PHD THESIS

New possibilities for combined therapy of breast cancer

Barbara Zsebik

Supervisor: György Vereb

UNIVERSITY OF DEBRECEN
DEPARTMENT OF BIOPHYSICS AND CELL BIOLOGY
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1. INTRODUCTION

ErbB2 overexpressed on cell surface of breast carcinoma cells as a terapeutic target

ErbB2 is a member of the ErbB receptor tyrosine kinase family. Although it has no soluble high-affinity ligand, it fulfills a central role in the ErbB signal transducing network by increasing the ligand binding spectrum and affinity of ErbB1, -3 and -4, the other members of the family. Activation of the ErbB family members requires their homo- or heterodimerization which is followed by transphosphorylation and downstream signaling cascades leading to cell proliferation and survival. A most mitogenic heterodimer is formed of ErbB2 and ErbB3, the latter, though kinase deficient, serving as a ligand for neuregulins. ErbB2-containing heterodimers are internalized less efficiently and evade lysosomal degradation, an effect which is even more pronounced upon ErbB2 overexpression also leading to ligand-independent constitutive activation of ErbB2 homodimers.

ErbB2 is overexpressed in 25-30 % of human breast cancers, and is associated with very poor prognosis. Overexpressed membrane proteins, especially those, like ErbB2 that are scarce in differentiated cells are ideal targets of molecular therapy. ErbB2 was the first antigen on a solid tumor to be targeted with a humanized monoclonal antibody, trastuzumab (Herceptin). Other molecular therapies targeting ErbB2 include the humanized 2C4 antibody (omnitarg) that inhibits ErbB2 dimerization and the pan-ErbB kinase inhibitor CI-1033, that are now investigated in several clinical trials.

While the use of trastuzumab in antibody-based cancer therapy has dynamically expanded over the past years, it also had to be realized that as single agent, it is ineffective in 60-70% of ErbB2 overexpressing breast tumors. Even though trastuzumab, when combined with chemotherapy gives better initial results, continued administration of the antibody usually leads to secondary resistance. The molecular mechanisms of resistance to trastuzumab, similarly to its mode of action, are largely unknown and possibly include various factors. Autocrine production of EGF-like ligands or overexpression of insulin-like growth factor 1 receptor (IGF1R), leading to an ErbB2-independent means for the constitutive activation of the PI3K pathway, as well as

blocking of trastuzumab binding by MUC4, a cell surface mucin, have been implicated in trastuzumab resistance.

Trastuzumab binds to a membrane-proximal domain of ErbB2 and causes partial activation and internalization of the receptor. Although the internalization itself may not be a definitive requirement for the antiproliferative effect of trastuzumab, decreased cell surface ErbB2 levels could well be one distinct cause of decreased cell proliferation. Thus, in the clinical setting, it could be a reasonable approach to use alternative methods to decrease ErB2 levels of overexpressing, but trastuzumab resistant tumor cells.

The chaperon Hsp90, besides catalyzing the proper folding of newly synthesized client proteins into a stable tertiary conformation, has been implicated in the stabilization of a number of cellular proteins that play central roles in signal transduction processes. ErbB2, alone in the ErbB family, possesses a C-terminal sequence that is responsible for Hsp90 binding. Interestingly, binding of Hsp90 to ErbB2 not only serves to maintain its physiological conformation, but also to restrain ErbB2 from forming active signaling dimers.

Ansamycin antibiotics isolated from *Streptomyces hygroscopicus*, such as geldanamycin, were found to inhibit the growth of many cancer cell lines at nanomolar concentrations. Geldanamycin having a very narrow therapeutic window, more promising analogs such as 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) were synthesized. 17-AAG exhibited decreased toxicity and enhanced stability, and even though its binding to Hsp90 was weaker than that of geldanamycin, 17-AAG displayed an antitumor effect similar to geldanamycin. It also proved to be reasonably successful in phase I clinical trials in spite of formulation problems and side effects, especially in two cases of melanoma.

Among several signaling molecules (such as Raf-1, CDK4, Lck), 17-AAG was found to decrease ErbB2 levels in breast, prostate and ovarian cancer cells. However, it remains unclear to what extent down-regulation of ErbB2 levels is correlated with the antiproliferative effect of 17-AAG, and whether it would also be effective in the cases where trastuzumab does not effectively decrease cell surface ErbB2 levels and proliferation. Furthermore, it is of importance to learn whether in trastuzumab sensitive tumors 17-AAG and trastuzumab could be used together to potentiate each others effect.

In this study we have exploited the recently established first trastuzumab resistant breast cancer cell line, JIMT-1, that can be passaged in vitro and compared these cells to the known trastuzumab sensitive SKBR-3 line. We investigated baseline and stimulated dimerization and activation levels of ErbB2, and the effects of trastuzumab and 17-AAG alone and in combination on cell proliferation and apoptosis, as well as on ErbB2 expression and phosphorylation. Our results indicate that while baseline activation and amenability to activation and downregulation by trastuzumab is much lower in the resistant line, its proliferation is more prone to inhibition by 17-AAG. In both cell lines, the antiproliferative effect of 17-AAG was correlated with the downregulation of ErbB2. The usual therapeutic dose of trastuzumab did not change the IC_{50} of 17-AAG on the proliferation of either cell line, but nevertheless decreased overall ErbB2 phosphorylation and at low doses of 17-AAG further decreased cell growth in the sensitive SKBR-3.

Photodynamic therapy and cysteine proteinase inhibitors in cancer therapy

In spite of the wide spectrum of available tumour treatment modalities, cancer therapy is still a major clinical challenge and none of the therapies can be considered fully effective when applied alone. In most cases, chemotherapy proved to be quite powerful. It is often combined with radiotherapy and surgery. In spite of radical surgery, tumour cells may remain in the surrounding area, which often requires the application of a postoperative adjuvant therapy. Both chemotherapy and irradiation can have various undesired side effects.

A relatively new therapeutic modality for both neoplastic and non-neoplastic diseases is photodynamic therapy (PDT), which involves light-driven activation of photosensitizers to produce molecular oxygen and other free radicals. PDT can cause the damage of malignant cells and/or tumour vasculature, which leads to necrosis and/or apoptosis of tumour cells. Since most cancer cells selectively accumulate the photosensitizer, the technique is supposed to be rather selective in destroying tumour cells. Although the serum level of various biologically active molecules is known to change after PDT, proper design of therapy provides for avoiding serious side effects. Hence it is a potentially effective and relatively safe adjuvant therapy in complementing surgery, radiation- and chemotherapy.

