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Combined effect of radionuclide tumour targeting and lysosomotropic substances

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Abstract

A combination of targeted therapy and lysosomotropic substances, such as 20 mM ammonium chloride (NH_4Cl), enhanced the uptake and retention of ^{125}I-EGF in the EGF-rich human squamous carcinoma cell line A431. The combined treatment with 20 mM NH_4Cl and ^{211}At-EGF in the same cell line resulted in a tenfold decrease in survival compared to cells treated with ^{211}At-EGF and 0 mM NH_4Cl. Combining 20 mM NH_4Cl with a ^{125}I-labelled affibody, binding the HER2-receptor, also had some effect on uptake and retention of the labelled affibody in the two HER2-rich cell lines SKOV-3 and SKBR-3.

Keywords

Targeted therapy, ErbB-receptors, radionuclides, lysosomtropic substances

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Sara Brolin

Sammanfattning

I målriktad tumörterapi används molekyler som binder specifikt till proteiner på tumörcellens yta. Exempelvis används antikroppar eller ligander som binder receptorer på ytan med hög specificitet. Genom att koppla cytotoxiska substanser, t.ex. radioaktiva nuklider, till tumörsökarna kan den toxiska effekten styras till att verka på utvalda celler.

Vissa tumörtyper med ursprung från epitelceller har ett överuttryck av ErbB-receptorer på cellernas ytor. Ligandbindning till dessa receptorer bidrar till cellsignallering som leder till celltillväxt och delning. Ett överuttryck av ErbB-receptorer kan därför leda till okontrollerad tumörtillväxt. Vid ligandbindning tas ligand/receptor-komplexen upp i cellen. Som ett led i regleringen av cellsignallering startar en nedbrytningsprocess där komplexen slutligen bryts ned i cellens lysosomer inom 20-30 minuter. Radioaktiva nuklider som kopplats till liganden kan då frigöras och diffundera ut från cellen. Tiden som nukliden tillbringar i cellen är avgörande för den toxiska effekten.

I denna studie har svaga baser använts kombinerat med målsökande terapi. De svaga baserna kan fördröja den snabba nedbrytningen genom att höja pH i lysosomerna. Studier med ligander till två av ErbB-receptorerna har visat att den svaga basen ammoniumklorid höjer upptaget samt förlänger tiden som radioaktivt märkta ligander blir kvar i cellerna. Detta har visats ge större toxisk effekt vid målsökande experiment med cell-linjer och den radioaktiva nukliden astat 211 (^{211}At).

Examensarbete 20p i Molekylär bioteknikprogrammet

Uppsala Universitet Juni 2004

CONTENTS

1 Introduction and theoretical background

External radiation can often be used in treatment of solid tumours but it has limitations when it comes to smaller tumours and migrating tumour cells. In these cases it would be appealing to use targeted therapy, where tumour-seeking “missiles” carrying radioactive nuclides with short range are intended to bind specifically to the tumour cells and deposit the cytotoxic radioactivity in the absolute vicinity of these cells. The carrier of the radioactivity should be one that binds to or is taken up by no other cells than the tumour cells to give as little effect as possible on the healthy, surrounding tissue.

Several types of cancers of epithelial origin such as lung, breast and bladder as well as head and neck cancer often have an increased expression of ErbB-receptors (1). These are a family of receptor tyrosine kinases (RTKs) located in the plasma membrane. The elevated expression of receptors makes these cells interesting for targeted therapy. Known ligands to these receptors are e.g. growth factors and neuregulins, who could be used as carriers of radioactive nuclides. Ligand binding triggers homo- or hetero dimerisation of the ErbBs, which then become active, and may activate an intrinsic signalling network. In some cases the activation also accelerates endocytosis as well as degradation of the receptor/dimer complexes. This is believed to be of importance in regulation of the signalling network (2). The specific binding of these ligands makes epidermal growth factor (EGF) and other ligands to the ErbB-receptors candidates for radionuclide labelling and targeted therapy. An advantage in using these ligands in targeted therapy could be their size. EGF is a quite small protein of about 6 kDa and might be easily distributed in tissues. A problem is the rapid endocytosis and degradation of the receptor/ligand complexes and the release of EGF-bound radioactivity. In this thesis project, different substances have been tested for their ability to slow down this degradation process.

1.1 The ErbB family of receptor tyrosine kinases

The mammalian ErbB family consists of four known receptors, all receptor tyrosine kinases (RTKs) that may be activated by binding of different ligands. The receptors are all involved in extensive mitogenic cell signalling and have been found to be overexpressed or mutated in several types of cancers and tumours of epithelial origin (1). The fact that these receptors are abundant in different tumour cells and that their ligands bind them with high specificity, make them interesting as targets in targeted therapy. A schematic drawing of the ErbB receptors is shown in figure 1.

1.1.1 The ErbB receptors and their ligands

The most studied member of the ErbB family is the epidermal growth factor receptor (EGFR), along with its ligand, the epidermal growth factor (EGF). Upon EGF-binding the receptor forms homo- or heterodimers with other receptors in the ErbB family, leading to autophosphorylation of the cytoplasmic parts of the complex. This event is important in cell signalling, making other proteins prone to interact with the complex. The interaction has been shown to involve both the MAP-kinase pathway and the IP3 pathway among others (3). The MAP-kinase pathway involves many different proteins in a signalling cascade that eventually activate transcription factors, stimulating the transcription of DNA. In a similar way the IP3 pathway ultimately results in a cell survival response. Activation of the signalling networks involved therefore eventually affects cell survival, growth and proliferation (4).

Another member of the ErbB family is ErbB-2, also known as HER-2. This receptor is often overexpressed in breast cancers, but no natural ligand to HER-2 has been found. Even though it has no ligand it is prone to dimerise with other members of the ErbB family. Heterodimers containing HER-2 have been shown to be the far most potent in signalling, making HER-2 a dangerously active oncogene product (5, 6). The HER-2 antibody Trastuzumab has in combination with external radiotherapy shown promising results in the treatment of breast cancer in clinical studies (7). Preclinical studies also focus on finding other proteins that could be used for targeted radiotherapy against HER-2. A bivalent affibody binding to HER-2 has been constructed and tested at our laboratory (Ann-Charlott Steffen, non-published results). Affibody molecules are stable three-helix bundles consisting of 58 amino acids derived from one of the IgG binding domains of the staphylococcal protein A – the Z domain, in which 13 positions have been randomized. The bivalent affibody called $(Z_{HER2})_2$ has been shown to bind to HER-2 and is believed to be a good candidate for targeted therapy and its properties are investigated in extensive studies.

Neuregulins (NRGs) are the most common ligands to HER-3 and HER-4. Neuregulins are small proteins known to mainly be involved in signalling that stimulates synthesis of membrane proteins on muscle cells. The HER-3 receptor has a dysfunctional tyrosine kinase domain and therefore fails to autophosphorylate. NRG-binding to the receptor still makes it able to dimerise, and in combination with another ErbB receptor it is active in signalling (8). HER-4 is also known to be activated by NRG and some other growth-factor-like ligands.

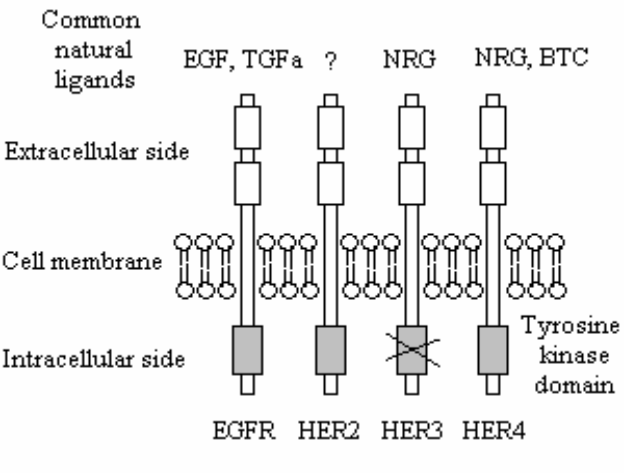

Figure 1 Schematic drawing of the four known ErbB receptors. Their most common ligands are indicated above each receptor. HER-2 has no known natural ligand and the tyrosine kinase domain of HER-3 is inactive.

1.1.2 Endocytosis and degradation of cell surface receptors

Molecules and proteins from the cell surface may be internalised into the cell by a process called endocytosis. Receptors involved in cell signalling, i.e. receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs), are located in the cell membrane and are often internalised by receptor-mediated endocytosis. The internalisation of active receptors regulates the signalling, and the receptors may then be recycled back to the cell surface, or sorted to degradation in the lysosomes (3).

