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Targeting energy metabolism of cancer cells: Combined administration of NCL-240 and 2-DG

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Abstract

Cancer cells increase their metabolism to produce the energy and biomolecules necessary for growth and proliferation. Thus, energy metabolism pathways may serve as targets for anti-cancer therapy. NCL-240 is a second generation anti-cancer drug belonging to the P1Tenins class of PI3K-Akt inhibitors. Our analysis suggested that NCL-240 caused disruptions in mitochondrial oxidative phosphorylation and up-regulated glycolysis, as evidenced by the loss of NMR peaks for the amino acid products derived from the TCA cycle along with presence of only lactate peaks and the loss of glucose peaks. NCL-240 was combined with 2-deoxy-D-glucose (2-DG) in early proof-of-concept studies on multiple cell lines. 2-DG enhanced cell death response to NCL-240 administration, with cytotoxicity results similar to those under hypoglycemic conditions. In further studies, NCL-240 encapsulated in phosphatidylcholine/cholesterol liposomes was combined with freely dissolved 2-DG. Cell cycle analysis of sensitive and resistant strains of A2780 cells treated with combinations of NCL-240/2-DG pointed to a G0/G1 phase arrest for 80–90% of the total, indicating an inability to grow and divide. Cytotoxicity studies with *in vitro* cancer cell monolayer models confirmed the results of cell cycle analysis. Significant improvements in cytotoxicity with combination treatments over control and individual treatments were seen in multiple cell lines. NCI/ADR-RES cancer cell spheroids further demonstrated the effectiveness of a NCL-240/2-DG combination.

Graphical abstract

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Keywords

Cancer cell energy metabolism; NCL-240; 2-deoxy-D-glucose (2-DG)

1. Introduction

Targeting the energy metabolism of cancer cells has significant promise to be an effective method of inhibiting tumor growth and proliferation (Zhao et al., 2013). The underlying reason to focus on cancer cell metabolism relies on the observation of Warburg that cancer cells consumed a higher fraction of glucose and produce more lactate compared to normal cells. The ‘Warburg effect’ posited that cancer cells rely on a relatively inefficient method of ATP production, i.e. via the glycolytic pathway (Warburg, 1956a, b).

Since solid tumors have an ill-defined vasculature (Torchilin, 2011), they have nutrient- and oxygen-deficient cells with altered energy metabolism. Consequently, the cancer cells over-express glucose transporters like GLUT1 and GLUT3 and hexokinases I and II (Bos et al., 2002; Burt et al., 2001). The hypoxic cancer cells (farther from blood capillaries) are predisposed to ATP production by glycolysis, because of hypoxia inducible factor 1- α (HIF1- α) up-regulation (Semenza, 1998). Nonetheless, even the malignant cells that grow under normoxic conditions (closer to blood capillaries) tend to have constitutively up-regulated aerobic glycolysis. The levels of aerobic glycolysis are dependent on the aggressiveness or invasiveness of the cell line (Gatenby and Gillies, 2004). Although anaerobic glycolysis produces less ATP compared to oxidative phosphorylation, it is rapid and it prepares invasive cells for the harsh conditions of tumor environment.

Recent studies (Feron, 2009; Pavlides et al., 2009) have also revealed that there are more crucial mechanisms that affect the energy needs of cancer cells. In fact, in solid tumors, there appears to be a parasitic relationship between cancer cells and the surrounding normal stroma. Cancer cells rely on their own oxidative phosphorylation for bioenergetic and biosynthetic functions. The mitochondria of cancer cells are active and are largely responsible for producing energy via oxidative phosphorylation. Indeed, the epithelial cancer cells have functional mitochondria and oxidative phosphorylation. In a tumor, the cancer-associated normal cells (such as fibroblasts) have dysfunctional mitochondria and the glycolysis in those cells is up-regulated. As a result, their lactate production increases and is utilized by the cancer cells as a fuel for the oxidative phosphorylation. Thus, the mitochondrial pathways for ATP and macromolecule production can certainly be exploited for therapeutic effects.

In this study, NCL-240, belonging to a new class of small molecule drugs known as PITenins, was used for targeting cancer metabolism. The PITenins are inhibitors of the phosphoinositide 3-kinase (PI3K) pathway (Maehama and Dixon, 1999; Vivanco and Sawyers, 2002), which regulates multiple cancer cell functions like cell metabolism, survival and apoptosis. The first-generation molecule of PITenins, DM-PIT-1, (N-[[[(2-hydroxy-5-nitrophenyl)amino]thioxomethyl]-3,5-dimethyl-benzamide), blocked the binding of PIP₃ to the PH domains of Akt and PDK1. Chemical modifications of the DM-PIT-1 and structure activity relationship studies have led to a second generation analog with a triazole moiety, NCL-240 (Figure 1). Because of its poor aqueous solubility (logP = 5.17), NCL-240 was encapsulated into lipid-based nanocarriers such as micelles and liposomes for delivery into cells. These formulations have been shown to be efficient anti-cancer agents while sparing normal cells (Kommagalla et al., 2014; Pattni et al., 2016; Riehle et al., 2016). New insights into the effects of NCL-240 indicate a metabolic change in treated cancer cells and complete dependence on glycolysis even when grown in presence of O₂. This paper describes such an effect in multiple cell lines.

