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Activation of alternative HER receptors mediates resistance to EGFR tyrosine kinase inhibitors in breast cancer cells

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The response rate to EGFR inhibitors may be poor and unpredictable in cancer patients with EGFR expression itself being an inadequate response indicator. There is limited understanding of the mechanisms underlying this resistance. Here we have provided a molecular mechanism of alternative HER receptor activation (ErbB receptor family members) in mediating resistance to EGFR TKIs in breast cancer cells. Using both Förster Resonance Energy Transfer (FRET) which monitors in situ HER receptor phosphorylation as well as classical biochemical analysis, we have shown that the specific tyrosine kinase inhibitors (TKIs) of EGFR (HER1), AG1478 and Iressa (Gefitinib) decreased EGFR and HER3 phosphorylation through the inhibition of EGFR/HER3 dimerization. Consequent to this, we demonstrate that cleavage of HER4 and dimerization of HER4/HER2 occur together with reactivation of HER3 via HER2/HER3, leading to persistent HER2 phosphorylation in the now resistant, surviving cells. These drug treatment-induced processes were found to be mediated by the release of ligands including heregulin and betacellulin that activate HER3 and HER4 via HER2. Whereas an anti-betacellulin antibody in combination with Iressa increased the anti-proliferative effect in resistant cells, ligands such as heregulin and betacellulin rendered sensitive SKBR3 cells resistant to Iressa. These results demonstrate the role of drug-induced autocrine events leading to the activation of alternative HER receptors in mediating resistance to EGFR tyrosine kinase inhibitors (TKIs) in breast cancer cells, and hence specify treatment opportunities to overcome resistance in patients.

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Combined targeting of DNA repair and AKT survival pathways enhance temozolomide therapeutic activity in melanoma

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Background: Melanoma, the most fatal skin cancer, has increased in incidence by 15-fold in the past 40 years, the fastest rate increase of any human malignancy. This disease metastasizes rapidly and is highly resistant to chemotherapy and other treatments. At this time, a new oral alkylating agent, Temozolomide (TMZ) is an effective drug with modest therapeutic activity for the treatment of melanoma, however, intrinsic and acquire resistance is the major cause of the reduction in effectiveness and in improving overall survival by this single agent. Although many combination treatments using immunotherapy, chemotherapy, and bio-chemotherapy have been tested in clinical studies, nearly all trials of combination therapies have failed. Thus, it is imperative to develop more targeted approaches to augment substantial benefit of treatment.
Methods: In this study, using melanoma cell lines we examined therapeutic activity of targeting based combinations: (i) combining methoxyamine (MX), an inhibitor of base excision repair (BER), with TMZ to block the repair of DNA adducts that are account for about 80% DNA lesions produced by TMZ, (ii) combining API-2, a small-molecule Akt inhibitor, with TMZ to inhibit TMZ-induced activation of AKT pathway, an important molecular event implicated in tumor cell survival and chemo resistance. We hypothesized that the combination of TMZ with MX and API-2 would synergistically enhance anti-tumor effect of TMZ through targeting two major resistant factors: DNA repair and AKT mediated anti-apoptosis.
Results: MX enhanced TMZ cytotoxicity in A375, WM9 and WM164 melanoma cells in vitro and in xenografts setting. The potentiation of TMZ by MX was through its activity to specifically bind to an abasic site, which turns the repairable DNA damage into a lethal lesion, leading to DNA strand breaks and apoptosis. However, we found that AKT was activated in response to either TMZ alone or in combination with MX, showing the induction of phosphorylated AKT. Thus, the combining with API-2 (1 microM) efficiently inhibited AKT activation and significantly increased apoptosis, 4 to 5-fold higher than TMZ alone. The increased apoptotic death was mediated by Bax-activation. Similarly, siRNA-mediated reduction of AKT expression sensitized melanoma cells to TMZ-cytotoxicity.
Conclusion: These results strongly support the hypothesis that clinical benefit could be obtained by combining TMZ with blocker of DNA repair and inhibitor of the AKT pathway.