The far most studied RTK is EGFR, and it has been shown that EGF-binding accelerates endocytosis of the receptor-ligand complexes in clathrin coated pits and sorting to the lysosomal degradation pathway (9). Active receptor/ligand complexes can be selectively recruited into these clathrin coated pits. The pits fuse with early endosomes and the receptors are further transported through the endosomal compartments and finally to the lysosomes. It is

believed that the receptors have an “endocytic code” in their cytoplasmic parts, targeting them for the endosomal pathway. Many motifs have been suggested to be important in the sorting, for example domains interacting with clathrin adaptor proteins (APs).

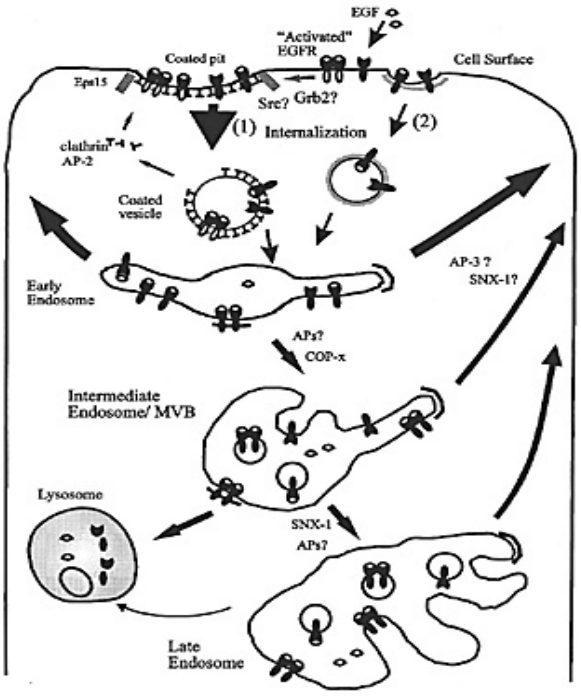

Figure 2 Endocytosis and intracellular trafficking of EGFR upon EGF binding. Activated EGFR-EGF complexes rapidly induce the formation of clathrin-coated pits (1) or caught in non-clathrin dependent pits (2). The complexes are then further transported through the endosomal compartments until eventually degraded in the late endosomes and lysosomes. At all stages the complexes can be recycled back to the cell surface. The thickness of the arrows indicates the possibility of recycling from the compartment. Picture used with permission of professor Alexander Sorkin.

The intravesicular pH drops along the endosomal pathway from about 6.5 in early endosomes to about 4.5 in late endosomes and lysosomes (10). The low pH is needed for the activity of the lysozymes i.e. enzymes responsible for degradation of proteins and peptides in the lysosomes. In cell cultures, EGFR/EGF complexes have been detected in early endosomes after about 2-5 minutes after activation and in late endosomes (where the degradation starts) after about 20 minutes (9). Thus the degradation is quite fast and that causes problems when using EGF for targeted therapy. An elongation of the time between uptake and degradation of radiolabelled EGF could make EGF more suitable for radionuclide labelling and thereby for use in targeted therapy.

Unoccupied receptors can internalize slowly in a clathrin-independent way and are often rapidly recycled back to the cell surface. Receptor-ligand complexes can also be recycled but ligand binding makes the rapid clathrin-dependent degradation pathway more efficient. The recycling rate is then two to three times slower than for the unoccupied receptors (9). Rapid degradation leads to down-regulation of the number of receptors, which attenuates their signalling. Over-expression of receptors might make the cells unable of efficient down-regulation leading to elevated mitogenic signalling and tumourogenic activity (9).

It seems as if the different ErbB-receptors take different routes when it comes to their internalisation. Studies have indicated that HER-2, HER-3 and HER-4 do not take the same endocytic pathway as EGFR, described above. The endocytic route also seems to be dependent on the ligand. EGFR is internalised upon EGF binding but has not been shown to take the same route in complexes with other ligands (11). The other receptors in the ErbB-family seem unable to interact with the clathrin-adaptor proteins (APs), which are important in the assembly of clathrin-coated pits. Instead, the complexes seem to be internalised in some other way and instead of getting sorted for degradation the complexes are recycled back to the

cell surface (1, 6). The receptors can still be interesting targets in targeted therapy. Usage of antibodies or short peptides directed to the receptors could work as targeting agents. The ErbB receptors are, as previously mentioned, possible candidates as targets for targeted therapy, as they are overexpressed in several types of epithelial tumour cells. EGFR is internalised into the cells and therefore it could be suitable to use a radioactive nuclide with short range, coupled to EGF. One problem is that after internalisation of the receptor/ligand-complexes, many of the complexes are targeted for rapid degradation. Dissociation of the radioactive agent and the degraded protein makes the radioactive nuclide free to diffuse out from the cells. It might then not have the time to harm the tumour cell, but instead harm surrounding cells.

Inhibition of the rapid degradation process could be a way to prolong the time the radioactive nuclides spend in the cells. One way to solve this problem could be to elevate the lysosomal pH, and thereby inhibit the activity of the lysosomal degradation enzymes. Lysosomotropic substances, such as weak bases, have this elevating effect on pH and might be used in combination with targeted therapy to enhance the therapeutic effects.

1.2 Substances that may effect the cellular retention of EGF/EGFR-complexes

1.2.1 Lysosomotropic effects of weak bases

Several substances such as chloroquine (a malaria prophylaxis drug), lidocaine, amantadine, thioridazine and ammonium chloride (NH_4Cl) among others have been considered for their potential to inhibit the rapid degradation of EGF or other proteins (12). These lysosomotropic substances all have the ability to elevate the lysosomal pH, and thereby inactivate the lysosomal enzymes responsible for degradation of proteins and peptides.

Recent studies with radiolabelled EGF (^{125}I-EGF) in the glioma U343MGaC12: 6 cell line, showed that addition of lysosomotropic substances such as 5 mM lidocaine or 30 μM chloroquine markedly enhanced the cell-associated radioactivity compared to controls (12). One study at our laboratory, (performed by Peter Lönn) showed that addition of 30 μM chloroquine increased the cell-associated activity from ^{125}I-EGF in A431 cells, 7.9-fold compared to the controls (0 μM), after 24 h incubation (results not published).

Similar effects on cell-associated ^{125}I-EGF have been shown with thioridazine and NH_4Cl in HeLa cells (13). The results indicate that both substances had an elevating effect on the lysosomal pH and are therefore believed to cause inhibition of lysosomal degradation. Incubation for 24 h with 3 mg/ml thioridazine or 20 mM NH_4Cl increased the lysosomal pH to 5.28 and 6.85, respectively, compared with 5.15 in the controls.

The use of substances such as lidocaine and chloroquine in combination with targeted therapy is attractive because of their enhancing effect on cell-associated radioactivity and the fact that they are wellknown drugs, already used in other therapeutic contexts. NH_4Cl is cheap and accessible and might also be a good lysosomotropic candidate in targeted therapy.

1.2.2 The RTK-inhibitor gefitinib

As activation of EGFR has been shown to stimulate cell cycle progression in tumours, attempts have been made to prevent the activation of this RTK. Gefitinib [“Iressa”, (trademark of AstraZeneca group of companies) ZD1839] is one of the most well known

RTK-inhibitors that act on EGFR. Gefitinib blocks the ATP-binding site and thereby inhibits receptor activation. It has already been tested in clinical studies with promising results (4). Previous studies at our laboratory have shown that gefitinib can increase the uptake of radiolabelled EGF in the glioma cell line U343 and in the squamous carcinoma cell line A431. The compound also seems to inhibit cell growth in A431 cells but not in U343 cells (4, 14). In this study gefitinib was tested for effects on uptake of ^{125}I-EGF in A431 cells, in combination with weak bases such as NH_4Cl and chloroquine.

1.3 Radiotherapy and nuclides

Radiation occurs spontaneously and naturally when unstable compounds decay to form more stable conformations. Naturally occurring or manufactured radioactive nuclides are unstable in their composition of protons and neutron and can use different approaches to loose excess energy and decay to more stable daughter nuclides.

Many substances can serve as cytotoxic agents in targeted therapy. The cytotoxic agent is then coupled to a carrier that binds specifically to a structure on the target cell. Different drugs, such as cytostatics, are commonly used as therapeutics but have the disadvantage that cells might develop drug resistance. Another disadvantage is that drugs often have to be internalised to affect the targeted cell. In this case all cells have to be targeted for the drug to have a curative effect (4).

