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Combination of KRAS Gene Silencing and PI3K Inhibition for Ovarian Cancer Treatment

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Abstract

The phosphoinositide 3-kinase (PI3K) and RAS signaling pathways are frequently co-activated and altered during oncogenesis. Owing to their regulatory cross-talk, the early attempts of targeting only one pathway have mostly ended up promoting the development of drug resistance. Here, we propose using small interfering RNA (siRNA) therapeutics to directly target the undruggable KRAS (siKRAS) in combination with the pan-PI3K inhibitor GDC-0941 (GDC) to simultaneously block both PI3K and RAS signaling, thereby exerting synergistic anti-tumor effects on ovarian cancers with *PTEN* deficiency and *KRAS*^{G12D} mutation. For successful delivery of siKRAS, tGC/psi—nanoparticle formulation of polymerized siRNA and thiol-modified glycol chitosan nanoparticle—was used for KRAS specific inhibition *in vitro* and *in vivo*. GDC or siKRAS monotherapy each impede downstream signaling, leading to some delay in cell proliferation and migration. When combined, however, they engender much higher inhibition of PI3K signaling and stimulation of apoptosis in an ovarian allograft model compared to monotherapies. Our results show the feasibility of developing new combination strategies for the management of multiple oncogenic mutations activating PI3K and RAS signaling.

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Associated content

The authors declare no competing financial interests.

Appendix A. Supplemental data

Keywords

PI3K; Combination Therapy; KRAS; siRNA; Ovarian Cancer

1. Introduction

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that have a central role in intracellular signaling thus controlling many physiological functions and cellular processes. The PI3K pathway is commonly mutated in human cancers resulting in dysregulated cell proliferation, growth, survival, motility and metabolism [1, 2]. Because of their encouraging therapeutic effects in preclinical studies, the early inhibitors of PI3K, the so-called pan-PI3K inhibitors that target all isoforms, were tested in clinical trials, but have proven to be less than optimal due to significant toxicities and potential acquired resistance [3]. Several groups are investing to further understanding of basic molecular mechanisms underlying these problems, seeking combination approaches that reduce toxicities and avoid resistance [4–7]. PI3Ks transduce important signals from various upstream inputs such as receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs) [8]. Often blocking PI3K activity in cells transformed by activated receptors is sufficient to block cell growth. However, even though oncogenic RAS mutants signal in part via PI3K, PI3K inhibition has been ineffective in RAS transformed cells; and KRAS mutation has found to be a common resistance mechanism for PI3K inhibition [9]. Similar to PI3K pathway, the RAS signaling pathway is a common mechanism for regulating a variety of cellular activities such as cell proliferation, differentiation, survival, motility and metabolism, in response to different extracellular cues [10, 11]. Notably, PI3K and RAS intensively cross-talk to regulate each other and co-regulate downstream functions. Thus blocking only one pathway induces compensatory signaling in the other that eventually leads to treatment failure and relapse [10, 12–14]. In a previous study, simultaneous inhibition of RAS and PI3K signaling achieved maximal effect in antitumor activity in an animal model. *In vivo* combination of a pan-PI3K inhibitor and a MEK inhibitor exhibited synergistic regression in a *KRAS*^{G12D} mutated lung tumor model [2]. In the case of RAS mutant cancers with acquired resistance to MEK inhibitors, dual-inhibition of PI3K and KRAS signaling led to mTOR pathway inhibition causing cell death [15]. Also, dual-treatment with inhibitors of the PI3K and RAF/MEK/ERK pathways effectively reduced proliferation in RAS-addicted leukemia xenograft model [16]. There have been many other studies utilizing co-inhibition of the PI3K and RAF pathways for improved therapeutic effect in various cancer models [2, 17–21].

Combination therapies that simultaneously inhibit several signaling pathways have been proposed as effective therapeutic strategies [22]. Since the combination of inhibitors also increases risk of on target toxicities, co-inhibition of PI3K and KRAS using an alternative targeting approach with lower toxicity is of considerable interest [23]. Notably, most mutant forms of KRAS itself are considered to be undruggable due to the lack of suitable binding sites for small-molecule inhibitors. Since direct pharmacologic targeting of KRAS protein is extremely challenging, most investigators have explored indirect therapeutic approaches to target mutant KRAS such as using inhibitors of proteins upstream of KRAS or inhibitors of

downstream RAS effectors. One way to overcome the toxicity and resistance of small molecule inhibitors—the most prevalent type of cancer treatment agent—is to use biological drugs that are biocompatible and degradable in the organism, thereby in theory having less cytotoxicity. Here, we used an siRNA that targets pre-existing mRNA molecules to down-regulate RAS expression and, thus, impede cellular signals for proliferation. This siRNA is a small non-coding double strand RNA that operates via RNA interference (RNAi) mechanisms to downregulate specific gene expression by complementary binding to target mRNAs which are degraded via the dicer complex. siRNA therapeutics hold advantages over conventional treatments due to ability to target problematic proteins at the root of disease origins without respect to their conventional druggability, flexibility in customizing drug targets, and safety for clinical use [24]. The use of siRNAs as targeted therapeutic agents has been under investigation, and multiple examples have entered clinical trials for their highly sequence-specific gene silencing ability [25]. Even with the strong advantages of siRNA therapeutics, efficient delivery to the disease site still remains as a major challenge [26]. Because of instability and immediate degradation of siRNA in serum, many groups have investigated ways to increase the molecular weight, neutralize the negative charge, or couple modifications to the siRNA to optimize delivery protection to the disease site. In our approach, polymerized and modified siRNAs are assembled into delivery complexes to help localize them into disease site to elicit therapeutic effect. As an example, polymerized-siRNAs (poly-siRNA or psi) were generated via disulfide crosslinking that self-assemble into glycol chitosan nanoparticles, or thiolated glycol chitosan (tGC), as a delivery nanoparticle system specific for tumor sites [27]. This particular siRNA delivery complex has been shown to passively localize at tumor sites in animal models, when administered via tail vein injection, and has demonstrated anti-cancer effects in a prostate cancer model [28]. In comparison to chemotherapeutic agents or drugs, siRNA is a safe therapeutic option that inhibits expression of any oncogenic driver at its origin.

In this study, the pan-PI3K inhibitor GDC and siKRAS were simultaneously administered to ovarian cancer cell lines *in vitro* and in an allograft ovarian cancer model *in vivo* to test their potential as a combination drug therapy (Fig 1). Ovarian cancer is the most lethal gynecological malignancy and is the fifth most common cause of cancer death in women. We showed that when GDC is given to an ovarian cancer cell line carrying a KRAS mutation, the subsequent reduction in PI3K signaling results in over-expression of KRAS due to an apparent feedback loop. When both GDC and siKRAS were given to ovarian OSE cell lines or an allograft ovarian cancer model, inhibition of both the PI3K and RAS pathways resulted in a synergistic anti-cancer effect. Here, we demonstrate that inhibition of both pathways impeded cell proliferation and migration and additionally induced apoptosis in tumors *in vivo*.