Cystein proteinases of tumour cells play a crucial role in the proteolytic cascade resulting in destruction of the neighbouring extracellular matrix components such as laminin, collagen and elastin. The increased activity of cathepsin, a member of the cystein proteinase family is associated with the vascularization and an invasive fronts of carcinomas. Inhibitors of these proteinases (CPI) produced by the organism participate in the mechanisms of defense against cancer progression. The balance between the activity of cystein proteinases and their inhibitors may determine the aggressiveness of cancer cells. In normal tissues, extracellular proteolysis is regulated by endogenous inhibitors; in pathogenic process the balance between proteinases and inhibitors is believed to be disturbed. The cystatin isolated from egg white was one of the first inhibitors of cystein proteinases to be identified and characterized. It belongs to the family II of cystatins which consist of 120 amino acids with two disulphide bridges. Since cystatins isolated from egg white effectively inhibit cystein proteinases from cancer cells, these molecules are potential new-generation drugs in "inhibitor-therapy".

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is essential for the growth of solid tumours and the appearance of metastases. This process can be stimulated by hypoxia. Several types of cells respond to hypoxia by producing different angiogenic factors, such as TNF, FGF and VEGF. After binding of VEGF to its type 2 receptor, endothelial cell migration and proliferation is induced, while the activation of VEGF receptor-1 by this factor enhances cell migration and secretion of tissue factor by endothelial cells. Overexpression of VEGF has been associated with tumour progression and poor prognosis in several tumours. The best characterized of the VEGF family members, VEGF-A (also known as vascular permeability factor) is associated with key events in physiological angiogenesis. VEGF as a potent vascular permeabilizing agent enhances microvascular permeability. The hyperpermeability of tumour vessels to plasma proteins is attributable to VEGF secretion by tumour cell leading to the transformation of the normally antiangiogenic stroma of normal tissues into a proangiogenic environment. VEGF has been shown to inhibit necrosis and upregulate antiapoptotic proteins Bcl-2 and stimulate the phosphatidylinositol 3´-kinase/Akt pathway that promote endothelial cell survival despite apoptotic stimuli.

In this work the efficacy of the combined application of photodynamic therapy and cystatin was investigated on a solid mammary carcinoma transplanted in Wistar rats. The combined therapy was significantly more effective than either treatment alone, and not only caused the decrease of tumour size by induction of necrosis and inhibiting cysteine proteinases, but it also diminished the level of neovascularization, partly by inhibiting VEGF production.

2. AIMS

In our studies, trastuzumab and photodynamic therapy, both used clinically for tumour therapy, were combined with novel small molecule inhibitors to test the efficacy of their combination in the treatment of breast cancers. In this context, following questions were posed:

- Could the Hsp90 chaperon inhibited by 17-AAG alter the activation process of ErbB2? Can this alteration influence the proliferation of tumour cells?

- Under *in vitro* conditions, could the effect of trastuzumab be potentiated with the combination of 17-AAG in the case of trastuzumab sensitive and resistant cells?

- Could the effect of the photodynamic therapy used for i*n vivo* treatment of solid breast adenocarcinoma be enhanced by the hindrance of cysteine proteinases that are active participants in the destruction of the extracellular matrix?

- In the case that the combination of photodynamic and inhibitor therapies proves effective, what are the optimal conditions? Could the decrease of VEGF levels be part of the mechanism of action?

3. MATERIALS AND METHODS

Cell cultures and animal models

In our *in vitro* experiments ErbB2–overexpressing trastuzumab resistant JIMT-1 and sensitive SKBR-3 cell lines were used. For i*n vivo* studies, HER2-negative solid breast adenocarcinoma was implanted into Wistar rats.

Antibodies, chemicals, treatments

For hetero- and homodimerization studies ErbB2 was labeled by 2C4, ErbB1 with 528 antidbody that were isolated from the hybridoma supernatant. Antibodies were conjugated with Cy3, Cy5 or AlexaFluor 488. Phosphorylated ErbB2 was labeled directly or indirectly with Ab18 against aa1242-1255 of the receptor. A488-Gamig (Fab) was used as a secondary antibody. Cells were stimulated by murine EGF. Trastuzumab (Herceptin) was from Hoffman-La Roche. 17-AAG was a kind gift of Kosan Biosciences and stored as a 10mM stock solution in DMSO. Cells adhered to the Petri dish were treated with trastuzumab (0, 2, 10, 40, 100 and 200 µg/ml), or 17-AAG (0.0005, 0.005, 0.05, 0.5 and 1.7 uM) and the combination of all 17-AAG doses with 40 µg/ml trastuzumab (the clinical dose).

Cysteine proteinase inhibitor (CPI) isolated from egg white was administered to the tumour-bearing rats by infiltrating the peritumour space. Hematoporphyrin derivative (HpD) and chlorin e6 (Ce6) were used as photosensitizers. Both of them were dissolved in physiological saline pH 7.2, containing 0.05 M NaOH and injected IP at doses of 10 or 20 mg/kg. After 24 hours the accumulated photosensitizer in unmasked tumours was irradiated for 7 minutes by a Penta (Teclas) lamp. Excitation wavelength ranges were selected with bandpass filters: 630 ± 20 nm for HpD and 654 ± 20 nm for chlorin e6. Light intensity was adjusted to 230 mW/cm^2, and total light dose was 90 J/cm^2.

Determination of ErbB-expression and phosphorylation with flow cytometry

$8 \cdot 10^5$ cells from each treatment group were trypsinized, washed with HBS, and divided into two aliquots, one for background control, the other for labeling. Extracellular ErbB2 was labeled with 20 µM Cy5-2C4 for 10 min at 4 ºC. After washing in HBS,

samples were fixed in formaldehyde, followed by incubation in HBS with 1% milk powder, 0.1% saponin and 1 mM sodium-ortho-vanadate. P-ErbB2 was labeled with 10 μM Alexa-488 conjugated Ab-18 for 40 min in the same buffer. Fluorescence of 10,000 cells was measured in a FACSCalibur flow cytometer in the FL1 and FL4 channels. ErbB2 expression was calibrated using the known expression level of viable SKBR-3 cells, which was determined to be 800,000 using QIFIKIT (DakoCytomation, Glostrup, Denmark).

Measuring ErbB2 association with acceptor photobleaching FRET

Cells grown to 80% confluence on coverslips were treated with 40 μg/ml trastuzumab or 50 nM EGF at 37 ºC for 7 min, or with 0.5 μM 17-AAG in 50 μl HBS for 2, 5 or 10 min. Coverslips were washed, fixed, and cell were labeled. For measuring ErbB2-ErbB2 homoassociation, ErbB2 was labeled with 20 μM Cy3-2C4 as donor and 20 μM Cy5-2C4 as acceptor (mixed in advance) at 4 ºC for 10 min. For ErbB1-ErbB2 heteroassociation, the same procedure was used, but as donor 20 μM Cy3-528 was used against ErbB1. Finally the samples were washed again and mounted in 5 μl Mowiol.