When using radioactive nuclides as cytotoxic agents, the range of the emitted particles determines the affected area. Depending on the range of the emitted particles, a radioactive nuclide can affect the targeted cell from outside the membrane and it might even affect neighbouring cells. Other nuclides have a really short range and have to be internalised into the cells to have a lethal effect. Thus, the range of emitted particles has to be taken in consideration when choosing a radioactive nuclide as a cytotoxic agent in targeted therapy.

1.3.1 Ionizing radiation and biological effects

When using radiation therapy on tumour cells, the intention is to cause lethal damages in the DNA of the tumour cells. Double strand breaks (DSBs) are the most important lesion produced in chromosomes and is the most difficult one to repair effectively. The DNA molecule has a diameter of about 2 nm and to produce a DSB, at least two ionizing events must occur within this range. The density of ionizing events varies with each nuclide. High doses of radiation might lead to several DSBs in a cell as well as other types of lesions (15).

Alpha-emitters, beta-emitters and Auger electron-emitters are all radioactive nuclides that can be considered for tumour targeting since they may cause lethal DNA lesions. Alpha particles have a high RBE (relative biological effectiveness) as they have high energy and short range of about 50-100 μm (16). This gives them a high linear energy transfer (high-LET). A problem with alpha-emitters, such as ^{211}At and ^{213}Bi, is their poor availability at a reasonable cost (17). Beta-particles often have a longer range and are suitable for therapy in clusters of cells up to about 0.5 cm in diameter. With this range beta-emitters work at a distance and also have effects on surrounding cells (16). A problem in using these radioactive nuclides is that they also might affect surrounding healthy cells. Auger-electrons have the shortest range of the radionuclides considered for targeted therapy. In order to cause the cell lethal damages these nuclides have to be in the closest vicinity of the cells DNA (16). Radioactive nuclides with long range emission, e.g. photon emitters are often used for diagnosis and imaging of

tumours as well as for detection in the laboratory. The radioactive nuclides used in this study are further presented below.

1.3.2 ^{125}I for detection

^{125}I is a commonly used radioactive nuclide in the laboratory. It is suitable for studies of radioactive uptake and is most commonly used for detection. As ^{125}I has a half-life of 60 days the measurements are easily performed and therefore decay of the nuclide occurring during the experiment does not need to be considered. ^{125}I is also easily used in labelling of proteins, both in direct reactions, as in the chloramine-T method, and in indirect labelling methods such as ATE-labelling. These methods are further described in 2.2.

In the decay of ^{125}I, the unstable nucleus attracts an electron from the K-shell, which is joined with a proton, creating a neutron. The nuclide then decays to the excited daughter nuclide ^{125}Te. The uptake of an electron to the nucleus creates a "hole" in the K-shell. Electrons from outer shells can fill this hole. As they "fall" from outer shells they loose energy, which can be released as emission of photons (gamma radiation) or Auger electrons (16).

The low energy Auger-electrons emitted in the decay of ^{125}I has a range of just a few micrometers and may therefore be suitable for therapy of single tumour cells. As described above the Auger-electrons would have to be emitted in close vicinity to the cells DNA. The gamma radiation emitted from ^{125}I makes it unsuitable for targeted therapy as it irradiates the surrounding tissue. In this study ^{125}I is used for detection of uptake and retention of ^{125}I-labelled EGF and ^{125}I-affibody in tumour cells.

1.3.3 ^{211}At - a cytotoxic alpha-emitter

The decay of ^{211}At involves emission of alpha-particles. These particles, consisting of two neutrons and two protons, have high energy. Because of the short range of the particles, all the energy is deposited along a short track, causing many ionizing events. The high energy cause complex damages in the DNA of treated cells (15). In this study ^{211}At was coupled to EGF and the uptake and retention of ^{211}At-EGF was studied. The cytotoxic effect of ^{211}At-EGF in A431 cells was also studied.

1.4 Aim of project

The aim of this project was to investigate the effects of weak bases, such as ammonium chloride (NH_4Cl) and chloroquine, on the uptake and retention of radiolabelled ligands targeting the EGFR and HER-2 receptors. Ligands to these receptors might be potential agents for targeted therapy.

EGF was used in experiments with EGFR, a receptor highly abundant in several tumour cells of epithelial origin. In this study all EGF/EGFR experiments were carried out in cultured A431 cells. EGF is a quite small protein (6 kDa) and has a strong affinity to EGFR. This makes it interesting as a radiolabelled agent. SKBR-3 and SKOV-3 cells were used in experiments involving HER-2. The effects of NH_4Cl on uptake and retention of the receptor was investigated with the HER-2 binding dimeric affibody $(Z_{HER2})_2$. The use of this affibody is under extensive investigation in our laboratory. As it is much smaller (15.5 kDa) than already used antibodies (Trastuzumab appr. 180 kDa) and seem to bind HER-2 with good affinity it might be a good candidate for targeted therapy. This study might also bring some light upon whether the HER-2/affibody complex is internalised into the cells and thereby if

the internalisation is affected by addition of weak bases. The dimeric affibody $(Z_{HER2})_2$ will in the following text simply be called the affibody.

The effect of NH_4Cl was also tested in combination with the weak base chloroquine and the RTK-inhibitor gefitinib in A431 cells. These substances may all be toxic to cells when used in high concentrations. By using combinations, lower concentrations of the substances might be used, leading to less toxic effects on the cells.

To further investigate the therapeutic possibilities in using NH_4Cl in targeted therapy the substance was tested with two different concentrations of ^{211}At-EGF in cell growth assays with A431 cells. These experiments could also show whether ^{211}At could be a good nuclide candidate in targeted therapy against these cells.

2 Materials and Methods

2.1 Cell cultures

The EGFR-rich cell line A431 (ATCC, CRL1555, Rocksville, MD, USA) was used in cellular experiments with radioactively labelled EGF. These human squamous carcinoma cells, originating from vulva, have approximately $2x10^6$ EGFR per cell. Cells were cultured in HAM´s F10 medium (Biochrome KG), supplemented with 10% foetal calf serum (Sigma), L-glutamine (2 mM) and PEST (penicillin 100 IU/ml and streptomycin 100 μg/ml), both from Biochrome KG.

In cellular experiments with the radioactively labelled affibody, two cell lines rich in HER-2 were used. SKOV-3 and SKBR-3, both originating from breast cancer tumours, have an elevated expression of the HER-2 receptor. The cells were cultured in Mc Coy´s medium (Biochrome KG), supplemented with 10% foetal calf serum (Sigma), L-glutamine (2 mM) and PEST (penicillin 100 IU/ml and streptomycin 100 μg/ml), both from Biochrome KG. In all cellular experiments, cells were incubated in 37 °C, 5% CO_2.

2.2 Radiolabelling of proteins

Different labelling methods were used in the experiments. The choice of method depended on the properties of the protein that was to be labelled and the radioactive nuclide. Proteins and peptides containing tyrosine can be directly labelled with a halogen agent like ^{125}I, while other may need a linker between the nuclide and the protein. This indirect labelling method is also used when labelling proteins with ^{211}At.

2.2.1 ^{125}I-labelling with the Chloramine-T method

In all experiments involving uptake and retention in A431 cells, human EGF, labelled with ^{125}I (Amersham Pharmacia Biotech, UK) was used. The 6 kDa protein was labelled directly using the chloramine-T method. First 10 MBq ^{125}I was added to 2 μg protein and the reaction was started by adding 10 μl chloramine-T solution (2 mg/ml in phosphate buffer). After 1 minute of mixing, the reaction was terminated by addition of 25 μl sodium metabisulfite solution (2 mg/ml in phosphate buffer). The labelled protein was separated from the low molecular weight chemicals and unattached ^{125}I on a NAP-5 column (Sephadex G-25, Pharmacia, Uppsala Sweden). The labelled EGF was eluted in phosphate buffer and measured with a NaI detector (MINI Instruments) to determine the specific activity of the protein.

2.2.2 ^{125}I-labelling using the ATE method

The 15.55 kDa affibody was indirectly labelled using the ATE method. Typically10 MBq ^{125}I was added to 5 μl N-succinimidyl-4-[tri-methylstannyl] (ATE), (1 mg/ml, 5% AcOH in MeOH) together with 10 μl 0.1% AcOH and 10 μl chloramine-T solution (4 mg/ml in phosphate buffer) to first label the ATE molecules. The reaction was terminated by addition of 25 μl sodium metabisulphite solution (8 mg/ml in phosphate buffer), before addition of the affibody and 80 μl borate buffer. The solution was then mixed for 45 minutes before separation and calculation of specific activity as described above. EGF was also labelled with this method for cellular experiments to exclude differences in uptake and retention depending on the different labelling methods.