Since NCL-240 causes cancer cell dependence on glycolysis, agents that target glycolysis should complement NCL-240 and lead to effective anti-cancer therapy. 2-deoxy-D-glucose (2-DG) is a glycolytic inhibitor that has been the subject of multiple studies (Boutrid et al., 2008; Kurtoglu et al., 2007b; Maher et al., 2004; Zhang et al., 2006), including clinical trials that emphasize the safety of using 2-DG in humans as well as the drug's role in combination anti-cancer therapy (Dwarakanath et al., 2009; Mohanti et al., 1996; Raez et al., 2013). As a glucose analog, 2-DG competitively blocks glycolysis by interfering with the reactions of hexokinase and phosphoglucose isomerase (Figure 2). In normoxic conditions, cells can use mitochondrial phosphorylation for ATP production. However, hypoxic cells are highly sensitive to the action of 2-DG (Liu et al., 2002). Recent investigations indicate that 2-DG promotes cell death in normoxic cells by interfering with N-linked glycosylation (Kurtoglu et al., 2007a), made possible by structural similarity of 2-DG to mannose. 2-DG competitively inhibits mannose in lipid-linked oligosaccharide synthesis leading to dysfunctional N-linked glycosylation, misfolded proteins and the related unfolded protein response (UPR) as well as endoplasmic reticulum (ER) stress (Xi et al., 2014). If the cells are unable to recover from the unchecked ER stress, they undergo autophagy (Xi et al., 2011). Thus, the use of 2-DG is extremely advantageous in solid tumors where it can cause cell death in hypoxic cells and ER stress-related autophagy in normoxic cells. Additionally, 2-DG inhibits metastasis (Valastyan and Weinberg, 2011) and angiogenesis (Merchan et al., 2010) and at high doses it activates the immune system (Andresen et al., 2012; Beneteau et al., 2012). In this paper we explore the effects of a combination of NCL-240 and 2-DG on cancer cell killing. The novelty of the current study is that we explore here recently understood mechanisms of NCL-240 and exploit the anti-cancer potential of its combination with 2-DG, which was not done previously. The drug combination used in our study is different from recently proposed work based on carboxyamidotriazole (Ju et al., 2016).

2. Materials and methods

2.1. Materials

2.1.1. Drug delivery systems—L- α -phosphatidylcholine (Egg; ePC) and 1,2- dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Absolute ethanol, cholesterol (CHOL) and cholesterol hemisuccinate (CHEMS) were purchased from Sigma Aldrich (St. Louis, MO). The DM-PIT-1 analog, NCL-240, was synthesized at the National Chemical Laboratory (Pune, India). 2-deoxy-D-glucose (2-DG) was purchased from Cayman Chemical (Ann Arbor, MI).

2.1.2. Cell culture—A375, A2058, Malme3M, 501mel, SK2 and SK30 cells were obtained from Dr. Philip Hinds, Tufts University Medical School (Boston, MA) and Dr. Hans R. Widlund, Brigham and Women's Hospital (Boston, MA). A2780, H69AR, and 4T1 cells were acquired from the ATCC (Manassas, VA). NCI/ADR-RES cells were obtained from the National Cancer Institute, Frederick, MD. A2780ADR cells were purchased from Sigma Aldrich (St. Louis, MO). A375, A2058, Malme3M, 501mel, SK2, SK30, 4T1 and NCI/ADR-RES were cultured in DMEM medium (CellGro, Kansas City, MO). A2780, A2780ADR, H69AR were grown in RPMI medium (CellGro, Kansas City, MO). The media were supplemented with 10% Fetal Bovine Serum (FBS) and a 1% antibiotic solution (10,000 IU/ml penicillin, 10,000 μ g/ml streptomycin, and 25ug/ml amphotericin B) to obtain serum-containing complete media. H69AR cells required RPMI medium supplemented with 20% FBS. The CellTiter-Glo® Luminescence assay was purchased from Promega, Madison, WI, and the Accumax® Cell Dissociation solution was from Innovative Cell Technologies, San Diego, CA. Hoechst 33342 dye was obtained from Molecular Probes, Inc., Eugene, OR.

