Annual Meeting of the Working Group for Pharmacology in Oncology and Hematology (APOCH) of the Central European Society for Anticancer Drug Research (CESAR) during the Symposium “Novel Approaches for the Discovery and the Development of Anticancer Agents”

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Overview of presentations

The Annual Meeting of the CESAR-APOCH (Arbeitsgruppe Pharmakologie in der Onkologie und Hämatologie), the Working Group for Pharmacology in Oncology and Hematology, was held this year at the University of Vienna from July 7 – 9, 2005 within the framework of the CESAR symposium “Novel Approaches for the Discovery and the Development of Anticancer Agents” and offered clinical and pediatric oncologists, clinical pharmacologists, clinical pharmacists and chemists opportunity for in-depth discussion of their latest scientific results. The sessions included plenary lectures, oral and poster presentations selected from submitted abstracts.

Thursday afternoon and Friday morning focused on targeted therapy which was rounded off by a critical review given by H. Calvert covering new strategies to overcome chemoresistance and new strategies and targets in dealing with cancer. The scientific program complemented by the award ceremonies for the Dietrich Schmähle Preis and the CESAR PhD Fellowship. Friday afternoon’s program was dedicated to “Tissue Pharmacology” within the framework of an APOCH workshop with lectures given by R. Port, M. Müller, K. Kletter and R. Strecker. Saturday’s agenda started with the business meeting of the CESAR-APOCH and was followed by the AWO/APOCH meeting with “Translational Research” as the central topic. Subsequently, the opportunity for free communications was given. In parallel, a COST D20 WG005 meeting on “Intracellular and Extracellular Targets for Antitumor Activity and Toxicity of Ruthenium Compounds” was held.

A broad spectrum of topics was covered during the meeting by the 59 speakers ranging from clinical studies on anticancer agents in different phases, targeted therapy, overcoming chemoresistance, tissue pharmacology, translational research and metal complexes in cancer therapy. The following extended abstracts summarize the contents of 19 presentations.

The next annual CESAR-APOCH meeting will take place in Essen from June 16 – 17, 2006. The CESAR-APOCH and CESAR-AWO (Arbeitsgruppe Wirkstoffentwicklung in der Onkologie), the Working Group for Drug Development in Oncology, are open for all scientists working on pharmacological aspects in oncology and hematology and on preclinical anticancer drug development, respectively.

Further information regarding membership can be obtained from the chairsman Dr. Ralf A. Hilger (CESAR-APOCH), Universitätsklinikum Essen, Innere Klinik und Poliklinik (Tumorforschung), Westdeutsches Tumorzentrum, Hufelandstraße 55, 45147 Essen, Germany, ralf.hilger@uni-essen.de, and Prof. Dr. Dr. Bernhard K. Keppler (CESAR-AWO), Institut für Anorganische Chemie – Bioanorganische, Umwelt- und Radiochemie, Universität Wien, Währinger Straße 42, 1090 Wien, Austria, bernhard.keppler@univie.ac.at. Further information on CESAR can be found at www.cesar-ewiv.org or obtained from the secretariat of CESAR (CESARiat), cesar@wienkav.at.
Pemetrexed: mRNA expression of the target genes TS, GARFT and DHFR correlates with the in vitro chemosensitivity of human solid tumors

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Introduction

Pemetrexed (Alimta®) – a novel antifolate – is clinically active in various solid tumors and has recently been approved for the treatment of NSCLC and, in combination with cisplatin, also of mesothelioma [Hanauske et al. 2001, Hazarika et al. 2004, Robinson et al. 2004, Zhao and Goldman 2004]. Thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycaminide ribonucleotide formyltransferase (GARFT) are key enzymes in nucleotide synthesis and DNA-replication. Inhibition of these enzymes is believed to constitute the primary mechanism of action of pemetrexed.

Pemetrexed is one of the best substrates presently known for folylpolyglutamate synthetase (FPGS). Polyglutamated pemetrexed has a substantially higher potency for inhibition of GARFT and TS.

The hypothesis of the present investigation was that mRNA levels of TS, GARFT, and DHFR in human tumor cells are pivotal for the chemotherapeutic effectiveness of pemetrexed [Salonga et al. 2000]. Therefore, correlation of gene expression of the target enzymes of pemetrexed with the in vitro chemosensitivity of freshly explanted human tumor specimens was studied. The objective was to differentiate subsets of tumors with high and low probability of response. Such correlations may help develop a rational hypothesis for the design of clinical studies using gene expression patterns as a predictor of treatment outcome and to enable individualized, targeted therapy to be carried out.

Material and methods

Cell biology

Freshly biopsied tumor cells (solid tumors, pleural effusions and ascites) were used for soft-agar cell cloning experiments [Hanauske et al. 2004]. Cells were exposed to final concentrations of 1, 10 and 100 μg/ml pemetrexed for 1 h or a 21-day (continuous) incubation at 37 °C, and clonogenic tumor growth was evaluated after three weeks of cultivation. Aliquots of the same tumor specimens were shock-frozen immediately after removal from the patient.

Molecular biology

Total RNA was isolated using Trizol reagent. Reverse transcription was performed using 1 μg RNA, random decamers and a 100 uMMLV-RT in a 20 μl reaction. Quantitative real-time PCR was performed as multiplex PCR using Taqman-Probes labeled with TexasRed (β-actin), Fam (TS), Hex (GARFT) or Cy5 (DHFR). Standard curves were used to quantify the results. Standards from plasmid DNA were HPLC-calibrated and PCR tubes were coated storage-stable with 10² – 10⁸ molecules/tube. Results from gene expression experiments were normalized to 10⁴ copies of β-actin transcripts.

Statistical methods

SPSS 12.0 was used for data analysis. Significance levels were calculated using the Mann-Whitney test.
Results

In vitro colony formation was evaluable in 102 tumor samples. A variety of tumor types was investigated, including mesothelioma, melanoma, lymphoma, NSCLC, SCLC, cancer of the thyroid, kidney, pancreas, stomach, breast, head-neck, liver, colon, cervix, gall bladder, prostate and tumors were the primary site was not known. Tumors were defined as resistant if colony survival was > 65% compared to untreated controls.

On continuous exposure to pemetrexed, concentration-dependent inhibition of colony formation was observed in 24/95 specimens (25%) at 1 µg/ml, in 30/96 (31%) at 10 µg/ml and 44/97 (45%) at 100 µg/ml. On short-term exposure to pemetrexed (1-hour) experiments, concentration-dependent inhibition was observed in 7/60 (12%) at 1 µg/ml, in 18/60 (30%) at 10 µg/ml and in 27/59 (46%) at 100 µg/ml.

Gene expression of the enzymes TS, GARFT, DHFR, and FPGS was investigated in 73 tumor samples. A clear difference in TS, GARFT, and DHFR mRNA expression between pemetrexed-sensitive and -resistant specimens was observed after 1 h exposure. Low levels of mRNA expression of the enzymes TS, GARFT, and DHFR were correlated with chemosensitivity to pemetrexed after 1 h exposure. The differences in gene expression between pemetrexed-sensitive and pemetrexed-resistant specimens were statistically significant (Table 1, Figure 1).

A small but not statistically significant difference was observed in FPGS mRNA expression between pemetrexed-sensitive and -resistant specimens.

Colony survival after prolonged exposure (21 days) to pemetrexed did not correlate with gene expression of the target enzymes.

Table 1. Median of the relative mRNA expression in tumors grouped according to sensitivity or resistance to 1 × ppc (peak plasma concentration = 100 µg/ml) pemetrexed after 1 h exposure in HTCA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative mRNA expression</th>
<th>n = 20</th>
<th>n = 25</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>59</td>
<td>215</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>GARFT</td>
<td>20</td>
<td>41</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>DHFR</td>
<td>22</td>
<td>38</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>FPGS</td>
<td>170</td>
<td>236</td>
<td>0.087</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. TS (A), GARFT (B), and DHFR (C) gene expressions in 45 tumor specimens grouped according to their sensitivity to pemetrexed (1 × peak plasma concentration = 100 µg/ml) in 1 h exposure.
Conclusions

The mRNA expression levels of TS, GARFT, and DHFR in samples taken directly from patients correlated with the predict antitumor activity of pemetrexed in vitro. Our studies may help to identify potentially clinically sensitive tumors for further development of pemetrexed. These results form a rational basis for the design of clinical trials to evaluate TS, GARFT, and DHFR expression as predictors of treatment outcome using pemetrexed.

References


In vitro investigation on the selectin binding mechanisms in tumor cell metastasis and their inhibition by heparin

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Introduction

Tethering and cell rolling along the vessel wall are 2 of the earliest steps in the adhesion cascade of leukocytes mediated by selectins – a family of 3 adhesion receptors (E, P and L). This first interaction allows a subsequent integrin-dependent stable arrest and diapedesis of leukocytes to sites in inflamed tissue. Besides their function in the immune response of the organism, selectins play a crucial role in cancer metastasis. Cells detached from a primary tumor might adhere to endothelial cells by interacting with selectins. However, the molecular mechanisms of this process are unknown and might differ from leukocyte extravasation in inflammation. Due to its rapid expression on platelets and on endothelial cells, P-selectin appears to be the most important candidate in the mediation of multiple interactions involving platelets, leukocytes, endothelium and carcinoma cells [Borsig et al. 2001]. To simulate metastasis-relevant binding processes in vitro and to evaluate a P-selectin involvement, we investigated three human tumor cell lines (HT-29 colon carcinoma, HepG2 liver carcinoma and NW 1539 melanoma) with regard to selectin ligand expression, binding ability to P-selectin, cell adhesion and interaction with platelets.

