Pre-clinical development of U3-1784, a novel FGFR4 antibody against cancer, and avoidance of its on-target toxicity

Authors’ names
René Bartz1*, Keisuke Fukuchi2*, Toshiaki Ohtsuka2, Tanja Lange1, Katrin Gruner1, Ichiro Watanabe3, Shinko Hayashi2, Yoko Oda2, Reimi Kawaida2, Hironobu Komori5, Yoshinori Kashimoto5, Peter Wirtz1, Jan-Peter A. Mayer1, Mauricio Redondo-Müller1, Shuntaro Saito9, Mizuki Takahashi5, Hiroyuki Hanzawa5, Emi Imai5, Alberto Martinez6, Masaharu Hanai4, Dieter Häussinger7, Roger W. Chapman8, Toshinori Agatsuma2, Johannes Bange1 and Reimar Abraham1,†

Authors’ affiliations
1 U3 Pharma GmbH/Daiichi-Sankyo, Martinsried; Germany
2 Biologics & Immuno-Oncology Laboratories, Daiichi Sankyo Co., Ltd., Tokyo, Japan
3 Modality Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo, Japan
4 Global Project Management Department, Daiichi Sankyo Co., Ltd, Tokyo, Japan
5 Daiichi Sankyo RD Novare Co., Ltd., Tokyo, Japan
6 Daiichi Sankyo Development, Gerrards Cross, UK
7 Heinrich-Heine-University, Düsseldorf, Germany
8 University of Oxford, Oxford, U.K.
9 Analytical & Quality Evaluation Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo, Japan
† Present address: Lead Discovery Center GmbH, Planegg, Germany

Running title: Efficacy and toxicology of U3-1784, a novel FGFR4 antibody

*These authors contributed equally to the work
# Corresponding author: abraham@lead-discovery.de
Lead Discovery Center GmbH
82152 Planegg, Germany
Tel.: +49 89 954586202
Financial Information

This work was supported by Daiichi-Sankyo

Conflict of Interest Statement

The authors declare no potential conflicts of interest.
Abstract

The FGFR4/FGF19 signaling axis is over-activated in 20% of liver tumors and currently represents a promising targetable signaling mechanism in this cancer type. However, blocking FGFR4 or FGF19 has proven challenging due to its physiological role in suppressing bile acid synthesis which leads to increased toxic bile acid plasma levels upon FGFR4 inhibition. An FGFR4-targeting antibody, U3-1784, was generated in order to investigate its suitability as a cancer treatment without major side effects. U3-1784 is a high affinity fully human antibody that was obtained by phage display technology and specifically binds to FGFR4. The antibody inhibits cell signaling by competing with various FGFs for their FGFR4 binding site thereby inhibiting receptor activation and downstream signaling via FRS2 and Erk. The inhibitory effect on tumor growth was investigated in 10 different liver cancer models in vivo. The antibody specifically slowed tumor growth of models overexpressing FGF19 by up to 90% whereas tumor growth of models not expressing FGF19 was unaffected. In cynomolgus monkeys, intravenous injection of U3-1784 caused elevated serum bile acid and liver enzyme levels indicating potential liver damage. These effects could be completely prevented by the concomitant oral treatment with the bile acid sequestrant colestyramine, which binds and eliminates bile acids in the gut. These results offer a new biomarker-driven treatment modality in liver cancer without toxicity and they suggest a general strategy for avoiding adverse events with FGFR4 inhibitors.
Introduction

Fibroblast Growth Factor Receptors (FGFRs) are a family of 4 receptor tyrosine kinases that play diverse and important roles in development and adult physiology. Their deregulation contributes to various diseases, particularly cancer, and therefore several inhibitors are in development targeting these receptors. However, none of these inhibitors has to date been approved by the FDA.

In mammals, there are 22 Fibroblast Growth Factors (FGFs) that can bind to one or several distinct FGFRs. Ligand binding leads to receptor dimerization and activation of the tyrosine kinase which in turn phosphorylates proteins intracellularly thus initiating a signaling cascade with two important routes either mediated via Extracellular Signal Regulated Kinase (Erk) or Phospholipase-Cγ (PLCγ). Most of the FGFs require binding to heparin on the cell surface to mediate activation of the FGFRs and are therefore thought to act locally in paracrine or autocrine fashion. Interestingly, 3 FGFs - FGF19, FGF21 and FGF23- do not bind heparin well, resulting in a hormone-like distribution in the whole body. These FGFs require co-factors of the klotho family for receptor activation (1–3).

Liver cancer is the second highest cause of cancer related death worldwide and the 6th most prevalent cancer (4). However, due to the limited understanding of the driving mechanisms in this heterogeneous disease, the multi-kinase inhibitor sorafenib remains the standard of care for non-resectable liver cancers. Sorafenib prolongs patient lives on average only 3 months and is associated with considerable adverse effects. Since its approval in 2007, only regorafenib, another multi-kinase inhibitor, achieved approval in 2017 for the treatment of patients who failed sorafenib (5, 6).