Fluorescence resonance energy transfer (FRET) efficiency was measured using the acceptor photobleaching protocol. The molecular proximity of an acceptor fluorophor emission from the donor is decreased. Photobleaching the acceptor releases the donor from this quenching which results in increased fluorescence intensity. The FRET efficiency *E* in each pixel of the microscopic image can be calculated as,

$E_{(i,j)} = 1 - \frac{F^{I}_{D(i,j)}}{\gamma \cdot F^{II}_{D(i,j)}}$, where $F^{I}_{D(i,j)}$ and $F^{II}_{D(i,j)}$ are the background subtracted donor fluorescence values of the $(i,j)^{\text{th}}$ pixel in the entire image before (*I*) and after (*II*) photobleaching the acceptor. In the denominator, γ is a correction factor that takes into consideration the photobleaching of the donor during the whole protocol. It can be calculated as $\gamma = \left\langle F^{ref,I}_{D(i,j)} \right\rangle \Big/ \left\langle F^{ref,II}_{D(i,j)} \right\rangle$, where $\left\langle F^{ref,I}_{D(i,j)} \right\rangle$ and $\left\langle F^{ref,II}_{D(i,j)} \right\rangle$ are the mean, background-subtracted donor intensities in pixels (*i*,*j*) above threshold of a reference sample labeled with donor only, before (*I*) and after (*II*) running an identical acceptor photobleaching protocol.

The measurement protocol was implemented in an LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany). A custom C script was written and used with SCIL Image (TNO, Amsterdam, The Netherlands) to evaluate image sequences and calculate average FRET efficiency for pixels above threshold in each frame.

Assessing cell proliferation

The effect of 17-AAG, and trastuzumab alone and in combination on cell proliferation was tested in a protocol of daily treatment for 4 days, starting 24 hours after seeding 4,000 cells in 200 µl medium per well into 96-well plates. Doses of drugs were applied as mentioned above. Separate plates were prepared for measuring cell numbers after each day of treatment. A tetrazolium-based assay (EZ4U, Biomedica Medizinprodukte GmbH and Co KG, Wien, Austria) was used according to the manufacturer's instructions. Processing time was optimized for each cell type.

Quantifying apoptosis

10^4 cells/well were cultured in 8-well Nunc chamber slides. Cells were treated daily with 0; 0.005; 0.05 and 0.5 µM 17-AAG for 4 consecutive days. Annexin V-FITC Apoptosis Detection Kit (Sigma APO-AF) was applied as per instructions. After 10 min images of each well were taken with a 40× /0.6 NA objective using an LSM 510 laser-scanning microscope in tile mode. Excitation was at 488 and 543 nm, emission through 505-555 and 560-605 nm bandpass filters. The transmitted light image during 488 nm excitation was also recorded. Apoptotic (Anexin-V positive) and necrotic (only PI positive) cells were counted and normalized to total area.

Protocols for examination of combination of photodynamic therapy and cystatin in the case of solid mammary adenocarcinoma implanted into Wistar rats

Combination treatments were carried out under three different experimental protocols. *Protocol I.* The homogenized ground tumour tissue (1 ml) was diluted with 0.5 ml physiological saline containing 500 µg CPI and implanted subcutaneously into the left abdominal region of the rat (day 0.). On the same day, HpD was injected intraperitoneally at a dose of 10 or 20 mg/kg. The next day (day 1.), 500 µg CPI per animal was

administered between the normal and cancerous tissues, and the site of inoculation was light-irradiated. During the following 6 days, CPI was injected around the tumour every day. After an 8-day treatment, rats were sacrified, 200 μl blood was collected and sera were stored at -70 °C until the determination of VEGF levels with ELISA. Tumours were excised, formaldehyde-fixed and paraffin-embedded. 30 animals were used in this protocol. *Protocol II* was similar to *Protocol I*, except HpD was injected on the day after the inoculation (day 1.) and tumours were irradiated on day 2. 10 animals were used in this protocol. *Protocol III* differed from *Protocol I* in that tumour implantation took place 7 days before the start of treatments on day 0 and that the photosensitizer used was Ce6. 10 animals were used in this protocol.

Evaluation of histological sections

Four μm sections were prepared from paraffin embedded tumours and stained with hematoxylin-eosin. The extent of necrosis was determined from examining 4 complete sections of each tumour applying the usual pathological criteria and expressed as percentage of the tumour. Blood vessels were counted in 4 complete sections and their average number per field of view at 10x magnification for each tumour calculated.

Determination of VEGF levels in rat sera

Determination of serum VEGF levels was carried out according to the manufacturer's instructions using a Mouse VEGF Immunoassay Kit (Quantikine, R & D Systems, D) which also recognizes rat VEGF.

Statistical analysis

Since some data were non-normally distributed, differences among all treatment groups and all protocols were tested using 2-way ANOVA on ranks, followed by testing the difference between each treatment group and the control (Holm-Sidak method). Differences between various treatments were compared using 1-way ANOVA, followed by pairwise t-tests. Correlation between readout parameters (tumour size, necrosis, neovascularization, VEGF levels) was determined from Pearson's product moment correlation coefficient.

4. RESULTS

Cell proliferation and dimerization and activation status of ErbB2 in the presence of trastuzumab, 17-AAG, and their combination

Since dimerization or higher order oligomerization is accepted as a key event in receptor tyrosine kinase activation, we have first examined the homo- and heterodimerization of ErbB2 on both SKBR-3 and JIMT-1 cells. The microscopic acceptor photobleaching approach allowed investigating the cells in their native, adherent state. In JIMT-1 cells, ErbB2-ErbB2 homoassociation was very low, and EGF (50 nM) or trastuzumab (40 μg/ml) did not have any effect on it. SKBR-3 cells exhibited a higher basal level of ErbB2 homoassociation, which was further increased by EGF and even more so by trastuzumab treatment. ErbB1-ErbB2 heteroassociation was high on SKBR-3, and as low on JIMT-1 as ErbB2 homoassociation. Trastuzumab had no effect on the ErbB1-ErbB2 heterodimers on either cell lines.

Flow cytometric measurements indicated that the low dimerization level of ErbB2 in JIMT-1 was paralleled by a low level of ErbB2 phosphorylation, which increased to a small extent upon treatment with 40 μg/ml trastuzumab as measured by flow cytometry. On the other hand, in SKBR-3, ErbB2 tyrosine phosphorylation was higher, and increased to over 300% upon trastuzumab treatment.