2.2.3 Labelling of EGF with ^{211}At

Indirect labelling of EGF with ^{211}At via N- succinimidyl-4-[^{211}At]-4-astatobenzoate was carried out. A methylene chloride solution of ^{211}At was evaporated to dryness at 45° C under a gentle stream of argon gas. Acetic acid (10 µl, 0.1 % acetic acid in water) and N-succinimidyl-4-[tri-methylstannyl] (ATE), (4 µl, 1 mg/ml, 5% AcOH in MeOH) together with 10 µl 0,1% AcOH and 10 µl chloramine-T solution (10 µl, 3 mg/ml in water) was added to initiate the reaction. After 5 minutes the reaction was stopped by addition of sodium metabisulphite (10 µl, 6 mg/ml in water). EGF (40 µg) in 0.1 M borate buffer, pH 9.0 (80 µl) was added to the vial and the coupling reaction was allowed to proceed for 30 minutes in room temperature. Astatinated EGF was separated from low-molecular-weight compounds on a NAP-5 column (Sephadex G-25, Pharmacia, Uppsala Sweden).

2.3 Cellular experiments with ^{125}I-labelled ligands and NH_4Cl

All cellular experiments were carried out in vitro. Cells were cultured in flasks, dishes or in well-plates (Nunc) in a humidified incubator (37 ° C, 5 % CO_2).

2.3.1 Method for measuring cell-associated radioactivity

The same method was used in all cellular experiments. After various incubation times the cells, treated with labelled ligand and various complementary substances, were washed six times in cold serum-free medium to remove unspecific radioactivity. Cells were trypsinised in 0.5 ml trypsin, (trypsin/EDTA, 0.25%/0,02% in PBS, Biochrome KG) for about 10-15 minutes (37 °C, 5% CO_2) before resuspended in 1 ml complete medium. 0.5 ml of the single cell suspension was used for counting of cells in a cell counter (Coulter Z2) and 1 ml was used for measuring the cell-associated radioactivity in an automatic gamma counter. Cells treated with ^{125}I-EGF or ^{125}I-affibody were measured in a gamma counter (1275 Minigamma, LKB Wallace), while ^{211}At-EGF-treated cells were measured in an automated gamma counter (1480 Wizard, Wallac Sverige AB). The measuring results were in all cases corrected for the number of cells present.

2.3.2 Concentration study of ammonium chloride (NH_4Cl)

To estimate what concentration of NH_4Cl could be used, the uptake of radiolabelled EGF after 24h incubation was studied in A431 cells, as well as the number of surviving cells. Cells were grown in a 24-well plate to obtain approximately 120 000 cells per well. A 1:1 (ligand:receptor ratio) solution of ^{125}I-labelled EGF (2.4 ng/dish, specific activity 5.1 MBq/µg) was added to the wells in combination with different concentrations of NH_4Cl, dissolved in medium. Triplicates of each concentration (0, 3, 7, 15, 20, 30, 40, 50 mM) were used. The cells were then incubated for 24 h before washed 6 times in cold serum free medium, trypsinised, resuspended, counted and measured for cell-associated radioactivity as described above.

2.3.3 Effect of NH_4Cl on uptake and retention of ^{125}I-EGF in A431 cells

To follow the uptake of ^{125}I-EGF, A431 cells were grown in 3.5 cm dishes to a density of about 300 000 cells/dish. A 1:1 (ligand:receptor ratio) solution of ^{125}I-EGF in medium was added to all dishes (6 ng/dish, 5.1 MBq/µg). A solution of 20 mM NH_4Cl in medium was added to half of the dishes and the rest (controls) received the same volume of fresh medium. Dishes were incubated for indicated time points (1 to 24 h) before counted and measured.

Triplicates of dishes treated with ^{125}I-EGF + 20 mM NH_4Cl and controls were used for each time point.

The retention of radioactivity in A431 cells was studied after interrupted incubation with ^{125}I-EGF combined with 0 or 20 mM NH_4Cl. Cells were grown to a density of about 300 000 cells/dish. After this, 0 mM or 20 mM NH_4Cl was added to the dishes before addition of a 5:1 ^{125}I-EGF solution (6 ng/dish, 5.1 MBq/μg). In a control study, some dishes were also pre-incubated with 20 mM NH_4Cl for 24 h to see whether this had any further effect on the retention. All dishes were incubated for 1 hour and then washed 6 times with cold serum-free medium. The cells were further incubated after addition of fresh medium containing 0 or 20 mM NH_4Cl. Some dishes previously treated with 20 mM NH_4Cl were also incubated with 0 mM after the first incubation in order to investigate whether NH_4Cl must be present during retention. Triplicates of dishes were counted and measured at indicated time points (0 to 24 h).

To study whether the labelling method used (CAT or ATE), had any effect on the retention, A431 cells were cultured to approx. 300 000 cells/dish. The retention of radioactivity was measured after interrupted incubation with a fivefold excess (5:1 ligand/receptor ratio) of either CAT-labelled ^{125}I-EGF (77 ng/dish, 2 MBq/μg) or ATE-labelled ^{125}I-EGF (77 ng/dish, 1.5 MBq/μg). Cells were incubated with labelled EGF and 20 mM NH_4Cl for 1 h. After interrupted incubation the dishes were further incubated for 0 to 24 h with 20 mM NH_4Cl in fresh medium before counted and measured for retained radioactivity.

2.3.4 Uptake and retention of ^{125}I-affibody in SKOV-3 and SKBR-3

SKOV-3 cells were grown to a density of 560 000 cells/dish and treated with a 5:1 solution of ^{125}I-affibody (0.14 mg/dish, 0.125 MBq/μg). The uptake of ^{125}I-labelled affibody was measured with the addition of 0 or 20 mM NH_4Cl after incubation for indicated time points (1-24 h).

The retention of ^{125}I-labelled affibody in SKOV-3 cells was studied after interrupted incubation with the labelled affibody, as described for A431 cells in 2.3.2 above. Dishes with about 100 000 cells/dish were treated with 0 or 20 mM NH_4Cl and a 5:1 solution of the labelled affibody (25 ng/dish, 0.43 MBq/μg). After 1 hour all dishes were washed and further incubated with 0 or 20 mM NH_4Cl in fresh medium. As described above, cells were counted and measured after 0 to 24 hours.

The same methods used for SKOV-3 were also used for SKBR-3. For the study of uptake about 300 000 cells/dish were treated with a 5:1 solution of ^{125}I-affibody (77 ng/dish; 0.43 MBq/μg). The retention experiment was carried out with a 10:1 solution and the labelled affibody had a lower specific activity of about 0.15 MBq/μg. Each dish contained 100 000 cells and was given 51 ng labelled affibody.

2.3.5 Estimation of number of EGF and HER-2 receptors

To study the number of EGF and HER-2 receptors on the three cell lines respectively, labelled EGF and affibody were separately allowed to bind to the receptors. Incubation on ice prevented the ligand/receptor-complexes to internalise.

A431, SKOV-3 and SKBR-3 cells were seeded in 12-wells plates to obtain a density of about 200 000 cells/well. All cell lines were treated with ^{125}I-EGF (40 ng/dish; 2 MBq/μg) and ^{125}I-

affibody (0.1 mg/dish; 0,16MBq/μg) in separate wells, 10:1 (ligand:receptor ratio) for both ligands. 20 mM NH_4Cl was added to some dishes to study whether it had some impact on ligand binding. The incubation was carried out on ice in humidified air, 5% CO_2, to prevent internalisation. After 2 hours all cells were washed, counted and the cell bound activity was measured. The number of the different receptors/cell was then calculated from known data - A431 has $2x10^6$ EGFR and SKBR-3 has $2x10^6$ HER-2.

2.4 Cellular experiments with radiolabelled ^{125}I-EGF combined with weak bases and gefitinib

Combinations of lysosomotropic substances and the RTK-inhibitor gefitinib were tested for their effect on uptake and retention of ^{125I}-EGF in A431 cells.

2.4.1 Combination of ammonium chloride and chloroquine

A431 cells were grown in dishes to a density of 300 000 cells/dish. Combinations of different concentrations of ammonium chloride (0 to 30 mM) and chloroquine (0-30 μM) were added to dishes in triplicates. In addition, all dishes were incubated with tenfold excess of ^{125}I-EGF (60 ng/dish, 3 MBq/μg) for 24 hours before washed, counted and measured as described above.

