Abstracts

Oral Presentations at the Symposium

Novel Approaches for the Discovery of Anticancer Agents

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1st Session · GENOMIC APPROACH

L1

Predicting Drug Response Based on Gene Expression

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Prediction of drug response would bring an invaluable improvement of the management of chemotherapy. This would help the clinician to select the patients, avoiding thus to expose to unacceptable toxic symptoms the patients unlikely to benefit from the treatment. This would also help to select the most appropriate drugs on an individual basis.

The identification of mechanisms of resistance to anticancer drugs has led to the establishment of relationships between the tumor expression of given genes such as MDR1, and the clinical resistance. More difficult is the identification of genes involved in tumor sensitivity to anticancer drugs. Some approaches using serial analysis of gene expression or genetic suppressor elements may allow to discover such genes whose expression is required for drug effects. Progressively, the panel of genes known to be involved in drug resistance or drug sensitivity is growing and it would not be possible to test them all by classical approaches in order to evaluate the relationship between their level of expression and response to treatment.

The development of high throughput techniques for establishing drug expression profiles on cDNA microarrays have brought a new dimension to this problem. It allows both to quantify the expression of hundreds or thousands of genes suspected to be involved in drug response or resistance, but it especially allows to discover new, unexpected relationships between the expression of a gene and tumor cell sensitivity to a drug.

Such an approach has been implemented at the cellular level by the NCI, who has determined the gene expression profiles of the 60 human tumor cell lines of their panel and put these data in correspondence to the cytotoxicity data of standard anticancer agents and of developmental drugs, which had been obtained on this screen. Mining this database may prove highly profitable for establishing correlations between the activity of a given compound and the expression of a gene, and for identifying, as a consequence, the genes involved in cellular response or resistance to this molecule. Some correlations thus observed were expected, for instance the negative correlation between fluorouracil cytotoxicity and the level of expression of the gene of dihydropyrimidine dehydrogenase, the enzyme able to inactivate this drug intracellularly. In addition, numerous significant unexpected correlations, both positive and negative, were found, and they constitute a huge reservoir of potential research. Similar cell line-based approaches have been developed by several groups in the world.

Based upon this principle, it is now possible to put in correspondence the gene expression profiles of human individual tumors and the clinical response obtained with an anticancer agent. At present, many studies have determined the diagnostic and prognostic values of gene expression profiles established in human tumors, but it seems premature to conclude about prediction of response to treatment from this still experimental innovative techniques. There is no doubt that such results will be soon available. The individual design of chemotherapy will probably become a routine practice in oncology in the next future.

L2

Gene Expression Profiles for Molecular Diagnostics and Therapy

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Within the last decade enormous efforts have been made to analyse the expression of genes in malignant tissues, aiming at identifying novel disease-related genes and disease-specific expression patterns that allow to distinguish between good or bad prognosis and improve diagnostics in different cancer tissue types. A number of novel gene expression technologies like e.g. SAGE (1) enable nowadays the in depth analysis and identification of

so far unknown genes. At present, results of a series of SAGE libraries from several diseased and healthy tissues are online available at the NCI. Due to the digital nature of such results a comparison of new libraries to already existing ones represents a promising approach for identifying novel – previously unknown – disease-related genes. Furthermore, with the advent of the microarray technology it became possible to perform global, parallel gene expression analysis (e.g. diseased vs. healthy tissue) in a high throughput manner. This technology will greatly impact our understanding of the pathogenesis of malignant diseases. Several hallmark studies have already demonstrated, that gene expression patterns derived from microarray based approaches allow a better responder/non-responder prediction towards specific drug treatments. In addition, these new prognostic gene expression parameters have been shown to outperform current existing clinical parameters in predicting disease outcome for certain tumor types (2–4).

At memorec both expression profiling platforms – SAGE™ (licensed from GMO) and PIQORTM microarray analysis – have been established several years ago. Currently, in one of our gene expression profiling programs, we are combining these platforms in order to identify drug target candidate genes and diagnostic/prognostic marker genes linked to skin diseases. Together with a clinical network of experts encompassing the dermatological departments of the universities of Aachen (Prof. Dr. Merk), Bonn (Prof. Dr. Dr. Bieber), and Cologne (Prof. Dr. Krieg) we are performing gene expression analysis of about 800 patient biopsies afflicted with inflammatory skin diseases (atopic-, contact dermatitis, and psoriasis) and skin cancer (malignant melanoma, basaliom, and spinocellular carcinoma). All genes identified in the SAGE™ based genome wide screen specific for normal and diseased skin are cloned, sequenced and used to complete an already existing PIQORTM Skin array comprising about 1300 genes. These arrays are currently used to generate expression profiles of abovementioned patient biopsies. The profiles are stored in a data base (SkinSAYS DB) and are analysed by state of the art bioinformatics to identify the most relevant genes/gene patterns for the respective disease.

Critical issues and requirements for setting up the SkinSAYS data base (such as SOPs for experimental design, quality control management of biopsies, amount of starting material for analysis etc.) as well as first results will be discussed in the talk.

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L3

RNAi: From Genome-Wide Screening to a New Class of Therapeutics

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Our team specializes in the use of RNAi technologies to accelerate and improve research and the early steps of drug development, from target discovery and validation to entry into the clinic. Our goal is the creation of a new class of RNAi-based therapeutics for high-need oncology and viral indications. We have thereby established a number of platforms covering a wide range of RNAi-based applications, falling into three primary categories:

- high throughput genome-wide screening for target discovery,
- high content phenotypic assays for target validation in human cells and mice, and
- efficient, streamlined development of pharmaceutical compositions for use as RNAi-based therapeutics.

The presentation will review the current status of our anti-proliferative discovery pipeline, which was initiated as a genome-wide RNAi screen for new genes involved in animal cell division in *C. elegans*. The use of time-lapse DIC and fluorescence digital video microscopy recordings of early embry-

onic divisions offered a relatively low throughput but very high content read-out, resulting in a good level of 'baseline validation' for identified candidate target genes. The screen has now reached a coverage of over 99% of all predicted worm genes, yielding over 560 cell division genes, ~220 of which have clear mammalian orthologues and ~40 of which had never before been implicated in cell proliferation. Human orthologues of 16 of these genes represent candidate therapeutic targets for anti-proliferative intervention, and are therefore being subjected to a series of validation experiments including RNAi-based assays in human cells and mouse models. Current results from these experiments will be reviewed, illustrating our overall approach towards achieving potent, reproducible and cost-effective high throughput RNAi experimentation in mammalian systems, including our efforts towards increased integration of target discovery and validation via genome-wide screening in human cells.

L4 Statistical Issues in Design and Analysis of Clinical Studies Using Functional Genomic Data

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The arrival of functional genomic data in the sequel of the human genome project on an individual patient basis has nourished the desire for use of this wealth of data for the development of new drugs and new treatment schedules which offer 'the right medicine for the right patient at the right time'. Genotyping patients and identifying genes which are differentially expressed between treatment and control group can now be integrated into the therapeutic research question. Using microarrays of oligonucleotides or cDNAs one can simultaneously quantify the expression of thousands of genes in diseased and in normal patient tissue in different organs. This information is useful for the definition of prognostic subgroups but has also a capacity to predict treatment success / drug resistance, or the chance of the occurrence of adverse events. Ideally gene expression analysis can be used in conjunction with conventional prognostic tests to guide pharmaceutical treatment in such a way that efficacy is maximized and adverse reactions are minimized. Furthermore analyzing microarray data can help to find subsets of genes or gene signatures for clinical screening.

Many important statistical questions arise if these data are collected for and analyzed in a clinical trial. Quality and, in particular, quantity of the new genomic data has therefore not only challenged a wave of new research on statistical data analysis but has also lead within a few years to a large number of different approaches pushed forward by an increasing number of research groups originating from very different fields ranging from bioinformatical engineering to theoretical statistics.

We discuss a range of statistical issues arising in the design and analysis of clinical trials when individual patient microarray gene expression data are collected and analyzed with the perspective of their practical applicability for preclinical and clinical anticancer drug research. Despite the diversity of clinical goals the corresponding statistical questions can be characterized by two main topics – univariable screening and multivariable prediction. For univariable screening we consider statistical methods to rank genes with regard to differential expression dependent on the scale of the endpoint: binary, categorical, continuous and censored continuous. Sampling variability in the gene rankings can be quantified using the selection probability function. Adjusting for confounding effects of clinical covariates may be necessary in some situations. Multivariable prediction is done by using prognostic models with respect to minimization of the prediction error. Restrictions on model complexity are needed to avoid overfitting. Model selection and validation should be done by resampling techniques like bootstrapping and k-fold cross-validation.

In order to get reliable results microarray studies require a clear identification of their objective, a valid study design, the definition of patient selection criteria, sample size calculations, and the development of an appropriate strategy for the statistical analysis.

2nd Session · BIOINFORMATIC APPROACH

L5 Integromics™: Integrating Genomics, Proteomics and Bioinformatics for Cancer Drug Discovery

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The new, highly multiplexed genomic and proteomic technologies, for example microarrays, pose novel challenges to the pharmacologist. In part, those challenges relate to experimental issues; in part they relate to what happens after the experiment has been finished. The first challenge after the experiment is to analyze the data statistically. The second is to interpret the resulting lists of genes biologically and pharmacologically. The third is to integrate the data with other types of molecular and pharmacological information ('Integromics™'). We have developed a number of practical software tools for meeting those challenges: **MedMiner** (1), which speeds up 5–10 fold the organization of biomedical literature on genes and drugs; **CIM Maker** (2, 3), which flexibly produces Clustered Image Maps ('heat maps'); **MatchMiner** (4), which translates fluently among the many types of gene and protein identifiers; **GoMiner** (5), which leverages the Gene Ontology for discovery of functional order in lists of genes; **MethMiner**, which organizes patterns of sequence information from DNA methylation studies; **LeadScope/LeadMiner** (6), which links genomic and proteomic information to the molecular substructures of potential drugs; **AbMiner**, a compendium of information on antibodies available for proteomic studies; and **GEEVS**, which integrates multiple types of molecular information at the DNA, RNA, protein, and pharmacological levels.

Development of these computer resources has been motivated by our studies of the 60 cancer cell lines (the NCI-60) used by the NCI DTP to screen >100,000 chemical compounds since 1990. These cells provide detailed information about mechanisms of drug action and resistance (7, 8). We and our many collaborators have generated multi-faceted molecular target profiles of the NCI-60 using 2-D gel electrophoresis (4), 'reverse-phase' protein microarrays (9), cDNA microarrays (10, 11), Affymetrix oligo chips (12), array-CGH, SKY, SNP chips, and DNA methylation-sequencing. Clinical molecular markers identified have been validated by tissue microarray (9). In the 'omic' era of biology (13–15), such integrated databases will have a great impact on diagnosis, prognosis, and therapy of cancer.

L6 Virtual Drug Screening Initiative of Natural Products

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With the new concept of the 'Virtual Natural Products Pool' we want to create a new tool in searching for biological active compounds. This project will exist beneath the well known 'Natural Products Pool' and will give access to the very high diversity of natural occurring structures by a new entrance.

The new starting point is the structure. This will be made available for the different arts of in silico screening. Afterwards followed by a biological assay to get a first hint of a real activity. As consequence the success depends on the availability of the real existing sample. With the background of the 'Natural Products Pool' we know that there is a large number of structures which could not yield benefit until now because of their little amount. This projects will give the opportunity for these unconsidered structures to become a hit.

Compounds which will enter to the 'Virtual Natural Products Pool' must be available in a minimum amount of 500 µg stored either by the supplier (national) or by the HKI (international). This will secure a short access time. The database will delivered once a year hopefully with 10,000 entries. Companies of interest will have access to this database. The principle object will be a bilateral collaboration between the industry and the scientific supplier, so the HKI acts only as a broker.

Nevertheless it needs money for this work. The companies have to pay for the database and in the case of interest in specific compounds the HKI will customize the selected structures in sample plates with an amount of 100 µg per sample. The price for this will be oriented on rare commercial chemicals.

The database will contain the structure, the names of the compound, the identity of the supplier, references, origin and remarks like known biological activities. The exchange of the data will be executed only via electronic data transfer as databases or as common transfer formats (e.g. 'sdf'). The industry will get only information on the structure including the related data but no information on the supplier. This will secure that the HKI and the suppliers will get the expense allowance.

As a bonus for good cooperation the suppliers will get the database for their own work as a dereplication tool. Unpublished structures could be handled confidential, that means no scientific partner will get the structures. This project will close the gap between

- databases containing not available structures of isolated natural compounds,
- assay based screenings with sample collections of natural products in a large amount (like the Natural Products Pool) and
- the in silico screening of large databases of commercial synthetic compounds.

L7

Virtual Screening for Anticancer Agents: A Data Mining Approach

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Experimental screening for anticancer agents has led to a substantial amount of data (e.g. chemical structures, biological activities, gene expression profiles). It is tempting to use this information to predict the activities of untested compounds (virtual screening). Unfortunately the extraction of knowledge from databases of that size and complexity is far beyond the capabilities of human experts. Traditional QSAR techniques are also not applicable, because they are restricted to structurally similar compounds and small datasets.

The extraction of predictive models from experimental data is essentially a data mining problem. We will present techniques that are capable of discovering hidden regularities and predictive models in large and complex databases. Special emphasis will be given to methods that are flexible enough to deal with various types of information (e.g. chemical structures, biological activities, transcriptomics) and that are able to provide understandable and interpretable results. The resulting models can be applied for a variety of purposes, for example for virtual screening of compound libraries and the selection of candidates for physical testing, for computer aided drug design, but also for the generation and validation of scientific hypothesis.

3rd Session · CHEMICAL PROTEIN INTERACTION APPROACH

L8

Small Molecule Drug Discovery on Chemical Microarrays

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Graffinity has developed a novel small molecule screening technology combining chemical microarrays and label-free affinity detection based on surface plasmon resonance (SPR) imaging. The proprietary drug discovery and development platform called RAISE® (Rapid Affinity Informed Structure Evolution) can be applied to any number of targets in a true Chemical Genomics approach.

The technology is applied to the screening of lead-like, fragment-based compound collections against protein targets providing direct access to comprehensive structure-affinity maps. The ability to start from weak but specific binding provides an early entry point for SAR analysis. Mapping and understanding such affinity fingerprints of molecular interactions is facilitated by chemistry oriented data visualization and analysis tools. We will present this novel approach, demonstrate the microarray data analysis and show how information driven screening results in potent leads with a systematic optimization history.

Figure: The SPR signal of a chemical microarray presenting 1,200 immobilized fragments is shown as a function of molecular weight (A). The corre-

sponding affinity fingerprint of the target protein probed against the chemical fragment microarray is shown as a grayscale with stronger binders in dark (B).