The inhibition of the selectin function decreases metastasis as described in many in vivo studies [Kim et al. 1998]. Recently, experimental studies and indirect clinical evidence suggest that low molecular weight heparins have positive effects on the survival of cancer patients [Altinbas et al. 2004, Klerk et al. 2005, Lee et al. 2005]. This effect might be attributable, at least partly, to the P-selectin blocking activity of heparin. However, heparin binding to selectins is controversially discussed. Heparin is a natural complex polysaccharide mixture and detailed structure-activity-rela-

Material and methods

Cell lines

HepG2 and U937 were grown in RPMI 1640 medium supplemented with 10% FCS, 50 µg/ml streptomycin and 50 U/ml penicillin G. NW1539 were maintained in DMEM containing 10% FCS, 50 µg/ml streptomycin, 50 U/ml penicillin G and 365 µg/ml L-glutamine. HT-29 were grown in DMEM supplemented with 10% FCS, 50 µg/ml streptomycin, 50 U/ml penicillin G, 365 µg/ml L-glutamine and 1% non-essential amino acids.

Flow cytometry

Cell suspensions of each cell line were incubated with anti-human CD162 and FITC-conjugated anti-mouse IgG1 (ligand expression) or with soluble P-selectin Fc chimera and FITC-conjugated anti-human IgG (binding ability to P-selectin). Platelets were stimulated with TRAP-14 and incubated with FITC-conjugated anti-human CD62P (P-selectin expression). For the investigation of platelet-cell interaction, platelets...
were stained with Calcein-AM, TRAP-14-activated and incubated with the cells.

Flow experiments at immobilized P-selectin

Tumor cell adhesion experiments and investigation of P-selectin inhibition with PSGL-1-carrying U937 cells were performed using the dynamic model system as described previously [Vogel et al. 1998].

Heparins and semisynthetic glucan sulfates

Five different batches of heparin (two unfractionated heparins, MMWH – middle molecular weight heparin, LMWH – low molecular weight heparin, VLMWH – very low molecular weight heparin) and a series of semisynthetic β-1.3 linked glucan sulfates (phycarin sulfates) with differing degrees of sulfation (DS 0 – 2.8) were tested.

Experimental melanoma lung metastasis

Mice received 400 µg phycarin sulfate/20 g (control: NaCl) i.v. 30 min prior to B16F10 injection. The metastatic foci were investigated after 11 days.

Results

The results of the FACS experiments revealed that the three tumor cell lines do not express P-selectin glycoprotein ligand-1 (PSGL-1). However, in spite of the lack of PSGL-1 expression, the cells were able to bind to soluble P-selectin. Therefore, one can postulate that the interaction with P-selectin is mediated via ligands distinct from PSGL-1.

In the next step we investigated whether the principle ability to bind P-selectin leads to cell adhesion via P-selectin under flow conditions. For this purpose, a flow chamber was used simulating physiological shear rates present in postcapillary venules. Although the cells were able to interact with P-selectin, adhesion or rolling analogue to leukocytes could not be observed. Thus, the molecular mechanisms of tumor cell adhesion must be different from the mechanisms of leukocyte extravasation.

We therefore examined the influence of platelets, which expressed P-selectin upon activation. We observed that activated platelets interacted with cell surface molecules of the tumor cells. This might lead to a bridging of tumor cells and endothelial cells. But under dynamic flow conditions, tumor cells did not adhere to immobilized P-selectin in the presence of activated platelets.

To determine whether the positive effects of heparins on the survival of tumor patients, as described in the literature, might be selectin-dependent, we investigated P-selectin inhibition under dynamic flow conditions using different batches of heparin in comparison with semisynthetic phycarin sulfates. It could be shown that heparin is only a weak P-selectin inhibitor, whereas the semisynthetic glucans block the P-selectin function efficiently in a DS-dependent manner (Figure 1).

One of the phycarin sulfates was tested in a murine model of experimental melanoma lung metastasis and was found to dramatically decrease the number of metastatic foci.

Conclusion

This study was carried out to investigate the selectin binding mechanisms in tumor cell metastasis. The results disprove the hypothesis that a direct P-selectin-mediated tumor cell binding exists, similar to leukocyte rolling along the vessel wall. Since many in vivo studies show that selectin inhibition attenuates metastatic rates, other mechanisms of selectin involvement can be assumed, such as the formation of microemboli consisting of platelets, possibly leukocytes (via L-selectin), and tumor cells. These microthrombi...
could be trapped in the microvasculature, leading to new tumor cell colonization. Another important aspect is the protection from natural killer cells of the innate immune system by becoming surrounded by platelets. These postulations are in accord with several preclinical and clinical findings on the antitumor effects of heparin. Recent clinical data confirm that fractionated heparins improve the survival of cancer patients, and this might be a selectin-dependent process. To obtain an insight into the structural requirements of heparin in P-selectin inhibition, heparins and semisynthetic glucan sulfates were compared. The data revealed that the nature of the selectin antagonism by heparin is not as expected suggesting that additional mechanisms, such as interaction with integrins and release of TFPI (tissue factor pathway inhibitor) have to be assumed. Glucan sulfates were much more effective P-selectin inhibitors in vitro and to had antitumor properties in the murine model. Consequently, blocking of selectins appears to be a promising antitumor strategy where semisynthetic glucan sulfates are promising drug candidates.

References


Individual variation in factors affecting the steps between dose application and effects of antineoplastic agents

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Key words

The problem

The mean maximal tolerated dose is used in the cytotoxic treatment of malignancies to achieve efficacy in as many patients as possible. Typically, not all patients experience sufficient therapeutic benefit. However, attempts to intensify the treatment often result in both an increased efficacy and increased toxicity. For instance, the replacement of the COPP/ABVD regimen by a more intensive treatment using the BEACOPP polychemotherapy scheme in elderly patients with Hodgkin’s disease resulted in an improved disease-specific freedom from treatment failure, but overall survival was not improved, because higher toxicity canceled out the better efficacy [Ballová et al. 2005]. However, in individual patients, toxicity of antineoplastic agents is often correlated with good efficacy. Thus, overall reduction in mean toxicity might not be desirable. In contrast, identification of the individual maximal tolerated dose can improve the risk/benefit ratio of antineoplastic therapy. With this aim, sources of interindividual variability need to be identified and their effects quantified.

Sources of variability in antineoplastic treatment

Sources of variability may affect systemic drug exposure, susceptibility of the healthy tissue, local drug exposure and susceptibility of the malignant cells.

Systemic pharmacokinetics of antineoplastic agents depend on the same factors as other drugs, including plasma protein binding, pharmacogenetic differences in metabolism, inhibition and induction of drug-metabolizing enzymes and drug transporters, age, body weight, sex, renal function etc. In some cases, it is possible to identify the main source of variability. For instance, it has been shown that the activity of the cytochrome P450 enzyme 3A4 explains more than 50% of the variability in the individual exposure towards irinotecan [Mathijssen et al. 2004] and docetaxel when given at a standard dose. Individual drug exposure is related to drug effects. We showed recently that individual pharmacokinetic parameters of cyclophosphamide contribute to the variability in the decrease of platelet counts following BEACOPP polychemotherapy [Wilde et al. 2004]. Likewise, genetic variants of thio purine S-methyltransferase (TPMT), an enzyme involved in the elimination of 6-mercaptopurine, has an effect on the treatment of childhood acute lymphoblastic leukemia [Stanulla et al. 2005].

Currently, it is hardly possible to predict which patients will experience severe toxicity on a given systemic exposure. Interindividual differences with respect to local biotransformation in bone marrow, the HLA system, DNA repair and susceptibility to allergic reactions may influence susceptibility of healthy tissue to antineoplastic drugs. However, there are no valid molecular diagnostic procedures available for modification of drug therapy to take into account the properties of healthy tissue.

Sources of variability applicable to tumor exposure include tumor vascularization, tumor architecture and interstitial pressure. Altered blood vessels and high interstitial pressure result in a heterogeneous drug exposure in most solid tumors where the degree of exposure is often lower than in healthy tissue. Tumor exposure can be measured directly using microdialysis [Hunz et al. 2001] and may be a better predictor of therapeutic response than systemic exposure.

Sources of variability in antitumor effects have been associated with differences in intracellular pharmacokinetics of malignant cells (drug transporters, metabolism, protein binding), expression/modification of target molecules, signal transduction, DNA and protein synthesis, oxidative stress damage and repair, regulation of the cell cycle, apoptosis etc. Today, extensive characterization of malignant cells on the DNA and protein level is possible.
in the case of many malignant diseases, e.g. in malignant lymphomas. This may lead to a very detailed diagnosis which is known to be reflected in therapeutic response. The net effect of all these processes on the susceptibility of tumor cells can be quantified in the cells of individual patients by ex vivo chemosensitivity assays [Sharma et al. 2003].

**Strategies for individual treatment optimization**

Susceptibility of malignant cells and systemic exposure are the 2 sources of variability which can be addressed by treatment individualization. The former can be addressed by selection of the drug, the latter by selection of the dose of a given drug.

A more detailed diagnosis, beyond the initial clinical and histopathological diagnosis, to identify tumor subtypes is an important step towards an individualized treatment. The variability of drug response in the more specific subgroups of a disease based on additional DNA and protein diagnostics is lower than in diseases based on clinical and conventional laboratory diagnostics only and allows to select drugs with a better mean efficacy/toxicity ratio. A recent example of molecularly targeted cancer therapy is trastuzumab, a humanized recombinant monoclonal antibody that recognizes the extracellular domain of HER2 transmembrane protein (a protein that also belongs to the EGFR family) expressed in some breast cancers and which is only given to patients over-expressing this target protein. Furthermore, measuring tumor susceptibility directly by chemosensitivity assays may enable the selection of a drug with an optimal efficacy in the individual patient. In an open study in patients who had received intensive pre-treatment for recurrent ovarian carcinoma, the overall response rate of chemotherapy optimized using an ATP-based tumor chemosensitivity assay was 61%, which was markedly superior to that in histological controls [Sharma et al. 2003].