As a next step, 17-AAG was used to assess whether inhibiting Hsp90 can revert this low dimerization and activation state of ErbB2 in JIMT-1. 0.5 μM 17-AAG significantly increased ErbB2 homoassociation both in JIMT-1 and SKBR-3 cells; the effect peaked at 5 min after adding the drug and was maintained at least for another 5 min. These observations indicate that 17-AAG could be used to promote activation and internalization of ErbB2 not only in SKBR-3, but also in the trastuzumab resistant JIMT-1.

Dose-response curves of trastuzumab and 17-AAG were determined for the proliferation of both SKBR-3 and JIMT-1 cells. 2-200 μg/ml dose of trastuzumab was added to the cells daily, while 17-AAG was given at a concentration of 0.0005-1.7 μM, also for 4 consecutive days. In the case of SKBR-3, concentrations as low as 40 μg/ml for trastuzumab and 0.05 μM for 17-AAG inhibited cell-proliferation starting from the third

day. According to expectations, trastuzumab had negligible effect on the proliferation of JIMT-1 cells at the therapeutic 40 μg/ml concentration, and had only little effect compared to SKBR-3 even at 200 μg/ml. As opposed to this, 17-AAG at 0.05 μM concentration decreased proliferation of JIMT-1 even more than in SKBR-3. In the case of SKBR-3, the effect of smaller doses (0.0005, 0.005 μM) of 17-AAG were enhanced by 40 μg/ml trastuzumab, whereas higher doses were not significantly influenced by combining with them the antibody, whereas in JIMT-1, the addition of trastuzumab to the 17-AAG regime appeared to cause no change in the proliferation rates.

To reveal the mechanisms behind the antiproliferative effect of 17-AAG, the amount of Anexin-V positive and propidium idodide (PI) positive cells was determined after 4 days of culture in the presence of 0.005 μM, 0.05 μM and 0.5 μM 17-AAG and compared to the control. Cells that displayed Anexin-V staining in the membrane were considered apoptotic regardless of PI positivity, whereas PI positive cells with no Anexin-V labeling were counted as necrotic. 17-AAG promoted both apoptosis and necrosis in a dose-dependent manner in both cell lines. Of the two processes, apoptosis was the dominant.

Dose-dependence and correlation of cell proliferation, ErbB2 membrane levels and ErbB2 phosphorylation upon 17-AAG treatment

To determine the half-effective concentration of 17-AAG, cells were treated for 4 days, administering 17-AAG daily in the dose range 0.0005 μM to 1.7 μM, either alone, or in combination with 40 μg/ml trastuzumab. At day 5, the number of cells per well was determined using a tetrazolium-based colorimetric assay, and the number of ErbB2 molecules in the membrane of cells, as well as the amount of phospho-ErbB2 in the cells was determined by flow cytometry. The dose-dependence of measured parameters showed typical sigmoid curves that were fitted to the Hill-equation to obtain IC_{50} values. JIMT-1 cells were more sensitive to 17-AAG than SKBR-3 in terms of proliferation, with IC_{50} values of 0.011 μM as opposed to 0.069 μM in the latter. Addition of therapeutic dose of trastuzumab appeared to cause a minor increase in the IC_{50} of 17-AAG. In the case of SKBR-3, IC_{50} of 17-AAG was not changed by adding trastuzumab to the cells, but at low 17-AAG doses, where the effect of the chaperon inhibitor was not very

pronounced, 40 µg/ml trastuzumab caused a significant decrease of proliferation as compared to treatment with 17-AAG alone.

The cell surface levels of ErbB2 were equally well decreased by 17-AAG in SKBR-3 and JIMT-1, yielding IC_{50} values of ~0.03 µM in both. ErbB2 numbers in JIMT-1 at 0 µM 17-AAG concentration were somewhat below 50% of those in SKBR-3, in coherence with previous data and with Western blotting experiments (data not shown). Addition of 40 µg/ml trastuzumab to the cells had negligible effect on IC_{50} of 17-AAG in SKBR-3, but doubled it in JIMT-1. Also, trastuzumab alone decreased cell surface ErbB2 levels in both cell lines, even though it had no effect on the proliferation of JIMT-1.

Somewhat surprisingly, 17-AAG caused an increase in total phospho-ErbB2 levels in a dose-dependent manner both in SKBR-3 and in JIMT-1, in spite of the overall downregulation of membrane ErbB2. The half-effective doses were similar in both cell lines, ~ 0.04 µM. Upon addition of trastuzumab, phospho-ErbB2 levels did not change in JIMT-1 as compared to 17-AAG-only treated cells. However, ErbB2 phosphorylation decreased to a great extent in SKBR-3, and the efficiency of 17-AAG in promoting phosphorylation decreased by an order of magnitude.

Using the antiproliferative effect (decrease of cell number normalized to control), the decrease of ErbB2 expression normalized to control levels, and the increase of phospho-ErbB2 level normalized to control as readout parameters at the end of the 5-day treatment period, the correlation between these parameters after treatment with trastuzumab, 17-AAG, or their combination was investigated. In both SKBR-3 and JIMT-1, the decrease of membrane ErbB2 levels correlated positively ($R^2 > 0.8$, $p < 0.05$) with the antiproliferative effect of 17-AAG, regardless of the presence or absence of trastuzumab. Trastuzumab alone inhibited the proliferation of SKBR-3 and decreased ErbB2 levels to ~40%. In coherence with this, addition of trastuzumab to 17-AAG treated SKBR-3 cells shifted the ErbB2 decrease vs. antiproliferative effect plot to greater starting values, but did not change its slope. While trastuzumab hardly inhibited the proliferation of JIMT-1 at therapeutic concentration, it did decrease ErbB2 levels on these cells similarly to SKBR-3. In accordance with this, when 40 µg/ml trastuzumab was added to 17-AAG treated JIMT-1 cells, the starting point of the ErbB2 decrease vs.

antiproliferative effect plot did not change in terms of relative decrease in cell number, but was shifted towards more downregulated ErbB2 values.