The uptake of ^{125}I-EGF was then studied with 20 mM NH_4Cl and 30 μM chloroquine, separately and in combination. Fresh medium was used for controls. Fivefold excess of ^{125}I-EGF was used (40 ng/dish, 2 MBq/μg) in all dishes. At indicated time points (1-24 hours) cells were washed, counted and measured for cell-associated radioactivity.

In retention studies 20 mM NH_4Cl and 30 mM chloroquine was used. Controls were incubated in fresh medium. 200 000 cells/dish were incubated with ^{125}I-EGF (40 ng/dish, 3 MBq/μg) for 1 hour. All dishes were washed 6 times and further incubated with the same concentrations of substances in fresh medium. At indicated time points (0-24 h) triplicates of dishes were washed, counted and measured for radioactivity.

2.4.2 Combination of ammonium chloride, chloroquine and gefitinib

The cell-associated radioactivity after 24 h of incubation with combinations of NH_4Cl (0 or 30 mM), chloroquine (0-30 μM) and gefitinib (0 or 1 μM) was measured as well as the number of cells surviving the incubation. Combinations of the substances were added to cells grown in wells (60 000 cells/well) on a 24-well plate together with ^{125}I-EGF (12 ng/well, 3 MBq/μg). After 24 h of incubation all cells were washed, counted and measured.

2.5 Cellular experiments with ^{211}At-EGF

Experiments with ^{211}At-labelled EGF were carried out to study the effect of lysosomotropic NH_4Cl on the uptake and retention of astatinated ligand in A431 cells. A growth assay was used to find out whether 20 mM NH_4Cl had any enhancing effect on targeted therapy with ^{211}At-EGF.

2.5.1 Uptake and retention of ^{211}At-EGF in A431 cells

A431 cells were cultured to about 300 000 cells/dish. The uptake and retention of ^{211}At-EGF with 0 or 20 mM NH_4Cl was measured as described for ^{125}I-EGF. The uptake was studied with 25:1 ^{211}At-EGF (150 ng/dish) and 5:1 ^{211}At-EGF (30 ng/dish) as well as in cells blocked

with 500:1 cold EGF (3 μg/dish) together with 5:1 ^{211}At-EGF. The retention of radioactivity in cells was studied after incubation with a 25:1 ligand/receptor ratio (150 ng/dish) of ^{211}At-labelled EGF.

2.5.2 Growth assay of ^{211}At-EGF-treated A431 cells

In order to estimate the effect of combined ^{211}At-EGF and NH_4Cl treatment, the growth of treated cells were studied. Triplicates of dishes, 300 000 cells/dish was used in the growth assay. These cells were treated with 25:1 ^{211}At-EGF (150 ng/dish), combined with 0 or 20 mM NH_4Cl. Cells treated with 0 or 20 mM NH_4Cl in fresh medium, were used as controls. After 24 h the dishes were washed six times with serum-free medium and fresh medium was then added. The cells were cultured in flasks in complete medium and counted once a week. The effect of 20 mM NH4Cl was studied in the same way for cells treated with 5:1 ^{211}At-EGF (30 ng/dish). Cells grown in 0 or 20 mM NH_4Cl served as controls (normal growth rate) as well as ^{211}At-EGF-treated cells blocked with 500:1 cold EGF (3 μg/dish). The approximate dose given to the cells could be calculated from the results of ^{211}At-affibody uptake. Cells were counted every week to follow cell growth.

3 Results

3.1 Effect of NH_4Cl on uptake and retention of ^{125}I-EGF

Different concentrations of NH_4Cl (0-50 mM) were first tested for their effect on the cellular uptake of ^{125}I-EGF in A431 cells (after 24 hours incubation). Counting of the cells also showed effects on cell growth and survival after the incubation. The results indicated that higher concentrations of NH_4Cl (30-50 mM) had some lethal effect on A431 cells but also elevated the uptake of radiolabelled EGF (figure 3). Use of 20 mM NH_4Cl had a very small effect on cell number but a 7.3-fold increase in amount of the cell-associated ^{125}I-EGF. These data indicated that NH_4Cl prolonged the retention of ^{125}I-EGF in A431 cells and that the cells tolerated a concentration of 20 mM NH_4Cl quite well. As 20 mM NH_4Cl also had enhancing effect on the cell-associated activity it was chosen for further experiments *in vitro*.

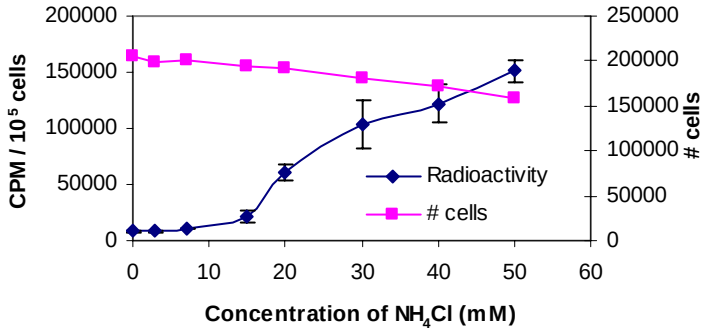

Figure 3 The cell-associated radioactivity after 24 h incubation with ^{125}I-EGF was shown to be affected of the concentration of NH_4Cl added. Higher concentrations of NH_4Cl also seemed to have a lethal effect on cells, thus making them improper in therapeutic means. The maximum error is presented for the cell-associated radioactivity.

In the study of the uptake of ^{125}I-labelled EGF over time it was shown that the uptake in the controls was highest during the first hour (figure 4A). After this, the loss of cell-associated radioactivity was greater than the further uptake for the controls. In cells treated with 20 mM NH_4Cl the uptake of ^{125}I-EGF was prolonged and the cell-associated radioactivity continued to increase until about 8 hours of incubation. After 8 hours the cells treated with NH_4Cl contained 4.7 times more radioactivity than the controls not treated with NH_4Cl (figure 4A).

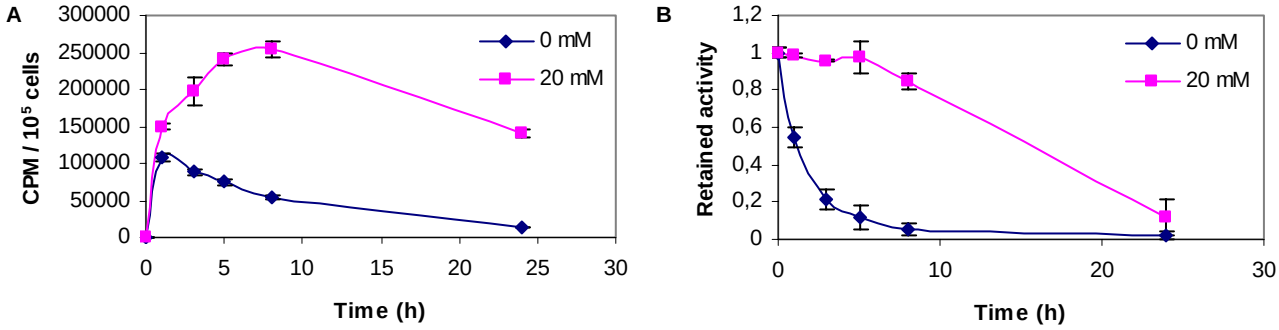

Figure 4 Chart A shows the uptake of ^{125}I-EGF in A431 cells with 0 or 20 mM NH_4Cl. Addition of 20 mM clearly affected and prolonged the uptake of ^{125}I-EGF. The cellular retention (B) of ^{125}I-EGF was also affected by 20 mM NH_4Cl. The maximum error is shown for each time point.

The results from interrupted incubation with ^{125}I-EGF also indicated that NH_4Cl prolonged the retention of ^{125}I in the cells. The retained cell-associated radioactivity after 3 hours was 6.7 times greater in the NH_4Cl-treated cells than in the controls (figure 4B). Cells pre-incubated with 20 mM NH_4Cl for 24 hours before ^{125}I-EGF treatment, showed no significant difference in retained radioactivity compared to the controls (results not shown). Cells treated with NH_4Cl in the uptake, but incubated with pure medium after interrupted incubation, lost the radioactivity at the same rate as the controls (results not shown). This indicated that NH_4Cl must be present during the retention time to affect the retention

Comparison of ^{125}I-EGF labelling methods (CAT or ATE) showed that the labelling method used does affect the retention of radioactivity. A larger portion of the radioactivity was retained in cells treated with ^{125}I-EGF indirectly labelled with the ATE method than ^{125}I-EGF directly labelled using CAT (results not shown).