According to current practice, once an antineoplastic drug regimen has been selected, a standard dose (which is often adjusted to body surface area even when there is a lack of evidence that this parameter influences pharmacokinetics) is given, which is reduced if toxicity occurs. In contrast, a more uniform drug exposure can be achieved by identification and quantification of cofactors associated with interindividual variability in exposure and subsequent dose adjustment. The dose adjustment of carboplatin according to renal function is a simple example which is applied clinically. In addition, the more than ten-fold differences in individual activities of drug-metabolizing enzymes can be quantified using pharmacogenetic diagnostics with whole blood, if there is genetic polymorphism, and by phenotyping involving administration of a probe drug metabolized by this enzyme. Subsequently, the relationship between enzyme activity and clearance of the therapeutic agent can be transformed to a dose resulting in a more uniform area under the concentration vs. time profile [Kirchheiner et al. 2005]. If no cofactors explaining pharmacokinetic variability can be identified, dose adjustment can be based on therapeutic drug monitoring.

In summary, individualization based on the properties of both the malignant cells and the host holds promise for an improvement in the risk/benefit ratio of antineoplastic treatment.

**References**


Rational development of oxaliplatin analogues – synthesis and preliminary structure-activity relationships

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Abstract

Introduction

Colorectal cancer is the second-leading cause of cancer-related deaths in developed countries. Worldwide, there were approximately 1,000,000 new cases and 530,000 cases of death in the year 2002. Treatment of metastatic colorectal cancer (MCRC) has made considerable progress during the last 30 years. Without treatment, the overall survival time is about 6 months. The standard treatment of MCRC over the last decades has been based on 5-FU (5-fluouracil). The median survival time is about 12 months when 5-FU and leucovorin (LV) are given together. The introduction of irinotecan (Camptostar®) and use in combination with 5-FU/LV prolonged the survival time to about 15 months (IFL regimen). Substitution of irinotecan by the metal-based complex oxaliplatin extends the median survival time to 19.5 months (FOLFOX regimen). In 2004, 2 monoclonal antibodies, bevacizumab (Avastin®) and cetuximab (Erbitux®), were approved for use together with cytotoxic regimens, resulting in a prolongation of survival time beyond 21 months. In the light of cost-effectiveness, a critical point is reached: a doubling of the median survival time from 12 to > 21 months is accompanied by a dramatic increase in drug costs. In only the initial 8 weeks of treatment, drug costs have exploded from $63.– to $30,790.–. Therefore, there is still interest and need for small molecule chemotherapeutics like oxaliplatin [Galanski et al. 2005a, 2005b].

Oxaliplatin (Eloxatin®) is now approved in many countries throughout the world as a first line treatment for metastatic colorectal cancer. At present, oxaliplatin is not only the standard treatment option in MCRC, but also for adjuvant therapy in cases of stage III primary colon cancer. Based on the assumption that the steric demand and/or the lipophilicity of the cyclohexane ring are structural requirements for the specific pharmacological properties of oxaliplatin, derivatization of the cyclohexane ring might result in a marked effect on antitumor activity. Following this concept and in order to explore structure-activity relationships, a series of novel oxaliplatin analogues were synthesized and characterized (Figure 1). Their in vitro antitumor activity in comparison to oxaliplatin has been tested in a panel of human cancer cell lines.

Material and methods

The novel mono- and dialkyl-substituted oxaliplatin derivatives were synthesized via three different pathways, as published recently [Galanski et al. 2004, 2005c, Habala et al. 2005], and characterized by NMR spectroscopy and elemental analysis. Their cytotoxic potency was investigated in different human tumor cell lines originating from the colon (SW480, SW620, HCT-15, COLO 205, HT-29), and ovarian carcinoma (NIH-OVCAR-3) as well as from leukemia (MOLT-4, HL-60) and melanoma (SK-MEL-5).

Results

Evaluation of different synthetic strategies finally resulted in the preparation of...
novel oxaliplatin analogues with a defined stereochemistry at carbon atoms 1, 2, 4, and 5. The 4-methyl-, cis-4,5-dimethyl-, and the 4,4-dimethyl-trans-cyclohexane-1,2-diamine(oxalato)platinum(II) complexes, which are the most promising derivatives so far, show a comparable or slightly better cytotoxic potency with respect to the parent compound oxaliplatin in most cell lines. These results are even more remarkable taking into account that racemic mixtures of the new derivatives have been tested. After enantiomer separation, an increase of the cytotoxicity by a factor of 2 – 3 is expected. Contrary, analogues with a bulky substituent at C(4) (propyl, phenyl, or tert-butyl) display a lower cytotoxicity in the cell lines under investigation.

In conclusion, it could be demonstrated in a series of human cancer cell lines as well as in preliminary animal experiments that an increase in anticancer activity and tolerability of new oxaliplatin derivatives is feasible.

References


Colorectal cancer (CRC) is still fatal in the majority of patients with the disease. Fortunately, new treatment options for CRC have been developed over the last few years. Treatment combination regimens consisting of chemotherapeutic agents such as fluoropyrimidines, irinotecan and oxaliplatin and monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) promise significant improvements in clinical outcome for these patients. However, enormous interindividual differences regarding toxicity and benefits of therapy (response rate, overall survival) still remain the norm with all therapy regimens. What can be done to identify the treatment regimen combining highest efficacy and lowest toxicity for the individual patient?

Variations in genetic factors i.e. so-called genetic polymorphisms, have been responsible for these interindividual differences (for a more detailed review see [Stoehlmacher 2005]). Based on the location of a polymorphism within a certain gene, varying levels of activity are created for the encoded protein. These differences can translate into variations in drug metabolism (e.g. activation, detoxification, efflux etc.), protein interaction (e.g. substrate binding) and alterations in repair of DNA damage. Finally, increased or decreased bioavailability of the administered drug may have an impact on efficacy and toxicity of a given drug combination.

Significant associations between functional genetic polymorphisms and clinical outcome have already been identified in the case of the main chemotherapeutic drugs currently used in the therapy of CRC.

TS represents a central enzyme of fluoropyrimidine metabolism and is the sole de novo source of thymidylate in the cell. 5-FU, which directly inhibits TS, still remains the most often used chemotherapeutic agent in CRC patients. So far, three functional genomic polymorphisms within the TS gene have been identified and linked to clinical outcome in CRC patients. Especially a TS promoter polymorphism consisting of either a double or triple repeat of a 28 bp sequence with an additional G>C SNP within the second repeat seems to have a major clinical impact in 5-FU-treated CRC patients [Marcuello et al. 2004]. Up to now, the impact of a 6 bp deletion polymorphism located in the 3’UTR of the TS gene is controversially discussed. Two polymorphic regions of the MTHFR gene (C677T and A1298C) are responsible for lower enzyme activity, resulting in lower levels of reduced folate 5,10-methylenetetrahydrofolate. The impact of these polymorphisms on clinical fluoropyrimidine efficacy and toxicity is presently being explored. DPD represents a main player in fluoropyrimidine metabolism, catalyzing > 80% of the drug in the liver. A G>A substitution in the invariant GT splice donor site flanking exon 14 (IVS14+1G>A) results in diminished enzyme activity and thus in severe toxicities. Although frequencies of the IVS14+1A variant are very low, with approximately 1% in Caucasians, this polymorphism should be considered, especially if other drugs with similar toxicity profiles like CPT-11 are added to the regimen.

CPT-11 and oxaliplatin have both been shown effective in the treatment of CRC. For both agents, predictive functional polymorphisms have been identified. Polymorphic genes of the DNA repair system (e.g. ERCC1, XPD) or glutathione S-transferases (GST’s) could be linked to the efficacy of oxaliplatin-based chemotherapy in CRC [Stoehlmacher et al. 2002, 2004]. The family of UDP-glucuronosyltransferases (UGT) is involved in activating processes of SN-38G, the active metabolite of CPT-11. Polymorphisms in UGT1A1, UGT1A7 and UGT1A9 have been shown to influence efficacy and toxicity of CPT-11-based chemotherapy in CRC [Carlini et al. 2005, Innocenti et al. 2004].

It is clear that the impact of known polymorphisms in EGFR and VEGF on clinical outcome in CRC patients treated with...
agents directed against these growth factors is not well understood (e.g. Cetuximab, Bevacizumab, Gefitinib). Nevertheless, initial studies on the correlation of polymorphisms and clinical endpoints in CRC patients have been reported. The number of the (CA)_n sequence repeat in intron 1 of the EGFR gene is related to the appearance and grade of skin toxicity in CRC patients treated with gefitinib [Amador et al. 2004].

The available data (mostly) from retrospective studies are promising and several associations have been confirmed in independent investigations. The next step toward a tailored treatment strategy in patients should be the inclusion of these parameters in clinical protocols to establish the level of toxicity to be expected and the efficacy. Despite the exciting implications of pharmacogenetic analyses for prediction of drug toxicity, more dynamic markers like gene or protein expression appear even more promising in predicting efficacy. This is supported by several studies in CRC (TS, DPD, ERCC1, etc.). Molecular predictors are an additional aid and improvement in establishing histopathological criteria. They will help to create an individual “toxicity and efficacy pattern” which will be useful in choosing the optimal substances from the growing armamentarium of effective drugs to treat a particular tumor and patient. There are still limitations affecting the integration of molecular parameters into daily routine such as the limited availability of experienced laboratories. Until the predictive character of certain molecular markers has been proven, their use in studies and the restriction of these analyses to specialized laboratories is warranted. We are only beginning to appreciate the power of molecular parameters in significantly improving the treatment of our patients. In this context, pharmacogenetics has achieved a central role. Over the last decades, our chances in developing individualized, improved treatment in CRC has never been better.

