Evaluation of EphA2 as a Therapeutic Target for Redirected T-cell Killing by DART® Bispecific Molecules

583

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Abstract

Introduction: EphA2 is a receptor tyrosine kinase that plays a critical role in cancer progression through both ligand-dependent and independent mechanisms. The broad overexpression in tumors, but limited normal tissue expression, of EphA2 makes it an attractive therapeutic target amenable for redirected T-cell killing via EphA2 x CD3 Dual-Affinity Re-Targeting (DART®) molecules designed to co-engage cytotoxic T cells with EphA2-expressing tumor cells.

Methods: Anti-EphA2 monoclonal antibodies (mAbs) were identified by target-specific screening of a library generated by whole-cell immunization with proprietary cancer cell lines, including models of cancer stem cells. The binding and signaling properties of the antibodies were characterized by ELISA, surface plasmon resonance (SPR), flow cytometry, and phosphorylation assays. Receptor binding regions were determined by ELISA-based competition assays and by utilizing human-mouse chimeric EphA2 molecules. Immunohistochemistry (IHC) was performed on frozen normal and tumor tissues. In vitro functional studies were performed with various cancer, as well as transfected cell lines, and primary human T cells or peripheral blood mononuclear cells (PBMCs). In vivo activity was evaluated in xenograft models in immune-deficient mice.

Results: EphA2 mAbs encompassing diversity in binding kinetics and effects on receptor phosphorylation were classified in 5 discrete binding groups. The majority interacted with the N-terminal ligand-binding domain of EphA2 and most mAbs within that group interfered with ligand binding. The majority of mAbs displayed little IHC reactivity with normal tissue, while strong staining of cancer tissues was observed, including colon, lung, pancreas, ovary, bladder, and breast cancers. Seven mAbs recognizing independent epitopes were selected for conversion into EphA2 x CD3 DART molecules that showed a range of potency in redirecting T cells to kill EphA2-expressing target cells. A lead EphA2 x CD3 DART molecule was selected based on potency and cross-reactivity with the cynomolgus monkey ortholog; this lead was engineered with a human Fc domain to confer an extended circulating half-life. The resulting Fc-bearing EphA2 x CD3 DART molecule demonstrated in vivo antitumor activity at doses as low as 20 µg/kg in NOD/SCID/IL-2 gamma chain null (NOG) mice co-implanted with activated human T cells and MDA-MB-231 breast cancer cells.

Conclusion: EphA2 is a potential cancer target for redirected T-cell killing applications independent of ligand-mediated mechanisms. Further preclinical assessment of EphA2 x CD3 DART molecules as a strategy for targeting EphA2-expressing malignancies is warranted.

Methods

- Binding group determination and ligand interference: Antibodies were tested in ELISA-based competition assays. Binding of EphA2 antibodies to immobilized recombinant EphA2 in the presence or absence of excess competing antibodies or EphA2 ligands ephrin A1 or ephrin A5 was measured by appropriate detection methods
- Antibody-induced receptor phosphorylation: Serum-starved G55 cells (a glioblastoma line) were exposed to EphA2 antibodies and the effect on receptor phosphorylation determined by EphA2 immunoprecipitation and Western Blot with anti-phosphotyrosine and anti-EphA2 antibodies
- In vitro cytotoxicity: EphA2-positive cell lines were exposed to EphA2 x CD3 DART molecules at increasing concentrations in the presence of human PBMCs or T cells (E:T = 10:1 to 30:1) for 24 or 48 hours. Cytotoxicity was measured by LDH release assay
- T-cell activation: Human PBMCs or T cells were exposed to increasing concentrations of EphA2 x CD3 DART molecules in the presence or absence of target-bearing cancer cells. Activation was measured by determining cytokine release (IFN-γ) by Luminex or expression of CD25 on T cells by flow cytometry
- In vivo activity: NOG mice were implanted subcutaneously with activated human T cells and target cells (E:T = 1:5). EphA2 x CD3 DART molecules were administered by intravenous injection once daily for 4 consecutive days starting on Day 0 and the effect on tumor growth was monitored by caliper measurement twice weekly

Results

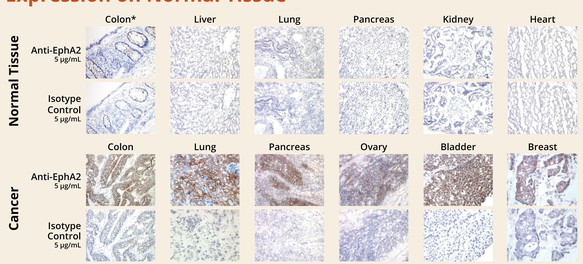
Generation of a Panel of Anti-EphA2 Reactive Antibodies with Diverse Binding and Functional Properties

