

# Impact of Binding Epitope and Antigen Size on the Cytotoxic Activity of MCSP-specific BiTE Antibodies for Treatment of Melanoma

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## Introduction

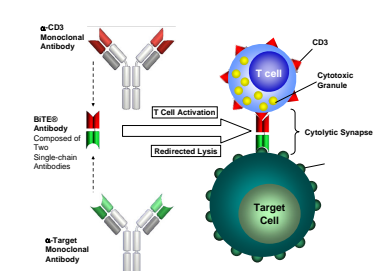
Melanoma chondroitin sulphate proteoglycan (MCSP; also called CSPG4, HMW-MAA, MSK16, MCSPG, MEL-CSPG, NG2, or gp240) is a surface antigen frequently expressed on human melanoma cells, which is involved in cell adhesion, invasion and spreading, angiogenesis, complement inhibition, and signalling. MCSP has therefore been frequently selected as target antigen for development of antibody- and vaccine-based therapeutic approaches. We have here used a large panel of monoclonal antibodies against human MCSP for generation of single-chain MCSP/CD3-bispecific antibodies of the BiTE (for bispecific T cell engager) class.

BiTE antibodies can transiently connect the T cell receptor subunit CD3 on T cells with a surface antigen on target cells leading to a highly efficient redirected lysis of target cells. This involves cytotytic synapse formation and delivery of perforin and granzymes. BiTE-engaged T cells are capable of serial target cell lysis, and are not affected by many immune escape mechanisms interfering with peptide antigen processing and presentation, or clonal T cell differentiation.

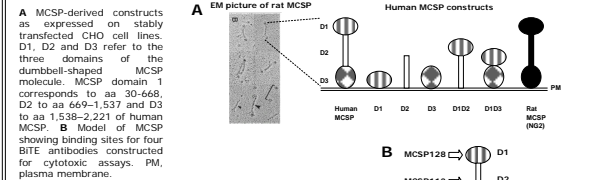
## Results

Despite similar binding affinity to MCSP, respective BiTE antibodies greatly differed in their potency of redirected lysis of CHO target cells stably transfected with full-length human MCSP, MCSP deletion mutants or fusion proteins. BiTE antibodies binding to the membrane proximal domain D3 of MCSP were more potent than those binding to more distal domains. This epitope distance effect was corroborated with EpCAM/CD3-bispecific BiTE antibody MT110 by using MCSP/EpCAM fusion proteins as surface target antigens. Generally, CHO cells expressing small surface antigens were better lysed than those with large antigens, indicating that antigen size is also an important factor for BiTE potency. This is in line with the observation that target cell lysis by membrane proximally-binding BiTE antibodies improved upon omission of more distal MCSP domains. The present study for the first time relates the size of surface antigens and positioning of binding domains to BiTE potency of target cell lysis via redirected cytotoxic T cells. In case of the MCSP antigen, this provides the basis for selection of a maximally potent BiTE antibody candidate for development of a novel melanoma therapy.

## BiTE® antibodies: Generation and mode of action

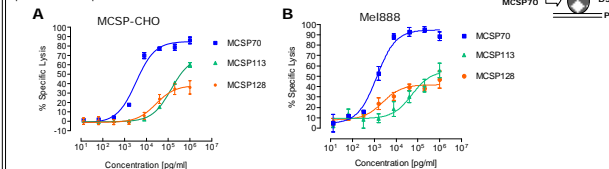


## MCSP constructs used to map antibody binding domains and to test BiTE activity



## The D3 domain-binding BiTE has the highest potency

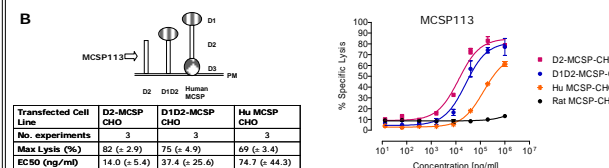
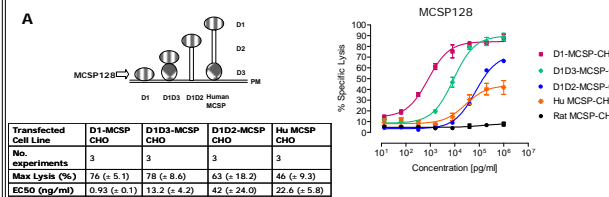
Redirected lysis of (A) human MCSP-expressing CHO line and (B) human melanoma cell line Mel888 by MCSP domain-specific BiTE antibodies. In a cytotoxicity assay 51Cr-labelled target cells were co-cultured for 18 h with stimulated human CD8+ T cells at an E:T ratio of 10:1 and the indicated BiTE-concentrations. **C** Maximal lysis and EC50-values from 3 different assays are shown. Each assay was performed in triplicates.



