study population 60.9% had a FIGO stage I, 9.4% FIGO stage II, 18.8% FIGO Stage III and 6.3% FIGO stage IV. 73.3% of all cases showed an Adenocarcinoma. 31.3% had a grade I tumour, 40.6% a grade II and 23.4% a grade III. A high expression of edi-1, edi-2 and edi-3 was in correlation to a significant shorter overall survival for edi-1 (p-value 0.011) and edi-3 (p-value 0.017). edi-2 was without a significant expression concerning to overall survival (p-value 0.0622). The expression was significant in correlation to recurrence free interval for all three samples. The p-value was 0.004 for edi-1, 0.014 for edi-2 and <0.001 for edi-3. In addition, NCBI blast-to-sequence analysis showed, that the initial sequence of edi-3, identified with differential display technique, is part of a hypothetical protein, named KIAA1434.
Conclusion: We found edi-1 and edi-3 in a significant correlation to Overall survival and recurrence free interval using Taqman-Assay. This analyses of differential displayed gene sequences using a second technique was done in a comparable study population. Edi-1 and edi-3 are valuable candidates for further investigations on tumour aggressiveness in endometrial cancer.

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Characterization of carbonic anhydrase 9 (CA9) overexpression: endogenous hypoxia marker and potential tumor-specific target

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Background: The transmembrane glycoprotein carbonic anhydrase 9 (CA9) has been discussed as an endogenous marker of tumor hypoxia and, due to the rarity of hypoxia in normal tissue, a target for tumor-specific treatment. Isolation of live CA9-positive cells from experimental tumors (Olive, Cancer Res 2001) may permit the separate analysis of treatment effects in normoxic and hypoxic cells. We characterized the conditions of CA9 overexpression *in vitro* and developed an improved FACS protocol for CA9 sorting.
Methods: HT 1080 human fibrosarcoma and FaDu human pharyngeal carcinoma cells were subjected to 20%, 5%, 1% or 0.1% O_2 for 10 min, 1 h, 6 h or 24 h (± reoxygenation up to 20 h; n=3–4). Treatment with 100 μM desferrioxamine (DFO) served as positive control. CA9 protein was measured by Western blotting of whole-cell lysates using the M75 antibody (Pastorekova, Virology 1992; CA9/β-actin ratio of DFO control = 100%). To evaluate the association of CA9 with radiobiologic hypoxia, cells were irradiated at the above O_2 concentrations with 2, 5 or 10 Gy (n=3–5). Modified oxygen enhancement ratios (OER') were calculated. A FACS protocol was developed using the anti-CA9 primary and FITC secondary antibodies and applied to known mixtures of normoxic and hypoxic (0.1% O_2) cells irradiated with 10 Gy before mixing. Mixtures were also plated for clonogenic survival.
Results: CA9 remained at aerobic baseline (20% in HT 1080, below 5% in FaDu) in the first 6 h of hypoxia, irrespective of O_2 concentration. At 24 h, equal CA9 overexpression of 100% was seen in HT 1080 treated with 5%, 1% or 0.1% O_2. In FaDu, 1% and 0.1% O_2 caused identical CA9 levels of 65% at 24 h (5% O_2: 37%). CA9 protein was stable over 20 h of reoxygenation. CA9 overexpression was modified by medium glucose concentration and cell density. OER' values were correlated with CA9 level in FaDu but not in HT 1080. Hypoxia (24 h, 0.1%) led to a 200-fold and 30-fold increase of anti-CA9-FITC fluorescence in HT 1080 and FaDu cell suspensions, respectively. The percentage of CA9-positive cells, as determined by FACS, in known mixtures of hypoxic/aerobic HT 1080 cells (1% to 99%) was well correlated with the known percentage of hypoxic cells and the clonogenic survival of mixtures after 10 Gy.
Conclusion: CA9 is a stable indicator of chronic hypoxia, being overexpressed already under mild hypoxia which may limit its use as a therapeutic target. The FACS protocol permits good separation of aerobic and hypoxic HT 1080 cells *in vitro*. The percentage of CA9-positive cells is correlated with hypoxic radiation resistance in mixtures of aerobic and hypoxic HT 1080 cells. The method appears suitable to study the treatment sensitivity of chronically hypoxic cells in tumors, e. g. to hypoxia-specific drugs or radiation.

