Abstract
559

Preclinical Characterization of the Bispecific EGFR/CD16A Innate Immune Cell Engager AFM24 for the Treatment of EGFR-Expressing Solid Tumors

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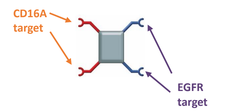
Introduction

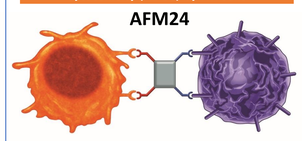
- Epidermal growth factor receptor (EGFR) is known to be overexpressed in several tumor types (e.g. colorectal cancer, non-small cell lung cancer, head and neck squamous cell carcinoma, glioblastoma, and triple-negative breast cancer). Additionally, mutations frequently arise in the EGFR signaling pathway, which may lead to increased tumor growth
- Current standard of care (SOC) therapies, including EGFR-targeting monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs), are limited on the one hand by intrinsic or acquired mutations that impact EGFR signal transduction, and on the other hand by associated side effects
- Affimed's fit-for-purpose Redirected Optimized Cell Killing (ROCK®) platform can be used to generate customized therapies against targets using a new mechanism of action (MOA) that activates innate immunity. Redirecting CD16A-expressing innate immune cells (NK cells/macrophages) to EGFR-positive tumor cells have the potential to activate a broad anti-tumor immune response
- AFM24 is a fully human tetravalent bispecific EGFR- and CD16A-binding antibody designed from the ROCK® platform to target EGFR-expressing solid tumors. AFM24's targeting capabilities through its novel MOA could overcome the resistance observed with other EGFR-targeting agents, and potentially offer an improved safety profile
- The preclinical characterization of AFM24 described below demonstrates encouraging results that AFM24 can redirect innate immune cells to EGFR-expressing malignancies and may act through a mutation-independent MOA and overcome resistance observed with current SOC therapies, while conferring a favorable safety profile

AFM24 is an EGFR-targeting innate cell engager inducing ADCC/ADP

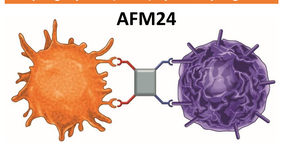
- **Developed by Affimed's fit-for-purpose ROCK® platform to target EGFR-expressing tumors**
- **Tetravalent bispecific antibody using a proprietary CD16A-binding domain that redirects NK cells and macrophages to EGFR-expressing tumors**



MOA: Antibody-dependent cellular cytotoxicity (ADCC) by NK cells



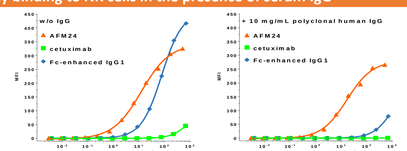
MOA: Antibody-dependent cellular phagocytosis (ADCP) by macrophages



AFM24 overcomes significant limitations of mAbs in binding to CD16A on NK cells

- **In contrast to cetuximab and Fc-enhanced immunoglobulin G (IgG), binding of AFM24 is virtually unaffected by IgG competition at physiological levels**

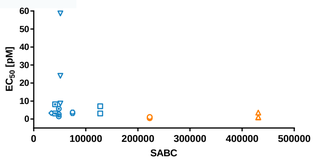
High affinity binding to NK cells in the presence of serum IgG



Primary human NK cells were incubated with serial dilutions of the indicated antibodies in the absence (left) or presence (right) of 10 mg/mL polyclonal human IgG at 37°C. Cell surface-bound AFM24 was detected by anti-AFM24 mAb 62-1-1 followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Biotinylated anti-EGFR IgG1 (cetuximab, Fc-enhanced [S239D, I332E] IgG1 with AFM24-derived anti-EGFR Fv domains) were detected with AlexaFluor488-conjugated streptavidin.

High cytotoxic potency of AFM24 *in vitro* is independent of EGFR density, BRAF and KRAS mutation

No correlation of EGFR density and origin of tested cell line for AFM24 potency



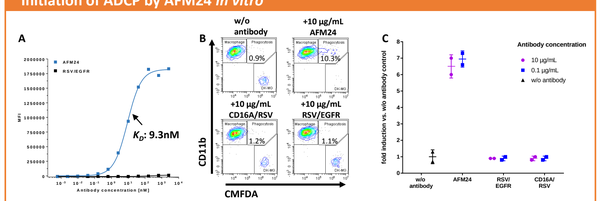
Cell line	Mean SABC	Mean EC_{50} [pM]	SD	n
A-431	431125	1.9	1.5	3
A-549‡	127574	5.1	2.8	2
COLO205†	51177	30.4	25.6	3
DK-MG	222648	0.8	0.4	4
HCT-116‡	33822	3.3	n/a	1
HT-29†	48174	3.3	2.2	3
LoVo†‡	74470	3.4	0.6	2
Panc 08.13‡	40980	5.7	3.6	2

Specific antibody-binding capacity (SABC) was determined using QIFIKIT® and anti-EGFR mAb H11. EC_{50} of AFM24 was determined by titration of AFM24 in 4 h calcein-release cytotoxicity assays with EGFR⁺ tumor cell lines (T) and primary human NK cells as effector cells (E) at an E:T ratio of 5:1. EC_{50} values were determined by non-linear regression/sigmoidal dose response. †: containing a BRAF mutation ‡: containing a KRAS mutation.