Cell Line	MCSP-CHO					Mel 888				
	BiTE	MCSP128	MCSP113	MCSP70	MCSP128	MCSP113	MCSP70	MCSP128	MCSP113	MCSP70
No. Experiments	3	3	3	3	3	3	3	3	3	3
Max. Lysis (%)	46 (± 9.3)	69 (± 3.4)	76 (± 7.6)	35 (± 9.9)	44 (± 16.3)	87 (± 11.3)	88 (± 11.3)	87 (± 11.3)	87 (± 11.3)	87 (± 11.3)
EC50 (ng/ml)	22.6 (± 5.8)	74.7 (± 44.3)	1.9 (± 1.3)	2.3 (± 0.15)	53.0 (± 0.17)	1.2 (± 0.16)	1.2 (± 0.16)	1.2 (± 0.16)	1.2 (± 0.16)	1.2 (± 0.16)

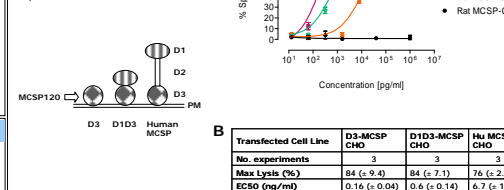
## Short distance of binding epitope to the target cell membrane improves BiTE potency

Redirected lysis of CHO lines expressing MCSP subdomains by MCSP-specific BiTE antibodies. Dose response analyses of redirected lysis were performed for A domain D1-specific BiTE antibody MCSP128 and B domain D2-specific BiTE antibody MCSP113 by a 18h 51Cr release assay. Effector cells: stimulated CD8+ T cells from healthy human donors; E:T: 10:1. Maximal lysis and EC50-values from 3 different assays are shown. Each assay was performed in triplicates.



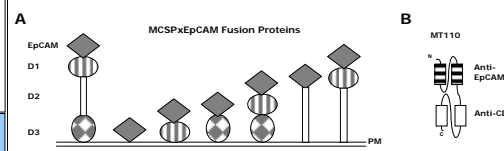
## Impact of antigen size on potency of D3-binding MCSP BiTE

Redirected lysis of CHO lines expressing different MCSP model molecules all of them containing the membrane-proximal D3 domain by D3-specific BiTE antibody MCSP120. **A** Dose response analyses were performed with stimulated human CD8+ T cells in an 18h 51Cr release assay. E:T: 10:1. **B** Maximal lysis and EC50-values from 3 different assays are shown. Each assay was performed in triplicates.



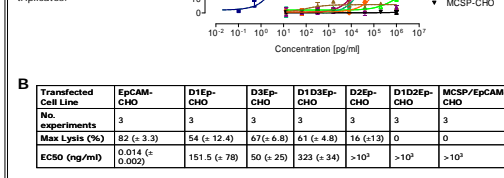
## The epitope distance effect was corroborated with EpCAM/CD3-bispecific BiTE antibody MT110 by using MCSP/EpCAM fusion proteins as surface target antigens

EpCAM/MCSP constructs used to test BiTE activity. **A** Schematic depiction of fusion proteins between EpCAM and MCSP. All fusion proteins use the transmembrane and cytoplasmic domain of human MCSP. PM, plasma membrane. **B** Structure of EpCAM/CD3-bispecific BiTE antibody MT110.



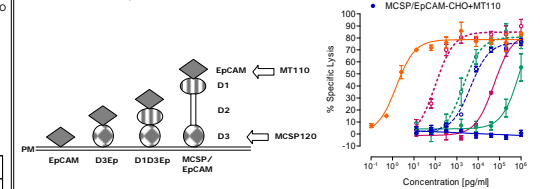
## Loss of MT110 potency with increasing distance to target cell membrane

Redirected lysis of CHO lines stably expressing EpCAM/MCSP fusion proteins by BiTE antibody MT110. **A** 51Cr-labelled target cells were co-cultured for 18 h with stimulated human CD8+ T cells at an E:T ratio of 10:1. **B** Maximal lysis and EC50-values from 3 different assays are shown. Each assay was performed in triplicates.



## Impact of antigen size and epitope distance on potency of BiTE molecules

Redirected lysis of CHO lines stably expressing EpCAM or MCSP/EpCAM fusion proteins by MT110 as well as D3-specific BiTE antibody MCSP120. All fusion proteins used contain the EpCAM ECD as well as the membrane-proximal D3 domain of MCSP. Dose response analyses were performed in an 18h 51Cr release assay with human stimulated CD8+ T cells from one healthy donor at an E:T: 10:1.



Transfected Cell Line	EpCAM-CHO	D3E-CHO	D1D3E-CHO	EpCAM/MCSP-CHO
BiTE	MT110	MCSP120	MT110	MCSP120
Max. Lysis (%)	78	n.d.	84	85
EC50 (ng/ml)	0.003	n.d.	54.95	0.101

## Summary

- MCSP-specific BiTE antibodies binding the most membrane proximal domain D3 of MCSP were most potent.
- Direct attachment of MCSP domains to the plasma membrane of target cells in isolation in each case increased the potency of BiTE antibodies.
- Stepwise increasing the distance of domains to the plasma membrane of target cells decreased BiTE potency.
- Stepwise increasing the size of the target antigen while keeping the epitope distance constant also decreased the potency of BiTE antibodies binding to a membrane proximal domain.
- CHO cells expressing full-length MCSP consistently required the highest BiTE concentrations for redirected lysis. In several experiments, both epitope distance and antigen size may have determined BiTE potency, since the two parameters were changed at the same time.

