inc., Mt. Pleasant, SC, and are shown in Figure 1 below. All the CFM compounds were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10–50 mM, and stored at –20 °C. Cisplatin, anti- β -actin mouse monoclonal antibody, and chemicals including 3-4,5-dimethylthiazol-2-yl-2.5-diphenyl-tetrazolium bromide (MTT) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). The cell culture grade non-essential amino acids (100 $\times$ stock) was purchased from Gibco Life Technologies, Grand Island, NY. The mouse monoclonal antibody for α -tubulin was obtained from Calbiochem (Billerica, MA). Anti-cyclin B1 and caspase-8 (1C12) mouse monoclonal antibodies, anti-RIPK1 (D94C12) rabbit monoclonal antibody, and rabbit polyclonal antibodies for PARP, phospho and total p38 α/β , phospho and total JNK1/2 SAPKs were obtained from Cell Signaling Technology (Beverly, MA). Anti-DEDD2 goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-FADD mouse monoclonal antibody was obtained from BD Pharmingen (San Jose, CA). Generation and characterization of the anti-CARP-1/CCAR1 rabbit polyclonal antibodies have been described elsewhere.⁷ Enhanced Chemiluminescence Reagent was purchased from Amersham Biosciences (Piscataway, NJ) and the Protein Assay Kit was purchased from Bio-Rad Laboratories (Hercules, CA).

Cells and Cell Culture

Human NSCLC cell lines A549, CALU-C3, H1299, and H460, human TNBC cell lines MDA-MB-468, MDA-MB-231, HCC1937, and Hs578T, as well as human neuroblastoma (NB) cell lines SK-N-BE(2) and SK-N-SH were purchased from ATCC, and were kindly provided by Drs. Sandro daRocha, Julie Boerner, Jing Li, and Yubin Ge, Departments of Chemical Engineering and Materials Science, Oncology and Pharmacology, Wayne State University, Detroit, MI. The NSCLC and NB cells were routinely cultured in RPMI 1640 medium while the human malignant pleural mesothelioma (MPM) cells H2373, H2461, H2714, and mouse mesothelioma AB12 cells were cultured either in RPMI 1640 or DMEM media essentially as described.^{13, 15–17} Routine culture of TNBC MDA-MB-231, MDA-MB-468, and Hs578T cells were carried out essentially as described before.^{9, 18} The HCC1937 TNBC cells were maintained in RPMI 1640 containing glucose (4.5 g/l), sodium bicarbonate (1.5 g/l), 10 mM HEPES, 1 mM sodium pyruvate and 1 $\times$ non-essential amino acids. SKBR-3 and MDA-MB-453 (human breast cancer cells that lack estrogen receptor, have mutant p53, and overexpress Her-2), human prostate cancer PC-3, human pancreatic cancer PANC-1, human follicular lymphoma WSU-FSCCL, human medulloblastoma (MB) Daoy, and HL-60 cells were routinely cultured as described by us before.^{9, 14, 19, 20} The human umbilical vein endothelial cells (HUVECs) and the *in vitro* angiogenesis assay kit was purchased from Lonza Inc. (Walkersville, MD) and Chemicon International Inc. (Temecula, CA), respectively. The HUVECs were maintained in a specified media (EGM Bullet kit; Lonza Walkersville Inc.) per the manufacturer suggested guidelines. All the cell culture media were also supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin, and the cells were maintained at 37 °C and 5% CO₂.^{13, 15–20}

Cell Viability, Apoptosis and Immuno-Precipitation (IP)-Western Blot (WB) Assays

The alterations in cell viabilities in the presence of CFMs were assessed by MTT reagent. Cells (5×10^3) were seeded in a 96-well culture plate and subsequently treated with respective CFMs or Cisplatin (Cis) at different concentrations and periods as mentioned. Control cells were treated with 0.1% DMSO in culture medium. After treatment, the cells were incubated with 1 mg/ml of MTT reagent at 37 °C for 2–4 hours and then MTT was removed and 50 μ L of DMSO was added, followed by colorimetric analysis using a multi-label plate reader at 560 nm (Victor3; PerkinElmer, Wellesley, MA, USA).

Apoptosis in tumor tissues was determined by staining for fragmented DNA utilizing terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay. TUNEL kits were purchased from Roche Diagnostics (Indianapolis, IN). The formalin-fixed tumor xenograft biopsies (see below) from the animals that were untreated or treated with CFM-4 NLF were paraffin-embedded and processed essentially following our previously described procedures.¹⁹ The tumor tissue slides were also stained for presence of CARP-1 using a 1:250 dilution of anti-CARP-1 (α 2) antibody. The fixed and labeled tumor tissues were photographed essentially as detailed in our previously described methods.¹⁹

For protein interaction (binding) and expression analysis, IP-WB experiments were carried out following our previously published methods. Briefly, the cells were either untreated or treated with different compounds for various times. Cells were lysed in cell lysis (10 $\times$) buffer (#9803; cell signaling) containing 0.1% of protease and phosphatase inhibitor cocktail (Sigma) for 20 min at 4 °C. The cell lysates were centrifuged at 14,000 rpm at 4 °C for 15–20 min to remove debris. Protein concentrations of the clarified cell lysates were determined using the Protein Assay Kit. IP was carried out by incubating ~1 mg of the protein lysate with appropriate antibodies, and the innunocomplexes were washed (3 $\times$) with cell lysis buffer. The immunoprecipitates or the cell lysates (50–100 μ g from each sample) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-rad, Hercules, CA) by standard procedures. The membranes were then hybridized with primary antibodies followed by incubation with appropriate secondary antibodies. The antibody-bound proteins were visualized by treatment with the chemiluminescence detection reagent according to manufacturer's instructions, followed by exposure to X-ray film (Denville Scientific Inc.). The membranes having protein lysates were re-probed with the anti- β -actin or anti- α -tubulin antibody, which was used as an internal control for protein loading.

Isolation of RNA and Microarray Analysis

The MDA-MB-468 TNBC cells were treated with 2 μ M, 5 μ M and 10 μ M dose of CFM-4 for 12 h in serum-free medium. At the end of treatments, the untreated and treated cells were harvested and total RNA were isolated, and purified using the RNeasy Mini kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's protocols. Alterations in CFM-4-treated TNBC cell gene expression were investigated by performing array hybridization experiments at the Genomic Core Facility, Karmanos Cancer Institute utilizing Illumina BeadChip Arrays essentially according to manufacturer's instruction (Illumina Inc., San Diego, CA). Briefly, 0.5 μ g total RNA was biotin-labeled and hybridized

with BeadChips. The signal was detected with streptovadin-Cy3 according to manufacturer's instruction (Illumina). The imaging of the BeadChips was conducted using a Bead Array Reader in conjunction with Bead Studio software (Illumina). Data normalization was carried out using a quantile based approach which transforms the raw data so that the resulting normalized expression values of each sample have the same distribution.²¹ Differential gene expression between the untreated and treated groups was tested using a moderated *t*-test. Genes were selected as significant using a cutoff of 0.1 on the nominal *p*-values and a cutoff of 1.5-fold change in expression between groups.

Cell Migration, Invasion, Clonogenic, and Angiogenesis Assays

Migration Assay—The effects of CFMs on migration of TNBC and NSCLC cells were measured by the “scratch” assay. Cells were grown in a 6-well plate (~10⁵ cells/well), and a scratch was created in the cell monolayer using sterile pipette tip. The cells were then allowed to grow in the absence (Control) or presence of the CFMs for a period of 72–96 h. The cells were photographed under different magnifications utilizing Zeiss microscope with attached 35 mm camera for recording the photomicrographs, and images were captured at regular intervals during and at the end of the treatment periods.

Clonogenic Assay—Cells were sandwiched between 0.6% and 0.3% agarose in DMEM medium containing 5% FBS in a six-well chamber (500 cells/chamber), and treated with buffer (Control), or respective CFM for 9 days at 37 °C humidified CO₂ incubator. The colonies from multiple random fields were counted, compared to control and photographed as above.

Invasion Assay—Basement membrane is a thin extracellular matrix (ECM) that underlies epithelia and endothelia and separates epithelial cancer cells from the stroma. Tumor cells produce proteases that degrade ECM to cross the basement membrane to invade stroma and establish distant metastases. The *in vitro* Boyden Chamber assay (Chemicon International, CA) using Matrigel is the most reliable, reproducible, and representative of *in vivo* invasion. Briefly, pre-warmed serum free medium (300 μ l) was used to hydrate the ECM layer of each chamber for 15–30 minutes at room temperature. Approximately 2–2.5 × 10⁵ HBC cells were seeded in the upper chamber in a serum-free medium without or with 10 μ M dose of the respective CFM. The lower chamber was supplied with medium containing 10% FBS that served as chemo-attractant to stimulate migration. After an interval, tumor cells present on the lower side of the membrane in the lower chamber were stained, and photographed as above. In addition, the stained cells from the lower side of membrane of some wells were dissociated, lysed in a buffer, followed by quantitation using a fluorescence plate reader with 480/520 nm filter set. The measurements were then plotted as bars in histogram as previously described.^{13, 14}

Angiogenesis Assay—Tubule formation by HUVECs, a process that generates new capillaries, is often used as surrogate for tumor angiogenesis. We determined whether CFMs interfered with the ability of HUVECs to form tubules to further evaluate whether these compounds possessed anti-angiogenic properties. Approximately 5000 cells from passage 5 were seeded onto a surface containing polymerized extracellular matrix (ECM). Cellular

network structures were allowed to develop in EGM medium (Control) for 12 hours, and subsequently either untreated or treated with 10 μM of respective CFM in serum-free EGM medium for 6 hours. Representative photomicrographs of control and treated cells were obtained as above.