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An inducible expression system to study the EGFR-T790M gefitinib-resistance mutation in a human lung cancer cell line

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Background: Lung cancer patients whose tumours harbour somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) respond to the reversible EGFR inhibitors gefitinib and erlotinib. One such mutation is the deletion of aminoacids 746–750, hereafter referred to as EGFR-Del. Resistance to gefitinib or erlotinib invariably develops and is often mediated by the appearance of the T790M mutation in EGFR. The T790M mutant is believed to be sensitive to irreversible EGFR inhibitors such as CL-387,785. To study the impact of EGFR-T790M expression in a relevant cell line, doxycycline (DOX)-inducible expression of EGFR-Del/T790M was established in the human lung cancer cell line HCC827, which expresses EGFR-Del and is very sensitive to gefitinib.
Materials and Methods: HCC827 cells capable of DOX-inducible expression of FLAG-tagged EGFR-Del (control) or EGFR-Del/T790M were produced by sequential transfection and selection with two different plasmids (Nat. Protoc. 1:803, 2006). Polyclonal pools of selected cells were cloned by plating at low density on 10-cm dishes. Clones expressing FLAG-tagged EGFR-Del ("D") or EGFR-Del/T790M ("T") in the presence of DOX were analysed for drug sensitivity using the MTT assay. Also, the effect of EGFR inhibitors on the activity of EGFR and downstream signalling molecules (Akt and Erk1/2) was analysed by western blotting.
Results: growth of T clones in the presence of DOX decreased the sensitivity of HCC827 cells to gefitinib more than 25 fold (figure 1). This was not observed in control D clones. Also, gefitinib was incapable of inhibiting the activation of EGFR, Akt and Erk1/2 in T clones grown in the presence of DOX. The sensitivity to CL-387,783 was also reduced when T clones were grown in the presence of DOX. However, D and T clones remained equally sensitive to the PI3 kinase (PI3K) inhibitor PI-103, irrespective of DOX treatment.
Conclusions: we have established and validated a robust system for inducible EGFR-T790M-mediated resistance to gefitinib in a relevant cell line. This is the first inducible expression system described for the T790M mutation in an established cancer cell line. Expression of EGFR-T790M also decreased the sensitivity of cells to an irreversible EGFR inhibitor. Our results suggest that targeting downstream signalling molecules (such as PI3K) might be a better strategy for overcoming T790M-mediated resistance in the clinic when compared to irreversible EGFR inhibition.

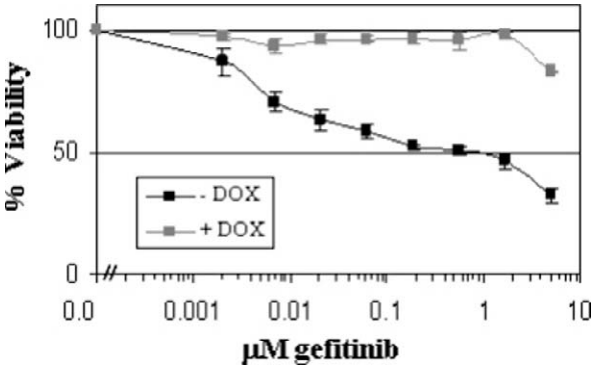

Figure 1. MTT assay – T clone.

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MDR-transporters, namely Pgp, MRP1 and vault protein LRP, as poor predictive markers of tamoxifen efficiency in estrogen receptor positive breast cancer tumours

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Background: Estrogen receptors (ER) are determinants of tamoxifen (Tam) sensitivity of breast cancer but the treatment are not effective in all the patients with ER-positive tumors. We supposed that among the