Aberrant FGFR4 signaling has been reported for several cancer types including breast, colon and pituitary carcinoma (7–15), current data suggests that FGFR4 is a cancer driver in hepatocellular carcinoma. FGFR4 is the dominant FGFR in hepatocytes and while hepatocytes do not express FGF19, about 15-20% of liver tumors express the ligand due to amplification of the FGF19 locus thus creating an autocrine loop that can fuel cancer cell growth (16, 17). Consequently, liver cancer patients with tumors overexpressing FGF19 have a shorter overall survival than patients whose tumors express lower levels or no FGF19 (18, 19). These data may therefore present a unique opportunity for liver cancer treatment since they identify -for the first time- a prevalent genetic lesion (amplification of FGF19) in this type of cancer. Recently, multiple efforts have been made to target FGFR4 or FGF19 in liver cancer by means of pharmacological inhibition. However, these efforts have proven difficult because pharmacological inhibitors may also interfere with the physiological role of FGFR4 in bile acid (BA) regulation. While FGFR4 knock-out mice grow and reproduce normally, they exhibit a larger BA pool than wild type mice (20). Subsequent studies revealed that BAs in the small intestine activate the transcription of FGF19 which travels back to the liver - presumably via blood in the portal vein - where it activates FGFR4. This results in the transcriptional repression of CYP7A1 the gene encoding Cholesterol 7a-hydroxylase which catalyzes the rate-limiting step in BA synthesis (21, 22). Consequently, interfering with the FGFR4/FGF19 axis has been shown to increase...
BA levels and to induce severe adverse effects including liver damage in cynomolgus monkey (23). Here, we report the development and characterization of U3-1784, an anti-FGFR4 antibody as well as a concomitant strategy to counteract antibody-mediated BA increases in cancer patients.
Material and Methods

Ethics statement
All rodent studies were conducted with approval and in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo (Tokyo, Japan). Studies in non-human primates (Cynomolgus monkeys, macaca fascicularis) were conducted at WIL Research Europe-Lyon (Saint Germain-Nuelles, France) according to a written study protocol and facility standard operating procedures in strict compliance with national legal regulations on animal welfare and accepted animal welfare standards. All other animal studies were conducted at selected CROs with respective guidelines in place.

Reagents and antibodies
FGFR1-4 (extracellular domain) from mouse and human was obtained from R&D Systems (Minneapolis, U.S.A.), FGFR4 (rat and rhesus monkey) from Sino Biologicals. Sorafenib was obtained from Bayer and Colestyramine from Bristol-Myers Squibb. U3-1784 was produced recombinantly either transiently expressed in FreeStyle 293-F cells (ThermoFisher) or in stably expressing CHO cells. Purification from the cell culture medium to more than 95% purity was achieved by Protein A affinity chromatography followed by size exclusion chromatography. The antibody preparations contained less than 0.5 EU/ml bacterial endotoxin as measured by the Limulus Amebocyte Lysate assay (Figure S1). For the determination of antibody concentrations, a molecular weight of 150 kDa was assumed for each antibody.

ELISA
ELISA plates coated with the extracellular domain of either FGFR4 (from rat, mouse, monkey and human) or FGFR1-4 (human) were incubated with U3-1784 (1:4 serial dilution), washed and binding was finally detected via an alkaline phosphatase coupled anti-human antibody (Jackson Immunoresearch, U.S.A.) and the AttoPhos® AP Fluorescent Substrate System (Promega, U.S.A.). For the FGF19 displacement assay recombinant human FGF19 was added at 120 ng/mL prior to incubation with antibodies and detection of bound FGF19 was achieved using a biotin-coupled anti-FGF19 antibody (R&D Systems, U.S.A.) followed by alkaline phosphatase-coupled Streptavidin (R&D Systems) and Attophos. For the detection of FGF19 protein in tumor lysates, a specific FGF19 ELISA was used (R&D Systems). All measurements were conducted with a Fluostar Omega fluorescent plate reader instrument from BMG Labtech (Ortenberg; Germany) and subsequent dissociation constants and IC50 values were calculated by non-linear regression using GraphPad Prism version 5.04 or 7.00 for Windows (GraphPad Software, La Jolla, CA; USA, www.graphpad.com).

For the peptide scan, linear peptides and non-linear CLIPS peptides were synthesized on PEPSCAN cards based on the amino acid sequence of FGFR4 domain2 using standard Fmoc-chemistry and deprotected using trifluoric acid with scavengers as described (24). The binding of antibody to each peptide is tested in a PEPSCAN-based ELISA. The 455-well credit card format polypropylene cards containing the covalently
linked peptides are incubated with U3-1784 and after washing with an antibody peroxidase conjugate. Binding of U3-1784 was revealed after incubation with the peroxidase substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 3 % H₂O₂ by means of CCD camera imaging (25). These experiments were performed at PEPSNAC, Lelystad, Netherlands.

**Signaling and Immunoblot**

For cellular assays, NIH 3T3 cells stably expressing FGFR4 were incubated overnight with U3-1784 or IgG control in serum free media. The next day, 300 ng/mL FGF19 or 20 ng/ml bFGF (both R&D Systems, U.S.A.) was directly added to the cells and incubated for 15 min. HuH-7 cells were incubated with U3-1784 or IgG control antibodies for 24 h, in the absence of additional FGF19. After incubations, cells were lysed. For the analysis of tumor samples, tumors were snap-frozen in liquid nitrogen and lysed later. This was followed by SDS-PAGE and immunoblotting using the Licor Odyssey system (LI-COR Biotechnology, Bad Homburg Germany) with antibodies specific to phosphorylated FGFR4 (Tyr642, Origene, Rockville, MD; U.S.A.), phosphorylated FRS2 (Tyr436, Cell Signaling Technology, Danvers, U.S.A) and α-tubulin (Santa Cruz Biotechnology, U.S.A.), phosphorylated STAT3 (Tyr705, Cell Signaling Technology, Danvers, U.S.A.) or pan-actin (Cell Signaling Technology) as loading controls. Staining intensities were quantified using the built-in Licor software system and normalized to the internal loading control. Results are displayed as values relative to control antibody.