Formulation of CFM-4 Nano Lipid Carriers (CFM-4-NLF)

The CFM-4-NLF was prepared by combining melt-emulsification and ultrasonication technique (Nanoease Technology, Filed Non provisional US patent # 61440977). Briefly, appropriate amounts of CFM-4, Compritol 888 ATO, Miglyol 812N and Geleol were blended and melted at 70 °C to form a uniform and clear oil phase. Meanwhile, the aqueous phase consisting of dispersing surfactant Tween 80 and Vitamin E TPGS in double distilled water was added drop wise to the oil phase at the same temperature by the aid of agitation at 5000 rpm for 5 min using tissuemiser. The coarse emulsion was then subjected to high pressure homogenization for 15 min. The resultant NLF was further processed with NanoDebee for about 5 cycles followed by probe sonication for 5 minutes to further decrease its size.²²

Surface Modification of CFM-4 NLF—For surface modification of NLF, appropriate amounts of the chitosan polymer (CP) was dissolved in water in order to form a series of concentrations (0.25%, 0.5%, 1%, 2%, w/v), and then mixed with CFM-4 loaded NLF dispersions.²³ In each case, an aliquot of NLF was mingled with an equal volume of CP by adding it drop wise under continuous agitation at room temperature (20 °C) for a 30-min incubation.

Drug Encapsulation Efficiency (EE)—Free drug (non-incorporated in the SM-NLF) was separated by ultrafiltration centrifugation technique.²⁴ Briefly, 1 mL of CFM-4 NLF colloidal solution was placed in the upper chamber of a centrifuge tube matched with an ultrafilter and centrifuged for 15 min at 4000 rpm. The total drug content in CFM-4 NLF was determined as follows: aliquots of 1mL SM–NLF dispersion were diluted appropriately by ethanol to dissolve the lipid ingredient and the resulting suspension was then filtrated through 0.45 μM membrane filters. The filtered solution was analyzed by Waters® Alliance e2695 HPLC using Symmetry® C18 column (250 mm×4.6 mm, 5 μM). The mobile phase was a mixture of Acetonitrile, Methanol, 10 mM KH_2PO_4 buffer (65:20:15 v/v) with pH adjusted to 2 and the flow rate was maintained at 1.0 mL/min. All the samples were analyzed at 248 nm using empower PDA software. The composition of CFM-4 NLF is summarized in Table I.

The encapsulation efficiency (EE) and drug loading was calculated by the following equations:

$$\text{EE (\%)} = \frac{W_{\text{Total}} - W_{\text{Free}}}{W_{\text{Total}}} \times 100$$

$$\text{Drug loading (\%)} = \frac{W_{\text{Total}} - W_{\text{Lipid}}}{W_{\text{Total}}} \times 100$$

where W_{Total} , W_{Free} , W_{Lipid} were the weight of total drug in NLF, the weight of un-entrapped drug in ultra-filtrate, and the weight of lipid added in system, respectively.

Particle Size and Zeta Potential Measurement—The average particle size, zeta potential and polydispersity index (PI) of SM–NLF was determined using zeta sizer at 25 °C after appropriate dilution with ultra-purified water.

In Vitro Drug Release Studies—*In vitro* drug release study was conducted in pH 7.4 PBS containing 1% volpo. Briefly, 20 mg of free drug or equivalent amount of CFM-4 NLF were placed in dialysis bag and kept in a basket which was immersed in 500 ml of release medium. Release studies were performed according to the USP type I basket method at 37 °C while stirring constantly at 50 rpm. Samples were withdrawn at different time points, centrifuged and drug content in the samples was analyzed by HPLC. The withdrawn samples were replaced by equal volumes of fresh medium maintained at the same temperature.

Pharmacokinetic Studies—The bio-availability kinetics of the CFM-4 NLF formulation, and CFM-4 free drug (FD) were conducted in rodents (Sprague Dawley Rats). Rats were fasted overnight before the start of the experiments and randomly divided into three experimental groups receiving CFM-4 FD and CFM-4 NLF at 40 mg/kg orally and CFM-4 solution (CFM-4 sol) at 5 mg/kg by intravenous route. After the drug administration, blood samples (250 μ l) were withdrawn from tail veins at predetermined time points (0, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h).

The blood samples were collected directly into heparinized microvet blood collection tubes and plasma was obtained by centrifugation at 10,000 rpm for 10 min and then stored at –80 °C until analysis. CFM-4 was extracted from the plasma by protein precipitation method and extracted samples were dissolved in mobile phase and samples were analyzed by HPLC. Oral bioavailability of CFM-4 FD and CFM-4 NLF along with their pharmacokinetic parameters such as area under curve (AUC), C_{max} , $t_{1/2}$, and t_{max} were estimated. Pharmacokinetic parameters were analyzed using non-compartmental techniques with WinNonlin® 5.0 software (Pharsight Corporation, Mountain View, CA, USA).

Murine Xenograft Experiments

The experiments involving xenograft studies were performed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committees at the Wayne State and Florida A&M Universities, and according to our previously published methods.^{22, 25} In the first instance, a maximal tolerated dose (MTD) for CFM-4 was determined in the SCID mice. A 20 mg/ml stock of CFM-4 was prepared in 10% DMSO/cermophor+dH₂O, and pH adjusted to 4.5. The SCID mice ($n = 4$) were administered 24–36 mg/kg dose of CFM-4, via tail vein injection, twice a day over a period of ten days. CFM-4 was generally well tolerated by the mice. Although a small (<5%) weight loss was noticed in some animals, no other adverse symptoms were noted. The mice were observed for subsequent three weeks post last treatment and did not show any latent toxicity including symptoms of diarrhea, dehydration, weight loss, hair loss, or any other discomfort.

Histologic as well as microscopic examination of various tissues (liver, kidney, heart, spleen, and lung) did not show any abnormalities (not shown).

Establishment of Sub-Cutaneous Tumors in SCID Mice—Three week-old, female, ICR SCID mice were obtained from Taconic Laboratories (German Town, NY). After a period of adaptation, 2 to 3 mice were subcutaneously (sc) injected on each flank with approximately 10^6 HBC SKBR-3, MDA-MB-231, MDA-MB-468, MDA-MB-453, prostate cancer PC-3, pancreatic cancer PANC-1, MPM H2461, H2714, AB12, MB Daoy, or follicular lymphoma WSU-FSCCL cells. When tumors developed, mice were sacrificed; tumors were dissected, cut into small fragments, and subsequently transplanted sc into similarly conditioned animals ($n=6$ for each group) by using a 12-gauge trocar. Mice were checked 3 times a week for tumor development. Once palpable tumors developed (usually <100 mg in size) by the day seven of xenograft implantation, groups of 6 mice were removed randomly for the efficacy trial using CFM-4. For treatment groups, each animal was injected 0.2 ml of CFM-4 solution via tail vein. The schedule and total dose of CFM-4 for each of the xenograft study are listed in Table II. The tumor measurements were carried out at multiple time points during the course of treatments and observation periods. Mice were observed for changes in weight and side effects followed by measurement of tumors three times per week.

The end points for assessing antitumor activity consisted of tumor weight, tumor growth inhibition (% T/C), and tumor cell kill Log 10. Tumor weight (mg) = $(A \times B^2)/2$ where A and B are the tumor length and width (in mm), respectively. Tumor growth inhibition (T/C) was the median tumor weight in the treated group (T) when the median tumor weight in the control group reached 750 mg. Results was expressed as percentage. According to NCI-accepted criteria, a treatment is considered effective if T/C is $<42\%$. Tumor growth delay ($T - C$) is the difference between the median time (in days) required for the treatment group tumors (T) to reach 750 mg and the median time (days) for the control group tumors to reach the same weight. Unlike T/C , animals that have no measurable tumor after treatment “cures” are excluded in this formula.

Tumor cell kill Log10 (Gross) = $(T - C)/(3.32)(Td)$ where Td = tumor doubling time (in days). Tumor cell kill Log 10 (Net) = $(T - C) - (\text{Duration of Rx in days})/(3.32)(Td)$.

Development of Orthotopic Breast Cancer Model in BALB/c Nude Mice—About 6–8-week-old female BALB/c nude mice were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, housed in isolated vented cages and allowed to acclimate for one week with standard chow diet. Briefly, 1×10^6 MDA-MB-231 TNBC cells were re-suspended in 200 μ l of serum-free Hank's balanced salt solution, and implanted in the mammary fat pads of female BALB/c nude mice using a 27-gauge needle. Tumors were allowed to grow unperturbed for 10–14 days and when the tumors became palpable (200 mm³), the mice were randomly assigned to treatment or control groups of six animals each. Mice were treated with placebo (Control) or CFM-4 NLF (60 mg/kg) by oral gavage (60 mg/kg) every second day for 4 weeks.