There was positive correlation ($R^2 > 0.85$, $p < 0.05$) between the increase of phospho-ErbB2 levels and the antiproliferative effect of 17-AAG both in SKBR-3 and JIMT-1, regardless of whether 40 µg/ml trastuzumab was present or not. Furthermore, decrease of ErbB2 levels and increase of ErbB2 phosphorylation were also positively correlated in both cells (not shown, $R^2 > 0.8$, $p < 0.05$). Trastuzumab alone slightly increased ErbB2 phosphorylation of JIMT-1 cells in a dose-dependent manner, but did not change the correlation plot of increase of phospho-ErbB2 vs. decrease of cell number in JIMT-1. In the case of SKBR-3, trastuzumab alone exerted a slight decreasing effect on phospho-ErbB2 levels which was independent of trastuzumab dose. Adding 40 µg/ml trastuzumab to 17-AAG treated cells had a most striking effect in reducing ErbB2 phosphorylation to below control levels in the case of 0.0005 and 0.005 µM 17-AAG, but also significantly decreasing phospho-ErbB2 at higher doses of 17-AAG, as compared to the same doses of 17-AAG applied alone. At the same time, the antiproliferative effect of 17-AAG was enhanced for low doses, and maintained for high doses.

Combination of PDT and CPI causes tumour necrosis and growth inhibition

Microscopic images taken of tumour samples showed that up to 85-90 % of the tumour tissue was necrotized after PDT with 20mg/kg PS combined with CPI injections, compared to ~28 % in the untreated control. The application of PDT or CPI alone caused less severe tumour damage resulting in ~35-45 % of tumour necrosis. The assessment of necrosis indicated that the most effective treatment was obtained with *Experimental Protocol I*. Nonetheless, in combined therapy, all protocols had significant ($p<0.001$) necrotising effect. In combined therapy, the higher dose of photosensitizer was somewhat more effective in necrotizing the tumour than the lower one ($p=0.06$). In the case of PDT or CPI monotherapies, 10mg/kg dose of PS had no effect ($p=0.58$), but the higher, 20mg/kg dose of PS as well as CPI caused some measurable increase of necrosis ($p=0.01$ and $p=0.06$). Nonetheless, the efficiency of either monotherapy alone was inferior to both the combination using low dose PS ($0.03<p<0.1$), and especially to the combination with high dose PS ($p=0.001$). In fact, combination therapies both with low and high dose PS

proved to be synergistic, improving, in protocol I, by 429% and 200%, respectively, the virtual total rate of necrosis formed as the sum of effect caused by the two agents alone.

Since the solid mammary carcinoma grew very rapidly, the injection of CPI directly after tumour implantation was chosen as the main approach (*Protocol I.*) to test. Here, 500 μg of CPI/animal and/or 10 or 20 mg/kg HpD were injected on the day of tumour implantation and irradiated 24 hours later. In the case of combined CPI-PDT therapy, where *Protocol I.* caused almost complete necrosis, the other protocols (*Protocol II*. with one day delay in PDT and *Protocol III* with tumour implantation 8 days prior to treatment) resulted in poorer responses, and were significantly ($p<0.05$) inferior to *Protocol I.* However, even in combined therapy all three protocols had a significant ($p<0.001$) necrotising effect. Also, 2-way ANOVA revealed no significant differences among the three protocols. Furthermore, pairwise comparison of parameters showed no significant difference in the efficacy of various protocols for any single or combined therapy, except for the aforementioned difference in necrosis after combined therapy. Thus, in subsequent analysis, efficiency of various treatments was also compared using data pooled for all three protocols.

Tumour volumes were determined by measuring three diameters of the tumour dissected on day 8. The combination of CPI and high dose PS which was most effective in inducing necrosis has caused a reduction of tumour volumes to ~ 2.5 cm^3, compared to the untreated control, which varied from 4.5 to 6 cm^3 ($p<0.001$). Combination therapy with the smaller dose of PS was also of beneficial effect, and significantly ($p=0.003$) reduced tumour size to an average of ~ 3.5 cm^3. The application of PDT alone caused a lesser but still significant decrease of tumour volumes to ~ 4.5 cm^3, ($p=0.03$, and $p=0.004$ for the lower and higher doses). CPI alone caused the smallest decrease of tumour volumes ($p=0.19$). Combination therapy was more effective than either monotherapy, for low dose PS $-0.003<p<0.06$, for high dose PS $0.001<p<0.003$. Combination therapies both with low and high dose PS were equally effective ($p=0.41$), and proved to be synergistic, improving by 118% and 33%, respectively, the virtual total rate of volume decrease estimated as the sum of effect caused by the two agents alone.

Serum VEGF levels are lowest in combined PDT and CPI, tumour vasculature is decreased by all treatment modes

Since overexpression of VEGF has been associated with tumour progression and poor prognosis, VEGF serum levels were measured upon termination of the experiments. In healthy animals not transplanted with tumour, serum VEGF level was 106.5±7.2 pg/ml, and this was elevated to the range of 134.8 to 170.4 pg/ml in carcinoma-bearing non-treated rats. After treatment with PDT, VEGF levels dropped only mildly ($p>0.15\%$), while CPI injection even seemed to elevate serum VEGF ($p=0.02$). The concentration of VEGF was the lowest in sera obtained from rats treated with PDT using 20 mg/kg PS combined with CPI, but the smaller dose of PS with CPI was also enough to reduce the level significantly compared to control ($p<0.001$ in both cases). Combination treatments were significantly more effective than monotherapies ($p<0.001$ for all comparisons), and showed a most striking synergism, changing the net elevation of 23% estimated from the sum of treatments into a drastic decrease of 86% in the case of 20 mg/kg PS, the corresponding proportions being 17% and 56% for 10 mg/kg PS. The higher dose of PS combined with CPI caused a significantly higher decrease of VEGF than the lower one ($p=0.001$).

The extent of vasculature was determined in samples stained with hematoxylin-eosin. The average number of vessels per field of view, as it was expected on the basis of decreased VEGF levels, was very much decreased by combination therapy ($p<0.001$ for both PS concentrations). In contrast to the other parameters assessed, in the case of vascularization also monotherapies had a significant decreasing effect ($p=0.001$). Nonetheless, combination therapies both with low and high dose PS outperformed every monotherapy ($0.02<p<0.1$ for low and $0.001<p<0.02$ for high doses), but do not differ much in their outcomes ($p=0.08$).

The Pearson's product moment correlation values were calculated for the four quantitated parameters. All correlations were significant at $p<10^{-4}$. Tumour necrosis negatively correlates with tumour volumes, VEGF levels and the number of vessels, whereas tumour volumes show positive correlation with VEGF levels and the number of vessels, and VEGF levels positively correlate with the number of vessels.