2.2. Methods

2.2.1. ^{13}C -NMR to study effects of NCL-240 on A2780 cells—A2780 cells were placed in glucose-free DMEM media (HyClone) supplemented with 10 mM [^{13}C] D-glucose (Cambridge Isotopes, Tewksbury, MA) and 10% dialyzed FBS (Sigma Aldrich, St. Louis, MO). Cells were treated with 10 μ M NCL-240 or 0.5% DMSO for 16 hr. Cells were lysed in 0.5 ml of cold methanol and further mixed by vortexing with 0.5 ml cold chloroform and 0.5 ml of water. Polar metabolite extracts were collected after 15 min of centrifugation at 14,000 rpm at 4 °C. Polar extracts were dried in SpeedVac and resuspended in 50 mM phosphate D₂O buffer pH 7.0 with 0.2 mM of NMR standard (DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid). ^1H NMR spectra of each sample were collected at 25 °C on a Bruker Avance 600 spectrometer using 128 scans and a NOE1D pulse sequence. ^{13}C NMR was collected using 20,000–40,000 scans, a 45° degree flip angle, a delay time of 0.5 sec. and an acquisition time of 0.47 sec. ^1H - ^{13}C 2D spectra were collected using an HSQC pulse sequence. Compounds were identified by matching 1D and 2D NMR data to the HMDB database (Wishart et al., 2009). The ^1H NMR data were processed and analyzed using CHENOMX NMR Suite 8.0 to identify the compounds present and to measure their concentrations.

2.2.2. Proof-of-concept: Effect of NCL-240 and 2-DG on different cell lines—Cancer cell lines like A375, A2058, Malme3M, 501mel, SK2 and SK30 were maintained in

DMEM/high glucose (HyClone) supplemented with 10% FBS and 1% antibiotic-antimycotic mix. Cells were treated in 96 well plates with NCL-240 (in 0.5% DMSO) alone or in combination with 2-DG for 24 h. Alternatively, cells were exposed to DMEM without glucose, supplemented with 0.5 g/l D-glucose and 10% dialyzed FBS. Cell viability was determined using CellTiter-Glo®.

2.2.3. Drug-loaded liposomes—ePC/CHOL/DOPE/CHEMS (molar ratio = 64/24/6/6) liposomes with or without NCL-240 were prepared by a thin-film hydration technique (Patel et al., 2011). Briefly, lipids in chloroform were added to round bottom flasks and the organic solvents were removed by rotary evaporation and freeze-drying resulting in the formation of a lipid film. The lipid film was hydrated using 1× PBS, pH 7.4 to prepare multi-lamellar vesicles (MLV) with lipid concentrations at 10 or 25 mg/ml. These rehydrated liposomes were sonicated briefly and incubated at 40 °C for 15–20 min followed by sequential extrusion through 0.2 µm and 0.1 µm polycarbonate membranes using a mini held-hand extruder (Avanti Polar Lipids Inc., Alabaster, AL). Simultaneously, a 2 mol% PEG₂₀₀₀-PE film was prepared. This film was hydrated with the liposomal solution and incubated overnight in a shaker at 37 °C to generate PEGylated liposomes by post-insertion. The liposomes were sterile filtered using 0.2 µm nylon membrane filters.

2.2.4. Characterization of liposomes—The size distribution and zeta potential of the liposomal samples were studied using a Zetaplus apparatus (Brookhaven Instruments Corporation, Holtsville, NY). Liposomal samples (10 µl) were diluted to 1 ml using distilled water, and the size and zeta potential were measured as per manufacturer's protocol.

The encapsulation efficiency of NCL-240 in the liposomes was analyzed using a Hitachi Elite LaChrom HPLC (Pleasanton, CA) and a reverse phase Xbridge C₁₈ (2.1 cm × 250 cm) column (Waters Corporation, Milford, MA) using a previously established method (Pattni et al., 2016). *In vitro* release of NCL-240 from liposomes was studied at 37 °C in 1× PBS (pH 7.4 and pH 5) containing 1% TWEEN-20 as a release medium. Drug-loaded micelles were prepared and the loading amount was estimated using HPLC. Volume equal to 200 µg of NCL-240 in micelles was added in dialysis bags with MWCO 1,000 Da and incubated in an orbital shaker at 37 °C and 150 rpm to achieve appropriate mixing. Samples were taken from the release medium and replaced with equal amount of fresh medium. After appropriate dilutions, the concentration of NCL-240 was measured using the HPLC. Free drug diffusion across the dialysis bag was analyzed as control. With the use of appropriate negative staining dyes, namely 1.5% PTA (phosphotungstic acid), the liposomal formulations (0.25 mg/ml) were mounted on a Formvar-carbon-coated film 300 mesh copper grid (Electron Microscopy Studies; catalog# FCF300-Cu). These formulation-mounted grids were placed in a JEM-1010 Transmission Electron Microscope (JEOL) to capture the TEM images.

