EGFR binding Fc domain-drug conjugates: stable and highly potent cytotoxic molecules mediate selective cell killing

Abstract: The exposition of cancer cells to cytotoxic doses of payload is fundamental for the therapeutic efficacy of antibody drug conjugates (ADCs) in solid cancers. To maximize payload exposure, tissue penetration can be increased by utilizing smaller-sized drug conjugates which distribute deeper into the tumor. Our group recently explored small human epidermal growth factor receptor 2 (HER2) targeting Fc antigen binding fragments (Fcabs) for ADC applications in a feasibility study. Here, we expand this concept using epidermal growth factor receptor (EGFR) targeting Fcabs for the generation of site-specific auristatin-based drug conjugates. In contrast to HER2-targeting Fcabs, we identified novel conjugation sites in the EGFR-targeting Fcab scaffold that allowed for higher DAR enzymatic conjugation. We demonstrate feasibility of resultant EGFR-targeting Fcab-drug conjugates that retain binding to half-life prolonging neonatal Fc receptor (FcRn) and EGFR and show high serum stability as well as target receptor mediated cell killing at sub-nanomolar concentrations. Our results emphasize the applicability of the Fcab format for the generation of drug conjugates designed for increased penetration of solid tumors and potential FcRn-driven antibody-like pharmacokinetics.

Keywords: antibody-drug conjugates; antibody engineering; drug delivery; Fc antigen binding fragments; transglutaminase; tumor penetration.

Introduction

The field of antibody-drug conjugate (ADC) has recently attracted attention with the approval of six new ADCs between 2019 and April 2021 suggesting that excessive research on this class of cancer therapeutics is finally bearing fruit (Boni et al. 2020; Mullard 2020, 2021; Syed 2020). ADCs consist of cytotoxic drugs covalently linked to monoclonal antibodies allowing for selective surface receptor binding on cancer cells followed by delivery of the drug into the intracellular compartment (Khongorzul et al. 2020). Beside conventional ADCs generated from full-length 150 kDa monoclonal antibodies (mAbs), smaller antibody-fragment based drug conjugates designed for enhanced tumor penetration and efficacy have been explored (Aubrey et al. 2018; Cao et al. 2020; Deonarain 2018; Glassman et al. 2020; Higgins et al. 2020; Liu et al. 2019; Nessler et al. 2020; Q. Li et al. 2019; Ruddle et al. 2019).

In this regard, we have recently described antigen binding Fc fragments (Fcabs) as a promising format for the generation of drug conjugates (Jäger et al. 2021). Fcabs are derived from the Fc fragment of human IgG1 antibodies by engineering the C-terminal structural loops of the CH3 domain to form an antigen binding site (Figure 1A, B). Hence, Fcabs combine Fc-mediated effector functions including neonatal Fc receptor (FcRn) binding with an antigen binding functionality but comprise only one third of the size of conventional IgGs (Lobner et al. 2016). The smaller size of the Fcab format promises to improve solid tumor penetration by enhancing the extravasation from the circulation into the interstitial space and increasing diffusion rates through the interstitium and tumor tissue (Thurber et al. 2008). Moreover, the half-life extending FcRn binding site delays systemic clearance of the Fcab (mouse terminal t1/2 Fcab 60–85 h (Leung et al. 2015; Wozniak-Knopp et al. 2010)) and thereby maintains a high plasma concentration that further drives penetration into...
tissues (Thurber et al., 2008). FcRn binding provides Fcabs with a significant advantage over other reported ≤50 kDa Fab- (Liu et al. 2019; Ruddle et al. 2019), scFv- (Aubrey et al. 2018; Higgins et al. 2020), diabody- (Q. Li et al. 2019) or single domain antibody-based drug conjugates (Cao et al. 2020; Glassman et al. 2020; Nessler et al. 2020) that lack an FcRn binding site and consequently suffer from a short half-life in vivo (mouse terminal t1/2 Trastuzumab-derived Fab 4.4 h (Z. Li et al. 2019)). In our initial feasibility study, we were able to show that the Fcab format is suitable to generate HER2 binding drug conjugates. Deploying an engineered microbial transglutaminase, we were able to site-specifically conjugate linker-payloads to the conserved Fc position Q295 resulting in stable and functional Fcab-drug conjugates with a drug-to-antibody ratio (DAR) of 2.0. Furthermore, we demonstrated in an in vitro spheroid experiment, that spheroid accumulation was higher for Fcabs compared to full-length antibody controls confirming the favorable penetration capability of Fcabs (Jäger et al. 2021).