AFM24 efficiently induces ADCP by human macrophages

- **AFM24 binds to human macrophages and efficiently induces ADCP of EGFR⁺ tumor cells**
- **ADCP is specific and dependent on both EGFR- and CD16A-binding of AFM24**

Initiation of ADCP by AFM24 *in vitro*

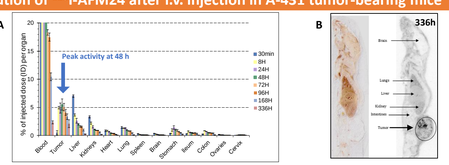


A) Binding of AFM24 to human macrophages generated from healthy donor peripheral blood mononuclear cells by *in vitro* culture in the presence of macrophage colony-stimulating factor (M-CSF). B) AFM24-induced phagocytosis of CMFDA-labeled, EGFR-expressing DK-MG cells mediated by unpolarized CD11b⁺ macrophages. Exemplary FACS plots showing phagocytosis of DK-MG cells upon 4 h co-culture with or without AFM24, CD16A/RSV, or RSV/EGFR antibody constructs at the indicated concentrations at an E:T ratio of 5:1. C) AFM24-induced phagocytosis of EGFR-expressing DK-MG after 4 h co-culture with macrophages at an E:T ratio of 5:1. Phagocytosis was assessed by flow cytometry quantifying CMFDA⁺CD11b⁺ cells as fold induction of control without antibody.

Tumor-specific accumulation of ^{125}I-AFM24 in mice after intravenous (i.v.) injection

- **Distribution pattern in normal tissue reflected the pattern of blood flow and blood circulation**
- **Gradual increase in ^{125}I-AFM24 activity in the tumor, peaking at 48 h; activity in tumor gradually decreased and accounted for 5.5% ID/organ at 96 h and 2.1% ID/organ at 336 h**

Distribution of ^{125}I-AFM24 after i.v. injection in A-431 tumor-bearing mice

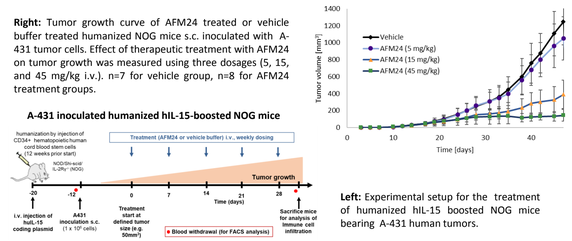


NOD-SCID mice were inoculated subcutaneously (s.c.) into the flank with $5x10^6$ A-431 tumor cells. When tumors reached a volume of 250 ± 50 mm^3, 32 mice were i.v. injected with a single dose of 0.5 mg/kg ^{125}I-AFM24 corresponding to about 10 µg and 15 µCi per mouse. (A) Tissue distribution and tumor uptake of ^{125}I-AFM24 was assessed at 8 time points over a period of 14 days following i.v. injection (left). (B) A supplementary mouse from the 336 hour time point was subjected to quantitative whole body autoradiography (right).

AFM24 shows dose-dependent *in vivo* tumor growth inhibition in a therapeutic setting

Efficacy of AFM24 against A-431 tumors in humanized NOG mice

Right: Tumor growth curve of AFM24 treated or vehicle buffer treated humanized NOG mice s.c. inoculated with A-431 tumor cells. Effect of therapeutic treatment with AFM24 on tumor growth was measured using three dosages (5, 15, and 45 mg/kg i.v.). n=7 for vehicle group, n=8 for AFM24 treatment groups.



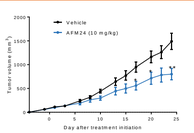
Left: Experimental setup for the treatment of humanized hIL-15 boosted NOG mice bearing A-431 human tumors.

AFM24 has anti-tumor efficacy and enhances tumor infiltration of immune cells

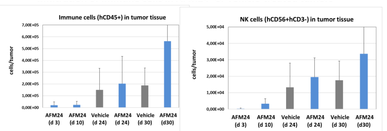
Tumor growth inhibition and tumor immune cell infiltration of AMF24 *in vivo*

Efficacy of AFM24 in a therapeutic setting

Right: Tumor growth curve of AFM24 or vehicle buffer treated humanized NOG mice inoculated s.c. with A-431 tumor. Therapeutic effect of AFM24 on A-431 tumor growth (10 mg/kg). Mean ± standard error of mean (SEM) of the tumor growth is represented for each group. n=6 for vehicle group, n=15 for AFM24 treatment group. Vehicle group vs AFM24 treated group *p<0.05, **p<0.01.