2.2.5. Cell cycle study by FACS—A univariate analysis of cellular DNA content after a treatment with the NCL-240/2-DG combination was carried out by cell staining with propidium iodide (PI) and deconvolution of the cellular DNA content frequency histogram. Briefly, A2780 and A2780-ADR cells were seeded in 6 well plates at a concentration of 4–5 × 10⁵ cells/well and incubated for 24 h at 37 °C and 5% CO₂. The media was replaced the next day and cells were treated with free 2-DG (5 or 10 mM), NCL-240 loaded liposomes

(2.5, 5 or 10 μ M) and combinations of free 2-DG and NCL-240 loaded liposomes for 24 h. The cells were harvested and centrifuged at 2,000 rpm for 5 min to obtain cell pellets. The supernatant was discarded and the cells were washed twice with ice cold $1\times$ PBS, pH 7.4. The samples were spun at 2,000 rpm for 5 min and the second supernatant was discarded. The cells were dispersed and fixed in 70% ethanol. Briefly, the cell pellets were then resuspended in 300 μ l cold deionized water and mixed well. Absolute ethanol (700 μ l) was added dropwise while shaking the tubes to form homogenous cell suspension. The samples were kept on ice for 1 h to fix the cells. After fixing, the samples were spun at 0.8 g for 8 min to stain the cells with FxCycle™ PI/RNase Staining Solution (Molecular Probes, Eugene, OR). The supernatant was discarded and cells were washed twice with ice cold PBS, pH 7.4. The samples were centrifuged at 0.8 g for 8 min for each wash step to ensure complete removal of ethanol. After the final wash, cells were resuspended in 250–300 μ l of PI/RNase staining solution and mixed well. Cells were incubated for 30 min in the dark at RT and cell fluorescence was subsequently analyzed using FACS. The Ex-Em of the PI bound to DNA was at 536–617 nm. This analysis was used to reveal the cell distribution in three phases of the cell cycle, G₁ vs S vs G₂/M. Cells, 10,000 per sample, were gated to obtain the sample data.

2.2.6. Spheroid formation—NCI/ADR-RES cells in T150 flasks were maintained at 70–80% confluence in an incubator (37°C 5% CO₂). The cells were harvested and a cell suspension was prepared in serum-containing media. Spheroids were developed by the non-adhesive liquid overlay method described previously (Perche et al., 2012). Each well of a 96-well flat bottom, tissue culture-treated clear plate was coated with 50 μ l of sterile 1.5% w/w agar (Fischer Scientific, New Haven, NJ) in serum-free media. The plates were allowed to cool at room temperature for 40 minutes under UV light in a sterile environment to form a non-adhesive concave coating. A $1 \times 10^4/100$ μ l cell suspension was seeded per well of the culture plates. The plates were centrifuged at 1500g for 15 minutes using an Eppendorf 5804 R centrifuge (Hamburg, Germany). Media in the 96-well plates was replaced every 2 days without disturbing the developing spheroid. The formation of dense spheroids was followed by visual inspection with a Nikon ECLIPSE E400 microscope and by light mechanical agitation using pipettes. SPOT 5.0 software was used to measure the size of the spheroids.

2.2.7. Cell viability assays—Viability of cell lines was evaluated with CellTiter-Glo®. Cells ($3-5 \times 10^3$ per well) were seeded in 96-well tissue culture-treated, clear bottom, black-well plates and incubated at 37 °C and 5% CO₂ for 24 h. The cells were treated with either NCL-240-loaded liposomes, free 2-DG or NCL-240-loaded liposomes supplemented with free 2-DG for 24 h. After treatment with the formulations, media was removed and cells were washed with fresh media followed by addition of 10 μ l CellTiter-Glo® in 50 μ l media. The plates were incubated in a shaker at RT for 10–15 min to allow complete lysis of the cells and release of intracellular ATP. The luminescence of samples was measured with a Biotek Synergy HT plate reader (Biotek, Winooski, VT). The cell viability was calculated relative to the luminescence of untreated cells (controls) and expressed as % cell viability.

Cell viability of the spheroids was also measured using CellTiter-Glo®. After the cultured NCI/ADR-RES spheroids reached their optimum compactness (day 4–5), they were treated

with one of the test formulations at 5 spheroids per replicate. After the 48 h incubation, five spheroids were collected into micro-centrifuge tubes per replicate (15 spheroids for n=3). The spheroids were dissociated into individual cells using 200 μ l of Accumax® Cell Dissociation solution and incubation at 37°C for 10–15 minutes under constant stirring. The spheroids were completely dissociated with pipetting. An equal volume of FBS or 1 ml of complete media was added to neutralize the Accumax®. The tubes were centrifuged at 2000 rpm for 5 minutes to form cell pellets and resuspended in a total of 200 μ l of a media-CellTiter-Glo® solution (1:1). The cells were lysed by vortexing to form a clear solution. A suspension from each replicate (100 μ l) was added to a 96-well black plate with clear bottom and luminescence was measured with a Biotek Synergy HT plate reader after 10 min. The cell viability was calculated relative to the luminescence of the untreated spheroids (controls) and expressed as % cell viability.