3.2 Effect of NH_4Cl on uptake and retention of ^{125}I-affibody

The results of ^{125}I-affibody uptake in SKBR-3 and SKOV-3 cells, indicated that NH_4Cl did have some effect on uptake and retention of ^{125}I-affibody. The difference appeared to be largest at the later time points. It also indicated that the effect was somewhat higher in SKBR-3 than in SKOV-3 (figure 5A and 5C).

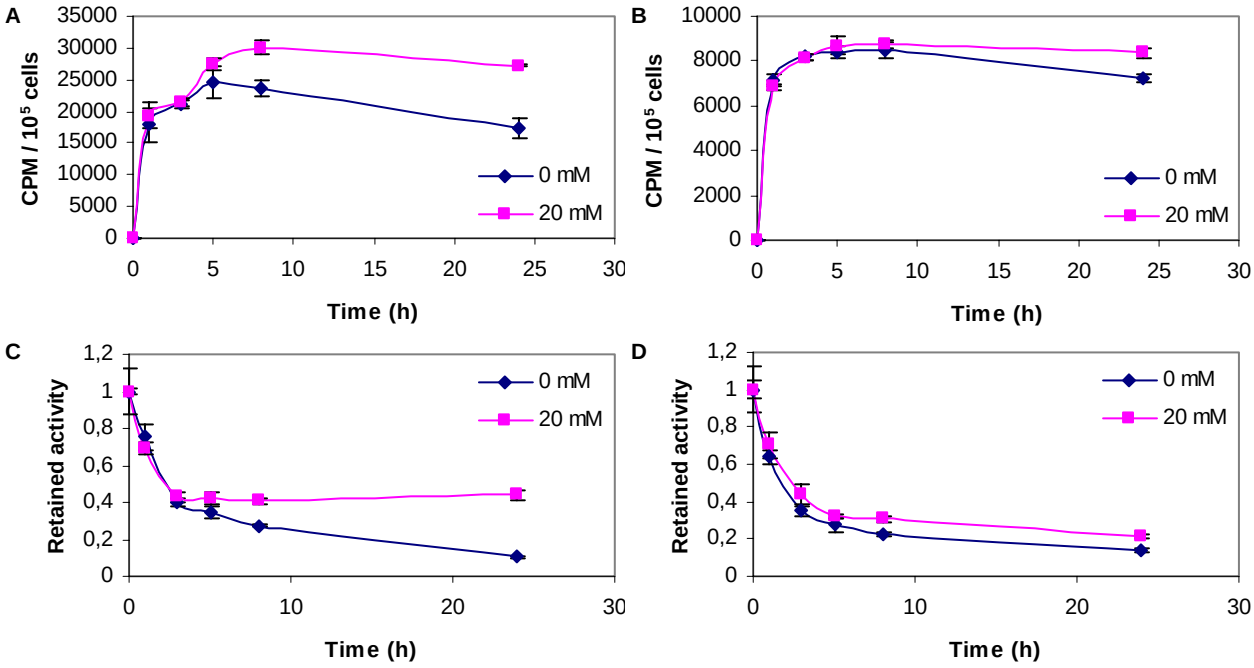

Figure 5 Upper charts show that the uptake of ^{125}I-affibody in (A) SKBR-3 cells and in (B) SKOV-3 cells with 20 mM NH_4Cl had some enhancing effect. The same tendency is seen in retention of the ^{125}I-labelled affibody (lower charts). Both SKBR-3 (C) and SKOV-3 cells (D) treated with 20 mM NH_4Cl had a prolonged retention of radioactivity from the ^{125}I-labelled affibody, compared to the controls. The maximum error is presented as error bars.

The uptake in controls seemed to be quite fast and the accumulated activity was held at a high level when cells were continuously incubated with ^{125}I-affibody. Interrupted incubation, on the other hand, showed that the cells lost most of the radioactivity within a few hours (figure 5B and 5D).

3.3 Combination of weak bases – ammonium chloride and chloroquine

It was of interest to find out whether a combination of weak bases, both separately affecting the lysosomal pH, could be used to enhance the retention of ^{125}I-EGF, using lower concentrations of each substance. Combinations of NH_4Cl and the malaria drug chloroquine were tested on A431 cells. Various combinations of NH_4Cl and the malaria drug chloroquine were tested for their effect on cell-associated radioactivity as well as on cell survival after 24 h incubation time.

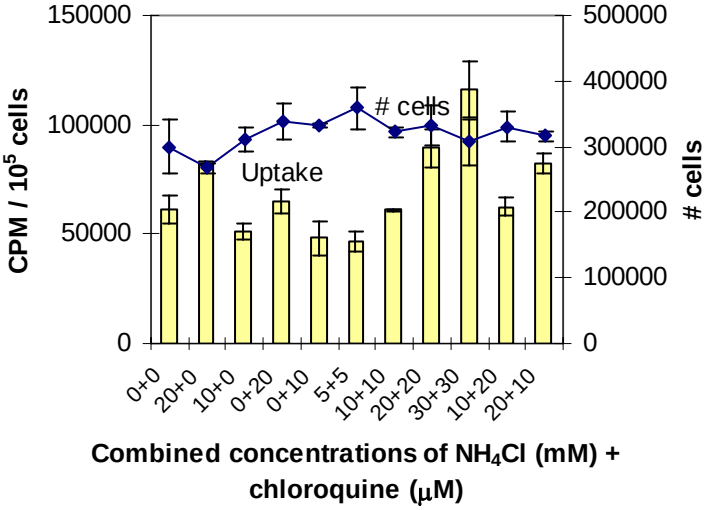

Figure 6 The cell-associated activity (uptake) of ^{125}I-EGF with various concentrations of NH_4Cl and chloroquine after 24 h incubation. The number of surviving cells for each combination is also indicated to reveal the cytotoxic effects of the substances. The maximum error is presented in each point.

The number of cells in treated dishes varied as well as the cell-associated radioactivity. 20 mM NH_4Cl seemed to be required to get a significant difference in uptake, compared to the controls (figure 6). Combinations of lower concentrations did not have the same effect as 20 mM NH_4Cl alone. Addition of chloroquine did not seem to have any great effect on the number of surviving cells. Using 30 mM NH_4Cl in combination with 30 mM chloroquine showed the greatest cell-associated radioactivity after 24 h of incubation. It had some effect on cell survival though. With the results from the other experiments with NH_4Cl it was decided that the uptake and retention of ^{125}I-EGF should be tested with 20 mM NH_4Cl in combination with 30 μM chloroquine.

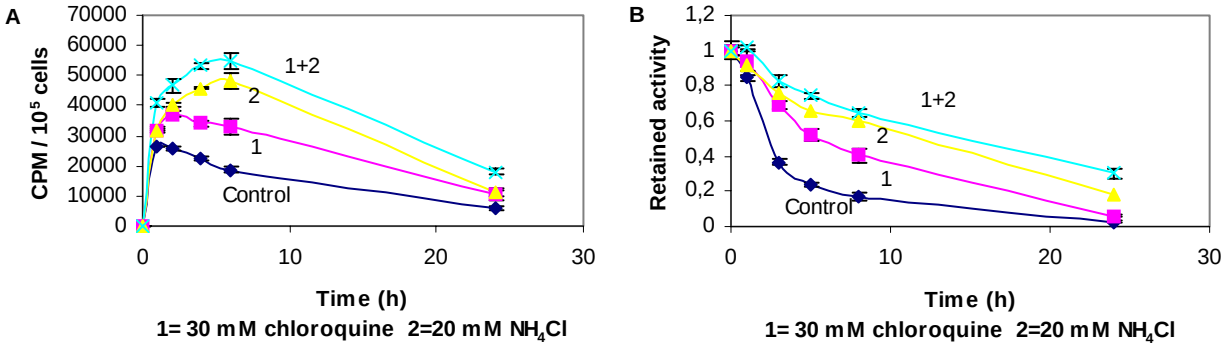

Figure 7 The uptake of ^{125}I-EGF with 20 mM NH_4Cl and 30 μM chloroquine, separately and in combination, is shown in chart A. Use of the substances in combination showed the greatest effects on the uptake. A combination of the two substances also had the greatest effect on retention of radioactivity (B).

Using a combination of the two substances had the greatest effect on uptake of ^{125}I-EGF (figure 7A). The combination and 20 mM NH_4Cl alone both prolonged the time of uptake to about 8 h, compared to 1 h for the controls and 2 h for cells treated with 30 μM chloroquine alone. At 8 h the cells treated with the combination had about 3 times higher uptake than the controls. The retained activity after interrupted incubation was also prolonged using the substances separately and in combination (figure 7B). Once again the combination had the greatest effect followed by 20 mM NH_4Cl.