**Xenograft experiments**

Animal studies were conducted at specific CROs as listed in Table S2. In general, six- to eight-week-old athymic BALB/c female mice were subcutaneously inoculated with cells or human tumor fragments. Prior dosing, all animals were weighed, and the tumor volumes were measured using a caliper. Mice bearing tumors of equivalent volumes (~150 mm³) were randomized into groups of 10 mice and treated with U3-1784 or vehicle control intraperitoneally twice weekly. Tumors were measured with an electronic caliper and volume was expressed in mm³ using the formula: TV = 0.5 a × b², where a and b were the long and short diameters of the tumor, respectively. Tumor growth inhibition was calculated using the formula: TGI = (1- (V_U3-1784-V_{Start}))/(V_{Vehicle}-V_{Start}) )*100, where V_U3-1784 and V_Vehicle are tumor volumes at the end of study and V_{Start} is the volume at study beginning.

**Toxicology studies**

Cynomolgus monkeys (*macaca fascicularis*) were chosen as the most appropriate animal model for assessing safety since rodents do not display apparent toxicologic effects upon pharmacologic alteration of the FGFR4/ FGF19 signaling axis whereas cynomolgus monkeys do (23). The toxicology study was
conducted in healthy, naïve monkeys weighing between 1.8 and 3.5 kg and 2-3 years of age. Animals were randomized into 2 groups (2 male, 2 female animals per group) and dosed with 100 mg/kg U3-1784 via slow IV infusions administered once every 7 days (day 0, 7, and 14) followed by a 4-week recovery period. Animals also received 350 mg/kg/day colestyramine (Quantalan®) via oral gavage twice daily at 8 hours (+/- 1 hour interval). Blood samples were taken at day 1, 8, 15, 21, 28, 35, and 42 days of the study; 2 pre-bleed samples (day -11 and day -5) were also obtained. Samples were analyzed for serum BA levels, liver enzymes (AST/ ALT) according to standard clinical chemistry protocols as well as FGF19 (R&D Systems FGF19 ELISA). Upper limit of normal (ULN) values were derived from historic measurements in healthy animals at WIL Research.

Gene expression analysis

For gene expression analysis, total RNA was isolated either from tissue (cynomolgus monkeys) or implanted cells (HUH-7 tumors). The tissues were resuspended in lysis buffer (Qiagen, Hilden; Germany) and disrupted mechanically by tissue dissociation (Milteny Biotech, Bergisch Gladbach; Germany) followed by RNA purification using the Total RNA Isolation Kit according to the manufacturer’s protocol (Qiagen). Messenger RNAs were reverse transcribed and gene expression was determined in duplicates using an ABI 7500 Fast Real Time PCR Systems (ThermoFisher, U.S.A.) and sequence specific Taqman probes (Table S1). Expression was normalized to that of the endogenous control GAPDH. Statistical analysis was performed by the Kruskal-Wallis non-parametric test, using Dunn’s multiple comparison for P-value correction in GraphPad Prism 8.11 for macOS.

Immunohistochemistry

Deparafinization and antigen retrieval was performed using Envision FLEX Target Retrieval Solution Low pH (Agilent, Santa Clara, U.S.A.). After blocking of endogenous peroxidase activity using Peroxidase-blocking solution (Agilent), sections were incubated for 30 min within Protein Block Serum-Free (Agilent). For KI67 staining, a suitable monoclonal antibody (Nichirei Biosciences, Japan) was used at 1:3 dilution for 60 min at RT. Indirect immunohistochemical staining was performed by the HRP labeled polymer method using a Histofine Simple Stain Mouse MAX-PO(R) (Nichirei Biosciences). TUNEL staining was performed using an TUNEL Apoptosis Detection kit, ApopTag ® Plus Peroxidase in Situ Apoptosis Kit (EMD Millipore, Darmstadt, Germany) according to the manufacturer’s instructions.

Crystal structure determination

FGFR4 D2 domain (residue 141-245, containing N-terminal His tag and 3c protease site) was overexpressed in E. coli and purified by Ni-affinity chromatography, followed by tag cleavage, reverse affinity chromatography and size exclusion chromatography. Antibody IgG was produced in HEK 293 cells by
transient transfection in suspension culture. Subsequently the antibody IgG was purified from supernatant using standard ProteinA chromatography. About 100mg purified antibody IgG was used to prepare Fab fragment by papain digestion at 37 °C at a ratio of papain/IgG 1:50. After IgG cleavage the Fab fragment was purified from IgG Fc part by using Lambda-Fab-Select column. And residual non-digested full IgG was removed by preparative SEC.

FGFR4 D2 domain / U3-1784 Fab complex was prepared by mixing D2 and Fab with 5:1 molar ratio and purified by size exclusion chromatography. The fractions containing FGFR4 D2 domain / U3-1784 Fab complex were collected and concentrated up to 8.9 mg/mL in the SEC buffer (50 mM Tris/HCl (pH7.5), 150 mM NaCl, 5% glycerol).