2.2.8. Statistical analysis—The data was generated in duplicates or triplicates for statistical analysis. The results are reported as the mean $\pm$ standard deviation. For statistical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison tests.

3. Results

3.1. Effect of NCL-240 on metabolism of cancer cells

Investigation of the NCL-240 mechanism of action on A2780 cells indicated that the drug played a major role in dictating the metabolic state of the affected cells. ^{13}C NMR results (Figure 3) show that the ^{13}C label for the DMSO control treatment ended in different compounds such as lactate, alanine, glycine and glutamate which are characteristic products of the aerobic glycolysis and citric acid cycle pathway. However, in case of the NCL-240 treated cells, only lactate was observed to carry the ^{13}C label. There was an absence of peaks for glucose and the products of the citric acid cycle pathways when compared to the bottom DMSO panel in Figure 3.

2-DG induced a glycolysis block and caused a cytotoxic effect in NCL-240 treated cancer cells (Figure 4 (a–c)). CellTiter Glo® assay measured the cell viability by a direct estimation of the amount of ATP in the metabolically active cells. The results represent the cytotoxicity of combinations of NCL-240 and 2-DG at 24 h on various cell lines. All the cell lines were cultured in DMEM. Figure 4(a) shows the cytotoxic effect of NCL-240 from 0.625 to 10 μ M concentration in DMEM containing 4.5 g/l glucose. Increased cell viability as seen in Figure 4(b) in glucose-deprived conditions was observed in control untreated cells as well as with lowest NCL-240 (< 2.5 μ M) dosed cells. Incubation in glucose-deprived media resulted in more growth inhibition of A375 and A2058 than other cell lines with NCL-240 (2.5 – 10 μ M). NCL-240 exposure in such conditions resulted in improvements of cytotoxic effects compared to glucose containing media. The addition of 10 mM 2-DG to NCL-240 (Figure 4(c)) improved cytotoxicity greatly with 2.5, 5 and 10 μ M NCL-240. 2-DG alone also had varying effects on the cell lines proving to be slightly cytotoxic to A2058 and 501mel. The effect of 10 mM 2-DG on the cells generally showed a cell death improvement similar to that of starvation of cells with glucose deprived media.

3.2. Characterization of NCL-240 liposomes

The liposomes were tested for their ability to encapsulate NCL-240. NCL-240 (4% w/w) was initially loaded into liposomes at a lipid concentration of 10 mg/ml (Table 1). Encapsulation was estimated using HPLC. The size distribution of the liposomes indicated that they were homogenous in size and <200 nm in diameter.

In vitro studies were performed to estimate NCL-240 release from liposomes under sink conditions. Free NCL-240 rapidly equilibrated across the dialysis membrane with 100% release achieved at 24 h. In PBS pH 7.4, the release rate of NCL-240 was slower than in PBS pH 5. NCL-240 (73.2%) was released from liposomes by 24 h in PBS pH 5 while 53.8% cumulative release occurred in PBS pH 7.4. Since the liposomes had CHEMS and DOPE in the bilayer, higher release of NCL-240 at pH 5 was expected. Appropriate NCL-240 release studies from the liposomes were thus established and, a higher release was seen at pH 5.

TEM images of NCL-240 loaded liposomes (Figure 5) were captured at 25,000 $\times$ magnification using a 1.5% PTA negative stain. As seen in the image, the size distribution was homogenous and the diameter was largely below 200 nm consistent with the readings taken by dynamic light scattering.

Three batches of NCL-240 liposomes (4% w/w, 25 mg/ml lipid) were prepared to study their stability under storage conditions of 4 °C. The liposomes were characterized for the loss of drug encapsulation and size over the period of time (Table 2). The size of the liposomes remained constant at around 180–220 nm throughout the storage. There was no aggregation of the liposomes due to steric hindrance associated with PEGylation of the liposomes and repulsion of adjacent liposomes by a strongly negative zeta potential. The zeta potential of the samples also remained consistent for the storage period. The liposomes lost about 15% of the encapsulated NCL-240 at the end of 1 month storage. At the end of 2 month storage, liposomes lost between 25 to 44% of drug.

3.3. Cell cycle study by FACS

Cell cycle analysis of A2780 and A2780-ADR cells after 24 h treatment with NCL-240/2-DG combination was carried out by a FACS-based PI/RNase method. The total cells gated in FACS for untreated A2780 and A2780-ADR had similar cell cycle proportions with most cells in G0–G1 phase (62–65%), and similar amount of cells in both, the S and G2/M phases. These phases were unperturbed with plain liposomes. The addition of 2-DG (5 and 10 mM) to A2780-ADR caused a significant arrest of cells in G0–G1 phase (>80%). A2780 cells were not affected by either 5 or 10 mM 2-DG, with only 66% and 72% cells in G0–G1 phase respectively. However, both the cell lines showed a dose-dependent arrest in the G0–G1 phase with NCL-240 liposomes (2.5, 5 and 10 μ M). Cells were then exposed to combinations of NCL-240/2-DG (2.5/5 and 5/10 μ M/mM). NCL-240/2-DG at 2.5/5 μ M/mM and 5/10 μ M/mM caused an 80% and 90% G0–G1 phase arrest in the A2780 cells. This outcome corresponded to a 28.8% and 45.4% increase in the number of cells at the G0–G1 phase compared to the untreated controls. Similarly, both the combination treatments resulted in G0–G1 phase arrest for around 90% of the total A2780-ADR cells. The number

