**Supplementary methods**

**Identification of FGFR2 antibody BAY** **1179470**

Antibody lead discovery was carried out by phage display technology using the n-CoDeR® Fab library (1,2) from BioInvent International AB (Lund, Sweden). All recombinant proteins used for panning were obtained from R&D Systems as Fc-fusion proteins in carrier-free preparations. Prior to selection and depletion, proteins were biotinylated using an excess of biotin-LC-NHS (Pierce; Cat. No. 21347) according to manufacturer’s instructions. For lead antibody identification, three different panning strategies were performed using different combinations of selections over four rounds. Target was either presented as biotinylated recombinant FGFR2 (different splice variants and species) or as FGFR2 on the surface of KATO III cells (ATCC HTB-103). For each selection, clones were picked from rounds 2 to 4 for analysis in phage ELISA to identify phage pools with favorable target specificity.

For the generation of soluble Fab fragments (sFabs), phagemid DNA from pools of selection rounds 3 and 4 were isolated and digested with restriction enzymes EagI (Fermentas, FD0334) and EcoRI (NEB, R0101L) in order to remove the gene III sequence according to provider’s instructions. The resulting fragment was re-ligated and constructs were transformed into chemically competent *E. coli* Top10 using standard methods. Single clones were selected and sFab-expression was induced for screening of target binding in sFab-ELISA. After sequence analysis of positive clones, unique sequences were obtained and corresponding sFabs were purified in small scale for further characterization in ELISA on FGFR2 splice variants and murine orthologues as well as for cell binding by FACS analysis on KATO III cells. The CAS registry number for BAY 1179470 is 1634620-63-5 (3).

**Determination of BAY 1179470 selectivity**

To assess the selectivity of BAY 1179470, the binding properties of the Fab precursor of BAY 1179470 to recombinant FGFR variants were determined by ELISA. All FGFR variants used were present as Fc fusion proteins in carrier-free preparations. Proteins were biotinylated using an approximately 2-fold molar excess of NHS-LC-Biotin (Pierce; Cat. No. 21347) according to manufacturer´s instructions and desalted using ZebaTM desalting columns (Pierce; Cat. No. 89889). For ELISA, 96-well plates pre-coated with streptavidin (Pierce, 15500) were coated over night with 1 µg/mL biotinylated protein at 4 °C. Coating with biotinylated TRAIL-Fc served as a reference. On the following day, plates were washed 3 times with PBS (phosphate-buffered saline) and Tween 20 (PBST), treated with blocking reagent, and washed again 3 times with PBST. The purified Fab (1 µg/mL) was added and plates were incubated for 1 h at room temperature, followed by washing 3 times with PBST. An anti-hIgG (Fab-specific) coupled to HRP (1:2500 diluted; Sigma; A 0293) was added and plates were incubated for 1 h at room temperature. Color reaction was developed by TMB (Invitrogen, 2023) and stopped after 5-15 min with H2SO4 (Merck, 1120801000). Colorimetric reaction was recorded at 450 nM using a plate reader (Tecan). Wells containing TRAIL-Fc were used as background values and the signal to background ratios were calculated.

**Internalization and localization of FGFR2-Ab**

In order to visualize internalization of the FGFR2-Ab BAY 1179470 and the isotype control Ab BAY 1138806, the antibodies were lysine conjugated with a two molar excess of CypHer5E mono NHS ester (GE Healthcare) at pH 8.3. After the conjugation, the reaction mixture was purified by chromatography (PD10 desalting column 1-2.5 mL, GE Healthcare) and concentrated (VIVASPIN 500, Sartorius Stedim Biotech). In addition to the pH-dependent fluorescent dye CypHer5E, the permanent dye Alexa 488 was used. The dye load of the antibody was determined with a spectrophotometer (NanoDrop™) and calculated with the formula D/P = Adye εprotein/ (A280-0,16Adye) εdye. The affinity of the labeled antibodies was confirmed in a cell-binding assay. Prior to treatment, cells (2 x 104/well) were seeded in 100 μL medium in a 96 well plate. After 18 h incubation at 37 °C / 5% CO2, medium was changed and labeled FGFR2-Ab or isotype control Ab was added at various concentrations (5, 2.5, 1 μg/mL) and incubated for 0.5, 1, 2, 3, 5, or 24 h. The fluorescence measurement was performed with the InCellAnalyzer 1000 (GE Healthcare). Granule counts and total fluorescence intensity were measured in a kinetic fashion.