FGFR4 D2 domain / U3-1784 Fab complex was crystallized by the vapor diffusion method with 1:1 mixture of protein solution and reservoir solution (1.9 M ammonium sulfate, 0.1 M sodium acetate, pH 4.5). Plate-like crystals were obtained by the seeding method and flash-frozen with the cryo protectant containing ~20% glycerol in the reservoir solution. Diffraction data were collected at PF-AR NW12 beamline (Tsukuba, Japan) at cryogenic temperature and processed by XDS (26). Phases were determined by molecular replacement using Phaser (27) with the Fab model structure mutated to the U3-1784 sequence. Refinement and model building were performed by REFMAC5 (28) and Coot programs (29). Coordinate and statistics for the FGFR4 D2 domain / U3-1784 Fab complex are available from the PDB using accession code 6J6Y.
Results

Figure 1A shows the screening strategy for obtaining a fully human antibody against FGFR4 starting from a BioInvent n-CoDeR® Fab fragment library. Selection of clones involved several rounds of enrichment using recombinant FGFR4 extracellular domain (positive selection), as well as negative selection steps using FGFR1-3 extracellular domains (30). Subsequently, we investigated whether FGFR4-specific Fab fragments inhibited FGF17-mediated Erk stimulation and inhibitory Fab’s were converted into their human IgG1 format, expressed recombinantly in 293-F cells and purified to more than 95% homogeneity (Figure 1A, S1).

Further characterization of eight antibodies regarding binding affinity, specificity and signal inhibitory activity led to the choice of U3-1784 for further studies. The antibody variable domain sequences are depicted in Figure 1B. To identify FGFR4 amino acid sequences involved in U3-1784 binding to the receptor, a peptide mapping was performed. These experiments identified the amino acid sequence RYNY at the C-terminal end of domain 2 as part of the epitope (Figure S2A). To precisely map the entire U3-1784 binding site, we additionally solved the crystal structure of FGFR4 domain 2 bound to U3-1784 at a resolution of 2.1 Å. This structure showed the detailed interaction of the antibody’s hypervariable loops with the C-terminal area of the domain (Figure 1C). As suggested by the peptide mapping, the sequence RYNY of FGFR4 contributed to the binding epitope (Figure S2B-C).

U3-1784 exhibited excellent specificity towards FGFR4 with no binding to the other FGFRs, including isoforms IIIb and IIIc that exist of each FGFR1-3 (Figure 1D). With a KD of around 0.3 nM, the interaction between U3-1784 and FGFR4 is of high affinity. Furthermore, the affinity of U3-1784 towards FGFR4 of different species including human, mouse, rat and monkey was virtually the same ranging from 0.36 nM (mouse and human), 0.44 nM (rat) to 0.41 nM (monkey) increasing the confidence for the prediction of human effects based on mouse and monkey models that are treated with U3-1784. Additionally, we tested whether U3-1784 was able to bind to FGFR4 on cells. For this, HuH-7 cells were incubated with U3-1784 and binding was determined. The antibody exhibited a binding Kd of 0.5 nM supporting previous in vitro results (Figure 1E).

Next, we characterized the capacity of U3-1784 to inhibit FGFR4 activation by its ligands. First, we tested whether U3-1784 can competitively displace FGFR4-bound FGF19. Figure 2A shows results from ELISA experiments that measured binding of FGF19 to FGFR4 with increasing concentrations of U3-1784 resulting in reduced FGF19 binding to the receptor with an IC\textsubscript{50} value of 2 nM U3-1784 (95% CI: 1.2 to 3.4 nM). In cell based systems, U3-1784 also inhibited FGFR4 activation by exogenously added bFGF and FGF19 in NIH-3T3 cells overexpressing FGFR4 (Figure 2 B-E) and it reduced FGFR signaling in HuH-7 liver cancer cells harboring constitutive FGFR4 phosphorylation because of endogenous FGF19 expression (Figure 2 F-G) (17)).

Having established the inhibitory activity of U3-1784 \textit{in vitro}, the anti-tumor effects of U3-1784 in several animal cancer models were evaluated. A total of 10 different liver cancer models were tested with U3-1784
(Table 1) and Figure 3 A-C shows representative mouse models harboring either cell lines or patient-derived xenograft tumors which have been treated with U3-1784. Administration of U3-1784 led to tumor growth inhibition of up to 90%. Furthermore, a strong correlation between FGF19 tumor expression and tumor growth inhibition was observed as none of the FGF19-negative models were sensitive to U3-1784 while U3-1784 inhibited tumor growth in 6 out of 7 FGF19-positive models. (Table 1, Figure 3A-C). The effect on tumor growth was accompanied by a greatly reduced number of cycling cells and an increased number of apoptotic cells as shown by reduced staining for the cell proliferation marker Ki67 and an increased staining of TUNEL-positive cells in the HuH-7 model (Figure 3D).