3.4 Combination of weak bases and the RTK-inhibitor gefitinib

In this experiment, the treatment with weak bases was further combined with addition of the RTK-inhibitor gefitinib. The uptake of ^{125}I-EGF and the number of surviving cells after 24 h incubation time was measured. The concentrations used were: NH_4Cl – 30 mM, chloroquine – 30 μM and gefitinib – 1 μM. The results showed that the substances all affected the cell-associated radioactivity after 24 h incubation, when used separately (figure 8). The effect was also shown to be greater when using ammonium chloride in combination with some of the other substances. These combinations also had greater effect on the survival of the cells. Combining chloroquine and gefitinib though, enhanced the retention of radioactivity but had less effect on the survival. As all these three substances were combined, the number of cells was heavily reduced.

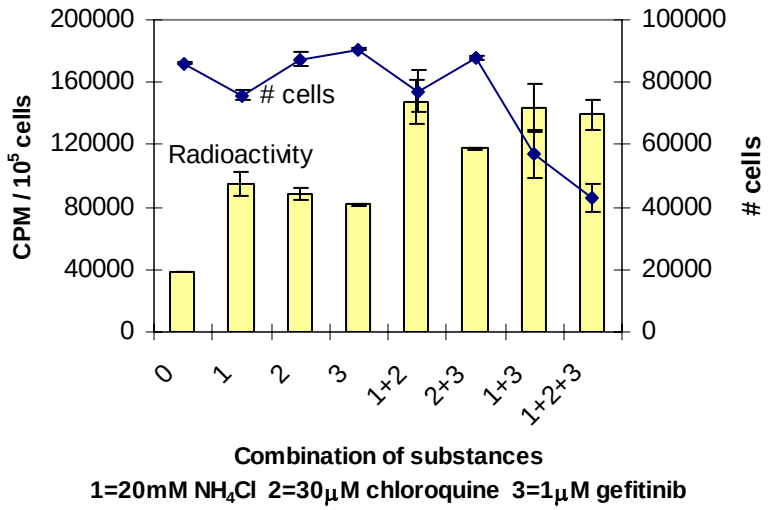

Figure 8 Cell-associated radioactivities after 24 h incubation with different combinations of 20 mM NH_4Cl, 30 μM chloroquine and 1 μM gefitinib. Combinations including NH_4Cl showed greatest effects on cell-associated radioactivity. Addition of 1 μM gefitinib indicated low survival in cells when combined with NH_4Cl but not in combination with chloroquine.

3.5 Effect of ^{211}At-EGF and NH_4Cl in A431 cells

The uptake and retention of ^{211}At-EGF followed the same patterns as ^{125}I-EGF. The results indicate that 20 mM NH_4Cl prolonged both the uptake and the release of radioactivity in A431 cells treated with ^{211}At -EGF (figure 9). Blocked cells had a very low uptake when treated with 5:1 ^{211}At-EGF.

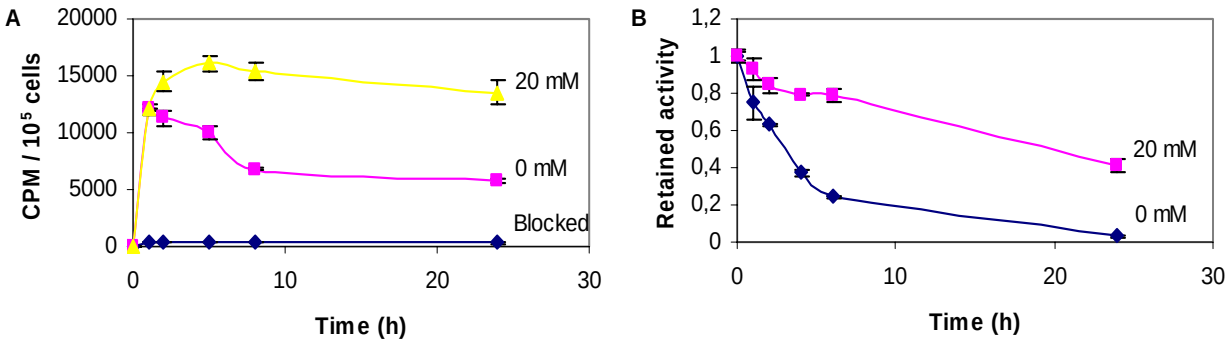

Figure 9: Chart A shows the uptake of ^{211}At-EGF in A431 cells with a 5:1 ligand/receptor ratio. Cells treated with 20 mM NH_4Cl and blocked with 500:1 cold EGF had a lower uptake compared to unblocked cells treated with 0 or 20 mM NH_4Cl. Blocked cells were not tested for retention. Addition of 20 mM NH_4Cl prolongs the retention of ^{211}At-EGF (B). Error bars represent the maximum error.

Cells treated with 5:1 ^{211}At-EGF showed that ^{211}At had great effects on survival of cells treated with either 0 or 20 mM NH_4Cl (figure 10). With this concentration blocked cells almost instantly regained their normal growth rate. Unblocked cells with either 0 or 20 mM NH_4Cl were heavily reduced in cell numbers during the first weeks. The growth delay of cells treated with ^{211}At-EGF and 0 mM NH_4Cl was in this case about 25 days. Two of the flasks with cells treated with ^{211}At in combination with 20 mM NH_4Cl did not regain normal growth rate. In the third flask cells started to grow after about 20 days as indicated in figure 10. It was possible to calculate the survival in percent for cells in this flask by extrapolate the growth curve back to the y-axes. The survival was then 0.000003%, indicating that only 1 cell out of 300 000 in the flask recovered and regained normal growth rate. Cells treated with ^{211}At-EGF and 0 mM NH_4Cl had about ten times higher survival than the cells treated with 20 mM NH_4Cl. Cells treated with ^{211}At-EGF in combination with 0 mM NH_4Cl experienced a dose of about 112 decays/cell while cells treated with ^{211}At-EGF in combination with 20 mM NH_4Cl experienced a dose of about 191 decays/cell. The growth assay revealed that cells treated with 25:1 ^{211}At-EGF had a very low survival independent of addition of 20 mM NH_4Cl (results not shown).

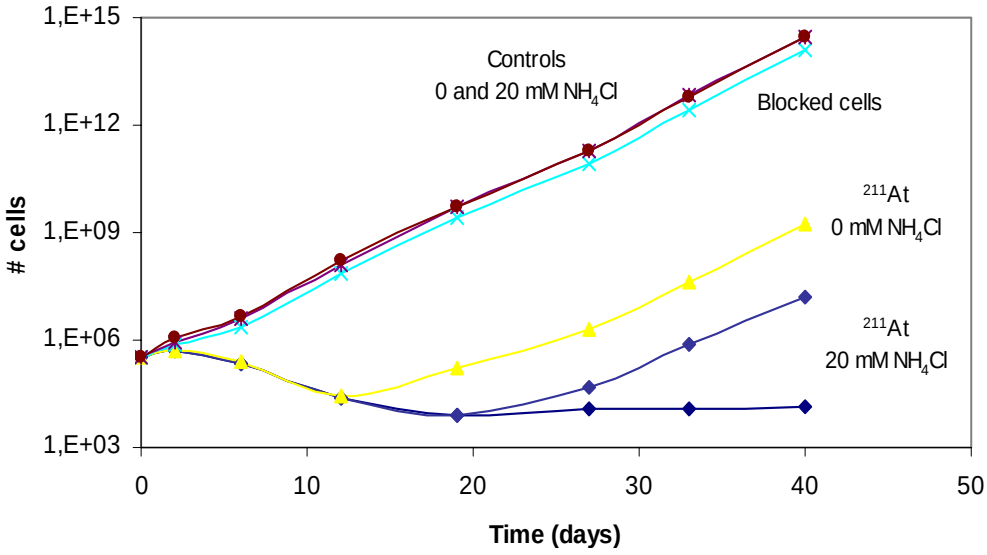

Figure 10 Growth curves of A431 cells treated with ^{211}At in combination with 0 or 20 mM NH_4Cl and controls blocked with 500:1 cold EGF. The most upper curves are the controls grown in medium containing 0 or 20 mM NH_4Cl. The controls grew with normal rate while astatinated cells had some growth delay. Two of three cell flasks treated with ^{211}At in combination with 20 mM NH_4Cl did not recover to grow at a normal rate.