L9

Evolutionary Drug Discovery in Oncology

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In the past, the chemical synthesis of natural-like molecules has been very difficult and could easily take 20 steps or more for a single compound. Such structures are however not only very complex but can also serve as templates for active hits, thereby increasing the chance to find active medications for the treatment of cancer.

Strategies other than the classical medicinal chemical synthesis were sought to find new molecules. Morphochem has established an evolutionary drug discovery approach using multi-component chemistry that is also applied to projects in oncology. This 'drug discovery engine' delivers compounds specifically designed for different targets including novel cytotoxic drugs that mimic naturally occurring molecules, serine proteases including factor Xa inhibitors and protein-protein interaction inhibitors such as ras pathway modulators. Morphochem's chemistry platform and examples from the respective projects will be presented.

Morphochem's drug discovery engine effectively allows the identification of diverse methods for finding medications for various cancer treatments. Oncology is an extremely difficult indication – thereby introducing more levels of complexity from the developmental perspective. Both chemistry and medicine need to establish new creative strategies to significantly increase the survival time of cancer patients and improve their quality of life.

L10

IDN 5390: An Oral Taxane Candidate for Protracted Treatment Schedule

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In the field of antineoplastic chemotherapy taxanes represent an important class of compounds, with broad activity in solid tumors. Taxanes exert cytotoxic action through microtubule stabilization. Moreover, paclitaxel (PTX) and docetaxel have been described as antiangiogenic agents, mainly for their ability to inhibit endothelial cell motility and to down regulate angiogenesis-related growth factors. Among compounds of a novel series of C-seco taxanes, IDN 5390 was originally selected on the basis of its potent antimotility activity and poor cytotoxicity on endothelial cells. Preclinical studies indicate peculiar pharmacological feature of IDN 5390 compared to PTX since, in spite of a lack of antitumor activity when delivered according the optimal PTX conditions (i.v. route and intermittent high-dose schedule), IDN 5390 was effective by oral route and was characterized by optimal tolerability following continuous protracted administration. Indeed, in mice total doses up to 4500 mg (about 20-fold higher than those of PTX) could be administered, without sign of toxic effects, when the compound was delivered for a very prolonged period (up to 9 weeks). Using the metronomic schedule (1 or 2 daily treatments for prolonged time) IDN 5390 resulted re-

markedly active against a variety of human tumor xenografts, either by s.c. or oral administration. In such conditions, IDN 5390 antitumor effects were comparable to the best effects of PTX. In addition, IDN 5390 achieved relevant tumor growth inhibition even in PTX-resistant tumors. In particular, in the INT-ACP/PTX human ovarian carcinoma, resistant to both cisplatin and PTX, IDN 5390 induced a strong antitumor effect (~ 85% TW inhibition versus control tumors). The treated tumors presented a reduced number of microvessels compared to control tumors, thus indicating a role for antiangiogenic effects in IDN 5390 antitumor activity. The high tolerability of IDN 5390, allowed a continuous treatment, and a prolonged control of tumor growth. Pharmacokinetics studies in mice showed a good oral bioavailability associated to rapid clearance and short half-life. In conclusion, IDN 5390 can be considered the prototype of a new series of well-tolerated, orally available, taxanes particularly suitable for metronomic scheduling of chemotherapy.

L11

$\alpha_v\beta_3$ -Integrin Targeted Camptothecin Conjugates: Configuration-Dependent Integrin Binding and Cleavage by Tumor-Associated Enzymes and its Impact on the Biological Activity

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Tumor angiogenesis is a complex process involving the coordinated expression of both tumor and host derived cell surface and soluble proteins that guide the formation of new blood vessels and remodeling of basement membrane proteins. $\alpha_v\beta_3$ integrins are adhesion proteins found highly expressed in certain tumor cells and particularly in remodeling neovasculature. They play an important role in tumor induced angiogenesis. In addition, matrix degrading enzymes such as matrix metalloproteases and elastase are also involved in tissue remodeling, particularly during tumor growth and metastasis.

To utilize the disease-associated functions of these different protein classes in a beneficial manner, we have undertaken a strategy for targeted cancer therapy utilizing a novel class of hybrid-molecules. We have developed an $\alpha_v\beta_3$ integrin antagonist linked to the cytotoxic agent camptothecin via peptide sequences cleavable by tumor associated proteases such as MMP-2 or elastase. Tumor selectivity of such conjugates should be improved by the targeting moiety of a potent integrin antagonist and by utilizing tumor-associated enzymes for toxophore release.

A novel class of peptidomimetic sulfonamides has shown high affinity to $\alpha_v\beta_3$ integrins with IC50 values in the single digit nanomolar range, as measured in an [¹²⁵I]-echistatin β_3 SPA competition assay. In subsequent synthetic efforts chemical space was identified where spacer linked camptothecin derivatives can be attached to provide hybrid molecules retaining strong integrin binding. The configuration of the stereocenter of the integrin antagonist moiety strictly determines the binding affinity of the disintegrin itself and of the respective hybrid molecules.

In a second step the peptide spacers were optimized for MMP-2 or elastase cleavage. To afford MMP-2 cleavable substrates, hexapeptide sequences were employed as spacer groups, whereas to provide elastase cleavable substrates, only tripeptide spacers are required. Respective diastereoisomers with D-configured amino acids at the cleavage site are stable and served as controls to determine the enzyme specificity of toxophore release.

Selected compounds effect tumor regressions or stasis in melanoma and renal xenograft models, which favorably compares to the effect of camptothecin alone and other reference compounds from this class. These results demonstrate the potential of such hybrid molecules in cancer treatment.

4th Session · TARGET DIRECTED APPROACH

L12

NCI Target-Directed Drug Discovery

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The National Cancer Institute, USA Developmental Therapeutics Program (DTP) after consultation with a blue ribbon panel of advisors has adopted the position that its future cancer drug discovery and development efforts should focus on the elucidation of agents which affect specific targets important in the pathogenesis or progression of cancer. Such agents ideally would be advanced to clinical trial in populations of patients known to possess a molecular abnormality in the relevant target or in pathways affecting or affected by the target. Further, early development of such agents would ideally assess not only traditional measures of toxicity and pharmacology, but also whether the agent in question was eliciting an effect on is intended target. With this general philosophy, NCI funds a number of investigators working at extramural sites across the country who have successfully competed for funding with this general goal. Selected project areas will be illustrated; a listing of the funded investigators is listed at <http://dtp.nci.nih.gov> (see 'Grants and Contracts Operations Branch'). NCI's in house drug discovery and development program, managed by the Developmental Therapeutics Program likewise has shifted to emphasize agents that are directed at a defined molecular target. Compounds with a known target are examined in NCI's 60-cell line anti-cancer drug screen, and verification that the pattern of activity either conforms to the expected correlation with the target's expression or action is sought through computational algorithms. Compounds active in the screen but without a known mechanism of action are advanced in development only if a reliable definition of the target can be ascertained, or a surrogate for compound action in molecular terms defined. Examples of recent compounds advanced in development with a defined target or pathway of relevance include the proteasome inhibitor PS-341, the benzoquinoid ansamycin 17allyl-amino 17-demethoxygeldanamycin, the arylhydrocarbon receptor activating aminoflavone and benzothiazoles, the paullone class of cyclin-dependent kinase inhibitors and the tyrphostin adaphostin. Lead optimization strategies can then be undertaken where successive refinements in the molecule can be introduced with the structure or action of the target directing optimization activities, and behavior in the screen serving to prioritize for further in vivo studies. Finally NCI DTP has implemented molecularly based primary screens with a goal of detecting novel structures whose suitability for further development will be tied to the ability to disrupt important pathways relevant to cancer. Interim results from two such screens, one directed against the activation of the hypoxia-inducibility factor (HIF), and the other the transcription factor CEBP α will be presented.

L13

Critical Evaluation of the Clinical Impact of a Target-Directed Approach

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A normal cell can survive the complete inhibition of a signaling pathway because other pathways take over all or some of the functions, thus maintaining homeostasis. Tumor cell growth is more dependent on particular overactive signaling pathways which suggests that cancer cells are preferentially vulnerable to signal pathway inhibitors. Thus, if a drug selectively blocks a pathway that is activated by an oncogene it should be possible to reverse the drug's effects while leaving normal cell functions unaffected. A paradigmatic situation of a well established targeted therapy is anti-hormonal therapy in breast cancer. Although anti-hormonal therapy is based on the interaction with a very simple pathway, the steroid hormone pathway, it took more than one century for its exploration and eventual clinical application. However, while widely applied, clinical success is far from influencing or even curing all patients receiving hormone treatment. The following therapies have been developed at differing levels of clinical use, realizing the concept of a targeted approach:

Trial	Indication	Phase and design	Target	Target
Trastuzumab (Herceptin®)	Breast cancer metastatic	Response (in combination with chemotherapy, non-randomized), survival (sign.) (randomized), (registered)	High	erbB2
Trastuzumab (Herceptin®)	CML, PM chronic phase CML, acute phase CML, blast crisis	Survival (sign.) (randomized), Response (survival) (non-randomized), Response (survival) (non-randomized) (registered)	Extremely high	BCR-ABL
Trastuzumab (Herceptin®)	Locally advanced, metastatic	Response (non-randomized), Survival (non-randomized) (registered)	Very high	erbB2
Cetuximab (Erbitux™)	Colorectal cancer metastatic	Time to progression (sign.) in combination with chemotherapy, randomized, (not registered yet)	Tested in preselected target positive patients only	EGFR
Trastuzumab (Herceptin®)	Colorectal cancer metastatic	Survival (sign.) in combination with chemotherapy, random, (not registered yet)	—	erbB2
Gefitinib (Iressa®)	NSCLC metastatic, 2nd line	Response rate (randomized phase II) (registered)	Weak to lacking	EGFR

To date, a variety of substances undergo early clinical testing and many interesting candidate substances have been identified that have not reached clinical use yet.

Common features of targeted therapeutic approaches are a) almost complete lack of cures, b) only a small percentage of them yields objective tumor regressions and symptom relief, c) development of resistance compromises long term antitumor effectiveness.

Shortcomings of most targeted therapies could be reduced/overcome by a) combining them with conventional antitumoral therapies, b) combining them with other targeted therapies, c) optimizing the right sequence of application, d) using them as continuous therapies of low toxicity, e) identifying better surrogate parameters, f) developing techniques substituting for serial tumor tissue biopsies and g) selecting candidate patients disposing on the relevant target.

L14

Targeting Ligand-Activated erbB2 / her2 in Solid Tumors

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The ErbB or HER receptor network is frequently dysregulated in a number of solid tumors. ErbB2/HER2 is a ligand-less member of the ErbB receptor family that functions as a co-receptor with EGFR, ErbB3 and ErbB4. Breast cancers containing ErbB2 overexpression can be treated with the ErbB2 antibody, trastuzumab (Herceptin). In these cases, ErbB2 overexpression is the outcome of a specific molecular alteration, i.e., ErbB2 gene amplification that results in excess protein synthesis. The accumulation of excess ErbB2 at the plasma membrane results in constitutive receptor activation due to receptor self-association. Constitutive ErbB2 signaling is thought to drive tumor growth. The availability of ErbB2 at the tumor cell surface allows the receptor to be targeted by trastuzumab. However, trastuzumab is only effective against high ErbB2 expressing tumors. A number of studies suggest that inappropriate ligand-dependent ErbB2 activation may also play a major role in driving tumor growth or survival pathways. In this presentation, I will provide an overview of our program to target ErbB2's role as a co-receptor using a monoclonal antibody, pertuzumab (humanized version of the murine antibody 2C4; Omnitarg™) which sterically hinders ErbB2's recruitment into ErbB ligand complexes. Inhibition of ligand-dependent ErbB2 signaling by pertuzumab occurs in both low and high ErbB2 expressing systems. Since the ErbB3 receptor contains an inactive tyrosine kinase domain, pertuzumab is very effective in blocking heregulin-mediated ErbB3-ErbB2 signaling. In the case of EGFR, which is fully functional as a tyrosine kinase, blocking the recruitment of ErbB2 into EGFR-ligand complexes does not ablate signaling but dampens the diversity, intensity, and duration of signaling. The *in vitro* and *in vivo* growth of several breast, prostate, lung, colon, and ovarian tumor models is inhibited by pertuzumab treatment. The Genentech Protein Engineering Department has recently solved the three dimensional structure of ErbB2 in complex with a Fab fragment derived from pertuzumab. A comparison of this structure to the ErbB2-trastuzumab structure will be discussed. Using site-directed mutagenesis, key residues in the ErbB2 extracellular domain have been identified that ablate pertuzumab binding and ErbB2's ability to heterodimerize. Potential therapeutic benefit of pertuzumab treatment does not require the antibody to contain an intact Fc region. Recently, the results of a Phase 1 clinical trial with pertuzumab were reported at ASCO. It was

concluded from this study that pertuzumab was well tolerated at doses up to 15 mg/kg q 3 wk and clinical activity was observed. Phase 2 studies in four different indications are planned.

L15

Modulators of Heat Shock Protein 90 – Targeting Multiple Signal Transduction Pathways

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The heat shock protein 90 (hsp90) plays an important role in mammalian cells as it is involved in the correct folding of a range of different proteins, many of which are associated with tumorigenesis. Examples of client proteins of hsp90 can be classed into three groups: steroid hormone receptors, serine/threonine or tyrosine kinases and unrelated proteins. They include the kinases c-raf1, erbB2, ATK (PPKB) and cdk4 as well as mutant p53 and the catalytic subunit of the telomerase enzyme hTERT. Inhibition of hsp90 results in proteasomal degradation of client proteins via ubiquitination. Broad spectrum antitumor activity is seen when hsp90 activity is inhibited. The benzoquinone ansamycins (e.g. geldanamycin) were the first compounds to be shown to modulate hsp90 by binding to the ATP-binding site of the heat shock protein. At therapeutic dose levels of geldanamycin, however, there were unacceptable liver toxicities which subsequently prevented the compound from being developed further. Through studies at the US-NCI it was discovered that the geldanamycin derivative 17AAG (17-allylamino-geldanamycin) retained activity against hsp90 whilst showing significantly less hepatotoxicity. Due to difficulties in formulation of 17AAG further efforts were made from which 17DMAG (17-(dimethylaminoethylamino)-17-demethoxygeldanamycin) emerged. 17DMAG is the first watersoluble hsp90 inhibitor. This compound shows promising results in that it is more potent than 17AAG, both *in vitro* and *in vivo*. In a panel of over 50 tumors, the overall IC₇₀, as measured by clonogenic assay, of 17DMAG was a third of the value determined for 17AAG (24nM versus 77nM). Responsive tumor types were melanomas, breast, renal, colon, sarcomas and pancreas carcinomas. 4 s.c. growing models, 2 17-DMAG sensitive (MEXF 276, MEXF 989) and 2 resistant (MEXF 514, MEXF 462), were chosen for nude mouse testing and evaluation of Hsp90 inhibition. If treated i.v. the growth of MEXF 276 (T/C = 10%) and MEXF 989 (T/C = 6%) melanomas was markedly inhibited, whilst MEXF 514 and MEXF 462 (T/Cs > 60%) showed no response. Furthermore, in the MEXF 276 model, oral 17-DMAG administration led to sustained growth inhibition over 4 weeks (T/C = 27%). The profile of response determined by the clonogenic assay was strikingly similar between 17AAG and 17DMAG suggesting that the two compounds show the same mode of action. Analysis of the effects of 17AAG and 17DMAG on the target protein hsp90 as well as a range of client proteins verified this observation. Both agents reduced the levels of erbB2, c-raf1 and phosphorylated AKT in the sensitive melanoma cell line MEXF 276L whilst these proteins were unchanged in the resistant line MEXF 514L.