5. DISCUSSION

I. Combination therapy with trastuzumab and 17-AAG

As opposed to SKBR-3, a known trastuzumab sensitive breast tumor cell line, the newly established trastuzumab resistant line JIMT-1 exhibits low basal levels of ErbB2-ErbB2 homo- and ErbB1-ErbB2 heterodimerization and ErbB2 activation. While in SKBR-3 trastuzumab or EGF increased ErbB2 homoassociation and phosphorylation, in JIMT-1 no significant changes of ErbB2 dimerization could be measured by fluorescence resonance energy transfer, and phospho-ErbB2 levels increased only to a small extent. The data on phosphorylation obtained using *in situ* immunofluorescent labelling and flow cytometry correlate well with Western blot measurements. Trastuzumab had no effect on ErbB1-ErbB2 heterodimerization in either cell lines, coherent with recent findings using trastuzumab beads and measuring ErbB1 phosphorylation as a readout for activation, which, taken together, hint that trastuzumab probably does not favor ErbB heterodimer formation.

0.5 μM 17-AAG caused a similar time-dependent increase of ErbB2 homoassociation in both cell lines. This is coherent with the present concept that in JIMT-1 the decreased effectiveness of trastuzumab is probably owed to masking of the trastuzumab-binding extracellular epitope on ErbB2, but otherwise ErbB2 is fully functional and can be activated if this steric hindrance is overcome, for example by using high local concentrations of trastuzumab coupled to paramagnetic microspheres. The data also substantiate previous findings that show increased heterodimerization of ErbB2 upon inhibition of Hsp90 by geldanamycin or 17-AAG and support the notion that Hsp90 restrains ErbB2 from forming active dimers. Taken together, 17-AAG could be used to promote activation and internalization of ErbB2 not only in SKBR-3, but also in the trastuzumab resistant JIMT-1.

Consequently, we have tested the effect of 17-AAG, trastuzumab, and their combination on the proliferation time-curves of SKBR-3 and JIMT-1 cells, and found that 17-AAG decreased cell proliferation in both cell lines by promoting both apoptosis and necrosis in a dose-dependent manner. Of the two processes, apoptosis was the dominant. JIMT-1 cells were more sensitive to 17-AAG than SKBR-3, with IC_{50} values

of 0.011 μM as opposed to 0.069 μM in the latter. 17-AAG also decreased the number of cell surface ErbB2 molecules in both cells with equal IC_{50}. In spite of the overall downregulation of membrane ErbB2, 17-AAG caused an increase in total phospho-ErbB2 levels in a dose-dependent manner, with similar half-effective doses in both SKBR-3 and JIMT-1. There was significant positive correlation between the increase of phospho-ErbB2 levels and the antiproliferative effect, as well as between the decrease of ErbB2 levels and the antiproliferative effect in both SKBR-3 and JIMT-1 treated with 17-AAG. In spite of these positive correlations, the fact that the half-effective concentrations of 17-AAG are the same for the two cell lines in terms of regulating ErbB2 levels and phosphorylation, but are quite different in terms of antiproliferative effect indicates that we are probably looking at a dual effect. On the one hand, ErbB2 is released from inhibition by Hsp90, gets activated and downregulated. On the other hand, activated ErbB2 may also initiate survival and proliferation signals via the PI3K/Akt and MAP kinase pathways. Experimental evidence supporting this notion includes mutating the Hsp90-binding site of ErbB2, which allows higher rates of spontaneous ErbB2 activation and causes enhanced cell proliferation as compared to the wild type ErbB2 when transfected into NIH-3T3 cells. Thus, the antiproliferative effect of 17-AAG likely relies on the downregulation of many other signaling molecules in addition to ErbB2, and in the case of higher levels of ErbB2 expression, such as SKBR-3 cells vs. JIMT-1 cells, the more abundant survival and proliferation signals originating form Hsp90-free ErbB2 could cause the increase of IC_{50} values. Consequently, increased ErbB2 phosphorylation and decreased membrane ErbB2 levels are a good readout of dose-dependent 17-AAG effect, but cannot be the single cause of decreased proliferation. The phenomenon also calls for a more detailed investigation of how the different time schedules of administration would change the balance between antiproliferative and activating effect of 17-AAG, as this clearly would have ample clinical significance.

Trastuzumab alone did not affect the proliferation of JIMT-1 cells as expected based on its initial characterization, but inhibited the growth of SKBR-3 in the 2-200 μg/ml range. While trastuzumab at therapeutic dose had negligible antiproliferative effect in JIMT-1, it nevertheless decreased the cell surface ErbB2 expression and slightly increased ErbB2 phosphorylation in a dose-dependent manner. This finding may be

related to a subpopulation of JIMT-1 that has much higher trastuzumab-binding capacity than average, and ErbB2 is therefore more effectively eliminated from its surface during trastuzumab treatment.

Addition of therapeutic dose of trastuzumab did not decrease the IC_{50} of 17-AAG on the proliferation of JIMT-1, in consistence with the negligible antiproliferative effect of trastuzumab in this cell line. Rather, a small increase of IC_{50} was observed in combination treatment. Furthermore, trastuzumab appeared to decrease the effectiveness of 17-AAG in downregulating ErbB2 on these cells, perhaps because trastuzumab bound to ErbB2 can recycle to the cell membrane avoiding immediate degradation.

In the case of SKBR-3, trastuzumab alone exerted a slight decreasing effect on phospho-ErbB2 levels, which was independent of trastuzumab dose. Adding 40 μg/ml trastuzumab to 17-AAG treated cells had a most striking effect in reducing ErbB2 phosphorylation to below control levels in the case of 0.0005 and 0.005 μM 17-AAG, but also significantly decreasing phospho-ErbB2 at higher doses of 17-AAG as compared to the same doses of 17-AAG applied alone. In terms of half-efficient dose, the ability of 17-AAG to promote ErbB2 phosphorylation in the presence of trastuzumab has decreased by an order of magnitude, while IC_{50} of 17-AAG on proliferation has not been changed. In fact, the antiproliferative effect of 17-AAG was enhanced for low doses, and maintained for high doses.

II. Combination therapy with PDT and CPI

In our previous study, increased survival times upon treating solid mammary adenocarcinoma with the combination of photodynamic therapy and cystein protease inhibitor (CPI) have been revealed. In the present study, our aim was to characterize some of the pathological processes upon single and combined PDT and CPI treatment, and to use these characteristics to establish beneficial doses and determine the nature of interaction between the two drugs. As readout parameters, tumour size was measured, tumour necrosis and vascularization determined based on HE stained sections and serum VEGF levels measured using an ELISA kit.

Pairwise comparisons of treatments revealed that the single application of either PDT or CPI resulted in increased tumour necrosis as compared to the untreated controls,

and this was further increased by combining PDT with CPI in each of 3 time-schedule protocols. This means that the combination therapy using PDT and CPI may be a valuable clinical option.

