In contrast, all neuroblastoma cell lines analyzed proved to be infectable with H-1PV, with complete viral replication yielding virus titer increase of up to 10.000-fold within 48 to 96 h after infection. Lytic infection was observed at TCIDs50 between 0.001 and 10 pfu/cell. Cell killing was independent of the status of MYCN amplification in the respective cell lines.
As with neuroblastoma cells, all medulloblastoma cell lines were efficiently infectable with H-1PV, displaying a dose-dependent cytotoxicity with TCIDs50 between 0.001 and 5 pfu/cell. Similar to the results with neuroblastoma cells, efficient viral replication could be shown in medulloblastoma cells by an increase of virus titers in a range of two log-steps within 72 to 96 h after infection.
In both, neuroblastoma and medulloblastoma cells, H-1PV induced a G2-arrest and subsequent apoptosis.
Conclusions: The data infer that application of oncolytic H1-PV may be a promising treatment option for embryonic tumours of neuroectodermal origin. This, however, has to be substantiated by a pre-clinical evaluation of the therapeutic efficacy for these tumours *in vivo*.

217 Testing of a poly(ADP-ribose) polymerase (PARP) inhibitor on human BRCA2 heterozygous cell lines

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The PARP inhibitor, AZD2281, olaparib®, is a promising targeted anti-cancer agent for patients with specific DNA-repair defects such as found in BRCA1 and BRCA2 mutation carriers. Early clinical trials suggest that this targeted drug is effective on tumour cells and well tolerated by normal tissues in mutation carriers.
Materials and Methods: Response to olaparib, was tested on three heterozygous mammary epithelial cell lines derived from carriers of a 999del5 BRCA2 founder mutation (A176, A240, A256) and one non-BRCA cell line (D492) transformed in the same way, as well as the commercially available BRCA2 deficient pancreatic cell line Capan-1 and the mammary cell line MCF7. Heterozygous cell lines were examined for BRCA2 allel loss using TaqMan qRT-PCR and copy number changes on CGH arrays (385K aCGH; NimbleGen Systems). CellTiter 96® Aqueous One Solution Cell Proliferation assay (MTS assay) was used to estimate survival and determine the maximum tolerated dose of olaparib and IC_{50} values for all cell lines. Cell death was also assessed with annexin-V and probidium iodide staining. Immunostaining for Rad51 and γH2AX was carried out to evaluate DNA double stand breaks and DNA repair.
Results from olaparib testing using the MTS assay show that the heterozygous cell-lines A176, A240 and A256 have similar IC_{50} values as both the non-BRCA cell lines, D492 and MCF-7. Whereas, Capan-1 shows increased sensitivity to the inhibitor. Annexin-V and PI staining show that the Capan-1 cell line goes through apoptosis at low dosages. Only at exposure to high dosages did the heterozygous cell lines show PI staining. Immunostaining with γH2A.X and RAD51 antibodies indicates that the Capan-1 cell-line has loss of γH2AX/RAD51 colocalization after treatment with olaparib, whereas the heterozygous BRCA2 cell-lines show colocalization after treatment.
In conclusion: Human mammary cell lines heterozygous for a BRCA2 mutation that have retained the second BRCA2 allel are not more sensitive to PARP inhibitor olaparib treatment than non-BRCA2 mammary cell line controls.

218 L-Asparaginase-loaded red blood cells: a promising therapy in solid tumours

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Tumour cells deficient in asparagine synthetase (ASNS) are unable to synthesize enough L-asparagine (L-Asn) to meet metabolic demands and, therefore, depend upon circulating L-Asn in the plasma for survival. L-asparaginase (L-aspa), which depletes L-Asn via deamination, has been used in the treatment of acute lymphoblastic leukemia (ALL) for over 40 years, since the majority of such tumours are deficient in ASNS. Patients able to tolerate more than 25 weeks of L-aspa experience significantly increased survival, but side effects often preclude that duration of use. However, in a phase II clinical trial, entrapment of L-aspa inside red blood cells (GRASPA®) has been found to strongly reduce side effects and also to improve L-aspa pharmacokinetics. Recent evidence suggests that subsets of solid tumours (head and neck, ovarian and pancreatic) are deficient in ASNS, providing rationale for testing L-aspa against such tumours.
To demonstrate the utility of L-aspa in solid tumours, we investigated:
- Expression of ASNS on human cell lines and tumour samples by immunohistochemistry and western blot
- IC50 and GI50 of L-aspa and cell viability in L-Asn deprived medium
- *In vivo* studies in subcutaneous and intraperitoneal metastasis models.