Taken together these results indicate Our data indicate that 17DMAG has a more potent antitumor activity than 17AAG which warrants further studies and especially clinical evaluation.

L16

A Fully Integrated Technology Platform for Drug Discovery Targeting Protein Kinases in Cancer

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ProQinase GmbH is a wholly owned subsidiary of the Tumor Biology Center, Freiburg. The biotech company was founded in 2001 with the goal of developing next generation protein kinase inhibitors for cancer therapy. The basis for its drug discovery program is ProQinase's *Integrated Technology Platform Protein Kinases*. This platform has been developed by scientists at the Tumor Biology Center over several years and has been successfully partnered with pharmaceutical companies in the past. A joint venture between the Tumor Biology Center, Novartis and Schering AG led to the identification and development of an anti-angiogenic VEGF-R receptor tyrosine kinase inhibitor, currently enrolling into Phase III clinical studies. The *Integrated Technology Platform Protein Kinases* features all necessary modules for protein kinase directed early drug discovery in oncology. Pro-

teomics, genomics and functional cloning approaches are taken in order to identify novel protein kinase sequences relevant for human cancer. 'Matched pairs' of human tumor tissues (i.e. cancerous and healthy biopsies from patients) obtained from the University Hospital in Freiburg are analyzed by differential proteomics and cDNA chip approaches. Protein kinase sequences that are over-expressed in a significant number of the analyzed samples are further validated in in-vivo validation models. These therapeutic validation models aim at blocking a given kinase activity and/or expression by inducibly expressing either dominant-negative protein kinase mutants or siRNAs in an already established mouse tumor. Alternatively, tumors are conditionally generated in nude mice by inducibly expressing a wildtype protein kinase sequence and by evaluating the effect of turning off the protein kinase expression in the animal. Both in-vivo approaches have been validated using protein kinase sequences known to be involved in human cancer. Currently, 10 additional protein kinase sequences are under clinical and therapeutic validation that are either novel kinase sequences or have not been implicated in cancer etiology so far.

ProQinase aims at developing highly specific protein kinase inhibitors directed against several of the identified protein kinases. Therefore, novel as well as public domain protein kinases are cloned and produced recombinantly in order to have as many protein kinases at hand as possible. Up to now ProQinase has cloned and expressed more than 50 human protein kinases, making it one of the most comprehensive collection of recombinant kinases in one company and available at HTS quantities. ProQinase is using its protein kinase panel for parallel high-throughput screening of chemical libraries using multiple protein kinase targets from different stages of tumor initiation and progression. Hits from the screening campaign are rapidly profiled using all available 50+ protein kinases, resulting in broad inhibition profiling of a large number of compounds in very short time frames. Compounds exhibiting inhibitory activities towards selected protein kinase targets from different tumor stages are further characterized using ProQinase's cellular and in-vivo tumor models.

Currently, ProQinase is pursuing 8 drug development programs – 3 in-house projects, 3 academic collaborations and two programs together with biotech companies. Recently, ProQinase and 4SC AG (Martinsried, Germany) announced a co-development partnership with the goal of developing multi-target specific protein kinase inhibitors up to early clinical testing. Negotiations with a US based company for a similar co-development deal are currently under way.

From its in-house programs ProQinase has identified a promising multi-target specific protein kinase inhibitor that specifically targets two protein kinases involved in cell proliferation and tumor angiogenesis, while showing no inhibitory activity towards 50 other protein kinases tested. For this compound a patent has already been issued.

As a second basis for its commercial success ProQinase is offering parts of its *Integrated Technology Platform Protein Kinases* on a fee-for-service basis to pharmaceutical and biotech companies worldwide. The revenues created by these activities are used for the support of internal R and D activities. Since ProQinase's foundation in 2001 the company has established itself as one of the leading service providers in the field of protein kinase directed drug discovery. Customers include major pharmaceutical and biotech companies worldwide. Currently 27 people are working for ProQinase.

L17

In Vivo Target Validation Employing Orthotopic Tumor Models and RNA Interference

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Many late stage tumors exhibit inactivating mutations in the tumor suppressor PTEN. PTEN is required for the controlled and transient activation of the phosphatidylinositol (PI) 3-kinase pathway that regulates a variety of cellular responses. Loss of PTEN function correlates with increased metastatic cell growth through hyperactivation of the PI 3-kinase signaling pathway. To identify downstream effector targets in this pathway, we applied independent 'gene silencing' technologies including RNA interference and antisense to inhibit PTEN expression. Genome wide gene expression profiling was performed and several PI 3-kinase downstream targets were identified. In order to validate these candidate genes as potential drug targets their functional role in tumor formation and metastasis had to be established.

Here we demonstrate the application of different gene silencing technologies to analyse the loss of function phenotypes in cell based assays and in

different mouse models. In addition, we describe the development and application of a new inducible RNA interference expression system that is based on the tetracycline repressor and eukaryotic RNA polymerase III promoters (U6, 7SK). For proof of concept we selectively inhibited expression of two catalytic subunits of the PI 3-kinase, p110 α and p110 β , by using vector-derived short hairpin RNAs (shRNAs). Stable pools of human prostate cancer cells (PC-3) expressing these shRNAs in an inducible fashion were established and analysed for their invasive potential *in vitro* and in an orthotopic (xenogeneic) metastatic mouse model. The application for RNA interference in combination with orthotopic mouse model will allow for a more comprehensive analysis of genes involved in tumor formation and/or metastasis.

L18

Novel Inhibitors of Cyclin Dependent Kinases Derived from Indirubin, the Active Constituent of an Antileukemia Medication Clinically Used in China

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The 3,2'-bisindole indirubin has been identified more than 3 decades ago as the active principle in a traditional Chinese medicine herbal preparation utilized against certain types of leukemias. Indirubin has been demonstrated in clinical studies to exhibit marked efficiency against chronic myelocytic leukemia and only minor toxicity. Several potential mechanisms of action have been proposed. We have recently discovered that indirubins are potent inhibitors of cyclin-dependent kinases (CDKs), by competing with ATP for binding to the catalytic kinase site. Since aberrant CDK activity and the associated disturbance of cell cycle control is closely associated with a wide spectrum of human cancer pathologies, CDK inhibition is one of the most promising molecular targets for innovative therapy. The 3,2'-bisindole core of indirubin offers an attractive lead structure of natural origin with already clinically established activity. Structure activity studies were based on crystal structure analysis of CDK2-inhibitor complexes and on studying effects on isolated kinases as well as molecular events in tumor cells, effects on the cell cycle, growth inhibition and apoptosis induction. These studies have lead to the synthesis of highly promising novel candidates for clinical testing.

L19

Bisimidazoacridones and Related Compounds: Potent New Agents Against Gastrointestinal Cancers

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Bisimidazoacridones and the closely related bistrizoloacridones (collectively termed BIAs) are synthetic compounds that bind to DNA. Some of these bifunctional agents are very effective in arresting the growth of tumor cells in culture and in vivo. One of these compounds, WMC26 was found to be particularly active against colon cancers, where it caused complete growth arrest at low nanomolar concentrations. The arrest occurred at both the G1 and G2-M cell-cycle checkpoints, and the most sensitive cell lines had wild-type p53 tumor suppressor gene. Removal of the drug resulted in the resumption of growth. However, if the growth-arrested cells were treated with the cdk inhibitors UCN-01 or flavopiridol, cell death by apoptosis occurred within 24 hrs. The data are consistent with DNA-damage induced up-regulation of p53 and p21, resulting in growth arrest. A closely related compound, the bistrizoloacridone derivative, temacrazine, had lower anti-tumor activity but showed extremely potent and highly selective activity against the HIV-1 virus, where it blocked the initiation of transcription of the integrated viral genome. Biophysical studies on the mode of binding of BIAs to DNA revealed that one of the planar imidazoacridone moieties was intercalated into DNA while the other, together with the linker between them, was located in the minor groove of the nucleic acid. The data on these compounds suggested that the mode of action of the BIAs involved the reversible binding of the compounds to DNA followed by the capture of a critical protein involved in repair and/or transcription. The unsymmetrical mode of binding suggested that the bifunctional agents did not have to be symmetrical. Consequently, a second generation of compounds

was prepared wherein one of the imidazoacridones moieties was replaced by different, potentially intercalating residue. One of these agents, WMC79, whose structure consisted of a imidazoacridone moiety at one end of a linker and a 4-nitronaphthalimide moiety at the other, was found to be highly cytotoxic to various GI tract human tumor cell lines including colon, pancreatic and liver cancers. In p53 wild type tumors, death by p53-mediated apoptosis was evident but other pathways were invoked in mutated p53 or p53-null tumors. The compound showed potent activity against colon cancer in vitro and in vivo and excellent activity against pancreatic cancer. It also showed very potent in vitro and in vivo activity against orthotopic liver cancer in the rat. The molecular targets of WMC79 appear to be topoisomerase I and topoisomerase II, where its activity appears to be stronger than that of the standard agents, camptothecin and doxorubicin. Thus, WMC79 is a novel agent that is a potent inhibitor of two critical enzymes that are involved in transcription and replication. WMC79 appears to be well tolerated and no systemic toxicities were observed during the in vivo experiments.

L20

Xenograft Tissue Microarrays: Applications in Characterization and Validation of New Molecular Targets

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Recent advances in oncogenomics particularly by using DNA microarray technology have led to the discovery of a vast amount of cancer related genes. Latter might prove valuable therapeutic targets or markers for diagnosis and prognosis of cancer. However, their characterization and validation is still very time-consuming. To accelerate the evaluation of gene expression in multiple tumor types and to examine target modulation, we have employed tissue microarrays of human tumor xenografts and cell lines. The Freiburg xenograft (XF) collection comprises more than 400 human tumors of over 20 different organs which grow subcutaneously in serial passage in nude mice [1]. Patient characteristics such as tumor grade, stage and survival as well as the response to standard therapeutic agents in patient and mouse are known. Microarrays were assembled from >200 formalin-fixed, paraffin-embedded tumor xenograft samples using a tissue arrayer (Beecher Instruments). 4 µm sections of the resulting microarray block were cut and transferred onto glass slides by employing the paraffin-sectioning aid system (Instrumedics) [2]. The expression of 2 established (c-erb-B2/her2, VEGF) and 2 experimental molecular markers/targets (cathepsin B, HSP 90) was studied using standard immunohistochemistry procedures (Histostain SP kit, Zymed).

We found differential expression patterns in 20 tumor types for all antigens examined. Significantly higher c-erb-B2/her2 levels were observed in adenocarcinomas of the lung, colon cancers and breast tumors compared to small cell lung cancers or pleural mesotheliomas. Thus, confirming published data, namely that breast cancers and adenocarcinomas of the lung are good target tumors for anti-her2 therapies e.g. Herceptin™. Interestingly, the chaperon protein Hsp90 which activates erbB-family members was co-expressed with c-erb-B2/her2 (Pearson correlation coefficient, $r = 0.5$). Hsp90 was down-regulated in biopsies taken from 6 different 17-AAG treated tumors growing s.c. in nude mice compared to vehicle controls. Moreover, the decrease in Hsp90 expression was dependent on duration of 17-AAG administration. These data suggest that the measurement of Hsp90 levels could serve as surrogate marker for evaluation of clinical efficiency.

A high cathepsin B content was observed in pleural mesotheliomas, colon and bladder cancers, whilst the protease was low in breast and small cell lung tumors. Many chemical target approaches such as polymer therapeutics employ the expression of tumor-associated proteases for pro-drug cleavage and activation. Such agents might best be evaluated in colon and bladder cancers and pleural mesotheliomas.

Tissue microarrays can accelerate the transition from basic scientific knowledge into clinical applications by means of high throughput target validation and parallel analyses of multiple tumor types.

- 1 Fiebig et al., in: Relevance of tumor models for anticancer therapy. Contrib Oncol, vol 54, pp 29–50, 1999.
- 2 Kononen et al., Nat Med 1998;4:844–847.

5th Session • CELLULAR AND TARGET SCREENS

L21

The US-NCI Cell Based Screen: The Role of Target Expression Profiling in Developmental Therapeutics

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Following a shift in emphasis to advance the discovery and development of molecularly targeted agents, the Developmental Therapeutics Program of the US National Cancer Institute has continued to focus efforts on the molecular profiling of 60 cell lines in its anti-cancer drug screen in order to characterize expression of potential cancer related targets. Moreover, the success of the COMPARE approach in identifying mechanism of action from drug response profiles was extended to the hypothesis that expression of cellular targets or molecules might also allow for associations between drug/target patterns in addition to the well-defined drug/drug patterns. This approach was validated by the high correlations between expression of the *mdr1* gene and cellular resistance to drugs included in the multi-drug resistant phenotype. Since then, a variety of other associations have been explored following measurement of expression (protein or gene) of certain target including regulators of apoptosis, transporters, repair proteins, metabolizing enzymes, activated oncogenes and a growth factor receptor. Moreover, using two different microarray technologies, general transcription profiling was undertaken to compare basal expression of thousands of genes across the cell line panel and attempt to recognize possible relationships with available drug profiles.