In the present study, we expand the concept of Fcab-based ADCs from HER2 to EGFR binding Fcab-ADCs and demonstrate the versatility of this antibody format for the generation of site-specific, stable and highly potent drug conjugates. We first demonstrate that the selected EGFR binding Fcabs are suitable for an ADC approach based on selective cellular uptake data using heterogenous conjugates carrying a pH dependent fluorescent dye. We then employed site-specific enzymatic conjugation to attach the microtubule inhibitor monomethyl auristatin E (MMAE) to position Q295 but also to the novel position Q311 and Q438 allowing to reach higher DARs. The drug conjugates showed retained EGFR and FcRn binding properties and possessed excellent stability in mouse and human serum. Finally, we show EGFR-mediated sub-nanomolar cytotoxicity of our Fcab-drug conjugates on different cancer cell lines.

Materials and methods

Preparation of antibody fragments

Amino acid sequences of Fcabs were taken from literature (Tuna et al. 2018). Sequences of Fcabs (no core hinge region, D265A) are given along with huFc (D265A) and Cetuximab sequences (SrtA tag) in the SI. Encoding sequences were ordered as codon-optimized versions and cloned into pT5 vector for mammalian expression (GeneArt, Thermo Fisher Scientific). Fcabs and huFc controls were expressed by transient transfection of Expi293F™ cells following the manufacturer’s instructions and the supernatants were harvested after five days post transfection. C-Fab contained a His-tag for purification and was dialyzed against phosphate-buffered saline (PBS) pH 7.4 before purification by immobilized metal affinity chromatography (1 mL HisTrap™ HP, GE Healthcare) using an AKTA Pure device (GE Healthcare). Fcabs, huFc and C-IgG were purified by protein A affinity chromatography using HiTrap™ Mab Select SuRe 5 mL columns (GE Healthcare) and subsequently formulated in PBS pH 6.8 using HiPrep™ 26/10 desalting columns. Antibody purity was confirmed by intact mass analysis by LC-MS using an Exion LC and TripleTOF® 6600+ mass spectrometer (AB Sciex). Proteins were concentrated using Ultra centrifugal filter units (3 K MWCO, Amicon®), sterile filtered and protein concentration was determined by UV–VIS spectroscopy at 280 nm. Proteins were snap-frozen in liquid nitrogen and stored at −80 °C.

Preparation of MMAE conjugates

Fcabs and huFc were conjugated to drug-linker Gly3-Val-Cit-PAB-MMAE (1, Levena) using a genetically engineered mTG (Dickiesser et al. 2020). mTG-mediated antibody conjugation was performed using 5 mg/mL Fcabs/huFc, 20 molar equivalents of drug-linker and 60 U/mL mTG in PBS pH 6.8 with up to 10% DMSO. Reaction mixes were incubated at 37 °C for 18 h with gentle shaking, chilled to 10 °C and purified by preparative size exclusion chromatography (SEC).

For SrtA conjugation, C-IgG or C-Fab (5 mg/mL) were formulated in 150 mM NaCl, 50 mM Tris-HCl, 5 mM CaCl2 pH 7.5. SrtA (Chen et al. 2011) was added to a final concentration of 13 µM along with 10

Figure 1: Fcab structure and pHAb-dye labeling. (A) Schematic representation of homodimeric Fcab with engineered antigen binding site at the C-terminus of the Cα3 domain. (B) Human IgG1-Fc monomer depicting the Cα3 AB, CD and EF loops that were engineered in the selected EGFR-binding Fcabs (PDB ID 5JI). Glycosylation is not shown for clarity. (C) Schematic representation of pHAb-dye labeling.
equivalents of Gly3-Val-Cit-PAB-MMAE (1) per SrtA recognition motif. The reaction mixture was incubated for 90 min at 25 °C, stopped by the addition of EDTA (final 10 mM) and purified by preparative SEC.