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Eradication by induced apoptosis of chemoresistant infiltrating ductal carcinoma (IDC) characterised by HDAC2 overexpression and 5′CpG island hypermethylation of the FHIT, RARb2, BRCA1, APC, p16(CDKN2A), RASSF1A, CDH1(Ecadherin), stratifin, MDG1 and HIC1 oncosuppressor genes after combined treatment consisting of immunochemoconjugate of anti-DNMT1/HDAC2 bispecific F(ab)2-bsAb linked with cleavable disulfide onto vinorelbine (I-VRL)

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IDC accounts for approximately eighty percent of all cancers. Despite complete surgical resection, up to one half of patients die from recurrent disease within five years. IDC is characterised by intrinsic resistance to chemotherapy and mechanisms of this clinical resistance are poorly known and understood. One of the reasons is deacetylation and DNA methylation which causes silencing of tumour suppressor genes. To improve the prognosis of patients with IDC, a better understanding of the molecular changes involved in its pathogenesis is essential. DNMT1 interacts with HDAC2 to repress transcription of tumour suppressor genes. IDC cells obtained by surgical excision from a patient were analysed by IHC, PCR-based LOH, ChIP assay, methylation-specific PCR, RT-PCR and Northern blot. There was loss of FHIT expression, LOH at FHIT and 5′CpG island methylation of the FHIT gene. Furthermore, there was transcriptional silencing of the following tumour suppressor genes: BRCA1, RARb-2, APC, p16(CDKN2A, RASSF1A, CDH1(E-cadherin),14–3-3-s (stratifin), MDG1 and HIC1. There was overexpression of DNMT1 and HDAC2 suggesting a link between histone deacetylation, cytosine methylation, local chromatin condensation and subsequent transcriptional repression. This chemoresistant IDC was defined as (CIMP+) CpG island-methylator-phenotype positive. We treated IDC cells with immunochemoconjugate of anti-DNMT1/HDAC2 bispecific F)ab)2-bsAb linked with cleavable disulfide to vinorelbine termed as immunovinorelbine (I-VRL). Post-treatment, there was inhibition of HDAC2 and DNMT1 blocking the 5′CpG island methylation of the tumour suppressor genes resulting to transcriptional activation by upregulation of their mRNA. Furthermore, there was histone hyperacetylation which opens chromatin structure in which the DNA is more loosely wrapped around the histones making it more receptive to interaction with transcription factors. Overexpression of the tumour suppressor genes combined with the microtubule depolymerizing action of vinorelbine inhibited metabolic activity and DNA synthesis of tumour cells according to MTT and BrdU assays, respectively. Immunological analysis exhibited antibody-directed cytotoxicity (ADCC). There was induction of apoptosis in IDC cells according to TdT-mediated-DUTP-biotin nick end labeling (TUNEL) method and transmission electron microscopy(TEM). A large number of tumour cells exhibited condensed chromatin and membrane-bound small bodies (apoptotic bodies) which were phagocytosed by adjacent tumour cells leading to a bystander killing effect. Concluding, this therapeutic approach with immunochemoconjugate anti-DNMT1/HDAC2 bispecific F(ab)2-bsAb linked onto vinorelbine (I-VRL) may revolutionize IDC treatment adding significantly to the current clinical armamentarium due to potential advantages offered by I-VRL over conventional therapy such as well defined mode of action, selectivity and mainly circumvention of chemoresistance by causing DNA demethylation and histone hyperacetylation reactivating transcriptionally silenced oncosuppressor genes.

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The role of NAD(P)H: quinone oxidoreductase 1 (NQO1) in geldanamycin, 17AAG and 17AG metabolism

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Geldanamycin and related quinones 17AAG and 17AG exhibit antitumor activity primarily through the inhibition of HSP90. Previous work by others has demonstrated that NQO1 metabolizes geldanamycin and related quinones and expression of NQO1 in cancer cell lines generally increased sensitivity to these agents. We have extended these studies to examine in greater detail the role of NQO1 in geldanamycin, 17AAG and 17AG metabolism. In studies using purified recombinant human NQO1 (rhNQO1) an approx. 1:1 stoichiometric relationship was observed between NAD(P)H oxidation and quinone reduction. Oxygen uptake studies revealed only trace levels of O2 consumption during the metabolism of these compounds by rhNQO1 indicating the formation of hydroquinones resistant to autooxidation. In addition, very low levels of oxygen consumption were also detected when geldanamycin, 17AAG or 17AG was incubated with NAD(P)H in the presence of either mouse or human liver microsomes suggesting that these quinones do not rapidly undergo redox cycling reactions. The reduction of geldanamycin, 17AAG and 17AG by rhNQO1/NAD(P)H to the corresponding hydroquinones was confirmed by tandom LC-MS. To examine the role of NQO1 in 17AAG metabolism in cells we utilized the NQO1-null human breast cancer cell line MDA468 and MDA468/NQ16, a stably transfected clone that expresses high levels of NQO1 protein. Following treatment with 17AAG the MDA468\NQ16 cell line was 20-fold more sensitive to growth inhibition compared to the MDA468 cell line. The increased sensitivity of the MDA468\NQ16 cell line to 17AAG could be abolished if the cells were pretreated with a mechanism-based inhibitor of NQO1. HLPC analysis of intact cells in culture treated with 17AAG demonstrated higher concentrations of 17AAG hydroquinone in MDA468\NQ16 cells compared to MDA-468 cells and interestingly MDA468\NQ16 cells also contained greater concentrations of 17AAG. These results demonstrate that geldanamycin, 17AAG and 17AG do not undergo redox cycling reactions that generate large quantities of reactive oxygen species. Additionally, the hydroquinone formed following