of A2780-ADR cells in the G0–G1 phase increased by 40% for both the combination treatments as compared to untreated controls. The number of cells in the S and G2/M phases declined for both cell lines when treated with the combination formulations as compared to the untreated or vehicle treated cells.

3.4. Cytotoxicity effect of NCL-240/2-DG liposomes

A combined treatment of NCL-240 and 2-DG was carried out for 24 h on several cell lines grown under normoxic conditions. The treatments comprised free 2-DG (10 mM), liposomal NCL-240 (5 or 10 μ M), and liposomal NCL-240 (5 or 10 μ M) supplemented with free 2-DG (10 mM) added to the media. Plain/empty liposomes were used as controls. The combination of NCL-240/2-DG induced an inhibition in the cell division and proliferation with significant number of cells arrested in the G0–G1 phase. Such cell cycle phase arrest translated into significant cell death for the combination treatments over the controls as well as the individual drug treatments for both cell lines, A2780 (Figure 7(a)) and A2780-ADR (Figure 7(b)). Similarly, significant improvements in cytotoxicity were also observed for Adriamycin-resistant strains of small cell lung cancer cells, H69-AR (Figure 7(c)), mouse breast cancer cells, 4T1 (Figure 7(d)), as well as Adriamycin-resistant ovarian cancer cells, NCI/ADR-RES (Figure 7(e)).

Finally, since all the above studies were carried out in normoxic conditions, i.e. monolayer cell culture, where the nutrients and oxygen are readily available to the adherent cells, almost all cells were likely to be metabolically active. To understand the effect of the drug combination on a cancer cell model that better replicated the features of the *in vivo* tumor conditions, the cytotoxicity studies were carried on with NCI/ADR-RES spheroids. NCI/ADR-RES spheroid growth and dynamics have been described in previous studies (Pattni et al., 2016; Riehle et al., 2016). Combination treatments were tested for 48 h on these spheroids and cell viability was analyzed by the CellTiter-Glo® assay. Different concentrations of both NCL-240 and 2-DG were analyzed using the spheroids (Figure 8). For all the combinations, a significant increase in cell death was observed over the individual drug treatments in a model that more closely resembles solid tumor *in vivo*.

4. Discussion

Rapidly growing and dividing cancer cells utilize nutrients more than normal cells. Bioenergy and biosynthesis pathways are at the center of any cell's function. In cancer cells, they are up-regulated to meet the demands of growing tumors. Thus, targeting the energy metabolism pathways is a clear option for anti-cancer therapy. In this paper, we have described the use of one such drug, NCL-240, which induces mitochondrial stress and results in an increased dependence on glycolysis. Moreover, in combination with a glycolytic inhibitor, NCL-240 caused significant cell death. The NCL-240 treated cells underwent a metabolic change with a dependence on glycolysis (Figure 3). NMR results with ^{13}C -labeled glucose indicated that the ^{13}C label ended up in different products of the glycolysis and citric acid cycle including alanine, glycine, and glutamate along with lactate in the control cells. In comparison, ^{13}C ended up in only lactate as a product of glycolysis when the cells were treated with NCL-240. An absence of peaks for alanine, glycine and

glutamate indicate a down-regulation of the biosynthetic functions associated with oxidative phosphorylation. Also, the peak for glucose is absent in the NCL-240 treated cells as compared to the control cells indicating higher uptake of glucose by the NCL-240 affected cells. Such an observation indicates that on NCL-240 treatment, the cells, even when grown in normoxic conditions, are able to utilize the glycolysis based-pathway for energy production. NCL-240 may have affected the mitochondrial oxidative phosphorylation, prompting the cells to produce energy (ATP) via glycolysis. Glycolysis acting as a compensatory mechanism to produce ATP in cancer cells with damaged mitochondrial function has been discussed previously (Carew and Huang, 2002). It is also important to point out that the tumor microenvironment can also play a role in increased glycolysis due to limited availability of nutrients and oxygen, and the up-regulated HIF1- α . Thus, it was hypothesized, if glycolysis in the NCL-240 treated cells is obstructed, then the cells could essentially ‘starve to death’.

