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Suppress KRAS oncogenic activity
Sensitize cancer cell to Chemoradiotherapy
Enrichment of nanoparticle in tumors
Suppress tumor growth
Targeting Oncogenic KRAS in Non-small Cell Lung Cancer with EGFR Aptamer-conjugated Multifunctional RNA Nanoparticles

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Short title: Silencing KRAS with mutation-targeting RNA nanoparticles
Abstract:

*KRAS* mutations are one of the most common oncogenic driver mutations in human cancers, including non-small cell lung cancer (NSCLC), and have established roles in cancer pathogenesis and therapeutic resistance. The development of effective inhibitors of mutant *KRAS* represents a significant challenge. Three-way junction (3WJ)-based multi-functional RNA nanoparticles have the potential to serve as an effective *in vivo* siRNA delivery platform with the ability to enhance tumor targeting specificity and visualize biodistribution through an imaging moiety. Herein, we assembled novel EGFR$_{\mathrm{apt}}$-3WJ-si*KRAS$^{G12C}$* mutation targeted nanoparticles to target EGFR-expressing human NSCLC harboring a *KRAS$^{G12C}$* mutation to silence *KRAS$^{G12C}$* expression in a tumor cell-specific fashion. We found that EGFR$_{\mathrm{apt}}$-3WJ-si*KRAS$^{G12C}$* nanoparticles potently depleted cellular *KRAS$^{G12C}$* expression, resulting in attenuation of downstream MAPK pathway signaling, cell proliferation, migration/invasion ability, and sensitized NSCLC cells to chemoradiotherapy. *In vivo*, these nanoparticles induced tumor growth inhibition in *KRAS$^{G12C}$* NSCLC tumor xenografts. Together, this study suggests that the 3WJ pRNA-based platform has the potential to suppress mutant KRAS activity for the treatment of KRAS-driven human cancers, and warrants further development for clinical translation.
**Introduction**

Kirsten rat sarcoma viral oncogene homolog (\textit{KRAS}) is a member of the human Ras gene family, encoding a small GTPase membrane-bound protein. \textit{KRAS} functions as a binary molecular switch, cycling between an GDP-bound inactive and GTP bound active state. \textit{KRAS} mutations are wide-spread in human cancers, especially in three of the most lethal cancers: lung cancer, colorectal cancer and pancreatic cancer.\textsuperscript{1} Missense mutations of \textit{KRAS}, most commonly at codons 12 and 13, aberrantly attenuate GTPase activity, resulting in accumulation of GTP-bound activated \textit{KRAS}. The constitutively activated \textit{KRAS} oncoproteins initiate downstream cellular signal transduction cascades irrespective of input from extracellular signals including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, leading to uncontrolled cell proliferation and abnormal cell survival, which are hallmarks of cancer.\textsuperscript{2-5} Lung cancer is the leading cause of cancer-related deaths worldwide, with NSCLC accounting for 84\% of all lung cancer diagnoses.\textsuperscript{6} \textit{KRAS} mutations occur in 20-25\% of NSCLC (most commonly in adenocarcinoma subtype), with \textit{KRAS}\textsuperscript{G12C} mutation being the most frequent mutation variant, accounting for approximately 39\% of all \textit{KRAS} mutations in NSCLC.\textsuperscript{7-10} A large body of research has reported that the presence of \textit{KRAS} mutations promotes tumor cell-autonomous-mediated resistance to cancer therapeutics. For instance, cetuximab and panitumumab (monoclonal antibodies to EGFR) and EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, are ineffective in \textit{KRAS} mutant colorectal cancer and NSCLC, respectively, restricting their use to \textit{KRAS}-wildtype colorectal cancer and EGFR-mutant NSCLC.\textsuperscript{11-17} While erlotinib is approved in combination with gemcitabine for pancreatic adenocarcinoma treatment, the combination produces minimal benefit in
outcomes (only 2 weeks improved survival), likely due to the presence of KRAS mutations in 90-95% of tumors leading to constitutive signaling downstream of the point of inhibition at EGFR. In radiotherapy, a preclinical study in 1988 first documented that hyperactivation of Ras isoforms (including KRAS) leads to tumor-intrinsic radioresistance. Emerging clinical evidence supports that KRAS mutations are associated with radiation resistance in colorectal and lung cancer. Our recent studies and that of others have detailed some of the molecular mechanisms involved in KRAS activation promoting radiation resistance in colorectal and lung cancer.

Due to the high prevalence and importance of KRAS mutations in human cancer, pharmaceutical companies and academic laboratories have tried for decades to identify small molecule inhibitors of KRAS mutation, but little progress had been achieved and many had labeled KRAS as “undruggable”. However, beginning in 2013, Shokat and colleagues reported the identification of covalent small molecule compounds to target the reactive cysteine-12 of KRAS<sub>G12C</sub>. Based on this finding, Janes et al. developed a covalent KRAS<sub>G12C</sub> inhibitor ARS-1620 which could induce NSCLC tumor regression in in vivo models. Furthermore, Canon et al. expanded on the success of ARS-1620 to develop the compound named AMG 510, which is the first molecule to enter the clinic (NCT03600883) and ultimately receive FDA approval for the treatment of KRAS<sub>G12C</sub> tumors. Recently, several other KRAS<sub>G12C</sub> covalent inhibitors are also in phase I/II clinical trials, such as MRTX849, ARS-3248, and LY3499446 (NCT03785249, NCT04006301, NCT04165031). As these covalent inhibitors require KRAS<sub>G12C</sub> to be in the GDP bound state, drug resistance could be induced by disabling the GTPase activity or promoting the guanine exchange of GDP for GTP. Currently, small molecular inhibitors that can target
the plethora of other \textit{KRAS} mutations (e.g. G12D, G12V, G12R, G13D, etc.) are either
under-studied are in preclinical development.