In comparing the three time-schedule protocols in terms of necrosis caused, they gave similar results for all treatments except that *Protocol I.* was superior to the others in the case of 20 mg/kg PS with 500 µg CPI, causing almost complete necrosis of the tumours. This observation has possible clinical relevance, as it hints that CPI injected immediately after tumour removal combined to PDT could enhance the killing effect of PDT on leftover tumour cells better than in the case of a delayed application, and thereby could be more efficient in preventing local recurrence or metastasis formation.

Therapeutic effects on necrosis were well correlated with decreases of tumour volumes, combination of CPI and PDT being the most effective. Interestingly, at the higher, 20mg/kg dose of PS, combined therapy was more effective in further enhancing necrosis (beyond that caused by the lower dose) than in promoting the shrinkage of the tumour. However, it is well possible that observation periods beyond our 8-day schedule would reveal a volume decrease proportional to necrosis upon the higher dose combination treatment as well.

The combination of PDT and CPI therapies proved to be highly synergistic, not only in terms of evoking necrosis, but also in their efficiency in reducing tumour volumes. Photosensitizers accumulate in lysosomes and cause lysosomal disruption after irradiation. Lysosomal photodamage can then initiate mitochondrium-mediated cell death by subsequent activation of Bid, pro-caspase-9 and -3, while pro-caspase-8 is not activated. The release of cytochrome C occurs simultaneously with Bid cleavage, which indirectly results in cell death.. One possible reason for CPI synergistically enhancing the effect of PDT is the inhibition of the activity of various proteases - especially cathepsins, legumain and calpain - by CPI. Cathepsin B and L play an important role in matrix degradation and cell invasion, thus contributing to the overall aggressiveness of the tumour. Leugumain, an asparaginyl endopeptidase was demonstrated in membrane-associated vesicles concentrated at the invadopodia of tumor cells and on cell surfaces where it colocalized with integrins. Its abundance conveys enhanced migratory and invasive properties possibly mediated by increased extracellular matrix degradation,

resulting from activation of zymogens such as progelatinase. Thus, the administration of CPI inhibiting cathepsins and legumain can cause the arrest of cell invasion into the peritumour stroma. The lack of invasion and concomitant vascularization probably promotes cell death in the tumour tissue, which is further enhanced by PDT. Inihibition of calpain in myoblasts hindered migration and caused a rounded morphology, loss of membrane extensions, disorganization of stress fibers and major defect in new adhesion formation as well as cell cycle arrest. Similar effects operating in our model could also contribute to the synergism of CPI with PDT.

Synergistic action of PDT and CPI can also be a result of PDT enhancing the anti-invasive effect of CPI. According to a previous study, the amount of VEGF in tumour-bearing BALB/c mice was considerably decreased 24 and 96 hours after PDT. Others, however, had reported short-term increases of VEGF production. In our studies, VEGF level was elevated in carcinoma-bearing non-treated rats compared to healthy rats. After treatment with PDT, VEGF levels dropped only mildly, while CPI injection alone even seemed to slightly elevate serum VEGF, probably as a feedback response of the tumour mass to decreased invasion and neovascularization. However, when CPI was complemented with PDT, the concentration of serum VEGF dropped to levels even below that of healthy animals. These combination treatments were significantly more effective than PDT alone and showed a very strong synergism. Also, the higher dose of PS combined with CPI caused a significantly greater decrease of VEGF than the lower one. The reduction in the level of VEGF is expected to result in decreased tumour neoangiogenesis, an effect synergistic with CPI which hampers local invasion, and the two globally leading to the inhibition of tumour growth and progression. In support of this reasoning, our results clearly show the positive correlation between decreased VEGF levels and decreased vascularization, between VEGF levels or vascularization and tumour growth, and, conversely, the negative correlation between VEGF levels or vascularization and tumour necrosis.

As a further possible mechanism of synergy between CPI and PDT, one needs to consider the lysosomal localization of cathepsin B, a main target of CPI inhibition (Joyce *et al.* 2004). Since photosensitizers are also accumulated in lysosomes, the irradiation damages the lysosomes resulting in reduced levels and activity of cathepsin B. As in

tumours the increased activity of cathepsin B is associated with neoangiogenesis and the invasive fronts of carcinomas, the effective inhibition of its activity by the combined addition of CPI and PDT can be a major factor in preventing tumour growth.

Tumour vascularization was a highly sensitive parameter which was decreased considerably by all treatments. While combination therapies outperformed every monotherapy, the differences were not extremely large. Since the decrease of vascularization exceeded that of VEGF levels in PDT monotherapy, it is likely that the damaging effect of photodynamic therapy was not only manifested in the cancer cells, but also in the endothelial and mesenchymal cells forming new blood vessels. It is also possible that PDT decreases the production of factors driving vessel growth other than VEGF. Similarly, the disparate effect of CPI on VEGF levels and vascularization can be explained by the negative feedback of cathepsin inhibition boosting VEGF production, while at the same time CPI causing the necrosis of tumour vasculature parallel to the necrosis of tumour cells themselves. The positive correlation of tumour volumes with serum VEGF levels substantiate the notion that at least part of the serum VEGF could originate from the tumour tissue. At the same time, the partly divergent effects of treatments on VEGF levels and vascularization explain why the correlation coefficient between these two parameters is relatively low.

It is interesting to note that the decrease by CPI of neovascularization alone could reduce the efficacy of PDT, and therefore other effects of CPI directly exerted on the tumour cells might also play a role in the synergy of CPI and PDT. Such effect could be the antagonism of CPI with TGFβ, which can decrease the proliferation of mammary carcinoma cells.

Taken together, our results indicate that cell damage caused by PDT as well as inhibition of tumour invasion by CPI could hinder tumour angiogenesis not only directly, but also via diminishing serum VEGF concentrations. As PDT and CPI acted very efficiently in synergy upon tumour volume, necrosis and VEGF levels at concentrations where they alone had no or little effect, we suggest that their combination could possibly gain clinical application, especially since during the treatment of animals no side effects were observed even at the most effective doses. While PDT has already been established in clinical practice, applicability of CPI is yet to be substantiated. However, some recent

advances are encouraging. Cegnar et al. have optimized uptake of cystatin into target cells using cystatin-loaded nanoparticles that effectively inhibited cathepsin B activity. Such approach using paramagnetic particles could even provide for site-directed delivery of CPI using magnetic field gradients. It may also be possible in the future to enhance CPI expression using transfection approaches. Overexpression of the CPI stefin A in human esophageal squamous cell carcinoma cells was found to inhibit tumour cell growth, angiogenesis, invasion, and metastasis. Moreover, adenovirus-mediated cystatin C overexpression in host mice efficiently reduced lung metastasis of an experimental human fibrosarcoma. Nonetheless, cautions also need to be taken, since cystatins were lately found to have not only antiproliferative but in a few cases also proliferative effects, depending on the system investigated.