4 Discussion

4.1 NH_4Cl in use of targeted therapy in EGFR-rich cells

The results from this study indicated that use of ammonium chloride (NH_4Cl) had enhancing effects on the uptake and retention of radioactively labelled EGF in A431 cells. NH_4Cl seemed to slow down the degradation of the EGF/EGFR complexes by preventing lysosomal degradation enzymes action. These enzymes are dependent on low pH in the degradation compartments. In A431 cells large differences in the uptake of ^{125}I-EGF were observed, in the absence or in the presence of 20 mM NH_4Cl. The uptake was continuous for up to 8 h when cells were treated with 20 mM NH_4Cl. In the controls the uptake was highest after 1 h of incubation and the cell-associated radioactivity then decreased over time (figure 4A). This indicates that the control cells degraded EGFR/EGF-complexes quite efficiently and thereby lost the radioactivity. The degradation of proteins targeted for lysosomal degradation is believed to start 20-30 minutes after internalisation (9). The results presented in figure 4B indicate that the controls have efficient degradation, and that ^{125}I was released from the cells even during the first hour after interrupted incubation with ^{125}I-EGF. In contrast, the radioactivity in cells treated with 20 M NH_4Cl was retained efficiently during the first eight hours after interrupted incubation with the labelled EGF though.

The uptake depends on the efficiency of internalisation and the formation of clathrin-coated pits. In this study the accumulated cell-associated radioactivity in the cells was measured and nothing can be said about in which compartments the activity is retained. Most probably the non-degraded complexes accumulate in late endosomes or lysosomes. The location of internalised EGFR/EGF-complexes in combined treatment with the weak base thioridazine was investigated in another study (13). The presented results from this study indicate that EGFR/EGF-complexes accumulate in the late compartments of the lysosomal degradation pathway. As it is believed that the weak bases have the same effects it is reasonable to assume that NH_4Cl also makes the complexes accumulate in the late compartments.

Pre-treatment with 20 mM NH_4Cl did not further enhance the uptake of ^{125}I-EGF, indicating that NH_4Cl has a rapid action of the lysosomal pH. The small molecule might be able to diffuse freely into the cells and can be given to the cells at the same time as the radiolabelled EGF. The effect of simultaneous incubation with 20 mM NH_4Cl and ^{125}I-EGF did not last when fresh medium (0 mM NH_4Cl) was added after interrupted incubation. This also indicates that there must be a continuous supply of NH_4Cl; otherwise it will be rapidly diluted.

4.2 NH_4Cl has little effect on internalisation of the HER-2-affibody

It was not really clear whether NH_4Cl would have any effect on the uptake and retention of the HER-2 affibody. As mentioned in the theoretical background it seems, as HER-2 does not take the same endocytic route as EGFR. There is no experimental data on the receptors behaviour after binding of the affibody or whether the receptor is activated. It seems that 20 mM NH_4Cl has some effect on the retention of ^{125}I-labelled affibody though. Differences are larger at late time points. This might indicate that the internalisation of these receptors is slow.

The affibodies were labelled using the ATE-method and in this study it has been shown that ATE-labelling have some advantage compared with CAT-labelling when it comes to the

retention of labelled EGF in A431 cells. If the affibody could be directly labelled, an experiment could have been done in SKBR-3 and SKOV-3 cells to investigate whether the different labelling methods have the same effect in these cells. Direct labelling of the affibody has however been shown to disturb the binding capacity of the affibody (results not published).

A suggestion to why NH_4Cl seemed to have some effect on HER-2 over-expressing cells was that some of the affibody-bound receptors might dimerise with EGFR. An experiment was performed to test whether the greater effect of 20 mM NH_4Cl in SKBR-3 cells might be related to a higher number of EGFR than in SKOV-3 cells. As NH_4Cl seemed to have greater effect on EGFR than on HER-2, a possible explanation could have been that the affibody might make HER-2 dimerise with EGFR and thus have an effect on cells with elevated expression of both receptors. If SKBR-3 had a higher number of EGFR, compared to SKOV-3, the dimerisation theory could have been applied. The results showed that SKBR-3 has the lowest number of EGFR, compared to A431 and SKOV-3 cells (results not shown). The greater retention of ^{125}I-labelled affibody effect did not correspond to a higher possibility of HER-2/EGFR dimerisation and the results can not be further explained from this study.

The affibody is still interesting as an agent for targeted therapy as it seem to efficiently bind to HER-2. The uptake of radioactivity in controls of SKOV-3 shows that the cell-associated radioactivity stayed quite high when cells were continuously incubated with the labelled affibody (figure 5B). On the other hand the cells lost the radioactivity quite fast when the incubation was interrupted, as seen in the retention experiment for SKOV-3 (figure 5D). To have radiation effects on these cells there seemed that there was a requirement of continous supply of labelled affibodies.

4.3 Effects of combinations of NH_4Cl, chloroquine and gefitinib

As drugs and other substances often affect cells in more ways than the desirable one, it is of interest to use as low concentration of a drug as possible. Treatment of A431 cells with various concentrations of NH_4Cl showed that the number of surviving cells decreased with an increase of concentration (figure 3). In this *in vitro* study, cell survival after 24 h incubation with 20 mM NH_4Cl was decided to be acceptable. It also had a distinct effect on the cell-associated ^{125}I-EGF after 24 h of incubation in A431 cells.

Results from other studies have shown similar effects in the use of chloroquine (12) and the RTK-inhibitor gefitinib (8) in other cell lines. When studying their effects in A431 cells, the results indicate that 20 mM NH_4Cl had the greatest effect on the cell-associated radioactivity. 30 μM chloroquine or 1μM gefitinib also showed distinct effects on the cell-associated radioactivity respectively. The use of these two substances, at these concentrations, did not seem to have the same negative effect on survival though (figure 7). Even when using 30 μM chloroquine and 1 μM gefitinib in combination there was no indication of reduced cell survival. Previous experiments with 1 μM gefitinib in A431 cells showed that gefitinib inhibited cell growth (4). Combinations including 20 mM NH_4Cl seemed to be the most toxic ones, especially when combined with gefitinib. Why these substances have different effects in these combinations is not known. Further experiments are needed to investigate these effects and to find suitable concentrations for clinical use.

4.4 ^{211}At seems to be a potent radionuclide for targeted therapy

The α–emitter ^{211}At was in this study shown to be a potent radionuclide for targeted therapy. These experiments showed an enhanced uptake of ^{211}At-EGF in A431 cells, when combined with 20 mM NH_4Cl. As for ^{125}I-EGF, the uptake continued for about 8 hours, compared to about 4 hours for the controls (figure 8A). The prolonged uptake in controls differs from the uptake of ^{125}I-EGF. This could be due to that ATE-labelling was used for ^{211}At-EGF. Results in this study showed that the different labelling methods have effects on the uptake and retention of ^{125}I –EGF in A431 cells (results not shown). These results may also be valid for ^{211}At-EGF. The retained activity was also elevated with the largest differences at 8 hours after interrupted incubation (figure 8B). The survival of the controls treated with ^{211}At-EGF and 0 mM NH_4Cl was about ten times higher than for cells treated with 20 mM NH_4Cl. In this experiment it was very likely that only one out of 300 000 cells in one of the flasks treated with 20 mM NH_4Cl was able to start grow and divide.

In the growth assay different controls were used. Cells not treated with ^{211}At-EGF, grown in either 0 or 20 mM NH_4Cl, showed a usual growth curve (figure 10). Cells blocked with 500x cold EGF was used to show that unspecific radiation (from outside of cells) has no great effect on survival and growth. The 25:1 ^{211}At-EGF-treatment was a bit too efficient in killing un-blocked cells. It was not possible to detect any difference for ^{211}At-treated cells treated with either 0 or 20 mM NH_4Cl since no re-growth or survival of cells could be detected. The idea was that cells incubated with the combination of ^{211}At-EGF and 20 mM NH_4Cl would have a longer growth delay compared to cells treated with ^{211}At-EGF alone. This study only showed that ^{211}At efficiently kills cells, when used in targeted therapy directed towards the EGFR.

When instead using 5:1 ^{211}At-EGF, blocked cells regained normal growth rate after just a short period of time (figure 10). Cells treated with ^{211}At-EGF alone were at first heavily reduced in cell number but recovered after about 12 days and seemed to regain normal growth rate. ^{211}At-EGF combined with 20 mM NH_4Cl experienced a great loss of cells after the treatment and had a longer growth delay compared to cells treated with ^{211}At-EGF alone. Only one of the three flasks with cells treated with ^{211}At-EGF in combination with 20 mM NH_4Cl regained normal growth rate. These results indicated that addition of the weak base NH_4Cl might enhance the therapeutic effects in use of targeted therapy. Further experiments should be done to investigate the possibility of using weak bases, separately or in combination, as enhancers of targeted therapy.

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Thank you!

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