For confirmation by flow cytometry, SNU-16 cells were incubated with 10 µg/mL of FGFR2-Ab (BAY 1179470) or the isotype control Ab (BAY 1138806) for 5 h at 37 °C. Membrane-bound FGFR2 was detected using the murine FGFR2 antibody 740-18F5H1 (generated by Diaclone) as primary antibody not competing with BAY 1179470 for the FGFR2 binding site, and a PE (phycoerythrin)-labeled goat anti-mouse IgG1 (Dianova) as secondary antibody.

In addition, the measurement of FGFR2 degradation was performed with FGFR2 ELISA using SNU-16 cells.The cells were treated with an isotype control Ab or FGFR2-Ab BAY 1138922 for 96 h. BAY 1138922 is a predecessor antibody of BAY 1179470. To obtain BAY 1179470, positions 1-3 of the N-terminal heavy chain sequence in BAY 1138922 were changed from QVE to EVQ and the C-terminal lysine amino acid position K452 in the heavy chain was removed.

In whole cell lysates, total FGFR2 was detected with the DYC665 ELISA kit from RD Systems.

In order to explore the internalization pathway of the FGFR2-Ab BAY 1179470, co-staining experiments with markers for endosomal compartments (early, late, recycling) and lysosomes were performed. SUM-52PE cells were incubated with CypHer5E labeled FGFR2-Ab (red stain) for 6 h prior to fixation of the cells with methanol. Subsequently, cells were stained with specific antibodies (green stain) recognizing lysosomal-associated membrane protein 1 (LAMP-1, a lysosome/late endosome marker), mannose-6-phosphate receptor (M6PR, a late endosome marker), Rab7 (an early endosome marker), and Rab11 (a recycling endosome marker) (4). Hoechst 33342 (Invitrogen, H3570) DNA stain was used to indicate the nucleus.

**Amnis® imaging flow cytometry**

To determine co-localization of FGFR2-Ab with markers of endosomal compartments and lysosomes in more quantitative way experiments were performed with an amnis® FlowSight® imaging flow cytometer (Merck Millipore). The FGFR2 antibody was labeled with CypHer5e as described above. 5x105 SUM-52PE cells were incubated with CypHer5E-labeled FGFR2-Ab (1µg/mL) at 37 ° and 5% CO2. After 5.5 h incubation, CytoPainter Lysosomal Staining kit components resulting in green fluorescence (Abcam ab 112138; final concentration: 1:2000 dilution) were added and the incubation time was extended to 6 h. Cells were then dissociated with Accutase (Sigma Aldrich), washed and resuspended in 100 µL cold FACS buffer (PBS + 3% FCS, 4 °C). To discriminate living and dead cells propidium iodine (final concentration: 0.5 µg/mL) was added before the start of the flow cytometer analysis. A representative selection of cell images (derived from 15,000 events) was evaluated using the IDEAS® (Millipore) software package. An isotype control antibody was prepared and analyzed in parallel.

**Preparation and characterization of FGFR2-ADC BAY** **1187982**

N,N-dialkyl auristatin W derivatives have been reported as highly efficient microtubule-disrupting agents (5). The payload employed in FGFR2-ADC BAY 1187982 (CAS registry number 1708947-48-1) is a novel 1,2-oxazinane auristatin W amide derivative alkylated at the amino terminus with a hexanoic acid linker, which is utilized for attachment to lysine side chains of the antibody. This linker chemistry provides non-cleavable ADCs with high stability (6).

ADC synthesis was accomplished by coupling of 10 equivalents of the activated N-hydroxysuccinimide ester precursor molecule to the FGFR2 antibody in DPBS phosphate buffer. For animal studies, ADC concentrations of >10 mg/kg were adjusted in DPBS buffer without any issues regarding solubility or aggregation.