reasons of Tam resistance discussed in literature expression of transporters associated with multidrug resistance mechanism (MDR-transporters) can be important one. Taking into account data about Tam binding to Pgp we suggested that in ER-positive breast tumors MDR-transporters localized on plasma membrane and sub-cellular organelles could compete with nuclear and nongenomic estrogen receptors for binding to Tam. As a result, an "active" intracellular Tam concentration binding to ER and Tam efficiency have to decrease in ER-positive breast cancer with MDR-phenotype. To confirm the existence of such Tam resistance mechanism we investigated the antiestrogen influence on monoclonal antibody (mAb) binding to Pgp, MRP1 and LRP.
Materials and Methods: JurKat, HeLa and A549 human cells expressed Pgp, MDR-transporters MRP1 and LRP, respectively and cells obtained from surgical specimens of breast cancer tumors were studied. Fluorescence of specific mAb bound to Pgp, MRP1 and LRP (UIC-2, QCRL-3 and LMR5 respectively) was estimated by flowcytometry. Mean fluorescence of Pgp-, MRP1- and LRP-labelled cells was calculated over fluorescence area of isotypic controls.
Results: It was shown that Tam decreased specific mAb binding to the all studied MDR-transporters – Pgp, MRP1 and LRP. The effect was revealed in both in vitro and ex vivo experiments. Under 50 μM of Tam action the parameters of mAb binding to MDR-transporters (mean fluorescence, peak mean fluorescence as well as number of the mAb-labelled cells) decreased up to more than 3 times. Binding of isotypic antibodies to the tumor cells was not change under Tam action. Conclusion. Decrease in specific mAb interaction with Pgp, MRP1 and LRP under Tam action revealed in different models, including tumor cells of cancer patients, let's conclude a competition between MDR-transporters and ER for binding to Tam and as the result – decrease an "active" intracellular concentration of Tam binding to ER and thereby decrease Tam efficiency in ER-positive breast cancer tumors. Thus, expression of MDR-transporters, namely Pgp, MRP1 and LRP, has to be considered as a poor predictive marker of Tam efficiency in ER-positive breast cancer tumors.
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Casein kinase II modulates topoisomerase II alpha nuclear export and drug sensitivity of multiple myeloma cells

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Background: In previous studies we demonstrated that topoisomerase II alpha (T2A) is exported from the nucleus of human multiple myeloma cells by a CRM1-dependent mechanism when grown at densities similar to those in myeloma patient bone marrow. We have identified the nuclear export signals for T2A at amino acids 1017–1028 and 1054–1066 using mutated FLAG-T2A protein and immunofluorescence microscopy. Nuclear export of T2A conveys drug resistance to T2A inhibitors because the enzyme is not in contact with DNA and thus unable to induce DNA damage. We have shown that blocking nuclear export with the CRM1 inhibitor ratjadoneC or by using CRM1 siRNA sensitizes myeloma cells to T2A inhibitors by increasing nuclear T2A.
Materials and Methods: It is unknown what signals CRM1 mediated nuclear export of T2A. Protein phosphorylation has been shown to modulate export of nuclear proteins; therefore, we investigated the phosphorylation status of T2A in both the nucleus and cytoplasm using highly sensitive proteomic technologies. T2A was isolated from the cytoplasm and nucleus of human myeloma cells by immunoprecipitation (IP), digested with trypsin, concentrated on TiO2 columns, and subjected to LTQ-Orbitrap MS. Based on the Orbitrap data, we produced T2A mutants to block nuclear export. In addition, a casein kinase 2 (CK2) inhibitor or CK2 siRNA was used to block T2A export and sensitize cells to T2A inhibitors doxorubicin and VP16.
Results: Comparing the phosphorylation sites of nuclear and cytoplasmic T2A, we found that cytoplasmic T2A, but not nuclear T2A, was highly phosphorylated at serines 1106 and 1524 (published CK2 motifs). Using site-directed mutagenesis, we converted serine 1106 and 1524 to alanine and found by immunofluorescence microscopy that mutated recombinant FLAG-T2A export was reduced compared to wild-type FLAG-T2A. Since CK2 phosphorylation sites were implicated, we used the CK2 inhibitor tetrabromobenzotriazole (TBB) and found that T2A nuclear export was blocked in high-density cells. Co-IP data indicated that blocking CK2 phosphorylation inhibited T2A binding to CRM1. TBB data were duplicated using a CK2 siRNA to knockdown CK2 expression. In addition, we found that blocking nuclear export of T2A with TBB or CK2 siRNA sensitized drug-resistant myeloma cells to the T2A inhibitors doxorubicin and VP16.
Conclusions: CK2 phosphorylation may regulate T2A nuclear export. CRM1 and CK2 inhibition may have clinical implications in the treatment of multiple myeloma.