We also wanted to establish how the anti-tumorigenic effect of U3-1784 compares to the standard of care for non-resectable liver cancer, the multi-kinase inhibitor sorafenib. To test end, we treated the HuH-7 tumor model with U3-1784 and sorafenib as single regimen or in combination. Figure 4A shows that treatment with U3-1784 or sorafenib alone yielded the same tumor growth inhibition of around 70% in this study, but only sorafenib and not treatment with U3-1784 alone led to body weight loss of the mice (Figure 4B). Since the combination of both agents led to body weight reduction too, it suggests that toxicity is mediated by sorafenib. Importantly, the combination of U3-1784 and sorafenib increased the tumor growth inhibition to more than 90%, a statistically significant improvement over the corresponding single agent activities.

To assess target engagement by U3-1784, we investigated the expression of the FGFR4 downstream gene expression target CYP7A1 in the tumors. Since expression of CYP7A1 is repressed upon FGFR4 activation by FGF19, blockade of FGFR4 by U3-1784 is expected to lead to increased CYP7A1 expression (20–22). Indeed, we observed a strongly increased CYP7A1 expression in the HuH-7 tumor when mice were treated with U3-1784 but not when mice were treated with sorafenib. Concomitantly with the increased Cyp7A1 expression we observed strongly reduced expression of the immediate early gene and known FGFR4 transcriptional target Egr-1 in a U3-1784-specific way corroborating the U3-1784 mediated inhibition of FGFR4. Interestingly, the small tumors that remained after treatment with U3-1784 but not the tumors treated with sorafenib alone exhibited a lower level of FGF19 protein (Fig. 4C). In addition, we detected decreased phosphorylation of the known FGFR4 down-stream target STAT3 (31, 32) when the tumors where treated with U3-1784 (Fig. 4D, E).

Mice that were treated with U3-1784 in our xenograft studies did not show any signs of toxicity and body weight was not affected. However, it was reported that pharmacological inhibition of the FGFR4/FGF19 axis results in serious adverse events (e.g. liver damage) in cynomolgus monkeys due to increased BA levels, hence suggesting that this would be the appropriate toxicology species for further testing (23). We therefore investigated whether U3-1784 treatment elevated BAs in these animals would subsequently lead to toxic effects. Indeed, treatment with anti-FGFR4 antibody resulted in an increase in FGF19 and BA levels (Figure 5A and B, respectively) leading to increased plasma levels of the liver enzymes Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST, Figure 5C and D). The increase in ALT...
levels was stronger than the increase in AST consistent with changes caused by hepatotoxicity. Within the same experiment we also tested whether capturing and removing BAs during U3-1784 treatment could prevent this increase. When animals were treated with U3-1784 in presence of the BA sequestrant colestyramine, an anion exchange resin that binds BAs in the gut and promotes their excretion, a complete reduction in the elevation of BAs, FGF19 and liver enzymes was observed (Figure 5). Although in this study, we used a high dose of U3-1784 (100 mg/kg), increased BA’s and ALT levels were also detected in a separate study at doses as low as 10 mg/kg (Figure S3). To further delineate the treatment effects on a molecular level, we investigated the expression of key genes involved in BA homeostasis in the liver and the ileum. As expected, U3-1784-mediated inhibition of liver FGFR4/FGF19 signaling elevated the expression of Cyp7A1 when administered both as a single treatment and in combination with colestyramine (Figure 6A). Liver FGFR4 expression did not change (Figure 6B) while expression of FGF19 in the liver was not detectable (Figure S4A). In contrast, FGF19 expression in the ileum strongly increased with the U3-1784 single treatment in line with BAs driving FGF19 expression in the ileum. Co-administration of colestyramine blunted this effect in parallel with the reduction of BA levels (Figure 6C). Ileal FGFR4 expression was lower than in the liver and unchanged by the treatments (Figure 6D).
Discussion

Fibroblast Growth Factor Receptors have long been implicated in various forms of cancer either through activating mutations in the receptor or through overexpression of the receptor and its ligands. Compared to FGFR1-3, stimulation of FGFR4 with various FGF’s leads to a less robust mitogenic effect (33). Therefore, FGFR4 was considered a less promising target for cancer intervention. However, recently, convincing data showed the capacity of FGFR4 to strongly activate signaling and to drive cancer progression in a sub-set of hepatocellular carcinoma that express the FGFR4 ligand FGF19 thus leading to constitutive FGFR4 activation (16, 17).

As potential therapeutic indication for U3-1784 we therefore focused on hepatocellular carcinoma. One reason for this was due to the previously described cancer driver function of the FGFR4/FGF19 autocrine loop which had been demonstrated in vitro only (17, 34). To establish a possible correlation between FGF19 expression and treatment success, both FGF19-positive and –negative tumor models were evaluated. U3-1784 was tested in 10 different liver cancer models. However, growth inhibition was achieved in FGF19-expressing models only (Table 1). Tumor growth was reduced in 6 out of 7 FGF19-expressing models and 3 of them showed an almost complete inhibition, while none of the 3 FGF19-negative models was sensitive. From our expression data, it was apparent that the 2 proteins which interact with FGF19, FGFR4 and β-Klotho, are expressed at similarly high levels across all cancer models and hence sensitivity to FGFR4 inhibition can be best attributed to the altered FGF19 levels. This suggests FGF19 expression as a patient selection biomarker for the treatment with U3-1784. The only FGF19-expressing model that did not show growth inhibition – JHH7 – exhibited a more than 10-fold higher expression than the other FGF19-positive models. This may suggest that extremely high levels of FGF19 may not be overcome by the antibody doses that we used. Together, these data clearly demonstrate the cancer driver function of the FGFR4/FGF19 axis in the liver cancer models.