2. Materials and Methods

2.1. Materials

Glycol chitosan (MW=250kDa, degree of deacetylation 82.7%), 4',6-diamidino-2-phenylindole (DAPI), ethidium bromide (EtBr), Ellman's reagent 5,5'-Dithiobis(2-nitrobenzoic acid) and crystal violet powder were purchased from Sigma Aldrich (St. Louis,

MO, USA). Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (Sulfo-LC-SPDP) was purchased from Pierce (Rockford, IL, USA). Nap-10 column is manufactured by GD Healthcare, (Piscataway, NJ). Dithiothreitol (DTT), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and ethylenediaminetetraacetic acid (EDTA) were obtained from Biosesang Inc. (Gyeonggi, Korea). Low molecular weight DNA ladder (N3233) was purchased from New England Biolabs® (Ipswich, MA, USA) Lipofectamine RNAiMAX, and NIRF Flamma FPR-675 were obtained Invitrogen (Carlsbad, CA, USA) and BioActs (Incheon, Korea), respectively. Cell culture media DMEM/F12, OPTI-MEM and 4-chamber slides were purchased from Life Technologies (Carlsbad, CA). RIPA buffer, micro BCA protein quantification kit, YOYO-1 intercalating dye and SYBR™ Green Master Mix were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). CCK-8 from Dojindo Molecular Therapeutics Inc. (Japan) was for in vitro experiment. For real-time PCR, RNeasy mini kit and iScript Reverse Transcription Supermix for RT-qPCR were purchased from QIAGEN (Venlo, Netherlands) and Bio-rad (Hercules, CA, USA), respectively. Primary antibodies of p-Akt, Akt, p-S6, S6, p-FoxO1, Cyclin D1, p-ERK1/2, p-MEK1/2, p-P70 S6K, Cleaved Caspase 3, ki67, and PARP from Cell Signaling Technology (Danvers, MA, USA), Vinculin from Sigma Aldrich (St. Louis, MO, USA), and Ras from Santa Cruz Biotechnology (Dallas, TX, USA) were purchased for immunoblot assay. Goat anti-rabbit and rabbit anti-mouse immunoglobulins-horseradish peroxidase was purchased from Enzo (Farmingdale, NY, USA). Biotinylated secondary antibodies, avidin-biotin complex solution, and 3,3'-diaminobenzidine were purchased from Vector Labs (Burlingame, CA, USA). Pan-PI3K inhibitor GDC-0941 was purchased from Selleckchem (Houston, TX, USA). Murine siRNA sequences are following: KRAS siRNA 5'-CCAUUAUAGAGAACAAAUU-3' and scrambled siRNA 5'-UGAAGUUGCACUUGAAGUC-3' were 5'-modified with thiol groups from IDT (IO, USA). The commercially designed primer-pairs of KRAS and β -actin were ordered from Ambion Inc. (now Life Technologies).

2.2. OSE Cell Culture

Previous study has isolated, primary cultured, and immortalized the OSE cell lines prior to use for the experiment [29]. The cells were cultured in DMEM/F12 supplemented with 4% (vol/vol) FBS, 1% penicillin/streptomycin, 10 ng/ml EGF, 5 ug/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml sodium selenite at 37 °C and 5% CO₂ atmosphere. 1×10^5 cells were seeding in 6-well culture plate for western blot and real-time PCR experiments. For protein and RNA analysis, cells were incubated with siKRAS in OPTI-MEM medium for 12 hours prior to replacement with culture medium. The protein and RNA were harvested from cell lysates after 48 hours of incubation for analysis. 5×10^3 cells were seeded on 4-chamber slides for fluorescence cellular uptake assay. The fluorescently labeled siKRAS was incubated in cells for 12 hours before formaldehyde fixation and counterstaining with DAPI solution.

2.3. Cellular Viability and Proliferation Assays

CCK-8 and crystal violet assay were used to measure cell viability and proliferation. For CCK-8 assay, 5×10^3 cells were seeded in 96-well plate and colorimetric intensity was measured at 450 nm 2 hours after addition of CCK-8 solution. For crystal violet assay, cells were seeded in 12 well plates at a density of 1×10^4 cells per well. At indicated time points,

cells were washed with PBS and fixed in 4% formaldehyde solution for 10 min. 0.1% crystal violet solution was added and incubated for 20 min, followed by washing with distilled water for 3 times. The plates were air-dried for image data collection. The crystal violet stain was released using 10% acetic acid and colorimetric value was measured at 450 nm using a plate reader.

2.4. Real time PCR and Western Blot

Real-time PCR was performed with lysates of confluent OSE cell treated with GDC-0941 and/or siKRAS. Cell lysis for the purification of mRNA of cells was done using RNeasy mini kit. cDNA was synthesized using iScript and amplified for SYBR green detection for Real-time PCR system (ABI7300). KRAS mRNA levels were normalized to β -actin mRNA using respective primers. For western blot assays, both harvested tumors and OSE cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, protease inhibitors). Protein concentration of lysates were normalized using Micro BCA Protein Assay Kit. Following primary antibodies were used for western blot analysis in cells and tumors—p-Akt, Akt, p-S6, S6, p-FoxO1, Cyclin D1, p-ERK1/2, p-MEK1/2, p-P70 S6K, Cleaved Caspase 3, PARP, Vinculin, and RAS.

2.5. Preparation of poly-siRNA formulated with tGC

Synthesis of poly-siRNA, abbreviated as psi, and thiolated glycol chitosan is prepared according to previously published protocol [27]. Dithiol-group modified siRNA was dissolved in 100 μ l of 10 mM HEPES buffer (pH 8.0, 1 mM EDTA). NAP-10 column was used to purify siRNA and the solution was lyophilized overnight and resuspended in 10 mM HEPES buffer (pH 8.0) for more than 24 hours. Synthesized poly-siRNA was verified via 8% polyacrylamide gel electrophoresis (PAGE) and ethidium bromide detection. The degree of thiolation of poly-siRNA was determined by reacted thiol groups with Ellman's reagent and measuring the absorbance at 412 nm. Glycol chitosan was also thiol group functionalized using sulfo-LC-SPDP (Thermo Fisher) to produce thiolated glycol chitosan (tGC). Lyophilized tGC (1 mg) was probe sonicated in 400 μ l of 10 mM HEPES (pH 8.0, 1 mM EDTA) and gently mixed with poly-siRNA for self-assembly. The poly-siRNA to tGC weight ratio ranged from 1:1, 1:5, to 1:10 for gel retardation assay on 8% PAGE. Poly-siRNA and psi/tGC were incubated in 10 mM 1,4-dithiothreitol (DTT) solution for 10 minutes for thiol group reduction. Optimized ratio of 1:10 of poly-siRNA to tGC was used for further experiments. The average particle size, polydispersity (μ^2/T^2), and surface charge density of tGC/psi formulation was measured using dynamic light scattering (DLS) (He-Ne Laser, Zetasizer Nano ZA, Malvern Instrument Ltd., Worcestershire, UK) The morphology of the particle was observed using transmission electron microscopy (CM 200 electron microscope, Philips, Eindhoven, Netherlands).