Regulators of apoptosis are considered important targets in cancer therapy that may significantly impact drug sensitivity and a variety of them were profiled in the 60 cell lines. Inhibitors of apoptosis were measured both at the gene and protein level, but there were no meaningful correlations of these profiles with drugs of known mechanism. In contrast, BID protein expression (a BCL2 agonist) correlated with sensitivity to ribonucleotide reductase (RR)-inhibiting drugs, and in confirmatory experiments, over expression of BID in a model tumor cell line by gene transfection resulted in increased sensitivity to apoptosis induction by a RR inhibitor. Although the 60-cell line pattern of Fas-associated phosphatase-1 (FAP-1) protein expression did not correlate with fas-induced apoptosis, when the data was broken down by disease type, there was a strong association between FAP-1 expressions with fas-induced apoptosis in ovarian cell lines. This may play an important clinical role as almost all ovarian tumors evaluated from tissue arrays expressed the FAP-1 protein. In an attempt to correlate drug response with expression of DNA repair proteins, the cellular levels of the two mismatch repair proteins most commonly mutated in human cancers; MLH1 and MSH2 were evaluated by Western blot. MLH1 deficiency was more common than MSH2 deficiency and was always associated with a high degree of temozolomide resistance, but there was no significant correlation with sensitivity to any standard anticancer drug. Furthermore, several nucleotide excision repair proteins XPB, XPD, XPA and ERCC-1 that have been implicated in anticancer drug resistance in human tumor cell were measured, but only XPD was found to have an association that indicated XPD expression might confer resistance to alkylating agents.

In an effort to facilitate identification of anticancer agents whose activity can be altered via cytochrome P450 (P450) metabolism, P450 enzyme expression patterns were determined. All 60 tumor cell lines displayed significant P450 activity, as well as P450 reductase activity, and significant negative correlations were found by COMPARE between the patterns of P450 activity and cell line chemosensitivity to a group of topoisomerase inhibitors, to 10 standard anticancer agents (including 6 alkylating agents) and 55 investigational compounds, suggesting a role for P450 metabolism in the inactivation of these agents. In contrast, there were no relationships observed between the pattern of expression of eight known nucleoside and nucleobase transporters in 50 of the cell lines, and cytotoxicity, based upon known interactions between certain nucleoside analogues and transporter proteins, suggesting that expression of individual transporters may not be a significant determinant of the cytotoxicity of these drugs.

Associations between target expression and drug response were also illustrated by the correlation of expression of ras mutations with drug response patterns for 1-beta-D-arabinofuranosylcytosine, 2,2'-O-cyclocytidine, and topoisomerase II inhibitors indicating drug sensitivity in the cell lines harboring ras mutations compared to the tumor lines with wild-type ras alleles. Additional investigation revealed that activated ras oncogenes enhanced the sensitivity of human tumor cells to these drugs by potentiating an apoptotic response. Moreover, the HER 1 expression profile, an important

receptor involved in success of clinical responses to the HER1 (EGF receptor) inhibitors, showed, as expected, that low HER1-expressing cell lines were insensitive to HER1 inhibitors. However, although low concentrations of HER1 inhibitors potentially inhibited both HER1 phosphorylation and the mitogen-activated protein kinase pathway in cell lines with high HER1 expression, this inhibition did not always correlate with cellular arrest. Further study into the mechanism of resistance led to the conclusion that insensitivity to HER1 inhibitors in a large subset of cancer cells was independent of mitogen-mediated signaling.

In order to provide more extensive expression data, transcription profiling of all 60 cell lines was undertaken both with cDNA microarrays (8,000 gene transcripts) and oligonucleotide arrays (6,817 genes represented). The data from the cDNA arrays was used to correlate gene profiles from the cell lines with those from other tumor material of similar origin, and clinical tumor material. Moreover, it gave the opportunity to integrate large databases of gene expression and molecular pharmacology, and exemplify how variations in the transcript levels of particular genes relate to mechanisms of drug sensitivity and resistance. These data are available in a searchable format on the DTP website at (http://dtp.nci.nih.gov/mtargets/mt_index.html). In addition, the gene signatures generated from the oligonucleotide arrays suggested that at least for a subset of compounds, genomic approaches for chemosensitivity prediction were feasible. Moreover, a comprehensive suite of tools, developed within the Screening Technologies Branch of DTP, called 3d Mind Tools, and available at <http://spheroid.ncifcrf.gov/>, attempt to incorporate the biochemical screening and bioinformatic data generated from the 60 cell lines into the process of drug discovery. This approach uses screening data to build two dimensional maps which lend themselves to aid in biochemical understanding and hypothesis generation, and are readily expanded with other information such as known protein ligands and cellular gene expression.

Thus in the last few years, the focus of the NCI 60 cell line screen has shifted from the chemosensitivity patterns of new compounds in the 60 cell lines, to the profiling of molecular targets in these lines, but a combination of both these data sets has led to some valuable and comprehensive conclusions about the relationship between basal target expression and drug sensitivity or resistance.

L22

A Cellular High Throughput Screen for Anticancer Drug Discovery

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Modern anticancer drug discovery is based on primary screens in high throughput format in which thousands of compounds can be analyzed within a few days. Isolated target-oriented enzyme linked assays, cell proliferation assays, cytotoxicity assays and others are being used as tools to screen huge compound libraries for substances with anticancer activity.

For a large scale primary screening, we have developed an *in vitro* screening procedure based on permanent human tumor cell lines, representing various tumor types (colon, gastric, lung, mammary, melanoma, ovary, renal, and others). Importantly, most of these cell lines were derived from our tumor xenografts (see HH Fiebig, abstract L26), resulting in some significant differences compared with the NCI cell line panel. For example, most of our cell lines display relatively slow growth with doubling times of between 30 and 48 h. Moreover, they resemble the parental tumor xenografts and the original patient tumors in their histology and in their sensitivity to cytotoxic drugs. In order to maintain these characteristics we restrict the number of *in vitro* passages to a maximum of 20. We have also modified the testing procedures used at the NCI. The incubation period of cell lines with the test substances was extended to between 4 and 6 days. As a read-out, we use propidium iodide (PI)-based fluorescence (propidium iodide assay) which is a measure for the number of viable cells. Results obtained with the propidium iodide assay are comparable to the results obtained with the sulforhodamine B assay, which is used at the NCI. In conclusion, our screening procedure differs in important respects from the procedure used at the NCI. As a consequence, both screens are not redundant and will likely identify different sets of anticancer substances.

Based on the panel of 24 xenograft-derived cell lines, a three stage strategy of compound identification and profiling was established at Oncotest. In a first screen, compounds are tested at one high concentration (eg 1–10 µg/ml) in a panel of 8–10 cell lines, with a throughput of >2000 compounds per week. Compounds that are active in this first screen are next tested in a

panel of 12 cell lines at 5 different concentrations. Main evaluation criteria are anticancer activity (IC₇₀) and tumor selectivity. In a third phase, following tests with additional cell lines, the tumor cell line selectivity profiles of compounds will be compared with the profiles of well established anticancer agents such as standard cytotoxic drugs or compounds in clinical trials. Moreover, anticancer activity of test substances will be correlated with the status of 35 cancer related drug targets and with the gene expression pattern of 12000 genes represented on the Affymetrix chip. For a sound comparison of tumor selectivity patterns of compounds a panel of at least 24 cell lines needs to be tested. The most promising compounds are further investigated in our clonogenic assay (tumor stem cell assay) and in *in vivo* therapy studies in our patient-derived xenografts growing in nude mice (see HH Fiebig, abstract L26). This testing procedure is currently used for the large scale screening of natural products.

L23

Robotic Screening for the Identification of Novel Cytotoxic Compounds

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Cancer chemotherapy began with the development of the alkylating agent Cyclophosphamide (Endoxan®) in 1952, an oxazaphosphorin pro-drug activated preferentially in the tumor. Based on the concept of a 'transport form and active form' by Druckrey and Brock, the therapeutic index of Cyclophosphamide was 5–10 times higher as for cytostatic drugs available at that time. Since these landmark achievements in cancer therapy, molecular biology changed our understanding of cancer fundamentally. Today cancer is described as a disease involving dynamic changes of the genome leading to acquired functional capabilities, namely limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion from apoptosis, sustained angiogenesis and finally tissue invasion and metastasis. As a consequence of the overwhelming success of technological achievements in molecular biology, the strategy of anti-cancer drug discovery and development also changed. Molecular targets with a causal pathophysiological relevance were defined and, by using biochemical and target-dependent cellular assays, compounds were selected either by robotic screening of diverse compound archives or by manual screening. In this regard, a landmark was the approval of the bcr/abl kinase inhibitor Gleevec (Imatinib®) for the treatment of chronic myelogenous leukaemia (CML). CML with the Philadelphia chromosome translocation is a rare example for a monocausal genetic aberration leading to malignancy and therefore perfectly suited for a targeted approach. In parallel biologicals became apparent as a drug format for cancer therapy with Trastuzumab (Herceptin®) and Rituximab (Rituxan®) targeting the receptor tyrosine kinase erbB2 and the CD20 B-cell antigen, respectively, as examples.

The driving force for developing target specific cancer drugs again was a higher therapeutic index and an option for therapy of cancer patients resistant to standard chemotherapy. Without doubt the strategy is successful but there are several pitfalls. Experimental validation of targets is a cumbersome task, and finally a target is validated by the clinical success of a drug. Specificity is a critical issue if the target is a member of a multiprotein family or involved in crucial signaling pathways. Inhibitors of protein kinases or small GTPase like ras are two examples and selectivity in biochemical assays does not necessarily predict selectivity in cells. Typically, the molecular alterations within tumors are multiple and complex. Thus, combination therapy is the standard in the clinic and molecular targeted drugs have to show additive or synergistic efficacy in standard treatment regimens. In this regard, the failure of the EGF receptor kinase inhibitor ZD1839 / Iressa® to show clinical benefit in combination with Gemcitabine / Cisplatin or Carboplatin / Paclitaxel in NSCLC patients demonstrated the risks of this approach.

Cytotoxic drugs are an integral part of current cancer therapies and will stay so in the future. To be innovative, novel cytotoxic drugs need to have a high therapeutic index, a new mode of action, preferable oral bioavailability, a simple chemical structure (small molecule) and efficacy in cancer indications with a high unmet medical need. Histone deacetylase inhibitors like SAHA currently in phase II clinical trials and the proteasome inhibitor PS-341 / Velcade® approved for relapsed/refractory multiple myeloma are two recent examples. Thus, search for novel cytotoxic drugs is reasonable and balancing the risk of today's targeted approaches. We decided to screen randomly the ALTANA Pharma compound collection (currently about

250,000 diverse compounds) in cell based assays for activators of the effector caspase 3 and inhibitors of proliferation in the A549 and H460 NSCLC and RKO colon carcinoma cell lines. For caspase 3 activation, a non-lytic assay based on a cell permeable Z-DEVD-R110 substrate was established and validated with apoptosis inducers like Staurosporin, Etoposide and TRAIL. The proliferation assay is based on reduction of Alamar Blue (Resazurin) to the pink and highly fluorescent product Resorufin and quantifies cellular dehydrogenase activity. Compared to the caspase 3 assay, this approach is broad and compounds with cytotoxic or cytostatic activity are selected.

The HTS campaigns were done in a 384-well format with all compounds available at that time. In the Alamar blue proliferation assay the Z factor was 0.46 (H460 cell line) and primary hit rates were 3.5% (8731 hits) and 1.4% (3135 hits) in the H460 and RKO cell lines, respectively (inhibition $\geq$ 70% after 72h). In the caspase 3 activation assay the overall primary hit rate was 1.2% (1914 hits) in the A549 and RKO cell lines (caspase 3 activation $>150\%$ at 6h and $>200\%$ at 20h). In both assays, all hits were retested and a dose-dependency was determined for qualified hits. A key success factor was selection for compounds having the desired activity profile: cell cycle and tumor cell specificity. As an integral part of the robotic secondary screening, selection filters and profiling screens were included: caspase 3 activation in normal human bronchial epithelial (NHBE) cells, cytotoxicity towards arrested (non-proliferating) cells and caspase dependent cytotoxicity. For selecting compounds active only towards cycling cells, a genetic engineered RKO cell line with inducible expression of the cell cycle inhibitor p21^{waf1} was applied (Oncogene 19: 2423–2429, 2000). Switching on p21^{waf1} by addition of Ponasteron A causes complete cell cycle arrest in G1 and G2. In this cellular system, drugs dependent on cell cycle progression like Taxol, Etoposide or 5-FU display specificity factors of 50 up to >3000 . In contrast unspecific cell poisons like Arsenic, Quabain or Amanitin have specificity factors around 1, meaning that they kill cycling and quiescent cells at equal doses. By using this profiling screen, unspecific cell poisons are discarded. For the caspase 3 activator HTS campaign, the secondary screening is completed. About 150 selected compounds were studied in more detail in defined target-specific assays to cluster compounds according to known mode-of-actions. The aim was to select compounds with a anti-mitotic, taxane-like activity profile but a target distinct from tubulin. The in-vitro profile of such a lead compound should be (i) sub μ M and broad anti-proliferative cellular activity, (ii) cell cycle specificity >50 , (iii) arrest in the M-phase of the cell cycle (high mitotic index) and (iv) potent caspase-specific induction of apoptosis. Regarding medicinal chemistry the lead compound should be selected from a cluster of structurally related compounds of low molecular weight and simple chemistry. Applying the biological criteria, 8 compound classes have been selected and by including objectives from the medicinal chemistry, 6 unpublished compound classes remain to be evaluated with high priority. As an example, an unpublished compound from cluster C010D is active in the Alamar blue assay towards A549 and RKO cell lines with $IC_{50} = 0.06 \mu$ M (A549) and 0.61μ M (RKO) in a caspase dependent manner, a specificity factor of 55 (RKO p21^{waf1} system), causes profound DNA fragmentation, raises the mitotic index more effectively as Taxol at $<0.3 \mu$ M concentration and does not interfere with tubulin.

In summary by using a cellular phenotypic HTS screening approach together with well defined robotic and manual secondary assays, compound clusters were selected with a very interesting cytotoxic activity profile. These clusters are currently evaluated for tolerability and efficacy in-vivo as well as studies to define the relevant molecular target or signalling pathway. Presumably these compound classes have targets relevant for mitotic progression and higher tumor cell specificity as standard chemotherapeutics. In this regard a significant higher therapeutic index as Taxol would be a major achievement in cancer chemotherapy.

L24

Three Dimensional Cellular Models and Hypoxia Selective Toxins

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The presence of hypoxia in tumours as a result of a poor or temporally occluded blood supply has profound implications for both tumour biology and cancer therapeutic outcome. Historically there has been considerable interest in the development of drugs that can eradicate hypoxic cells (radiosensitizers and bioreductive drugs) and more recently, there is considerable interest in developing agents that can interfere with hypoxia mediated cell

signalling pathways (via the HIF1 transcription factor). The key requirements for an effective hypoxia targeted therapy include; drug target interactions (eg activation by specific reductases), selectivity for hypoxic cells or cells rich in biochemical target expression, ability to eradicate non proliferating cells and the drug must not be adversely affected by other properties of the microenvironment (such as low extracellular pH). In addition, it is essential that hypoxia mediated therapies are able to penetrate through the aerobic fraction to reach the target cell population and that pharmacokinetic parameters are adequate to allow sufficient time for the drug to reach hypoxic cells. Many of these requirements can be assessed using cell free or monolayer culture techniques but multicell *in vitro* models (such as spheroids and multicell layers) are essential when addressing questions such as drug delivery.

