Abstract 3681: Internalization of the dual-specific immunotoxin D2C7-(scdsFv)-PE38KDEL in malignant glioma cell lines

Chris J. Hedegaard[1], Charles Pegram[2], Darell D. Bigner[2], Hans Skovgaard Poulsen[1]

[1]*Department of Radiation Biology, Section 6321, Finsen Center, Copenhagen National University Hospital, Capitol Region, Denmark*

[2]*Preston Robert Tisch Brain Tumor Center, Duke University Medical Center, Durham, NC 27710, USA*

email: chris.juul.hedegaard@rh.regionh.dk

www.radiationbiology.dk

Background

Glioblastoma multiforme (GBM) is the most severe brain cancer and is characterized by:

- Overexpression of, dysregulated activity of- and mutations in the epidermal growth factor receptor (EGFR).
- Expression of the constitutive active mutant EGFRvIII, which comprises a new epitope that is tumor-specific.

A immunotoxin has been created based on the dual specific anti-EGFR/EGFRvIII antibody D2C7 and the Pseudomones Exotixin A (see Fig. 1), which has the potential for use in anti-GBM therapy.

AIM: To investigate the potential of the EGFR/EGFRvIII dual specific D2C7-immunotoxin (D2C7-IT) in targeted anti-GBM therapy.

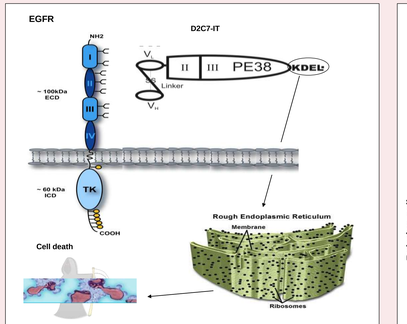

Figure 1: The strategy behind the D2C7-IT.
D2C7-(scdsFv)-PE38KDEL immunotoxin (D2C7-IT) is composed of the single-chain (sc) Fragment variables (FV) of both light chain (V_L) and heavy chain (V_H) originated from the dual-specific anti-EGFR/EGFRvIII antibody D2C7. The 'sc Fv' are disulphide bridge stabilized (ds; collectively scdsFv), and are fused by a 15 amino acids linker ((Gly$_4$Ser)$_3$). The V_L-moiety is merged to a 38 kDa fragment of the Pseudomonas Exotoxin A, including the endoplasmic reticulum retention sequence KDEL (collectively: PE38KDEL).
Because of the affinity for both EGFR and EGFRvIII, D2C7-IT will, upon binding, internalized together with EGFR/EGFRvIII, then relocate to the endoplasmic reticulum (by a KDEL-binding receptor) where the PE38 moiety causes cell death by disrupting protein synthesis.

D2C7-IT exerts EGFR/EGFRvIII-specific cytotoxicity

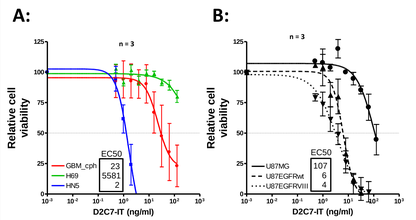

Figure 2: The cytotoxicity of D2C7-IT depends on the level of EGF- and EGFR expression.
(A): The HN5 (EGFR^{high}) head and neck cancer cells and the H69 (EGFR-) small cell lung cancer cells were used as positive and negative controls, respectively. The GBM_CPH cell line (EGFR^{high}) were cultured in neurobasal media supplemented with 10 ng/ml EGF; in contrast to the other cell cultures that were cultured in DMEM supplemented with 10% serum. **(B):** U87MG cell lines (EGFR^{low}) and its derived cell lines U87EGFRwt (EGFR^{high}) and U87EGFRvIII (EGFRvIII^{high}). Each D2C7-IT concentrations have been tested in triplicates and each data point are represented as mean(±SEM) of three assays. EC50 values are presented for each of the cell cultures.

Results

The EGFR expression level and EGF influence cell viability during incubation with D2C7-IT

- D2C7-IT reduces cell viability predominantly in cell cultures expressing high levels of EGFR (HN5, GBM_CPH and U87EGFRwt) and EGFRvIII (U87EGFRvIII) (Fig 2).
- D2C7-IT is much less potent in cultures with no EGFR expression (H69) or low EGFR expression (U87MG).
- Unspecific cytotoxicity by D2C7-IT above 16 ng/ml was observed in H69 cultures (Fig. 2A).
- D2C7-IT is slightly less potent in the GBM_CPH cell cultures (Fig. 2A), due to competition with culture media supplemented EGF .

D2C7-IT binds only to EGFR/EGFRvIII expressing cells

- The level of EGFR expression (Fig. 3A) coincided with the level of bound D2C7-IT (Fig. 2B): more D2C7-IT detected in lysate from high EGFR expression cells (HN5 and U87EGFRwt) as compared to low EGFR expression cells (U87MG). No D2C7-IT was found in the EGFR negative H69 cells (Fig. 3A+B).
- D2C7-IT binds at a lower concentration to U87EGFRvIII cells than to U87EGFRwt and HN5 cells (Fig. 3B).
- Activation of EGFR, observed by phosphorylation of tyrosine1173 (pTyr1173), was not affected by neither serum starvation nor of D2C7-IT in HN5 and U87EGFRvIII cell cultures; but increasing D2C7-IT levels resulted in increasing pTyr1173 in U87MG and U87EGFRwt cell cultures (Fig. 3C).
- D2C7-IT mediates pTyr1173 in EGF-starved GBM_CPH cell cultures, which has no intrinsic pTyr1173 (Fig. 3D).
- Pre-incubation with EGF (10:1 surplus) resulted in inhibition of D2C7-IT binding to U87EGFRwt cells (Fig. 3E).