2.6. Scratch Migration Assay

OSE cells were seeded at 2×10^4 cells/well in 6-well culture dishes incubated for more than 24 hours. When the cell confluency reached approximately 90–100%, wounds were made with 1 ml pipette tip on monolayer of cells. The cells were then incubated in GDC and siKRAS containing serum-starved medium at 37°C for 10 hours. The images of scratch of PKO monolayer were obtained using inverted phase contrast microscope (Olympus, Tokyo,

Japan) at 0 hour and 24 hour. The migration was quantified compared to control groups using Image J software.

2.7. Mouse Tissue Histopathology

The subcutaneous tumors of OSE-KP were collected and fixed in paraformaldehyde in phosphate buffer saline. The tissues were sent to Dana-Farber/Harvard Cancer Center (DF/HCC) Histopathology Core Facility for hematoxylin & eosin (H&E) staining and histopathological evaluations.

2.8. Immunohistochemical Analysis

Immunohistochemical staining was performed on paraformaldehyde-fixed, paraffin-embedded sections according to the standard protocol. The sections were deparaffinized, followed by antigen retrieval in boiling sodium citrate solution. The tissue sections were then incubated overnight with the following primary antibodies: p-AKT, RAS, Ki67, and Cleaved Caspase 3. Biotinylated secondary antibodies, avidin-biotin complex solution, and 3,3'-diaminobenzidine were used to detect proteins of interest against Meyer's hematoxylin counterstaining.

2.9. Ovarian Allograft Transplantation Therapy Study.

All experiments with live animals were performed in compliance with the relevant laws of institutional guidelines of Korea Institute of Science and Technology (KIST). OSE-KP cell line was grown *in vitro* to about 90% confluency before trypsinization and cell counting. Total of 2×10^6 cells were mixed with matrigel in 1:1 ratio and injected subcutaneously onto flanks of Balb/c nude mice. Mice were closely monitored for tumor formation or any signs of illness. For *in vivo* drug studies, GDC-0941 was dissolved in distilled H₂O supplements with 2% DMSO, 30% PEG300, and 5% Tween 80. GDC-0941 was orally administered at 100 mg/kg (p.o.) once per day and siKRAS was intravenously administered at 1.5 mg/kg (i.v.) once every three days. Tumor volume was calculated using caliper with formula $(\text{length} \times \text{width}^2)/2$. The mice were sacrificed to harvest tumor tissues in formalin solution for histopathological evaluation.

2.10. Statistical Methods.

Two-tailed T-test (two groups) and two-way analysis of variance (ANOVA) tests (multiple groups) were used, and calculated with built-in analysis of Prism 6.0 (GraphPad). Data are represented as mean $\pm$ standard error mean (s.e.m.), otherwise noted. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns is not significant

3. Results and Discussion

3.1. Effect of GDC-0941 on OSE cells on Signaling and Viability

We first tested the effect of the pan-PI3K inhibitor, GDC-0941(GDC), which effectively inhibits all isoforms of the p110 subunit of Class IA PI3Ks in OSE cells *in vitro*. OSE cell lines were derived from tumors formed in ovaries of female mice harboring *LSL-KRAS*^{G12D/+} or both *LSL-KRAS*^{G12D/+} and *Pten*^{loxP/loxP} alleles as described in previous studies [29]. Both OSE-K and OSE-KP cell lines carry activated oncogenic *KRAS*^{G12D},

whereas only OSE-KP cells additionally have deleted both copies of the *PTEN* gene. These cell lines were chosen to accurately compare the molecular network under the influence of PTEN deletion. GDC was given to cells in varying dosages from 0 to 5 μ M to assess its effect on cell signaling via the PI3K pathway. In both OSE-K and OSE-KP cells, GDC significantly inhibited AKT phosphorylation in a dose dependent manner (Fig 2a). The ribosomal protein S6 is phosphorylated by p70S6 kinase acting downstream of PI3K. When GDC is present, ribosomal protein S6 activity was inhibited as is reflected in its phosphorylation level. FoxO1, a direct substrate of AKT, also shows lower phosphorylation as GDC concentration increases. The cell viability of OSE cells was measured via cell counting (CCK-8) over a five day of period in comparison to the fibroblast cell line, NIH3T3 (Fig 2b). The NIH3T3 cell line immediately reacted to GDC with 19% decrease in viability compared to OSE-K which showed an inhibition in cell viability of up to 39%. Interestingly, the OSE-KP cells showed less sensitivity to GDC over 6-day period with a minimal inhibition of viability (~3%). Activity of downstream proteins in the PI3K pathway and RAS pathway were assessed at longer incubation times with GDC (Fig 2c). Here, the overall expression of phosphorylated AKT, indicating activity of PI3K signaling, was more effectively inhibited in OSE-K harboring wild type PTEN over five-day period in comparison to consistent increase seen of OSE-KP cells. Interestingly, RAS expression showed 1.6-fold increase in OSE-KP cells upon GDC incubation over the six-day period. In addition, the relative mRNA levels of KRAS increased as GDC incubation time was increased in OSE-KP cells (Fig 2d). Here, we were able to observe over-expression of KRAS induced by inhibition of the PI3K pathway, possibly suggesting a feedback relationship with RAS Pathway. Compared to OSE-K, OSE-KP cells bearing PTEN deletion showed a recovery in their cell proliferation response to GDC-0941 from day 4, which might be explained by the increased mRNA level of KRAS observed 3 days after the initiation of the GDC treatment (Fig 2b and 2d). Here, we hypothesize that PTEN deficiency may be contributing to enhanced activation of the PI3K pathway that in turn leads to increased KRAS expression resulting in enhanced cell survival upon addition of GDC in ovarian cancer. Other cancer types may demonstrate alternative results regarding the KRAS activation in response to PI3K kinase inhibitors.