The purpose of this presentation is to provide a specific example of how more complex 3-dimensional *in vitro* models can be integrated into a bioreductive drug development program. The benefits and limitations of such an approach will be illustrated using quinone based bioreductive drugs based on the compound EO9.

L25

Spherogenex™: Cellular Assays of Angiogenesis Inhibitor and Stimulator High Throughput Screening

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Endothelial cells (EC) are routinely grown in plastic monolayer culture which provides a suitable and readily available system to study EC functions under defined conditions. Yet, standard monolayer culture is less suitable for the analysis of more specialized EC functions as they relate to organotypic and vessel caliber specific differentiation and angiogenesis. We have developed a three dimensional organoid spheroidal EC cell culture system that offers a unique model to study vascular differentiation and angiogenesis (1, 2).

In addition to using EC spheroids as a cellular differentiation model, we developed a novel collagen gel based three dimensional in vitro angiogenesis assay based on the use of EC spheroids as a cellular delivery device. Gel embedded EC spheroids act as focal starting points for the sprouting of lumenized capillary-like structures that can be induced to form complex anastomosing networks. Formation of capillary anastomoses is associated with directional sprouting of outgrowing capillaries towards each other. To analyze, if directional sprouting is dependent on cytokine gradients or tensile forces exerted through the extracellular matrix, we designed a 'tension trimmer' that enables the application defined tensional forces on the extracellular matrix. Based on this assay, we present causal evidence that tensional forces on a fibrillar extracellular matrix, such as type I collagen but not fibrin, is sufficient to guide directional outgrowth of endothelial cells (3, 4).

The spheroid based angiogenesis assay has proven to be one of the most reliable and robust in vitro angiogenesis assays with little intraassay and interassay variation. The robustness of the assay allows reliable and reproducible IC_{50} determinations of angiogenesis inhibitors and has, thus, become a useful tool in the optimization process of angioinhibitory lead compounds. Likewise, we have taken advantage of the unique properties of the assay to develop an automated quantitative image analysis system that allows the screening of up to 1,000 compounds within a few weeks.

Screening of a chemical library of 900 compounds identified 83 angioinhibitory and 19 angiostimulatory compounds. Of these, 46 inhibitors and 12 stimulators were identified as endothelial cell selective compounds employing a secondary fibroblast gel invasion screen. These 48 compounds were tested for their specificity against a panel of 14 kinases identifying 12 compounds that had activity against individual or multiple kinases. Ongoing experiments are aimed at further validating the compounds employing an orthotopic in vivo tumor angiogenesis model.

Corresponding experiments have been performed to utilize the spheroid based angiogenesis assay as a screening platform for the genome wide search for novel genes of angiogenic endothelial cells employing VEGF and FGF-2 as stimulatory cytokines. This transcriptome analysis served to further validate the assay based on the pattern of known genes of angiogenic EC expressed in the spheroid model and yielded a total of 1,400 novel candidate genes of angiogenic endothelial cells. Validation of these candidate genes is presently pursued by scaling up RNAi throughput in EC for a systematic knockdown of all novel candidate genes in the spheroid angiogenesis assay.

In conclusion, the experiments have led to the implementation of a high efficacy screening and validation platform for the analysis of novel pro- and antiangiogenic compounds and EC molecules. Additionally, the transcriptome analysis of monolayer and spheroid EC provides a database pool for future vascular systems biology approaches and vascular pharmacogenomics.

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6th Session - IN VIVO MODELS AND THEIR CHARACTERIZATION

L26

Patient and Cell Line Derived Human Tumor Xenograft Models for the Discovery of Anticancer Agents

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For all major solid human tumor types experimental models have been developed by engrafting patient tumors or permanent human cell lines into immunodeficient mice. In 1989, the author transferred the NCI-60-cell line panel as xenografts into nude mice, 47 out of 58 cell lines showed a take and 35 grew regularly and were suitable for in vivo drug testing. The success rate of the nude mouse xenografting was further improved at the NCI and SRI to 82% (49 out of 60 cell lines, Plowmann et al. 1997). By histological examination 85% of the cell lines present in vivo an undifferentiated or very poorly differentiated picture without the typical tumor architecture seen in e.g. adenocarcinomas. On the average cell line-derived xenografts grow faster than patient-derived xenografts. When standard cytotoxic agents were evaluated, activity in tumor cell line-derived xenografts was mainly seen with alkylating agents.

During the past 15 years my group implanted more than 1600 solid tumors directly from patients, resulting in the establishment of more than 400 permanent xenograft models. The percentage of tumors established in serial passage was highest (38–51%) for melanomas, non-small cell lung and colon cancers as well as soft tissue sarcomas, intermediate (20–35%) for cancers of the head and neck, small cell lung, testicular, stomach, bladder and kidney, and low (5–18%) for cancers of the breast and prostate. Sixty tumors were characterized in detail for their sensitivity to standard cytotoxic agents as well as with regard to the status of 35 molecular drug targets and expression profiles of 12,600 genes present on Affymetrix chips. The response of established tumors treated in both the nude mouse and in the patient with cytotoxic standard agents was very similar, 90% (19/21) in sensitive and 97% (57/59) in resistant tumors. The high correct predictivity validates the xenograft system for drug development.

More recently, also target-directed compounds were identified as active. EGF-R and HSP-90 inhibitors (EMD72000 and 17-AAG) were able to induce remission and T/C values <10%. A CDK-inhibitor from a Chinese plant and the HuMV833 antibody against VEGF effected a tumor stasis with T/C values between 15–29%. Modulation of the respective target has been demonstrated by immunohistochemistry and Western blots. The combination of an EGFR inhibitor with Taxol in a NSC lung model and of HuMV833 with Gemcitabine in a pancreas model resulted in a marked synergism.

At Oncotest we follow 2 approaches for the discovery of novel anticancer agents. First, for the target-directed approach the presence of the target of interest in Oncotest's tumor collection is analysed at the gene expression level using data for 12600 genes represented on Affymetrix DNA arrays and/or at the protein level using immunohistochemistry and tissue microarray techniques (see abstract L20 Wirth et al). Second, for the random screening of compound libraries an initial testing is done in a cellular high throughput screen in a monolayer assay (see abstract L22 Kelter et al).

Next, to identify sensitive tumors, compounds of interest are assessed in the tumor stem cell assay. Finally, compounds will be tested in vivo in the most sensitive tumors expressing the target of interest. Examples of the evaluation of novel compounds will be presented.

In vivo activity is essential before novel compounds should be developed for clinical trials. Patient derived xenograft models are excellent models for studying the tumor biology of human cancer and for the discovery of target directed novel agents.

L27

Anticancer Drug Response and Expression of Molecular Markers in Early-Passage Xenotransplanted Colon Carcinomas

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Introduction: Novel rational approaches for a more selective cancer therapy need suitable preclinical test procedures resembling the clinical situation. Tumor cell lines used in vitro or in vivo only partially mimic tissue specificity, they are mainly undifferentiated and indicative for a general antiproliferative activity of a novel agent. On contrary, xenografts directly established from patient's material in immunodeficient mice maintain a close similarity with the clinical tumor and are useful models for regulation studies on genetic or protein markers. **Material and methods:** Surgical samples of colorectal carcinomas were directly transplanted to nude mice. After successful growth and serial passaging they were characterized for response to 5-FU (30mg/kg i.p. qd 1–5), 5-FU+Leucovorin (40+10 mg/kg i.p. qd 1–5), Irinotecan (15 mg/kg i.p. qd 1–5), Oxaliplatin (5 mg/kg i.p. qd 1–5). Therapeutic efficacy in mice determined as treated/control values was compared with the clinical course of disease. Molecular markers like EpCAM, E-cadherin, CEA, Ki67, Topoisomerase I and II, p53, and K-ras mutations were determined on protein or RNA level. **Results:** In total, 35 surgical samples from colorectal carcinomas (TNM classification T2 to T4, 33 from primary lesions, 2 from liver metastases) were s.c. transplanted to immunodeficient mice. 15 (43%) grew progressively over more than 3 passages and were used for further characterization and therapeutic experiments. The doubling times in vivo ranged from 4.2 to 32.9 days. Both take and growth rate in mice did not correlate with the clinical tumor stage or presence of metastases.

The response rate of the xenografts to chemotherapeutic treatment was as follows:

5-FU	5/15 (33%)
5-FU + Leucovorin	5/10 (50%)
Irinotecan	14/15 (93%)
Oxaliplatin	8/14 (57%)

Five out of 15 patients got chemotherapy for treatment of synchronous metastases. The clinical response correlated very clearly with chemosensitivity profile of the xenografts (see Table). For instance, one patient (V.D.) was treated with 5-FU/Leucovorin as first line therapy. Due to a lack of response, second line therapy of Oxaliplatin and Irinotecan was given resulting in a decrease in tumor marker CEA and shrinkage of liver metastases. The corresponding xenotransplanted tumors were found to be 5-FU resistant but sensitive to both Oxaliplatin and Irinotecan.

All tumors expressed the glycoprotein EpCAM and the carcinoembryonal antigen (CEA) on their surface. During passaging for not more than 10 generations in mice the portions of cells expressing these markers and the histologically malignant areas increased while clearly the architecture and the characteristics of the original tumor were maintained.

All xenografted tumors demonstrated nuclear positivity for the proliferation marker Ki67 and 9/14 (64%) were positive for the tumor suppressor protein p53. Although a positive relationship was registered for Ki67 and p53 expression (R = 0.69), no correlation was found between these markers and xenograft growth rate or clinical tumor staging.

The high levels of topoisomerase I alpha expression correlated well with the high response rate of the xenografts to Irinotecan, a Topo I inhibitor.

We were interested in the detection of potential K-ras mutation in the transplantable colorectal carcinomas and analysed it with a specific genotyping test kit. Point mutation were detected in 5/15 xenografts in codon 12

(concerning the amino acids serine, asparagine or valine) and 2/15 xenografts in codon 13 (asparagine). Xenografts containing mutations in codon 12 had a poor response to Oxaliplatin.

Table: Course of disease and chemotherapeutic response of patients with synchronous metastases

Patient	Date	Clinical course of disease	Treatment	Response	Xenograft result
S.K.	03/89 05/89 18/89 18/89 01/93	2 months head/neck CT rectum and liver resection progression of liver metastases resection of liver metastases alive with stable disease	5-FU 1 1	progression of liver metastases complete remission complete remission	5-FU - ++++
V.D.	11/88 03/89 02/91	bowel resection resection death	6 months 5-FU / LV 4 months O / I 4 months I / 5-FU / LV	progression of liver metastases partial remission partial remission	5-FU - +++ 0++
E.H.	06/89 16/89 04/92 01/93	sigmoid resection progression of liver metastases progression of liver metastases alive with stable disease	13 months 5-FU / LV intravenous 3 months Capecitabine O / 5-FU	stable disease stable disease partial remission	5-FU (+) 0++
W.H.	06/89 06/89 01/91	rectum resection lung metastases death	6 months 5-FU / LV intravenous 3 months 5-FU / LV	partial remission of liver metastases progression	5-FU - 5-FU / LV -
G.L.	07/89 04/92 18/92	rectum resection lung metastases alive with stable disease and local relapse	4 months O / 5-FU / LV 7 months 5-FU	complete remission of lung partial remission of liver metastases progression	5-FU - 0+++

- no activity ** TIC 21-35% *** TIC 5-20% **** TIC 0-5%
CT Chemotherapy O Capecitabine I Irinotecan 5-FU 5-Fluorouracil LV Leucovorin

Conclusion: Our study on the molecular characterization of colon cancer xenografts in early passages revealed a high coincidence in histology and chemotherapeutic response with the original patient tumors. The well characterized models are useful tools for the rational preclinical development of novel target oriented therapeutic approaches and for investigating translational research aspects.

L28
Imaging Tumor Progression *in vivo*

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Mouse models of metastatic cancer with genetically fluorescent tumor cells that can be imaged in fresh tissue, *in situ*, as well as externally have been developed. These models have opened many new possibilities including real-time tumor progression and metastasis studies on internal organs and real-time drug response evaluations. The green fluorescent protein (GFP) gene, cloned from bioluminescent organisms, has now been introduced into a series of human and rodent cancer cell lines *in vitro* to stably express GFP *in vivo* after transplantation to metastatic rodent models. Techniques were also developed for transduction of tumors by GFP *in vivo*. With this fluorescent tool, tumors and metastasis in host organs can be imaged down to the single cell level. GFP tumors on the colon, prostate, breast, brain, liver, lymph nodes, lung, pancreas, bone, and other organs have also been visualized externally, transcutaneously by quantitative whole-body fluorescence imaging. Real-time angiogenesis has also been imaged and quantified using GFP technology. The GFP technology enables a fundamental advance in the visualization of tumor growth and metastasis in real time *in vivo*.

L29
Models for Prostate Cancer: Their Use in Fundamental and Preclinical Research

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Basic and preclinical research of prostate cancer has long been hampered by the limited availability of relevant model systems. Although much knowledge has been obtained with such models like the rat ventral prostate and the Dunning rat model system, the need for human-derived models was obvious. Since 1976, the department of Urology of the Erasmus MC has put a lot of effort in establishing human prostate cancer cell lines and xenografts. In 1991 a number of seven new human prostate cancer xenografts could be added to the already existing panel of 4 xenografts. With the addition of some sublines originating from one of the xenografts the present panel consists of 13 xenografts of androgen responsive and unresponsive human prostate cancer (see table below and reference van Weerden et al.). Unfortunately, so far only from two xenografts permanent *in vitro* cell lines could be established: PC-346C (derived from the PC-346 parental line) and PC-346mAR (derived from the PC-346I). This androgen responsive PC-346 system has proven very powerful as from the PC-346C cell line several treatment-resistant, androgen independent sublines have been created.

Both xenograft models and cell lines have been very instrumental in a wide variety of basic and preclinical research. Although some of the xenografts are strictly androgen dependent and represent the early stages of prostate cancer, and others are already androgen independent, the PC-346 model is androgen dependent, but progresses in time towards androgen independency, creating a model system to study the mechanisms of prostate cancer progression. The establishment of PC-346C treatment-resistant sublines in culture has resulted in an interesting panel of sublines with different mechanisms of androgen independent growth which further adds to the possibilities to study the various mechanisms of tumor progression in this tumour system.