D2C7-IT internalizes in EGFR/EGFRvIII expressing cells

Internalization of the D2C7-IT (as a result of D2C7-IT mediated activation of EGFR) was investigated by fluorescent microscopy:

- D2C7-IT associates with HN5 and GBM_CPH cells (Fig. 4A+B) but not with U87MG cells (Fig. 4C).
- D2C7-IT resides on the cell surface on HN5 cells after 1 hour of incubation (Fig. 4A), but appears already to be internalized in U87vIII cells (Fig. 4D).
- Internalized D2C7-IT results in more spatial staining, as compared to surface staining, observed in U87vIII (Fig. 4D) and U87wt cell cultures (Fig. 4E).
- Blockage of internalization (by the EGFR-kinase inhibitor AG1478) results in D2C7-IT surface staining (Fig. 4A+B).
- Some co-localization between D2C7-IT and transferrin receptor (endosomes) was observed after 4 hours in U87vIII (Fig. 4D): mean co-localization coefficient at 0.34 0.11; in U87wt: 0.14 0.08 (not shown).
- Very little co-localization between D2C7-IT and LAMP-1 (lysosomes) was observed: U87vIII (0.03 0.02); U87wt (0.02 0.02).

D2C7-IT binds to the EGFR/EGFRvIII and subsequently activates EGFR

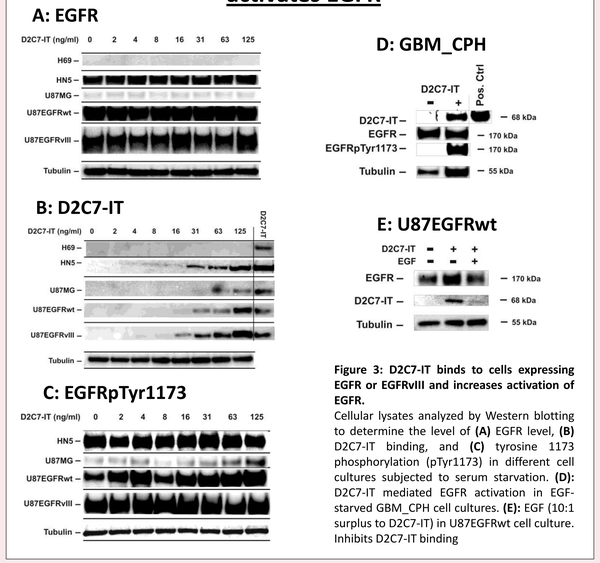

Figure 3: D2C7-IT binds to cells expressing EGFR or EGFRvIII and increases activation of EGFR.
Cellular lysates analyzed by Western blotting to determine the level of **(A)** EGFR level, **(B)** D2C7-IT binding, and **(C)** tyrosine 1173 phosphorylation (pTyr1173) in different cell cultures subjected to serum starvation. **(D):** D2C7-IT mediated EGFR activation in EGF-starved GBM_CPH cell cultures. **(E):** EGF (10:1 surplus to D2C7-IT) in U87EGFRwt cell culture. Inhibits D2C7-IT binding

D2C7-IT internalization depends on tyrosine kinase activity

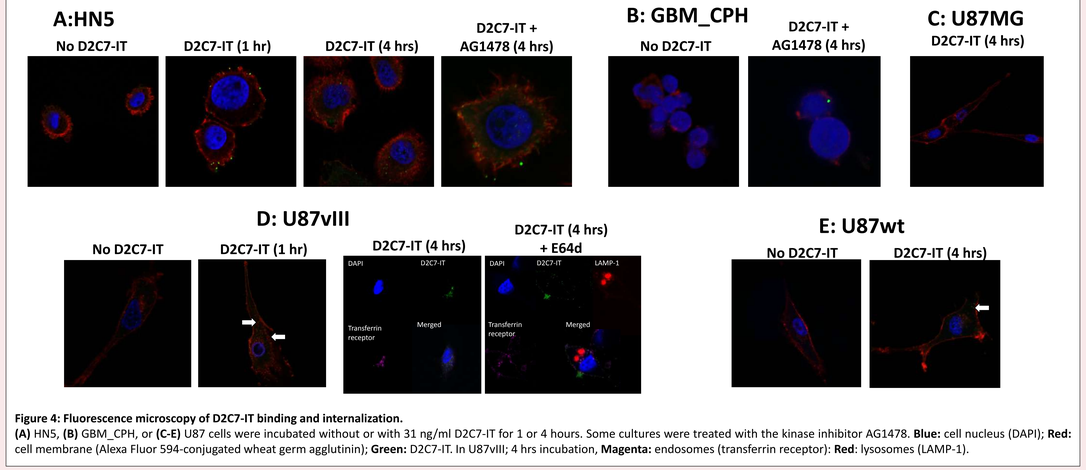

Figure 4: Fluorescence microscopy of D2C7-IT binding and internalization.
(A) HN5, **(B)** GBM_CPH, or **(C-E)** U87 cells were incubated without or with 31 ng/ml D2C7-IT for 1 or 4 hours. Some cultures were treated with the kinase inhibitor AG1478. **Blue:** cell nucleus (DAPI); **Red:** cell membrane (Alexa Fluor 594-conjugated wheat germ agglutinin); **Green:** D2C7-IT. In U87vIII; 4 hrs incubation, **Magenta:** endosomes (transferrin receptor): **Red**: lysosomes (LAMP-1).

Conclusion: D2C7-IT exerts specific cell death by binding to EGFR/EGFRvIII thereby mediating activation of EGFR, which facilitates internalization into the tumor cell.

Thus D2C7-IT shows a potential for use in EGFR/EGFRvIII targeted anti-GBM therapy.