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Tight junctions as a limit to Herceptin penetration?

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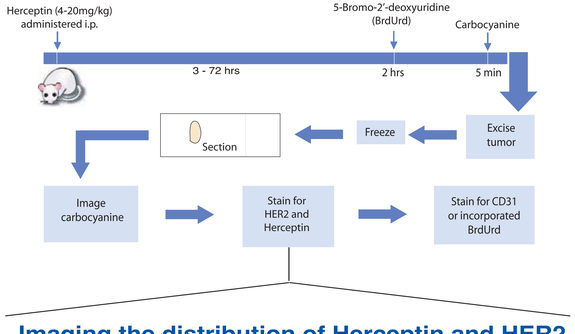
Summary

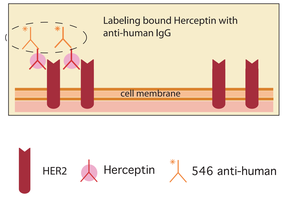
The tumor microenvironment presents many barriers to drug penetration, including abnormal microvessel structure and function, deficient or absent lymphatics and variable extracellular matrix composition. Using immunohistochemical mapping of MDA-435/LCC6^{HER2} xenografts we have found the extravascular distribution of trastuzumab (generic, Herceptin®), to be incomplete and highly heterogeneous. To characterize properties of the tumor microenvironment that govern trastuzumab penetration, we extended these studies using HER2 over-expressing MCF-7 breast cancer (MCF-7^{HER2}) cells and a tight junction marker, ZO-1. Additionally, we used multilayered cell cultures (MCCs) in conjunction with transmission electron microscopy (TEM) to assess trastuzumab penetration through MCF-7^{HER2} tumor cells *in vitro*.

Experimental Procedure

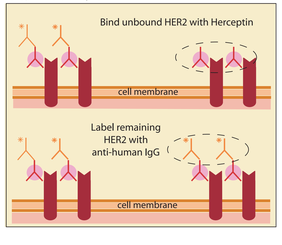


Imaging the distribution of Herceptin and HER2

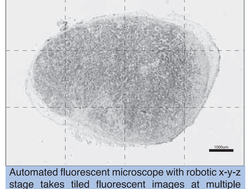
Step 1. Staining for bound Herceptin



Step 2 . Staining for HER2

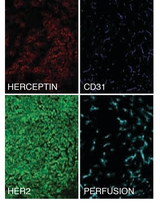


Step 3. Imaging

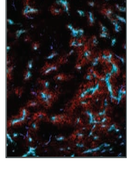


Automated fluorescent microscope with robotic x-y-z stage takes tiled fluorescent images at multiple wavelengths to create a composite image of whole tissue sections.

Fluorescent imaging of multiple markers

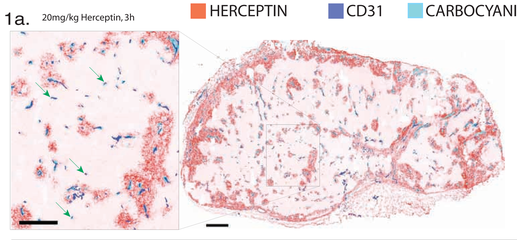


Step 4. Mapping and quantitation: Overlay of tiled composite images



Xenograft Tumour Mapping

1a. 20mg/kg Herceptin, 3h



1b. 20mg/kg Herceptin, 26h

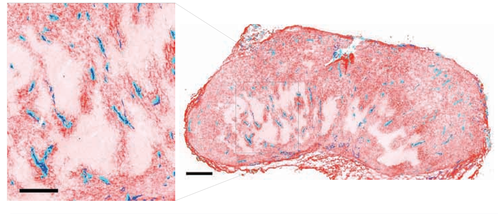
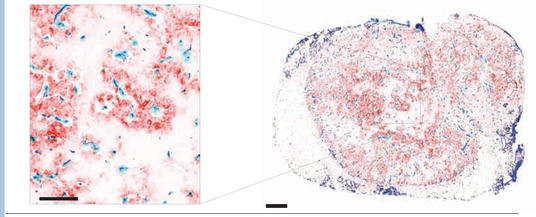


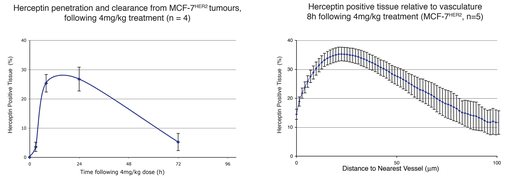
Figure 1. (LEFT): Heterogeneous distribution of Herceptin. Mice were dosed with 20mg/kg Herceptin i.p. and harvested at 3h (**1a**) or 26h (**1b**). Herceptin extravasates in a heterogeneous pattern in all regions of the tumors at 3h (**1a**); inset illustrates the presence of many perfused vessels not associated with Herceptin (**arrows**). At 26h tissue with no bound Herceptin can be found on cryosections (**1b**) in areas demonstrating both a high density of perfused vessels (**1b, inset**) and low density of perfused vessels. All scale bars represent 200μm.

Figure 2. (RIGHT) Similar heterogeneous distribution of Herceptin in MCF-7^{HER2} tumours administered a lower dose, 4mg/kg Herceptin, and harvested 24h (2a). HER2 is highly expressed throughout the tumour (image not shown). Bound herceptin is shown to increase in tumours 4-24h following 4mg/kg i.p. dosing, after which clearance is observed (**2b left**), 8h after dosing, little Herceptin staining is observed at distances further than 100μm from blood vessels (**2b right**). All values are mean ± standard error.

