Comprehensive analyses of the intracellular and \textit{in vivo} disposition of Fab-siRNA conjugate to identify key issues to improve its \textit{in vivo} activity

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Running Title Page

a) Running title.
Intracellular and in vivo disposition of Fab-siRNA conjugate

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d) A list of nonstandard abbreviations.
siRNA, small interfering RNA.
EGFR, epidermal growth factor receptor.
PCR, polymerase chain reaction.
LC/MS, liquid chromatography with mass spectrometry.
RISC, RNA-induced silencing complex.
GalNAc, N-acetylgalactosamine.
ADCs, antibody and cytotoxic drug conjugates.
KRAS, Kirsten rat sarcoma viral oncogene homolog.
HPLC, high-performance liquid chromatography.
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
BSA, bovine serum albumin.
PBS, phosphate-buffered saline.
FITC, fluorescein isothiocyanate.
MFI, mean fluorescence intensity.
ELISA, enzyme-linked immunosorbent assay.
QC, quality control.
IP, immunoprecipitation.
CL\text{total}, total clearance.
V_{ss}, steady-state distribution volume.
t_{1/2}, plasma half-life.
LLOQ, lower limit of quantification.
ASGPR, asialoglycoprotein receptor.
Abstract

Comprehensive analyses of intracellular disposition and in vivo pharmacokinetics were performed for small interfering RNA (siRNA) conjugated with the Fab fragment of Panitumumab, a fully humanized anti-epidermal growth factor receptor (EGFR) monoclonal antibody. The Fab-siRNA conjugate was internalized into EGFR-expressing cancer cells in an antigen-dependent manner. Intracellular disposition was quantitatively evaluated using fluorescent-labeled Panitumumab and confocal microscopy. The majority of internalized Panitumumab was suggested to be transferred into lysosomes. In vivo pharmacokinetics were evaluated in EGFR-expressing tumor-bearing mice. Intact Fab-siRNA was measured by immunoprecipitation using anti-Fab antibody followed by quantitative polymerase chain reaction. The Fab portion was measured by a ligand binding assay. Intact Fab-siRNA concentrations rapidly decreased in the plasma and tumor, although the Fab portion concentration remained high, suggesting extensive degradation in linker-siRNA portion. After incubation of Fab-siRNA in mouse plasma, samples were digested with proteinase K, and extracted siRNA tagged with Fab-derived peptide was subjected to an ion-pair reversed-phase liquid chromatography with mass spectrometry analysis. Results suggested that hydrolysis from the 3’-end of the antisense strand of siRNA is the major metabolizing pathway. Based on these findings, endosomal escape and stability in lysosomes, blood and tumor are key factors to improve to achieve efficient target gene knockdown in tumors and stabilizing the 3’-end of the antisense strand was suggested to be most efficient. Our approaches clearly identified the key issues of Fab-siRNA from a pharmacokinetics aspect, which will be useful for improving the in vivo activity of siRNA conjugated with not only Fab but also other immunoproteins.
Significance Statement

The intracellular and in vivo disposition of Fab-siRNA conjugate was comprehensively investigated using various approaches, including newly developed analytical methods. This study clearly shows that improvements in siRNA stability in lysosomes, blood and tumor are needed for target gene knockdown in tumors. The major metabolic pathway of Fab-siRNA is 3' exonuclease degradation, suggesting that optimization of the conjugation site to Fab might help improve stability.
Introduction

Small interfering RNA (siRNA) comprises a 20- to 25-mer RNA duplex that cleaves the target mRNA in a sequence-dependent manner. The antisense strand of siRNA binds to a RNA-induced silencing complex (RISC) in the cytosolic region and recognizes the complementary sequence in the target mRNA for cleavage (Agrawal et al., 2003). siRNA is expected to be a useful new modality for next-generation therapeutics, since it can target undruggable targets for small molecules and antibodies. Small molecules need a functional pocket on the target protein to bind and modify the function, and antibodies can only access proteins in blood or on the cell surface. siRNA can theoretically target any protein since it can reduce the target protein level by reducing its mRNA.

However, siRNA requires a drug delivery system to work in vivo. siRNA is generally unstable in blood, and its large molecular size and hydrophilic features prevent its cellular uptake in the target tissue. Onpattro (Patisiran), the first FDA-approved RNA interference drug, uses a lipid nanoparticle that can effectively accumulate in the liver (Kristen et al., 2019). In addition, N-acetylgalactosamine (GalNAc) conjugated siRNAs were developed for delivery to the liver. Givlaari (Givosiran) and Oxlumo (Lumasiran) have already been approved (Scott, 2020), (Scott and Keam, 2021). However, drug delivery systems targeting solid tumors are still under development.

For siRNA delivery to tumors, lipid and polymer-based nanoparticles are the major approach. However, a number of hurdles have yet to be addressed, including the poor delivery efficiency to tumors and strong immunostimulatory effect; therefore, no drugs have yet been approved (Charbe et al., 2020). Antibody conjugate is another attractive approach. Antibody and
cytotoxic drug conjugates (ADCs) have shown great promise in clinical studies, and more than 10 ADCs have been approved by the FDA thus far (Tong et al., 2021). Several groups have reported tumor delivery of siRNA using conjugates with antibody or antibody fragments. These reports indicated the potential of such immunoprotein-siRNA conjugate. However, the dosing amount of immunoprotein-siRNA conjugate for an anti-tumor effect in tumor-bearing mice is still higher than that of ADCs due to the partial knockdown activity (Cuellar et al., 2015), (Lu et al., 2016), (Tsuchida et al., 2018). Further improvement is needed to fully realize immunoprotein-siRNA conjugates as anti-cancer therapeutics.

Immunoprotein-siRNA conjugate must overcome a number of hurdles to achieve an antitumor effect, including tumor accumulation being dependent on antigen binding, tumor penetration from blood vessels, efficient internalization into cancer cells, endosomal escape to avoid lysosomal degradation, and complex formation with a RISC (Figure 1B). To compound matters, siRNA is degraded in circulating blood by ribonucleases within a few hours (Haupenthal et al., 2006). Therefore, to improve the efficiency of tumor delivery of immunoprotein-siRNA, it is necessary to understand the disposition of immunoprotein-siRNA conjugate in vivo. However, due to the high technical difficulty of evaluating the disposition of immunoprotein-siRNA conjugate, there have been very few reports on such efforts.

In a report by Tan et al. (Tan et al., 2012), the serum concentration of antibody-siRNA conjugate rapidly decreased compared to that of total antibody after intravenous administration in mice, suggesting degradation of siRNA from the antibody. Understanding the degradation pathway will be very helpful for improving the stability, but it is unclear maybe due to technical difficulty to evaluate degradation pathway of siRNA conjugated to immunoprotein. In general,
the ion-pair reversed-phase liquid chromatography with mass spectrometry (LC/MS) method is used for the metabolite analysis of siRNA, but immunoprotein-siRNA conjugate cannot be subjected to this method due to its large molecular weight.