Preparative SEC was performed using either a Superdex™ 200 Increase 10/300 GL, Superdex™ 75 10/30 GL or a Superdex™ 200 prep grade 16/60 column in a 1260 liquid chromatography system (Agilent Technologies) or an ÄKTA Avant device (GE Healthcare) with PBS pH 6.8 as running buffer. Purified conjugates were concentrated using Ultra centrifugal filters units (10 K MWCO, Amicon™), sterile filtered and protein concentration was determined by UV–VIS spectroscopy at 280 nm. The purified conjugates were subjected to analysis by SE-HPLC and DAR determination (reversed phase (RP) HPLC, LC-MS) as described elsewhere (Dickgiesser et al. 2020), snap-frozen in liquid nitrogen and stored at −80 °C.

Preparation of pHAb-dye conjugates

Fcabs, huFc and Cetuximab controls were formulated in 10 mM sodium-bicarbonate buffer pH 8.5. pHAb amine reactive dye (10 mg/mL 1:1 (v/v) DMSO/H2O, Promega) was either added at a 2:1 molar ratio (pHAb-antibody) (Fcab-1, Fcab-2, Fcab-3, C-IgG, C-Fab) or a 10:1 molar ratio (huFc), excised by incubation at 25 °C, 450 rpm for 1 h in the absence of light. Excess dye was removed by Dulbecco’s phosphate buffered saline (DPBS) equilibrated Zeba™ Spin desalting columns (ThermoFisher Scientific) according to the manufacturer’s instructions. Aggregation of pHAb-dye conjugates and fluorescence degree of labeling (DOL™) were determined by an SE-HPLC method described in the SI.

Peptide mapping

Fcab-1 and Fcab-1-MMAE were deglycosylated with GlycINATOR (Genovis) according to the instruction manual. Deglycosylated molecules were then reduced with 10 mM dithiothreitol (DTT) for 30 min at 56 °C and alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark. Ten microgram protein was digested with 0.5 µg trypsin (mass spectrometry grade, Promega) at 37 °C overnight. LC-MS analysis was performed using an Exion HPLC system coupled to a TripleTOF 6600+ mass spectrometer (Sciex). 7.5 µg peptide solution was loaded onto an Aeris PEPTIDE XB-C18 column (Phenomenex, part no. 00F-4506-AN) and eluted with a linear gradient from 5 to 50% buffer B (acetonitrile, 0.1% formic acid; buffer A: water, 0.1% formic acid) within 49 min. Data were acquired with positive polarity and in a TOF-MS mass range from 350 to 2500 m/z and a TOF-MS/MS mass range from 50 to 2500 m/z. Other instrument settings were as follows: ion spray voltage 5.5 kV, source temperature 450 °C, accumulation time 0.25 s for TOF-MS and 0.08 s for TOF-MS/MS, gas1 45 psi, gas2 45 psi, curtain gas 35 psi, declustering potential 80 V, and collision energy was set to dynamic. Data were processed with Genedata Expressionist.

Cell culture

Human cancer cell lines were obtained from the American Type Culture Collection (EGFR positive: MDA-MB-468, A431; EGFR negative: MCF-7) and maintained according to standard culture conditions (37 °C, 5% CO2, 95% humidity). A431 and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium pyruvate. MDA-MBA-468 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. For subculturing, adherent grown cells were detached by adding 0.05% trypsin-EDTA, diluted with fresh medium and transferred into a new culturing flask.

Cellular uptake

Cells were centrifuged at 500 × g for 5 min, the supernatant was discarded, and cells were resuspended in the respective medium without phenol red at 300,000 cells/mL. The cell suspension (40 µL/well) was seeded into a black 384 clear bottom plate followed by incubation (37 °C, 5% CO2) in a humid chamber overnight. pHAb-dye labeled proteins were supplemented with 0.3% Tween-20 (final), diluted to 3 µM and added to the cells in triplicates (final 100 nM) using a D300e digital dispenser (Tecan). The cells were immediately transferred to a Cytation 5 cell imaging reader (BioTek) equipped with DAPI and RFP filter cubes and a BioSpa 8 automated incubator (BioTek). Brightfield (objective: 10×, LED intensity: 10, integration time: 13 ms, camera gain: 24) and RFP channel images (ex.: 531 nm, em.: 593 nm, LED intensity: 10, integration time: 50 ms, camera gain: 24) were taken every 2 h over a period of 26 h. About 30 min before the 26 h measurement, the plate was removed from the BioSpa 8 device and 1 µg/mL Hoechst 33,342 (ThermoFisher Scientific) was added via a Tecan D300e digital dispenser for an additional 26 h endpoint DAPI image. Images were processed by the BioTek gen5 data analysis software. The sum of the integrated pHAb dye fluorescence intensities of each image was normalized to the number of cells determined in the DAPI channel and subtracted by the sum of the integrated RFP signal at 0 h (background signal). The cell number and background normalized intensities were divided by the pHAb-dye DOL™ of each construct and plotted against the time. Normalized data was fitted by linear regression in GraphPad Prism (GraphPad Software, Inc.) and intracellular accumulation rates (slopes) were derived. Finally, the relative intracellular accumulation was calculated for each construct with respect to the highest intracellular accumulation rate (here, C-IgG-pHAb on MDA-MB-468 was set 100%).