2a. 4mg/kg Herceptin, 24h



2b.



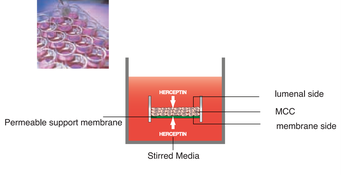
Multilayered Cell Cultures

Figure 3. Herceptin Penetration through Multilayered Cell Cultures (MCCs)
A multilayered cell culture system was used to assess the penetration of Herceptin through tumour tissue *in vitro*. In this assay, MCF-7^{HER-2} cells are seeded into standard tissue culture inserts and incubated for 15 h to allow the cells to attach. The cultures are then incubated for 2 days in custom built growth vessels to form MCCs ~150-μm in thickness (**3a**).

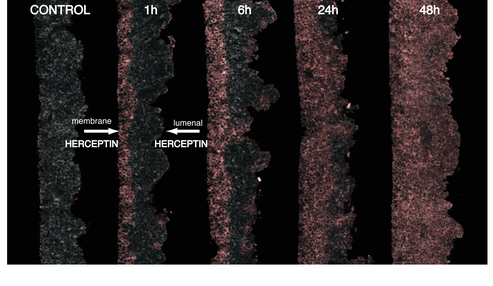
For Herceptin treatment, 60μg/mL Herceptin was added to the vessel, allowing access to the culture from both sides. The cultures were removed following treatment and immediately prepared for cryosectioning. Frozen sections were stained and imaged in a similar manner as tumour sections.

Strikingly, herceptin has limited penetration from the lumenal (top) side of the culture (**3b**) and penetrates through the membrane (bottom) side of the culture at a faster rate. This effect is quantified by analysing the average intensity of Herceptin staining across the culture after a 6h exposure (**3c**).

3a.



3b.



3c.

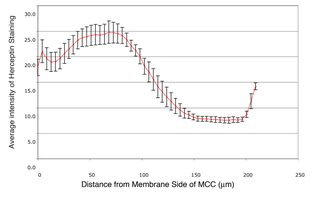
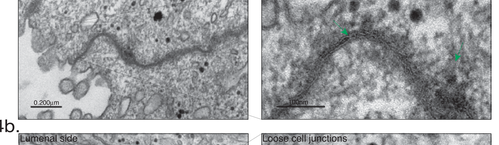


Figure 4. Transmission Electron Microscopy of MCF-7^{HER2} Multilayered Cell Cultures
Untreated MCF-7^{HER2} MCCs were removed from the growth apparatus and fixed in gluteraldehyde. The fixed MCCs were then treated with osmium tetroxide and embedded in epoxy resin. Ultra-thin 60nm sections were imaged at both the lumenal (top) and membrane (bottom) side of the MCC.

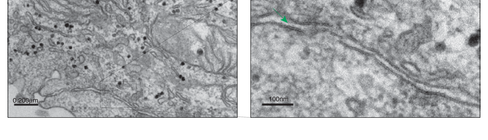
At the lumenal side of the MCC cells junctions appear to be contiguous and tight (**4a**). Examples of 'kiss points' at tight junctions, and desmosomes are seen (**arrows, 4a, inset**).

On the membrane side of the MCC cell junctions are more spacious. Extracellular space can frequently be seen between cells (**4b**). Tight junctions and desmosomes are observed less frequently. The asymmetry of tight junctions was also observed in MCF-7 wild type MCCs, but not in HCT-116 colorectal carcinoma MCCs (data not shown).

4a



4b.

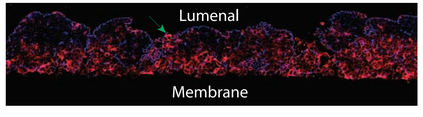


Tight Junctions

Figure 5. Tight Junctions limit the penetration of Herceptin *in vitro*
An MCC treated for 6h with Herceptin, before sectioning and immunostaining for the tight junction marker ZO-1 (blue) and Herceptin (red). Here, the lumenal side displays a clear wall tight junctions and is occluding Herceptin from penetrating the culture.

MCCs were additionally stained for HER2 and expression is high and homogenous across the MCC. Herceptin can be seen penetrating the lumenal side of the culture in spots of discontinued ZO-1 stain (arrow).

5.



Hypothesis

We propose that the activity of Herceptin *in vivo* may be related to its ability to penetrate through the extravascular compartment of solid tumours. Herceptin exits preferential tumour vasculature; the properties which support Herceptin extravasation may be related to the expression of tight junctional complexes and/or vessel maturity.

Conclusions

- Herceptin does not fully distribute throughout solid MCF-7^{HER2} and MDA-435/LCC6^{HER2} xenograft tumours.

- Herceptin extravasates from tumour vasculature selectively, leading to heterogeneous microregional distribution throughout tumours.

- Tight junctions restrict the penetration of Herceptin through multilayered cell cultures *in vitro*.

- Paracellular transport is required for Herceptin to effectively penetrate tumour tissue.

- Variables in tumour vessels with regards to the presence of tight junctions may affect the ability of Herceptin to reach target cells far from vasculature.

Acknowledgments

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