U3-1784 single treatment resulted in the same tumor growth inhibition as sorafenib, the standard of care treatment but without the body weight loss induced by sorafenib. A combination of U3-1784 with sorafenib therefore appears possible without increasing its toxic effects and indeed the combination of both agents further decreased tumor growth without resulting in additional toxic effects as monitored by body weight. For this reason, patients whose tumors harbor FGF19 expression, might benefit from U3-1784 treatment instead of sorafenib or in combination with this drug.

The observation that FGF19 levels in the HuH-7 tumors are strongly reduced in the remaining tumor after U3-1784 treatment may suggest that tumor cells which harbor high FGF19 production are preferentially eliminated by U3-1784. An alternate intriguing possibility could be that the FGFR4/FGF19 loop feeds FGF19 production and therefore inhibition of this loop accelerates tumor growth inhibition by undercutting the loop itself.

FGF19 binds to FGFR4 with high affinity exhibiting a Kd of 0.3 nM for the interaction (9). For this reason, an antibody that aims to inhibit FGF19 interaction with FGFR4 by competing with ligand binding should
have an equal or higher affinity to the receptor or ligand to effectively prevent the binding. With an affinity of about 0.3 nM and an IC50 of 2 nM for FGF19 displacement from the receptor U3-1784 fulfills this requirement explaining the excellent tumor growth inhibition that was observed in several models. These data are in line with the determined FGFR4 epitope of U3-1784 at the C-terminal end of IgG-like domain 2 since it was shown for other FGF receptors that ligand binding involves domain 2 and 3 (1, 35). To our knowledge, the herein reported crystal structure of FGFR4 domain 2 is the first structure of an extracellular domain of this receptor.

The FGFR4/ FGF19 receptor ligand pair drives cancer progression in tumor patients and is essential in regulation of plasma BA levels under physiological conditions. Therefore, targeting receptor or ligand in cancer cells will also lead to an increased production of BAs, which can be toxic since high levels may lead to the disruption of cell membranes (23). Due to the recirculation of BAs from the intestine to the liver via the portal vein, intestinal and liver cells are particularly exposed to high BA levels. Consequently, patients who harbor increased BA levels due to cholestatic liver disease or BA malabsorption exhibit liver damage and diarrhea (36–38).

Liver damage can be life threatening and its detection may necessitate discontinuation of therapy. This is particularly true when treating liver cancer patients who already harbor a liver with compromised function and further damage should be avoided. We have identified an on-target toxicity effect upon administration of U3-1784 due to inhibition of the FGFR4/ FGF19 signaling pathway and subsequent alterations of BA homeostasis. BA sequestration with colestyramine not only prevented liver injury but also identified elevated BA as the root cause of the observed liver injury after U3-1784 administration. Therefore, other mechanistic explanations for the liver injury, including antibody-dependent cytotoxicity, appear highly unlikely. Gene expression data further showed that the BA sequestrants do not interfere with FGFR4 inhibition in the liver since colestyramine co-administration did not alter the expected increase in liver Cyp7A1 expression that was observed upon treatment with U3-1784. In line with BA-induced FGF19 expression in the ileum, U3-1784 alone increased ileal FGF19 expression in parallel with increasing BA levels whereas colestyramine co-administered with U3-1784 reduced BA and subsequent FGF19 levels (Figure 5, 6). Additionally, an increased expression of the bile salt export pumps MRP2 and BSEP was observed in the liver of animals treated with U3-1784 while expression of the bile salt import pump SLC10A1/NTCP was unchanged explaining the observed increased BA’s. This effect was reduced when combining U3-1784 with colestyramine (Figure S4 B-D). This corroborates the findings that BA regulate the expression of their transporters and may help in reducing the toxicity of high bile acid concentrations in hepatocytes (36).

Of note, elevated levels of ALT and AST were observed in cynomolgus monkeys while the same was not true in rat toxicology studies. Despite an increased liver CYP7A1 expression mediated by U3-1784 treatment, elevated liver damage signals were not detected. The lack of apparent phenotypic effect is also
seen in FGFR4-/- mice which are healthy despite increased BA levels (20). It is concluded that differences in BA metabolism appear to render rodents less sensitive to FGFR4 inhibition and monkeys are a better and more relevant model for elucidating toxic effects upon FGFR4 inhibition.

The combination with a BA sequestrant may be particularly promising for FGFR4 inhibitors that are administered intravenously such as the antibody U3-1784 given that BA sequestrants may bind oral drugs in the intestine altering their absorption or elimination. However, since the sequestrant does not cross into the blood circulation, it will likely not interfere with an antibody that is administered by intravenous or subcutaneous routes. Future clinical studies may show if the combination of bile acid sequestrants with U3-1784 is required and if they have an impact on the anti-tumor activity of the antibody.

In summary, a highly-specific FGFR4-directed inhibitory human recombinant antibody has been developed that holds special promise for the treatment of FGF19-positive liver cancer. U3-1784 is expected to cause minimal toxicity in patients since the only toxic effect that was observed in animal experiments, BA-induced liver injury, can be prevented with concomitant treatment of BA sequestrants.
Acknowledgements

We would like to thank Tetsuo Aida at the Medicinal Safety Research Laboratory of Daiichi-Sankyo, Tokyo, for expert advice. In addition, we thank Dale Shuster, Shinya Tokuhiro and all members of the Daiichi-Sankyo U3-1784 development team for their numerous contributions to the project. We also thank Prof. Toshiya Senda of the Institute of Materials Structure Science and the staff at the Photon Factory for their assistance in the use of the synchrotron beamline. Finally, we would like to acknowledge the singular contributions of Axel Ullrich (Max-Planck-Institute of Biochemistry, Germany) in starting this project.
References


Tables

Table 1. Overview tumor mice models.