FcRn and EGFR binding

Kinetic parameters of Fcabs, Cetuximab variants and their respective MMAE conjugates were determined by BLI using the Octet® RED96 system (FortéBio, Pall) at 30 °C and 1000 rpm agitation speed.

For EGFR binding analysis, Fcabs variants (10 µg/mL in DPBS), C-IgG (2.5 µg/mL in DPBS) and respective MMAE conjugates were loaded onto anti-human IgG Fc capture biosensors (AHC) for 60–180 s. C-Fab (2.5 µg/mL in DPBS) was loaded onto anti-human Fab-CH1 second Generation biosensors (FAB2G) for 180 s. Biosensors were then transferred into kinetics buffer (DPBS pH 7.4, 0.02% Tween-20 and 0.1% bovine serum albumin) and incubated for 60 s followed by an association step to EGFR-His6 (produced in-house). EGFR-His6 was serially diluted in kinetics buffer in a concentration range varying from 20 to 0.313 nM. Association was monitored for 180, 240 or 300 s followed by a dissociation step in kinetics buffer for 600 s to determine $k_a$ and $k_d$ values. EGFR-His6 was replaced by kinetics buffer, serving as a negative control and reference. Respective non-binding huFc was...
used as negative control in each experiment. The buffer reference measurement (control curve) was subtracted from antibody measurements for data fitting and kinetics parameter were determined by using FortéBio data analysis software 12.0 applying a 1:1 global full-fit binding model after Savitzky-Golay filtering.

The FcRn binding assay was performed as described elsewhere (Jäger et al. 2021).

Serum stability

The serum stability assay was conducted as previously described (Dickgiesser et al. 2020) applying some minor modifications: MMAE conjugates were incubated at a final concentrations of 5 µM conjugated MMAE (considering the DAR of each construct) in human and mouse serum. Moreover, serum samples were supplemented with 5 µM deuterated D8-MMAE internal standard prior to 96 h serum incubation.

Cell proliferation assay

For the evaluation of C-IgG-, C-Fab- and Fcab-MMAE conjugates and related compounds, 40 µL of viable cell suspension were seeded into opaque 384-well plates (MDA-MB-468: 2500 cells/well, A431: 9000 cells/well, MCF-7: 5000 cells/well) followed by incubation (37 °C, 5% CO2) in a humid chamber overnight. Test compounds were added using a D300e digital dispenser (Tecan). Free MMAE, and protein/protein-conjugate solutions were supplemented with 0.3% Tween-20 according to the manufacturer’s instructions. Luminescence values were normalized to luminescence of non-treated cells and dose-response was fitted using the asymmetric (five parameter) fitting function of GraphPad Prism (GraphPad Software, Inc.) to derive IC50 values.

Results and discussion

Preparation of Fcabs, controls and pHAb-dye labeled constructs

As a starting point, we selected three different EGFR-binding Fcabs (Fcab-1, Fcab-2, Fcab-3) from literature. For all three, single-digit nanomolar binding affinities to EGFR have been described (KD 0.7–2.6 nM) (Tuna et al. 2018). Fcabs were modified by removing the core hinge region (TCPPCP). As a negative control, we included an unmodified human Fc (huFc) fragment. As EGFR-binding references, a Cetuximab-based full length IgG (C-IgG) and a Cetuximab-derived Fab (C-Fab) fragment, both equipped with a sortase A (SrtA) recognition motif (LPETG) at the C-terminal end of the light chain, were included. Fcabs and huFc were expressed with a D265A mutation (Baudino et al. 2008; Shields et al. 2001) to avoid Fcy receptor (I, II, III) mediated cytotoxicity (Mahalingaiah et al. 2019) and purified by affinity chromatography (Figure S1). Mass spectrometry analysis (LC-MS, Figure S2) confirmed the identity of all molecules. Differential scanning fluorimetry showed lower unfolding transition midpoints (Tm) for Fcabs (e.g. Tm,fluorescence at the acidic pH in endosomal and lysosomal compartments (Figure S6) (Nath et al. 2016). Successful pHAb-dye labeling and the absence of aggregates were confirmed by analytical size exclusion chromatography (SE-HPLC) (Figure S8). To ensure comparability between individual fluorescence intensities resulting from differently labeled constructs, the fluorescence signals were normalized. A comprehensive description of the underlying experimental procedures can be found in the Supporting Information (SI).