the reduction of 17AAG by NQO1 is relatively stable to autooxidation and formation of the 17AAG hydroquinone may allow for increased levels of 17AAG intracellular drug accumulation via a slow autooxidation process. Another implication of the data is that the 17AAG hydroquinone may represent an alternative conformation for binding to HSP90 (supported by CA51210).

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Para-amino benzoic acid (PABA) modulation of chemotherapy – evolution from preclinical work to a Phase I study. A report of early clinical activity including pharmacokinetic and pharmacodynamic studies

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Background: B16F10 melanoma cells cultured in media containing PABA resulted in a rapid reduction in pigmentation compared to cells grown PABA deficient media. *In-vivo* murine studies with a B16F10 melanoma xenograft model treated with daily PABA IP at 50 mg/kg and paclitaxel (P) treatment alone or in combination showed that the PABA + P combination was significantly different from the control ($p = 0.016$) and P groups. Patients (pts) with metastatic melanoma who had failed first line therapy with dacarbazine routinely receive platinum and/or taxane agents as second line therapy. We have translated these findings into a Phase I study of combination PABA, P and carboplatin(C) in metastatic melanoma patients who had failed first-line therapy.
Methods: Both C and PABA dose were fixed (AUC 5 mg/ml, IV; 2 g/d, po) with P escalated over 4 dose levels ranging from 100 to 175 mg/m2. PABA was administered on days 1–10 with chemotherapy on day 6. Three patients were enrolled at each dose level. Toxicity was assessed at day 21 of the 1st cycle. Correlative studies include the measurement of PABA, P and C PK, monitoring for shed collagen cryptic epitopes, molecular pathology assessment on accessible metastatic lesions, and documentation for vitiligo development. We plan to correlate these findings with the clinical response of these pts.
Results: Fifteen pts have been enrolled to date with 14 eligible for assessment. All pts at DL I treated with only PABA and C showed no toxicities or response. For DL II and III, (P 100 and 125 mg/m2), no toxicities have been seen. Three of 6 pts have shown clinical responses. The second pt on DL IV (P 150 mg/m2), developed Grade III neutropenia. DL IV was expanded to 6 pts. 4 evaluable pts had no toxicity and 1 not yet evaluated. Two of 5 pts on DL IV, had clinical responses. We have observed inflammatory responses in responding cutaneous lesions. PABA PK for DL I-IV showed a mean Cmax of 14 μg/ml ± 7.9 μg/ml at 0.5 hour, and an elimination HL that ranged from 0.2–13 h. In 6 out of 11 pts, a mean 4-fold increase of the AUC was observed 24 h following the administration of P. Assessments of the lack of toxicity with P PK are ongoing. Data on the C AUC, shed cryptic epitopes, and histopathologic markers for antibodies to β1 integrin, BRCA-2 and CDC-25A are in progress.
Conclusions: This regimen demonstrates activity with low toxicity in refractory melanoma pts. A phase II efficacy trial is planned.