3.2. Characterization of psi/tGC and Cellular Uptake in OSE cells

In order to counteract the activation of the RAS pathway resulting from addition of a PI3K inhibitor, we designed an siRNA delivery complex targeting KRAS encapsulated into nanoparticles with thiolated glycol chitosan (tGC). The well-known lack of clinically acceptable inhibitors for RAS renders it a great target for siRNA that can regulate RAS activity at the level of mRNA expression. Our previous studies have demonstrated that poly-siRNAs exhibit enhanced biocompatibility and easier formation into nanoparticles with synthetic polymers and proteins, due to their increased molecular weight and stronger negative charge [27, 30–32]. Fig 3a and 3b show a general illustration of poly-siRNA synthesis and formation of the siRNA delivery complex, tGC/psi, using thiol-modified siRNA and glycol chitosan. The self-assembly of siRNA and glycol chitosan is initially induced via the electrical attraction of anionic siRNA with the cationic chitosan, thereby allowing exposed thiol groups to form disulfide bonds resulting in stable crosslinking. In order to verify synthesis of poly-siRNA or psi, we loaded psi with monomer siRNA (msi)

onto 8% polyacrylamide gel to verify crosslinking of the psi (Fig 3c). Self-polymerized siRNA resulted under mildly oxidative and basic conditions in HEPES buffer as was seen in the ladder-like band pattern of molecular weights representing various lengths of poly-siRNA. In previous studies, the intensity of gel bands was quantified and more than 70% of poly-siRNA were above 300 bp in length and the average anionic charge of the poly-siRNA was 12 times of that of psi [27]. Addition of the strong reducing agent DTT resulted in dissociation of the disulfide crosslinking of the poly-siRNA as shown in Fig 3c. Ellman's thiol assay measurement was used to assess the number of thiol bonds in the siRNA to further verify and quantify disulfide bonding of poly-siRNA in comparison to monomeric siRNA. As the amount of siRNA increased, the measured thiol bonds also increased in a proportional manner as shown in Supplemental Fig 1. Based on this thiol quantification, the number of exposed thiol groups on monomeric siRNA was 15-fold greater in total as compared to cross-linked poly-siRNA (Fig 3d). The gel electrophoresis and thiol assay results verify that the poly-siRNA was formed via disulfide-bond based chemical crosslinking capable of dissociation in a reducing environment such as the cytosol. To form a stable delivery complex for poly-siRNA, glycol chitosan (GC) was chosen as a biocompatible and biodegradable nanocarrier. The tumor-homing ability of GC nanoparticles has been previously demonstrated in tumor-bearing mouse models. The thiolated GC facilitates condensed particle formation with siRNA in addition to the electrostatic interaction, resulting in particle sizes of ~300 nm. Whereas, the unmodified GC with poly-siRNA leads to charge-charge interaction-based assembly only, resulting in particle size bigger than 500 nm which will cause liver retention [27]. Similar to poly-siRNA, tGC synthesis was confirmed using nuclear magnetic resonance spectroscopy (NMR) analysis in Supplemental Fig 2. After verification of thiol group modifications on poly-siRNA and tGC, varying amounts of tGC was self-assembled with poly-siRNA to determine an optimal ratio for *in vitro* and *in vivo* applications. Poly-siRNA was successfully encapsulated in tGC in all weight ratios tested: 1:1, 1:5, and 1:10, as measured by particles bound on PAGE wells. Disulfide bonds of poly-siRNA and psi/tGC were readily reduced back to thiol groups in monomeric siRNA, as shown in the single band at monomer size in presence of DTT (Fig 3e). When psi/tGC nanoparticles were synthesized with various amounts of tGC, the ratio of 1:10 psi/tGC was observed to have an optimal hydrodynamic diameter of around 300 nm [27]. The size distribution of psi/tGC measured by dynamic light scattering (DLS) and transmission electron microscope (TEM) image confirmed expected morphology and size of psi/tGC with average diameter of 232.58 ± 25 nm with polydispersity index (PDI) of 0.309 (Fig. 3f). Furthermore, dissociation of tGC/psi nanoparticle by DTT was confirmed by showing small-scale decomposed particle size and morphology to demonstrate that the tGC/psi was bound by a reversible thiol chemical linkage (Supplemental Fig 3).

The cellular uptake of psi/tGC was monitored in both OSE cell lines using confocal microscopy (Supplemental Fig 4). Although specific cellular uptake mechanism was not examined in OSE cells, glycol chitosan derivative nanoparticles have been proven to be taken up by cells via more than one mechanism. Using endocytic inhibitors such as chlorpromazine, filipin III and amiloride, glycol chitosan was found to internalize through clathrin-mediated and caveolae-mediated endocytosis along with other independent

pathways [33]. tGC was labeled with the near infrared fluorescence (NIRF) dye, FPR675, and poly-siRNA was labeled with the intercalating YOYO-1 dye. The fixed cells were stained with DAPI for nuclear visualization prior to confocal imaging. Here, we confirmed that fluorescently labeled psi/tGC as successfully taken up by cells to induce RNAi mediated effects on RAS.

3.3. siKRAS Inhibition and Signaling Influence in OSE cells

Polymerized siRNA that specifically targets both wild-type and mutated endogenous KRAS mRNA, abbreviated as siKRAS, was administered to OSE cell lines to test its specific inhibition of KRAS expression in comparison to control cells treated with scrambled siRNA. The RAS protein levels were down-regulated in presence of 0.1 and 0.2 μ M of siKRAS in serum-free media (Fig 4a). No more than 0.2 μ M siRNA were given to cells as more than 5 μ g siRNA highly increases pro-inflammatory IFN- α level, thus, inducing an immune response [34]. Relative mRNA levels of KRAS were also measured when cells were treated with siKRAS, as shown in Fig 4b. The real-time PCR data shows that a more than 30% reduction of KRAS mRNA occurred both in OSE-K and KP cell lines. The magnitude of down-regulation of KRAS gene expression in mRNA also correlates the concentration of siKRAS. After KRAS specific inhibition was confirmed, various proteins involved in the PI3K and RAS signaling pathways were immunoblotted to test for significant alterations in signaling due to siKRAS. Treatment with siKRAS largely reduced RAS expression and reduced levels of phosphorylated ERK1/2 in both OSE-K and KP cells, and also showed a significant downregulation PI3K pathway signaling including phosphorylation of both AKT and the S6 Ribosomal protein. Some expression and activity inhibition were observed at different concentration of siKRAS, such as S6 ribosomal protein phosphorylation was most inhibited by higher concentration of siKRAS and RAS expression showed greater reduction at lower siKRAS concentration. The discrepancy of RAS expression level from Fig 4a may be due to prolonged incubation time for siKRAS causing greater inhibition of KRAS in OSE-KP cells in Fig 4c. These reductions confirm that the RAS expression was successfully down-regulated with the expected influences on KRAS related pathways including PI3K (Fig 4c).