Development of Orthotopic A549 Lung Tumors in Nude Mice—The animals were anesthetized with Isoflurane and a 5 mm skin incision was made to the left chest, ~5 mm from scapula. A 100 μ l of cell suspension (1×10^6 A549 NSCLC cells) in a Hamilton syringe was directly inserted through the 6th intercostal space into the lung to a depth of 3 mm.²⁶ Wounds were closed using surgical skin clip. Tumors were allowed to grow for two weeks and then the mice were randomly assigned to treatment or control groups (six animals per group). The three groups consisted of placebo (Control), CFM-4 FD (40 mg/kg), and CFM-4 NLF (40 mg/kg). The formulations were administered by oral gavage every second day for four weeks.

The tumor volume, tumor weight and body weight were measured regularly during the study. At the end of study, the tumors were harvested from the euthanized mice, and a part of each tumor tissue was fixed in formalin for IHC & HE studies while the remaining tissue was utilized to assess the drug distribution in the tumor.

The tissue/tumor samples were extracted by following a modification of a previously described procedure.²⁷ In brief, accurately weighed 0.5 g of tissue was minced and homogenized with 500 μ L of acetonitrile and methanol (1:1) for 10 min using tissue homogenizer to completely solubilize the drug in organic phase. All the samples were then centrifuged at 15000 rpm for 15 min. The supernatant was collected and evaporated at 40 °C under a stream of nitrogen followed by reconstitution with 250 μ L of mobile phase, mixed by vortexing for 30 sec and passed through a filter with 0.45 μ M pore size (Millipore). All the samples were then analyzed by HPLC to determine the drug content.

Statistical Analysis

In some instances, statistical analysis was performed using unpaired Student's *t*-test. A *p*-value less than 0.05 between treatment groups was considered significantly different.

RESULTS

Novel Analogs of CFM-4 Suppress Growth of Human NSCLC and TNBC Cells

In an attempt to develop CFM-4 as a potential anti-cancer agent, we performed preliminary structure activity relationship (SAR) studies. In the first instance, we identified six additional compounds, named as CFM-4.1 to 4.6, that were structurally similar to our lead compound CFM-4 (Fig. 1). Each compound was utilized in an *in vitro* fluorescence polarization assay (FPA) and respective IC₅₀ values were determined essentially as described by us before.⁹ The FPA revealed IC₅₀ of 0.31 μ M, 0.27 μ M, 1.21 μ M, 1.13 μ M, 13.5 μ M, and 3.96 μ M for CFM-4.1, CFM-4.2, CFM-4.3, CFM-4.4, CFM-4.5, and CFM-4.6 respectively. We next performed MTT-based *in vitro* assays to determine the extent CFMs 1, 4, and 5, and CFM-4.1–4.6 compounds affected the viabilities/growth of cancer cells. Our previous studies have revealed HBC, prostate cancer, pancreatic cancer, colon cancer, MPM, MB, and NB cell growth inhibitory effects of CFMs 1, 4, and 5.^{9, 13, 14, 28} Here we undertook further studies to determine whether the CFM compounds and CFM-4 analogs inhibit growth of NSCLC and TNBC cells, and investigated the molecular mechanisms involved. Consistent with our observations in other cancer models, CFMs 1, 4, and 5, and CFM-4 analogs

inhibited growth of a number of NSCLC and TNBC cells. With reference to NSCLC, a 48 h treatment with various doses of CFM-1, -4, -5, and Cisplatin caused loss of cell viability. Treatments with various doses of Cisplatin or CFM-1 generally elicited a 20–50% loss of viabilities of all the NSCLC cells (Fig. 2(A)). CFM-5 exposure resulted in a somewhat higher loss of cell viability in the case of H1299, H460, and A549 cells when compared with their loss of viabilities noted following treatments with CFM-1 or Cisplatin. Calu-C3 cells however were highly sensitive to 10 and 20 μ M doses of CFM-5 when compared with their CFM-1 or Cisplatin-treated counterparts (Fig. 2(A)). All the doses of CFM-4 however were able to elicit ~80–90% loss of viability of all the NSCLC cells (Fig. 2(A)). A time course analysis further revealed that a 10 μ M dose of CFM-4 for 12 h or 24 h periods caused ~50–60% reduction in the viabilities of A549, H1299, and H460 NSCLC cells, while this CFM-4 dose and treatment periods failed to significantly impact the viabilities of CALU-C3 cells (Fig. 2(B)). Since A549 and H1299 cells were affected to similar extent by various treatment periods or doses of CFM-4, we next investigated the extent CFM-4 analogs would also affect the viabilities of these two NSCLC cells. Both the NSCLC cells were independently treated with a 10 or 20 μ M dose of each of the CFM-4, CFM-4.1, -4.2, -4.3, -4.4, -4.5, and -4.6 compounds for a period of 24 h. As shown in Figure 2(C), CFM-4 and CFM-4.6 compounds elicited a significant loss of viabilities of both the NSCLC cells, while a modest, ~20–30% inhibition was noted in CFM-4.1, 4.2, and 4.3-treated A549 cells. Majority of the CFM-4 analogs in general were unable to stimulate a robust growth inhibition of the NSCLC cells. The data in Figure 2 suggest that although CFM-4 had greater potency when compared with Cisplatin, its analog CFM-4.6 was equally effective in inhibiting viabilities/growth of the NSCLC cells *in vitro*.

In light of the data in Figure 2 that indicated CFM-4.6 was potent in inhibiting growth of NSCLC cells, we further determined whether this analog was also an effective growth inhibitor of other cancer cell types. For this purpose, we utilized MPM (H2373 and H2461) and NB (SK-N-BE(2) and SK-N-SH) cell lines as well as TNBC MDA-MB-468, MDA-MB-231, HCC1937, and Hs-578T cells. Each of the cell line was separately treated with 10 μ M dose of each compound for 24 h, and cell viabilities were determined as above. Although CFM-4.4 and -4.5 did not affect MPM and NB cells, and were moderately active in TNBC cells, both the CFM-4 and CFM-4.6 treatments provoked a robust ~80–90%, ~70–90%, or ~50–60% loss in viabilities of MPM, TNBC, or NB cells, respectively (Fig. 3). CFM-4.1 and CFM-4.3 also induced ~60–80% reduction in MPM cell viabilities (Fig. 3(B)) while CFM-4.1 caused ~50–60% reduction in viability of all the TNBC cells (Fig. 3(A)). Interestingly, although CFM-4.1, -4.2, and -4.3 compounds caused an absent to moderate loss of NB cell viabilities (Fig. 3(C)), NB cells were generally less sensitive to treatments with 10 μ M dose of each CFM-4, -4.1, -4.2, -4.3, and -4.6 over a 24 h period when compared with TNBC and MPM cells (Fig. 3). The data in Figures 2 and 3 collectively suggest that although compounds CFM-4.1, -4.2, -4.3 have different levels of cytotoxicity towards TNBC, NB, and MPM cells, CFM-4 and CFM-4.6 caused a similar and high levels of cytotoxicity in NSCLC, MPM, and TNBC cells while both the compounds were moderately active in NB cells. Importantly, the reduction in viabilities of all the cell lines that were treated with CFM-4.6 was generally similar, if not higher, than their CFM-4-treated counterparts.

CFM-4 and Its Analog CFM-4.6 Suppress Growth of Human NSCLC and TNBC Cells in Part by Stimulating Apoptosis

In an attempt to further elucidate molecular mechanisms of cell growth inhibition by CFMs, we first conducted a gene-array-based analysis. MDA-MB-468 TNBC cells were either untreated or separately treated with CFM-4 as described in Methods. The RNAs from each group were hybridized with gene-array chips, and the data were analyzed to identify genes that had a significant 1.5-fold or higher altered expression following CFM-4 treatments. A select subset of these genes is indicated in Table III. The array data revealed down-regulation of a majority of genes while a small number of gene were up-regulated. Among the genes that were down-regulated included those involved in DNA damage repair, ubiquitination and transcriptional regulation to maintain genomic stability, and cell cycle control. In particular, CFM-4 treatments caused reduced expression of DNA repair regulating BRCA1 associated RING domain 1 (BARD1) and DEAD (Asp-Glu-Ala-Asp) box helicase (DDX)1 that is involved in repair of double strand breaks (DSBs), co-activation of p65/RelA subunit of NF- κ B, and RNA processing. In addition, cell cycle regulatory Cyclin A2 as well as eukaryotic translation initiation factor (EIF) 4A2 expression was also down-regulated in CFM-4-treated TNBC cells (Table III). Among the genes that were up-regulated included the tumor necrosis factor alpha inhibitory protein (TNFAIP)3-interacting proteins (TNIP) 1 [also known as A20-binding inhibitor of NF- κ B activation (ABIN) 1], as well as the death effector domain containing (DEDD) 2 proteins. CFM-4-dependent increased expression of ABIN1/TNIP1 in TNBC cells is consistent with our earlier studies showing increased expression of ABIN1 in MB cells following treatments with CFM-4.¹⁴ Interestingly, CFM-4 exposure resulted in down-regulation of ABIN1 in MPM cells while in NB cells the ABIN1 expression was regulated by CFM-4 in a cell-type manner.^{13, 28} Moreover, although CFM-4 treatments also caused reduced expression of RelA-regulator DDX1 (Table II), our recent studies revealed that transcriptional activities of NF- κ B were nonetheless enhanced in TNBC cells following their exposure to CFM-4, ADR, or TNF α .²⁸