The next aim of this work was to check combinations of NCL-240 with 2-DG in multiple cell lines. Treatment of cells with NCL-240 in media containing glucose resulted in cell death at NCL-240 concentrations from 2.5 – 10 μ M. Cell Viability assay (CellTiter-Glo) used for these studies measures cellular ATP levels. Cancer cell metabolism is characterized by plasticity, allowing the cells to rapidly adapt to specifics of microenvironment. Low glucose conditions (Fig 4(b)) prompted a metabolic shift towards mitochondria-based ATP synthesis, which would represent a distinct metabolic state characterized by different ATP levels. As we saw in Fig. 4b, in some but not all melanoma cells this shift appeared to lead to increased levels of ATP. However, even in these cells, effective dose of the NCL-240 promoted efficient loss of viability. 2-DG combination with the NCL-240 treatment enhanced cytotoxicity across all the cell lines. While the improvement in cytotoxicity was similar to that observed in starving conditions, the mechanism of action of 2-DG may be different from just an impairment in glycolysis and lack of production of ATP by cells. Since, the cells were cultured in normoxic conditions, impairment in N-linked glycosylation and ER stress are also expected (Xi et al., 2014). Since NCL-240 exposure made the cells dependent on glycolysis, both of the above mechanisms of 2-DG action could play a role in the observed cell death.

NCL-240 was successfully encapsulated in liposomes. We expect that the liposomes can deliver their content to the cells in several ways, as has been explored by other researchers too. Generally, the liposomes can fuse with the bilayer of the cells to unload their content into the cytoplasm or can be endocytosed into endosomes. Liposomes used in this study had CHEMS/DOPE in their bilayer and it has been shown that CHEMS/DOPE can destabilize the bilayer of liposomes and also the endosomes because of acidic environment (Cho et al., 2008; Ellens et al., 1984). In this manner, NCL-240 was expected to release efficiently into the cytoplasm of the cells.

The cell cycle study also indicated the benefits of using a NCL-240/2-DG combination. Tumors present a higher fraction of cells in S phase and G2/M phase because of uncontrolled cell division. In comparison normal tissues typically have fewer cells in the S and G2/M phases. Relatively, the number of cells in G0–G1 phase is significantly less in cancerous tissues (<70%) compared to a normal tissue (>90%) (Rais et al., 1999). In this

study, most cells were arrested in the G0–G1 phase of the cell cycle when treated with the combinations. NCL-240 may have affected the macromolecule synthesis and the combination of 2-DG would have contributed to reduce ATP synthesis. Hence, the percentage of total cells in the macromolecule- and ATP-dependent S and G2/M phases also declined. A number of cells undergoing division and proliferation was obstructed by this treatment combination. A cell cycle blockade in G0/G1 and G2/M phases with a disappearance of S-phase in SKOV3 and MSTO-211H cells because of 2-DG treatment had been reported (Zhang et al., 2006). Similarly, another triazole-based compound, carboxyamidotriazole, was shown to inhibit the mitochondrial respiration of cancer cells, causing disturbance in their energy metabolism and inducing glucose uptake and lactate production. When treated in combination with 2-DG, carboxyamidotriazole resulted in a G2/M phase arrest (Ju et al., 2016). Previous studies have also described the use of 1 to 10 mM 2-DG that caused inhibition of cell culture (Barban and Schulze, 1961; Halicka et al., 1995). Furthermore, the concentrations of glucose and mannose in serum are 4–6 mM and 100 μ M, respectively. Thus, it has been suggested that a 2-DG dose above 5–6 mM should inhibit both glucose and mannose, while a 1–2 mM dose would effectively inhibit only mannose (Xi et al., 2014). Hence, for cytotoxicity studies with NCL-240/2-DG combinations, it was fitting to administer free 2-DG at 5, 10 and 20 mM with NCL-240 in liposomes. The combination of NCL-240 and 2-DG were significantly more cytotoxic than the control empty liposomes or individual drug treatments. As observed so far, 2-DG enhanced the cytotoxicity of NCL-240 by blocking glycolysis in the glycolysis dependent cells. Such an enhancement was observed in cells grown using monolayer cell culture techniques.

As compared to monolayer cell culture, spheroids have cells growing at different rates from the periphery (with direct access to nutrients and oxygen) to the core (often hypoxic and necrotic). H&E staining performed on NCI/ADR-RES spheroid sections have shown such differences (Pattni et al., 2016). Thus, the cells in the spheroid are at different cell cycle stages, with many cells in a senescent stage in the core of the spheroid and others proliferating faster at the periphery. Even with the cells growing in different rates in a simulated *in vivo* solid tumor environment, 2-DG significantly enhanced the activity of NCL-240.

Thus, a glycolytic inhibitor such as 2-DG is likely to significantly enhance the effects of NCL-240 drug targeting the energy metabolism of cells. Unlike drugs that attack specific stages of the cell cycle, like paclitaxel primarily targeting actively dividing cells, NCL-240 can be used for cancer cells in different cell cycle phases. Similarly, other glycolytic inhibitors may also be effectively combined with NCL-240. Examples include inhibitors like lonidamine, 3-Bromopyruvate, imatinib and oxythiamine (Pelicano et al., 2006). Overall, NCL-240 is an effective option to target cancer energy metabolism in combination with glycolysis inhibitors, and further animal-based studies can be performed to affirm the benefits of targeting energy metabolism in tumors.