3.4. Dual Inhibition of PI3K and KRAS using GDC and siKRAS *in vitro*

Next, GDC was tested in combination with siKRAS as well as the MEK 1/2 inhibitor AZD 6244 (AZD). GDC was dosed in combination with siKRAS or AZD and cell viability was visualized via crystal violet staining (Supplemental Fig 5). For both OSE-K and OSE-KP cell lines, GDC used in combination with siKRAS showed a decrease in cell viability compared to either single drug. However, when MEK 1/2 was the target of inhibition instead of KRAS, the combined therapeutic effect was found to be negligible in comparison to GDC treatment alone. This indicates direct inhibition of RAS activity using siKRAS is critical in targeting the two cross-talking pathways. Here, GDC and siKRAS were simultaneously given to OSE cells, and both signaling and viability were measured. In Fig 5a, immunoblots of key downstream proteins in cells under treatment with GDC show reduction in PI3K signaling at the 0.5 and 1 μ M doses. When siKRAS is given in combination with GDC, the phosphorylation of AKT, p70S6 kinase and S6 ribosomal protein all show more complete inhibition compared to when GDC is used alone. Cell proliferation of OSE cells was

visualized and measured using crystal violet staining and CCK-8. As the concentration of GDC increases, cell proliferation is delayed regardless of status of PTEN. When siKRAS was given to cells in combination with GDC, cells proliferated at a much slower rate compared to cells treated with GDC alone (Fig 5b). Throughout the 7-day period, OSE cell viability was quantified via colorimetric assay in presence of GDC or siKRAS alone or in combination as shown in Fig 5c. GDC was given to cells daily, and the arrows indicate the days when cells were treated with siKRAS in serum-free media. Both OSE-K and OSE-KP presented similar results showing the lowest cell viability when GDC and siKRAS were given together. In addition to cell proliferation, cell migration of OSE cells was tested using a scratch wound migration assay. The cells were grown to around 90–100% confluency and then treated with GDC and or siKRAS overnight. Micropipette tips were used to create a gap on the cellular monolayer and the migration of cells was observed and measured after 24 hours (Fig. 5d and 5e). In presence of siKRAS alone, the gap area of wound was re-filled by the migration of cells by 82% and 70% in OSE-K and KP cells, respectively, indicating that the siKRAS has negligible effect on the migration ability of OSE cell lines. However, when GDC was given to both cells, the migration decreased by about 25% in both OSE cells. Interestingly, GDC and siKRAS were given together to OSE cells and migration was inhibited to below 45% compared to when siKRAS and GDC were given alone. This indicates that usage of GDC in combination with siKRAS further induced anti-proliferation effect rather than single agent treatment approach.

3.5. Combination treatment using GDC and siKRAS in the OSE-KP allograft mouse model

In previous studies glycol chitosan derivative nanoparticles, or CNPs, have shown sufficient stability to maintain nanoparticle structure during blood flow and circulation through narrow sinuses in the spleen. During circulation, CNPs of suitable particle size passively accumulate in tumor site because of leaky vasculature in the tumor environment, an effect also known as the enhanced permeation and retention (EPR) effect [28]. In addition, innate protonation of amine groups of CNPs in the acidic tumoral environment promotes fast binding and uptake in tumor tissue [35]. Thus, glycol chitosan serves as an exceptional delivery tool for drug agents compared to other nanoparticles. In this experiment, we generated siKRAS, a glycol chitosan delivery system for siRNA to target KRAS, to use in collaboration with a PI3K inhibitor to test the anti-tumor effect of the combination. Ideally, GDC and siKRAS might be combined in one formulation; however, the hydrophilic tGC/psi and the hydrophobic kinase inhibitor mixture adversely alters the chemical formulation and pharmacokinetics of the delivery system. Here, the combination of siKRAS in tGC/psi and GDC were given to allograft mouse models generated with OSE-KP tumors. OSE-KP cells increase expression their activated allele of KRAS under the influence of GDC, due to an apparent feedback loop in the PI3K pathway. For this reason, an allograft mouse model of OSE-KP was chosen for *in vivo* experiments. A mixture of OSE-KP cells and matrigel was injected into the flanks of Balb/c nude mice to generate ovarian tumor allografts. The mice carrying these tumors were divided into four groups—a control group and groups treated with GDC, siKRAS, and GDC + siKRAS. All groups were started on the treatment regimen with GDC and siRNA once the tumors reached a size of 40 to 50 mm³. The group that received both GDC and siKRAS showed significantly decreased tumor growth compared to either the control group or those treated with GDC and siKRAS alone (Fig 6a). When GDC and

siKRAS were administered as single agents, tumor growth was still quite significantly reduced compared to the control group. After 30 days of monitoring tumor sizes, tumors were harvested for detailed examination. Tumors were taken for immunoblotting and histology experiments to seek a mechanistic explanation the animal results. Fig 6c shows the protein expression in individual tumors of all experimental groups. As expected, mice administered with GDC everyday were observed to have reduced PI3K activity as measured by phosphorylated AKT levels (Fig 6c). Phosphorylation of another downstream target protein, the S6 ribosomal protein, was also reduced by GDC. Compared to the control samples, treatment with siKRAS mildly reduced phosphorylation of S6 ribosomal protein, which indicates interdependent relationship between RAS and PI3k pathway. RAS expression was also visualized to confirm specific down its regulation by siKRAS. Interestingly, GDC alone reduced RAS expression to an extent similar to what was seen when siKRAS was given. However, when both GDC and siKRAS were given to the animals, tumors showed nearly complete loss of RAS expression. Cleaved poly ADP ribose polymerase (PARP) and cleaved caspase 3 are biological markers that indicate apoptotic tumor cell death. Both apoptotic markers are highly expressed in tumors of the GDC and siKRAS combination treatment groups and interplay of MAPK and PI3K has been shown to induce apoptosis in published reports [36]. Similar to the *in vivo* results, a previous study demonstrated that cancer patients bearing KRAS and PI3K mutations showed BIM-induced apoptosis when treated with an PI3K/mTOR dual-inhibitor [21]. Also, Cho's group has shown that ERK activation may be associated with apoptosis and negatively regulated by AKT signaling, which signifies the interplay occurring between the AKT signaling and MAPK activation in apoptosis [37]. Also another group demonstrated that apoptosis was induced by JNK/MAPK pathway and PI3K Akt pathway via regulation of Bax/Bcl-2/ caspase-3 activation [38]. Histological immunohistochemistry (IHC) was used to further assess PI3K and RAS signaling along with cell proliferation and apoptosis markers (Fig 6d). Phosphorylation levels of AKT were visualized to measure GDC inhibitor action in tumors. As expected, GDC and GDC + siKRAS groups showed reduction in phosphorylated AKT level in tissue sections. RAS showed higher expression in groups that did not receive siKRAS. The Ki-67 antigen is a common indicator of cell proliferation. In presence of both GDC and siKRAS, this cell proliferation marker is noticeably reduced compared to all other groups. In addition, the apoptosis marker cleaved caspase 3 is more prevalent in tumors treated with both GDC and siKRAS, parallel to the immunoblotting results. Furthermore, the heart, kidney, liver, lung, and spleen organs were harvested from ovarian cancer models treated with siKRAS and low and high dose of GDC to evaluate acute toxicities and biomarkers indicating liver function. In supplemental Fig 6a, the H&E staining of five organs are shown to demonstrate acute toxicities. Here, the liver section shows moderate to severe vascular and sinusoidal congestion in comparison to that of control and siKRAS group. In addition, markers for liver toxicity—Aspartate transaminase (AST), Alanine transaminase (ALT), and Alkaline phosphatase (ALP)—were quantified in serum level (Supplemental Fig 6b). The acute toxicity of liver increased as given amount of GDC increased, thereby supporting the usage of poly-siRNA as an alternative therapeutic agent. Notably the *in vivo* results demonstrate a more highly encouraging therapeutic effect in comparison to cellular experiments.