We further investigated the molecular mechanisms of TNBC and NSCLC growth inhibition by CFM-4 and its analog CFM-4.6. The cells were separately treated with 10 μ M or 20 μ M dose of each of the compound for 12 h. In addition, the cells were treated with 16.7 or 33 μ M dose of Cisplatin for 12 h. Expression of CARP-1, cyclin B1, cleaved PARP, activation of pro-apoptotic SAPK p38, and levels of DEDD2 and actin proteins were determined by WB. As shown in Figure 4, with the exception of TNBC MDA-MB-231 cells, cisplatin treatments provoked an increase in CARP-1 expression in NSCLC (H1299 and A549) and the TNBC MDA-MB-468 cells. Cisplatin treatments caused PARP cleavage in NSCLC and MDA-MB-468 TNBC cells (Figs. 4(A)–(C)). Treatments with CFM-4 or CFM-4.6 however caused increased expression of CARP-1, activation of p38 and JNK1/2 SAPKs, and cleavage of PARP in both the NSCLC as well as TNBC cells (Figs. 4 and 5). Interestingly, as also demonstrated in Figure 5, activation of p38 precedes that of JNK1/2 and PARP cleavage in the CFM-4.6-treated TNBC cells. Since SAPKs are not known to cross-activate each other during apoptosis signaling, activation of p38 before JNK1/2 indicate a pleiotropic signaling by CFM-4.6. In light of our gene-array data in Table III showing elevated expression of DEDD2, and the facts that DEDs are protein–protein interaction modules that occur in a family of proteins that are primarily involved in control of apoptosis and proliferation

mediated by the TNF α receptor/death receptor (DR) family,^{29, 30} we further clarified whether TNBC and NSCLC cell growth suppression by CFM-4 or CFM-4.6 involved stimulation of DEDD2 expression. Consistent with promotion of apoptosis by CFM-4 or CFM-4.6 in TNBC and NSCLC cells, WB analysis revealed elevated expression of DEDD2 in both the NSCLC (H1299 and A549) and MDA-MB-231 TNBC cells (Figs. 4(A), (B), (D), 5(B)). In addition, levels of mitotic cyclin B1 were diminished in NSCLC and TNBC cells that were treated with CFM-4 or CFM-4.6 (Fig. 4), and were corroborative of our previous studies that indicated CFM-4-dependent loss of cyclin B1 in MB, MPM, and NB cells.^{9, 13, 14, 28}

CARP-1 is Part of an Intracellular, Pro-Apoptotic Sub-Complex

We previously noted that activation of caspase-8 that is often regulated by TNF α receptor/DR family was required for apoptosis signaling by CFM-4,⁹ and since both the caspases-8 and -10 contain two DED each, DEDD2 has been thought to play a critical role in transducing death receptor-induced apoptosis by targeting caspases-8 and -10 to the nucleus.²⁹ Since CARP-1 is a perinuclear protein⁷ and although the precise mechanisms of CARP-1 shuttling between the nuclear and cytoplasmic compartments are yet to be elucidated, we next investigated whether, in addition to elevated expression of DEDD2 and CARP-1 proteins during apoptosis, CARP-1 also interacts with DEDD2. This interaction would facilitate a pro-apoptotic complex of CARP-1-DEDD2-caspases-8 and -10 to regulate activation of these caspases in cytosol. Although both the DEDD2 and CARP-1 have nuclear localization signals for nuclear import, their interaction and formation of pro-apoptotic complex with caspases could also facilitate efficient transport of activated caspases to the nucleus. To test this hypothesis, we conducted an IP-WB experiment, and found that CARP-1 binds with DEDD2, Receptor-Interacting Protein Kinase (RIPK)1, Fas-Associated via Death Domain (FADD), and cleaved caspase-8 proteins (Fig. 6). Given that caspase-8 is often activated at the intra-cellular, death-inducing signaling complex (DISC) following activation/ligation of TNFR/DR family members,³⁰ and FADD, RIPK1, and caspase-8 have previously been proposed to be parts of a pro-apoptotic subcomplex that transduces DR signaling and caspase-8 activation,³¹ the interaction of CARP-1 with DEDD2, cleaved (p43/41) caspase-8, FADD and RIPK1 proteins strongly suggests that CARP-1 is a novel component of the intracellular complexes that regulate caspase-8 activation. Further studies would be necessary to determine whether and to the extent CARP-1 binding with DEDD2, FADD, and/or RIPK1 is required for apoptosis transduction by CFM compounds.

Oral Administration of the CFM-4 NLF Formulation Suppresses Growth of Xenografted Human NSCLC and TNBC Cells

To further explore the potential of CFMs as anti-cancer molecules, we investigated whether CFMs altered biological properties of the cancer cells and to the extent CFM-4 was effective in suppressing growth of xenografted tumors in mice. In the first instance, *in vitro* studies were carried out utilizing HBC and NSCLC cells to determine inhibition of cell migration, colony formation and invasion by CFMs. Treatments with CFM-1, -4, -5, or -4.6 prevented MDA-MB-468 and H1299 cells from robustly growing in the areas of wound created by a scratch when compared with their untreated counterparts as well as caused significant reduction in size and number of colonies formed by these cells in soft agar (Figs. 7(A)–(D)).

We next examined whether CFMs blocked the ability of HBC cells to invade through matrigel-coated membranes as detailed in methods. As expected, treatments of HBC cells with CFM-1, CFM-4 or CFM-5 caused reduced number of cells that were able to invade through the matrigel-coated membranes (Fig. 7(E)). Additionally, an *in vitro* tubule formation assay was utilized to determine the extent CFMs possessed anti-angiogenic properties. As shown in Figure 8, presence of each compound disrupted formation of tubules by HUVECs when compared with the untreated controls.

Next, subcutaneous tumor xenografts derived from different cancer cells were generated in SCID mice and efficacy and potency of CFM-4 was first tested by intravenous (Tail vein injection) administration as detailed in methods. Although growth of the SKBR-3 HBC cell-derived xenografts was affected by the CFM-4 treatments in an initial experiment (not shown), subsequent experiments failed to yield a therapeutic *T/C* values for this agent when used in animals bearing xenografts derived from a variety of cancer cells including the SKBR-3 HBC cells. The data from these set of xenograft studies are summarized in Table II. Since all the xenograft studies involved intravenous administration of CFM-4 that was dissolved in DMSO plus cremophor, we suspected that its systemic metabolism likely contributed to its lower levels in serum that could have resulted in its lack of xenograft inhibitory effects. We clarified this possibility as detailed below.

The nanostructure lipid carriers (NLF) have been developed to increase the physicochemical stability of both incorporated drug molecules and particulate system, and have a higher loading capacity and lower drug leaching upon storage. NLF-based delivery formulations widely utilize Chitosan, a natural biocompatible cationic polysaccharide extracted from crustacean shells and capable of efficient drug and gene delivery. Chitosan has been found to have many beneficial effects like anticoagulant activity, wound-healing and antimicrobial properties.^{32–34} To test the extent serum bioavailability of CFM-4 contributed to its lack of efficacy in the above xenograft studies, we first generated and characterized surface modified NLF and CFM-4 NLF as detailed in methods. Optimized, unmodified CFM-4 NLF carries -2.58 ± 0.24 mV surface charge with 183.67 ± 7.40 nm size, and PI of 0.23 ± 0.03 and EE = $91.01\% \pm 7.17$. The surface of these NLF with negative charge was modified using chitosan polymer. The particle size and positive charge on the surface of NLF were found to increase gradually from 183.67 ± 7.40 to 224.72 ± 19.46 and -2.58 ± 0.24 mV to $+18.05 \pm 0.22$ mV respectively, when the polymer concentration was increased from 0% to 2% (w/v) due to the molecular layers of chitosan coating on the surface of NLF. Chitosan at 1% concentration was found to be optimal as there was no significant increase in the charge beyond this concentration. There might be a saturation of NLF surface with chitosan at 1% concentration as a result since beyond this concentration an increase in NLF particle size was found rather than the charge (Fig. 9(A)). *In vitro* drug release studies further demonstrated a superior dissolution of CFM-4 in CFM-4 NLF formulation when compared with CFM-4 FD. The drug release was sustained up to 72 h in the case of CFM-4 NLF (Fig. 9(B)). The total cumulative amount of drug released after 24 h from CFM-4 FD was 13.03% whereas from CFM-4 NLF it was 61.78%, which was a 4.75-fold more than the free drug.