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Abbreviations

ATP	Adenosine triphosphate
HIF1-α	Hypoxia inducible factor 1-α
GLUT	Glucose transporter
DM-PIT-1	N-[(2-hydroxy-5-nitrophenyl)amino]carbonothioyl-3,5-dimethylbenzamide
PI3K	Phosphoinositide 3-kinase
PIP₃	Phosphatidylinositol-3,4,5-triphosphate
PKB/Akt	Protein kinase B
PH	Pleckstrin-homology
2-DG	2-deoxy-D-glucose
DMEM	Dulbecco's modified Eagle's medium
PEG	Polyethylene glycol
ePC	Egg phosphatidylcholine
CHOL	Cholesterol
DOPE	1,2- dioleoyl-sn-glycero-3-phosphoethanolamine
CHEMS	Cholesterol hemisuccinate
EPR	Enhanced permeability and retention
PTA	Phosphotungstic acid
DMSO	Dimethyl sulfoxide
PI	Propidium iodide
EPR	Enhanced permeability and retention

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Figure 1.
Structure of DM-PIT-1 and NCL-240.

Figure 2. 2-deoxy-D-glucose (2-DG) mechanism of action in cancer cells. As a glucose and mannose mimetic, 2-DG blocks glycolysis and N-linked glycosylation respectively.

Figure 3.

^{13}C NMR on A2780 cells treated with NCL-240. DMSO was used as control. In DMSO-treated cells, ^{13}C label was present in glucose, lactate, alanine, glycine and glutamate, representing several active bioenergetics and biosynthetic pathways of A2780. In NCL-240-treated cells, all ^{13}C label ended in lactate indicating drug-induced effect on biosynthetic pathways (oxidative phosphorylation), and increased glycolysis as seen by absence of glucose peaks.

Figure 4.

(a) Cytotoxicity of NCL-240 in various cancer cells grown in DMEM containing 4.5 g/l glucose; (b) Cytotoxicity of NCL-240 in various cancer cells grown in DMEM with 0.5 g/l glucose; (c) Cytotoxicity of combination of NCL-240 and 10 mM 2-DG in various cancer cells grown in DMEM supplemented with 4.5 g/l glucose. Indicated by 4 (a) and 4 (b), NCL-240-treated cells in glucose-deprived media were killed more than in glucose-containing media. Blocking glycolysis by competitive inhibition using 2-DG provided with similar cell-killing abilities [4 (c)].

Figure 5.
TEM analysis of NCL-240 liposomes (magnification: 25,000×).

Figure 6.

Cell cycle analysis after NCL-240 and 2-DG treatment in (a) A2780 and (b) A2780-ADR cells. 2-DG and NCL-240 (N) concentrations are given in mM and μ M respectively. For both cell lines, the combinations of 2-DG and NCL-240 caused a higher G0–G1 phase arrest compared to controls.

Figure 7. 24 h Cytotoxicity of combination NCL-240 and 2-DG treatment on (a) A2780, (b) A2780-ADR, (c) 4T1, (d) H69-AR, and (e) NCI/ADR-RES cells. (n=3, Mean $\pm$ S.D, ** p 0.01, *** p 0.001). A significant improvement in cell death was observed across all cell lines with combination treatment over the controls and the individual drugs.

Figure 8.
Cytotoxicity of combination NCL-240/2-DG treatments on NCI/ADR-RES spheroids (48 h, n=3, Mean ± S.D, *** p 0.001)

Table 1

NCL-240 liposome characterization

NCL-240 initial loading (w/w)	NCL-240 loading (mg/ml)	Size (nm)	PI
Empty		175.3	0.063
4%	0.315	151.1	0.054

Table 2

Storage stability of 4% w/w NCL-240 liposomes.

Initiation of study				After 1 month				After 2 months			
Batch no.	Initial drug loading (µg/ml)	Size (nm)	Zeta (mV)	Loss of drug (%)	Size (nm)	Zeta (mV)	Loss of drug (%)	Size (nm)	Zeta (mV)	Loss of drug (%)	Size (nm)
A	303.45	225.2 ± 5.1	-41.8	15.27	205.2 ± 3.8	-40.45	25.51	175.2 ± 1.8	-43.54		
B	361.03	215.7 ± 2.1	-42.86	14.75	196.4 ± 5.4	-43.64	43.67	176.0 ± 3.7	-42.88		
C	340.18	222.2 ± 2.4	-51.58	14.69	226.1 ± 3.6	-48.71	36.47	241.4 ± 2.7	-49.06		
Empty	-	215.5 ± 2.1	-43.89	-	186.2 ± 2.9	-42.1	-	191.6 ± 4.8	-44.25		