Binding Group	Name	Isotype	Ligand Interference	Induces Phosphorylation
A	MG.A1	IgG1	–	N
A	MG.A2	IgG1	+	N
A	MG.A3	IgG1	+	Y
A	MG.A4	IgG1	–	Y
A	MG.A5	IgG2b	++	Y
A	MG.A6	IgG2b	++	N
A	MG.A7	IgG2b	++	N
A	MG.A8	IgG2a	+++	Y
A	MG.A9	IgG2a	+	N
A	MG.A10	IgG2a	++	N
A	MG.A11	IgG2a	+	Y
A	MG.A12	IgG2a	+	N
A	MG.A13	IgG1	–	Y
A	MG.A14	IgG1	–	Y
A	MG.A15	IgG1	+	Y
A	MG.A16	IgG2a	++	Y
A	MG.A17	IgG1	++	Y
A	MG.A18	IgG1	+	N
A	MG.A19	IgG2a	+++	Y
A	MG.A20	IgG2a	+	Y
A	MG.A21	IgG1	++	Y
B	MG.B1	IgG1	–	N
B	MG.B2	IgG2a	–	Y
C	MG.C1	IgG1	–	N
C	MG.C2	IgG1	–	N
C	MG.C3	IgG2a	–	N
D	MG.D1	IgG2a	–	N
D	MG.D2	IgG2a	–	N
E	MG.E1	IgG2a	–	Y
E	MG.E2	IgG1	–	Y
E	MG.E3	IgG1	–	Y
E	MG.E4	IgG1	–	Y

EphA2 Extracellular Domain (ligand binding domain, sushi, EGF, fibronectin 1+2 domains)

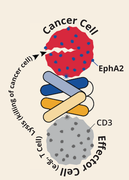
- A panel of anti-EphA2 mAbs was identified by target-specific screening of a library of purified mAbs generated by whole-cell immunization with proprietary cancer cell lines, including models of cancer stem cells
- EphA2 antibodies belong to 5 distinct binding groups
- The majority of antibodies interacts with the N-terminal ligand-binding domain of EphA2 (binding group A) and most mAbs within that group interfere with ligand binding
- All antibodies cross-react with cynomolgus monkey EphA2
- The EphA2 antibodies show diversity in their effect on receptor phosphorylation

EphA2 is Highly Expressed on Tumors but Shows Limited Expression on Normal Tissue



* Colon sample subjected to BLOXALL blocking and mAb used at optimal concentration.

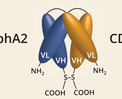
Redirected T-cell Killing Through DART Molecules



- DART molecules engage T cells via anti-CD3 and cancer cells through tumor cell-associated antigens, such as EphA2
- Cancer cell recognition is independent of TCR/MHC cognate interaction: virtually any T cell can be recruited to kill cancer cells
- Co-engagement of both targets is required for T-cell activation and target cell lysis
- Monovalent binding to CD3 avoids target independent T-cell activation
- Lead candidates from the EphA2 antibody panel were chosen to represent all binding groups and a variety of binding kinetics and assembled with an anti-CD3 into DART molecules

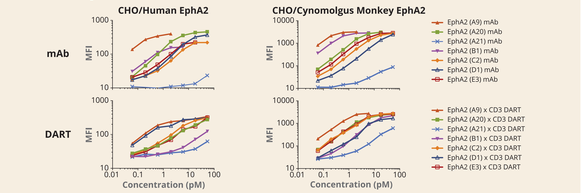
EphA2 x CD3 DART Molecule Panel — Binding and Activity

mAb (by binding group)	DART (by binding group)	Affinity to Immobilized EphA2 K_D (nM), k_a ($M^{-1}s^{-1}$)/k_d (s^{-1}) mAb	Affinity to Immobilized EphA2 K_D (nM), k_a ($M^{-1}s^{-1}$)/k_d (s^{-1}) DART	CTL (EC_{50} ng/mL) Transfected CHO Human	CTL (EC_{50} ng/mL) Transfected CHO Monkey	CTL (EC_{50} ng/mL) DU145	CTL (EC_{50} ng/mL) MDA-MB-231
MG.A9	A9 x CD3	16.7 8.4e4/1.4e-3	7.8 5.4e4/4.2e-4	0.63	2.75	0.20	4.99
MG.A20	A20 x CD3	12.5 1.2e5/1.5e-3	6.2 5.5e4/3.4e-4	0.57	0.87	0.41	14.20
MG.A21	A21 x CD3	83.3 1.8e4/1.5e-3	Not calculated (low capture)	6.94	5.60	3.62	36.77
MG.B1	B1 x CD3	28.8 3.2e5/9.2e-3	116.0 7.7e4/8.9e-3	1.05	3.07	1.78	14.10
MG.C2	C2 x CD3	10.0 7.4e4/7.4e-4	Not calculated (low off-rate) 3.6e4/<1.0e-5	1.46	0.67	0.66	0.76
MG.D1	D1 x CD3	3.1 3.2e5/1.0e-3	6.3 1.6e5/1.0e-3	0.16	5.88	0.09	0.18
MG.E3	E3 x CD3	4.6 1.6e5/7.4e-4	14.9 4.3e4/6.4e-4	4.64	2.96	12.67	6.87