References


Identification of new genes involved in cisplatin resistance and their expression profile in 18 human tumor cell lines

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Introduction

Cisplatin plays a central role in the treatment of a wide variety of human cancers such as testicular, ovarian, cervical, head and neck and non-small-cell lung cancer [Wang and Lippard 2005]. Unfortunately, its effectiveness is limited by intrinsic and acquired resistance. Many genes involved in cisplatin resistance have already been identified. They are involved in drug uptake and efflux, detoxification, DNA repair and inhibition of apoptosis [Siddik 2003]. Since the known genes do not account for all of the observed cisplatin resistance in tumor cells, the multifactorial and complex phenomenon of cisplatin resistance needs further elucidation and a search for further candidate genes predicting chemosensitivity of cisplatin. The aim of this study was to identify new genes involved in cisplatin resistance and to examine their expression characteristics after cisplatin treatment in a panel of different tumor cell lines originated from different human tissues. The results of this study will help to add further candidate genes to the list of genes predicting the chemosensitivity of cisplatin and may eventually help to identify new targets to circumvent cisplatin resistance.

Suppression Subtractive Hybridization (SSH)

SSH was performed as previously described [Gosepath et al. 2004].

Methods

Cell lines and cytotoxicity assay

18 human cell lines were analyzed for their chemosensitivity against cisplatin and for changes in gene expression upon treatment with cisplatin. Cell lines were obtained from the European Collection of Cell Cultures (ECACC, United Kingdom: A2780, A2780cis, 1321N1-WT, HT-29, U937) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany: CAL27, CAL27cis, Kyse-510, K-562, Hek293-WT, Hep-G2, CaCo-2). 2102 and 2102KLE were kindly provided by Dr. W. Vogt (University of Halle, Germany), DU-145, MCF-7 and T24 were a gift from Dr. R. Hartmann (University of Saarbrücken, Germany), and NW1539 cells were kindly obtained from Dr. R. Ludwig (University of Frankfurt, Germany). The cisplatin-resistant cell line CAL27cis was established by repeated exposure to 10 μM cisplatin. Cell lines were either preserved in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, 50 U/ml penicillin G and 365 μg/ml L-glutamine (2102, 2102KLE, A2780, A2780cis, DU-145, Hep-G2, K-562, Kyse-510 and U937) or in DMEM supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, 50 U/ml penicillin G and 365 μg/ml L-glutamine (1321N1-WT, NW-1539, CaCo-2, CAL27, CAL27cis, Hek293-WT, HT-29, MCF-7, T24) (all: Sigma Chemical, Taufkirchen, Germany). IC50 values of cisplatin were determined by an improved MTT assay as previously described [Mueller et al. 2004].

qRT-PCR

Confirmation and quantification of differential gene expression of candidate clones from SSH identified by sequencing and BLAST was performed by qRT-PCR using SYBR Green I dye-based detection on an Opticon Monitor (MJ Research, Waltham, MA, USA). PCR primers were designed with Primer3 [Rozen and Skaltsky 2000]. Data were normalized to internal control genes (housekeeping genes) which were determined using the geNorm program according to Vandesompele and colleagues [2002] and quantified by the ΔΔCT-method [Livak and Schmittgen 2001].
Results

Two SSH performed after treatment with cisplatin, one comparing the cisplatin-sensitive ovarian cancer cell line A2780 with the resistant A2780cis (resistance factor 4.5), the second comparing the cisplatin-sensitive tongue cancer cell line CAL27 with the resistant CAL27cis (resistance factor 6) [Gosepath et al. 2004], yielded a number of clones from which 10 genes (listed in Table 1) were identified and confirmed by qRT-PCR to be differentially expressed in A2780 versus A2780cis or CAL27 versus CAL27cis. To evaluate the 10 candidate genes plus 4 closely related genes in terms of their significance for chemosensitivity of tumor cells against cisplatin, their expression levels upon treatment with cisplatin was estimated by qRT-PCR in 18 human cancer cell lines derived from various tissues. As exemplified in Figure 1 for the aldoke reductase AKR1C1 and holding true for all examined candidate genes, no quantitative correlation between the degree of resistance against cisplatin and the expression level of the candidate genes after treatment with cisplatin was found. However, certain cancer cell lines show a strong regulation of the candidate genes upon treatment with cisplatin: AKR1C1 was ~8-fold up-regulated in Kyse-510 and 1321-N1, whereas it was ~10-fold down-regulated in MCF-7 cells (Figure 1). Fold changes for the 14 candidate genes varied between 0.1- and 277-fold (Table 1).

Conclusions

The combination of SSH and subsequent qRT-PCR led to the identification of 10 candidate genes possibly involved in chemosensitivity against cisplatin and so far not described in the context of cisplatin resistance. Most candidate genes show a marked change in expression upon treatment with cisplatin (up to 277-fold) in certain cell lines, thus assuming a role in chemosensitivity against cisplatin in certain cancer types. Further studies using siRNA are under investigation.

References

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Siddik ZH 2003 Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 22: 7265-7279

Table 1. Ten candidate genes identified by SSH plus 4 additional genes (*) and their ranges of fold change in expression upon treatment with cisplatin over 18 tumor cell lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Range of fold change</th>
</tr>
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<tr>
<td>AKR1B1</td>
<td>0.7 – 43.6</td>
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<tr>
<td>AKR1C1*</td>
<td>0.1 – 8.4</td>
</tr>
<tr>
<td>CSTA</td>
<td>0.4 – 90.9</td>
</tr>
<tr>
<td>CTSB*</td>
<td>0.1 – 4.1</td>
</tr>
<tr>
<td>DDX21</td>
<td>0.2 – 4.1</td>
</tr>
<tr>
<td>DKK1</td>
<td>0.4 – 108.8</td>
</tr>
<tr>
<td>EGR1*</td>
<td>0.1 – 136.4</td>
</tr>
<tr>
<td>EMP1</td>
<td>0.3 – 277.2</td>
</tr>
<tr>
<td>HF1</td>
<td>0.1 – 3.5</td>
</tr>
<tr>
<td>p53*</td>
<td>0.6 – 5.2</td>
</tr>
<tr>
<td>PI15</td>
<td>0.2 – 227.3</td>
</tr>
<tr>
<td>RPL5</td>
<td>0.3 – 2.5</td>
</tr>
<tr>
<td>THBS1</td>
<td>0.2 – 81.2</td>
</tr>
<tr>
<td>TRA1</td>
<td>0.2 – 1.3</td>
</tr>
</tbody>
</table>
Interactions of a novel ruthenium-based anticancer drug (KP1019 or FFC14a) with serum proteins – significance for the patient

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Introduction

The discovery and development of tumor-inhibiting ruthenium(III) complexes has been initiated with the intention of overcoming the serious side effects of established platinum-based drugs and to increase the spectrum of tumors which can be effectively treated. Indazolidine trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019 or FFC14a; see Figure 1) [Galanski et al. 2003] and (H3-im)trans-[RuCl3(DMSO)(Him)] (Him = imidazole; NAMI-A) [Sava et al. 1999] are currently undergoing clinical investigations. KP1019, in particular, has successfully been through a phase I clinical trial, has shown evidence of activity without severe side effects (stable disease) [Dittrich et al. 2005]. It exhibits its activity in a chemically induced autochthonous colorectal tumor model that resembles human cancer and in a broad range of primary explanted human tumors [Reisner et al. 2005]. Its binding to plasma proteins is thought to be a step of utmost importance for exerting the antitumor activity. Specifically, there is much experimental evidence that the Ru moiety is transferred into the tumor predominantly via the transferrin pathway [Pongratz et al. 2004].

In this report, more informative analytical schemes i.e., capillary electrophoresis (CE) [Timerbaev et al. 2005], electrospray ionization mass spectrometry (ESI-MS) and two-dimensional high-performance liquid chromatography (HPLC)-inductively coupled plasma (ICP)-MS [Sulyok et al. 2005] are described for the determination of binding constants and reaction kinetics of KP1019 with albumin and transferrin and to determine drug distribution between the main binding molecules in patient serum.

Methods

The binding constants were assessed by CE (fused-silica capillary: 40 cm × 75 μm i.d.; UV detector: 200 nm) under conditions similar to extracellular fluid i.e., using solutions and incubation electrolytes containing 10 mM phosphate buffer, pH 7.4, 100 mM NaCl, at 37 °C. The stoichiometry of KP1019 binding to 2 plasma proteins was determined by ESI-MS at various molar ratios and for varying incubation periods. Before analysis, low concentrations of formic acid (0.5 – 2%), methanol and acetonitrile were added to the incubation mixtures in order to facilitate the spraying and ionization processes. A two-dimensional HPLC scheme consisting of size-exclusion (SEC) and ion-exchange (IEC) columns was developed. Detection was achieved by a combination of an UV/vis detector (480 nm) and an online-coupled ICP-mass spectrometer equipped with a dynamic reaction cell. The analyzed plasma samples from a cancer patient being treated for solid tumors and involved in a phase I clinical study were compared with albumin and apotransferrin standards.