The field of RNA nanotechnology has advanced rapidly during recent decades. It was first
introduced in 1998, and the field encompasses the design, fabrication, and application of
nanometer scale RNA architectures.\textsuperscript{28,29} RNA nanoparticles have the simplistic
characteristic of DNA canonical base pairing, while containing the structural flexibility and
functional diversity characteristics of proteins. Noncanonical base pairing, base stacking,
and elaborate networks of tertiary contacts further expand RNA structure versatility while
also increasing thermodynamic stability.\textsuperscript{29} Yet the dynamic nature of RNA allows RNA
nanoparticles to remain deformative and motile to lead to high tumor accumulation and
rapid renal clearance for maximized therapeutic dose delivery and reduced toxicities.\textsuperscript{30,31}

Three-way junction (3WJ) packaging RNA (pRNA) is a novel type of RNA nanotechnology derived from the packaging RNA of the bacteriophage phi29 DNA packaging motor. 3WJ pRNA has been extensively studied to fabricate various RNA nanoparticles with precise control of shape, size, and stoichiometry. The extending arms of 3WJ pRNA structures could be intelligently replaced with siRNAs, miRNAs, riboswitches, and RNA aptamers, and conjugated with fluorescent probes or other moieties to construct multi-functional pRNA nanoparticles.\textsuperscript{32} Due to their great plasticity and stability, 3WJ pRNA nanoparticles are emerging as a highly desirable \textit{in vivo} delivery system for targeted gene therapy in human cancers.\textsuperscript{28,29,32}

In the current study, we report the development of a multifunctional RNA nanoparticle,
\textit{EGFR}_{apt}-3WJ-Alexa647-si\textit{KRAS}^{G12C}, to explore the potential of 3WJ pRNA nanoparticles
to deliver siRNA targeting \textit{KRAS}^{G12C} into NSCLC cells. Overexpression of EGFR has been
reported in many human cancers, including NSCLC.\textsuperscript{33} Thus, we have engineered EGFR targeting RNA aptamers (EGFR\textsubscript{apt}) to further enhance the tumor-specificity of the technology. We find that our EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} nanoparticles exhibit high thermostability and demonstrate high efficiency to specifically silence KRAS\textsuperscript{G12C} and suppress downstream signaling pathways. Importantly, these nanoparticles sensitized NSCLC cells to chemotherapy and radiation therapy, inhibited proliferation, migration, invasion, and suppressed tumor growth by systemic delivery in a heterotopic mouse model. This platform technology has the potential to be adapted to other RAS mutations, enabling high tumor specificity.

**Results**

**Construction and Characterization of EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} Nanoparticles.**

Utilizing the three-way junction of Phi29 pRNA (3WJ pRNA) as the core scaffold, multifunctional EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} pRNA nanoparticles were constructed, harboring EGFR-targeting RNA aptamer, therapeutic KRAS\textsuperscript{G12C} siRNA, and Alexa-647 as the imaging module (Figure 1A). When the four nanoparticle component strands were mixed in equal molar ratio in TMS buffer, the pRNA nanoparticles assembled with very high efficiency as indicated by agarose gel shift assays, showing stepwise assembly of the pRNA nanoparticle (Figure 1B). Dynamic light scattering (DLS) analysis determined the diameter of the EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} RNPs as 7.2 ± 0.5 nm (Figure 1C), compared to 4.2 ± 1.1 nm for the 3WJ pRNA core scaffold\textsuperscript{34}. To assess thermodynamic parameters, the Alexa647 labeled EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} RNPs were subjected to temperature gradient gel electrophoresis (TGGE). Alexa647 labeled strands were used to determine the percentage of remaining intact nanoparticles as temperature gradient increased from 25°C
to 80°C. We determined the Tm of EGFR$_{\text{apt}}$-3WJ-siKRA$_{G12C}$ nanoparticles as 51.77°C (Figure 1D), indicating that the constructed RNPs with all functional modules are thermostable.

**EGFR$_{\text{apt}}$-3WJ-siKRA$_{G12C}$ Nanoparticles Efficiently Bind and Deliver KRA$_{G12C}$ siRNA to NSCLC cells.**

We next examined the EGFR-targeting capabilities of Alexa647-conjugated EGFR$_{\text{apt}}$-3WJ-siKRA$_{G12C}$ nanoparticles in KRA$_{G12C}$ NSCLC cell lines H2030 and H2122. Flow cytometric analysis illustrated a significantly higher level of target cell association using the EGFR$_{\text{apt}}$-3WJ- siKRA$_{G12C}$ nanoparticles as compared to 3WJ- siKRA$_{G12C}$ nanoparticles and scrambled aptamer conjugated pRNA nanoparticles (Figures 2A, S1A). To further confirm whether the observed improvement in pRNA nanoparticle uptake is dependent on EGFR aptamer, we evaluated the binding ability of pRNA nanoparticles to NSCLC after silencing EGFR expression by siRNA. We found that compared to the non-targeting silencing control, the EGFR silencing induced by siRNA (confirmed in Figure S1B) reduced the binding ability of EGFR$_{\text{apt}}$-3WJ- siKRA$_{G12C}$ nanoparticles by 30% and 38%, in H2030 and H2122 cell lines respectively (Figures 2B, S1C). Interestingly, EGFR depletion also slightly reduced binding of SCR$_{\text{apt}}$-3WJ- siKRA$_{G12C}$ nanoparticles by 20% and 15% in H2030 and H2122 cell lines respectively, perhaps indicating that there are other non-EGFR-dependent properties of the aptamers that confer increased binding to these NSCLC cell lines.

