C6-Ceramide (C6-Cer) to Induce Sensitivity to Cetuximab (Cet) in KRAS Mutant Colorectal Cancer (Crc)

Menendez A[1,2], Haidemenos G[2], Curzake D[3], Luo L[2] and Wanebo H[1,4]*

[1]Hematology and Medical Oncology, Indiana University School of Medicine, USA

[2]Department of Medicine, Roger Williams Medical Center, USA

[3]University of Rhode Island College of Pharmacy, USA

[4]Division of Surgical Oncology, Landmark Medical Center, USA

Abstract

Background: Cet is beneficial for patients with metastatic KRAS Wild type (WT) CRC. C6-Cer can act synergistically with chemotherapy to induce apoptosis. The aim was to compare growth inhibition percentage (GIP) of cytostatics 5-FU, oxaliplatin (Ox) and Cet with or without C6-Cer in KRAS WT and KRAS mutant (KRAS Mut) CRC cell lines (SW48 and SW480, respectively).

Methods: Cells were incubated with IC50 (0.8µM for 5-FU, 0.04µM for Ox, 25 µg/mL for Cet, and C6-Cer concentrations ranged from 5 to 10 µM). Cell survival was assessed 72h after using 0.4% Trypan Blue.

Results: C6-Cer's GIP was 78.3% for SW-480 (*vs.* 33.33% for SW-48). Addition of C6-Cer increased GIP with an especially significant effect on SW-480. Addition of 5 and 7.5µM resulted in GIP of 75% and 86.25%, respectively, *vs.* 32.5% of 5-FU + Ox + Cet alone. The greatest effect was seen when 10µM of C6-Cer was added (92.5%). Same concentration of drugs increased GIP for SW-48 to 93.5%.

Conclusion: C6-Cer appears to have direct inhibitory properties, especially on KRAS Mut cells. When added to Ox, 5-FU and Cet, C6-Cer reversed the apparent insensitivity of KRAS Mut to Cet. Also, the study showed C6-Cer can provide additional synergism to their cytostatic properties in KRAS WT CRC cell lines. The effect of isolated C6-Cer on KRAS Mut raises possibility of a different pathway that could bypass EFGR pathway.

Keywords: C6-Cer; Cet; Crc; KRAS

Introduction

CRC is the third most common cancer in the United States and the second leading cause of cancer-related deaths [1]. Metastatic disease is found in 40-50% of patients at the time of diagnosis, with 25% of newly-diagnosed patients found to have liver metastases [2]. Management of patients with (mCRC) is variable, and depends on a number of factors, including performance status and whether the tumor expresses a mutation to KRAS. KRAS mutations have been documented in as high as 35-40% of mCRC patients [3], with a substitution of glycine for aspartate at codon 12 being the most common mutation [4,5]. KRAS mutations cause inhibition of the guanosine-5'-triphosphate (GTP)-ase activity, thereby causing a buildup of GTP-bound KRAS, which is the active form of the protein [5]. Because KRAS is a protein in the pathway between the Epidermal Growth Factor Receptor (EGFR) and oncogenic effects such as cell proliferation and transformation, accumulation of the active form of KRAS allows cells expressing Mut KRAS to promote these effects independent of EGFR activation, which renders drugs that inhibit EGFR much less effective [5]. Due to the fact that surgical resection is rarely a viable option in Stage IV and recurrent colorectal cancer, chemotherapy is the mainstay of treatment for these patients. Combinations of 5-FU with leucovorin, irinotecan or oxaliplatin with or without bevacizumab are all considered standard chemotherapeutic regimens for metastatic disease, and in 2004 the EGFR inhibitor Cetuximab (Cet) received FDA approval after emerging as a promising therapy for patients with metastatic colorectal cancer. Cet is a monoclonal antibody that binds to EGFR to inhibit signal transduction that was approved as first-line treatment in combination with FOLFIRI (leucovorin, 5-FU and irinotecan) for patients with KRAS WT mCRC [6,7]. Subsequent studies however proved Cet to be ineffective on KRAS-Mut mCRC [8]. Ceramide is a sphingolipid metabolite that can induce cancer cell death.

Table 1: Dosing of the combination of oxaliplatin, 5-FU and Cet.