Results

The protein-binding parameters were measured in vitro by monitoring the time- and concentration-dependent changes in CE peak-area responses for the reaction components. Comparison of the apparent rate constants (k) revealed that KP1019 binds to transferrin much faster than to albumin: k = 39.5 × 10⁻⁴ and 3.3 × 10⁻⁴ s⁻¹, respectively. The corresponding association constants in-
icate a moderate metal-protein binding with a higher affinity of KP1019 towards albumin than apotransferrin (9900 and 6500 M$^{-1}$, respectively). This result was proven by IEC-ICP-MS analysis of a KP1019-transferrin adduct formed by interaction with albumin where there was a transfer of the Ru moiety to the protein. In in vivo studies performed by SEC-ICP-MS, the drug was found to bind entirely to the protein fraction of 60 – 80 kDa (i.e., to the proteins under examination). The SEC/IEC-ICP-MS approach delivered the stoichiometric data on the KP1019-protein binding through acquiring the Ru/S molar ratio. In plasma samples taken from a cancer patient treated with the drug, the concentration of the transferrin adduct was below the limit of quantification of the method and the overall Ru content was below 1%. The KP1019-to-albumin stoichiometry in the plasma samples, the largest value being 1.4, could be correlated with Ru plasma concentrations over 26 days. The corresponding KP1019-per-transferrin loading ranged from 0.3 – 0.5, thus falling in the expected range for a sufficient drug uptake via the transferrin pathway [Pongratz et al. 2004]. The binding stoichiometry was additionally characterized by ESI-MS. The presence of methanol (or acetonitrile) and formic acid did not impair the binding but improved the mass spectra obtained. Of all transferrin : KP1019 ratios tested in the incubation the highest binding ratio was found at a ratio of 1 : 2.

**Conclusions**

The binding of KP1019 with transferrin is kinetically favored, whereas the albumin adduct is thermodynamically more stable than the respective transferrin conjugate. Correspondingly, the Ru moiety was bound predominantly to albumin in vivo. The measured ruthenium-to-albumin ratios in patient plasma samples over time resemble the pharmacokinetics of KP1019 in plasma. The fact that adducts no larger than bisadducts were identified in ESI-MS spectra shows that only the specifically loaded Ru moieties were attached to the protein. The KP1019-to-transferrin molar ratio estimated in in vivo experiments indicates that only a fraction of transferrin was involved in KP1019-adduct formation. This finding appears important from the clinical point of view because cellular uptake studies revealed increased Ru concentrations in the cell when transferrin was loaded even to a minor extent with KP1019.

**Acknowledgment**

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References


A new oxygen-enriched solution enables human tumor tissue transport without cell devitalization

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Introduction

Inquiries by patients requesting long-time storage of their excised tumors are increasing. Conserved tumor tissues/biopsies might enable patients to benefit from new discoveries in cancer research because it might allow for optimization of their cancer treatment. Therefore, transport and handling of patient material (tumors and associated normal tissue) for analyses in genomics and proteomics are crucial.

Recently, a new medium called Liforlab solution (LS) was developed for improved tumor sample transport at room temperature (RT). LS is a physiological, oxygen-enriched solution, containing inorganic salts, amino acids, vitamins, adenosine, cholesterol, glucose, dextran 70, the growth factors EGF, VEGF and HGF and low protein. As stated, LS allows tumor sample storage and transport without any affecting cell proliferation and viability of the transported tissue. Moreover, LS contains a high and non-physiological high concentration of oxygen fixed on nano-particles which donated to the tissue when needed.

Furthermore, cryopreservation of tumor samples in LS was shown to be feasible without addition of cryoprotective agents like DMSO and HES. Tumor samples were frozen using a special procedure adequate for LS, without any ice crystallization during freezing/thawing.

In this study, we examined

– the viability of tumor samples (cells/biopsies) stored in LS at RT and in liquid nitrogen;
– the tumor activity of biopsies after long-term incubation in LS;
– the content and quality of total RNA and DNA in tumor samples stored in LS at RT and –196 °C;
– the generation of primary cell cultures from tumor biopsies after incubation in LS at RT and –196 °C.

Materials and methods

Tumor samples: Tissues/biopsies were obtained from patients with carcinoma of prostate, colon, bladder, stomach and kidney as well as from patients with lung, gastric, thyroid and esophageal cancer. All patients gave their informed consent. In addition, more than 30 different cell lines (parental and resistant) were used in this study: HCT8 and PC3 cells were used for examination of cell viability after long-term conservation in LS at –196 °C.

PSA values were determined using the fluorescence immunoassay. Staining of PSA expression was done by immunohistochemistry.

Generation of tumor cell lines: Tumor biopsies were incubated in LS or regular medium at RT for up to 72 hours with subsequent enzymatic preparation using tissue dissociation medium (mixture of collagenase A, dispase grade III and DNAse I). In addition, prostate cancer and melanoma fragments were cryopreserved in LS for up to one year, thawed, subsequently prepared as single cell suspensions and cultured in regular medium.

RNA and DNA isolation was performed according to standard procedures using cell lines pre-incubated in LS for up to 72 hours at RT and for up to one year at –196 °C. Tumor biopsies were stored in LS at RT for 5 days with subsequent RNA extraction.

Results

Human cancer tissue fragments of thyroid, prostate, lung, kidney and sarcoma were incubated in LS for up to 72 hours at RT and subsequently transferred to a primary cell culture in regular medium. In contrast, prostate cancer samples stored at RT in regular me-
dium (up to 72 hours) showed reduced cell proliferation and loss of viability.

In addition, the correlation between the tumor marker PSA and tumor activity was determined in prostate cancer biopsies cultured in LS as single cell suspensions, with high PSA levels up to 50 days. Moreover, about 40% of prostate cancer cells of biopsies transferred in LS at RT, stored at −196 °C in the same solution for one year, thawed and cultured in regular medium, still possessed membrane located PSA.

Furthermore, tumor cell lines incubated in LS for more than 96 hours were less strongly attached to the flask, compared to control cell lines, but did not show reduced viability. These cells re-attached in regular medium. Long-time incubation of tumor tissues in LS at RT (more than 5 days) resulted in increased uptake of LS and a slight reduction of tissue homogeneity.

Total RNA and DNA from tumor samples (lung and gastric cancer as well as carcinoma of the thyroid, esophagus and colon) stored in LS for up to 8 days at RT and 4 °C, respectively, did not show reduced viability. In addition, LS had no effect on the quality of RNA/DNA in cell lines cryopreserved for up to one year.

Moreover, tumor samples (cells/biopsies) cryopreserved in LS (for up to one year) without cryoprotectors were regrown after thawing (at 37 °C for 5 min). Cancer cells (HCT8 cells) stored in LS at −196 °C were recultured in regular medium, and primary cell cultures were established from frozen cancer tissues (prostate) (Figure 1).

Conclusions

LS allows storage and transfer of human tumor cells/biopsies for up to 72 hours at RT without loss of viability. LS conserves cell lines and cancer tissues (prostate/melanoma) at −196 °C without DMSO and HES. Generation of primary cell cultures was feasible after incubation of tissue samples in LS for up to 72 hours at RT and for up to one year at −196 °C (prostate/melanoma). Moreover, storage of tumor samples in LS did not affect DNA or RNA stability.

References

Pharmacokinetics (PK) of a liposomal encapsulated fraction containing doxorubicin and of doxorubicin released from the liposomal capsule after intravenous infusion of Caelyx™/Doxil®

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Introduction

The clinical utility of doxorubicin, the most widely used anthracycline, is limited by toxicity that may preclude adequate dosing and diminish its therapeutic effect on relapse, or lead to drug resistance. Caelyx™ (Doxil®), a doxorubicin formulation of polyethylene glycol-coated liposomes, has demonstrated activity against solid tumors with mild myelosuppression, minimal hair loss and a low risk of cardiotoxicity. For this new form of doxorubicin, a superior pharmacokinetic profile over that of free doxorubicin is postulated. It is also theorized that there is minimal drug-leakage of these long-circulating particles, because the concentration of doxorubicin in the liposomes exceeds the water solubility of doxorubicin.

Purpose

It was the aim of this study to analyze the plasma levels as well as the cellular uptake of encapsulated and free doxorubicin. For this purpose, Caelyx was administered i.v. to patients with various refractory malignancies at doses of 30, 40, 50, and 60 mg/m² (equivalent to doxorubicin HCl) within one hour, once every 28 days, for as long as patients responded and tolerated the treatment.

Methods

To answer the question of a possible accumulation of Caelyx, plasma and urine analysis was performed on days 1, 2, 3, 4, 5, 8, 15, 22 and 28 of each cycle. Additionally, spontaneous urine fractions were collected for the first three days, time point and volume of each fraction were documented and a 100 µl aliquot of urine was separated for quantification. Free doxorubicin of either plasma or urine was bound to silica gel. After centrifugation, the liposomes (upper phase) were separated from the silica-bound doxorubicin fraction (lower phase) and the fractions extracted with acidified methanol. For the quantification of doxorubicin, doxorubicinol, and 7-deoxy-doxorubicinolon, volumes of 200 µl of each fraction were injected to the following validated RP-HPLC system: a Waters 717 Plus Autosampler connected to a Spectra Physics Ternary Gradient Pump and a Waters 474 Scanning Fluorescence Detector (excitation wavelength: 470 nm, emission wavelength: 550 nm); solvent system: 1 ml/min 28% acetonitrile and 72% aqua (20 mM di-potassium hydrogen phosphate, 20 mM 1-heptanesulfonic acid sodium salt, pH = 7.50 with ortho-phosphoric acid) at 0 min to 40% acetonitrile and 60% aqua within 30 minutes at RT; column: Waters Symmetry, 250 × 4.6 mm; software: Waters Millennium Chromatography Manager. Blood and urine samples were analyzed for PK calculations by the use of Kinetta 2000 (InnaPhase Sarl, Champs-sur-Marne, France). In addition, cellular uptake of doxorubicin was analyzed in single intraindividual comparisons.