We next examined the ability of EGFR$_{\text{apt}}$-3WJ-siKRA$_{G12C}$ nanoparticles to silence the expression of KRA$_{G12C}$ in H2030 and H2122 cells, and used KRA$_{\text{wild-type}}$ H1299 NSCLC cells as a negative control. To optimize the dose of pRNA for *in vitro* treatment,
we treated H2122 and H2030 cells with increasing doses of EGFR_{apt-3WJ-siKRAS^{G12C}} pRNA nanoparticles for 48 hrs. We found that KRAS expression was suppressed in a dose-dependent manner, with 50 nM of pRNA nanoparticle serving as the lowest, most effective dose (Figure S2). To further determine the optimal pRNA treatment time, we treated H2122 and H2030 cells with 50 nM of EGFR_{apt-3WJ-siKRAS^{G12C}} pRNA nanoparticles and collected cells at various time points after treatment. We found that KRAS mRNA knockdown efficacy is approximately equivalent between 48 and 72 hrs after treatment with 50 nM of pRNAs, with slightly improved efficacy over 24 hrs (Figure S2). Thus, in this study, we chose 50 nM of pRNAs for 48 hrs as the optimal treatment conditions for functional assays. Sanger sequencing of KRAS exon 2 was performed to confirm KRAS mutation status in all of these cell lines, with H2122 and H2030 harboring homozygous KRAS^{G12C} mutations as expected (Figure S3). Then, we treated H2122 and H2030 cells with 50 nM of EGFR_{apt-3WJ-siKRAS^{G12C}} nanoparticles along with control RNPs (PBS, EGFR_{apt-3WJ-siScramble}, 3WJ-siKRAS^{G12C}) for 48 hrs. We found that 3WJ-siKRAS^{G12C} nanoparticles significantly suppressed KRAS mRNA expression in KRAS mutant cells, and that EGFR aptamer conjugated EGFR_{apt-3WJ-siKRAS^{G12C}} nanoparticles significantly further suppressed KRAS expression in KRAS^{G12C} NSCLCL cell lines (Figure 2C). However, neither 3WJ-siKRAS^{G12C} or EGFRapt-3WJ-siKRAS^{G12C} nanoparticles were able to significantly decrease KRAS expression in KRAS wild-type H1299 cells (Figure S4, Top panel). Furthermore, by immunoblotting we confirmed that EGFR_{apt-3WJ-siKRAS^{G12C}} nanoparticles suppressed protein expression of KRAS and activation of its downstream signaling components p-MEK-1/2, p-ERK-1/2 in KRAS^{G12C} NSCLC cells (Figure 2D), but not KRAS wild-type NSCLC cells (Figure S4, bottom panel). These data demonstrated
that EGFR\textsubscript{apt-3WJ-siKRAS}\textsubscript{G12C} nanoparticles efficiently silence \textit{KRAS}\textsubscript{G12C} expression only in \textit{KRAS}\textsubscript{G12C} mutant NSCLC cells.

\textbf{EGFR\textsubscript{apt-3WJ-siKRAS}\textsubscript{G12C} nanoparticles suppress KRAS activity, cell growth, migration, and invasion capabilities of NSCLC cells.}

To investigate whether \textit{KRAS}\textsubscript{G12C} specific knockdown might result in reduced KRAS activity, we treated H2122 and H2030 cells with 50 nM of EGFR\textsubscript{apt-3WJ-siKRAS}\textsubscript{G12C} nanoparticles along with control RNPs (PBS, EGFR\textsubscript{apt-3WJ-siScramble}, 3WJ-siKRAS\textsubscript{G12C}) for 24 hrs followed by assessment of KRAS functional activation. In both two cell lines, we confirmed that 3WJ-siKRAS\textsubscript{G12C} can suppress KRAS activity, which was maximal when cells were treated with EGFR\textsubscript{apt-3WJ-siKRAS}\textsubscript{G12C} nanoparticles (Figure 3A), which is consistent with immunoblotting data showing EGFR\textsubscript{apt-3WJ-siKRAS}\textsubscript{G12C} nanoparticles suppressed activation of MEK-1/2 and ERK-1.2 (Figure 2D).

Oncogenic KRAS results in persistent stimulation of its downstream signaling intermediates, which results in many of the phenotypic hallmarks of cancer including increased proliferation, and metastasis. To assess the effects of depletion of oncogenic \textit{KRAS}\textsubscript{G12C} by siRNA delivered our RNPs on cell growth, IncuCyte cell proliferation assays were carried out. As shown in Figure 3B, 3WJ-siKRAS\textsubscript{G12C} nanoparticles suppressed NSCLC cell growth, and the presence of EGFR aptamer maximally suppressed cell growth, while the control (scrambled) siRNA RNPs had no effect on cell proliferation. To investigate the influence of \textit{KRAS}\textsubscript{G12C} knockdown on NSCLC cell metastasis, migration ability and invasion ability were evaluated by transwell assay and Matrigel assay, respectively. As shown in Figure S5, and Figure 3C (top panels), the number of cells that migrated or invaded into the lower chamber decreased significantly in cells treated with
EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles compared to the control siRNA group. The calculated invasion index also confirmed that KRAS knockdown decreased cell invasion ability independent of differences in migration (**Figure 3C**, bottom panels).

**EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles sensitize NSCLC cells to chemotherapy and radiation therapy in vitro.**

It has been well documented that hyperactivation of KRAS can lead to development of intrinsic chemotherapy and radiation therapy resistance in tumor cells.\textsuperscript{19,20,35} To test whether KRAS suppression by EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles sensitize NSCLC to radiation therapy, we performed radiation clonogenic assays using 2 Gy ionizing radiation in H2122 and H2030 cell lines at 24 hrs after pRNA nanoparticles treatment. As shown in **Figure 4A**, 3WJ-siKRAS^{G12C} nanoparticles significantly sensitized cells to radiation treatment as observed by lower cell surviving fraction after 2 Gy, compared to the treatment with pRNA nanoparticles containing control siRNA. EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles further sensitized NSCLC cells to radiation therapy. We further investigated whether EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles could sensitize cells to cisplatin, a common first-line chemotherapy drug for NSCLC. Cells were pretreated with EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles for 24 hrs, followed by 72 hrs cisplatin treatment. AlamarBlue cytotoxicity assays were used to determine whether the RNPs could alter the IC50 values to cisplatin. As shown in **Figure 4B**, EGFR_{apt}-3WJ-siKRAS^{G12C} nanoparticles significantly lowered the IC50 values to cisplatin compared to control siRNA RNPs, decreasing from 7.6 \mu M to 3.8 \mu M and 6.6 \mu M to 1.3 \mu M in H2030 and H2122 cell lines, respectively. This finding was corroborated by clonogenic assays (**Figure 4C**), revealing reduced cell surviving fraction in both cell lines during treatment with increasing dose of...
cisplatin and EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> nanoparticles. Together, these data support that KRAS mutation-specific silencing by EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> nanoparticles sensitizes NSCLC cells to chemoradiation therapy in vitro.

EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> nanoparticles are preferentially taken up by tumors and significantly attenuate tumor growth in vivo

We further investigated the potential therapeutic effects of EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> nanoparticles in vivo. First, to determine the in vivo siRNA delivery effects of EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> nanoparticles, we established heterotopic tumor xenografts with H2122 cells in nude mice. To decrease photo-toxicity, we conjugated pRNA nanoparticles with Alexa750, and systemically delivered Alexa750-conjugated pRNA nanoparticles in TES via tail vein injection. At 24 hrs post-injection, IVIS lumina imaging system was utilized to assess pRNA biodistribution in live animals, and in ex vivo organs. Live imaging demonstrated that Alexa750-conjugated 3WJ-siKRAS<sup>G12C</sup> nanoparticles exhibited intense fluorescence in the areas of the tumors, and the presence of EGFR aptamer further increased the accumulation of RNPs in the tumor (Figure 5A, left top). Further, ex vivo organ biodistribution analysis confirmed that EGFR aptamer facilitated pRNA nanoparticles accumulation in the tumors, with minimal uptake in some normal organs including lung, spleen, and heart (Figure 5A, left bottom). We also compared the in vivo biodistribution of EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> and SCR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> pRNA nanoparticles. As shown in Figure S6, EGFRapt-pRNA can more efficiently accumulate in tumor xenografts compared to SCRapt-pRNA. In order to identify the optimal dose of RNPs for in vivo treatment, serial doses of EGFR<sub>apt</sub>-3WJ-siKRAS<sup>G12C</sup> nanoparticles were intravenously administrated to mice bearing H2122 xenografts every 2 days, four times.
over 7 days, and tumors were isolated for extraction of total RNA and protein (Figure 5B).

We found that KRAS RNA (Figure 5C), and ERK activation (Figure 5D) were suppressed in a dose-dependent manner. One micromole per kilogram dose (1 mmol/kg) of pRNA nanoparticle was the most effective dose for suppression of KRAS in vivo. To assess whether systemically delivered EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} nanoparticles could suppress tumor growth, mice bearing H2122 or H2030 tumors were injected with 1 µmol/kg of the indicated pRNA nanoparticles twice a week for 3 weeks. We noted suppression of tumor growth with EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C}, but not 3WJ-siKRAS\textsuperscript{G12C} nanoparticles (Figures 5E, 5F). These data confirm that EGFR\textsubscript{apt}-3WJ-siKRAS\textsuperscript{G12C} nanoparticles are more effective RNPs for targeting KRAS\textsuperscript{G12C} NSCLC cells in vivo relative to 3WJ-siKRAS\textsuperscript{G12C} RNPs, in alignment with the results obtained from our in vitro cell-based experiments.

Discussion

For more than 30 years, development of effective therapeutics targeting oncogenic RAS has eluded the field and RAS was thought to be “undruggable”.\textsuperscript{27} Although breakthroughs have been made recently with the development of covalent inhibitors targeting KRAS\textsuperscript{G12C}, such as AMG510 (sotorasib), there are still challenges and questions regarding the development and prevention of drug resistance, and the ability to successfully target other KRAS mutations.\textsuperscript{27} Other strategies targeting KRAS have been attempted. For example, systemic delivery of KRAS small interfering RNA (siRNA) in nanoparticles suppressed pancreatic, lung and colorectal cancer xenografts in various mouse models.\textsuperscript{36-39} SiG12D-LODER, is a specific siRNA targeting KRAS\textsuperscript{G12D}, and is in a phase II trial to evaluate the clinical efficacy of siG12D LODER in combination with gemcitabine and nab-paclitaxel in pancreatic cancer patients with KRAS\textsuperscript{G12D} mutation (NCT01676259).\textsuperscript{27,40} AZD4785, a
chemically-modified antisense oligonucleotide (ASO), can significantly deplete cellular 
KRAS mRNA and protein in a preclinical study, but failed to suppress KRAS expression 
in patients in a clinical trial (NCT03101839). Further studies are ongoing to improve 
delivery efficiency, uptake and internalization in order to make this approach more 
effective.

In the current study, we took a mutation-specific gene silencing approach by constructing 
novel multifunctional EGFR$_{appr}$-3WJ-siKRAS$^{G12C}$ nanoparticles and explored the potential 
of these RNPs carrying KRAS$^{G12C}$ siRNA to target KRAS in NSCLC cells in a mutation-
specific fashion. Furthermore, we added EGFR$_{appr}$ to the RNP to enhance tumor cell 
targeting. Our results demonstrate that EGFR$_{appr}$-3WJ-siKRAS$^{G12C}$ nanoparticles were 
successfully delivered to (and enriched in) NSCLC cells both in vitro and in vivo. These 
RNA nanoparticles effectively silenced KRAS expression only in KRAS$^{G12C}$ mutant cells, 
attenuated activation of MAPK signaling pathway, suppressed cancer cell migration and 
invasion properties, enhanced chemotherapy and radiotherapy sensitivity, and showed 
some tumor growth inhibitor properties in tumor xenograft mouse models.

Currently, a variety of nanocarriers are being investigated for siRNA delivery, such as 
polymers, dendrimers, liposomes, exosomes, and RNA nanoparticles. Among 
them, 3WJ pRNA nanoparticles are showing great potential as an efficient siRNA delivery 
system, harnessing beneficial characteristics which include a relatively uniform nanoscale 
size, precise stoichiometry, ultrastability, and a high degree of biocompatibility compared 
to other therapeutics (e.g. liposomal nanoparticles). In addition, the branches/arms of the 
3WJ motif could be easily modified with different subunits, such as multiple sequence-
independent siRNAs, different RNA aptamers to improve specificity, or fluorescent dyes
without disrupting the stability and conformation. This technology enables multiple functional units for targeting, therapy and tracking which can all be combined into one nanoparticle. In our study, EGFR<sub>apt-3WJ-siKRAS<sup>G12C</sup></sub> nanoparticles are ultracompact with 7.2±0.5 nm diameter, and thermostable with a T<sub>m</sub> of 51.77°C. Alexfluor647 was conjugated the RNP to form Alexa647-EGFR<sub>apt-3WJ-siKRAS<sup>G12C</sup></sub> nanoparticles for <i>in vitro</i> cellular binding and <i>in vivo</i> biodistribution analysis. Alternatively, multiple units of the same function, such as different siRNAs targeting the same gene or different genes, can be combined on the same 3WJ pRNA nanoparticle for enhanced therapeutic effects. For example, as resistance to AMG510 has been shown to be associated with intratumoral heterogeneity and evolution of new non-G12C mutations, one could envision putting on multiple mutation-specific siRNAs targeting <i>KRAS G12C</i>, <i>G12D</i>, <i>G12R</i>, <i>G12V</i>, etc. to suppress development of resistance to these RNPs. Conversely, we could fashion these EGFR<sub>apt-3WJ-siKRAS<sup>G12C</sup></sub> nanoparticles to be more efficient at suppressing <i>KRAS<sup>G12C</sup></i> activity, by adding another <i>KRAS<sup>G12C</sup></i> siRNA on another branch of 3WJ pRNA to increase dose of gene silencing.