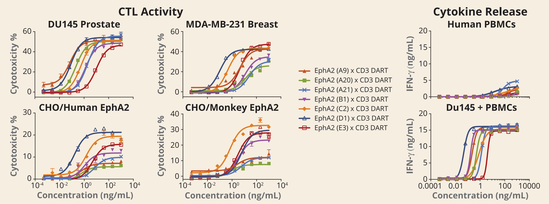
- Affinities were determined by SPR analysis for mAb (bivalent model) and DART molecules (monovalent model)
- DART molecule-induced T-cell mediated cytotoxicity was determined to compare potency across different cancer cell lines, as well as against human and cynomolgus monkey EphA2

Binding of EphA2 mAb and DART Molecules to Cell Surface EphA2



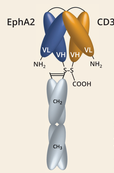
- Cell-surface binding of EphA2 mAbs to CHO cells expressing human or cynomolgus monkey EphA2 reveals range in binding affinities and species cross-reactivity
- EphA2-target cell recognition by mAb and DART molecules can vary depending on molecule characteristics (mono- vs. bi-valent)

EphA2 x CD3 DART Proteins Mediate EphA2 Target-dependent Cell Killing and Cytokine Release

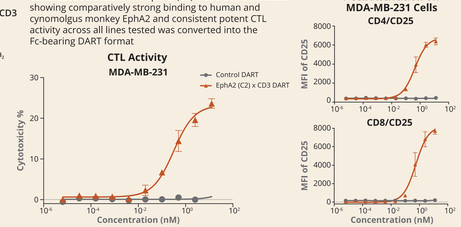


- EphA2 x CD3 DART molecules support range in CTL-mediated cancer cell cytotoxicity, with certain EphA2 targeting specificities (primarily binding group A) demonstrating cell line-dependent effects independent of EphA2-target cell recognition
- Evaluation of CTL activity against human and cynomolgus monkey EphA2-expressing CHO cells likewise reveals epitope-specific activity
- Cytokine release was minimal with human PBMCs alone, but significant upon co-engagement with EphA2-positive DU145 prostate cancer cells

An EphA2 x CD3 Fc-bearing DART Molecule Mediates CTL and Target-dependent T-cell Activation

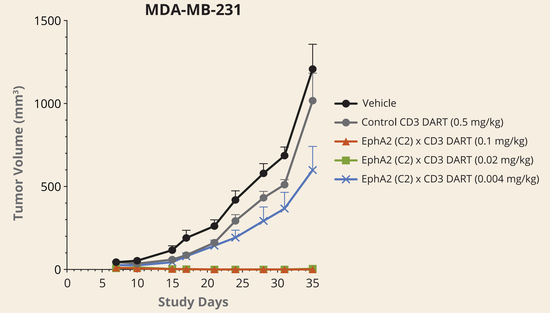


Candidate selection: The EphA2 (C2) x CD3 DART molecule showing comparatively strong binding to human and cynomolgus monkey EphA2 and consistent potent CTL activity across all lines tested was converted into the Fc-bearing DART format



- The EphA2 (C2) x CD3 Fc-bearing DART molecule shows CTL activity against MDA-MB-231 cells and target-dependent T-cell activation

Antitumor Activity of EphA2 x CD3 Fc-bearing DART Molecule Against MDA-MB-231 Cells in a Mouse Winn's Xenograft Model



- The EphA2 (C2) x CD3 Fc-bearing DART molecule shows strong antitumor activity in vivo

Conclusions

- Five distinct EphA2 monoclonal antibody groups were identified based on binding localization
- EphA2 antibodies display a range of binding kinetics and effects on receptor phosphorylation
- EphA2 x CD3 DART molecules recognizing the ligand binding domain of EphA2 show variation in CTL activity independent of cell-surface target expression
- EphA2 x CD3 DART molecules trigger cytokine release by T lymphocytes only in the presence of target cells
- An Fc-bearing EphA2 x CD3 DART molecule shows potent antitumor activity in vitro and in vivo

EphA2 is a potential cancer target suitable for redirected T-cell killing applications independent of ligand interference

Presented at the 2016 American Association for Cancer Research Annual Meeting, April 16–20, 2016, New Orleans, Louisiana