The number of drug molecules per antibody (DAR, drug-antibody ratio) of the FGFR2-ADC BAY 1187982 was determined after deglycosylation with PNGase F using a combination of HPLC and ESI-Q-TOF consisting of an Agilent 1200 Series Capillary-HPLC System (Agilent Technologies) for sample desalting and separation and a MicroToFQ mass spectrometer (Bruker Daltonik, Bremen) for analysis of the complete antibody mass. After deconvolution, each signal of the different FGFR2-conjugate species was integrated and the DAR was calculated as Σ (Peak area \* drug load) /Σ (Peak area).

Purity and homogeneity of the ADC was analyzed by size exclusion chromatography coupled with multi angle light scattering (SEC-MALS). SEC-MALS analysis was performed by an Agilent 1200 HPLC system monitored at 220 nm coupled to a miniDAWN Treos light scattering detector and an Optilab rEX interferometric refractometer (Wyatt Technology Corporation). A Superdex200 10/300 GL column (GE Healthcare) was operated at room temperature with an isocratic gradient using PBS at a flow rate of 0.5 mL/min. Dimer content was determined from integration results of UV-peaks of monomeric and dimeric species.

**ELISA for determination of EC50 of BAY** **1179470 and BAY** **1187982**

The binding affinities of FGFR2 monoclonal antibodies (mAb) BAY 1179470 (human IgG1) and FGFR2-ADC BAY 1187982 were compared *in vitro* by ELISA using the recombinant human FGFR2-Fc (rhFGFR2, R&D # 663-FR, 100 µg/mL) as an antigen. Briefly, a multititer plate (MTP, 384-well MaxiSorp, black, Nunc) was coated with 20 µL rhFGFR2 in a concentration of 0.08 µg/mL in 1x coating buffer, pH 9.6 (Candor Bioscience GmbH # 121125) at 4 °C overnight. After washing with 50 µL PBS-T [0.05% Tween-20 (Acros # 233360010) in 1xPBS], plates were blocked with 50 µL of 100% Smart Block (# 113500, Candor Bioscience GmbH) for 1 d at 4 °C. After three washing steps, 20 µL quadruplets of the FGFR2-mAb or FGFR2-ADC dilution series in 10% SmartBlock in PBST with a maximum concentration of 300 nM were added and the plates were incubated for 1 h at 20-22 °C and thereafter washed three times. For the detection of the bound FGFR2-mAb or FGFR2-ADC, 20 µL of anti-lambda-POD conjugate (Sigma # A5175) in a 1:10000 dilution in 10% SmartBlock in PBST was applied for 1 h at 20-22 °C. After three washing steps, 20 µL of 10 µM Amplex red substrate (Invitrogen #A12222) in 50 mM sodium hydrogen phosphate, pH 7.6, was added, incubated for 20 min at 20-22 °C in the dark and the fluorescence signal was detected using a Tecan M1000 fluorescence reader at 535/590 nm (excitation/emission). EC50 values were evaluated by fitting the data with the GraphPad Prism software (sigmoidal dose-response, variable slope, bottom set to background).

***In vitro* microtubule polymerization assay**

The potency in an *in vitro* microtubule polymerization assay was determined for BAY 1168650, the respective lysine-linker toxophore acid variant generated as intracellular metabolite in tumor cells in xenograft models. A cell-free assay was performed using the “Fluorescence-based Microtubule Polymerization Assay Kit” (Cytoskeleton, Denver, Colorado, USA; order number BK011) according to manufacturer’s instructions.

***In vitro* caspase 3/7 assay**

The apoptosis induction *in vitro* was determined with the Caspase-Glo 3/7 Assay (Promega, #G8093) which is a homogeneous, luminescent assay that measures Caspase-3 and -7 activities. Cells were plated in 96-well plates (SNU-16: 3000 cells/75 µL; MDA-MB-231 4000 cells/75 µL) and incubated at 37 °C and 5% CO2 for 24 h. After incubation, cells were either left untreated or treated with the FGFR2-ADC BAY 1187982, control ADC, vinorelbine, or paclitaxel at concentrations between 3x 10-11 M and 3x 10-7 M for 48 h. Thereafter, 100 µL of Caspase-Glo 3/7 reagent was added to each well and the plates were incubated for 45 min at room temperature and luminescence was recorded for 0.2 s/well on a Victor V instrument (Perkin Elmer). The EC50 values were calculated with the BELLA-DRC software.