Indicated models (xenograft or PDX) were treated with U3-1784 [25 mg/kg] and tumor growth inhibition was determined compared to control treated animals. Gene expression in tumor was determined using Taqman gene expression assays.
N.D.: not determined
B.D.L.: below the detection limit

<table>
<thead>
<tr>
<th>Model Name</th>
<th>Model Type</th>
<th>Tumor Growth Inhibition</th>
<th>Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FGFR4</td>
<td>FGF19</td>
</tr>
<tr>
<td>Huh-7</td>
<td>Cell line</td>
<td>83%</td>
<td>267</td>
</tr>
<tr>
<td>Huh-1</td>
<td>Cell line</td>
<td>48%</td>
<td>208</td>
</tr>
<tr>
<td>Hep3B</td>
<td>Cell line</td>
<td>58%</td>
<td>293</td>
</tr>
<tr>
<td>JHH7</td>
<td>Cell line</td>
<td>no inhibition</td>
<td>533</td>
</tr>
<tr>
<td>SNU-761</td>
<td>Cell line</td>
<td>90%</td>
<td>182</td>
</tr>
<tr>
<td>LI1078</td>
<td>PDX</td>
<td>30%</td>
<td>233</td>
</tr>
<tr>
<td>LI0574</td>
<td>PDX</td>
<td>87%</td>
<td>272</td>
</tr>
<tr>
<td>HepG2</td>
<td>Cell line</td>
<td>no inhibition</td>
<td>558</td>
</tr>
<tr>
<td>PLC/PRF-5</td>
<td>Cell line</td>
<td>no inhibition</td>
<td>620</td>
</tr>
<tr>
<td>LI0752</td>
<td>PDX</td>
<td>no inhibition</td>
<td>698</td>
</tr>
</tbody>
</table>
Figure Legends

Fig. 1 U3-1784 screening funnel, structural and molecular characterization. (A) Schematic diagram showing the antibody selection/screening steps for U3-1784. (B) Variable domain amino acid sequences of U3-1784 heavy and light chains; CDR: Complementarity Determining Regions were underlined. (C) Crystal structure of U3-1784 (FAB) binding to human FGFR4 domain 2. (D) FGFR isoform-specific binding of U3-1784 to recombinant FGFR ectodomains of indicated isoforms at 10 µg/ml antibody concentration. Data show mean and s.d. of 3 independent experiments (E) Binding of U3-1784 to living cells. U3-1784 binding to HuH-7 was determined by flow cytometry.

Fig. 2 U3-1784 inhibits ligand binding and FGFR4-mediated signaling. (A) Competitive displacement of FGF19 by U3-1784. Human, recombinant FGFR4 was coated to microtiter plates, washed and then incubated with saturating concentrations (120 ng/ml) of recombinant, human FGF19 in the presence of Heparin followed by incubation with indicated concentrations of U3-1784 or control human IgG. Binding of FGF19 was detected using a specific anti-FGF19 antibody. Data depict mean and SD of 3 independent experiments. (B-E) FGF19 and b-FGF mediated phosphorylation of FGFR4 in absence and presence of U3-1784. NIH-3T3 cells stably overexpressing human FGFR4 were incubated overnight with indicated concentrations of U3-1784 or human IgG (control) in the absence of FBS. The next day, FGF19 or b-FGF was added and after 15 min at 37°C the cells were lysed and subjected to SDS-PAGE followed by immunoblotting with an antibody specific to pFGFR4. Tubulin served as a loading control. Staining intensities were quantified, normalized and displayed in (C, E). (F, G) Signaling inhibition in liver cancer cells endogenously expressing FGFR4, FGF19 and b-klotho. Huh-7 cells were incubated with indicated concentrations of U3-1784 or hIgG for 16 h, lysed and then subjected to SDS-PAGE. Detection of phosphorylated FRS2 in immunoblot occurred with a specific antibody and Actin served as loading control; results from quantification are displayed in (G). All quantifications depict the mean and SD of 3 independent experiments.

Fig. 3 Anti-tumor activity of U3-1784 in vivo. (A-C) Inhibition of tumor growth by U3-1784 in animal models. Six- to eight-week-old athymic BALB/c female mice were subcutaneously injected with either 1x10^7 (SNU-761) or 5x10^6 (HuH-7) cells. The PDX model LI0574 was implanted with human tumor fragments. After estimated tumor volume reached about 150 mm³, animals were randomized into groups of 10 mice and then the treated with 25 mg/kg bw U3-1784 twice weekly i.p. or vehicle control. Data depict mean and S.E.M. (D) Histological assessment using HE-, Ki-67- and TUNEL-staining of paraffin-embedded HuH-7 tumor at the end of the study. Apoptotic TUNEL positive cells were indicated by arrows.