The presence of unlimited sources of pure human cancer specimens (either hormonally manipulated or not) make the xenografts valuable tools for the microarray technique. CGH arrays have been performed to detect common areas of chromosomal changes and gene arrays compare genetic expression patterns of androgen dependent and androgen independent xenografts. The xenograft models are also valuable for preclinical research in which new compounds or new drug modalities are tested for their suitability in the clinical setting. Since the xenografts represent the different stages of prostate cancer, a model system can be selected from the panel that resembles the patient group most. Also, the presence of xenograft models secreting PSA (prostate specific antigen) increase their use for preclinical evaluation of new therapies. An experimental study may not only evaluate the efficacy of the compound, but is also able to show whether tumour volume changes coincide with plasma PSA changes. If PSA follows tumour volume changes under treatment, this would allow to use PSA as an indicator of treatment effect and thus be regarded as a surrogate end point for clinical phase II studies. This would significantly reduce the time needed for such trials where at present progression-free survival is the end point (see reference Schröder et al.).

The availability of the PC-346C cell line system also allows orthotopic growth studies. This is especially relevant for those new drug developments that are target-directed and need a critical (prostate) micro-environment. We have developed a transrectal ultrasonographic technique for the mouse prostate which allows us to follow prostate tumour growth in one animal on a twice weekly basis (see reference Kraaij et al.). In addition, the orthotopic technique allows metastatic spread to be studied. Although the PC-346C cells have proven to spread to lymph nodes and lung, no macroscopic outgrowth can be observed within the life span of our animals as yet. Also, we have not been able yet to establish a subline with an increased metastatic ability.

The PC-346C orthotopic model has recently been used to evaluate the role of dietary supplements in prostate cancer. Initial studies using vitamin E and lycopene as supplements have been positive and indicate that plasma PSA may indeed (in this study) be used as a marker for treatment effect. Further studies are now focusing on the possible mechanisms of growth delay caused by such supplements. Also, other (soya-based) supplements are being included for testing.

The Rotterdam Prostate Cancer Xenograft Panel

Tumour model	Established	Origin ¹	AD/AI ²	AR ³	PAP ⁴	PSA ⁵
PC-82	1977	PC	+	+	+	+
PC-133	1981	bone	-	-	-	-
PC-135	1982	PC	-	-	-	-
PC-295	1990	LN	-	-	-	-
PC-310	1991	PC	+	+	+	+
PC-324	1991	TURP	+	+	+	+
PC-329	1991	PC	+	+	+	+
PC-338	1991	TURP	-	-	-	-
PC-346 ⁶	1991	TURP	+	+	+	+
PC-346i			+	+	ND ⁸	+
PC-346B			+	+	ND	+
PC-346B1			+	+	ND	+
PC-374	1992	SSM	- ¹⁰	+	+	+

¹PC = primary prostate tumour; LN = lymph node metastasis; TURP = transurethral resection of the prostate; SSM = scrotal skin metastasis. ²AD = androgen dependence (+); AI = androgen independence (-). ³AR = human androgen receptor. ⁴PAP = prostate acid phosphatase. ⁵PSA = prostate specific antigen. ⁶PC-324 expresses wild type AR, but the AR pathway is inactive and unresponsive. ⁷PC-346 is derived from a non-progressed TURP patient and meanwhile 3 sublines have been derived from this xenograft. ⁸AR mutation (T877A). ⁹ND = not determined. ¹⁰PC-374 is still sensitive to androgen treatment.

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L30

The Role of Neurotransmitters in Metastasis Development and their Inhibition by Specific Receptor Antagonists

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Malignant tumors of solid tissues initially arise as localized diseases which can be often successfully treated by surgery if detected early. Because of these successful operative procedures, complications in cancer treatment have shifted; over 95% of patients suffering from cancer do not die from the primary tumour but from the development of metastases. Due to the spreading of metastatic tumour cells, the chemotherapy, a more systemic approach, has been developed to destruct tumour cells anywhere in the body. Chemotherapy has thus led to great successes in the treatment of cancer. Many tumour cells, however, are unaffected by this therapy, known as dormant cells, which are able to grow into a recurring tumour months or even years later. Thus, an important aim in the treatment of a diagnosed cancer must be the inhibition of metastasis development and the inhibition of tumor cell migration as a prerequisite for this. Whereas long-standing theories have addressed the genetic predisposition of tumor cells to migrate, recent focus has shifted to the study of epigenetic factors regulating migration. The most prominent of these are ligands to serpentine receptors, e.g. chemokines and neurotransmitters, which are vital in the regulation of migration and localization of leukocytes. The involvement of psychosocial factors in the incidence and progression of cancer, which has been discussed for decades, is further supported by the large variety of serpentine receptors which are known to be expressed on tumor cells. The classic stress hormone norepinephrine, for example, is known to induce the migratory activity of cancer cells of various tissue origin. This migration can be inhibited in vitro by beta-blockers, which have been established for the treatment of cardiovascular disease. Most importantly, the norepinephrine-induced migration of tumor cells is directed. Such chemotactic movement might explain the preservative pattern of metastasis localization. In conclusion, the understanding of the regulatory involvement of neurotransmitters in migration and the availability of specific receptor antagonists may deliver new tools for the chemoprevention of tumor cell migration and metastasis development.

L31

Hollow Fiber Assay

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The goals of pre-clinical *in vivo* model studies include the prioritization of compounds for eventual clinical test. Commonly used *in vivo* models include transplantable rodent tumors syngeneic with their host, and human tumor cells propagated in athymic mouse xenografts. In either case, the cost of putting compounds through multiple models, as well as the time required for the completion of each model prompted NCI to consider alternate strategies to prioritize compounds for subsequent more detailed *in vivo* evaluations. With the goal of achieving a short assay time, capacity for relatively large throughput, conservative compound consumption, and low false negative rate, the hollow fiber assay was developed. Tumor cells derived from *in vitro* cell cultures are introduced into 1 mm internal diameter polyvinylidene fluoride (PVDF) hollow fibers permeable to substances of $M_r < 500,000$, and heat sealed at 2 cm intervals. Following cultivation *in vitro*, the fibers are introduced into the peritoneal and subcutaneous compartments of athymic mice, and test substances routinely administered by the intra-peritoneal route. Multiple fibers containing different cell lines can be studied simultaneously in different compartments of the same animal. Following dosing over a period of four days, fibers are removed, and the viable cell mass ascertained by MTT dye reduction. The initial scoring system (2 points assigned for >50% growth inhibition is a single fiber; 48 test conditions resulting from 12 cell lines tested against two dose levels of compound in two sites, peritoneal and subcutaneous, of fiber placement) defined as active compounds with the bias of not having allowed discard of an agent with subsequent demonstration of *in vivo* activity in a training set of compounds. Correlations of *in vitro* activity with hollow fiber activity have revealed that activity *in vivo* hollow fibers tends to increase with increasing *in vitro* potency, such that compound with a 50% growth inhibitory concentration (GI₅₀) > 1 μM had < 10% likelihood of hollow fiber activity, and that compounds with activity in >7 fibers have a 20% likelihood of activity in >33% of xenograft models, a degree of *in vivo* activity that correlates with approximately 50% likelihood of activity in >1 clinical phase II trial. As of 2003, there have been 3398 tests of compounds in the hollow fiber assay. Of these 554(16%) revealed activity recommending further study. The hollow fiber assay therefore usefully prioritizes compounds for further evaluation. Modifications of the hollow fiber assay have been proposed to allow the fibers to evoke an angiogenic response, as well as to permit the study of engineered cell lines possessing luciferase reporter elements in an effort to track compound activity through sensitive light detectors and thus be responsive to dynamic changes in cellular function.

L32

Multiple Approaches in Anti-Angiogenesis: Preclinical Models

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Preclinical studies and emerging findings from early clinical trials indicate that cancer treatment based on the inhibition of angiogenesis need to be optimized in combination with conventional therapies. We have explored this aspect on human tumor xenograft models that express different levels of angiogenic growth factors and malignant behavior. Using stable transfectants from the human HEC-1-B human endometrial adenocarcinoma line that differ for VEGF and/or FGF2 expression, we show the concomitant expression of FGF2 and VEGF resulted in fast growing tumors characterized by limited necrosis and high blood vessel density (Giavazzi et al.: *Cancer Res.* 2001 and *Am. J. Path.* 2003). The inhibition of FGF2 and/or VEGF production resulted in tumor growth inhibition and decreased vascularization and vessel functionality. The addition of paclitaxel in the treatment protocols further affected the growth of the xenografts and the final outcome. The relevance of these findings has been investigated in preclinical trials based on low molecular weight molecules with high affinity for growth factor receptors. We show that SU6668 a tyrosine kinase receptor (VEGFR2, FGFR1 and PDGFR-β

inhibitor delayed the growth of VEGF-producing human ovarian carcinoma xenografts in the peritoneal cavity of nude mice. The addition of paclitaxel resulted in improved antitumor response and increased the mice survival. This was accompanied by a significant decrease in ascites formation and tumor spread in the organs of the peritoneal cavity. The relevance of the tumor stage at the beginning of the treatment, the schedule/dose of treatment and the type of combination will be discussed. These findings provide a rationale for the clinical evaluation of combination therapies affecting multiple biological targets in malignant tumors.

L33

Marine Anticancer Compounds

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In spite of the great progress made in the understanding of the crucial molecular biology alterations occurring in different human neoplasms only in few cases it has been possible to obtain a significant anticancer response by inhibiting specific molecular pathogenetic reactions.

The multiplicity and complexity of the abnormal regulation mechanisms make it more realistic either to develop the combination of different compounds acting at different level or to use natural products which have a multiplicity of mechanisms of action and favorable pharmacological properties.

Although most of natural products currently used in cancer chemotherapy are terrestrial, there is a growing interest for marine natural products.

The marine world offers great resources for novel compounds because of the high biodiversity and the enormous number of different micro and macro organisms living in the sea.

It is reasonable to hypothesize that among the hundreds of millions of different marine natural products there are some with antitumor properties. This view is supported by the fact that the recent research in this area has lead to the identification of several novel compounds which possess antitumor activity.

Our laboratories at Mario Negri Institute have collaborated for more than 10 years with the researchers of the Spanish Company Pharma Mar to investigate the mode of action and the pharmacological properties of many marine compounds.

In my lecture I'll overview our major findings obtained on the mode of action of several novel marine natural products including Thiocoraline, Isohomalichondrin B, variolins, didemnins, ecteinascidins. The peculiar features that make these compounds distinct from those of other anticancer drugs will be highlighted.

For the two compounds which are in a more advanced stage of clinical development, i.e. Yondelis (Ecteinascidin-743, ET-743) and Aplidine, the data of the pharmacological and biological studies will be complemented with the results obtained in the clinical investigations.

Yondelis is the only anticancer compound that appears to act specifically altering the transcription of induced genes. In addition, its cytotoxicity appears to be influenced by the integrity of Nucleotide Excision Repair mechanisms. For the other DNA alkylators NER deficiency confers a dramatic increase in the sensitivity whereas for Yondelis it causes resistance. Whether this unique property of Yondelis has an implication for the clinical efficacy/toxicity of this compound is a matter of speculation.

By using microarray technology it was discovered that Aplidine interferes with the autocrine VEGF loop in some human leukemia cell lines, although other mechanisms and probably more relevant to explain the cytotoxicity against other cancer cells.

The complexity of these molecules make it difficult to find a precise relation between the inhibition of a specific target and the biological effect. Future studies should be addressed at identifying patterns of gene expression changes associated with the drug effects in order to develop more rational methods to investigate these compounds in clinic.

7th Session · NOVEL DRUGS IN DEVELOPMENT

L34

Antitumour Activity of rViscumin *in vitro* and *in vivo*

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RViscumin is a new biological entity (NBE) which can be categorized in the type II ribosome inactivating protein family. rViscumin is the recombinant in *E. coli* produced counterpart of the mistletoe lectin I plant protein. rViscumin is a heterodimer consisting of a carbohydrate binding B-chain and an enzymatically active A-chain. The B-chain was shown to bind specifically to the CD75s motif, bearing a terminal sialic acid residue. CD75s is found on the surface of many cancer tissues (e.g. colon) which was shown by pathological analysis of patient biopsy material. The extent of CD75s on tumor cells is increased compared to healthy tissue. The expression of CD75s on tumor cells correlates with the extent of their malignancy. The A-chain is a N-glycosidase and induces a depurination within the 28S rRNA. In consequence, translation of the target cell is irreversibly stopped and the cell undergoes apoptosis.

The intracellular effects of the translation inactivator rViscumin are mediated by signal transduction mechanisms like the stress kinase pathway (SAPK and p38) and the caspase cascade (activation of caspase-1, -3, -8). In various experiments it was shown, that rViscumin-induced apoptosis is independent of the state of apoptosis regulators p53 or NFkB. rViscumin shows cytotoxic activity against target cells independent of the cell cycle. Cancerostatic activity of rViscumin was assessed in different experimental approaches *in vitro* and *in vivo*. rViscumin was found to be strongly cytotoxic towards human tumour cell lines or xenografts. The mean IC50 value in a sulforhodamin B assay was 0.16 ng/ml (IC70 0.4 ng/ml) for a set of 20 cell lines derived from different tumour entities (Freiburg panel) and 0.9 ng/ml (IC70 3 ng/ml) for 42 human xenografts as determined in a clonogenic assay.

Interestingly, strong cancerostatic activity was observed towards cell lines which were resistant to standard therapeutic agents, such as adriamycin and vindesine, showing that multi-drug resistance mechanisms do not affect its cytotoxicity. In the NCI 60 cell line panel the mean GI50 for rViscumin (NSC D-716789-A) was 0.9 ng/ml, the mean TGI was 6.4 ng/ml and the mean LC50 was 24 ng/ml (average of 2 experiments). A comparison of the data obtained with rViscumin using the NCI 60 cell line panel revealed no correlation of the mode of action of rViscumin with any other cytotoxic and cytostatic agent tested (COMPARE algorithm). rViscumin was shown to have a unique mode of action, acting independent of typical cellular targets. Pronounced *in vivo* antitumour activity of rViscumin was seen in a set of about 20 preclinical models, such as B16 melanoma, two different bladder cancer models, tumour colonisation models for metastasis inhibition, and human xenografts in immune compromised mice. In conclusion, these findings implicate that rViscumin is a promising new anticancer drug. Therefore, currently we set out to

investigate the potency of rViscumin in several clinical trials (phase 1/2).

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L35

Vesicular Phospholipid Gels: The Basis for a Novel Family of Anticancer Therapeutics?

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The *in vivo* pharmacokinetics (PK), biodistribution (BD) and antitumor activity of vesicular phospholipid gel (VPG) -based formulations of gemcitabine (Gem-Lip), and vincristine (VCR-Lip), is presented. VPGs are cream-like formulations consisting of densely packed small phospholipid vesicles (liposomes) of good drug loading capacity and storage stability. Upon dilution, they were found to contain liposome-bound and free drug in the ratio of 40/60 (Gem-Lip) or 55/45 (VCR-Lip).