Cellular uptake studies

To assess the general suitability of EGFR-binding Fcabs for intracellular drug delivery, their uptake and accumulation into cancer cells were studied. Therefore, pHAb-dye labeled constructs were incubated on EGFR overexpressing (MDA-MB-468, A431) and EGFR negative (MCF-7) adherent cells and fluorescence was continuously measured over 26 h (Figure S9A). Fluorescence intensities were normalized to account for individual cell numbers and fluorescence intensities of labelled constructs. These intensities were plotted against the time to determine intracellular accumulation rates by linear regression (Figure S9B). All accumulation rates were expressed relative to the rate of C-IgG-pHAb (C-IgG-pHAb on MDA-MB-468) which showed the fastest uptake (Figure 2). Differences in accumulation between MDA-MB-468 and A431 cells directly correlated with the differences in specific EGFR expression density. Remarkably, all Fcabs underwent selective, EGFR-mediated cellular uptake. Interestingly, bivalent and monovalent controls C-IgG-pHAb and C-Fab-pHAb exhibited most pronounced internalization, which may arise from different epitopes addressed by Cetuximab than by the Fcabs (Tuna et al. 2018). To measure the therapeutic threshold (IC50) for respective Fcab-drug conjugates, Fcabs were conjugated to cytotoxic payloads and tested for their cell killing activity.
**Generation of Fcab-drug conjugates**

Fcab-1, Fcab-2, Fcab-3 and huFc were conjugated by an engineered microbial transglutaminase (mTG) (Dickgiesser et al. 2020) targeting Q295 with Val-Cit-PAB-MMAE possessing a triple glycine handle (1) (Figure 3) (Table 1) and purified by preparative size exclusion chromatography (Figure S10). C-Fab and C-IgG were conjugated to 1 by SrtA (Chen et al. 2011; Gébleux et al. 2019) reaching DARs ranging DAR 0.8–1.1. Analytical SE-HPLC of purified conjugates showed the absence of high molecular weight species (Figure S11A) (Table 1). Surprisingly, analyses via reversed phase chromatography (RP-HPLC) (Figure S11B), hydrophobic interaction chromatography (HI-HPLC) (Table S2) and LC-MS (Figure S12) revealed that all three Fcabs showed an elevated DAR between 2.7 and 2.9. This was not observed for huFc (DAR 2.0) indicating that substrate 1 had been coupled to additional residues in the Fcab scaffold only (Table 1). The additional conjugation sites could subsequently be identified via LC-MS peptide mapping as Q311 and Q438 in the constant CH2 and CH3 region (Figures 3A and 4). The disappearance of peaks assigned to unconjugated peptides containing Q295 in the digested Fcab-1-MMAE mixture (Figure 4; peak a, b) indicated that Fcabs were nearly completely conjugated at position Q295 while only partially conjugated at position Q311 and Q438 (total 18–23% conjugated based on DAR 2.7–2.9). As previous mTG conjugation to the same constant region in HER2 Fcabs or native IgGs did not lead to Q311 or Q438 conjugation (Dickgiesser et al. 2020; Jäger et al. 2021), accessibility for mTG and altered conjugation has most likely been driven by structural changes or electrostatic effects in adjacent regions. These changes must be induced by the EGFR paratope inserted to the CH3 region or by the missing core hinge region of the EGFR Fcabs. All three conjugation positions, namely Q295, Q311 and Q438 lie within the solvent exposed exterior of the Fcab. Consequently, conjugation could theoretically affect accessibility for serum proteases and interfere with FcRn and EGFR binding. To assess this notion, Fcab-1-MMAE, Fcab-2-MMAE and Fcab-3-MMAE were subsequently tested for FcRn and EGFR binding and serum stability.