Infiltration of immune cells in AFM24- or vehicle-treated tumors

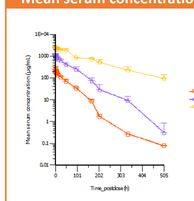


Left: Fluorescence-activated cell sorting (FACS) analysis of tumor tissues from AFM24- and vehicle-treated mice. Treatment started when tumor size has reached a volume of 50–100 mm^3, weekly i.v. administration (4 times maximum). Number of animals n=3 (d3, d10, and d24, satellite mice) and n=6 (d30).

Pharmacokinetics (PK) of AFM24 in cynomolgus monkeys

- **Male cynomolgus monkeys received a single 2-hour i.v. infusion of AFM24 (three dose levels)**
  - **Terminal half-life ranged from 33.4 to 154 h, which approximates to half-lives observed for cetuximab and panitumumab**
  - **Systemic CL decreased and terminal half-life increased at higher doses**
  - **Volume of distribution indicated that AFM24 is mainly located in the plasma volume**

Mean serum concentrations and pharmacokinetic parameters of AFM24

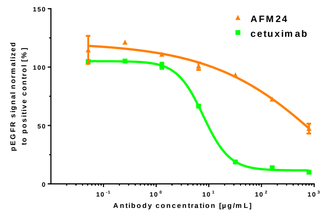


Dose (mg/kg)	8	25	75
C_{max} (µg/mL)	265	1295	2593
AUC_{last} (h*µg/mL)	10028	63881	304704
AUC_{inf} (h*µg/mL)	10034	64157	321203
CL (mL/h/kg)	0.807	0.398	0.237
Vz (mL/kg)	61.7	24.2	35
$t_{1/2}$ (h)	53	43.1	106

Three groups (n=3) of male cynomolgus monkeys received a single i.v. dose of 8, 25, or 75 mg/kg AFM24. Samples were collected pre-dose and at the time points indicated (semi logarithmic scale). AFM24 serum concentrations were determined by an Electrochemiluminescence-Based Ligand Binding Assay (Meso Scale Discovery). PK parameters (mean) were determined by Non-Compartmental Analysis using Phoenix® WinNonlinTM (Pharsight Corporation, Mountain View, California 94040/USA).

AFM24 shows reduced inhibition of safety-relevant EGFR signaling

Inhibition of EGFR signaling by AFM24 at higher concentrations compared with cetuximab



Aliquots of $5x10^4$ A-431 cells were seeded in individual wells of a 96-well micro plate and starved for 4 h before incubation for 30 min with the indicated antibodies. After 10 min stimulation with 100 ng/mL EGF, cells were lysed, and phosphorylated EGFR was quantified using a phosphotyrosine EGFR enzyme-linked immunosorbent assay (ELISA) (RayBiotech). Individual values were normalized to positive control (recombinant EGF without antibody) and plotted against antibody concentration.

AFM24 was well tolerated in cynomolgus monkeys

- **Animals were dosed weekly (q7d x 28d) by a 2-hour infusion for 4 weeks including a 5-week recovery phase**
- **All animals were systemically exposed as shown by toxicokinetic analysis**
- **Transient-marked elevation of circulating interleukin-6 levels at all dose levels 2–4 hours after the first dose; fully reversible to baseline after 24 hours**
- **Transient reduction in absolute NK cell counts (CD3-CD20-CD159+) and CD69+ activated NK cells in peripheral blood at a dose ≥24 mg/kg 7 days after first administration**
- **AFM24 did not induce systemic or local toxicity and was well tolerated up to the highest dose of 75 mg/kg. By contrast, cetuximab revealed moderate to severe toxicity at identical dose levels with skin as the primary target organ**

28 days pilot toxicity study in cynomolgus monkeys with AFM24

Group description	Dose level (mg/kg)	Dose volume (mL/kg/h)	Animals/Group Males	Animals/Group Females	Necropsy after 4 weeks	Necropsy after 9 weeks
Control	0	5	1	1	1M/1F	
Low	8	5	1	1	1M/1F	
Medium	25	5	1	1	1M/1F	
High	75	5	2	2	1M/1F	1M/1F

Summary & Conclusions

- Innate cell engagers developed from the ROCK® platform enable targeting of the clinically validated tumor antigen EGFR that with the current SOC, has limitations in efficacy and/or shows dose-limiting toxicities
- AFM24 is designed to overcome limitations of SOC therapy by addressing resistant patient populations and potentially offering an improved safety profile
- AFM24 is based on the novel MOA of redirecting CD16A-bearing innate immune cells.
- AFM24 demonstrated an ability to bridge NK cells and macrophages to EGFR-expressing tumors, independent of KRAS and BRAF status, and induced tumor lysis through ADCC and ADCP, respectively
- AFM24 elicited *in vivo* anti-tumor efficacy through dose-dependent tumor inhibition and enhanced tumor infiltration
- AFM24 showed reduced inhibition of EGFR phosphorylation relative to cetuximab and demonstrated a favorable safety profile in cynomolgus monkeys (relevant species), potentially indicating significantly lower toxicities compared with SOC

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