The *in vivo* efficacy of Gem-Lip was studied using the s.c. growing human soft tissue sarcoma SXF 1301 and the orthotopically growing human bladder cancer BXF 1299T. For VCR-Lip the s.c. growing human small cell lung carcinoma LXFS 650 and the human mammary carcinoma MX1 were used. PK and BD were evaluated using radiolabeled drug and lipid in SXF 1301 (Gem-Lip) or LXFS 650 tumor-bearing (VCR-Lip) nude mice.

Gem-Lip was highly active in SXF 1301 at a gemcitabine dose of 6–9 mg/kg (days 1, 8 and 15; dose near the MTD). In the 6-mg/kg groups, complete tumor remissions were observed in seven of eight mice. Equimolar doses of the conventional formulation of gemcitabine (Gem-Conv) resulted in only moderate tumor growth inhibition. Even at equitoxic doses (360 mg/kg given on days 1, 8 and 15, or 120 mg/kg on days 1, 5 and 8) Gem-Conv was less active than Gem-Lip. Furthermore, Gem-Lip was active in the orthotopically growing BXF 1299T bladder cancer model at 6 mg/kg and prevented distant organ metastasis. In the PK study, Gem-Lip achieved a 35-fold higher plasma AUC (1680 mg h/ml) than Gem-Conv (47.6 mg h/ml). The serum half-lives were 0.15 h for free gemcitabine and 13.3 h for liposomal gemcitabine (6 mg/kg each i.v.). Moreover, gemcitabine levels in tumors were fourfold higher upon injection of Gem-Lip than of Gem-Conv.

VCR-Lip at a dose of 1.0 mg/kg (dose near MTD) led to partial remissions in the MX1 tumor xenograft model (T/C 53.9%). The conventional vincristine formulation (VCR-Conv) at an equitoxic dose of 0.6 mg/kg produced only a tumor growth inhibition (T/C 57.0%). In LXFS 650 tumor-bearing mice, VCR-Lip was highly active at doses of 0.75 (T/C 50.7%) and 1.0 (T/C 50.0%) mg/kg, and complete tumor regressions were observed. In contrast, equitoxic doses of VCR-Conv (0.6 mg/kg) resulted only in less pronounced tumor remissions (T/C 54.1%). The PK study revealed that VCR-Lip achieved an over 10-fold higher plasma AUC (22.6 µg h/ml) than VCR-Conv (2.16 µg h/ml). Moreover, tumor drug levels were 2.3-fold higher when VCR was injected as VCR-Lip in comparison to VCR-Conv.

VPGs were shown to be a highly effective therapeutic system resulting in superior pharmacodynamics and PK in the case of gemcitabine and vincristine. The enhanced *in vivo* efficacies are explained by sustained release of the drug and passive tumor targeting due to enhanced permeability of the vasculature and retention of the particulate.

Keywords: vesicular phospholipid gel, liposome, human tumor xenograft, pharmacokinetics, metastases, gemcitabine, vincristine

8th Session - CESARs PROOF OF PRINCIPLE

L36

Indazolium Trans-[Tetrachlorobisindazolruthenate(III)] and Tris(8-quinolinolato)Gallium(III): Two New Metal Complexes Exhibiting Promising Antitumor Activity

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Ruthenium and gallium complexes have attracted attention as potential non-platinum antitumor agents. Of these the *Hind*[trans-RuCl₄(ind)₂] (KP1019), FFC14 and tris(8-quinolinato)gallium(III) (KP46, FFC11) are currently entering phase I clinical trials. Both compounds show a promising antitumor activity in different preclinical models *in vitro* and *in vivo*. High antitumor activity could be observed for both compounds in a panel of primary explanted tumors. The Ruthenium complex in particular turned out to be highly active against an autochthonous colorectal tumour model which reassembles the human situation very closely. Low toxicity of the compound at effective doses, which stems presumably from a transferrin-mediated accumulation of the ruthenium complex in the tumour cells is another advantage of this compound. Improved bioavailability and sufficient plasma concentration is an advantage of the compound KP46. The compound exhibits better antiproliferative properties and superior capacity of apoptosis induction *in vitro* compared to simple gallium salts.

L37

Heterocyclic Complexes of Ruthenium(III) Induce Apoptosis in Colorectal Carcinoma Cells by Oxygen Stress Induced Mechanisms

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The ruthenium complex salt indazolium *trans*-[tetrachlorobisindazole-ruthenate(III)] (KP1019) and its analogous sodium salt (KP1339) are effective tumor-inhibiting drugs in experimental therapy of autochthonous col-

orectal carcinomas in rats. The analogous imidazolium complex (KP418) shows much weaker activity.

KP1019 and KP1339 are efficiently taken up into colorectal tumor cells, where they cause dose-dependent DNA strand breaks as well as oxygen radical formation. Uptake of KP418 was 10-fold lower correlating with its lower cytotoxic efficiency.

The compounds induce apoptosis as demonstrated by morphological criteria, depolarization of mitochondrial membranes, downregulation of bcl₂ and activation of caspase 3. This effect can be partially inhibited by N-acetyl-cysteine indicating involvement of oxidative stress in apoptosis induction as well as DNA damage. Inhibition by NAC is much more effective in non-malignant adenoma cells pointing to a potential for protection of normal tissues by antioxidants.

Based on their cellular mechanisms that *trans*-[tetrachlorobisindazole-ruthenate(III)] complex salts could be promising candidate drugs in the second line treatment of colorectal cancers resistant to other cytostatic drugs, that should be introduced into clinical trials.

L38

Influence of Drug Resistance Mechanisms on the Antitumor Activity of New Metal Compounds

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Platinum compounds belong to the most frequently used chemotherapeutic agents. Generally, a major obstacle to a successful treatment of cancer by chemotherapeutics including platinum compounds is the development of tumor cell resistance. During the last decades new heavy metal compounds with promising pharmacological characteristics have been developed. Some of these compounds are close to clinical approval currently. For a successful application as anticancer agents, knowledge about the potential mechanisms of cellular resistance are of utmost importance. Currently, we are investigating the impact of various multidrug resistance (MDR) and anti-apoptotic mechanisms on the cytotoxic activity of six heavy metal complexes synthesised at the Institute for Inorganic Chemistry, Vienna University. They all have proven cytotoxic activity and potential applicability in anticancer therapy. The investigated substances include ruthenium (KP1019), gallium (KP1089, KP46), lanthan (KP 772), cer (KP 776), and bismuth (KP1255) complexes. Cytotoxic and cytostatic activity against diverse cell models comprising drug-sensitive parental cell lines and drug-resistant sub-lines have been established. Cell models over-expressing either P-glycoprotein, MRP1, MRP2, MRP5, LRP, or temperature-sensitive variants of p53 have been employed. Impact of the particular resistance proteins were further characterised by applying specific chemosensitizing agents (including P-gp, MRP and glutathione-depleting agents and others) together with the studied compounds. Additionally, cell lines resistant to the investigated drugs have been established by stepwise selection and the underlying resistance mechanisms are currently investigated. Results demonstrate that different heavy metal compounds may be influenced by different resistance mechanisms. Besides that also enhanced activities of some compounds against drug resistant cell lines were detected (collateral sensitivity). In summary, the presented data add to a better understanding of the anticancer activity of new heavy metal compounds and should facilitate their successful application in the clinics.

L39

A Phase I Study with Sodium Trans-[Tetrachlorobis(1H-Indazole)Ruthenate(III)]/Indazolhydrochloride (Ffc14a) in Patients with Solid Tumors

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Based on preclinical screening of a number of Ruthenium complexes, FFC14A was chosen for clinical investigations because of its high therapeutic index due to its significant antitumor efficacy and low toxicity. A multicentre phase I study in patients with solid tumors will be performed by CESAR-EWIV in collaboration with Faustus Forschungs Compagnie, Leipzig and is now ready to start. The essentials of the study protocol are outlined here. Primary objectives are the determination of the maximum

tolerated dose (MTD) and the toxic side effects including the dose limiting toxicity (DLT) to recommend a safe dose for phase II studies and the evaluation of the pharmacokinetics of different doses of FFC14A and its metabolites. Secondary objectives are safety and tolerability as well as anti-tumor efficacy. Female and male patients older than 18 years with histologically proven malignant solid tumors without alternative established treatment options with a life expectancy of more than three months can participate in the trial after providing written informed consent. Exclusion criteria are standard with respect to bone marrow and organ function, concomitant diseases and treatment-free interval. The application of FFC14A will be performed according to an accelerated titration design with one patient per dose level at 100% increase unless there is no $\geq$ grade 2 toxicity according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC). Thereafter, two more patients are to be treated on the same dose level and further dose escalation will be performed according to Fibonacci. The drug will be dissolved to a final concentration of 0.42 mg/ml based on the Ruthenium complex in isotonic common salt solution containing a slight excess indazol hydrochloride and applied as continuous intravenous infusion at a fixed infusion rate of 10 ml/min twice weekly for three weeks with a flat starting dose of 25 mg. According to the results of the final examination on day 26–28 treatment may be repeated on day 29 in case of acceptable tolerability and at least stable disease.

9th Session - CLINICAL STUDIES OF TARGET SPECIFIC DRUGS

L40

Clinical Evaluation of New Target-Specific Drugs (TSD)

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After proof of principle a number of TSD have been introduced into the clinics and more and more new small-molecule inhibitors of or monoclonal antibodies against molecular targets crucial in signal transduction, regulation of cell cycle, cell growth, cell differentiation and apoptosis, as well as tumorigenesis, are on their way from bench to bed. In contrast to conventional cytotoxic drugs (CCD) mainly acting by proliferation inhibition due to DNA alkylation, DNA destruction or inhibition of mitosis, TSD are cytostatic. In general, TSD are less toxic than CCD with a different panel of potential toxic side effects without dose-limiting myelosuppression but mucocutaneous and cutaneous toxicity being more pronounced. Thus, the strategy for clinical testing of new antitumor drugs has to be reconsidered. In clinical phase I studies the determination of the maximal tolerated dose (MTD) and the dose-limiting toxicity (DLT) is no longer essential and should be advantageously replaced by the minimal effective dose (MED) with respect to inhibition of the target. As before, concomitant clinical pharmacology is mandatory. Furthermore, the development of methods for the determination of surrogate parameters or biomarkers is necessary for the proof of principle and the determination of the MED. These may be performed by the determination of the inhibition of the target by the TSD or by the measurement of the binding of the TSD to the target in somatic cells, e.g. leukocytes from peripheral blood or skin tissue or, ideally, in tumor cells. Other methods include the determination of glucose uptake, thymidine metabolism or blood flow by positron emission tomography (PET) and vascular permeability or blood flow by magnetic resonance tomography (MRT). Accelerated dose escalation schemes are recommended instead of Fibonacci's dose escalation to reduce the number of patients treated in an ineffective dose range. Strategies for clinical phase II studies include the randomized discontinuation design. Crucial endpoint for clinical trials with TSD is the time to progression (TTP).

L41

Why Do We Need Surrogate Parameters for Early Clinical Trials?

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More anticancer drugs are being tested than ever before but several of them have failed in phase III trials despite promising preclinical data and encouraging results of early clinical trials. This seems to be due to the fact that the development of anti-cancer drugs is currently undergoing funda-

mental changes. Due to the increasing understanding of the mechanisms relevant to the genesis of cancer, we are experiencing a transition from disease to target-oriented therapy. Thus the challenge of bringing new therapeutics to clinical application and registration now focuses on the molecular target expressed by the malignant cell rather than treatment of the histopathological entity itself.

The new concepts, however, bring along major problems for the set-up of early clinical trials. One major issue is the limited availability of predictive in vitro models on the one hand, and of the need to perform early clinical trials usually in heavily pretreated patients with a considerable tumor load on the other. Maximum tolerated dose (MTD) and response rate, however, are no longer the only decision-making endpoints of phase I/II trials. The new generation of compounds usually exerts only limited toxicity and stabilizes the tumor rather than leading to massive reduction of tumor load. Therefore, endpoints different from those we are experienced to use for cytotoxic drugs need to be developed in phase I/II trials.

The definition of biological surrogate markers is crucial and a chance to allow proof of principle in early clinical trials in order to move rapidly to phase III and registration. This aspect has not always been taken into account when designing clinical trials and may have caused a considerable prolongation of drug development and thus enormous additional costs. On the other hand, agents may have failed in the past due to inappropriate trial design and missing evaluation of surrogate markers.

The inclusion of surrogate markers may have several aims. It allows us to follow the drug until it reaches the target and define potential effects at the tumor site. Furthermore, it can help to define subpopulations of patients who profit or do not profit from therapy. These markers partially differ among the various approaches of targeted therapy and are under continuous development. In general major effort should be undertaken to monitor target expression and its potential inhibition by the experimental drug.

Whenever the immune system is involved, immune responses need to be monitored, preferentially at the tumor site. It is well known that the immune system of cancer patients may express functional defects on various levels and most trials with anti-cancer vaccines thus included very heterogeneous patient populations. While determination of cytotoxic T cells in the peripheral blood has shown to be of only very limited value, biopsies taken before and during therapy can provide detailed information on what is going on at the tumor site.

Potential effects of anti-angiogenic compounds can be monitored by functional imaging. Vascular permeability is a surrogate for angiogenesis and can be assessed by MRI scanning. Positron emission tomography (PET) is a tool to follow the experimental drug throughout the body.

Recently proteomics performed with two-dimensional electrophoresis has shown to be a tool to discriminate between benign and malignant lesion in ovarian tumors. This technique also holds promise for the monitoring of functional effects of investigational agents.

In summary, the application of surrogate markers in early clinical trials is required and represents a fascinating challenge for both academia and industry. Biotech research is an extremely competitive field, and rational drug development is not only essential for scientific and ethical reasons but will also reduce developmental costs of new drugs

L42

Anti-Angiogenic Therapy with Vascular Endothelial Growth Factor Receptor (VEGF-R) Inhibitors in Patients with Advanced Cancer

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Angiogenesis is responsible for the growth and metastasis of malignant tumors. The tumor vessels are responsible for the supply of oxygen and nutrition that was provided by diffusion from healthy tissue before. Increasing hypoxia is causing the so called angiogenic switch characterized by the production of pro-angiogenic factors from tumor tissue and the increase of specific receptors on the surface of the surrounding endothelial cells. The most important factor in tumor angiogenesis is VEGF (vascular endothelial growth factor). Therefore, the inhibition of tumor-angiogenesis by blocking the VEGF receptors on endothelial cells is a promising new clinical approach in cancer therapies.