Overexpression of EGFR has been reported in many human malignancies and is detected in up to 85% in NSCLC patients. EGFR monoclonal antibodies including cetuximab and necitumumab have been used to treat NSCLC patients combined with cytotoxic chemotherapeutics. Recently, RNA aptamers are emerging as promising targeting moieties, analogous to monoclonal antibodies. EGFR aptamers are selected through SELEX methods (systematic evolution of ligands by exponential enrichment), and have been utilized to decorate nanocarriers for more targeted delivery. In our study, the addition of EGFR<sub>apt</sub> to 3WJ-si<i>KRAS<sup>G12C</sup></i> showed obvious enhancement on cellular uptake,
and gene knockdown in both *in vitro* and *in vivo* experiments. Though EGFR depletion by *EGFR* siRNA didn’t completely abolish EGFR apt pRNA binding (Figure 2B), this observation might be due to insufficient *EGFR* knockdown, or more likely that EGFR apt may nonspecifically bind to the membrane or other membrane receptors/components. This represents an opportunity to further optimize EGFR apt binding specificity in the future through re-engineering of EGFR apt or utilizing other targeting moieties.

Upregulation of KRAS mediated signaling pathways is one of the mechanisms of chemotherapy resistance. For example, KRAS mutations have been shown to activate the anti-oxidant NRF2 pathway in NSCLC, thereby decreasing cisplatin-induced reactive oxygen species within the tumor cells, and ultimately leading to cisplatin resistance. Studies have also shown KRAS mutations render patients resistant to gefitinib in NSCLC, and cetuximab resistance in colorectal cancer. In addition, KRAS mutations are also associated with radiation resistance in various cancers, including NSCLC, colorectal cancer. Herein, we document that EGFR apt-3WJ-siKRAS<sup>G12C</sup> nanoparticles can sensitive NSCLC cells to radiation therapy or the first-line chemotherapy agent cisplatin (Figure 4). The results show the potential of 3WJ RNPs for allowing reduction in the doses of chemotherapy or radiotherapy leading to reduced normal tissue toxicity, or conversely, allowing enhancement of therapeutic efficacy if doses are kept the same.

In conclusion, we have demonstrated that EGFR apt-3WJ-siKRAS<sup>G12C</sup> RNPs efficiently suppressed KRAS mRNA expression, resulting in suppression of downstream effector pathways in KRAS<sup>G12C</sup> NSCLC cells, leading to suppression of tumor cell proliferation, migration/invasion ability, and sensitized NSCLC cells to chemoradiotherapy. Furthermore, our animal modeling showed excellent biodistribution of the RNPs to the
tumor, effective KRAS silencing, and subsequent tumor growth inhibition. In addition, the 3WJ pRNA motif is universal and can be easily applied to target other KRAS mutant variants. Overall, these data support that 3WJ pRNA is an attractive RNA nanotechnology platform to deliver KRAS mutation-specific siRNA for the treatment of KRAS-driven human cancers that could lead to a widened therapeutic index by being a more tumor-selective therapy.

Materials and Methods

Construction of EGFR_{apt}-3WJ-siKRAS^{G12C} RNA nanoparticles

Multifunctional 3WJ pRNA nanoparticles (RNP) were prepared using a bottom-up self-assembly approach as previously described.\textsuperscript{51-53} The EGFR_{apt}-3WJ-Alexa647-siKRAS^{G12C} consisted of four components attached to the 3WJ core motif (Figure 1A), harboring EGFR targeting RNA aptamer (EGFR_{apt}) as a targeting ligand; AlexaFluor 647 or 750 (ThermoFisher, Waltham, MA) as a fluorescent imaging module; a KRAS^{G12C} sense strand, and a KRAS^{G12C} anti-sense strand, as the therapeutic module. The controls include RNPs without targeting EGFR_{apt} ligand (denoted as 3WJ-siKRAS^{G12C}), with scramble aptamer ligand (denoted as SCRapt-3WJ-siKRAS^{G12C}), without therapeutic module (denoted as EGFR_{apt}-3WJ-siScramble), or without therapeutic and targeting modules (denoted as 3WJ).

The sequences of the four strands of EGFR_{apt}-3WJ-siKRAS^{G12C} RNPs are as below: strand “a” (5’ - UUG CCA UGU GUA UGU GGG AGU UGG AGC UGU UGG CGU AGU U-3’); strand “b” (5’ – CCC ACA UAC UUU GUU GAU CC - EGFR_{apt} - 3’); strand “c” (5’ – GGA UCA AUC AUG GCA A -3’); strand “d” (5’- CUA CGC CAC AAG CUC CAA C -3’). The complete pRNA sequences are provided in Supplementary Table S1. The KRAS^{G12C} siRNA sequence is derived from a previous publication which demonstrated that
this sequence could knockdown oncogenic KRAS but not wild-type KRAS. The RNA fragments were either synthesized via standard phosphoramidite chemistry by ourselves, or purchased from Trilink (San Diego, CA), and strands a, b and c are 2’-F modified at cytosine (C) and uracil (U) nucleotides to make the RNP\textquotesingle s resistant to RNase degradation. For the experiments involving the detection of RNPs (binding assay), strand c was conjugated to fluorophore Alexa647 at the 3’ end. The RNP was formed through one-step self-assembly by mixing the four RNA module strands at equal molar ratios in annealing buffer (10 mM tris, pH 7.5-8.0, 50 mM NaCl, 1mM EDTA), and heated to 95°C for 5 min and slowly cooled to 4°C over 45 minutes. Step-wise assembly of RNPs was verified on a native 10% PAGE running in 1x TBE buffer (89 mM Tris-borate, 2 mM EDTA), and imaged by Typhoon FLA7000 (GE Healthcare) under the ethidium bromide channel. The self-assembled \textit{EGFR}_{apt}-3WJ-Alexa647-si\textit{KRAS}^{G12C} RNPs were purified from 8M urea-containing PAGE and stored at -80°C until use. The RNPs were freshly reconstituted in PBS before each use.