Results

Results are based on analysis of 30 patients, receiving 57 administrations of Caelyx, resulting in 551 administrations of Caelyx, 319 urine samples and 313 cell samples.
Calculations were performed using the pharmacokinetic data analysis system Kinetica (InnaPhase Corporation). The pharmacokinetics of Caelyx and free doxorubicin are well described by a one-compartment distribution. Although the metabolite 7-deoxydoxorubicinol was detected in several samples, pharmacokinetic calculations are not available up to now. Free doxorubicin but no Caelyx was excreted in the urine (data not shown).

Pharmacokinetics are linear for the encapsulated doxorubicin formulation as well as for doxorubicin released from Caelyx, with a dose-dependent increase of AUC and $C_{\text{max}}$.

There is no evidence for an accumulation of the free doxorubicin in plasma up to a dose of 50 mg/m² Caelyx every 4 weeks. The mean (terminal) half-life for Caelyx was calculated as 3 days, mean residence time (MRT) about 5 days, the $V_{ss}$ 1.5 l, and clearance was less than 1 ml/min. There was no detectable renal excretion ($CL_{\text{ren}}$) of the intact liposomes. The calculated PK parameters for the released doxorubicin indicated an increased area under the concentration/time curve (AUC) and MRT and a decreased $C_{\text{max}}$ and clearance (CL) in comparison with PK parameters of conventionally administered free doxorubicin previously published. Cellular uptake of doxorubicin after application of Caelyx was greater than the uptake of doxorubicin from an equivalent conventional dose of doxorubicin.

**Conclusions**

In addition to the indirect tumor targeting using long circulating pegylated liposomes, the slow release of adequate amounts of doxorubicin into plasma enables potential (micro)metastases to be treated and increases the antiangiogenic properties of doxorubicin [Drevs et al. 2004]. This formulation can also be used as an option in the management of leukemia.

**References**

Early results from a phase I study on orally administered tris(8-quinolinolato)gallium(III) (FFC11, KP46) in patients with solid tumors – a CESAR study (Central European Society for Anticancer Drug Research – EWIV)


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Introduction

Gallium nitrate has shown notable anticancer activity in phase II trials in lymphoma and bladder cancer [Chitambar 2004], but its efficacy is compromised by nephrotoxicity (when administered as a short infusion) and there are occasional cases of optical neuropathy and blindness (when given as a protracted continuous infusion). In an attempt to overcome these limitations, the oral administration of gallium chloride has been examined but the results have been discouraging because of an unsatisfactory bioavailability [Collery et al. 2002].

FFC11 (KP46) is a novel, orally bioavailable non-charged octahedral gallium complex comprised of three bidentate 8-hydroxyquinolinato ligands [Jakupec and Kepller 2004]. FFC11 was selected from a series of complexes because of its higher oral bioavailability in animal models compared to gallium chloride. Higher serum and tissue gallium concentrations were achieved after a smaller number of administrations of equimolar doses in mice. Furthermore, the pattern of organ distribution was comparable to that observed after gallium chloride administration.

FFC11 exerts its antitumor activity by replacing iron in the R2-subunit of ribonucleotide reductase resulting in a loss of activity at the catalytic site of the enzyme and consequently in a reduction in cellular dNTP pools.

Moreover, cells are arrested in the S-phase and induction of apoptosis has been demonstrated in Walker carcinoma-256 cells. In preclinical models, FFC11 proved to be a stronger anticancer agent than gallium nitrate and it was effective in a model of tumor-associated hypercalcemia.

Material and methods

An open-label dose-escalation phase I clinical trial was initiated in order to determine the maximum tolerated dose of FFC11, the dose-limiting toxicity (DLT) and a suitable dose for phase II trials. Female or male patients (18 – 75 years) with histologically or cytologically verified malignant tumor were eligible if the tumor had failed prior treatment or if no standard therapies existed. The patients were required to have stopped previous treatment at least four weeks prior to the study and to have a life expectancy of at least three months.

An accelerated titration design (one patient per dose level) with initial escalation steps of 100% was chosen with a starting dose of 30 mg/m² [Simon et al. 1997]. In the event of toxicity NCI-CTC grade ≥ 2, two more patients had to be treated at the respective dose level. Accelerated dose escalation was halted if 2 out of 3 patients had toxicity grade ≥ 2 or if one patient experienced a DLT, defined as follows: febrile neutropenia grade 4 or neutropenia grade 4 lasting for ≥ 7 days, thrombocytopenia grade ≥ 3, non-hematological toxicity grade ≥ 3.
Detailed pharmacokinetic profiles were obtained on Days 1 and 14; trough levels were measured additionally on Days 2, 3, 4, 8 and 11. Gallium concentrations in plasma were determined using a validated graphite furnace atomic absorption spectrometry method.

Results

A total of 7 patients were entered at the following dose levels: 30 mg/m² (n = 3), 60 mg/m², 120 mg/m², 240 mg/m² and 480 mg/m² (n = 1, each). Localizations of the primary tumors were: head and neck, stomach, kidney (n = 4) and ovaries. The first patient treated at the lowest dose level exhibited grade 2 leukopenia, and 2 more patients were treated at that dose. No further adverse event grade ≥ 2 was recorded and dose escalation was continued up to 480 mg/m². FFC11 was well tolerated up to the highest investigated. The study was terminated for feasibility reasons. Adverse events, where there was a relationship to the study medication that could not be definitively ruled out were: vomiting grade 2 and nausea grade 1 (n = 1; 30 mg/m²), stomatitis grade 1 (n = 1; 60 mg/m²), acne grade 1 and headache grade 2 (n = 1; 240 mg/m²) and fatigue grade 1 (n = 2; 120 and 480 mg/m²).

After the first treatment cycle, the disease stabilized in 3 out of 4 patients with renal cell carcinoma who had documented progression at the time of entry to the study and treatment was continued in these cases on compassionate grounds. In one of these patients there was a partial remission after the second cycle. The longest duration of stable disease was 49 weeks in a patient treated with the lowest dose level of 30 mg/m².

Samples from Patient 1 (30 mg/m²) and 7 (480 mg/m²) have already been analyzed and the pharmacokinetic parameters (Table 1) indicate that gallium is bioavailable after oral administration of FFC11. Peak plasma levels were reached 5 – 7 h after intake. $C_{\text{max}}$ and AUC increased with increasing dose, CL/F and $V_{\text{ss}}$/F were high and terminal half-life was relatively long. Data to show that gallium has linear pharmacokinetics after administration of FFC11 are not yet available.

Conclusions

FFC11 is a lipophilic gallium complex suitable for oral administration. It shares the mode of action of other gallium compounds but exhibits greater efficacy in preclinical studies. FFC11 was well tolerated up to the highest investigated dose level of 480 mg/m². No toxicity greater than NCI-CTC grade 2 was observed even after administration periods of up to 49 weeks. Gallium pharmacokinetics after FFC11 is characterized by a long terminal half-life, a high CL/F and a large V/F. The preliminary evidence for efficacy in patients with renal cell cancer indicates that phase II trials should be started.

References

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<table>
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<tr>
<th>Patient</th>
<th>Dose [mg/m²]</th>
<th>$C_{\text{max}}$ [ng/ml]</th>
<th>$t_{1/2 z}$ [h]</th>
<th>AUC [µg x h/ml]</th>
<th>CL/F [l/h]</th>
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<td>15.31</td>
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<tr>
<td>7</td>
<td>480</td>
<td>62.65</td>
<td>121.5</td>
<td>11.22</td>
<td>11.1</td>
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</table>

Table 1. Pharmacokinetic parameters of gallium after oral administration in two patients.
Molecular targets of indirubins

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Key words

Introduction

The 3,2′-bisindole indirubin has been identified as the biologically active ingredient of a herbal medicine used in Traditional Chinese Medicine for the treatment of chronic myelogenous leukemia (CML). Indirubins have been shown to act as potent inhibitors of isolated recombinant cyclin-dependent kinases (CDKs) [Hoessel et al. 1999] as well as of immunoprecipitated enzyme preparations after incubation of intact human tumor cells [Marko et al. 2001]. Indirubin derivatives effectively inhibit the growth of various human tumor cell lines in the low micromolar range [Hoessel et al. 1999]. Growth inhibition is also observed in vivo at non-toxic doses in the human xenograft tumor LXFL529 [Fiebig et al. 2001]. Furthermore, indirubins cause a concentration-dependent cell cycle arrest at G1/S and G2/M phases in a wide spectrum of human tumor cell lines [Hoessel et al. 1999]. In most cases, cell cycle arrest is followed by induction of apoptosis [Marko et al. 2001]. A highly attractive feature of indirubin derivatives lies in the fact that by appropriate molecular substitution pattern of the parent indirubin molecule inhibition of a broad spectrum of cellular targets can be achieved. This includes glycogen synthase kinase-3β (GSK-3β) [Leclerc et al. 2001] and vascular endothelial growth factor receptor-2 (VEGFR-2/KDR) [Siemeister et al. 2002]. Therefore, we have synthesized novel 5,3′-substituted indirubin derivatives with improved properties, offering perspectives towards multi-mode anti-signaling molecules, potentially leading to highly promising antineoplastic drugs.