**Receptor binding properties of Fcab-drug conjugates**

Fcab-MMAE conjugates were analyzed along with controls and non-conjugated parent molecules for their binding affinity to the target receptor EGFR and half-life extending FcRn (Table 2) (Figure S13) (Tables S3 and S4). Biolayer interferometry (BLI) measurements did not reveal impaired binding ($K_D$) of conjugates to EGFR and FcRn with differences in $K_D$ beyond the assay variability.
suggesting that attached payloads at positions Q 2 9 5d on o ti m p a t b i n g f u n c t i o n a l i t i e s o f b o t h r e -
ceptors. However, for Q311 and Q438 it should be noted
that effects on EGFR and FcRn binding cannot be assessed
robustly with our BLI assay because of just minor conju-
gation (18–23%).

Serum stability of Fcab-drug-conjugates

Several studies have shown, that conjugate pharmacoki-
netics can strongly be influenced by premature cleavage of
the linker that connects the protein with the cytotoxic drug
(Dorywalska et al. 2015; Shen et al. 2012; Strop et al. 2013).
The Val-Cit linker motif is especially prone to cleavage by a
carboxylesterase (mCes1c) that is present in mouse serum
but absent in human serum (Dorywalska et al. 2016). The
extend of this instability heavily depends on the chosen
conjugation sites. When Fcab-MMAE conjugates were
incubated in mouse and human serum for 96 h, no free
MMAE could be detected for all constructs. This indicates
that the Val-Cit linker motif is not accessible for mCes1c
neither at position Q295 nor at the novel positions Q311 and
Q438, hence all positions protect the conjugate from being
cleaved prematurely (Table 2).

**Table 1: Overview of conjugated Fcabs and controls.**

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Size [kDa]</th>
<th>Site</th>
<th>Technique</th>
<th>DAR</th>
<th>SE-HPLC purity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fcab-1-MMAE</td>
<td>52.02</td>
<td>Q295, Q311, Q438</td>
<td>mTG</td>
<td>2.9</td>
<td>96.7</td>
</tr>
<tr>
<td>Fcab-2-MMAE</td>
<td>51.07</td>
<td>Q295, Q311, Q438</td>
<td>mTG</td>
<td>2.7</td>
<td>98.5</td>
</tr>
<tr>
<td>Fcab-3-MMAE</td>
<td>51.93</td>
<td>Q295, Q311, Q438</td>
<td>mTG</td>
<td>2.8</td>
<td>99.7</td>
</tr>
<tr>
<td>huFc-MMAE</td>
<td>53.00</td>
<td>Q295</td>
<td>mTG</td>
<td>2.0</td>
<td>100.0</td>
</tr>
<tr>
<td>C-Fab-MMAE</td>
<td>50.49</td>
<td>LC-C-LPETG</td>
<td>SrtA</td>
<td>0.8</td>
<td>99.5</td>
</tr>
<tr>
<td>C-IgG-MMAE</td>
<td>155.37</td>
<td>LC-C-LPETG</td>
<td>SrtA</td>
<td>1.1</td>
<td>99.5</td>
</tr>
</tbody>
</table>

Size refers to unconjugated protein including the most abundant
glycosylation pattern as measured by LC-MS. DAR is given as a mean
from RP-HPLC and LC-MS analysis. SE-HPLC purity refers to the final
drug conjugate and was analyzed after a freeze-thaw cycle. LC-C –
light chain C-terminus.