We next tested bio-availability of the surface modified CFM-4 NLF formulation and CFM-4 FD following their administration in Sprague Dawley rats as detailed in methods. Oral

administration of CFM-4 NLF resulted in a significant increase in the bioavailability when compared to CFM-4 FD (Fig. 9C)). The plasma C_{max} concentration of CFM-4 FD was found to be $1.19 \pm 0.03 \mu\text{g/ml}$, while in the case of CFM-4 NLF it was $4.37 \pm 0.14 \mu\text{g/ml}$, which was a 3.67 fold increase when compared with CFM-4 FD. The AUC was also found to be significantly higher in the case of CFM-4 NLF when compared to CFM-4 FD (Fig. 9(D)). The AUC for CFM-4 FD was $2.82 \pm 0.04 \mu\text{g} \cdot \text{h/ml}$, whereas for CFM-4 NLF it was $16.84 \pm 1.17 \mu\text{g} \cdot \text{h/ml}$. The AUC for CFM-4 NLF was 5.97-fold more compared to CFM-4 FD. A 2.92-fold increased plasma half-life of CFM-4 NLF suggests for a sustained release behavior of CFM-4 NLF. Our studies therefore indicate that the improved pharmacokinetic parameters such as increased C_{max}, *t*_{1/2} and AUC in the case of CFM-4 NLF led to its overall improved bioavailability over CFM-4 FD by a 2.49-fold.

The surface modified CFM-4 NLF formulation and CFM-4 FD were then utilized to treat BALB/c nude mice bearing xenografted tumors derived from A549 NSCLC cells as described in methods. Oral administration of both the CFM-4 NLF and CFM-4 FD caused reduction in lung tumor weight, number of tumor nodules, and lung tumor volume when compared with the placebo-treated controls (Figs. 10(A)–(C)), although CFM-4 NLF in general was more efficacious than CFM-4 FD. Both the agents caused a similar but modest change in overall lung weight and were almost equally distributed throughout the normal lungs of the treated animals (Figs. 10(D), (E)). CFM-4 NLF formulation however had a much higher distribution in the lung tumors and was also present in higher concentrations in the plasma when compared with the CFM-4 FD (Figs. 10(F), (G)). These data together with the absence of any gross histological changes in the lung tissues of the treated animals (not shown), suggest that both the CFM-4 formulations although had equal distribution throughout the lungs and were effective in inhibiting tumor growth and numbers, CFM-4 NLF had a much higher plasma levels and distribution in lung tumors, underscoring its increased efficacy when compared with CFM-4 FD (Figs. 10(A)–(C)).

In an analogous experiment, MDA-MB-231 TNBC cells were xenografted in the mammary fat pads of the female BALB/c nude mice and when tumors developed, the mice were treated with placebo (Control) or CFM-4 NLF formulation as in methods. Oral administration of CFM-4 NLF resulted in reduced breast tumor volume and overall tumor weights (Figs. 10(H), (I)). Immunohistological analysis of a representative NSCLC and TNBC tumors from the animals that were treated with placebo (control) or CFM-4 NLF revealed increased staining for TUNEL and CARP-1 protein (Fig. 11). Altogether, the data in Figures 9–11 demonstrate that NLF formulation of CFM-4 enhance its bioavailability, serum levels, and anti-tumor efficacy. In addition, CFM-4 inhibited xenografted NSCLC and TNBC tumors in part by inducing apoptosis, and this finding would be consistent with our *in vitro* observations from the current and previous studies where CFM-4 treatments stimulated apoptosis in a variety of cancer cell types including NSCLC and TNBC origins.^{9, 13, 14, 28}

DISCUSSION

In an attempt to discover novel transducers of apoptosis-inducing effects of chemotherapy, CARP-1 was identified as a perinuclear protein that was required for apoptosis by DNA damaging agents adriamycin (ADR) and etoposide, as well as EGFR targeting therapeutic

such as Iressa.^{4, 5, 7} Although CARP-1 was also found to function as a co-activator of the steroid-thyroid receptor superfamily of proteins,⁴ we have focused our efforts to exploit apoptosis signaling by CARP-1 for discovery and development of novel agents for use as inhibitors of cancer cell growth. In this regard, our recent chemical biological studies yielded a number of novel, small molecule compounds, termed CFMs that function in part by binding with CARP-1 and stimulating apoptosis in a number of cancer cell types.⁹ The lead compound CFM-4 binds with CARP-1, causes increase in CARP-1 levels, induces apoptosis and inhibits growth of breast and other cancer cells. CFM-4 however did not increase CARP-1 levels or apoptosis in immortalized breast epithelial MCF-10A cells.⁹ Here we tested our working hypothesis that CFM-4 and its analogs are suitable inhibitors of TNBC and NSCLCs *in vitro* and pre-clinical animal studies.

Our current SAR studies involving CFM-4 analogs revealed that although a somewhat limited number of analogs were identified and tested, and CFM-4.6 elicited levels of cell growth suppression and activated molecular signaling that were generally similar to that of CFM-4, a 10 μ M dose of the compounds CFM-4.1 or -4.3 caused ~60–80% loss of viabilities of HBC and MPM cells, a moderate ~40% inhibition of NB cells, and modest to absent inhibition of NSCLC cells (Figs. 2, 3). The compound CFM-4.2 was also active in suppressing viabilities of the HBC and NB cells but caused a moderate loss of viabilities of NSCLC and MPM cells. It is therefore conceivable that further rational medicinal chemistry modifications of these compounds could yield additional novel small molecule compounds that may have greater potency and selectivity in inhibiting TNBC, NSCLC, MPM, or NB cancers. Our current data nonetheless indicate that CFM-4 and its analog CFM-4.6 are effective inhibitors of NSCLC and TNBC cells *in vitro*.

Our studies presented here also show that CFM-4 and CFM-4.6 function in part by elevating CARP-1 levels, activating pro-apoptotic SAPKs p38 and JNK1/2, reducing levels of mitotic cyclin B1 and stimulating PARP cleavage. These data are consistent with our previous reports where CFMs, particularly CFM-4, -5, were found to inhibit growth of MPM and NB cells by causing G2M cell cycle arrest, loss of mitotic cyclin B1, activating SAPKs p38 and JNK, and increasing CARP-1 expression.^{13, 14} Although CFM-4 and CFM-4.6 have IC₅₀ values of 0.75⁹ and 3.96 μ M (Fig. 1), respectively, it remains to be determined whether CFM-4.6 also binds with CARP-1. Interestingly, both the compounds elicit a generally similar level of inhibition of the NSCLC and TNBC cells *in vitro* and seem to utilize overlapping molecular mechanisms of action (Figs. 2–5).

A novel finding of our current studies pertains to up-regulation of apoptosis transducing DEDD2 protein in TNBC and NSCLC cells following their treatments with CFM-4 or CFM-4.6 compounds. Additionally, our studies revealed for the first time that CARP-1 binds with DEDD2, RIPK1, FADD, and p43/41 fragment of cleaved caspase-8, and suggest that CARP-1 likely is a component of apoptosis-transducing intracellular sub-complexes that regulate caspase-8 activation following stimulation of TNFR/DR family of proteins. Our cDNA-based high-throughput array hybridization studies revealed a number of genes including DEDD2 that were modulated in CFM-4-treated MB¹³ and HBC cells (Table III), and thus provided a rationale for further investigating whether DEDD2 expression was increased in cells exposed to CFM-4 or CFM-4.6. Moreover, the facts that (a) DEDD2

interacts with caspases-8 and -10 via their respective DED domains, (b) DEDD2 facilitates activation of both the caspases as well as translocation and activation of caspase-6 to the nucleus,²⁹ (c) caspase-8 activation was necessary for apoptosis signaling by CFM-4,⁹ (d) CARP-1 binds with DEDD2 (Fig. 6), and (e) both CFM-4 and -4.6 elevate expression of CARP-1 and DEDD2 proteins suggest that CARP-1 binding with DEDD2 and its consequent co-localization in the cytosolic and nuclear compartments is a novel mechanism of apoptosis signaling by these compounds and would also be consistent with previously noted peri-nuclear presence of CARP-1 in TNBC cells.⁷ Although CARP-1 expression was required for apoptotic functions of CFM-4,⁹ whether and to the extent CARP-1 is also necessary for DEDD2-dependent activation of caspases-8 and -10 in the presence of CFM-4 or CFM-4.6 is a subject of our on-going studies.

It was estimated that about 40–70% of all new chemical entities entering drug development programs possess poor aqueous solubility.³⁵ Even CFM-4 was not an exception and has a poor aqueous solubility of less than 1 mg/mL. This poor aqueous solubility leads to poor dissolution which ultimately results in poor absorption and oral bioavailability. Hence a lipid based nano drug delivery system of CFM-4 was prepared to enhance its dissolution and oral bioavailability.³⁶ A nano lipid carrier of CFM-4 offered the following advantages

- a. reduction or elimination of positive food effect and
- b. improved dissolution resulting in enhanced absorption

which was also evident by the improved pharmacokinetic profile when compared with the CFM-FD.

Since CFM-4 had very good lipid solubility, we prepared CFM-4 NLF to promote its absorption through the lymphatic route in association with the chylomicrons and very low density lipoproteins (VLDL) which offers the opportunity to enhance its oral bioavailability by avoiding the first pass effect.³⁷ The CFM-4 NLF comprise of a combination of two high melting solid lipid carriers (Compritol & Geleol) which were also responsible for its sustained release. Geleol is also a good emulsifier and was helpful in binding with compritol and other components of the formulation. Vitamin E TPGS was the co-surfactant (with Tween-80) that acted as stabilizer and permeation enhancer.