In the present study, we performed a comprehensive evaluation of the disposition of siRNA conjugated with the Fab fragment of the anti-EGFR antibody Panitumumab. We used dicer substrate siRNA, which is 25-27 nucleotides in length and further cleaved and processed by the dicer enzyme (Kim et al., 2005). To utilize the dicer substrate site as a linker, the 5’-end of the antisense strand was used for conjugation. Kirsten rat sarcoma viral oncogene homolog (KRAS) siRNA was chosen for the conjugation, since the KRAS mutation causes resistance to epidermal growth factor receptor (EGFR) blockade treatment. The combination of EGFR blockade and KRAS silencing is expected to overcome the resistance to EGFR blockade therapy. The cellular uptake of Fab-siRNA conjugate was evaluated in vitro using EGFR-expressing cancer cell lines. Intracellular localization was evaluated by confocal microscopic analyses. In vivo disposition and degradation were evaluated in tumor-bearing mice. The degradation pathway of Fab-siRNA was evaluated in mouse serum using a newly developed LC/MS method combined with proteinase K digestion.
**Materials and Methods**

**Materials**

A series of siRNAs were provided from Dicerna Pharmaceuticals Inc. (Lexington, MA, USA). The sequences of the siRNAs are shown below, where m, r and d correspond to 2’-O-methyl nucleotide, ribonucleotide and deoxyribonucleotide, respectively. KRAS siRNA: sense strand, 5’-rGrGrArGrGrCrUrUrUrGrUrGrUrArUrUrGdCdC-3’; antisense strand, 5’-mGmGmCmArArArUrArCrArCrArArArArAmGrCmCrCmUrCmCmCmC-3’. The scrambled sequence siRNA was used as internal standard in LC/MS. For conjugation with Fab, a thiol group was added at the 5’-end of the antisense strand (KRAS siRNA-SH). For the analyses using a fluorescent probe, Alexa Fluor 488 or Cy3 was labeled at the 5’-end of the sense strand of siRNA.

Panitumumab was purchased from Amgen Inc. (Thousand Oaks, CA, USA). Fluorescent-labeled Panitumumab was prepared using an Alexa Fluor 488 and Alexa Fluor 647 Protein Labeling Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. BM(PEG)3 (1,11-bismaleimido-triethyleneglycol), EZ-Link Maleimide-PEG2-Biotin and Alexa Fluor 488 C5 Maleimide were purchased from Thermo Fisher Scientific. Anti-human kappa light chain Goat IgG was internally produced and biotinylated using a Biotin Labeling Kit–SH (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer’s instructions. *Escherichia coli* strain W3110 was obtained from American Type Culture Collection (Manassas, VA, USA).
Preparation of Pani Fab-siRNA

Fab-siRNA was prepared using site-directed Cys introduction into Fab to conjugate with siRNA via a maleimide linker. This allowed 1-to-1 site-specific conjugation with siRNA. Fab fragment of IgG1-type Panitumumab with an A140C mutation in the heavy chain (Pani Fab) was prepared as described previously (Shiraishi et al., 2015). In brief, the plasmid pFLAG-CTS-Fab was designed to coexpress the light and heavy chains of the Fab fragment, which was cloned between the NdeI and Sall sites on pFLAG-CTS (Sigma-Aldrich, St. Louis, MO, USA). The hexahistidine tag sequence was added to the C-terminal of the heavy chain for affinity purification using an Ni column. Pani Fab was expressed in E. coli strain W3110 and purified using a protein G column. The buffer exchange for reaction buffer was accomplished using the Amicon Ultra-0.5 30K device (Merck Millipore, Billerica, MA, USA).

Pani Fab-BM(PEG)3-KRAS siRNA (Pani Fab-siRNA) was prepared using a two-step conjugation method (Figure 1A). First, Pani Fab was incubated with a 20-fold molar ratio of BM(PEG)3 in reaction buffer (20 mM sodium citrate (pH 6.0), 150 mM NaCl) for 2 h, followed by cation-exchange high-performance liquid chromatography (HPLC) purification on a Mono S 5/50 GL column (GE Healthcare, Piscataway, NJ, USA) using an ÄKTA purifier (GE Healthcare). The mobile phases comprised 20 mM sodium acetate (pH 5.0) (A); and 20 mM sodium acetate (pH 5.0), 1 M NaCl (B). The mobile phase B concentration was linearly increased from 0% to 60% over 45 min (flow rate: 4 mL/min). The purified Pani-Fab-BM(PEG)3 was buffer-exchanged for reaction buffer using an Amicon Ultra-0.5 30K device.

Pani Fab-BM(PEG)3 was then incubated with a 3-fold molar ratio of KRAS-siRNA-SH for 2 h at room temperature followed by anion-exchange HPLC purification on a Mono Q 5/50
GL column (GE Healthcare). The mobile phases comprised 10 mM Tris-HCl (pH 8.0), 150 mM NaCl (A); and 10 mM Tris-HCl (pH 8.0), 1 M NaCl (B). The mobile phase B concentration was linearly increased from 0% to 100% over 45 min (flow rate: 1 mL/min). The purified Pani Fab-siRNA was then buffer-exchanged for reaction buffer using an Amicon Ultra-0.5 30K device. The reaction mixture and the purified Pani Fab-BM(PEG)3-KRAS siRNA were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Multi Gel II mini 10/20; COSMO BIO, Tokyo, Japan) under non-reduced conditions. Pani Fab-Maleimide-PEG2-Biotin (Pani Fab-Biotin) and Pani Fab-Alexa Fluor 488 (Pani Fab-Alexa488) were prepared as in the same way as Pani Fab-BM(PEG)3.

**Cell lines and culture conditions**

The human pancreatic cancer cell line AsPC-1 and human lung cancer cell line A549 were purchased from American Type Culture Collection. Both cell lines are EGFR-positive. The cells were cultured at 37 °C in a humidified incubator in 5% CO₂ in the recommended media with 10% fetal bovine serum. Cell seeding was performed 18–24 h in advance, and cells were used at 50–80% confluency unless otherwise specified.

**mRNA knockdown**

For the *in vitro* mRNA knock-down assay, AsPC-1 cells were seeded in each well of 96-well plates at $1 \times 10^4$ cells/well and cultured overnight. Pani Fab-siRNA was diluted in medium to the appropriate concentrations and added directly to the cells. Naked siRNA was transfected using RNAiMAX according to the manufacturer's protocol. After incubating for 24 h, cells were
treated with a Cells-to-Ct kit (Thermo Fisher Scientific) and subjected to quantitative polymerase chain reaction (PCR). The transcript levels of KRAS and Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) were measured using a 7900FT Fast Real-time PCR system (Applied Biosystems). TaqMan primer sets for human KRAS (Code No. Hs00270666_m1) and human HPRT1 (Code No. Hs99999909_m1) were purchased from Thermo Fisher Scientific. The relative mRNA level of KRAS was normalized to HPRT1 by reverse transcription (RT)-PCR.

