Abstract No
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Highly cytotoxic EGFR/CD16A TandAbs specifically recruit NKcells to potently kill various types of solid tumors

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Introduction

Constitutive EGFR activation plays an important role in the pathophysiology of several solid cancers, such as colorectal, non small cell lung or squamous cell carcinomas of the head and neck. Tyrosine kinase inhibitors or monoclonal antibodies that prevent EGFR ligand binding, dimerization and activation have been approved for treatment of such cancers. However, despite demonstrated clinical efficacy, intrinsic or acquired resistance to such treatments has been described for a larger number of patients.

Natural killer cells (NK-cells) play a central role in the innate immune system and have the capacity to destroy neoplastic cells. To specifically utilize the cytotoxic potential of NK-cells for the elimination of EGFR$^+$ cancer cells, tetravalent bispecific EGFR/CD16A NK-cell TandAbs, with two binding sites for EGFR expressed on tumor cells, and two binding sites for CD16A expressed on NK-cells were developed.

Using antibody phage display, scFv recognizing novel epitopes in the extracellular domain of EGFR were identified and characterized. Bispecific EGFR/CD16A TandAbs were constructed and analyzed in terms of thermo-stability, binding and cytotoxicity. TandAbs containing our EGFR-specific domain were highly potent in cytotoxicity assays towards EGFR$^+$ tumor cell lines or transfected CHO cells with single digit picomolar or subpicomolar EC_{50} values. In contrast to NK-cell recruiting TandAbs containing the Fv sequences from cetuximab, TandAbs containing our EGFR-binding domain did not exhibit signs of thermal instability or aggregation. Our data suggest that EGFR/CD16A TandAbs are novel, highly potent drug candidates suitable for the treatment of EGFR expressing malignancies and overcoming intrinsic or acquired resistance to other drugs.

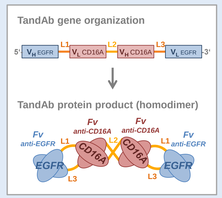
TandAbs are potent bispecific tetravalent antibodies

a. TandAb Features

- comprised of V_H and V_L domains
- expressed as a single gene product
- linkers favor intermolecular head-to-tail homodimerization

b. TandAb Properties

- Bivalent binding to antigens on target and effector cells
- No renal clearance due to MW >100 kDa
- Excellent drug-like properties (production and stability)



c. Mode of Action

- NK-cell recruitment (CD16A NK-cell TandAb Platform)

EGFR-specific Fv sequences were formatted into TandAbs

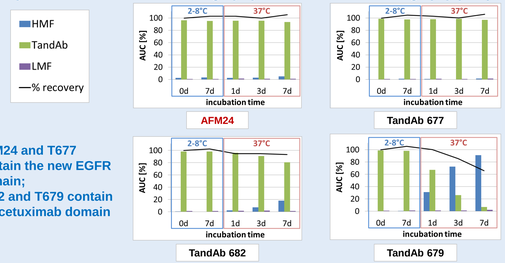
- Novel EGFR-specific scFvs were identified by phage display screening
- In addition, EGFR-binding Fv sequences of cetuximab (C225) were used to construct TandAbs as comparators
- All anti-EGFR domains were combined with previously described anti-CD16A domains to construct NK-cell recruiting TandAbs
- Various TandAbs differing in the order and position of both binding specificities (EGFR and CD16A) were generated and characterized

TandAbs with anti-EGFR in outer position

TandAbs with anti-EGFR in inner position

TandAbs generated with novel EGFR antibodies show excellent stability

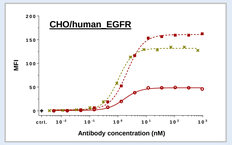
- Incubation of purified TandAbs at 37°C or 2-8°C in buffer (500 µg/ml) for 1, 3 and 7 days
- Analysis of molecular forms and integrity by size exclusion chromatography (SEC)



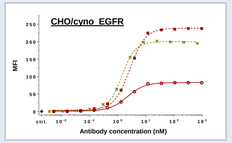
AFM24 and T677 contain the new EGFR domain;
T682 and T679 contain the cetuximab domain

TandAbs bind to human and cyno EGFR and CD16A

Binding of AFM24, T677 and cetuximab to CHO cells expressing human or cynomolgus EGFR:



Antibody	K_D [nM] (human)
TandAb677	1,7
AFM24	2,2
Cetuximab	1,1



Antibody	K_D [nM] (cyno)
TandAb677	2,2
AFM24	2,8
Cetuximab	1,4

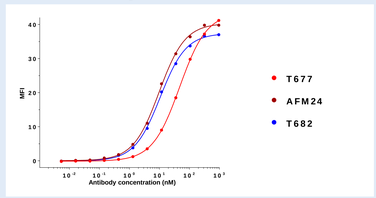
Binding of AFM24 and T677 to human or cynomolgus EGFR in ELISA:

Species	TandAB	EGFR-Fc KD [nM]	CD16A-Fc KD [nM]
Human	T677	0.257	0.9
	AFM24	0.084	0.5
Cynomolgus	T677	0.304	0.9
	AFM24	0.082	0.6

AFM24 shows comparable binding to human and cynomolgus EGFR

Binding to CD16A on NK-cells

NK-cell binding at 37°C

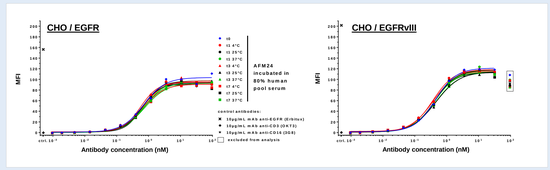


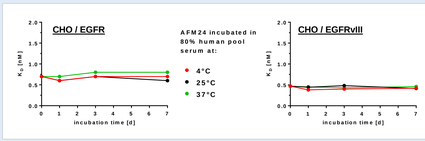
	K_D [nM]
T677	46.2
AFM24	9.8
T682	10.8

Binding on CD16A on NK cells may depend on domain order

High serum stability of AFM24 at 4, 25 and 37°C and binding to EGFR-expressing CHO cells

AFM24 was incubated in human pool serum at 4°C, 25°C, and 37°C for 0, 1, 3, or 7 days. Binding activity of stressed AFM24 samples was quantified by titration of samples on EGFR$^+$ and EGFRvIII$^+$ CHO cells and flow cytometric analysis. K_D values were determined by non-linear regression.