Due to the crystalline nature of matrix lipids in solid particles, it was understood that there would be limited space for the drug loading; hence the drug loading capacity of most of the lipid based formulations is generally less than 5–10%.³⁸ The other problem with the lipid based drug delivery systems is lipolysis due to lipid digestibility in GI tract which results in drug escape from lipid carriers followed by precipitation leading to decreased oral bioavailability. These issues were addressed by including liquid lipid “Miglyol” in the formulation. Due to the rearrangement of crystalline structure of lipid matrix in solid nano particles with miglyol, the space for drug loading increased to 10% of solid lipids used and with an entrapment efficiency of $91.01 \pm 7.17\%$. Because both the solid lipids used in the formulation were high melting lipids ($<50^\circ\text{C}$), the scope for lipolysis was low and in an event of slight lipolysis, the escaped drug would likely be self-emulsified due to the presence of miglyol and the surfactants.

Surface modification of NLF was done using chitosan, a natural biocompatible cationic polysaccharide as surface modified positively charged nanoparticles would improve drug availability in tumor due to better permeation effect of chitosan and charge interaction of positively charged NLF with negatively charged tumor surface. Improved dissolution was also observed with CFM-4 NLF compared to CFM-4 FD. The improved dissolution in turn resulted in improved absorption which was evident by the improved Cmax and AUC values of CFM-4 NLF when compared to CFM-4 FD.

Finally, our preclinical, xenograft-based studies convincingly demonstrate that CFM-4 holds therapeutic potential. Although, intra-venous administration of CFM-4 was well tolerated and had absent toxicities to the host, and was generally in-effective in suppressing growth of xenografts derived from a variety of cancer cell lines (Table II). However, a nano-lipid formulation of this compound not only enhanced its bioavailability and significantly improved its pharmacokinetics, oral administration of CFM-4 NLF formulation suppressed growth of xenografted TNBC and NSCLC tumors in nude mice. Further analysis revealed that oral administration of CFM-4 NLF resulted in significant and likely therapeutic, levels of CFM-4 in the xenografted tumors without eliciting toxicities to the animals. Whether the cancer cells are able to metabolize CFM-4 and to the extent any such metabolite(s) contributed to its inhibitory effects remain to be clarified. However, the fact that CFM-4 accumulated in the xenografted tumors following oral administration of the CFM-4 NLF indicate that the nano lipids likely prevent systemic metabolism of this compound by the host, and thus play a crucial role in enhancing its therapeutic effects. These findings also indicate that NLF formulation of this compound could be a suitable vehicle for development of this compound and its analogs in further exploring the therapeutic potential of this class of novel small molecules.

CONCLUSIONS

1. CARP-1 functional mimetics (CFMs) are a novel class of small molecule inhibitors of triple-negative breast and non-small cell lung cancers.
2. CFMs cause elevated levels of CARP-1 and death effector domain DEDD2 proteins. CARP-1/CCAR1 interacts with RIPK1, pro-apoptotic adaptor protein FADD, cleaved caspase 8, and DEDD2 proteins, and is likely a part of an intracellular, apoptosis-transducing subcomplex.
3. CFMs attenuate biological properties of cancer cell motility, migration, and invasion.
4. Nano-lipid formulation of CFM-4 (CFM-4 NLF) has superior bioavailability when compared with free drug, and oral administration of CFM-4 NLF suppresses growth of Xenografted TNBC and NSCLC tumor growth in nude mice.

Acknowledgments

Financial Support

This work was supported by the Department of Veterans Affairs Merit Review grant (AKR), the NCI/NIH Cancer Center Support Grant to Karmanos Cancer Institute, NIH Minority Biomedical Research Support (MBRS)-SC1 program (Grant # SC1 GM092779-01; MS) and National Institute on Minority Health and Health Disparities (NIMHD) NIMHD P20 (Grant # 1P20 MD006738-03; MS), Department of Defense (DOD) Breast Cancer Program grant number W81XWH-11-1-0211 (MS), and a grant from the National Plan of Science, Technology and Innovation, King Saud University, Riyadh, Saudi Arabia (Grant No. 10 MED-1188-02; AEA).

Authors gratefully acknowledge Dr. Prasanna Ekambaram, University of Pittsburg, PA, for providing HUVECs.

References

1. Jemal A, Siegel R, Xu J, Ward E. CA cancer statistics, 2010. *Cancer J Clin.* 2010; 60:277.
2. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, Pietenpol JA. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest.* 2011; 121:2750. [PubMed: 21633166]
3. O'Toole SA, Beith JM, Millar EK, West R, McLean A, Cazet A, Swarbrick A, Oakes SR. Therapeutic targets in triple negative breast cancers. *J Clin Pathol.* 2013; 66:530. [PubMed: 23436929]
4. Kim JH, Yang CK, Heo K, Roeder RG, An W, Stallcup MR. CCAR1, a key regulator of mediator complex recruitment to nuclear receptor transcription complexes. *Mol Cell.* 2008; 31:510. [PubMed: 18722177]
5. Rishi AK, Zhang L, Yu Y, Jiang Y, Nautiyal J, Wali A, Fontana JA, Levi E, Majumdar AP. Cell cycle and apoptosis regulatory protein [CARP]-1 is involved in apoptosis signaling by epidermal growth factor receptor. *J Biol Chem.* 2006; 281:13188. [PubMed: 16543231]
6. Ou C-Y, Chen T-C, Lee JV, Wang J-C, Stallcup MR. Coregulator CCAR1 positively regulates adipocyte differentiation through the glucocorticoid signaling pathway. *J Biol Chem.* 2014; 289:17078. [PubMed: 24811171]
7. Rishi AK, Zhang L, Boyanapalli M, Wali A, Mohammad RM, Yu Y, Fontana JA, Hatfield JS, Dawson MI, Majumdar AP, Reichert U. Identification and characterization of a Cell-Cycle and Apoptosis Regulatory Protein [CARP]-1 as a novel mediator of apoptosis signaling by Retinoid CD437. *J Biol Chem.* 2003; 278:33422. [PubMed: 12816952]
8. Jiang Y, Puliappadamba VT, Zhang L, Wu W, Wali A, Yaffe MB, Fontana JA, Rishi AK. A novel mechanism of cell growth regulation by cell cycle and apoptosis regulatory protein (CARP)-1. *J Mol Signal.* 2010; 5:7. [PubMed: 20594350]
9. Puliappadamba VT, Wu W, Bevis D, Zhang L, Polin L, Kilkuskie R, Finley RL Jr, Larsen SD, Levi E, Miller FR, Wali A, Rishi AK. Antagonists of anaphase promoting complex [APC]-2-cell cycle and apoptosis regulatory protein (CARP)-1 interaction are novel regulators of cell growth and apoptosis. *J Biol Chem.* 2011; 286:38000. [PubMed: 21903591]
10. Zachariae W, Nasmyth K. Whose end is destruction: Cell division and the anaphase-promoting complex. *Genes Dev.* 1999; 13:2039. [PubMed: 10465783]
11. Harper JW, Burton JL, Solomon MJ. The anaphase-promoting complex: It's not just for mitosis any more. *Genes Dev.* 2002; 16:2179. [PubMed: 12208841]
12. Lehman NL, Tibshirani R, Hsu JY, Natkunam Y, Harris BT, West RB, Masek MA, Montgomery K, van de Rijn M, Jackson PK. Oncogenic regulators and substrates of the anaphase promoting complex/cyclosome are frequently overexpressed in malignant tumors. *Am J Pathol.* 2007; 170:1793. [PubMed: 17456782]
13. Jamal S, Cheriyan VT, Muthu M, Munie S, Levi E, Ashour AE, Pass HI, Wali A, Singh M, Rishi AK. CARP-1 functional mimetics are a novel class of small molecule inhibitors of malignant pleural mesothelioma cells. *PLoS One.* 2014; 9:e89146. [PubMed: 24598827]
14. Ashour AE, Jamal S, Cheryan VT, Muthu M, Zoheir KM, Alafeefy AM, Abd-Allah AR, Levi E, Tarca AL, Polin LA, Rishi AK. CARP-1 functional mimetics: A novel class of small molecule inhibitors of medulloblastoma cell growth. *PLoS One.* 2013; 8:e66733. [PubMed: 23826121]
15. Bharatwaj B, Dimovski R, Conti DS, da Rocha SR. Polymeric nanocarriers for transport modulation across the pulmonary epithelium: Dendrimers, polymeric nanoparticles, and their nanoblends. *AAPS J.* 2014; 16:522. [PubMed: 24691741]