**Antibody binding assay using quantitative flow cytometry**

The number of antibodies bound per cell (ABC) was determined with the QuantiBRITE Fluorescence Quantitation System (Becton Dickinson Biosciences, NJ, USA). BAY 1179470-PE, obtained from Becton Dickinson Biosciences, contains only one molecule of phycoerythrin (PE) per BAY 1179470. Briefly, adherent cells were washed twice with PBS w/o Ca2+, Mg2+, pH 7.4 (Biochrom) and subsequently detached using a non-enzymatic dissociation solution (Sigma). Cells were then washed twice with PBS w/o Ca2+, Mg2+, pH 7.4. After centrifugation, cells were resuspended in FACS buffer (Biochrom) with 3% FCS (Biochrom), counted and aliquoted to 1x105 cells per well in a 96-well plate format. Cells were incubated with 20 µg/mL of BAY 1179470-PE for 1 h at 4 °C. After washing with FACS buffer, PE levels were measured using the FACSCanto™ instrument (Becton Dickinson Biosciences). For quantification, PE-labeled QuantiBRITE beads with defined PE-levels were used.

**Mode-of-action analysis of FGFR2-ADC in *ex vivo* tumor samples**

On day 21 after first treatment, mice bearing BR1115 tumors were administered with 7.5 mg/kg FGFR2-ADC BAY 1187982 or control-ADC, 10 mg/kg vinorelbine or PBS 4 hours prior to sacrifice. FFPE tumors from three independent mice were cut into 4 µm slices. Antigen retrieval was performed with citrate buffer, pH 6. The PathScan apoptosis and proliferation multiplex IF kit (#7851) from Cell Signaling technology was used for the staining. All three primary antibodies were applied onto the slides as cocktail. Three FFPE slides per tumor were stained and three different fields were analyzed under the microscope for number of (a) pHH3- or (b) cleaved PARP1-positve cells, (c) cells with -tubulin stained mitotic spindles, and (d) for number of cells with co-localization of -tubulin stained mitotic spindles with pHH3 as marker for G2/M phase chromosomes.

**Supplementary Data**

**Supplementary Figure S1.** The FGFR2-ADC BAY 1187982 inhibits microtubule polymerization resulting in induction of apoptosis*.* A. Activity of the FGFR2-ADC toxophore linker metabolite (BAY 1168650) in comparison to paclitaxel and monomethyl auristatin F (MMAF) in a microtubule polymerization assay. B-C, Activation of caspase 3/7 by FGFR2-ADC, control ADC, paclitaxel, and vinorelbine in FGFR2-positive SNU-16 human gastric cancer cells (B) and in FGFR2-negative MDA-MB-231 human breast cancer cells (C).

**Supplementary Figure S2.** The anti-tumor efficacy of different FGFR2-ADC BAY 1187982 (DAR 5.1) treatment schedules on MFM-223 human TNBC mouse model. Treatment with the indicated doses (n=8-12; n=54 in BAY 1187982 group) was started when tumors had reached a mean size of 74 mm3. Initially, a second randomization was planned for the group treated with BAY 1187982 at 5 mg/kg to evaluate the effect of a second treatment cycle in regrown tumors. However, no palpable tumors were observed after the first BAY 1187982 treatment and therefore, this approach was abandoned. A, Time course of tumor cell growth. Statistical analysis was performed by Kruskal-Wallis test, followed by Mann-Whitney U-test, \*\*\*, p < 0.001. B, Changes in tumor size represented as a percentage of the initial tumor size in each individual mouse. Progressive disease, mice exhibiting >20% tumor growth; stable disease, mice exhibiting <30% tumor shrinkage and <20% tumor growth; partial response, mice exhibiting >30% tumor shrinkage; complete response, mice with non-measureable tumors.

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