Internalization analyses of Pani Fab-siRNA

The internalization of Panitumumab, Pani Fab and Pani Fab-siRNA was determined by the previously described method with minor modification (Austin et al., 2004). To evaluate the number of EGFR molecules on the cell surface of AsPC-1 and A549, anti-human EGFR mouse monoclonal antibody (H11; Thermo Fisher Scientific) was added to the cells in a 12-well plate at 50 nmol/L and then incubated on ice for 1 h. After washing with PBS-0.1% BSA-Azide (0.1% bovine serum albumin [BSA] and 0.1% sodium azide in phosphate-buffered saline [PBS]), Fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG F(ab’)2 was added to each well and incubated on ice for 1 h. After washing with PBS-0.1% BSA-Azide, cells were detached from the plate with Cell Dissociation Buffer (Thermo Fisher Scientific) and analyzed using a FACS cantoII System (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The number of EGFR molecules on the cell surface was estimated using QIFIKIT (DAKO, Glostrup, Denmark) according to the manufacturer’s protocol.
For internalization analyses of Panitumumab, Pani Fab and Pani Fab-siRNA by a quenching method, four types of samples were prepared. In brief, cells were incubated with Alexa Fluor 488-labeled test article (10 nM) at 37 °C in 5% CO₂ for 1, 2, 4 and 6 h. After washing with ice-cold PBS-Azide (0.1% sodium azide in PBS), cells were incubated in PBS-Azide (Unquenched cells) or with Quenching antibody (Anti-Alexa Flour 488, Rabbit IgG; Thermo Fisher Scientific) (Quenched cells) on ice for 1 h. The quenching efficiency was corrected by Quench control cells; the cells were incubated with Alexa Fluor 488-labeled test articles (10 nM) for 1 h on ice. After washing with ice-cold PBS-Azide, cells were incubated with Quenching antibody on ice for 1 h. Non-treated cells were used as Blank cells. The cells were detached from the plate with Cell Dissociation Buffer (Thermo Fisher Scientific), and the mean fluorescence intensity (MFI) was measured by the FACScantoII System. Internalized ratios of test articles were calculated by the following equation: Internalized ratio = (MFI of Quenched cells − MFI of Quench control cells) × 100 / (MFI of Unquenched cells − MFI of Blank cells). The internalized test article was quantified by multiplying the internalized ratio by the number of antibodies bound to the cell surface.

Analyses of the internalization pathway by confocal microscopy

The internalization pathway of Pani Fab-siRNA was investigated in A549 cells. A549 cells were seeded on Lab-Tek II Chambered Coverglass and cultured overnight to attach. The cells were incubated with Pani Fab-siRNA-Cy3 (10 nM) in the presence or absence of non-labeled Panitumumab (200 nM) at 37 °C with 5% CO₂ for 4 h. Cells were washed once after the incubation and then analyzed by laser scanning confocal microscopy (LSM700; Zeiss,
Oberkochen, Germany). The internalization pathway of Pani Fab-siRNA was then evaluated using endocytotic pathway-specific markers, as follows: clathrin-mediated endocytosis marker, Alexa488-transferrin (50 μg/mL); caveolae-mediated endocytosis marker, Alexa488-cholera toxin (5 μg/mL); and macropinocytosis marker, FITC-Dextran (0.5 mg/mL). Each endocytotic pathway-specific marker was incubated with Pani Fab-siRNA-Cy3 at 37 °C in 5% CO₂ for 3 h. Cells were washed once after the incubation and then analyzed by laser scanning confocal microscopy. All endocytotic pathway-specific markers were purchased from Thermo Fisher Scientific.

**Lysosomal trafficking efficiency**

Lysosomal trafficking of Panitumumab was investigated by co-localization with a lysosome-staining marker. A549 cells were seeded in a Greiner µCLEAR 96-well plate and cultured overnight to attach. The cells were incubated with Alexa647-labeled Panitumumab (100 nM) at 37 °C in 5% CO₂ for 2, 4, 6, 8, and 24 h. Hoechst 33342 solution (Dojindo Laboratories) and Lysotracker green DND-26 (Thermo Fisher Scientific) were added to cells 15 and 3 min before the end of incubation, respectively. Cells were washed once after the incubation and then analyzed using a fully automated laser-scanning confocal cell imaging system (IN Cell Analyzer 6000; GE Healthcare). Image stack files were analyzed with a high-content image analysis software program (IN Cell Developer Toolbox; GE Healthcare). Internalized antibodies and lysosomes were automatically identified by their respective fluorescence and granular shape. The summation of fluorescence intensity of the internalized antibody and the internalized antibody
co-localized with lysosomes was separately quantified, and the lysosomal trafficking efficiency was determined.

Animals

C.B-17/ICR-scid/scidJcl male mice were purchased from Clair Japan (Tokyo, Japan). All animal studies were performed in accordance with the Standards for Proper Conduct of Animal Experiments at Kyowa Kirin Co., Ltd. (Tokyo, Japan), under the approval of the company’s Institutional Animal Care and Use Committee. Fuji Research Park of Kyowa Kirin Co., Ltd., is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (10-213).

Pharmacokinetic studies in xenograft mice

C.B-17/ICR-scid/scidJcl male mice were subcutaneously inoculated with 2 million AsPC-1 cells in a volume of 0.1 mL PBS per mouse. Mice were randomly grouped when the tumor size reached around 100 mm³ (n=3 per group). Animals in each group were intravenously administered Pani Fab or Pani Fab-siRNA at 5 mg/kg on day 0. Blood (approximately 0.3 ml) and tissues (tumor, kidney) were collected at 1, 6 and 24 h after drug administration under isoflurane anesthesia. For mice dissected at 24 h post-dose, blood samples were serially collected from the tail vein at 10 min and 0.5, 1, 2, 4 and 6 h. Heparinized plasma was isolated from blood via centrifugation at 13,000 rpm for 5 minutes and stored at −80 °C. The excised tissues were rinsed with saline, soaked in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan) and gently frozen in a gas phase of liquid nitrogen to obtain a frozen tissue block. Frozen tissue
blocks were cryosectioned at 5-μm thickness, placed on slide glasses and stored frozen at −80 °C. A portion of the tissue was frozen with liquid nitrogen for tissue concentration measurement. A 9-fold volume of 0.5% NP-40/PBS and metal cones were added to the tissue sample, followed by homogenization with a Multi-Beads Shocker (YASUI KIKAI Co., Ltd., Osaka, Japan). The obtained homogenates were stored at −80° C.

Quantification method for pharmacokinetic evaluations

Three quantification methods were used for determining plasma and tissue concentrations of Pani Fab, Pani Fab-siRNA and Pani Fab-Biotin.