Four pancreatic and 4 ovarian cell lines were studied, as well as MOLT-4, a lymphoblastic leukemia cell line used as a negative ASNS expressing control.
In several patient tumour samples, the healthy tissues highly expressed ASNS in opposite to cancerous tissues which weakly expressed ASNS. With cell lines, ASNS expression was variable regardless of the detection method used, but only MOLT-4 was totally negative.
L-aspa was efficient *in vitro* in all cell lines studied (IC50 between 0.1 and 0.6 IU/mL). In addition, we confirmed that cytotoxicity was essentially due to L-Asn deprivation from the medium.
In vivo, a pancreatic model in mice displayed sensitivity to GRASPA in combination or not with gemcitabine. However, this sensitivity was dependent on the treatment schedule.
The results indicate that L-Asn depletion can be an effective strategy for treating solid tumours. GRASPA®, a better tolerated L-aspa formulation is under evaluation in a phase I clinical trial for pancreatic cancers and a biomarker assay for ASNS expression is being developed for patient stratification.

219 Cytotoxicity and cell death signaling in stem cell like AML cells

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Background: Acute myeloid leukemia (AML) is a result of uncontrolled proliferation of white blood cells in the myeloid line of cell differentiation in the bone marrow. High dose chemotherapy is the current treatment option but a large proportion of the patients relapse within 1−2 years and the 5-year survival threshold is low, particularly in elderly patients. Thus, new effective therapeutic drugs are highly desirable. We evaluate the cytotoxic potential of two novel treatments, gemtuzumab ozogamicin (GO), a monoclonal antibody linked to a toxin causing DNA double strand breaks and J1, an alkalyting prodrug of melphalan, in AML cell lines of different maturation stages. The aim of this study was to understand if and how these novel agents may work in different AML maturation stages with comparison to the conventional therapies daunorubicin or cytarabine.
Material and Methods: Two AML cell lines with different maturation stages were used. The partly differentiated HL60 cells were compared to the immature Kasumi-1 cells, both expressing high levels of CD33 on their surface and both positive for CD13. The cytoxic responses of the agents were compared using MTT cell viability assay. Activation of the proapoptotic proteins caspase 3 and Bax were determined using activation specific antibodies and was evaluated using FACS analysis.
Results: Treating the AML cells with GO, J1 daunorubicin or cytarabine revealed significant differences in their cytotoxic potential in HL60 and Kasumi-1 cells. Thus, daunorubicin (100 nM) and cytarabine (1 μM) were found to cause 50% growth inhibition at 48 h post drug addition in HL60 cells whereas in Kasumi-1 cells no more than 20% growth inhibition was observed. This was consistent with a lack of Bax and caspase-3 activation in Kasumi-1 cells. Comparison of GO sensitivity revealed a 50% growth inhibition in HL60 cells after 48 h of treatment with 100 ng/ml GO while no inhibition was observed in Kasumi-1 cells. This was not caused by different expression of the target CD33 on the cells, but may be explained by less efficient activation of caspase-3. A comparison of J1-induced cytotoxicity in HL60 and Kasumi 1 cells revealed a 50% growth inhibition after 48 h treatment with 0.5 μM J1 in HL60 cells whereas the Kasumi-1 cells needed 5 μM to achieve similar growth reduction. Analysis of apoptotic signaling is ongoing.
Conclusions: We demonstrate that the maturation state of the AML cells influences their responsiveness to both novel and conventional chemotherapeutics. The immature Kasumi-1 cells were more resistant to all drugs tested and only J1 was able to induce a proper cytotoxic response. Our data suggest that the capacity to trigger apoptotic signaling may explain why the drugs have a diverse cytotoxic potential in different maturation stages of AML. Further analyses of Kasumi-1 cells may reveal novel therapeutic targets that might have profound therapeutic implications for AML.

220 Identification of resistance mechanisms to EGFR inhibitors in non-small cell lung cancer cells

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Non-small cell lung cancer (NSCLC) patients are routinely treated with small molecule EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib. These inhibitors compete reversibly with ATP to bind the intracellular catalytic domain

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