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Examination of the mechanisms associated with bortezomib-resistance in human multiple myeloma cell lines

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Targeting the proteasome as an anti-cancer therapy was first realized with bortezomib (VELCADE®), now one of the standards used in relapsed/refractory multiple myeloma and mantle cell lymphoma. Despite this advance, greater than half of patients have less than a partial response, while others initially respond and then progress, indicating the importance of innate or acquired resistance to bortezomib. The mechanisms by which plasma cells survive bortezomib therapy are not fully characterized, in part due to lack of appropriate model systems. We have created several multiple myeloma polyclonal cell lines with bortezomib-resistance (BR). Our studies into the changes associated with BR show that basal levels of proteasome activity are repressed in BR cells compared to wild-type cells. Assessment of transcriptional changes in BR myeloma cells revealed identification of several perturbations in cellular signaling (e.g. downregulation of TNF-alpha signaling), and increases in known stress response elements (e.g. heat shock proteins) and anti-apoptotic signaling pathways (e.g. PI3K), which validate reports from other groups on BR patient sample data and in other chemotherapeutic resistant cell lines with cross resistance to bortezomib. Activation of nuclear factor kappa B (NF-kappa B), which should be suppressed in bortezomib treated cells due to accumulation of inhibitor to NF-kappa B alpha (I-kappa B alpha) and the NF-kappa B p50 subunit processing, was found to be increased in BR cells compared to their wild type counterparts. These data indicate that BR cells are evading proteasome-mediated inhibition of NF-kappa B. A brief study into traditional chemotherapies that could be used to overcome BR revealed cross-resistance to a number of other drugs, including doxorubicin and melphalan; however, BR cells remained sensitive to arsenic trixoxide and camptothecin. Expression of multi-drug resistance pumps may explain some of the cross resistance, as treatment of BR cells with verapamil moderately abrogated BR cell viability to doxorubicin, indicating that other resistance mechanisms are active in these cells. Our future objectives are to characterize single cell clones to determine the multiple resistance mechanisms. Discovery of these resistance mechanisms will facilitate the development of strategies to circumvent them, and help to identify potential novel therapies for the treatment of patients with resistance to bortezomib.

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AF1q enhancement of doxorubicin induced apoptosis in human squamous carcinoma A431 cells

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Background: Drug resistance is one of the major obstacles in cancer therapy. To identify genes that regulate the drug resistance in human cancer cells, the gene expression profiles between human squamous carcinoma A431 cells and its doxorubicin-resistant subline, A10A, were compared by RT-PCR differential display. The gene *ALL1-fused gene chromosome 1q (AF1q)* was one of the clones found to be down-regulated in A10A cells. AF1q is an oncogenic factor involved in leukemia development, thyroid tumourigenesis and breast cancer metastasis. The involvement of AF1q in drug resistance has however not yet been demonstrated. The objective of this study was to elucidate the importance of apoptosis in AF1q regulation of drug resistance in cells.
Materials and Methods: The effect of AF1q on doxorubicin sensitivity was determined by MTT assay, while its effect on doxorubicin induced apoptosis was by DNA fragementation and Annexin V binding assays. The effect of AF1q on the apoptotic pathway was assessed by Western blotting, quantitative PCR and flow cytometry.
Results: Transient transfection with full length AF1q was found to increase the sensitivity to doxorubicin and also apoptosis induction in A431 cells. Similar results were shown in A431 AF1q stable transfected cells. In addition to the increase in doxorubicin induced apoptosis, a more than two fold increase in both mRNA and protein expressions of pro-apoptotic BAD, a member in the intrinsic apoptotic pathway, was also detected in the AF1q stable transfectants. Moreover, increases in drug induced mitochondrial membrane depolarization as assessed by flow cytometric analysis of cells staining with JC-1, mitochondrial cytochrome *c* release, activation of caspase-9 and caspase-3 as measured by Western blot analysis were also observed in the AF1q stable transfectants.
Conclusions: The study provides the first evidence that AF1q regulates the drug resistance and also the drug induced apoptosis in cells through the intrinsic apoptotic pathway.