A large group of selective VEGF-receptor tyrosine kinase inhibitors is actually under clinical development. The group of selective VEGF-receptor-2 (KDR) inhibitors is mainly represented by PTK787/ZK 222584. This compound is studied in patients with solid tumors and glioblastoma in different

clinical phase I trials. Until today, the maximal applied oral dose is 2000 mg on a once daily oral schedule. Worsening of preexisting hypertension and ataxia have been detected as drug related toxicities but no dose limiting toxicity have been observed. Having investigated the detection of serum levels of soluble pro-angiogenic factors, the detection of tumor blood flow by Color Doppler Imaging (CDI) and the detection of tumor permeability by dynamic contrast-enhanced magnetic resonance imaging (dce-MRI) as surrogate markers to detect biological activity, the optimal once daily dosing of PTK787/ZK 222584 was found to be at 1200 mg. Best response of PTK787/ZK 222584 was reported to be minor response or stable disease in 44–50% of the treated patients, lasting for a maximum of 15 month. In conclusion, PTK787/ZK 222584 is an orally bioavailable well-tolerated efficacious anti-angiogenic therapy in patients with solid tumors. Other potent VEGF-receptor tyrosine kinase inhibitors just entered clinical phase I studies.

L43 Raf Kinase Inhibitors

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Proliferation, differentiation, survival, and apoptosis of all eukaryotic cells is controlled by a highly interactive network of protein kinases and other signal messengers. Many receptor tyrosine kinases and cytokine receptors in association with G proteins are known to activate intracellular protein serine/threonine kinases termed mitogen-activated protein kinases (MAPKs). Although activated in the cytosol, the MAPKs translocate to the nucleus upon activation and phosphorylate a large number of nuclear proteins. The MAPK pathway represents a cascade of phosphorylation events including three pivotal kinases, namely Raf, MEK (MAP kinase kinase), and ERK (MAP kinase). These kinases present new opportunities for the development of novel anti-cancer drugs designed to be target-specific and probably less toxic than conventional chemotherapeutic agents.

The Raf family of serine/threonine-specific protein kinases comprises 3 members: A-Raf, B-Raf, and Raf-1. Experimental evidence supports a direct role for Raf-1 kinase in the development and maintenance of human malignancies. For instance, the Raf kinases are the direct downstream mediators of the Ras proteins, whose oncogenic version is associated with approximately 30% of human solid tumor types. The potential for Raf-1 to play a broad role in tumorigenesis is evidenced by its ability to become activated by either PKC or the antiapoptotic protein Bcl-2 in a Ras-independent manner. Furthermore, Raf mutations, especially mutations of the B-Raf gene were detected in a wide range of human tumors and in 66% of malignant melanomas. Independent of its mutation status, Raf is also activated in tumor cells containing enhanced growth factor signaling pathways, such as those induced by mutant or constitutively expressed EGF receptor family members. In addition, analysis of the transcriptional program induced by Raf in epithelial cells revealed autocrine activation of the EGF receptor to be responsible for the ability of Raf activation to protect transformed cells from apoptosis. Therefore, the collective evidence suggests that Raf is a viable anticancer drug target. To date, two different Raf-directed approaches have been published that entered clinical trials: Degradation of Raf-1 mRNA and inhibition of Raf.

ISIS 5132 is a phosphorothioate oligodeoxynucleotide antisense compound that targets the 3' untranslated region of the human Raf-1 gene. ISIS 5132 appears to mediate degradation of Raf-1 mRNA and thereby suppresses protein expression. Three phase I trials have been initiated using ISIS 5132 in patients with a range of solid tumors. This approach was generally well-tolerated, producing only mild side effects; these were attributed to the antisense technology rather than to sequence-specific effects. In one of the clinical trials, the levels of Raf-1 mRNA were found to be suppressed in peripheral-blood mononuclear cells of treated patients. Although no major responses were reported, a number of patients experienced prolonged stable disease. Furthermore, phase II data with single agent ISIS 5132 in patients with hormone-refractory prostate cancer and in patients with locally advanced or metastatic colorectal cancer, in patients with small-cell (SCLC) and non-small cell lung cancer (NSCLC) have been published recently. However, in the doses and schedules studied, ISIS 5132 failed to induce objective responses in these patients.

The drug BAY 43-9006 is a novel potent and selective small-molecule inhibitor of Raf kinase and the first compound in this class to undergo clinical testing. Anticancer activity has been shown in numerous tumor models with

K-Ras mutations. Antitumor activity was also demonstrated in tumor models that express wild type Ras, but overexpress growth factor receptors.

In the phase I clinical program, a total of 182 patients have been treated with the Raf kinase inhibitor BAY 43-9006 according to 4 different dosing schedules. Two confirmed partial responses have been observed. Five additional patients achieved tumor shrinkage of more than 20%, and 29 additional patients (25%) had disease stabilization for longer than 6 months and 9 patients (8%) for longer than 1 year. All of these patients received a daily dose of 200 mg bid or more. Toxicities were generally mild to moderate. Dose-limiting-toxicity was diarrhea and reversible skin toxicity (rash, palmo-plantar erythema). Substantial inhibition of PMA-stimulated ERK phosphorylation in peripheral blood lymphocytes (PBLs) from the treated patients was observed. The time course and extent of ERK inhibition in PBLs tended to parallel the dose level of BAY 43-9006 administered. Clinical studies with BAY 43-9006 in combination with other chemotherapy agents are in progress.

In summary, inhibition of Raf kinase is clinically feasible and safe. Although only few patients experienced major remissions across the BAY 43-9006 phase I clinical program, a considerable number of patients showed disease stabilization for longer than 6 months. Phase II studies are ongoing at 400 mg bid continuous dosing.

10th Session · SIGNAL TRANSDUCTION INHIBITORS EFFECTIVE IN THE CLINIC

L44 Glivec, a Targeted Therapy of Cancer

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Chronic myeloid leukemia (CML) is a clonal hematologic disorder characterized by a reciprocal translocation between chromosomes 9 and 22 known as the Philadelphia (Ph) chromosome. The molecular consequence of this inter-chromosomal exchange is the creation of the bcr-abl gene coding for a protein with elevated tyrosine kinase activity. The demonstration that the expression of Bcr-Abl is both necessary and sufficient to cause a CML-like syndrome in murine bone marrow transplantation models and the finding that the tyrosine kinase activity of Bcr-Abl is crucial for its transforming activity, has established the enzymatic activity of this deregulated protein as an attractive drug target addressing Bcr-Abl positive leukemias. Glivec® (Gleevec™, formerly STI571) is an orally bioavailable 2-phenylaminopyrimidine compound rationally designed to inhibit adenosine triphosphate binding to protein tyrosine kinases and thus block the activation of kinase-mediated intracellular signal transduction pathways. The drug has heralded rapid change in the pharmacotherapy of CML. Clinical results with Glivec® in the treatment of CML were striking from the outset of testing and extensive clinical data available in phase I to phase III studies indicate that the inhibition of Bcr-Abl can be achieved with Glivec® in human and translate into clinically meaningful patient benefit. Therapy with Glivec® has resulted in unprecedented rates of hematologic and cytogenetic response and a lower rate of progression to advanced phases of the disease (O'Brien et al. 2003, *N Engl J Med* 348:994–1004).

The efficacy and safety results have prompted investigations of the potential role of Glivec® as a therapy for other diseases with molecular targets known to be sensitive to this agent. In addition to inhibiting the Abl tyrosine kinase, Glivec® inhibits the unrelated c-Kit and platelet-derived growth factor (PDGF) receptors. These are members of the receptor tyrosine kinase family which possess five immunoglobulin-like motifs in the extracellular domain and a cytoplasmic split kinase domain. Dysregulation of c-Kit has been implicated in the aetiology of a number of cancers, including acute myelogenous leukemia (AML), small cell lung cancer, gliomas, testicular cancer and gastrointestinal stromal tumours (GIST). The majority of the latter tumours are characterized by mutations in c-Kit leading to the constitutive activation of this kinase. In clinical trials Glivec® demonstrated remarkable activity in the treatment of metastatic and/or unresectable GIST, with durable responses and prevention of disease progression achieved in approximately 80% of patients (Demetri et al. 2002, *N Engl J Med* 347: 472–480).

There is increasing evidence that the PDGF ligand/receptor system also plays an important role in tumorigenesis (Östman and Heldin 2001, *Adv Cancer Res* 80:1–38). Paracrine and/or autocrine activation of the PDGF receptor kinase has been postulated in numerous malignancies and the presence of PDGF autocrine loops is most well documented in gliomas.

There is relatively little known about ligand-independent activation of PDGF receptors. However, rearrangement of the PDGF- β receptor gene has been described in chronic myeloproliferative disorders. The best known of these is the t(5;12) chromosomal translocation in chronic myelomonocytic leukemia (CMML) where the PDGF- β receptor gene located on chromosome 5 is fused to the TEL (ETV6) gene on chromosome 12. Transformation of hematopoietic cells occurs through oligomerization of the TEL/PDGF- β receptor fusion protein, which causes ligand-independent constitutive activation of the PDGF receptor kinase. Glivec® inhibited the growth of cells expressing the TEL/PDGF- β receptor (Carroll et al. 1997, Blood 90:4947–4952) and in transgenic mice expressing the TEL/PDGF- β receptor, treatment with Glivec® inhibited tumor formation and prolonged survival of the animals (Tomasson et al. 1999, Blood 93:1707–1714). As with Bcr-Abl in CML and c-Kit in GIST, clinical proof of concept in a PDGF-R driven disease has been achieved with Glivec® in this rare hematologic malignancy (Apperley et al. 2002, N Engl J Med 347:481–487). Furthermore, recent case reports have described the reduction in tumor volume in patients with dermatofibrosarcoma protuberans (DFSP) treated with Glivec® (Maki et al. 2002, Int J Cancer 100:623–626, Rubin et al. 2002, J Clin Oncol 20:3586–3591). DFSP is associated with translocations which fuse the collagen 1A1 gene with the PDGF-B chain and lead to constitutive PDGF-R kinase activation through autocrine stimulation. Additional data for the use in diseases with mutationally caused changes in PDGF ligand and/or receptor expression come from the recent findings where patients with hyperosinophilic syndrome (HES) have been shown to respond to Glivec® therapy. Constitutive activation of PDGF- α receptor resulting from the fusion of the Fip1-like 1 (FIP1L1) gene to the PDGF- α receptor gene was identified as the cause of HES in 9 of 16 patients in a recent study (Cools et al. 2003, N Engl J Med 348:1201–1214). In summary, these data validate the initial hypothesis of this program, and underscore the importance of rationally selecting the target diseases to be considered in the development of targeted therapies such as Glivec®.

L45

The Epidermal Growth Factor Receptor-Tyrosine Kinase: A Promising Therapeutic Target in Solid Tumors

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The overexpression and aberrant function of the epidermal growth factor receptor (EGFR) and its ligands in several human carcinomas have provided a rationale for targeting this signaling network with novel treatment approaches. The epidermal growth factor receptor-tyrosine kinase (EGFR-TK) is a selective target for inhibiting cancer because it is activated in many tumor cells, yet is strictly controlled in normal cells. The EGFR-TK initiates a variety of signal transduction pathways in tumor cells with profound effects on the tumor's biology. Activation of the EGFR-TK provides signals that drive dysregulated proliferation, invasion and metastasis, angiogenesis, and enhanced cell survival. Therefore, the EGFR-TK is a promising drug target for many types of solid tumors and its inhibition has potential in both the treatment and prevention of these neoplasias. Based on the structure and function of the EGFR, several antireceptor therapeutic strategies have been developed. One important approach uses small molecules that compete with adenosine triphosphate (ATP) for binding to the receptor's kinase pocket, thus blocking receptor activation and the transduction of postreceptor signals. Early clinical studies suggest that this approach, either alone or in combination with standard anticancer therapies, is well tolerated or can induce clinical responses and tumor stabilization in a variety of common carcinomas.

ZD1839 (gefitinib, IRESSA®; AstraZeneca Pharmaceuticals, Wilmington, DE) and OSI-774 (erlotinib, TARCEVA®; Genentech Inc., South San Francisco, CA) are the EGFR-TK inhibitors that are furthest in clinical development, and they are currently being investigated in a variety of solid tumors.

L46

m-TOR Inhibitor CCI-779

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Rapamycin was isolated in the year 1970 from a bacterium (*Streptomyces hygroscopicus*) found in the soil of Easter Island and was initially shown to have fungicidal activity. Rapamycin was then developed as an immunosuppressant drug, actually registered in the US and Europe. The drug has subsequently been shown to have also antitumor activity. CCI-779 is an ester analogue of rapamycin developed by Wyeth Pharmaceuticals with improved water solubility. It selectively inhibits the mammalian target of rapamycin, known as mTOR, which is linked by signal pathways to the cell cycle progression from G1 to S phase. CCI-779 binds intracellularly to a protein called FKBP12 and forms a complex that inhibits the protein kinase activity of mTOR. mTOR controls the translation of specific mRNAs required for cell cycle traverse from G1 to S phase.

An antiproliferative effect of CCI-779 was seen against several tumor cell lines, such as various breast cancer and glioblastoma cell lines, rhabdomyosarcoma, osteosarcoma, colon-, pancreas- prostate – and small cell lung- cancer cells. Tumors derived from human glioblastoma, breast-, prostate-, colon cancer, lymphoma, and melanoma were sensitive to CCI-779 in the nude mouse xenograft model.

The drug is essentially well tolerated. Significant side effects seen in phase I and II studies included predominantly skin toxicity with rash, folliculitis and nail changes, as well as stomatitis and asthenia. Central nervous toxicity includes depression and even cases of reversible somnolence. Thrombocytopenia, anemia and leukopenia were described at various dose levels. Laboratory abnormalities included hypocalcemia, hypertriglyceridemia and hypercholesterolemia and elevated transaminases. A reversible decrease of testosterone levels was seen in some men.

Clinical schedules were developed in an attempt to avoid immunosuppression. One schedule used weekly 30 minute infusions, another schedule daily infusions for 5 days every two weeks. Major responses had already been seen in phase I trials including breast, renal NSCLC and colon cancer.

A phase II program included trials in renal and breast cancer. CESAR was involved in a European phase II trial in patients with pretreated metastatic breast carcinoma. The results in this heavily pretreated group of patients were encouraging. In this randomized phase II trial 2 doses of CCI-779 (75 mg and 250 mg weekly) were used. A total of 106 patients were treated. All patients were either pretreated with anthracyclines, taxane or both and 55% had received three or four prior regimens. 1 CR, 8 PRs and 43 SD lasting >8 weeks were observed (including 8 additional unconfirmed PRs).

In another randomized phase II study weekly doses of 25 mg, 75 mg and 250 mg were used in patients with renal cell carcinoma. Overall a 5% response rate was seen and a prolongation of median survival is suggested. Combination with interferon proved possible in another trial.

Future research including phase III trials will evaluate various combinations with hormonal or cytotoxic therapy as well as studying the predictive effect of high levels of Akt phosphorylation or loss of PTEN function.

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