SW-480	Average	Std Dev	p-value
5-FU + Ox + Cet + C6- Ceramide 5 µM	10.875	3.52288437	0.000232925
5-FU + Ox + Cet + C6- Ceramide 7.5 µM	6.75	2.49284691	0.000319356
5-FU + Ox + Cet + C6- Ceramide 10 µM	4.5	2	0.000665572
SW-48			
5-FU + Ox + Cet + C6- Ceramide 5 µM	16.375	6.186101934	7.92969E-05
5-FU + Ox + Cet + C6- Ceramide 7.5 µM	14.875	5.914570387	0.000180083
5-FU + Ox + Cet + C6- Ceramide 10 µM	10	8.451542547	5.83415E-05

It is generated endogenously by ionizing radiation or chemotherapy through the actions of sphingomyelinases. It can also be administered as short-chain analogs, also known as C6-Cer [9]. When exogenous C6-Cer is encapsulated in a nanoliposomal formulation, one in vitro study demonstrated increase in its potency and efficacy [10]. In this study, concentration of C6-Cer required to inhibit 50% of cells (IC50) decreased from 12µmol/L to 5µmol/L when integrated into a nanoliposome [10]. A separate study later found that the mechanism for nanoliposomal C6-Cer-mediated apoptosis was by inhibition of synthesis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [11]. *In vitro* analyses have been published regarding the ability of C6-Cer to act synergistically with chemotherapeutic drugs administered for the treatment of several types of cancer [12]. These studies have also shown that C6-Cer may have the potential to inhibit the mutated KRAS ERK/MAPK pathway, and thus reverse the resistance of cancer cells to certain cytotoxic drugs even those that might be EGFR dependent [13]. In another study it was found that C6-Cer inhibited growth of the CRC and induced apoptosis, an effect that was not seen in human mesenchymal stem cells [14]. It is the hypothesis of this experiment that C6-Cer can restore the anti-tumor effect of Cet in patients with the KRAS mutation.

Methodology

Cell cultures

The study is a test system measuring the cell growth of KRAS WT and KRAS Mut cells. KRAS WT SW-48 (SW48 [SW48] (ATCC° CCL231TM)) and KRAS-Mut CRC cells SW480 ([SW480] (ATCC° CCL228TM)) colorectal cancer cell lines (CRC cell lines) were obtained from American Type Culture Collection (ATCC). They were cultured in Dulbecco's modified Eagle's medium culture (DMEM; Gibco) with additional sodium bicarbonate (2 grams) (Gibco) and fetal bovine serum at 10% (Gibco). 100 units/mL Penicillin and 100 microgram/mL of Streptomycin were added to that mix at 1% concentration. Cells were cultured at 37°C in a humidified atmosphere including 5% CO2. Cells were grown in 24-well plates.

Drugs

Oxaliplatin, 5-FU and Cet were added to all plates. C6-Cer was used at 5mcg/cc. C6- Cer was obtained from Avanti Polar Lipids Inc, Alabaster, Alabama. Liposomal C6-Cer (5:88:718:0PEG2PE: DOPC: C6Ceramide) consisted of a phospholipid 1, 2-Dioleoyl-Sn-glycero-3- phosphocholine (DOPC) and a hydrophilic polymer polyethylene glycol (PEG) conjugated with another phospholipid (phophatidylethanolamine). Liposomes were reconstituted from the lyophilized powder using double distilled water. The drugs were dissolved in 100% dimethylsulfoxide (DMSO) and then diluted in the media for experiments. In all the experiments, control cells were
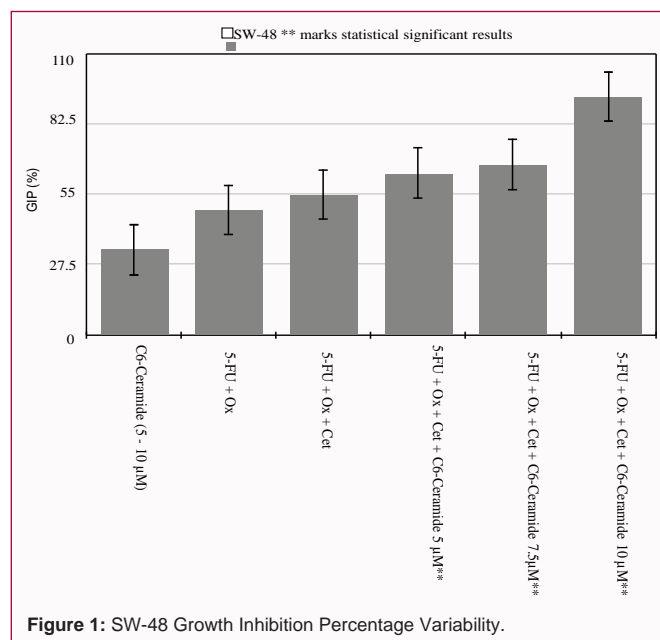
Figure 1: SW-48 Growth Inhibition Percentage Variability.