Material and methods

Effects on cell growth were determined according to the method of Skehan et al. [1990] with slight modifications as published earlier [Marko et al. 2001]. For in vitro inhibition of human recombinant CDK/cyclin complexes, human recombinant CDK/cyclins, expressed in Sf21 insect cells, obtained from Upstate, Lake Placid, NY, USA (CDK1/cyclin B: # 14-450; CDK2/cyclin A: # 14-448, CDK2/cyclin E: # 14-475) with a specific activity according to the lot number were used. The assays were performed following the manufacturer’s protocol, with 0.4 µCi [γ-32P]ATP per sample vial. Briefly, the respective CDK/cyclin assay mixture was incubated for 10 min at 30 ºC. Thereafter, the solution (20 µl) was spotted on P81 phosphocellulose squares. After washing (3 × 5 min with 0.75% phosphoric acid), the sheets were rinsed with acetone and dried. The squares were transferred into scintillation vials and subjected to β-counting. The IC₅₀ values were determined by linear regression of the data points comparing drug-treated samples with solvent control. Each measurement was done in duplicate in at least three independent experiments. To study effects on retinoblastoma protein (pRb), LXFL529L cells were arrested in G1 phase with pRb in the hypophosphorylated state by serum deprivation (0.2% FCS; 70% confluence) for 3 days. Incubation with substances was performed for 24 h in 10% FCS. After washing twice with ice-cold PBS, 200 µl of SDS-buffer (25 mM Tris/HCl, pH 6.8; 1% (w/v) SDS; 5% (v/v) glycerol; 50 mM NaF; 2 mM Na₃VO₄) were added, and dishes were frozen (at least 2 h at −80 ºC). Cells were scraped off and processed according to manufacturer’s instructions. The resulting supernatant was DNA-digested, and the protein level was determined by the Bradford assay; 20 µg of total protein were used for SDS-PAGE (8% acrylamide) and blotted afterwards onto a nitrocellulose membrane. Western blot detection was done using anti-phospho-Rb (Ser780), anti-phospho-Rb (Ser795), anti-phospho-Rb (Ser807/811) and Rb-C-terminal-control antibody (Cell Signaling Technology). Effects on non-receptor tyrosine phosphorylation (c-Src) and Stat-signaling using the breast carcinoma cell line MDA-MB-468 have been carried out as described by Nam et al. [2005].
Table 1. Inhibition of tumor cell growth by indirubin derivatives in vitro (IC_{50} values, μM).

<table>
<thead>
<tr>
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<th>LXFL529L</th>
<th>MCF-7</th>
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<td>E671</td>
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<td>E729</td>
<td>0.5</td>
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<td>E804</td>
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**Results**

We first studied effects of the novel 5,3’-indirubin derivatives E671, E729 and E804 (Figure 1) on CDK inhibition using recombinant human CDK/cyclin complexes, expressed in Sf21 insect cells. For most CDK/cyclin complexes, these compounds were more effective than the well-known CDK/cyclin inhibitor roscovitine, with the exception of CDK1/cyclin A and CDK2/cyclin A IC_{50} values for E671. Incubation of the intact human tumor cell lines LXFL529L and MCF-7 showed growth inhibition in the low micromolar range for all three indirubin derivatives (Table 1). Furthermore, we studied the influence of the indirubin derivative E671 on CDK4/CDK6/cyclin D-specific phosphorylation sites of the retinoblastoma protein in LXFL-529L cells. After 24 hours incubation, CDK4/CDK6-dependent phosphorylation of pRb was significantly reduced already at 1 μM. Total pRb levels were also significantly affected under these conditions [Eisenbrand et al. 2004]. Thus, although this compound had been found previously by us to exert only moderate inhibitory activity on recombinant CDK6/cyclin D in vitro, its inhibitory effect on pRb phosphorylation in intact cells is quite strong. This might result from E671 being effectively taken up into cells and might partially explain the potent antiproliferative effects of this compound.

To find out how indirubin derivatives may cause apoptosis in human tumor cells, effects on Stat signaling were measured, since especially Stat3 has been found to be constitutively active in many human tumor cells. Incubation of MDA-MB-468 cells with E804 for 4 hours resulted in a significant reduction of Stat3 phosphorylation already at 1 μM [Nam et al. 2005]. It was further found that this is the result of efficient inhibition of the non-receptor-tyrosine kinase c-Src (IC_{50} 0.43 μM). In consequence, the antiapoptotic proteins Mcl-1 and survivin, downstream targets of Stat3, are down-regulated and apoptosis is induced [Nam et al. 2005].

**Conclusion**

Novel indirubin derivatives have been developed showing improved tumor cell growth inhibition and CDK/cyclin inhibition. For E671, a significant reduction of pRb phosphorylation was achieved in large cell lung carcinoma cells. In addition to CDKs and depending on the substitution pattern of indirubin derivatives, further cellular targets aberrantly expressed in tumor cells can be affected. These include various serine/threonine kinases and receptor tyrosine kinases such as GSK-3β and VEGFR-2/KDR. In addition, the non-receptor-tyrosine kinase c-Src is potently inhibited by the indirubin derivative E804, leading to potent inhibition of Stat3 phosphorylation and Stat3 DNA-binding. As a consequence, antiapoptotic proteins Mcl-1 and survivin are down-regulated, resulting in apoptosis induction. The indirubin molecular template thus offers fascinating perspectives towards multi-mode anti-signaling molecules, potentially leading to highly promising antineoplastic drugs.

**Acknowledgment**

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KP1019 (FFC14A) from bench to bedside: preclinical and early clinical development – an overview

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Key words
ruthenium – transferrin – redox activity

Introduction and rationale

Though originally stimulated by the overwhelming success of the metal coordination compound cisplatin, the development of antitumor ruthenium complexes has grown into a prolific field of research in its own right, particularly since pharmacological properties distinctly different from platinum drugs have been recognized. Ruthenium complexes are capable of DNA cross-linking, but in contrast to platinum, several characteristics, such as the more iron-like coordination behavior and redox activity suggest that ruthenium is particularly suitable for making use of the pathophysiological conditions met in solid tumors, as described below. Indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019, FFC14A) has been selected for clinical development from a large series of ruthenium(III) complexes with azole ligands coordinated to the metal center through nitrogen, mainly because of its superior efficacy in preclinical tumor models.

Interaction with transferrin

KP1019 is capable of occupying the iron-binding sites of transferrins. In the model molecule lactoferrin (a close homologue of serum transferrin), it binds with a high affinity to His 253 in the specific N-lobe metal-binding cleft [Smith et al. 1996]. Furthermore, KP1019 is capable of being delivered to cells via the transferrin receptor-mediated endocytotic route. Uptake into human SW480 colon cancer cells is enhanced upon binding to human serum transferrin, provided that the protein is loaded with iron(III) to a normal physiological extent of ~ 30% [Pongratz et al. 2004]. Up-regulation of transferrin receptors as a consequence of the high iron demand is commonly found in tumor cells, rendering them susceptible to the cytotoxic effects of KP1019.

Redox activity and cytotoxicity

Reduction to ruthenium(II) species increases the reactivity towards target molecules and is thought to be favored under the hypoxic conditions prevailing in many solid tumors. Cytotoxicity of ruthenium(III) complexes is therefore expected to increase with increasing ease of reduction. In order to examine this hypothesis, redox potentials within a series of KP1019 analogues differing only in their number of indazole vs. chloride ligands were determined by cyclic voltammetry and calculated by Lever’s parametrisation method. Both confirm that a higher indazole-to-chloride ratio results in a higher reduction potential. Within this series, cytotoxicity correlates fairly well with redox potentials. Differences in the cellular uptake contribute to this correlation but cannot solely account for it. However, the nearly identical IC_{50} values of the tetra(1H-indazole) complexes of ruthenium(III) and ruthenium(II) suggest that the former is easily reducible under normoxic conditions. Thus, selecting a drug candidate only on the basis of cytotoxic potency might be misleading, since this will result in a loss of hypoxia selectivity, whereas the less easy reducibility of KP1019 is more likely to translate in a proper balance between cytotoxic potency and hypoxia selectivity [Jakupec et al. 2005].

Mode of action

KP1019 induces apoptosis via the mitochondrial pathway in human SW480 colon cancer cells, independently of p53. The
apoptotic process is characterized by depolarization of mitochondrial membranes, down-modulation of endogenous bel-2 and activation of caspase-3 [Kapitza et al. 2005a]. The cellular effects of KP1019 potentially triggering the apoptotic response include binding to DNA, oxidative damage due to the generation of reactive oxygen species [Kapitza et al. 2005b] or other yet unexplored effects. An interference with iron-dependent metabolic processes is likely, but remains to be elucidated experimentally.

**Antitumor activity and toxicity in vivo**

A remarkable therapeutic effect in the chemoresistant MAC15A murine colon cancer model (survival T/C > 300%) was the reason for evaluating KP1019 in chemically induced autochthonous colorectal tumors of the rat, which closely resemble colorectal cancer in humans. In the latter model, KP1019 inhibits tumor growth more efficiently and more reliably than 5-FU, resulting in a complete remission of one third of the tumors [Berger et al. 1989].

KP1019 is devoid of severe toxicities in applicable doses. Mild adverse effects reflecting an interference with erythropoiesis (reduced erythrocytes and hemoglobin) have been observed in mice and rats having received high cumulative doses of 21 and 17 mg/kg daily for 28 consecutive days, respectively.