16. Conti DS, Brewer D, Grashik J, Avasarala S, da Rocha SR. Poly(amidoamine) dendrimer nanocarriers and their aerosol formulations for siRNA delivery to the lung epithelium. *Mol Pharm.* 2014; 11:1808. [PubMed: 24811243]
17. Wang G, Edwards H, Caldwell JT, Buck SA, Qing WY, Taub JW, Ge Y, Wang Z. Panobinostat synergistically enhances the cytotoxic effects of cisplatin, doxorubicin or etoposide on high-risk neuroblastoma cells. *PLoS One.* 2013; 8:e76662. [PubMed: 24098799]
18. Stark K, Burger A, Wu J, Shelton P, Polin L, Li J. Reactivation of estrogen receptor α by vorinostat sensitizes mesenchymal-like triple-negative breast cancer to aminoflavone, a ligand of the aryl hydrocarbon receptor. *PLoS One.* 2013; 8:e74525. [PubMed: 24058584]
19. Zhang L, Levi E, Majumder P, Yu Y, Aboukameel A, Du J, Xu H, Mohammad R, Hatfield JS, Wali A, Adsay V, Majumdar AP, Rishi AK. Transactivator of transcription tagged cell cycle and apoptosis regulatory protein-1 peptides suppress growth of human breast cancer cells *in vitro* and *in vivo*. *Mol Cancer Ther.* 2007; 6:1661. [PubMed: 17513614]
20. Levi E, Zhang L, Aboukameel A, Rishi S, Mohammad RM, Polin L, Hatfield JS, Rishi AK. Cell cycle and apoptosis regulatory protein (CARP)-1 is a novel, adriamycin-inducible, diffuse large B-cell lymphoma (DLBL) growth suppressor. *Cancer Chemother Pharmacol.* 2011; 67:1401. [PubMed: 20809119]
21. Tarca AL, Carey VJ, Chen XW, Romero R, Draghici S. Machine learning and its applications to biology. *PLoS Comput Biol.* 2007; 3:e116. [PubMed: 17604446]
22. Zhang W, Liu J, Zhang Q, Li X, Yu S, Yang X, Kong J, Pan W. Enhanced cellular uptake and anti-proliferating effect of chitosan hydrochlorides modified genistein loaded NLC on human lens epithelial cells. *Int J Pharm.* 2014; 471:118. [PubMed: 24858387]
23. Tian B, Luo Q, Song S, Liu D, Pan H, Zhang W, He L, Ma S, Yang X, Pan W. Novel surface-modified nanostructured lipid carriers with partially deacetylated water-soluble chitosan for efficient ocular delivery. *J Pharm Sci.* 2012; 101:1040. [PubMed: 22102480]
24. Sun M, Nie S, Pan X, Zhang R, Fan Z, Wang S. Quercetin-nanostructured lipid carriers: Characteristics and anti-breast cancer activities. *in vitro Colloids Surf B Biointerfaces.* 2014; 113:15. [PubMed: 24060926]
25. Chougule MB, Patel AR, Jackson T, Singh M. Antitumor activity of Noscapine in combination with Doxorubicin in triple negative breast cancer. *PLoS One.* 2011; 6:e17733. [PubMed: 21423660]
26. Watson SA, Kumari R. Theoretical considerations in using animal models of metastasis and brief methodology for *in vivo* colorectal cancer models in SCID and nude mice. *Methods Mol Biol.* 2014; 1070:117. [PubMed: 24092436]
27. Ding Y, Lu C, Yang J, Jin X, Yang L, Wang C, Ma Z, Zhu Y, Ding L, Jia Y, Wen A. Application of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to the pharmacokinetics, tissue distribution and excretion studies of felotaxel (SHR110008) in tumor-bearing mice. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2012; 887:61.
28. Muthu M, Cheriyan VT, Munie S, Levi E, Frank J, Ashour AE, Singh M, Rishi AK. Mechanisms of neuroblastoma cell growth inhibition by CARP-1 functional mimetics. *PLoS One.* 2014; 9:e102567. [PubMed: 25033461]
29. Valmiki MG, Ramos JW. Death effector domain containing proteins. *Cell Mol Life Sci.* 2009; 66:814. [PubMed: 18989622]
30. Lavrik IN, Krammer PH. Regulation of CD95/Fas signaling at the DISC. *Cell Death and Differentiation.* 2012; 19:36. [PubMed: 22075988]
31. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: Nodes of coordination in immune signaling networks. *Nat Immunol.* 2009; 10:348. [PubMed: 19295631]
32. Panos I, Acosta N, Heras A. New drug delivery systems based on chitosan. *Curr Drug Discov Technol.* 2008; 5:333. [PubMed: 19075614]
33. Huang SS, Sung SH, Chiang CE. Chitosan potentiation of warfarin effect. *Ann Pharmacother.* 2007; 41:1912. [PubMed: 17925502]
34. Rossi S, Marciello M, Sandri G, Ferrari F, Bonferoni MC, Papetti A, Caramella C, Dacarro C, Grisoli P. Wound dressings based on chitosans and hyaluronic acid for the release of chlorhexidine diacetate in skin ulcer therapy. *Pharm Dev Technol.* 2007; 12:415. [PubMed: 17763146]

35. Gursoy RN, Benita S. Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. *Biomed Pharmacother.* 2004; 58:173. [PubMed: 15082340]
36. Charman WN, Rogge MC, Boddy AW, Berger BM. Effect of food and a monoglyceride emulsion formulation on danazol bioavailability. *J Clin Pharmacol.* 1993; 33:381. [PubMed: 8473554]
37. Khoo SM, Shackelford DM, Porter CJ, Edwards GA, Charman WN. Intestinal lymphatic transport of halofantrine occurs after oral administration of a unit-dose lipid-based formulation to fasted dogs. *Pharm Res.* 2003; 20:1460. [PubMed: 14567642]
38. Bunjes H. Lipid nanoparticles for the delivery of poorly water-soluble drugs. *J Pharm Pharmacol.* 2010; 62:1637. [PubMed: 21039547]

Figure 1.
Chemical structure and identities of CFM-1, -4, 5, and CFM-4 analogs CFM-4.1–4.6.

Figure 2.

CFMs reduce viabilities of the NSCLC cells. Cells were treated with vehicle (Untreated Control), or indicated period and dose of various agents. Determination of viable/live cells was carried out by MTT assay. The data in the histograms represent means of three independent experiments; bars, S.E.

Figure 3.

CFMs reduce viabilities of the TNBC (A), MPM (B) and NB (C) cells. Cells were treated with vehicle (Untreated Control), or indicated period and dose of various agents. Determination of viable/live cells was carried out by MTT assay as in Figure 2. The data in the histograms represent means of three-four independent experiments; bars, S.E.

Figure 4.

CFM-4 and -4.6 activate pro-apoptotic p38 SAPK, induce expression of CARP-1 and DEDD2, and promote PARP cleavage in NSCLC and TNBC cells. NSCLC H1299 (A), A549 (B), TNBC MDA-MB-468 (C), or MDA-MB-231 (D) cells were either untreated (Control) or treated with noted dose of respective CFMs or Cisplatin for 12 h. Levels of CARP-1, phosphorylated and total p38, DEDD2, cleaved PARP, cyclin B1, α -tubulin, and actin proteins were determined by WB as detailed in methods. The numbers on the right of each autoradiogram indicate migration of the protein molecular weight markers (Bio-Rad).

Figure 5.

Activation of pro-apoptotic p38 SAPK precedes JNK1/2 activation in CFM-4.6-treated TNBC cells. TNBC MDA-MB-468 (A) or MDA-MB-231 (B) cells were either untreated (Control) or treated with noted time and dose of CFM-4.6. Levels of CARP-1, phosphorylated and total p38 and JNK1/2 SAPKs, DEDD2, cleaved PARP, and actin proteins were determined by WB as detailed in methods. The numbers on the right of each autoradiogram indicate migration of the protein molecular weight markers (Bio-Rad).

Figure 6.

CARP-1 interacts with DEDD2, RIPK1, Caspase-8 (A), and FADD (B). Protein complexes from 1mg of total proteins derived from TNBC MDA-MB-231 cells that were untreated (noted as -) or treated with noted time and dose of CFM-4 (indicated as +) were subjected to IP using IgG or anti-CARP-1 antibodies. Fifty μ g of the respective whole cell lysates (noted as WCE) or immunoprecipitates were analyzed by WB with noted antibodies as described in methods. Presence of endogenous CARP-1, DEDD2, RIPK1, cleaved caspase-8, and FADD proteins is indicated by an arrowhead on the left side of each blot. Approximate location of various molecular mass markers is indicated on the right side of each blot. kDa, kilodalton; WCE, whole cell extract; α -CARP-1, anti-CARP-1 antibodies.

Figure 7.

CFMs inhibit TNBC and NSCLC Cell Growth in Soft Agar, motility and invasion. Cells were either untreated (Control), treated separately with 3 μM dose of indicated CFM for 96 h (A, B), 10 μM dose of respective CFMs for 14 days (C, D), or 24 h (E). The cells growth in the scratch assay in panels A, B or the number of colonies of cells in panels C, D were recorded by photography as described in Methods. Representative photomicrographs of untreated and CFM-treated cells are shown. (E) The HBC cells were separately seeded in chambers with matrigel-coated membranes, and treated with buffer Control (DMSO) or with 10 μM dose of respective CFMs as noted in Methods. Live cells migrating across the matrigel-coated membranes were dissociated, and quantitated by an MTT-based assay. The columns in histogram represent MTT OD of the CFM-treated HBC cells relative to untreated controls (*, %, $p < 0.05$ relative to respective untreated control).

Figure 8.

CFMs inhibit angiogenesis. HUVECs were seeded onto a surface containing polymerized extracellular matrix (ECM), and allowed to develop network structures in buffer (Control) or CFM-treated cells. The growth of tubules was monitored over a period of 12 hours. Representative photomicrographs of control and treated cells are presented.

Figure 9.