**Characterization of the assembled pRNA-3WJ nanoparticle**

The molecular size of assembled 3WJ pRNA nanoparticles was confirmed by native 10% PAGE gel electrophoresis. The hydrodynamic diameter of pRNA nanoparticles was assessed by dynamic light scattering (DLS) using a Zetasizer nano ZS (Malvern Instruments) at 25°C via a laser wavelength at 633 nm. The thermodynamic stability of the pRNA nanoparticles was studied using the temperature gradient gel electrophoresis system (TGGE, Biometra GmbH, Germany), as previously described. The apparent T\textsubscript{m} (melting temperature) of the pRNA nanoparticle was determined as the temperature at which 50%
of the pRNA nanoparticle remained assembled. Data shown are representative of three
independent measurements.

**Cell culture, chemicals, and antibodies**

Human NSCLC cell lines H2122, H2030 and H1299 were obtained from American Type
Culture Collection and maintained in RPMI 1640 medium (ThermoFisher), with 10% FBS
(GE Healthcare, Chicago, IL) and 1% penicillin/streptomycin (Life Technologies,
Carlsbad, CA). Cells were cultured in 37°C with 5% CO₂. Typically, cells were kept in
culture for a minimum of two passages prior to and a maximum of 20 passages during
experiments. The identity of all cell lines was confirmed by STR genotyping (Identifier
Kit, Applied Biosystems, Carlsbad, CA). For the detection of mycoplasma in cell culture,
the Universal Mycoplasma Detection Kit (ATCC) was used. Cisplatin (Sigma, St.Louis,
MO) was dissolved in dimethyl formamide (Sigma). Anti-KRAS antibody was purchased
from Santa Cruz Biotechnology (Dallas, TX); anti-p-MEK-1/2, p-ERK-1/2, EGFR, and
GAPDH primary antibodies were purchased from Cell signaling Technology (Danvers,
MA). Anti-rabbit, and anti-mouse secondary antibodies were purchased from LI-COR
Bioscience (Lincoln, NE).

**DNA extraction and KRAS mutation analysis**

Genomic DNA was isolated by using QIAamp DNA mini kit (Qiagen) according to the
manufacturer’s instruction. Exon 2 of *KRAS* gene was amplified by polymerase chain
reaction (PCR) using the following primers: Forward 5′-TGA CAT GTT CTA ATA TAG
TCA G-3 and reverse 5′-ACA AGA TTT ACC TCT ATT GTT G-3. PCR was performed
as previously described.35 PCR products were purified using a DNA purification kit (Zymo
Research, Irvine, CA) and direct Sanger sequencing was performed on capillary electrophoresis using Applied Biosystems 3730 DNA Analyzer (ThermoFisher).

**In vitro pRNA nanoparticle cellular binding assay**

Specific binding of pRNA nanoparticles to NSCLC cells was assessed *in vitro* by flow cytometry. In brief, 1x10⁵ H2122 and H2030 cells were resuspended in 100 ul PBS, and incubated with 50 nM of Alexa647 labeled EGFR<sub>apt</sub>-3WJ-siKRAS<sub>G12C</sub> and 3WJ-siKRAS<sub>G12C</sub> pRNA nanoparticles at 37°C for 1 h. After washing three times with PBS, the cells were subjected to flow cytometry analysis using the BD LSR Fortessa flow cytometer (Becton Dickinson). Data were analyzed using the FlowJo 7.6.1 software (Tree Star). For EGFR dependent selectivity assessment, cells were treated with EGFR-siRNA to knockdown EGFR expression, followed 24 hrs later by pRNA nanoparticle cellular binding assay. Data shown are representative of three independent experiment.

**Real-time quantitative PCR analysis**

KRAS gene silencing was detected by real-time quantitative PCR assay. Total cellular RNA was isolated using Trizol (ThermoFisher), and one microgram of total RNA was reverse-transcribed using Supercript reverse transcriptase (Bio-Rad). PCR was performed on iCYCLER real-time PCR machine (Bio-Rad) using SYBR-Green chemistry (Bio-Rad). The genes expression levels were normalized to housekeeping gene GAPDH. The primer sequences are as follows: KRAS forward: 5’-GAC TCT GAA GAT GTA CCT ATG GTC CTA-3’ and reverse: 5’-CAT CAT CAA CAC CCT GTC TTG TC-3’; GAPDH forward: 5’-AAC GGG AAG CTT GTC ATC AAT GGA AA-3’, and reverse: 5’-GCA TCA GCA GAG GGG GCA GAG-3’. Experiments were performed three times.

**Immunoblotting**
Immunoblotting was performed as described previously. Briefly, cell lysates were prepared using RIPA buffer (ThermoFisher) supplemented with 1x protease inhibitors (Complete, Roche, Indianapolis, IN) and phosphatase inhibitors (PhosSTOP, Roche) followed by protein quantification with the Dc protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated in 5% bovine serum albumin (BSA) in Tris buffered saline with 0.1% Tween-20 (TBST) blocking buffer for 1 hour at room temperature. Primary antibodies with dilution of 1:200-1000 were allowed to bind overnight at 4°C, or for 2 hours at room temperature. After washing in TBST, the membranes were incubated with immunofluorescent secondary antibodies at a 1:5000 dilution for 1 hour at room temperature. Membranes were washed with TBST and allowed to air dry prior to imaging via LI-COR Odyssey® CLx Imaging System (ThermoFisher). Immunoblotting data represents three independent experiments.