4. Conclusions

In summary, our results demonstrated the efficacy of combined inhibition of PI3K and RAS in *KRAS* mutated ovarian cancer cell lines and mouse models. As a potential strategy to overcome discouraging clinical outcome of kinase inhibitors, combination therapy targeting compensating pathways has the potential to revitalize therapeutic studies of kinase inhibitors. Single inhibitors, including the PI3K inhibitor used here, alone have been ineffective on RAS transformed cells and mutations in RAS have been found as an acquired resistance mechanism in preclinical studies [20]. In order to overcome such resistance, other studies have co-inhibited PI3K and RAS pathways to mitigate feedback mechanisms allowing cell proliferation and survival. Thus, a combination of PI3K and MEK inhibitors has been tested and various early-phase clinical trials are under investigation; However manageable toxicity, safety profiles and response rates of such combinations remain as challenges [39]. Due to lack of specific RAS inhibitors and resistance and side-effects from multi-drug use, we synthesized a polymerized siRNA delivery system targeting *KRAS* to use in combination with the pan-PI3K inhibitor GDC. Use of this GDC in *KRAS* mutated ovarian cancer cells resulted in a feedback loop enhancing the RAS expression as shown in our *in vitro* results. However, we found that simultaneous treatment with GDC and si*KRAS* results in synergistic effect of PI3K and RAS pathways in cells and animal models. The dual-inhibition of both pathways impeded cell proliferation and migration and induced apoptosis in animal model. The combination therapy showed more encouraging result in animal models, as siRNAs were markedly more effective *in vivo* than *in vitro* according to previous studies [27, 35, 40]. Thus, combination therapy using targeted therapies such as PI3K inhibitors and siRNA therapeutics may represent a promising strategy for tumors that rapidly develop resistance to targeted therapies alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- [1]. Vivanco I, Sawyers CL, The phosphatidylinositol 3-Kinase AKT pathway in human cancer, *Nat Rev Cancer*, 2 (2002) 489–501. [PubMed: 12094235]
- [2]. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, Maira M, McNamara K, Perera SA, Song Y, Chirieac LR, Kaur R, Lightbown A, Simendinger J, Li T, Padera RF, Garcia-Echeverria C, Weissleder R, Mahmood U, Cantley LC, Wong KK, Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers, *Nat Med*, 14 (2008) 1351–1356. [PubMed: 19029981]
- [3]. Janku F, Yap TA, Meric-Bernstam F, Targeting the PI3K pathway in cancer: are we making headway?, *Nat Rev Clin Oncol*, 15 (2018) 273–291. [PubMed: 29508857]
- [4]. Caumanns JJ, van Wijngaarden A, Kol A, Meersma GJ, Jalving M, Bernards R, van der Zee AGJ, Wisman GBA, de Jong S, Low-dose triple drug combination targeting the PI3K/AKT/mTOR

pathway and the MAPK pathway is an effective approach in ovarian clear cell carcinoma, *Cancer Lett*, 461 (2019) 102–111. [PubMed: 31319139]

- [5]. Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X, Targeting PI3K in cancer: mechanisms and advances in clinical trials, *Mol Cancer*, 18 (2019) 26. [PubMed: 30782187]
- [6]. Leong S, Moss RA, Bowles DW, Ware JA, Zhou J, Spoerke JM, Lackner MR, Shankar G, Schutzman JL, van der Noll R, Voest EE, Schellens JHM, A Phase I Dose-Escalation Study of the Safety and Pharmacokinetics of Pictilisib in Combination with Erlotinib in Patients with Advanced Solid Tumors, *Oncologist*, 22 (2017) 1491–1499. [PubMed: 28798270]
- [7]. Park YL, Kim HP, Cho YW, Min DW, Cheon SK, Lim YJ, Song SH, Kim SJ, Han SW, Park KJ, Kim TY, Activation of WNT/beta-catenin signaling results in resistance to a dual PI3K/mTOR inhibitor in colorectal cancer cells harboring PIK3CA mutations, *Int J Cancer*, 144 (2019) 389–401. [PubMed: 29978469]
- [8]. Zhao JJ, Cheng H, Jia S, Wang L, Gjoerup OV, Mikami A, Roberts TM, The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation, *Proc Natl Acad Sci U S A*, 103 (2006) 16296–16300. [PubMed: 17060635]
- [9]. Mazumdar T, Byers LA, Ng PK, Mills GB, Peng S, Diao L, Fan YH, Stemke-Hale K, Heymach JV, Myers JN, Glisson BS, Johnson FM, A comprehensive evaluation of biomarkers predictive of response to PI3K inhibitors and of resistance mechanisms in head and neck squamous cell carcinoma, *Mol Cancer Ther*, 13 (2014) 2738–2750. [PubMed: 25193510]
- [10]. Aksamitiene E, Kiyatkin A, Kholodenko BN, Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: a fine balance, *Biochem. Soc. Trans*, 40 (2012) 139–146. [PubMed: 22260680]
- [11]. Shaw RJ, Cantley LC, Ras PI(3)K and mTOR signalling controls tumour cell growth, *Nature*, 441 (2006) 424–430. [PubMed: 16724053]
- [12]. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, Majumder PK, Baselga J, Rosen N, AKT Inhibition Relieves Feedback Suppression of Receptor Tyrosine Kinase Expression and Activity, *Cancer Cell*, 19 (2011) 58–71. [PubMed: 21215704]
- [13]. Mendoza MC, Er EE, Blenis J, The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and Compensation, *Trends Biochem Sci*, 36 (2012) 320–328.
- [14]. Castellano E, Downward J, RAS Interaction with PI3K: More Than Just Another Effector Pathway, *Genes & Cancer*, 2 (2011) 261–274. [PubMed: 21779497]
- [15]. Wee S, Jagani Z, Kay XX, Loo A, Dorsch M, Yao YM, Sellers WR, Lengauer C, Stegmeier F, PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers, *Cancer Research*, 69 (2009) 4286–4293. [PubMed: 19401449]
- [16]. Gritsman K, Yuzugullu H, Von T, Yan H, Clayton L, Fritsch C, Maira SM, Hollingworth G, Choi C, Khandan T, Paktinat M, Okabe RO, Roberts TM, Zhao JJ, Hematopoiesis and RAS-driven myeloid leukemia differentially require PI3K isoform p110alpha, *J Clin Invest*, 124 (2014) 1794–1809. [PubMed: 24569456]
- [17]. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, Nye E, Stamp G, Alitalo K, Downward J, Binding of Ras to Phosphoinositide 3-Kinase p110a Is Required for Ras-Driven Tumorigenesis in Mice, *Cell*, 129 (2007) 957–968. [PubMed: 17540175]
- [18]. Ischenko I, Petrenko O, Hayman MJ, A MEK/PI3K/HDAC inhibitor combination therapy for KRAS mutant pancreatic cancer cells, *Oncotarget*, 6 (2015) 15814–15827. [PubMed: 26158412]
- [19]. Ebi H, Costa C, Faber AC, Nishtala M, Kotani H, Juric D, Della Pelle P, Song Y, Yano S, Mino-Kenudson M, Benes CH, Engelman JA, PI3K regulates MEK/ERK signaling in breast cancer via the Rac-GEF, P-Rex1, *Proc Natl Acad Sci U S A*, 110 (2013) 21124–21129. [PubMed: 24327733]
- [20]. Greger JG, Eastman SD, Zhang V, Bleam MR, Hughes AM, Smitheman KN, Dickerson SH, Laquerre SG, Liu L, Gilmer TM, Combinations of BRAF MEK, and PI3K/mTOR inhibitors overcome acquired resistance to the BRAF inhibitor GSK2118436 dabrafenib, mediated by NRAS or MEK mutations, *Mol Cancer Ther*, 11 (2012) 909–920. [PubMed: 22389471]
- [21]. Kim A, Lee JE, Lee SS, Kim C, Lee SJ, Jang WS, Park S, Coexistent mutations of KRAS and PIK3CA affect the efficacy of NVP-BEZ235, a dual PI3K/MTOR inhibitor, in regulating the