Formulation of surface modified CFM-4 NLC and evaluation of its pharmacokinetic parameters. (A) Table showing size (nm), PI and charge (mV) values of surface modified CFM-4 NLC formulation. (B) Kinetics of CFM-4 release from the CFM-4 FD and surface modified CFM-4 NLC preparations. The cumulative release of CFM-4 from each preparation was carried out at multiple time points and in three separate experiments following procedures detailed in methods. (C) HPLC analysis of rat serum levels of CFM-4 at the noted time intervals following oral administration of indicated dose of CFM-4 NLC (noted as CFM NLC), CFM-FD (noted as CFM Free drug) or intravenous (iv) administration of CFM-4 (noted as CFM). (D) Table showing indicated pharmacokinetic parameters for CFM-4 when administered orally as CFM-4 FD (noted as CFM-4 free drug) or CFM-4 NLC, or intravenously (CFM-4 IV). The data analysis and calculations were performed essential as detailed in methods.

Figure 10.

CFM-4 NLC suppresses growth of xenografted NSCLC (A–G) and TNBC (H, I) tumors. Histograms showing lung tumor weight (A), number of lung tumor nodules (B), lung tumor volume (C), Lung tissue weight (D), CFM-4 distribution in normal lungs (E) and lung tumors (F), plasma concentration of CFM-4 (G), breast tumor volume (H), and breast tumor weight (I) of the placebo-treated (indicated as Control), CFM-4 NLC, or CFM-4 FD treated, NSCLC (A549) or TNBC (MDA-MB-231) xenograft-bearing animals. The xenograft establishment, treatment and analysis procedures were carried out essentially as detailed in methods. The columns represent average values from a total of six animals in respective group, bars, SE, significant where * $p < 0.05$, ** $p < 0.001$ versus Control or FD.

Figure 11.

CFM-4 NLC treatments induce CARP-1 expression and apoptosis in NSCLC and TNBC tumor xenografts. NSCLC and TNBC tumor xenografts were generated in the nude mice and the animals were treated with oral administration of CFM-4 NLC formulation as in methods and Figure 10 above. A representative tumor tissue from the placebo-treated (noted as Control) or CFM-4 NLC treated animal was fixed in formalin, paraffin embedded, processed, and subjected to immuno-staining as detailed in methods. Photomicrographs (400× magnification) are shown to indicate for apoptosis (by TUNEL assay), and levels CARP-1 protein as noted in methods. Elevated apoptosis or CARP-1 levels are indicated by dark-brown spots in TUNEL-stained, CFM NLC panels or increased brown staining in CFM NLC panels stained with anti-CARP-1 antibodies.

Table I

Composition of CFM-4 NLF (1 ml).

Ingredient	Quality
CFM-4	10 mg
Compritol 888	75 mg
Geleol	25 mg
Vitamin E TPGS	20 mg
Tween 80	0.02 ml
Miglyol 821	0.1 ml
Water	0.75 ml
Total	1 ml

Table II
Summary of SCID mice xenograft studies indicating CFM-4 tumor response profiles.

CFM 4 tumor response profile					
Experiment	Tumor	Dose (mg/kg)	Route	Schedule	%T/C Log cell kill
3086	MDA-MB-231/13	470	IV	Qd 1–19	81 <0.5
3091	SKBR-3/TC15/11	867	IV	Qd 1–6 BID 7–16	28 1.3 (500 mg) 0.8 (750 mg)
3096	MDA-MB-468/1	1296	IV	BID 1, 6, 20, 21, 24–26 Qd 5, 7–19, 22, 23	77 0.08
3097	SKBR-3/TC15/12	864	SC	Qd 27–29	72 NA (tox trial)
			IV	Bid 1, 6, 20, 21	
3116	MDA-MB-231/18	28	IV	Qd 5, 7–19, 22, 23	TOXIC LD50 25 mg/ml stock used
3126	MDA-MB-231/10	601.5	IV	Qd 3–16	
				BID 17–23	60 (d) 0.6
				QD 3–9	65 (d)
3144	PC-3/268	676	IV	BID 10–18	>100 0.0
				QD 3–9, 25–28	55 (d27) 0.4
3146	MDA-MB-468/4	1129.6	IV	BID 10–24	60 (d38) 0.4
3157	HL-60 (SENS)/19	776	IV	Qd 1–2	>100 0.0
				BID 3–15	
3171	FSCCL	923.8	IV	Qd 7–38	0 ILS 0.0
3200	AB12	405.4	IV	Qd 1, 2, 8	85 0.19
				BID 3–7	
3223	H2714	759	IV	Qd 3, 5–7 >100	0.0
				BID 8–16	
3228	Pane-1	947	IV	Qd 3–13, 20–23	71 0.8
				BID 14–19	
3230	H2461	1055	IV	Qd 3–8	>100 0
				BID 7–19	
3239	MDA-MB-453	328	IV	Qd 1–3	>100 0.0

CFM 4 tumor response profile						
Experiment	Tumor	Dose (mg/kg)	Route	Schedule	%T/C	Log cell kill
3246	SKBR-3/TC15/21	1277.5	PO	BID 4–7	>100	0.0
				Qd 3–8		
				BID 9–13		
				Qd 3–12		
3295	Daoy	925.5	IP	Qd 3–13, 21–23	>100	0.0
				Qd 3–13, 21–23		
				BID 14–20		
				Qd 3, 4 BID 5–12		
3295	Daoy	765	IV	BID 13, 14 Qd 15, 16	60	0.45
				Qd 3, 4 BID 5–12		

Table III

List of select genes regulated by CFM-4 in TNBC MDA-MB-468 cells.

Symbol	Name	Ref. seq	Fold change	Adj.p.val	Direction
FAM125A	Family with sequence similarity 125, member A	NM_138401	1.588943282	0.174693673	UP
BARD1	BRCA1 associated RING domain 1	NM_000465.3	1.514832801	0.174693673	DOWN
DDX1	DEAD (Asp-Glu-Ala-Asp) box helicase 1	NM_004939.2	1.511935964	0.174693673	DOWN
EIF4A2	Eukaryotic translation initiation factor 4A2	NM_001967.3	1.605902961	0.177671962	DOWN
KCTD3	Potassium channel tetramerisation domain containing 3	NM_016121.3	1.514413437	0.177974394	DOWN
TSSC4	Tumor suppressing subtransferable candidate 4	NM_005706.2	1.626202118	0.178396144	UP
RPS2	Ribosomal protein S2	NM_002952.3	1.531935189	0.195766934	UP
PHIP	Plectstrin homology domain interacting protein	NM_017934.5	1.56161691	0.195766934	DOWN
HMGB1	High mobility group box 1	NM_002128.4	1.511086839	0.195766934	DOWN
NQO1	NAD(P)H dehydrogenase, quinone 1	NM_000903.2	1.584281719	0.195766934	DOWN
RPL7L1	Ribosomal protein L7-like 1	NM_198486.2	1.597780178	0.195766934	DOWN
TFPT	TCF3 (EZA) fusion partner (in childhood leukemia)	NM_013342.3	1.585676511	0.197592004	UP
MICA	MHC class polypeptide-related sequence A	NM_000247.1	1.577027021	0.197592004	UP
TNIP1	TNFAIP3 interacting protein 1	NM_001252385.1	1.584473705	0.197939416	UP
CCNA2	Cyclin A2	NM_001237.3	1.586874826	0.199494975	DOWN
CCDC167	Coiled-coil domain containing 167	NM_138493.2	1.516313201	0.200494228	UP
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	NM_002137.3	1.614955396	0.203768743	DOWN
C11orf74	Chromosome 11 open reading frame 74	NM_001276722.1	1.574034404	0.204610061	DOWN
MT1F	Metallothionein 1F	NM_005949.3	2.50233673	0.214168099	DOWN
GNG10	Guanine nucleotide binding protein (G protein), gamma 10	NM_001017998.3	1.608076651	0.219871845	DOWN
NUCKS1	Nuclear cecain kinase and cyclin-dependent kinase substrate 1	NM_022731.4	1.854262341	0.220225071	DOWN
1-Mar	Membrane-associated ring finger (C3HC4)7, E3 ubiquitin protein ligase	NM_001166373.1	1.520555627	0.220225071	DOWN
DPY30	dpy-30 homolog (c. elegans)	NM_032574.2	1.576671016	0.223777338	DOWN
MT1E	Metallothionein 1E	NM_175617.3	3.251817393	0.224872867	DOWN
WDR75	WD repeat domain 75	NM_032168.1	1.762265043	0.226819241	DOWN
NSMCE4A	Non-SMC element 4 homolog A (S. cerevisiae)	NM_001167865.1	1.763378117	0.228431257	DOWN
INHBE	Inhibin, beta E	NM_031479.3	1.518956801	0.22843516	UP
DEDI2	Death effector domain containing 2	NM_001270614.1	2.00908631	0.233522593	UP

Symbol	Name	Ref. seq	Fold change	Adj.p.val	Direction
MT1X	Metallothionein 1X	NM_005952.3	3.036197848	0.238026148	DOWN
HIL.PDA	Hypoxia inducible lipid droplet-associated	NM_001098786.1	1.794773803	0.238055718	UP
PIGY	Phosphatidylinositol glycan anchor biosynthesis, class Y	NM_001042616.2	1.64221563	0.245879947	DOWN
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	NM_001145443.1	1.794573901	0.256796384	UP
SNX11	Sorting nexin 11	NM_013323.2	1.534598432	0.256796384	UP