incubated with DMSO alone. The final concentration of DMSO was maintained at 0.2%.

Determination of chemotherapy dosing

To establish the dosing of the combination of oxaliplatin, 5-FU and Cet used in the experiment, an analysis of the IC50 was performed. For this analysis, both SW-48 and SW-480 CRC cells were cultured in 24-well plates, and then exposed to varying concentrations of each drug. The well-plates were incubated in varying concentrations of 5-FU and oxaliplatin alone. Using a microscope and Tryptan Blue stain at 0.4%, researchers counted the number of living cells in each of the wells 72 hours after incubating the cells in the drug combination. The percentage of cells killed by the combination was been calculated and the IC50 was determined. IC50 for this two-drug combination was 0.8 and 0.04µM, respectively. IC50s were used as a benchmark for the next phase of the experiment, where Cet was added to the combinations in order to determine the IC50 of the three-drug combination. In this experiment, 5-FU and oxaliplatin combinations used were 0.8 and 0.04µM, 0.4 and 0.02µM and 0.2 and 0.01µM, Cet concentrations used were 5, 12.5, 25, and 50 µg/mL. After 72 hours, cell counts indicated an IC50 of 0.8µM for 5-FU, 0.04µM for Oxaliplatin and Erbitux at 12.5µg/mL for SW-48 cells. For SW-480 cells, 0.8µM for 5-FU, 0.04µM for Oxaliplatin and Erbitux at 50µg/mL failed to improve the total percent of inhibition reached by 0.8µM of 5-FU and 0.04µM of Oxaliplatin. This difference was expected due to Cet established lack of efficacy in KRAS-Mut cells. Based on these

results, concentrations of 0.8µM for 5-FU, 0.04µM for Oxaliplatin and Erbitux at 12.5-50 µg/mL were chosen to be used in the experiment analyzing the effect of C6-Cer on the IC50 of combination therapy. C6-Cer was added at concentrations varying from 5 to 10 µM (Table 1).
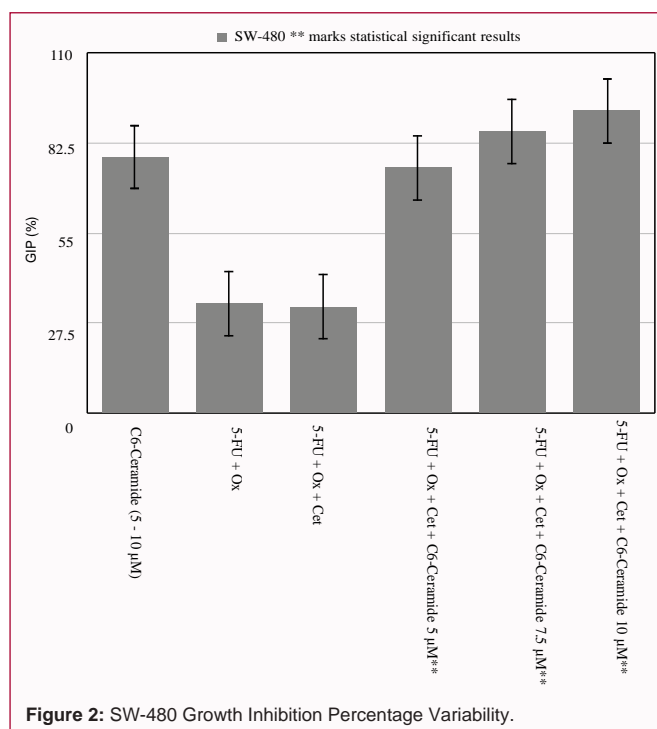
Results

