of the EGFR tyrosine kinase, and thereby inhibit its autophosphorylation. Patients with *EGFR* mutant tumours benefit most from this treatment and experience longer progression-free survival. Nevertheless, all patients eventually acquire resistance to this class of drugs. To date, two major mechanisms of resistance to EGFR small molecule inhibitors have been described. In about 50% of the patients, a second site mutation in the kinase domain of *EGFR* can be found, of which the amino acid change T790M is the most common. This change impairs the binding of the inhibitor to the ATP-binding pocket, and also enhances the affinity of the EGFR to ATP. In about 20% of patients, increased expression of the MET tyrosine kinase has been found, resulting in EGFR-independent activation of PI3kinase/Akt. Notably, some patients show both mechanisms at the same time. However, the mechanisms of resistance in the remaining set of patients are still unknown.
In this study, we aimed to identify novel mechanisms of acquired resistance to erlotinib using two approaches. Firstly, we established erlotinib-resistant NSCLC cells *in vitro*. Direct EGFR sequencing of several clones revealed that resistant cells harbour a mutation corresponding to T790M in a subset of *EGFR* alleles. However, these resistant cells still show increased resistance to an irreversible EGFR inhibitor to which T790M-expressing cells are expected to be sensitive, suggesting that additional factors contribute to resistance. We are currently performing microarray-based gene expression profiling to characterise these resistant cells in more detail. Secondly, we performed a genome-wide siRNA screen to identify genes that confer resistance to erlotinib in NSCLC cells. Validation of the 261 hits in an independent deconvoluted screen revealed 61 siRNAs that significantly altered the cell viability upon treatment with erlotinib. We are currently analysing a subset of these genes in greater detail, and evaluating their clinical relevance in patient samples. We expect these approaches to identify markers to select patients with primary resistance, or reveal potential targets to prevent acquired resistance.

221 Rapamycin effect upon glioblastoma-derived cancer stem cells

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We have characterized the effect of rapamycin upon patient-derived cancer stem cells (CSC).
CSCs-enriched cultures were derived from surgical resections. They were able to self-renewal and long-term proliferate as neurospheres expressing the neural stem cell markers CD133, nestin, Sox2, vimentin and nucleostemin. The CSCs were multipotential, as demonstrates by their ability to give rise to a differentiated progeny (neurons, astrocytes and oligodendrocytes) with limited proliferative potential. Once the CSCs were established and characterized, in vitro experiments were performed to study the effect of rapamycin in cell proliferation, cell death, CD133 expression, the ability of colony formation and mTOR (mammalian target of rapamycin) pathway inhibition.
Trypan blue exclusion and Annexin V/7-AAD-binding assay experiments showed the ability of rapamycin to reduce, in more than forty percent cell growth with a weak effect in cell viability. Also, flow cytometry analysis indicated partial depletion of CD133+ in cells treated with rapamycin. By western blot analysis of the proteins implicated in the mTOR pathway we detected depletions in the phosphorylated S6 ribosomal protein and STAT3 while increments in phosphorylated AKT protein. These results confirm that the mTOR pathway is affected by the drug. Soft-agar assays were performed in order to estimate the tumourigenic potential. Colony forming ability after rapamycin treatment diminished more than 5-fold when 1000 ng/ml concentration of the drug was used.
We had obtained two cell lines from human glioblastomas that fulfil the criteria to be considered CSCs. Our results suggest that rapamycin causes important modifications in the normal behaviour of the CSC. The mTOR pathway plays an important role in differentiation, migration, proliferation and cell survival. The results we present here could be explained by the inhibition of mTOR pathway.