Fig. 4 Anti-tumor activity of U3-1784 in combination with Sorafenib. (A) Anti-tumor effects of U3-1784 and Sorafenib in animal models. Six- to eight-week-old athymic BALB/c female mice were subcutaneously injected with either 1x10^7 (SNU-761) or 5x10^6 (HuH-7) cells. The PDX model LI0574 was implanted with human tumor fragments. After estimated tumor volume reached about 150 mm³, animals were randomized into groups of 10 mice and then the treated with 25 mg/kg bw U3-1784 (i.p. 3 injections in 10 days), 30 mg/kg bw Sorafenib (oral gavage, daily for 10 days) or a combination of both. Animals in control groups received either PBS or vehicle administrations. Data show mean and S.E.M. Statistical analysis was performed by one-Way ANOVA with Dunnett’s multiple comparison test comparing the U3-1784 group to Sorafenib or the combination of both (B) Body weight development under treatment. (C) Gene and protein expression analysis. At the end of the study, tumors were lysed and total RNA and protein isolated. Gene expression was determined using TaqMan assays. Expression was normalized to the geometric mean of GAPDH and ACTB. FGF19 amount was determined by ELISA. (D) western blot showing pSTAT3 and actin signals of 5 samples per group after lysis at the end of the study. (E) Quantification of western blot bands in all 10 samples per treatment group using an Odyssey imaging system (LI-COR). Each symbol in (C-D) represents data from one animal. Statistical analysis was performed by the Kruskal-Wallis non-parametric test and using Dunn’s multiple comparison for P-value correction. Significant differences (corrected P<0.05) vs. the PBS control group are marked.
Fig. 5 Colestyramine abrogates U3-1784-mediated adverse effects. Cynomolgus monkeys (n=4/group; 2 male, 2 female) were treated intravenously with 100 mg/kg bw U3-1784 on days 0, 7 and 14 (indicated by dotted vertical lines). Colestyramine was administered orally [175 mg/kg bw] twice daily. Blood samples for serum chemistry analyses of each animal were drawn at 5 min, 7, 14, 21, 28, 35, and 42 days post dose and indicated parameters serum bile acids (A), FGF19 (B), alanine aminotransferase (C) and aspartate aminotransferase (D) were determined using validated, specific ELISAs.

Fig. 6. Gene expression analysis of liver and ileum samples. Fresh tissue samples from liver (upper panel) or ileum were obtained from cynomolgus monkeys as described in Figure 4 (day 42 post-dose). RNA was isolated and gene expression was determined using a TaqMan assay with specific probes for Cyp7A1 (A), FGFR4 (B, D) and FGF19 (C). Expression of indicated genes was normalized to that of a control gene (GAPDH). Each symbol represents data from one animal (tested in technical replicates). Statistical analysis was performed by the Kruskal-Wallis non-parametric test using Dunn’s multiple comparison for P-value correction. Significant differences (corrected P<0.05) vs. the PBS control group are marked.
Figure 1

A

- 2885 phages
- DNA Sequence Analysis
- 222 unique clones
- Affinity Determination Selectivity vs. FGFR1-4
- 147 clones
- Inhibition of ligand-mediated signaling (FAB and IgG format)
- 8 candidate antibodies

B

Heavy chain variable region:
EVQLLESGGGLVQPGGSRLSCAASGGFTSDYMSWIRQAPGKGLEWV
STISGGSTYYADSVKGRFTISRDNSKNTLYQLMNSLRADTAAYCAR
LTAYGHVDWGGQLTVSS

Light chain variable region:
QSVLTQPPSAGTGPQRVTISCSGSSSNIGTNTVNWYQLPGTAPKLLY
RNYQRPSGVPDRFSGSKSASLASLRASEDEADYCCAADSLSGP
HVVFAGGTQLTVL

C

D

Binding to Huh-7 cells (%):

E

FGFR binding (RFU):

Downloaded from mct.aacrjournals.org on January 1, 2021. © 2019 American Association for Cancer Research.
Figure 3

A

SNU-761 (cell line)

tumor volume (mm$^3$) ± SEM

days

- Red: Vehicle control
- Blue: U3-1784 (25mg/kg, 2 x weekly)

B

HuH-7 (cell line)

tumor volume (mm$^3$) ± SEM

days

- Red: Vehicle control
- Blue: U3-1784 (25 mg/kg, 2 x weekly)

C

LI0574 (patient derived xenograft)

tumor volume (mm$^3$) ± SEM

days

- Red: Vehicle control
- Blue: U3-1784 (25mg/kg, 2 x weekly)

D

PBS

- H&E
- KL67
- TUNEL

U3-1784

- H&E
- KL67
- TUNEL
Figure 5

A. Bile acids

B. FGF19

C. Alanine aminotransferase

D. Aspartate aminotransferase

- U3-1784
- U3-1784 + Colestifyramine

ULN
Figure 6

(A) Liver Cyp7A1

(B) Liver FGFR4

(C) Ileum FGF19

(D) Ileum FGFR4
Pre-clinical development of U3-1784, a novel FGFR4 antibody against cancer, and avoidance of its on-target toxicity

René Bartz, Keisuke Fukuchi, Toshiaki Ohtsuka, et al.

Mol Cancer Ther Published OnlineFirst July 26, 2019.

Updated version Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-18-0048

Supplementary Material Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2019/07/26/1535-7163.MCT-18-0048.DC1

Author Manuscript Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2019/07/26/1535-7163.MCT-18-0048. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.