- PI3K/MTOR pathway in colorectal cancer, *Int J Cancer*, 133 (2013) 984–996. [PubMed: 23475782]
- [22]. Pons-Tostivint E, Thibault B, Guillermet-Guibert J, Targeting PI3K Signaling in Combination Cancer Therapy, *Trends Cancer*, 3 (2017) 454–469. [PubMed: 28718419]
- [23]. McNeill RS, Canoutas DA, Stuhlmiller TJ, Dhruv HD, Irvin DM, Bash RE, Angus SP, Herring LE, Simon JM, Skinner KR, Limas JC, Chen X, Schmid RS, Siegel MB, Van Swearingen AED, Hadler MJ, Sulman EP, Sarkaria JN, Anders CK, Graves LM, Berens ME, Johnson GL, Miller CR, Combination therapy with potent PI3K and MAPK inhibitors overcomes adaptive kinome resistance to single agents in preclinical models of glioblastoma, *Neuro Oncol*, 19 (2017) 1469–1480. [PubMed: 28379424]
- [24]. Ryther RC, Flynt AS, Phillips JA 3rd, Patton JG, siRNA therapeutics: big potential from small RNAs, *Gene Ther*, 12 (2005) 5–11. [PubMed: 15496962]
- [25]. Chakraborty C, Sharma AR, Sharma G, Doss CGP, Lee SS, Therapeutic miRNA and siRNA: Moving from Bench to Clinic as Next Generation Medicine, *Mol Ther Nucleic Acids*, 8 (2017) 132–143. [PubMed: 28918016]
- [26]. Shajari N, Mansoori B, Davudian S, Mohammadi A, Baradaran B, Overcoming the Challenges of siRNA Delivery: Nanoparticle Strategies, *Curr Drug Deliv*, 14 (2017) 36–46. [PubMed: 27538460]
- [27]. Lee SJ, Huh MS, Lee SY, Min S, Lee S, Koo H, Chu JU, Lee KE, Jeon H, Choi Y, Choi K, Byun Y, Jeong SY, Park K, Kim K, Kwon IC, Tumor-homing poly-siRNA/glycol chitosan self-cross-linked nanoparticles for systemic siRNA delivery in cancer treatment, *Angew Chem Int Ed Engl*, 51 (2012) 7203–7207. [PubMed: 22696263]
- [28]. Yoon HY, Son S, Lee SJ, You DG, Yhee JY, Park JH, Swierczewska M, Lee S, Kwon IC, Kim SH, Kim K, Pomper MG, Glycol chitosan nanoparticles as specialized cancer therapeutic vehicles: sequential delivery of doxorubicin and Bcl-2 siRNA, *Sci Rep*, 4 (2014) 6878. [PubMed: 25363213]
- [29]. Schmit F, Utermark T, Zhang S, Wang Q, Von T, Roberts TM, Zhao JJ, PI3K isoform dependence of PTEN-deficient tumors can be altered by the genetic context, *Proc Natl Acad Sci U S A*, 111 (2014) 6395–6400. [PubMed: 24737887]
- [30]. Lee SY, Ryu JH, Park K, Lee A, Lee SY, Youn IC, Ahn CH, Yoon SM, Myung SJ, Moon DH, Chen X, Choi K, Kwon IC, Kim K, Polymeric Nanoparticle-Based Activatable Near-Infrared Nanosensor for Protease Determination In Vivo, *Nano Letters*, 9 (2009) 4412–4416. [PubMed: 19842672]
- [31]. Lee SJ, Yook S, Yhee JY, Yoon HY, Kim MG, Ku SH, Kim SH, Park JH, Jeong JH, Kwon IC, Lee S, Lee H, Kim K, Co-delivery of VEGF and Bcl-2 dual-targeted siRNA polymer using a single nanoparticle for synergistic anti-cancer effects in vivo, *J Control Release*, 220 (2015) 631–641. [PubMed: 26307351]
- [32]. Lee SY, Huh MS, Lee S, Lee SJ, Chung H, Park JH, Oh YK, Choi K, Kim K, Kwon IC, Stability and cellular uptake of polymerized siRNA (poly-siRNA)/polyethylenimine (PEI) complexes for efficient gene silencing, *J Control Release*, 141 (2010) 339–346. [PubMed: 19836427]
- [33]. Nam HY, Kwon SM, Chung H, Lee SY, Kwon SH, Jeon H, Kim Y, Park JH, Kim J, Her S, Oh YK, Kwon IC, Kim K, Jeong SY, Cellular uptake mechanism and intracellular fate of hydrophobically modified glycol chitosan nanoparticles, *J Control Release*, 135 (2009) 259–267. [PubMed: 19331853]
- [34]. Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, *Nat Biotechnol*, 23 (2005) 457–462. [PubMed: 15778705]
- [35]. Na JH, Koo H, Lee S, Han SJ, Lee KE, Kim S, Lee H, Lee S, Choi K, Kwon IC, Kim K, Precise Targeting of Liver Tumor Using Glycol Chitosan Nanoparticles: Mechanisms, Key Factors, and Their Implications, *Mol Pharm*, 13 (2016) 3700–3711. [PubMed: 27654060]
- [36]. Gire V, Marshall C, Wynford-Thomas D, PI-3-kinase is an essential anti-apoptotic effector in the proliferative response of primary human epithelial cells to mutant RAS, *Oncogene*, 19 (2000) 2269–2276. [PubMed: 10822377]