When compared to DMSO 0.2% control plate, 0.8µM of 5-FU and 0.04µM of Oxaliplatin alone inhibited 33.5% of SW480 cells and 49% of SW48 cells from growing. Additionally, 0.8µM of 5-FU, 0.04µM of Oxaliplatin, and Erbitux at 12.5 and 25µg/mL, killed about 28.75%, and 32.5% respectively of KRAS Mut CRC cell lines (SW-480). Conversely, same concentrations inhibited up to 55% of the KRAS WT cell line (SW-48) (p<0.005) (Figure 1).

At the lowest C6-Cer concentration of 5µM with IC50 of chemotherapeutic regimen, cell growth inhibition increased from 32.5% to 75% in KRAS Mut, and from 55% to 63.3% in KRAS WT. When C6-Cer dose was increased to 7.5µM, the percentage increased to 86.25% and 66.6% respectively. Finally, when C6-Cer was raised to 10µM, inhibitory growth percentage reached 92.5% in KRAS Mut, and 93.3% in KRAS WT (p<0.005) (Figure 2).

C6-Cer was also placed individually with both cell lines at concentrations ranging from 5 to 10µM. It was noticed the experimental substance had stronger cytotoxic properties in KRAS Mut. Inhibitory growth percentage ranged from 70 to 85% in KRAS Mut cell line, and reached a high of only 40% in KRAS WT.

Discussion

Cet functions as a competitive antagonist to EGFR and may even lead to its degradation. Direct stimulation of EGFR induces a conformational change in its extracellular domain that promotes dimerization with other receptor tyrosine kinases (RTKs), activating its intrinsic kinase activity and leading to the autophosphorylation of tyrosine residues, resulting in increased cellular survival, proliferation, migration and angiogenesis [15]. By inhibiting EGFR, Cet prevents tumor growth and metastasis and can also induce apoptosis [16]. Cet received accelerated approval after showing it could significantly reduce the size of tumors and even delay tumor growth for a mean of 4.1 months when used in combination with irinotecan or even alone [17]. In the Cet combined with irinotecan in first-line therapy for mCRC study (CRYSTAL), the efficacy of Cet in combination with standard FOLFIRI therapy was assessed vs. FOLFIRI alone [8]. This open-label randomized controlled study showed that Cet in combination with FOLFIRI resulted in statistically significant PFS, but did not prolong OS [8]. However, in a retrospective analysis of the study, it was found that Cet produced greater benefits in patients with WT KRAS. In these patients, OS was increased (23.5 months vs. 19.5 months in the control group), as well as PFS (9.5 months vs. 8.1 months) and ORR (57% vs. 39%) [8]. This also showed that in patients with Mut KRAS, Cet did not improve any of these metrics which led to the indication for Cet in combination with FOLFIRI to be used as first line therapy in patients with WT KRAS mCRC only. The CECOG/CORE2 study reported interim results on the efficacy of Cet in mCRC patients. It was reported that KRAS WT mCRC showed an increased ORR, OS and PFS with use of a combination of Cet with FOLFOX4 in comparison to FOLFOX4 alone [18]. This benefit was not observed in patients with Mut KRAS, lending evidence to the confirmation that Cet is not effective in patients with Mut KRAS CRC [18]. And although it can still increase both OS and PFS if mutation is

Figure 2: SW-480 Growth Inhibition Percentage Variability.

present, it still remains inferior to the results observed in KRAS WT patients [19,20]. We witnessed this phenomenon when our results demonstrated SW-480's GIP was 33.5% in 5-FU+Ox vs. 32.5% in 5-FU+Ox+Cet at 25µg/mL (Figure 2). Additionally, we replicated the benefits for KRAS WT CRC, when GIP increased from 49% to 55% when a small dose of Cet was added to IC50 of drugs (Figure 1). All of these results were statistically significant and were reproducible in a second study. A recent pilot study performed at another institution showed good outcomes of pancreatic cancers, characteristic for having KRAS gene mutation, exposed to Gemcitabine and Paclitaxel plus Cet with C6-Cer. Although the effect of C6-Cer was found to be mainly additive, in vivo studies showed that the combination was better when it came to stabilization of the tumor volumes and the survival percentages.