222 Ofatumumab binds to a membrane-proximal epitope which comprises amino acids in the small and large loops of CD20

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Introduction: CD20 represents a well-established target for immunotherapy of B-cell malignancies. Ofatumumab (OFA; Arzerra ®) is a novel human CD20 monoclonal antibody currently approved for fludarabine- and alemtuzumab-refractory chronic lymphocytic leukemia (CLL). OFA is able to induce cell killing via Fc-mediated effector functions, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Based on previous studies we postulate that OFA's exceptional efficacy in activating these effector functions is related to its recognition of a unique membrane-proximal epitope, comprising amino acids in both the small (7 amino acid) and large (44 amino acid) loop of the CD20 molecule.
Materials and Methods: To map OFA's cognate epitope in detail, we generated mutants via site-directed mutagenesis and performed a structure function analysis. To ensure optimal surface expression, we employed a strategy in which we generated hybrid CD20 molecules using small loop sequences derived from the MS4A superfamily of which CD20 is a member, and which includes proteins sharing similar structure but limited sequence homology.
Results: Complete substitution of the CD20 small loop almost entirely abrogated OFA binding, whereas binding of rituximab (RTX) was not affected. A similar loss of OFA binding was observed when amino acids were replaced at three positions in the small loop (A74T, I76A, and Y77S), in which Y77, in particular, seemed to be crucial. Binding of OFA was also abrogated by mutations in the large loop (T159K, N163D and N166D). Notably, mutations affecting the cognate CD20 epitope for RTX (A170S, P172S) in the large loop did not affect OFA binding, confirming that OFA and RTX recognize completely distinct epitopes.
Conclusion: Our data show that binding of OFA, but not RTX, is disrupted by amino acid substitutions in the CD20 small loop. Small loop amino acids A74-I76-Y77 are most important and complement the previously identified large loop amino acids T159-N163-N166 in the OFA epitope. The specific involvement of the CD20 small loop, which comprises only 7 extracellular amino acids, presumably positions OFA very close to the membrane, which allows it to harness killing via Fc-mediated effector functions most efficiently.

223 Silencing of survivin enhances the sensitivity of cancer cells to chemotherapeutic agents

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Background: Current understanding of cancer genetics and biology has not been translated into significant improvement of the available treatments. Increased survival of cancer patients has resulted mainly from early detection and prevention. Since clinical application of conventional therapeutic anticancer approaches is usually limited by drug resistance and toxic side effects, the combination of chemotherapeutic agents with gene therapy appears as a promising strategy to overcome these issues. Several studies have shown that survivin is selectively expressed on the most common human cancers, suggesting that its expression may be the cause for enhanced cell viability and resistance to chemotherapeutic agents or radiation therapy. In this context, survivin can constitute a promising target for the development of successful anticancer strategies aiming at eliminating tumour cells, while sparing normal tissues.
Materials and Methods: Cancer cells were transfected with anti-survivin siRNA-based liposome formulations 24 h before treatment with the different chemotherapeutic agents. Cytotoxicity was assessed by Alamar blue assay 24 and 48 h after incubation with the chemotherapeutic agents. Survivin knockdown in the different cancer cell lines was evaluated by quantitative real-time polymerase chain reaction (RT-PCR) and Western blot analysis 48 and 72 h after transfection.
Results: Our data on the total levels of survivin, determined in a number of human cell lines, including HeLa, MCF-7, A549 and U373 cells, have shown that survivin is expressed in all these cell lines, while not at equivalent levels. A notorious protein knockdown could be observed after treatment of the cells with anti-survivin siRNAs while control siRNAs did not result in any decrease in survivin protein levels. A significant reduction of cell viability was observed for cells treated with anti-survivin siRNAs, but not for cells treated with control siRNAs. Most importantly, an enhancement of the therapeutic effect was observed when the survivin-silencing approach was combined with application of chemotherapeutic agents.
Conclusion: Survivin knockdown sensitizes cancer cells to the effect of conventional chemotherapeutic agents, thus allowing the use of much lower doses of the drugs. Therefore, the combination of a survivin-directed gene therapy strategy with a chemotherapeutic agent constitutes a valuable approach for cancer treatment.

224 A new lytic peptide for the treatment of cancer

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Background: A current approach for cancer treatment is the development and use of cationic peptides. Such peptides, which are known to have significantly higher toxicity to bacteria than to normal mammalian cells, have also been shown to exhibit a broad spectrum of cytotoxic activity against cancer cells. Most membrane active peptides bind rapidly to the plasma membrane of cancer cells and disrupt it, leading to cell death. The exact mechanisms of