Pani Fab concentrations were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as described below. 1) Capture antibody (goat anti-human kappa light chain antibody) was added to each well of a 96-well plate (Maxisorp immuno plate; Thermo Fisher Scientific) and incubated at room temperature for 1 h. 2) Incubation was then performed with blocking buffer (PBS containing 1% [w/v] casein) for 1 h. 3) Calibration standards, quality control (QC) and test samples were added in duplicate and incubated for 1 h. 4) Incubation was then performed with the detection antibody (biotinylated goat anti-human kappa light chain antibody) for 1 h. 5) Incubation was then performed with Streptavidin-HRP (Thermo Fisher Scientific) for 1 h. 6) The plate was then incubated with 1-step ultra TMB-ELISA (Thermo Fisher Scientific) for 5 min, followed by addition of 1 mol/L HCl. The UV light absorbance at 450 nm was measured using a plate reader (ARVO; PerkinElmer, Waltham, MA). Between the individual steps, plates were washed 3 times with PBS containing 0.05% Tween-20. For preparation of calibration standards (1-40 nM) and QC samples (2, 16 and 40 nM), Pani Fab was
diluted with blank matrix. This method was used to determine Pani Fab concentrations after intravenous (i.v.) administration of Pani Fab and total Pani Fab concentrations after i.v. administration of Pani Fab-siRNA. Total Fab includes intact Pani Fab-siRNA and its degraded metabolites, in which linker-siRNA portion is degraded.

The concentrations of intact Pani Fab-siRNA in the plasma and tissue samples were determined as described below. 1) Anti human IgG Fab antibody (Bethyl Laboratories, Montgomery, TX, USA) was immobilized to Dynabeads M-270 Epoxy beads (Thermo Fisher Scientific) to prepare immunoprecipitation (IP)-beads, according to the manufacturer's protocol. 2) IP-beads were added to calibration standards, QCs and test samples and incubated for 2 h at 4 °C. 3) After washing IP-beads with PBS twice, the samples were put on magnetic separator, and the supernatant was removed. 4) Stem-loop RT primer solution was added to IP-beads and incubated at 95 °C for 5 min and at 16 °C for 10 min and then held at 4 °C. 5) RT reaction mix (dNTPs, MultiScribe Reverse Transcriptase, RT buffer and RNase inhibitor) of a TaqMan Micro RNA RT kit (Thermo Fisher Scientific) was prepared according to the manufacturer's protocol and added to IP-beads. A reverse transcriptase reaction was performed (at 50 °C for 30 min, at 42 °C for 30 min and at 85 °C for 5 min and then held at 4 °C) to obtain cDNA. 6) Quantitative PCR mix (Forward primer, Reverse primer, TaqMan probe and TaqMan Fast Advanced Master Mix) was added to cDNA, and real-time quantitative PCR was performed according to standard protocols using a Real-Time PCR System ABI7000 (Applied Biosystems, Waltham, MA, USA). The reactions were amplified with the following sequence for 40 cycles: 50 °C for 2 min, 95 °C for 20 sec, 95 °C for 3 sec and 60 °C for 30 sec. For preparation of calibration standards (0.1-5 nM) and QC samples (0.1, 1 and 5 nM), Pani Fab-siRNA was diluted with blank matrix. This
method was used to determine intact Pani Fab-siRNA after i.v. administration of Pani Fab-siRNA.

The sequences of the stem-loop RT primer, forward primer and reverse primer were as follows:

- **Stem-loop RT primer** (GTCGATCCAGTGAGGGGTCCAGGATATTGCACCTGGATACGACGGCAAA)
- **Forward primer** (ATCGCGGGAGGGCTTTCTTTTGT)
- **Reverse primer** (GTGCAGGGTCCGAGGT)
- **Taqman probe** (CTGGATACGACGGCAAA)

Pani Fab-Biotin concentrations were determined by a sandwich ELISA as described below. 1) Capture antibody (goat anti-human kappa light chain antibody) was added to each well of a 96-well plate (Maxisorp immuno plate; Thermo Fisher Scientific) and incubated at room temperature for 1 h. 2) Incubation was then performed with blocking buffer (PBS containing 1% [w/v] casein) for 1 h. 3) Incubation was then performed with calibration standards, QCs and test samples in duplicate for 1 h. 5) Incubation was then performed with Streptavidin-HRP (Thermo Fisher Scientific) for 1 h. 6) The plate was then incubated with a 1-step ultra TMB-ELISA (Thermo Fisher Scientific) for 5 min, followed by the addition of 1 mol/L HCl. The ultraviolet light absorbance at 450 nm was measured using a plate reader (ARVO; PerkinElmer). Between the individual steps, we washed the plates 3 times with PBS containing 0.5% Tween-20. For preparation of calibration standards (1-40 nM) and QC samples (2, 16 and 32 nM), Pani Fab-Biotin was diluted with blank matrix. This method was used to determine Pani Fab-Biotin concentrations after i.v. administration of Pani Fab-Biotin.

A calibration curve was generated by log-log regression, except for the 0 ng/mL sample, using the SOFTmax PRO software program (Nihon Molecular Devices, Tokyo, Japan). The
concentrations of Pani Fab and Pani Fab-siRNA were calculated by substituting the signal intensity into the regression equation for each calibration curve.

**Pharmacokinetic parameters**

Pharmacokinetic parameters (CL$_{\text{total}}$, $V_{\text{ss}}$, and $t_{1/2}$) were obtained by a non-compartmental analysis of a series of plasma concentration data for individual animals using Phoenix WinNonlin (Certara, Princeton, NJ, USA) (CL$_{\text{total}}$: total clearance, $V_{\text{ss}}$: steady-state distribution volume, $t_{1/2}$: plasma half-life).

**Immunohistochemistry examinations**

Tissue sections were stained as described below. 1) Sections were fixed in 4% paraformaldehyde and then immersed in 50 mmol/L NH$_4$Cl/PBS. 2) Sections were washed with PBS and permeabilized by 0.1% TritonX-100/PBS. 3) After PBS washing, sections were blocked with 5% BSA/PBS. 4) Sections were then incubated with anti-human kappa light chain Goat IgG at room temperature for 1 h. 5) After PBS washing, sections were incubated with Alexa 488-labeled anti-Goat IgG rabbit antibody (Thermo Fisher Scientific) at room temperature for 1 h. 6) After PBS washing, sections were covered in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Newark, CA, USA) and then analyzed by laser scanning confocal microscopy (LSM700; Zeiss).
In vitro plasma stability

Pani Fab-siRNA and naked siRNA were spiked in mouse heparinized plasma at concentrations of 5 μmol/mL and 100 μg/mL, respectively. The spiked plasma samples were incubated at 37 °C for 1, 4 and 24 h. Stability samples were stored at −80 °C until measurement.