There has been an increase in the study of bioactive lipids as adjunct components in the field of cancer research. Ceramides are structural components of the natural cell membrane and they can induce apoptosis in cancerous cells and even aid in overcoming drug resistance [21]. It's most important component to induce cancer cell death is inhibited by its metabolizing enzyme Glucosylceramide synthase (GCS) which turns it to a neutral non-apoptotic metabolite [22]. As a result, several studies have focused on finding ways of inhibiting GCS, which is hyper-produced in malignant cells [23]. Others have hypothesized even just increasing exogenous C6-Cer concentrations as a substrate to initiate substantial apoptosis, especially following affirmations that exogenous C6-Cer administration might stimulate the generation of intracellular Ceramide [24].

C6-Cer (N-Hexanoyl-D-erythro-sphingosine) possesses advantageous physical characteristics such as intermediate hydrophobicity when compared to previously test larger chain ceramides such as C16-Cer, which are more structurally equivalent to natural ceramides. In a dose-dependent manner, the analog was found to activate a cytosolic serine/threonine protein phosphatase, an intracellular signaling pathway involved in cell differentiation and proliferation [25]. The nanoliposome triggered intracellular
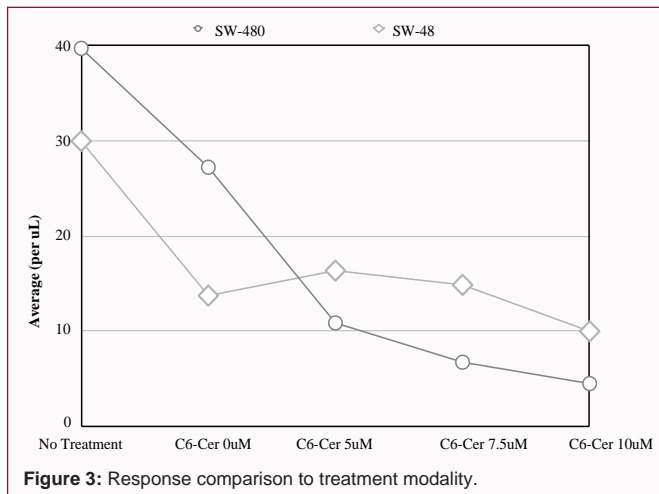
Figure 3: Response comparison to treatment modality.

phosphorylation of PI3K & PKCz and dephosphorylation of PKCa, resulting in downstream fiber depolymerization, adhesion disassembly and integrin modulation [26]. Also on par with these effects on cellular infrastructure, C6-Cer administration has shown direct modulatory effects on the activation of beta integrins-CD molecules involved in cell adhesion through phosphorylation inhibition [27]. Others affirmed ceramides can induce nuclear factor k-B (NF-kB) inhibition, caspase-3, ADP-ribose polymerase degradation and mitochondrial cytochrome c release, concluding that apoptosis occurred through both caspase activation and mitochondrial pathway. It should be noted, however, that the survival of these cells compared to control cell lines remained the same, prompting the authors to recognize that NF-kB inhibition did not modify the ceramide-induced apoptotic pathway [28]. To add additional evidence of C6-Cer directly affecting the mitogenic capability of cancer cells, the use and abstinence of serum growth factors in Molt-4 leukemia cells was studied [29]. They were able to provide evidence that exogenous C6-Cer was equivalent to the withdrawal of various serum factors on cell cycle arrest where nearly 80% of cells were arrested in G0/G1, with only 12% leading to apoptotic cell death. It was proposed that this may be the first preliminary evidence whereby an intracellular signal transduction pathway mediated cell cycle arrest, certainly touching on the importance of lipoid secondary messengers and the possible future target for cancer therapy. Through another molecular mechanism, C6-Cer has been shown to have an inhibitory effect on VEGF-induced endothelial cells [30]. Ceramide administration directly inhibited the endothelial cell formation and subsequent blood vessel formation. Additionally, it inhibits both RNA and protein expression of GADPH, an enzyme of the glycolytic pathway in a CLL model, utilized by cancer cells as part of their primary metabolism, otherwise known as the 'Warburg effect' [31]. This was assessed by concomitantly measuring GADPH and ATP levels after its administration. They were able to show decreasing the enzyme levels led to subsequent tumor regression by decreasing overall protein levels [18]. By inhibiting cancer cell glycolysis, C6-Cer depletes the cell of adenosine triphosphate (ATP), causing cell death. The study proved this mechanism by showing that cells that were pretreated with pyruvate, the end-product of glycolysis, did not suffer the cytotoxic effects of C6-Cer [18]. This reduction, however, was not seen in noncancerous peripheral blood mononuclear cells; a finding that provides evidence for the specificity of C6-Cer to cancer cells. Overall liposomes are biocompatible and fairly nontoxic to noncancerous cell lines [32].