6. SUMMARY

The main aim of our work was to enhance the effect of therapies targeting breast cancer in the clinics with another small molecule agent.

- Our *in vitro* experiments show that 17-AAG enhances ErbB2 homodimerization and phosphorylation on trastuzumab sensitive and resistant cells. In the long run, the drug causes down-regulation of the receptor and inhibition of cell proliferation. This phenomenon can be explained by the apoptosis and in the case of higher doses the necrosis caused by 17-AAG. Thus, 17-AAG can be used to treat also trastuzumab-resistant ErbB2-positive tumours.

- In trastuzumab sensitive tumours, especially with high levels of ErbB2 expression, the proliferation-inhibiting effect of 17-AAG is lower because of activation of the numerous ErbB2 receptors. In these cases, additional trastuzumab in the treatment regime can be beneficial in terms of inhibiting 17-AAG-evoked ErbB2 phosphorylation and also in enhancing the growth inhibitory effects of low doses of 17-AAG.

- In our *in vivo* studies the combined application of photodynamic therapy (PDT) and cysteine proteinase inhibitor (CPI) had a synergistic effect on the solid mammary adenocarcinoma transplanted into Wistar rats. CPI unequivocally enhanced the effectiveness of PDT, which can be explained by the increased tumour necrosis, decreased VEGF-level in sera and the intense inhibition of vascularization.

- The combination of PDT and CPI most effectively inhibits the progression of solid mammary carcinoma transplanted into Wistar rats with the application of 20 mg/kg HpD, 500 μg/animal cystatin and photon flux of 90 J/cm^2. The most promising results were achieved when the therapy was started simultaneously with tumor inoculation, which promises effectiveness of combined treatment of the scattered and residual cancer cells.

7. SIGNIFICANCE

The *in vitro* results carry dual practical significance. For one, the effectiveness of 17-AAG monoteraphy in the case of trastuzumab-resistant, but ErbB2-positive breast cancers is shown. On the other hand, results indicate that it might be worthwhile combining 17-AAG therapy of ErbB2-overexpressing breast cancer with trastuzumab in order to reduce the ErbB2 phosphorylation caused by 17-AAG as an adverse effect. This result appears to be especially important in the context of clinical applications since from the only clinical trial currently investigating the efficacy of 17-AAG in breast tumors those on concurrent trastuzumab therapy are excluded. In addition, ErbB2 being a potential target of 17-AAG, efforts have been made to synthesize 17-AAG – trastuzumab conjugates for specific delivery of the chaperone inhibitor to ErbB2 overexpressing tumors. Application of such conjugates also necessitates the proper knowledge of how 17-AAG and trastuzumab interact in tumors with various expression levels and diverse degrees of trastuzumab resistance.

Our *in vivo* results indicate that cell damage caused by PDT as well as inhibition of tumour invasion by CPI could hinder tumour angiogenesis not only directly, but also via diminishing serum VEGF concentrations. As PDT and CPI acted very efficiently in synergy upon necrosis, tumour volume and VEGF levels at concentrations where they alone had no or little effect, we suggest that their combination could possibly gain clinical application, especially since during the treatment of animals no side effects were observed.

8. PUBLICATIONS

In extenso publications included in the thesis:

Zsebik B., Citri A., Isola J., Yarden Y., Szöllősi J., Vereb Gy.: **Hsp90 inhibitor 17-AAG reduces ErbB2 levels and inhibits proliferation of the trastuzumab resistant breast tumor cell line JIMT-1** Immunulogy Letters (2006) 104(1-2):146-55
IF: 2,301 (2005)

Zsebik B., Symonowicz K, Saleh Y., Ziolkowski P., Bronowicz A., Vereb Gy.: **Photodynamic therapy combined with a cysteine protease inhibitor synergistically decrease VEGF production and promote tumour necrosis in a rat mammary carcinoma** Cell Proliferation (2006) in press
IF: 4,462 (2005)

Posters and lectures:

Zsebik Barbara, Fazekas Zsolt, Petrás Miklós, Szöllősi János, Vereb György (pozter)
ErbB2 receptorok homoasszociációja és tirozin foszforilációja humán emlőtumor sejtekben
XXXIII. Membrán-Trasnszport Konferencia, Sümeg, 2003. május 20-23.

Zsebik Barbara, Fazekas Zsolt, Petrás Miklós, Szöllősi János, Vereb György (pozter)
Az ErbB2 receptorok molekuláris kölcsönhatásai és foszforilációja Herceptin szenzitív és rezisztens emlőtumor sejtekben eltérőek
A Magyar Biofizikai Társaság XXI. Kongresszusa, Szeged, 2003. augusztus 24-27.

Barbara **Zsebik**, Miklós Petrás, János Szöllősi, György Vereb (poster)
Possible use of Hsp inhibitor 17-AAG for treating Herceptin resistant breast tumors
International School of Biophysics, Timisu de Sus-Predeal, Romania, 7-12 October, 2003

Zsebik Barbara, Horváth Gábor, Petrás Miklós, Szöllősi János és Vereb György (pozter)
Herceptin rezisztens emlőtumorok adjuváns terápiájának lehetőségei Herceptin és 17-AAG kombinációjával
IV. Magyar Sejtanalitikai Konferencia, Budapest, 2004. május 6-8.

Barbara **Zsebik**, Miklós Petrás, Jorma Isola, János Szöllősi, György Vereb (outstanding poster award)
ErbB2 homodimerization and activation in Herceptin resistant and sensitive cell lines
International Biophysics Congress, Montpellier, France, 22-27 May, 2004

Zsebik Barbara, Horváth Gábor, Petrás Miklós, Szöllősi János és Vereb György (pozter)
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XXXIV. Membrán-Transzport Konferencia, Sümeg, 2004. június 1-4.

Barbara **Zsebik**, Krzysztof Symonowicz, Yousif Saleh, Piotr Ziolkowski, Andrzej Bronowicz, György Vereb (pozter)
Photodynamic therapy combined with a cystein protease inhibitor synergistically decreases VEGF production and promotes tumour necrosis in a rat mammary carcinoma
14th Annual Conference of the German Society for Cytometry, Leipzig, October 19-22, 2005

Zsebik Barbara (lecture)
ErbB2 receptorok homoasszociációja és tirozin foszforilációja humán emlőtumor sejtekben
DE OEC Ph.D. és TDK Tudományos Diáktalálkozója, Debrecen, 2003. február 24-28.

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DE OEC Ph.D. és TDK Tudományos Diáktalálkozója, Debrecen, 2004. április 5-9.