For the analysis of naked siRNA, calibration standards were prepared by diluting naked siRNA with blank plasma. Calibration standards and stability samples were mixed with a 9-fold volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 5.2), followed by centrifugation. The upper aqueous layer was collected, and naked siRNA were precipitated by ethanol and Dr. GenTLE precipitation carrier (Takara Bio, Shiga, Japan). The dried pellets were reconstituted in RNase-free water and subjected to LC/MS. For the analysis of Pani Fab-siRNA, calibration standards were prepared by diluting Pani Fab-siRNA with blank plasma. Calibration standards and stability samples were digested by proteinase K in 6 M guanidine thiocyanate at 50 °C for 2 h. Digested Pani Fab-siRNA was then subjected to liquid-liquid extraction and ethanol precipitation with internal standard siRNA in the same manner as naked siRNA. Internal standard siRNA was only added for the quantitative analysis.

Extracted samples were analyzed by LC/MS. An ACQUITY TQD system (Waters, Milford, MA, USA) equipped with an ACQUITY UPLC system (Waters) was used. Antisense and sense strands of siRNA were separated on an ACQUITY UPLC Oligonucleotide BEH C18 Column (130 Å, 1.7 μm, 2.1 mm I.D. × 100 mm; Waters) by a gradient mode with 400 mM 1,1,1,3,3,3-hexafluoro-2-propanol/15 mM triethylamine (mobile phase A) and methanol (mobile phase B). The time course of the percentage of mobile phase B was 5% (0 min), 30% (9 min) and 5% (9.01–10 min) for naked siRNA, and 5% (0 min), 30% (8 min), 70% (8.01–9 min) and 5%
(9.01–10 min) for Pani Fab-siRNA. The column temperature was 70 °C. The antisense and sense strands of siRNA were ionized by negative electrospray ionization and detected by single-ion monitoring with the following Q1 ions: antisense strand 873.6 and sense strand 790.7 for naked siRNA and antisense strand 899.6 and sense strand 790.2 for Pani Fab-siRNA. The lower limit of quantification (LLOQ) was 30 μg/mL for naked siRNA and 0.3 μmol/L for Pani Fab-siRNA. In the metabolite analysis, a full mass scan was performed in the range of m/z 600-1000. The mass spectra of metabolite peaks were deconvoluted using the Microsoft Excel software program (Microsoft, Redmond, WA, USA).

**Results**

*Conjugation of siRNA with Pani Fab*

Pani Fab-siRNA was prepared with a two-step conjugation method, where Pani Fab was first conjugated with BM(PEG)3 followed by a second conjugation with KRAS siRNA-SH (Figure 1A). The reaction mixture of the second conjugation was purified by anion-exchange-HPLC (Supplemental Figure 1A). On SDS-PAGE under non-reduced conditions, Pani Fab-siRNA showed a single band at a reasonable molecular size (Supplemental Figure 1B), suggesting that Pani Fab-siRNA had been prepared and purified properly.

*mRNA knockdown*

Figure 1C shows the *in vitro* knockdown activity of Pani Fab-siRNA in AsPC-1 cells. Naked siRNA was added with transfection reagent and showed approximately 90% KRAS mRNA
knockdown at 10 nM. Pani Fab-siRNA was directly added to the cells and showed no knockdown activity up to 500 nM.

**Internalization analyses of Pani Fab-siRNA by flow cytometry**

Internalization of Pani Fab-siRNA was compared with that of Panitumumab and Pani Fab in AsPC-1 and A549 cells at a concentration of 10 nM. Figure 2A shows the time-courses of internalization of Panitumumab and Pani Fab in A549 cells. The internalization efficiency of Panitumumab and Pani Fab increased up to 6 h. To investigate the impact of siRNA conjugation, the internalization of Pani Fab-siRNA, Panitumumab and Pani Fab was evaluated at 4 h in AsPC-1 and A549 cells (Table 1). The numbers of internalized molecules were similar among Panitumumab, Pani Fab and Pani Fab-siRNA in each cell line, suggesting that Fab fragmentation and conjugation with siRNA did not markedly affect the internalization ability.

**Internalization analyses of Pani Fab-siRNA by confocal microscopy**

The internalization pathway of Pani Fab-siRNA-Cy3 in A549 cells was analyzed by confocal microscopy. Internalization of Pani Fab-siRNA-Cy3 (10 nM) was clearly observed on confocal images after 4 h of incubation and was completely inhibited in the presence of non-labeled Pani Fab, suggesting EGFR-mediated internalization of Pani Fab-siRNA (Figure 2B).

To investigate the internalization pathway, colocalization of Pani Fab-siRNA-Cy3 with typical internalization pathway markers (clathrin pathway: Alexa488-trasnferrin, caveolae pathway: Alexa488-cholera toxin, macropinocytosis: Fluorescein-dextran) was evaluated.
Colocalization of Pani Fab-siRNA-Cy3 and transferrin was clearly observed, suggesting that Pani Fab-siRNA was internalized mainly via the clathrin pathway (Figure 2C).

**Lysosomal trafficking of Panitumumab analyzed by confocal microscopy**

As an alternative to Pani Fab-siRNA and Pani Fab, which have a lower yield of fluorescent labeled material, lysosomal trafficking of Alexa 647-labeled Panitumumab was quantitatively analyzed by an IN Cell Analyzer 6000 (Figure 3A). Alexa 647-labeled Panitumumab (10 nM) was incubated with A549 cells, and lysosome was stained with Lysotracker green DND-26 before image observation. Panitumumab in lysosomes was identified by co-localization with Lysotracker green DND-26. Typical images are shown in Figure 3A. Co-localization of Panitumumab with lysosome was clearly observed at 24 h, although no co-localization was observed at 2 h. Internalized Panitumumab in lysosomes and the other endosomes was separately quantified. The time-courses of total internalized Panitumumab and Panitumumab in lysosomes are shown in Figure 3B. Total internalized Panitumumab increased in a biphasic manner, and the inclination observed in the second phase was smaller than that seen in the first phase. The lysosomal distribution of Panitumumab increased almost linearly up to 24 h, and the fraction of Panitumumab in lysosome against total internalized Panitumumab was calculated to be 52% at 24 h.

**Pharmacokinetics of Pani Fab and Pani Fab-siRNA in tumor xenograft mice**

The pharmacokinetic profiles of Pani Fab and Pani Fab-siRNA were evaluated in AsPC-1 tumor-bearing mice. Pani Fab or Pani Fab-siRNA was intravenously administrated to tumor-bearing
mice at 5 mg/kg. For the samples obtained after i.v. administration of Pani Fab-siRNA, intact Pani Fab-siRNA and total Pani Fab were separately measured. The total Pani Fab-siRNA included intact Pani Fab-siRNA and its degraded metabolites, in which the linker-siRNA portion was degraded. Only the concentration of intact Pani Fab-siRNA is related to the in vivo activity.