**KRAS activity assay**

As previously reported, KRAS activity was detected using a Ras Activation ELISA assay kit (Millipore) according to the manufacturer’s instructions. GST-Raf-RBD was used to pull-down RAS-GTP from 50 ug of cell lysate prepared the same way as for immunoblotting, and then a primary antibody for KRAS was added, followed by incubation with an HRP-conjugated secondary antibody. After addition of developing reagent, chemiluminescent reaction was determined with a Fluoroskan Ascent FL luminometer. Experiments were repeated three times.

**IncuCyte cell proliferation assay**
Cells were treated with various pRNA nanoparticles accordingly for 48 hrs, and then seeded at 1,000-2,000 cells per well in 96-well plates. Cell confluence as a measure of cell growth over time was monitored every 4 hours for up to 6 days using the IncuCyte ZOOM Live-Cell Imaging System (Essen Biosciences), until cells reach about 80% confluence. Cell proliferation curves were plotted using GraphPad Prism version 9.0 for Windows (La Jolla, CA). Experiments were repeated three times.

Cell migration and invasion assays
In the migration assay, cells were treated for 48 hrs treatment with nanoparticles, then 2 x 10^4 cells were resuspended in 300 μl cell culture medium with 0.5% FBS and placed in the upper transwell chamber (8 μm pore size, BD Biosciences). The upper chamber was placed in a 24-well culture dish containing 1 ml of complete cell culture medium (with 10% FBS). After 48 hrs incubation, non-migrated cells on the upper membrane were removed with a cotton swab. Migrated cells on the bottom surface were fixed with methanol (-20°C) and stained with 0.5% crystal violet. Four fields of each well were photographed, and the cell numbers were counted. In the invasion assay, Matrigel-coated transwell chambers (BD Biosciences) were used. Percentage invasion was calculated as the number of invaded cells in comparison with the number of migrated cells. All experiments were repeated three times.

Chemotherapy and radiation sensitivity assays
To study the effects of pRNA nanoparticles on chemoradiation sensitivity in NSCLC, clonogenic assay was performed as previously reported. Briefly, cells were treated with indicated pRNA nanoparticles for 24 hrs, and then harvested to generate single cell suspension and seeded onto 60 mm tissue culture plates in triplicate. After 24 hrs, for
radiation sensitivity assay, cells were irradiated with 0 Gy, or 2 Gy X-ray using Radsource RS2000 biological irradiator (RadSource, GA). For chemotherapy sensitivity assay, cells were treated with increasing dose of cisplatin (0, 0.5, 1 μM). Twenty-four hours later, the medium was changed to remove cisplatin. Ten to 14 days after IR or cisplatin treatment, colonies were fixed with methanol/acetic Acid, stained with 0.5% crystal violet, and the numbers of colony forming units (CFU) containing at least 50 cells were counted using a dissecting microscope (Leica Microsystems, Inc. Buffalo Grove, IL) and surviving fractions were calculated. Experiments were performed three independent times.

Cisplatin cytotoxicity was also assessed by alamarBlue™ assay (Bio-Rad). Briefly, cells were treated with pRNA nanoparticles as indicated for 24 hrs, and then collected and seeded in 96-well plates in 4 replicates at a density of 2000 cells per well in 100 μl medium. The next day (~48 hrs after adding pRNA), cells were treated with cisplatin at various concentrations. After 72 hrs, alamarBlue reagent was added to cells at 37°C for 4 hrs, and absorbance was measured at 490 nm. Half maximal inhibitory concentration (IC50) was determined using the nonlinear four-parameter regression function in GraphPad Prism. Experiments were performed three independent times.

Biodistribution and antitumor activity of pRNA nanoparticles in vivo

Animal studies were conducted in accordance with an approved protocol adhering to the IACUC policies and procedures at The Ohio State University. Six to eight-week-old male athymic nude mice (Taconic Farms Inc., NY) were caged in groups of five or less and fed with a diet of animal chow and water ad libitum. H2122 and H2030 cells were injected subcutaneously into the flanks of each mouse at 5 × 10^6 cells per injection. When the tumor size reached 150-200 mm³, the mice were randomly divided into four groups with 10 mice
per group and injected with pRNA nanoparticles via tail vein twice a week for 3 weeks. The tumor volume was monitored 3 times a week, and tumor size was calculated using the formula: $V = \frac{L \times W^2}{2}$, where $V$ (volume) is determined by length (L) and width (W). For in vivo pRNA nanoparticle targeting and tumor imaging, mice were tail vein injected with 100 µl of 20 µM of RNA Alexa750 labeled pRNA nanoparticle, and animal or tissue were imaged using the IVIS lumina imaging system with Living Images 3.0 software (Caliper Life Sciences).

**Data analysis**

Data were presented as the mean ± standard deviation (SD) or standard error of the mean (SEM), with a representative experiment from at least three independent experiments shown. The difference among groups was calculated using Student’s t test or one-way ANOVA analysis followed by Tukey’s post-hoc test (GraphPad Prism).

**Acknowledgments**

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**Authors’ contributions**

L.Y., Z.L., P.G. and T.W. designed the study, participated in the supervision and coordination of the study; L.Y., Z.L., and T.W. conceived and designed the experiments; L.Y. and Z.L. performed most of the experiments; All authors analyzed the data; All authors contributed to the writing, review, and revision of the manuscript. All authors read and approved the final manuscript.
Declaration of Interests: P.G. is the consultant of Oxford Nanopore Technologies; the cofounder of Shenzhen P&Z Bio-medical Co. Ltd, as well as the cofounder of ExonanoRNA, LLC and its subsidiary Weina Biomedical (Guangdong), LTD.

Data Availability Statement: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Keywords: RNA nanotechnology, pRNA, three-way junction, 3WJ, EGFR RNA aptamer, siRNA, KRAS mutation, KRASG12C, non-small cell lung cancer, NSCLC.

References

International Association for the Study of Lung Cancer 14, 876-889. 10.1016/j.jtho.2019.01.020.


Figure Legends

Figure 1. Construction and characterization of EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA nanoparticles. (A) Schematic of Alexa-647 labeled EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA structure. (B) Native PAGE showing stepwise assembly of EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA nanoparticles. (C) DLS assay to assess hydrodynamic size of EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA nanoparticles. (D) TGGE assay determined Tm value of EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA nanoparticles.

