FORSYS Partner Project “Predictive Cancer Therapy”
Project Meeting 01/09

P4

Ex Vivo Models of Human Lung Cancer

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Model substance Cetixumab

- Commercially available (Erbitux) antibody against EGFR (c-erbB-1)
- Approved for squamous cell carcinoma of head and neck and colorectal cancer
- Dosing schedule in vivo: once weekly initial dose of 400 mg/m²
  Subsequently 250 mg/m²
- Steady state plasma concentrations: maximum 170 – 240 µg/ml
  trough 40 – 90 µg/ml
Labelling strategies for Cetixumab

- Determination of concentrations in perfusion buffer and tissue
- Localization (and quantification) within tissue

**Digoxigenin approach**

- Labeling with digoxigenin active ester
- Quantification in plasma / homogenate by ELISA using monoclonal anti digoxigenin-alkaline phosphatase antibody
- Immunohistochemical staining of tissue sections using monoclonal anti digoxigenin-alkaline phosphatase antibody

**Fluorescent labeling approach**

- Labeling with fluorescent dye active ester
- Quantification in plasma / homogenate by ELISA or direct quantification by fluorescence reader
- Direct observation of fluorescence signal by confocal laser microscopy
Fluorescence labelling of Cetixumab

- Determination of useful wavelengths

Autofluorescence of lung tissue following different treatment

Ex 488 nm
Ex 633 nm

Fluorescence [AU] vs. wavelength $\lambda$ [nm]

- Alexa-488
- OregonGreen-488
- Alexa-680

n/c lung tissue
- 70% 2-propanol
- no fixation
- formalin fixation
**Alexa-488 labelled Cetuximab**

Binding to different cell lines (data from IZI)

- Commercially available Cetuximab
- Exchange of glycine buffer to PBS by ultrafiltration
- Labeling with Alexa-488 using kit

- Degree of labeling: 7 by a molar ratio dye : protein of 10
- Overall yield: 85%
- Maximum amount of antibody per reaction (use of purification column provided with kit): 2 mg

**Graphs:**
- A549
- NCI-H460
- Jurkat

- **Control**
- **Cetuximab Alexa-488**
OregonGreen-488 labelled Cetuximab

Binding to different cell lines

- Commercially available Cetuximab
- Exchange of glycine buffer to PBS by ultrafiltration
- Labeling with Oregon Green® 488 carboxylic acid succinimidyl ester, bicarbonate buffer, pH 8.3
- Purification of labelled Cetuximab by ultrafiltration

- Degree of labeling: 6 by a molar ratio of 15
- Overall yield appr. 80%
- Reaction size: 20 mg

A549 | NCI-H460 | LAMA84

IgG-control  |  Cetuximab OregonGreen-488
**OregonGreen-488 labelled Cetuximab**

Binding to different cell lines

- Incubate viable cells with 1 µg/ml Oregon Green-488 labelled cetuximab (20 min @ room temperature in dark)
- Wash with PBS with 1%BSA
- FACS

NSCLC isolated from a patient at Klink Schillerhöhe

NCI-H23

5707 AEM

8163 Fibroblast

Isolated from normal lung tissue of a patient at Klink Schillerhöhe

Incubate viable cells with 1 µg/ml Oregon Green-488 labelled cetuximab (20 min @ room temperature in dark)

Wash with PBS with 1%BSA

FACS
OregonGreen-488 labelled Cetuximab
Binding to different cell lines

- Incubate viable cells with 100 µg/ml Oregon Green-488 labelled cetuximab in medium (15 min @ 37°C in dark)
- Wash with PBS with 1%BSA
- Confocal laser microscopy

NCI H-23
Short term culture of tumour tissue slices

Procedure

Consent of the patient, surgery

Tumour tissue

Tissue punch

Slices:
- Thickness: 200µm
- Diameter: 5mm

24 well plate
1 slice/well

Staining of nuclei

Confocal laser microscopy

Routine diagnosis → histology (HE)

'Krumdieck tissue slicer'

'Krumdieck tissue slicer'

Short term incubation with Oregon Green-488 labelled Cetuximab
Oregon Green-488 labelled Cetuximab
Incubation of lung cancer tissue slices