The plasma concentration-time curves and pharmacokinetic parameters are shown in Figure 4A and Table 2, respectively. Clearance was similar between Pani Fab and Pani Fab-siRNA, but Pani Fab-siRNA showed a shorter half-life and smaller distribution volume than Pani Fab. In the Pani Fab-siRNA-treated group, the plasma concentration of intact Pani Fab-siRNA became lower than that of total Pani Fab-siRNA, suggesting degradation in the linker-siRNA portion during circulation in systemic blood (Figure 4A).

The tumor concentration of intact Pani Fab-siRNA in the Pani Fab-siRNA-treated group was lower and decreased more rapidly than that of Pani Fab in the Pani Fab-treated group (Figure 4B). The tumor concentration of total Pani Fab in the Pani Fab-siRNA-treated group increased up to 6 h and became comparable to that of Pani Fab in the Pani Fab-treated group.

The kidney concentrations were evaluated to measure the renal excretion of Pani Fab-siRNA. The kidney concentration of Pani Fab in the Pani Fab-treated group was much higher than those of intact Pani Fab-siRNA and total Pani Fab at 1 h post-dose but rapidly decreased (Figure 4C).

**Pharmacokinetics of Pani Fab-Biotin conjugate in xenograft mice**

To examine the stability of conjugation site (A140C) and BM(PEG)3 linker, we prepared Pani Fab-Biotin conjugate, and its pharmacokinetic profile was evaluated in AsPC-1 xenograft mice.
Plasma and tumor concentrations of Pani Fab-Biotin were compared to those of Pani Fab in the Pani Fab-treated group. There was no apparent difference in the plasma or tumor concentrations between Pani Fab and intact Pani Fab-Biotin for 6 h (Figure 5A, 5B).

**Immunohistochemical analyses of intra-tumor distribution of Pani Fab-siRNA.**

Intra-tumor distribution of pani Fab-siRNA was investigated by immunohistochemical analyses. As shown in Figure 6, Pani Fab-siRNA bound to the membrane of tumor cells at 1 h after administration and gradually penetrated a wide area of the tumor and internalized into cancer cells over time. There was no marked difference in the intra-tumor distribution between the Pani Fab- and Pani Fab-siRNA-treated groups (Supplemental Figure 2).

**Metabolite analyses of naked siRNA and Pani Fab-siRNA by MS**

The *in vitro* stability of Pani Fab-siRNA in mouse plasma was evaluated and compared with that of naked siRNA. To investigate the stability of the linker-siRNA portion of Pani Fab-siRNA, samples were digested by proteinase K, and released linker-siRNA with digested peptide was extracted and analyzed by LC/MS. Figure 7A shows the total ion chromatograms of naked siRNA and proteinase K-digested Pani Fab-siRNA. Sense and the antisense strands were separated well. In proteinase K-digested Pani Fab-siRNA, the antisense strand was detected with linker and Pani Fab-derived peptide (GGTCALG) (Figure 7B). Supplemental Figure 3A shows total ion chromatograms of naked siRNA before and after incubation in mouse plasma. The peaks of the sense and antisense strands gradually decreased over incubation time, and metabolite peaks (P1 and P2) appeared instead (Supplemental Figure 3A). Given the molecular
mass of P1: 8427.1 and P2: 7626.1, we suspected that both the sense and antisense strands underwent 3’exonuclease digestion in mouse plasma (Supplemental Figure 3B). The sense and antisense strands of naked siRNA showed a similar stability in mouse plasma, whereas the sense strand of Pani Fab-siRNA was more stable than the antisense strand (Figure 7C).

Discussion

In the present study, we performed a comprehensive evaluation of the intracellular and in vivo disposition of Fab-siRNA conjugate. A site-directed variant of Fab (A140C) was designed for simple and efficient conjugation. The thiol moiety of A140C has difficulty forming disulfide bonds with cysteine or glutathione in media. As a result, this allows for direct conjugation with a maleimide-linker without reductive pretreatment to activate the thiol (Shiraishi et al., 2015). Using Fab (A140C), Fab-siRNA conjugate was prepared with good purity.

An in vitro internalization study revealed that the cellular uptake of Fab-siRNA was similar to that of Panitumumab and Pani Fab and completely inhibited in the presence of excess Panitumumab. These results suggested that siRNA conjugation to Fab at the A140C position does not cause non-specific cellular binding or affect the antigen-mediated internalization of Fab. A previous paper reported non-specific cell binding of antibody-siRNA conjugate when double-stranded hybridization of the siRNA is not perfect (Lehot et al., 2021). In our conjugate preparation method, siRNA was pre-annealed after sufficient denaturation at 90 °C and then subjected to conjugation with Pani-Fab. This process allowed for perfect annealing and no non-specific cell binding.
The internalized molecules of Pan Fab-siRNA in AsPC-1 and A549 numbered approximately 30,000 at 4 h, which is much higher than the required number of siRNA molecules (300-1000 molecules) in cytoplasm for RNA interference (Jagannath and Wood, 2009). However, despite a more than sufficient number of internalized siRNA molecules, Pan Fab-siRNA did not show target gene knockdown (Figure 1C). To investigate the reason for this issue, lysosomal trafficking of fluorescent-labeled Panitumumab was quantitatively evaluated using confocal microscopy. The total amount of internalized Panitumumab increased in a biphasic manner, with a rapid initial phase followed by a slower second phase. Such biphasic internalization can be explained by the recycling process of EGFR. After rapid internalization (1st phase), the apparent internalization rate reaches a steady state (2nd phase) of internalization, recycling, and transfer from endosomes to other cellular compartments (e.g. lysosomes). In contrast, the amount of Panitumumab transferred in lysosomes increased linearly, and the slope was apparently parallel to that of the second phase of total internalized Panitumumab. This suggests that the majority of internalized Panitumumab in endosomes is transferred to lysosomes. It is generally known that acidification during the maturation process of endosomes leads to dissociation of immunoprotein-siRNA conjugates from the receptors, triggering endosomal escape (Springer and Dowdy, 2018) (Cuellar et al., 2015). Therefore, efficient endosomal escape and stabilization in lysosomes is needed for Pan Fab-siRNA conjugate to achieve target gene knockdown. This study suggested that Pan Fab-siRNA internalizes via a clathrin-mediated endocytosis pathway. Although the impact of the internalization pathway on mRNA knockdown activity has been discussed in depth by Cuellar et al. (2015), the mechanism of intracellular trafficking of each pathway remains to be fully elucidated. Further understanding the process of
the trafficking mechanism from endosomes to lysosome compartments will help to realize the
design of Fab-siRNA conjugates that improve endosomal escape efficiency. Alnylam’s GalNAc-
siRNA technology is a successful example of receptor-mediated delivery of siRNA. GalNAc-
siRNA is internalized into hepatocytes via asialoglycoprotein receptor (ASGPR) and shows great
target gene knockdown activity. The number of ASGPRs on hepatocytes is very high
(approximately 10^6 per cell), and its rapid uptake and recycling are thought to contribute to
GalNAc-siRNA delivery (Springer and Dowdy, 2018). According to Alnylam's research on its
intracellular dynamics, ligand-receptors are rapidly dissociated at low intracellular pH, and a
certain amount of GalNAc-siRNA is pooled in endosomes (Brown et al., 2020). In addition,
membrane-permeable peptides, such as R9 and TAT, are reportedly useful for improving
endosome escape efficiency (Varkouhi et al., 2011), (Teo et al., 2021). Structurally, our Pani
Fab-siRNA has the potential to additionally conjugate endosome escape elements, such as
peptides, in high uniformity (e.g. the 3'end of the sense strand). Further technological
development will be required to overcome the "dilemma" of improving intracellular endosome
escape efficiency while suppressing the non-specific uptake.