Figure 2. EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA nanoparticles target EGFR-expressing cells and suppress KRAS expression in human lung cancer. (A, B) Cellular uptake of AF647-labeled pRNA nanoparticles after 1 hr incubation was determined by flow cytometry assay. (C) KRAS mRNA expression was quantified by qRT-PCR assay 48 hrs post-treatment with pRNA nanoparticles. (D) KRAS protein expression and activation status of KRAS downstream pathway intermediates was assessed by immunoblotting assay 48 hrs post-treatment with pRNA nanoparticles. Error bars represent SD. Significance was calculated using Student’s t-test: **$P<0.001$.

Figure 3. Silencing of KRAS by EGFR$_{\text{apt}-3WJ}$-siKRAS$^{G12C}$ pRNA nanoparticles suppressed KRAS activity, cell growth and metastatic capabilities of NSCLC cells. (A) KRAS activity was determined by KRAS activation ELISA assay on H2030 and H2122 cells. (B) Effects of KRAS silencing by pRNA nanoparticles on cell growth was determined by Incucyte cell proliferation assay (cell number reflects percent confluency). (C) Quantification of transwell migration and invasion assays (top panels), and calculated invasion index (bottom panels). Error bars represent SD. Significance (A, C) was
calculated using Student’s t-test; significance (B) was calculated using one-way ANOVA analysis followed by Tukey’s post-hoc test: *P<0.05, **P<0.001.

**Figure 4.** EGFR\textsuperscript{apt-3WJ-siKRAS\textsuperscript{G12C} } pRNA nanoparticles sensitized NSCLC cells to radiotherapy and chemotherapy. (A) Radiation clonogenic assay was performed and normalized surviving fraction was calculated to evaluate sensitivity of NSCLC cells to radiation treatment after 48 hrs pRNA treatment. (B,C) Effects of 48 hrs treatment of EGFR\textsuperscript{apt-3WJ-siKRAS\textsuperscript{G12C} } pRNA nanoparticles on cisplatin cytotoxicity on NSCLC cells assessed by both alamarBlue (B), and colony formation assays (C). Error bars represent SD. Significance was calculated using Student’s t-test: *P<0.05, **P<0.001.

**Figure 5.** In vivo evaluation of EGFR\textsuperscript{apt-3WJ-siKRAS\textsuperscript{G12C} } pRNA nanoparticles on NSCLC tumor xenograft models. (A) IVIS lumina imaging system was used to study the biodistribution of Alexa750 labeled EGFR\textsuperscript{apt-3WJ-siKRAS\textsuperscript{G12C} } pRNA nanoparticles in live mice (top panels), and dissected organs ex vivo (bottom panels). (B) Dosing schematic of pRNA nanoparticle injection and tumor isolation for dose optimization study. (C, D) After 4 i.v. administrations of increasing dose of EGFR\textsuperscript{apt-3WJ-siKRAS\textsuperscript{G12C} } pRNA nanoparticles, KRAS mRNA expression (C) in tumor xenografts was quantified by qRT-PCR, and activation status of KRAS downstream MAPK pathway signaling was evaluated by p-ERK immunoblotting (D). (E, F) Tumor growth curve of H2122 and H2030 xenograft mouse models, which were treated with pRNA nanoparticles 2 times a week for 3 weeks (n=10 mice per group). Error bars represent SEM. significance was calculated using one-way ANOVA analysis followed by Tukey’s post-hoc test: **P<0.001.
A diagram shows a molecular structure with strands labeled a, b, c, and d. Strand a is labeled with Alexa 647, strand c has an EGFR aptamer, and strand d contains siRNA. The diagram also includes a marker with different mixtures of strands:

- Strand a
- Strand b
- Strand c
- Strand d
- a + d
- a + b
- a + c
- b + c
- a + b + c + d

B: The graph shows a DLS size distribution with a peak at 7.204 ± 0.523 nm.

C: A graph of intensity (A.U.) vs. diameter (nm) is shown, indicating a peak at 7.204 ± 0.523 nm.

D: A graph shows a melting curve with a Tm = 51.77°C and a peak at 79.6°C.
A

H2030

PBS

EGFRapt-3WJ-siCtr

3WJ-siKRAS

EGFRapt-3WJ-siKRAS

H2122

PBS

EGFRapt-3WJ-siCtr

3WJ-siKRAS

EGFRapt-3WJ-siKRAS

B

H2030

Cell viability (%) = 100

Log Concentration (Cisplatin nM)

EGFRapt-3WJ-siCtr

EGFRapt-3WJ-siKRAS

G12C

H2122

Cell viability (%) = 100

Log Concentration (Cisplatin nM)

EGFRapt-3WJ-siCtr

EGFRapt-3WJ-siKRAS

G12C

C

H2030

Relative surviving fraction (%) = 100

Cisplatin (µM)

EGFRapt-3WJ-siCtr

EGFRapt-3WJ-siKRAS

G12C

H2122

Relative surviving fraction (%) = 100

Cisplatin (µM)

EGFRapt-3WJ-siCtr

EGFRapt-3WJ-siKRAS

G12C

IC50

| EGFRapt-3WJ-siCtr | EGFRapt-3WJ-siKRAS
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A Heart
Liver
Spleen
Lung
Kidney
Tumor

B pRNA injections

2 days
2 days
2 days
1 day

C H2122 xenografts

KRAS mRNA relative expression

EGFRapt-3WJ-siKRASG12C (nmol/kg)

D 0 30nM 100nM 300nM 1000nM

p-ERK
ERK
GAPDH

E TES
H2122 xenografts

% Tumor Volume Change

0 5 10 15 20 25 30 35 40 45 50 55

Time (Days)
pRNA injections

F H2030 xenografts

% Tumor Volume Change

0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48

Time (Days)
pRNA injections
Terence M. Williams and colleagues assembled EGFR<sub>apt</sub>-3WJ-si\(KRA\)\(_{5}\)\(_{G12C}\) RNA nanoparticles, which efficiently suppressed KRAS expression in NSCLC, leading to suppression of MAPK pathway and tumor growth both in vitro and in vivo. The data support that 3WJ RNA nanoparticle is a potentially effective siRNA delivery platform for oncogene-driven human cancers treatment.