- [37]. Lee ER, Kim JY, Kang YJ, Ahn JY, Kim JH, Kim BW, Choi HY, Jeong MY, Cho SG, Interplay between PI3K/Akt and MAPK signaling pathways in DNA-damaging drug-induced apoptosis, *Biochim Biophys Acta*, 1763 (2006) 958–968. [PubMed: 16905201]
- [38]. Song F, Wang Y, Jiang D, Wang T, Zhang Y, Ma H, Kang Y, Cyclic Compressive Stress Regulates Apoptosis in Rat Osteoblasts: Involvement of PI3K/Akt and JNK MAPK Signaling Pathways, *PLoS One*, 11 (2016) e0165845.
- [39]. Jokinen E, Koivunen JP, MEK and PI3K inhibition in solid tumors: rationale and evidence to date, *Therapeutic Advances in Medical Oncology Ther Adv Med Oncol*, 7 (2015) 170–180.
- [40]. Garcia-Fuentes M, Alonso MJ, Chitosan-based drug nanocarriers: where do we stand?, *J Control Release*, 161 (2012) 496–504. [PubMed: 22480607]

Fig 1. Signaling cascade of PI3K and RAS.

Schematic illustration of the KRAS and PI3K pathways in presence of siRNA targeting KRAS, siKRAS, and a PI3K inhibitor, GDC-0941. Normal cells display well modulated signaling on MAPK and PI3K signaling pathways, whereas cancer cells with KRAS and PTEN mutation display constitutive activation of MAPK and PI3K pathways resulting in tumor growth. Combination therapy targeting KRAS and PI3K additively inhibits both signaling cascades, thus blocking a critical feedback loop, and inhibits tumor growth.

Fig 2. In vitro single-treatment of GDC-0941 on OSE cells.

Effect of pan-PI3K inhibitor, GDC-0941, on KRAS mutant ovarian cancer cells. OSE-K carries KRAS mutation alone and OSE-KP carries additional PTEN deficiency. (A) Immunoblot analysis of downstream proteins of PI3K pathway in presence of inhibitor. (B) Cell viability of ovarian cancer cells and NIH3T3 fibroblast cell measured by CCK-8 kit. (C) Immunoblot analysis of downstream proteins of PI3K and RAS pathway after one, two, and five days after addition of GDC. The protein expression level is shown below Ras bands. (D) Relative messenger RNA level of *KRAS* gene measured via real-time PCR in presence of PI3K inhibitor. The data points are not significant compared to the control. The values under the immunoblot indicates quantitative value measured using ImageJ.

Fig. 3. Characterization of poly-siRNA and tGC/psi nanoparticle.

Schematic illustration of (A) monomeric siRNA synthesizing to polymerized siRNA (psi) under oxidative stimulus via disulfide crosslinking. (B) Psi and thiolated glycol chitosan (tGC) self-assemble to psi/tGC nanoparticle delivery complex via initial electrostatic interaction and following intermolecular crosslinking of thiol residues. (C) Polyacrylamide gel electrophoresis (PAGE) analysis monomeric siRNA (msi) and psi in comparison to low molecular weight DNA ladder. A strong reducing agent, dithiothreitol or DTT, was added to poly-siRNA for disulfide bond crosslinking verification. (D) Ellman's reagent measured the

degree of thiolation of poly-siRNA, or psi, compared to msi. (E) PAGE analysis of psi/tGC formation in varying weight ratios. DTT was added to 1:10 ratio psi/tGC nanoparticle to ensure breakage of disulfide crosslinking. (F) Particle size distribution and morphology of psi/tGC nanoparticle measured by dynamic light scattering (DLS) and transmission electron microscopy (TEM), respectively. Scale bar = 200 μm .

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Fig. 4. Gene expression of OSE cells with single treatment siKRAS.

(A) Immunoblot analysis RAS expression to test siKRAS target and gene down-regulation ability. (B) Relative KRAS messenger RNA level measured by Real-time PCR. The error bars indicate standard deviation (S.D.) of the values. (C) Immunoblot analysis of downstream proteins of the PI3K and RAS pathway in presence of siKRAS. Amount of siKRAS for 1× is 100 nM and 2× is 200 nM. Control cells were treated with 200 nM of universal scrambled siRNA as control. The values under the immunoblot indicates quantitative value measured using ImageJ.

Fig. 5. GDC and siKRAS dual-treatment on OSE cells

(A) Western blot analysis of related proteins of PI3K pathway in OSE-K and KP cells in presence of both siKRAS (200 nM) and GDC in two different doses. (B) Crystal Violet staining of OSE cells in presence of GDC in difference concentration. (C) Cell viability of OSE-K and KP cells using CCK-8 kit. (D&E) Scratch wound migration assay and quantified measure to test migration of OSE cells in 24 hours.

Fig. 6. siKRAS and GDC treatment on allograft ovarian cancer model.

(A) Measurement and images of ovarian tumor sizes of control, GDC-0941, siKRAS, and GDC and siKRAS co-treated groups. Each group sample size is 4. GDC-0941 was administered 100 mg/kg orally (p.o.) once daily and siKRAS was given 1.5 mg/kg intravenously (i.v.) via tail vein every 3 days. (B) Tumor specific growth rate calculated from monitored tumor sizes of all groups of (A). (C) Immunoblot analysis of proteins harvested from tumors from (A) using indicating antibodies. (D) Immunohistochemistry (IHC) analysis of tumors harvested from each group to measure pAKT, RAS, Ki67, and cleaved caspase 3. Scale bar = 200 μ m.