**In vitro cytotoxicity of Fcab-drug conjugates**

Next, we evaluated selective cell killing capabilities of the
Fcab-drug conjugates in an in vitro cell proliferation assay
(Figure 5). All Fcab-drug conjugates showed similar sub-
nanomolar inhibitory activity on EGFR positive MDA-MB-
468 and A431 cells (IC₅₀ 0.18–0.22 nM and 0.23–0.32 nM,
respectively) while toxicity against EGFR negative MCF-7
cells was decreased by several orders of magnitude
(IC₅₀ > 100 nM) indicating strong target-dependent cell
killing. Non-targeting huFc-MMAE showed low toxicity as
well (MDA-MB-468: IC₅₀ > 300 nM; A431 and MCF-7:
IC₅₀ > 100 nM) confirming that Fcab-MMAE toxicity is pri-
marily driven by specific receptor-mediated uptake. In line
with higher cellular uptake on MDA-MB-468 cells compared
to A431 cells (Figure 2), Fcab-drug conjugates and Cetux-
imab controls showed higher activity on MDA-MB-468 cells
compared to A431 (Figure 5B). Furthermore, higher potency
Figure 4: Conjugation site identification.
LC-MS chromatogram of digested Fcab-1-MMAE shows conjugated peptides (e–i, k–m) that were not detected in the Fcab-1 preparation. Conjugated peptides eluted at higher retention times due to hydrophobic MMAE. Matched pairs of unconjugated peptides (a–d) eluted at lower retention times and peaks disappeared (a, b) or showed reduced intensity (c) in the Fcab-1-MMAE preparation compared to Fcab-1.

Table 2: Binding affinity and serum stability of Fcab-drug conjugates and controls.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>$K_D$ (EGFR) [nM]</th>
<th>$K_D$ (FcRn) [nM]</th>
<th>$K_D$ (EGFR) [nM]</th>
<th>$K_D$ (FcRn) [nM]</th>
<th>Free MMAE (%) Total MMAE</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fcab-1-MMAE</td>
<td>2.42 ± 0.01</td>
<td>381 ± 12</td>
<td>2.67 ± 0.02</td>
<td>309 ± 10</td>
<td>1.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Fcab-2-MMAE</td>
<td>1.07 ± 0.01</td>
<td>329 ± 13</td>
<td>1.37 ± 0.01</td>
<td>362 ± 12</td>
<td>1.1</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Fcab-3-MMAE</td>
<td>1.71 ± 0.01</td>
<td>589 ± 22</td>
<td>1.54 ± 0.01</td>
<td>337 ± 12</td>
<td>0.9</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>huFc-MMAE</td>
<td>0.0</td>
<td>n.d.</td>
<td>0.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>C-Fab-MMAE</td>
<td>0.82 ± 0.01</td>
<td>–</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>C-IgG-MMAE</td>
<td>1.25 ± 0.01</td>
<td>747 ± 24</td>
<td>0.60 ± 0.01</td>
<td>892 ± 31</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

Dissociation constants ($K_D$) were measured by BLI. Binding to recombinantly produced EGFR and FcRn were measured at pH 7.4 and 6.0, respectively. Errors are standard errors from fitting using FortéBio data analysis software 12.0. Twofold changes of $K_D$ are in the range of assay variability. Free MMAE was measured via LC-MS/MS after incubation in mouse and human sera at 37 °C for h (n = 3). Numbers show the released fraction relative to initially conjugated MMAE. n.d. – not determined.
of Fcab-drug conjugates on EGFR positive cells (IC50 0.18–0.32 nM) compared to C-Fab-MMAE (IC50 0.78–0.99 nM) or C-IgG-MMAE (IC50 0.44–0.50 nM) suggested that reduced intracellular accumulation of Fcabs (Figure 2) may be compensated by their higher DAR enabled by additional conjugation to Q311 and Q438 (2.7–2.9 vs. 0.8 and 1.1, respectively). Overall, the results indicate that in vitro potency of Fcab-drug conjugates is in the typical sub-nanomolar range of ADCs despite their monovalent binding mode.

**Conclusion**

We have recently demonstrated the suitability of the Fcab format for generation of drug conjugates using HER2 binding Fcabs in a first feasibility study (Jäger et al. 2021). Here, we have further expanded the concept of Fcab-drug conjugates to constructs that carry a higher drug load and are targeted against the therapeutically relevant solid cancer target EGFR. Fcabs produced in the scope of this study bound EGFR with nanomolar affinity and accumulated...
target-dependently in EGFR expressing cells. Site-specific conjugation to Q295 and the novel positions Q311 and Q438 via mTG yielded DAR 2.7–2.9 Val-Cit-PAB-MMAE conjugates without altered EGFR or FcRn binding affinities. Generated Fcab-drug conjugates exhibited high stability in human and mouse serum and showed EGFR-mediated cytotoxicity at sub-nanomolar concentrations similar to Cetuximab-based reference conjugates. The Fcab-drug conjugate candidates presented herein show favorable stability and potency profiles suggesting further animal studies to evaluate whether Fcabs’ smaller size and intermediate plasma half-life are beneficial for ADC exposure to solid tumors.

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