- AFM24 showed excellent serum stability at 4, 25, and 37°C when tested for cellular antigen binding
- AFM24 also demonstrated serum stability when tested for binding on NK-cells at 4, 25, and 37°C (data not shown)

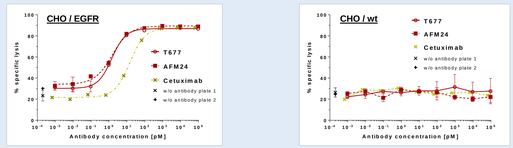
AFM24 binds to EGFR-expressing cell lines and NK-cells with high affinity and specificity, and induces strong cytotoxicity

Cells tested	Binding K_D [nM]	Cytotoxic activity EC_{50} [pM]
A431 (human epidermoid carcinoma)	1.2	5.9
CHO_{EGFR} (hamster ovary cells)	1.3	1.5
CHO_{WT} (target negative)	No binding	No cytotoxicity
DK-MG (human glioma)	0.7	4.5
A-549 (human lung carcinoma)	0.5	2.3
NK-cells (human)	10.9	n.a.

- AFM24 binds to human tumor cells with high affinity and specificity
- No binding was seen on target-negative CHO cells

No off-target activity of EGFR/CD16A TandAbs

- No AFM24-mediated NK-cell lysis of CHO_{WT} cells in the absence of EGFR antigen
- In contrast, AFM24 induces dose-dependent lysis by NK-cells in the presence of target cells



PKH67-labeled CHO cells were incubated with human NK-cells at an E:T ratio of 5:1 in the presence of increasing antibody concentrations. After 24h incubation target cell lysis was quantified by flow cytometry with prodidium iodide staining of dead cells. EC_{50} values for lysis were determined by non-linear regression/sigmoidal dose-response (n=2, Mean±SD).

High affinity binding of AFM24 enables efficient killing of EGFR$^+$ tumor cells and greater cytotoxicity than cetuximab

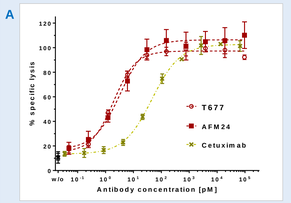
A431 tumor cells as targets and NK-cells as effectors

EC_{50} AFM24: 3.3 pM
EC_{50} T677: 1.9 pM
EC_{50} Cetuximab: 46.0 pM

DK-MG tumor cells as targets and NK-cells as effectors

EC_{50} AFM24: 2.5 pM
EC_{50} T677: 5.5 pM
EC_{50} Cetuximab: 52.3 pM

A



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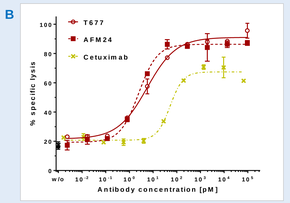

Calcein-labeled A431 (A) or DK-MG (B) target cells were incubated with enriched human NK-cells at an E:T ratio of 5:1 in the presence of increasing antibody concentrations. After 4 h incubation fluorescent calcein released from lysed target cells was quantified and used for the calculation of % specific lysis. Mean and SD of duplicates are plotted. EC_{50} values were calculated by non-linear regression/sigmoidal dose-response.

- AFM24 demonstrates highly potent and efficacious tumor cell killing
- AFM24 shows superior tumor cell killing compared to cetuximab as shown by the the lower EC_{50} values of AFM24 (13.9- or 20.9-fold higher for cetuximab)
- NK-cell recruitment results in superior anti-tumor efficacy AFM24 in direct comparison to cetuximab

Summary

- AFM24 shows excellent stability at 4°C, 25°C, and 37°C for up to 7 days in buffer and serum
- AFM24 binds specifically to human EGFR and to human CD16A, but not to human CD16B
- AFM24 binds with comparable affinity to EGFR of cynomolgus monkeys (relevant species)
- AFM24 demonstrates high affinity binding and specificity to several EGFR$^+$ cell lines from different tumor types, and most importantly no binding is seen to target-negative cells
- AFM24 shows superior killing of tumor cells in direct comparison to cetuximab

Conclusion

Despite demonstrated clinical efficacy of anti-EGFR therapeutics, intrinsic or acquired resistance has been described for a substantial number of patients. We developed tetravalent bispecific EGFR/CD16A NK-cell recruiting TandAbs to engage the cytotoxic potential of NK-cells for the elimination of EGFR-expressing cancer cells.

The combination of high affinity EGFR binding domains with potent NK-cell recruitment is hypothesized to overcome such therapeutic resistance and lead to improved tumor cell killing compared to EGFR-targeting IgGs such as cetuximab, or small molecule inhibitors such as erlotinib. NK-cells play a central role in the innate immune system and have the capacity to not only destroy neoplastic cells, but also to re-shape the tumor microenvironment as previously shown for AFM13 *in vivo*. Furthermore, combinations with check-point modulators may augment anti-tumor efficacy in solid tumors with high medical need.

Taken together, our data suggest that AFM24 is a novel, highly potent drug candidate suitable for the treatment of EGFR-expressing malignancies.