- Incubate viable tumor slices with 100 µg/ml Oregon Green-488 labelled cetuximab in medium
- Wash with PBS with 1% BSA
- Confocal laser microscopy

> Ratio labelled cells / all cells
OregonGreen-488 labelled Cetuximab
Stability of fluorescence label

- Incubation of 50 µg/ml Oregon Green-488 labelled cetuximab for 3 hours at 37°C in perfusion buffer
  - in the dark
  - under lab light
- Determination of fluorescence at 485 nm -> 535 nm
**OregonGreen-488 labelled Cetuximab**

**Human lung perfusion experiment**

**Procedure**

- Surgery of lung cancer patient
- Lobe preparation containing tumor
- Cannulation of pulmonary arteries
- Intubation of bronchus
- Connection to perfusion equipment (located nearby surgery theater)

Perfusion buffer:

- HCO$_3$$_{-}$/CO$_2$-buffer + 5% Albumin
- pH: 7.2 (regulated by CO$_2$ addition)
- Volume: 1L
- 10 μg/ml Oregon Green labelled Cetuximab

Right upper lobe, weight: 130 g
- Flow rate: 200 mL/min
- Perfusion duration: 120 min
OregonGreen-488 labelled Cetuximab
Human lung perfusion experiment – detailed procedure

- Lobe preparation from surgery
- Cannulation of pulmonary arteries
- Connection of bronchus to respiration tubing
- Rinsing of lobe preparation with 1L of perfusion buffer with separate system (decolorization shows perfusion)
- Perfusion buffer with cetuximab is equilibrated in reservoir of perfusion system
- Lung is connected to perfusion circuit
- Start of perfusion and ventilation
**OregonGreen-488 labelled Cetuximab**

**Human lung perfusion experiment**

Kinetics of cetuximab elimination from perfusion buffer
Quantification using fluorescence reader

![Graph showing the elimination of OregonGreen Cetuximab](image)

Conc = A * exp(k*time) + B

k = 0.12 min\(^{-1}\)
(95% CI: 0.09 – 0.15 min\(^{-1}\))

Calculated ratio tissue / buffer: 3
So far no quantification in tissue

ELISA SOP for tissue from Merck

Perfusion with doxorubicin glucuronide and gluc-inhibitor

![Graph showing the elimination of HMR1836](image)

k = 0.05 min\(^{-1}\)

ratio tissue / buffer: 0.6
**OregonGreen-488 labelled Cetuximab**

Human lung perfusion experiment

Localization of labelled cetuximab using confocal laser microscopy

Formalin fixed paraffin embedded tissue

Peripheral lung tissue

- Auto-fluorescence of collagen fibres
- Low fluorescence signal of cetuximab (10 µg/ml)

cryosections, IHC
Isolated perfusion of murine lungs

- Equipment is built up in Tübingen
- Permissions according law on animal welfare granted
Structure of blood vessels within tumour
Staining of blood vessels in tumour slices

- Very soft tumour
- Blood vessels: UEA-FITC
- Nuclei: Topro3
- Distance between sections: 1.7 µm
Structure of blood vessels within tumour

Staining of blood vessels in tumour slices

- Blood vessels: UEA-FITC
- Nuclei: Draq5

- Distance between sections: 1.3 µm
Outlook

Cetuximab
- Supply of high amounts of cetuximab from Merck (MTA signed)
- Supply of ELISA for quantification of cetuximab
- Labelling with digoxigenin and “secondary” antibody

Lung perfusion
- Perfusion experiments with 150 µg/ml Cetuximab
- Quantification in perfusion buffer and tissue using ELISA

Cell culture
- Determination of IC\textsubscript{50} using labelled and un-labelled cetuximab
- Correlation to EGFR-expression levels
- Investigate cancer associated fibroblasts for binding of cetuximab and determination of IC\textsubscript{50}

Tumour tissue slice culture
- Distribution of cetuximab
- Influence of cetuximab treatment on cell proliferation
- Staining of blood vessels (UEA, in addition: FITS- or PE-labelled anti CD34)