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A peptide inhibitor of t(4;11) leukemia

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Despite considerable progress in the treatment of leukemias, particularly in children, acute lymphocytic leukemia (ALL) in infants remains highly resistant to treatment. Infant ALL is characterized by reciprocal chromosomal translocations involving the mixed-lineage leukemia (*MLL*) gene at locus 11q23 and results in the in-frame fusion of *MLL* to a variety of different genes. In each case, the translocation leads to the expression of a chimeric protein with transforming properties. Investigations of the MLL fusion proteins and their interacting partners could provide reasonable targets of study to gain insights into mechanism of transformation. In particular, AF4 is a prime target as the MLL-AF4 fusion protein is expressed in most cases of ALL in infants.
We find that a small domain of AF4 interacts with the carboxy-terminus of another, relatively less common MLL fusion partner, AF9. The binding domains of AF4 and AF9 are maintained in leukemia-associated MLL fusion proteins, suggesting that AF4 and AF9 are capable of interacting in their native form and/or as MLL fusion proteins. The physical interaction of MLL-AF4 with AF9 may be important in leukemogenesis in cells with t(4;11)(q21; q23) translocations characteristic of infant leukemia and may serve as an important target in the development of a treatment for this disease. To this end, our lab has developed a small synthetic peptide based on the 14 amino acid residues of AF4 that are essential for the interaction with AF9. The ability of this peptide to disrupt the binding of AF4 to AF9 has been established using both *in vitro* and *in vivo* assays. Furthermore, we have shown that it specifically inhibits proliferation of t(4;11) leukemia cell lines and that this inhibition is mediated through apoptosis. Importantly, the peptide does not affect the proliferative capacity of hematopoietic stem cells as evidenced by methylcellulose-based assays to determine colony forming potential. Here we examine the relationship between AF4-AF9 binding and its resulting leukemogenesis potential. Future studies with this peptide or its derivatives could lead to the development of novel therapies specifically targeted to infant leukemia or other leukemias with t(4;11) rearrangements

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Combinatorial cancer therapy using a pharmacological inhibitor of indoleamine 2,3-dioxygenase (IDO), a target of the cancer suppression gene Bin1

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Mutations produce a dynamic substratum upon which modifier genes and the stromal microenvironment act to facilitate the development of a fully malignant state. Previous studies of Myc interactions have identified Bin1 as a candidate suppressor or negative modifier gene in cancer. Bin1 encodes a set of alternately spliced adapter proteins. Here we report that Bin1 loss can promote tumor formation by facilitating escape from host anti-tumor immunity, and ongoing mechanistic investigations have identified a unique pharmacological strategy to correct this. We have found that homozygous deletion of the Bin1-gene enhances tumor formation by Myc+Ras – transformed murine skin fibroblast and keratinocyte isografts. In striking contrast, the negative impact of Bin1 expression on tumor growth was either diminished or lost in immunocompromised mice, suggesting a cell-extrinsic effect on anti-tumor immunity. A candidate mediator of this effect, indoleamine 2,3-dioxygenase (IDO), is being investigated. IDO is an oxidoreductase that we have found to be genetically controlled by Bin1. Catabolism of tryptophan by IDO has previously been shown to locally attenuate the activation of T cells and promote immune tolerance. Consistent with the more aggressive growth of Bin1–/– isografts in immunocompetent hosts, Bin1–/– cells exhibit superinducible IDO. We have tested whether IDO blockade may have therapeutic benefit in the MMTV-neu transgenic mouse breast cancer model using the competitive bioactive IDO inhibitor 1-methyl-tryptophan (1MT). By itself 1MT had little effect on tumor growth. In contrast, 1MT elicited tumor cell death and regression when combined with cytotoxic agents that alone were inefficacious in the model. No synergy occurred in tumors engrafted into nude mice, supporting the expectation that combinatorial efficacy is immune-based. Results of additional studies examining mechanism, PK/ PD, and survival in response to treatment will be presented. Our findings 1) support the concept of Bin1 as a cancer suppression gene, 2) link Bin1 deficiency to a pharmaceutically tractable enzyme that may control anti-tumor immunity, and 3) identify IDO/cytotoxic combination therapy as a promising new strategy to treat cancer. One radical implication of this work is that combining immunotherapy and chemotherapy to treat cancer, which may seem counterintuitive in principle, may prove to be unexpectedly effective in practice.

Drug screening

102 POSTER

The synthetic lethal trap: a general approach for screening small-molecule protein inhibitors using genetic triangulation in the yeast Saccharomyces cerevisiae

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Our purpose was to develop a novel chemical-genetic screening strategy to identify small-molecule inhibitors of any non-essential protein using a surrogate synthetic lethal (SL) phenotype. Synthetic lethality is a form of genetic enhancement in which two mutations are lethal in combination, but the corresponding individual mutants are viable. We hypothesize that SL genetic interactions can be used as reporters for drug-based inhibition of any non-essential protein function. If protein inactivation using a small molecule is functionally equivalent to a loss-of-function mutation, then a specific protein inhibitor should satisfy all the genetic SL interactions associated with the corresponding gene. For example, if inactivation of