With increasing doses of C6-Cer, GIP increased for both cell lines although not exponentially. It appears SW-480 cells required less C6-Cer to potentiate the effect of Cet. C6-Cer effect is not only dose dependent but also cell density and cell type dependent, with an inverse proportion for the latter ones. Cellular differences between SW-480 and SW-48 were not studied and could explain the increased sensitivity of SW-480 cancer cells to C6-Cer, although this would not subtract importance to the study results. At the highest C6-Cer concentration of 10μM, GIP rose above 90% in both cell lines, supporting the theory of C6-Cer reverting the resistance of KRAS Mut CRC cells to Cet. Furthermore, we also perceived a potentiation of the Cet synergistic effect with Ox and 5-FU on KRAS WT cells. Initial analysis showed an increase in GIP from 49 to 55% with addition of Cet. When C6-Cer was added, GIP increased by 15% at the lowest dose, and by 69% when high dose C6-Cer was added.

C6-Cer was studied individually. At different concentrations, it did appear to have certain effect in GIP, especially in KRAS-Mut. Additional research is warranted to try to establish the molecular and structural changes in KRAS Mut CRC cells that would explain for this difference in susceptibility to C6-Cer. Even more, the effect of isolated C6-Cer in KRAS Mut CRC cell lines raises possibility of studying a different pathway for treating this type of cancer that would bypass EGFR receptor pathway. Further studies could include labeling exogenous C6-Cer within the liposomes to test where is it that it distributes itself. These results may allow lowering the current doses of the chemotherapeutics with the combinational therapy while still maintaining a significant therapeutic effect, resulting in fewer side effects and even decreased chemoresistance. Additional studies could include lowering the dose of C6-Cer with the cytostatic drugs, although the entire panel of C6-Cer adverse effects is yet to be completely understood.

Administration of C6-Cer as a suspension is unfeasible due to its hydrophobic nature. Encapsulating the substance in a liposomal formulation through optimized solvent evaporation technique has shown decrease cytotoxicity levels when compared to free C6-Cer. Liposomes are nano particles that enable the delivery of molecules to particular areas of the organisms through both passive and active targeting. Adding polyethylene glycol (PEG), a hydrophilic polymer that enables a shielding effect of those liposomes from the absorption of proteins, detection by digestive enzymes and the immune system, leads to longer circulating times, reduced clearance and greater half lives [33]. Finding a more effective *in vivo* drug delivering system for C6-Cer surges as another potential research field.

Conclusion

C6-Cer holds great promise in the future battle against malignancies. From significant effects on intracellular signaling and phosphorylation, to altering enzyme activation and ultimately aiding in programmed cell death, this analog appears to target multiple molecular sites. In our opinion the most important finding of this work is the demonstration that C6-Cer used in combination with 5- FU, oxaliplatin and Cet seems to revert the resistance of KRAS Mut CRC cells lines, although its potentiating effect on KRAS WT CRC cell lines raises a hypothesis of a secondary pathway other that KRAS leading to apoptosis of these cells. We believe the results of this study provide a starting point for clinical studies for C6- Ceramide in patients with relapsing or metastatic KRAS Mut CRC and KRAS WT CRC in combination with standard chemotherapy plus molecular target agents hoping they will translate into clinical benefit for this difficult to treat patient population.

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