The in vivo pharmacokinetics of Pani Fab and Pani Fab-siRNA were compared in AsPC-1
tumor xenograft mice. The half-life of Pani Fab was about 2 h, which was similar to previously
reported half-lives of Fab. The kidney concentration of Pani Fab was very high at earlier time
points. This is reasonable, since the major elimination pathway of Fab is urinary excretion.
Compared to Pani Fab, the kidney concentrations of intact Fab-siRNA were much lower,
possibly due to the higher molecular weight of Fab-siRNA (approximately 66 kDa), which
cannot undergo glomerular filtration. The serum concentrations of intact Fab-siRNA were lower
than those of total Fab-siRNA, suggesting degradation of the linker-siRNA portion during circulation in blood. The degradation of siRNA in antibody-siRNA was also reported previously (Tan et al., 2012). The clearance of Fab-siRNA was comparable to that of Pani Fab. However, the major elimination pathway of Fab-siRNA shifted from renal excretion to siRNA degradation. Tumor concentrations of Fab-siRNA were much lower than those of Pani Fab, although the clearance of Fab-siRNA was comparable to that of Pani Fab, possibly because the linker-siRNA portion of Fab-siRNA may also have been degraded in the tumor. This is also supported by the fact that total Fab-siRNA remained in the tumor for as long as 24 h after administration, whereas intact Fab-siRNA disappeared rapidly. An immunohistochemical analysis revealed a wide distribution of Fab-siRNA in the tumor, as expected from previous reports (Debie et al., 2020), (Nessler et al., 2020). Fab may be distributed more widely in tumor than intact antibody due to its smaller molecular size. Given the rapid disappearance of siRNA in the blood, it is a rational strategy to conjugate siRNA to Fab fragments, which also have a short half-life, and penetrate deep into the tumor in a short time.

**In vivo** pharmacokinetic studies revealed stability of linker-siRNA to be a key issue of Fab-siRNA. To improve the stability, it is necessary to clarify the degradation mechanism. The biotransformation studies of ADC are good references for investigating the metabolic pathway of Fab-siRNA. In the case of ADC, deconjugation and linker degradation were observed in circulating blood (Huang et al., 2021), (He et al., 2018). To investigate the possibility of deconjugation and linker degradation, **in vivo** pharmacokinetics of Pani Fab-Biotin were evaluated. As a result, BM(PEG)3 linker and conjugation site (A140C) were revealed to be
stable in both the plasma and tumor, indicating that siRNA degradation is the major degradation pathway of Fab-siRNA.

A metabolite analysis of siRNA is generally performed by ion-pair reversed-phase LC/MS (Jiao et al., 2012), (Ramanathan and Shen, 2019), (Sutton et al., 2021); however, Pani Fab-siRNA cannot be subjected to ion-pair reversed-phase LC/MS. To overcome this issue, we developed a novel LC/MS method by combining proteinase K digestion. In brief, Fab-siRNA was digested by proteinase K, and siRNA tagged with the small peptide fragment of Fab was purified by liquid-liquid extraction and then subjected to ion-pair reversed-phase LC/MS. As expected, siRNA tagged with the Fab-derived peptide was able to be detected by LC/MS. Based on a mass analysis, the Fab-derived peptide was identified as GGTCALG. Using this method, the stability and metabolites of the siRNA were investigated separately for the sense and antisense strands. In the case of Fab-siRNA, the antisense strand degraded much more rapidly than the sense strand, whereas the degradation rates were similar between the sense and antisense strands for naked siRNA. Metabolite analyses revealed that 3'-end hydrolysis was the major degradation pathway for both the sense and antisense strands. In our Fab-siRNA format, the 5’-end of the antisense strand is conjugated to Fab, and the 3’-end of the sense strand is spatially close to Fab. Therefore, steric hinderance by Fab may be the reason for greater stability of the sense strand than the antisense strand.

As mentioned in the Introduction section, Fab-siRNA must overcome multiple hurdles to achieve target gene knockdown in tumor, including antigen-dependent delivery to tumors, deep tumor penetration, efficient internalization and endosomal escape in tumor cells. In conclusion, the two factors of poor tumor accumulation and poor endosomal escape have therefore been
identified as important issues which need to be improved in the Fab-siRNA format. The poor tumor accumulation comes from the poor stability in blood and tumors, and the major degradation pathway in plasma was identified as 3’-end hydrolysis of the antisense strand. Therefore, stabilizing the 3’-end of the antisense strand was suggested to be the most effective way to improve the in vivo target gene knockdown activity. Improving the stability may also increase endosomal escape efficiency by improving the stability of siRNA during endosome-to-lysosome transition. Such a systematic approach to evaluating the intracellular and in vivo disposition will provide very important information to help improve the in vivo activity for not only Fab-siRNA but also other immunoprotein-siRNA conjugates.
Acknowledgments

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Authorship Contributions

Participated in research design: Toshima, Shiraishi, Enokizono.

Conducted experiments: Toshima, Shinmi.

Performed the data analysis: Toshima.

Wrote or contributed to the writing of the manuscript: Toshima, Enokizono, Kagawa.
References


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Fab Constant Region for Site-Specific Conjugation. Bioconj Chem 26:1032–1040, American Chemical Society.


Footnotes

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Figure Legends

**Figure 1.** Structure and properties of Pani Fab-siRNA. (A) Schematic representation of the Pani Fab-siRNA preparation process. Pani Fab and KRAS siRNA were combined using the BM(PEG)3-linker. The siRNA sequence of 2’-O-methyl nucleotide is underlined, while that of deoxyribonucleotide is italicized. (B) A proposed mechanism of action of Pani Fab-siRNA. (C) *In vitro* mRNA knockdown activity of Pani Fab-siRNA in AsPC-1 cells after 24 h of treatment. Each bar represents the mean ± S.D. (n = 3).

**Figure 2.** Internalization profile of Pani Fab-siRNA in A549 cells. (A) Time courses of internalized Panitumumab and Pani Fab in A549 cells (10 nM). (B) Internalization of Pani Fab-siRNA-Cy3 (10 nM) in A549 cells in the presence or absence of non-labeled Panitumumab (200 nM) (40×, bar = 20 μm). (C) Colocalization of internalized Pani Fab-siRNA-Cy3 (10 nM) and pathway-specific cargo after 3-h incubation (40×, bar = 10 μm).

**Figure 3.** Lysosome trafficking profile of Panitumumab. (A) Confocal imaging of intercellular disposition of Alexa 647-labeled Panitumumab in A549 cells (60×, bar = 10 μm). (B) Quantitative determination of internalization (Endosome+Lysosome) and lysosome trafficking amount of Alexa 647-labeled Panitumumab in A549 cells. Each symbol represents the mean ± S.D. (n = 4).
**Figure 4.** Results of a pharmacokinetic evaluation of Pani Fab and Pani Fab-siRNA after intravenous administration of Pani Fab and Pani Fab-siRNA to AsPC-1 xenograft mice at 5 mg/kg. The Pani Fab concentration was measured for the Pani Fab-treated group. In the Pani Fab-siRNA-treated group, total Pani Fab was measured in addition to the intact Pani Fab-siRNA. Total Pani Fab includes intact Pani Fab-siRNA and Pani Fab where the siRNA portion was degraded. Concentration-time profiles in plasma (A), tumor (B) and kidney (C) are shown. Each symbol represents the mean ± S.D. (n = 3)

**Figure 5.** Plasma (A) and tumor (B) concentration-time profiles of Pani Fab and Pani Fab-Biotin after single intravenous administration at 5 mg/kg in AsPC-1 xenograft mice. Each symbol and bar represents the mean ± S.D. (n = 3)

**Figure 6.** Results of an immunohistochemical analysis of *in vivo* tumor distribution of Pani Fab siRNA after single intravenous administration at 5 mg/kg in AsPC-1 xenograft mice. Upper row: ×10, bar = 100 μm, lower row: ×100, bar = 10 μm.

**Figure 7.** *In vitro* mouse plasma stability and metabolite estimation of naked siRNA and Pani Fab-siRNA by using LC/MS. (A) Total ion chromatogram (TIC) of naked siRNA (upper) and proteinase K digested Pani Fab-siRNA (lower). (B) MS spectra of the antisense strand of naked siRNA (upper) and Pani Fab-siRNA (lower). (C) *In vitro* mouse stability of naked siRNA and Pani Fab-siRNA.
## Tables

### Table 1. Quantification of internalized molecules of Panitumumab, Pani Fab and Pani Fab-siRNA (10 nM) in AsPC-1 and A549 cells after 4 h incubation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell line</th>
<th>EGFR Expression</th>
<th>Internalization efficiency (%)</th>
<th>Number of internalized molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panitumumab</td>
<td>AsPC-1</td>
<td>$2.3 \times 10^5$</td>
<td>14.4</td>
<td>$3.3 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>$1.5 \times 10^5$</td>
<td>20.1</td>
<td>$3.0 \times 10^4$</td>
</tr>
<tr>
<td>Pani Fab</td>
<td>AsPC-1</td>
<td>$2.3 \times 10^5$</td>
<td>20.1</td>
<td>$4.6 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>$1.5 \times 10^5$</td>
<td>23.9</td>
<td>$3.6 \times 10^4$</td>
</tr>
<tr>
<td>Pani Fab-siRNA</td>
<td>AsPC-1</td>
<td>$2.3 \times 10^5$</td>
<td>16.2</td>
<td>$3.7 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>$1.5 \times 10^5$</td>
<td>19.3</td>
<td>$2.9 \times 10^4$</td>
</tr>
</tbody>
</table>

a) Number of internalized molecules = Internalization efficiency (%) × EGFR expression

### Table 2. Plasma PK parameters of Pani Fab and intact Pani Fab-siRNA in AsPC-1 xenograft mice. Each value represents the mean ± S.D. (n = 3). AUC, area under the curve; MRT, mean residence time.

<table>
<thead>
<tr>
<th></th>
<th>t$_{1/2}$</th>
<th>AUC$_{0-\infty}$</th>
<th>CL</th>
<th>MRT</th>
<th>V$_{ss}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>h</td>
<td>ng*h/mL</td>
<td>mL/kg</td>
<td>h</td>
<td>mL/kg</td>
</tr>
<tr>
<td>Pani Fab</td>
<td>$2.07 \pm 0.30$</td>
<td>$55608 \pm 4502$</td>
<td>$90.3 \pm 7.3$</td>
<td>$1.04 \pm 0.04$</td>
<td>$93.6 \pm 6.3$</td>
</tr>
<tr>
<td>Intact Pani Fab-siRNA</td>
<td>0.93 ± 0.03</td>
<td>63057 ± 885</td>
<td>79.3 ± 1.1</td>
<td>0.62 ± 0.06</td>
<td>49.4 ± 3.9</td>
</tr>
</tbody>
</table>
Figure 1

A

Hc A140C
SH

Pani Fab

BM(PEG)3

Pani Fab-BM(PEG)3

KRAS siRNA-SH

Sense strand
5’-GGAGGGCUUUCUUUGUGUAUUUGCC-3’

Antisense strand
3’-CCCCUCCCGAAGAACACAUAAACGG-5’

Pani Fab-siRNA

B

recycling
internalization
endosomal escape
early endosome
lysosome

degradation
RNAi

C

![Bar chart](image)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Relative mRNA Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>500nM Pani Fab-siRNA</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>100nM Pani Fab-siRNA</td>
<td>1.2 ± 0.05</td>
</tr>
<tr>
<td>10nM naked siRNA/ RNAiMAX</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>NT</td>
<td>1.0 ± 0.05</td>
</tr>
</tbody>
</table>

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Figure 2

A

![Graph showing internalized efficiency (%) vs. incubation time (h) for Panitumumab and Pani Fab.]

B

**Pani Fab-siRNA-Cy3**

- **non-labeled panitumumab (-)***

- **non-labeled panitumumab (+)***

C

**Transferrin**  **Cholera toxin subunit B**  **Dextran**

**Pani Fab-siRNA-Cy3**
**Figure 3**

**A**

Red: Alexa 647 panitumumab  
Green: Lysosome  
Blue: Nuclear  
Yellow: Colocalization

2h  
24h

**B**

- Endosome + Lysosome  
- - Lysosome

Fluorescence intensity/cell

0  
200000  
400000  
600000  
800000  
1000000  
1200000

0  
4  
8  
12  
16  
20  
24

Incubation time (h)
Figure 6

Green: Fab, Blue: Nuclear
Figure 7

A

Antisense strand

Sense strand

+ BM(PEG)3

+ Pani Fab peptide

B

Naked siRNA

Pani Fab-siRNA

Proteinase K digest

C

<table>
<thead>
<tr>
<th></th>
<th>0h</th>
<th>4h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked siRNA</td>
<td>100</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>Pani Fab-siRNA</td>
<td>100</td>
<td>80</td>
<td>60</td>
</tr>
</tbody>
</table>

Remaining (%)