**References**


**Clinical phase I study**

A clinical phase I dose escalation and pharmacokinetic study in patients with solid tumors was conducted at the Ludwig Boltzmann Institute for Applied Cancer Research, KFJ Spital Vienna, Austria, and the West German Cancer Center, University of Essen Medical School, Essen, Germany [Dittrich et al. 2005]. Most patients included in this study were heavily pretreated, and all established therapeutic options have been exhausted. 8 patients received KP1019 by intravenous infusion twice a week over 3 weeks. Starting from 25 mg, the dose was escalated to 600 mg according to an accelerated titration design. Dose escalation was terminated because of feasibility reasons, and no dose-limiting toxicities have been observed. Of 6 evaluable patients, 5 experienced disease stabilization for 8 – 10 weeks. Outcome was not related to the dose. Toxicities related to treatment were generally mild, encouraging further clinical development of the drug.
Expression profile of copper transporters in sensitive and cisplatin/oxaliplatin-resistant tumor cell lines

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Key words

Introduction

Platinum complexes like cisplatin and oxaliplatin are among the most potent antitumor agents and play an important role in the treatment of numerous cancers [Siddik 2003, Wang and Lippard 2005]. The use of platinum complexes is however limited by acquired or intrinsic resistance of tumors. Resistance mechanisms of tumor cells against cisplatin are multifactorial and include reduced uptake, increased efflux, intracellular detoxification by glutathione and metallothionein, DNA repair by the nucleotide excision repair and/or base excision repair complexes, mutations in the mismatch repair repair complex and prevention of apoptosis [Siddik 2003, Wang and Lippard 2005]. Changes in uptake and efflux of cisplatin have recently been linked to the expression of copper transporters. The copper uptake transporter Ctrl seems to act as an enhancer of cisplatin uptake whereas the P-type ATPases ATP7A and ATP7B increase the efflux of copper and cisplatin [Kruh 2003]. The purpose of this study was to profile the expression of the copper transporters Ctrl, ATP7A, and ATP7B in three pairs of sensitive and cisplatin/oxaliplatin-resistant human tumor cell lines.

Material and methods

Cell lines and cytotoxicity assay

The human ovarian cancer cell lines A2780 and A2780cis (European Collection of Cell Cultures, United Kingdom) and the ileum cancer cell lines HCT-8 and HCT-8Ox (kindly provided by Dr. M. Heim, University of Essen, Germany) were preserved in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, 50 U/ml penicillin G and 365 μg/ml L-glutamine (all: Sigma Chemical, Taufkirchen, Germany). The head/neck (tongue) cancer cell line CAL27 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The cisplatin-resistant cell line Cal27cis was established by repeated exposure to 10 μM cisplatin. CAL27 and CAL27cis were maintained in DMEM supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, 50 U/ml penicillin G and 365 μg/ml L-glutamine (all: Sigma). IC₅₀ values of cisplatin and oxaliplatin were determined by an improved MTT assay as previously described [Mueller et al. 2004].

qRT-PCR

Quantification of gene expression of copper transporters was performed by qRT-PCR using SYBR Green I dye-based detection on an Opticon Monitor (MJ Research, Waltham, MA, USA). PCR primers were designed with Primer3 [Rozen and Skaletsky 2000]. Data were analyzed by the ΔΔCq-method using housekeeping genes [Livak and Schmittgen 2001].

Results

Resistance factors of all three cell pairs against cisplatin were between 2.5 and 5 whereas resistance factors in the case of oxaliplatin were between 2 and 12 (Figure 1). Cell lines selected against cisplatin showed a cross-resistance against oxaliplatin (A2780cis, CAL27cis) and vice versa (HCT-8Ox). Housekeeping genes identified by the geNorm program were GUS and RPL13 for A2780, HPR1 and HuPo for CAL27, and ACTB and GUS for HCT-8 cell lines [Vandesompele et al. 2002]. No quantitative correlation between the degree of resistance and the expression of copper transporters could be found. The pattern of copper transporter expression was distinct for the three cell pairs. Whereas a
treatment with cisplatin or oxaliplatin did not lead to differences in the expression of ATP7A and ATP7B between sensitive and resistant cell pairs compared to untreated sensitive/resistant controls, an up to five-fold lower expression of Ctrl1 in resistant versus sensitive A2780 cells was found upon treatment of both the resistant and the sensitive cell line with cisplatin or oxaliplatin (Figure 2). Treatment of A2780, CAL27, or HCT-8 cells (sensitive or resistant) with cisplatin or oxaliplatin did not affect the expression of Ctrl1.

Conclusion

Copper transporters seem to play a role in the uptake and efflux of platinum complexes. Cisplatin/oxaliplatin-sensitive/resistant tumor cell pairs derived from three different tissues (ovary, head-neck, and ileum) have been used to examine the expression profile of copper transporters. The uptake transporter Ctrl1 was down-regulated in platinum-complex-treated resistant versus sensitive A2780 cell lines confirming data on a reduced platinum uptake in resistant A2780cis cells [Zisowsky et al. 2003]. Furthermore, upon treatment with platinum complexes, Ctrl1 does not show a significant change in expression, neither in A2780, nor in CAL27 nor in HCT-8 cells. These results confirm the importance of copper transporters for platinum complex uptake but show, however, that copper transporters are unlikely to be involved in every tumor cell line.

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Proteomic analysis of chemoresistance to 5-fluorouracil in colon cancer in vitro

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Introduction

Resistance to the widely used anti-metabolite 5-fluorouracil (FU) is one of the most relevant obstacles to successful chemotherapy in colon cancer. We have recently investigated the pharmacogenomic alterations driving this process by gene expression profiling of different stages of resistance [Schmidt et al. 2004]. Interestingly, this data suggested that resistance to FU may not be explained by a combination of known mechanisms, but mainly involves a wide molecular repertoire such as e.g. signal transduction, cell adhesion and cytoskeleton. In comparison to expression profiling, only a few studies have focused on the protein level [Castagna et al. 2004]. Therefore, we set out to evaluate the correlation between transcription and translation by proteomic analysis. In the present study, the differential protein expression was assessed comparing the naïve colon cancer cell line CCL 227 with its highly resistant subclone grown under continuous exposure to 125 μM FU. Exploiting the advantages of this in vitro system, the proteomic analysis combined two complementary approaches: - the conventional approach, i.e. analysis of the total protein content, and - pulse-labeling experiments assessing the de novo protein synthesis during the logarithmic growth phase of the cells [Gerner et al. 2002].

Material and methods

Proteomic analyses were done by nano-LC ESI-MS/MS identification of peptides generated from proteins separated by two-dimensional PAGE as previously described [Zwickl et al. 2005]. In order to evaluate the metabolic activity, cells were exposed to a mixture of [35S]-methionine/cysteine for 2 hours prior to protein isolation. Thus, separated proteins were quantified both by fluorescence analysis (total protein content) and autoradiography (de novo protein synthesis). Results

We present results from a systematic comparison of results based upon the analysis of 165 identified protein spots in the 2D gels. The analysis based on total protein content measured by fluorescence intensities revealed that, out of the 165 identified proteins, 20 were down-regulated, whereas 10 were up-regulated. Among the differentially expressed proteins, we observed suppression of members of the S100 calcium binding protein family (S100A4, S100A9, S100A11) and the gelso-lin-like capping protein (CAPG), which was in good correlation with the results obtained by GeneChip analysis (Figure 1). Up-regulation of protein expression was observed for proteins such as the signal transducer factor cellular retinoic acid binding protein 2 (CRAPB2) or the important apoptosis regulator BH3 interacting domain death agonist (BID). While the latter was missed by gene expression profiling, cellular retinoic acid binding protein (CRAPB1) was found to be amongst the rare targets up-regulated in our gene expression data set. There was a good correlation between the results obtained by gene expression profiling and proteomics focusing on the total protein content of the cells.

Major differences were observed when including the de novo protein synthesis in this evaluation. Here, 108 out of 119 identified proteins were up-regulated, with a roughly five-fold higher incorporation rate on average. Importantly, the down-regulated S100 family proteins still showed significant suppression. Up-regulation was observed for components of the cytoskeleton (ACTB, TUBB2, TUBBA3), underlining our earlier notion that changes of the cytoskeletal organization might play a role in FU resistance. When focusing on targets with good correlation between all three methods, we were able to narrow down to the S100 proteins, which further strengthen our previous observation regarding this protein family.

Although the total protein content in the cells did not differ significantly, the [35S]-
methionine/cysteine uptake was more than five-fold higher in the chemoresistant cells indicating a highly accelerated protein turnover. It is important to note that the de novo protein synthesis was up-regulated in 92 out of 135 proteins where there was no change and in 8 out of 20 where there was even a down-regulation in total protein content.

Conclusion

In conclusion, proteomics may provide novel and very useful information about the underlying mechanisms of chemoresistance. In addition to transcriptional information, the combination of proteomics with metabolic rates clearly points at differential protein expression driven by dramatically enhanced protein turnover, which is associated with increases in both protein synthesis and protein degradation. In addition to the genetic instability often observed in aggressive phenotypes of cancer, this observation supports the view that energetic instability is the price for acquired drug resistance.

References


Flat dose (175 mg/weekly) paclitaxel: pharmacokinetics and clinical implications

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Introduction

Paclitaxel (PAC), an antineoplastic agent derived from the bark of the Pacific yew Taxus brevifolia, has become a major drug for the treatment of breast, ovarian, non-small-cell lung cancer and other cancer patients. Due to its chemical nature, lipophilic PAC can only be kept in solution for injection by use of polyoxethylated castor oil Cremophor EL [Van Zuylen et al. 2000]. Clinical problems include severe hypersensitivity reactions (HSR), severe neutropenia and cumulative sensory peripheral neuropathy (PNP). During the last 15 years, several problems in the use of paclitaxel have been solved or reduced. The HSR problem was reduced by a premedication with corticoids, and H1 and H2 antihistamins, but complete avoidance of adverse reactions was not achieved. The observed severe neutropenia was dose-dependent and dependent on the duration of the infusion but by reducing the duration of the infusion from 24 hours to 1 hour, it was possible to manage this problem. PNP and development of resistance are the only problems which can severely limit the use of PAC and these remain a clinical challenge [Gelderblom et al. 2002, Mielke et al. 2005, Mross et al. 1997, 2000, 2002].

Cytotoxic agents are most efficient when the size of the dose and duration of administration are optimized in the treatment schedules. The most effective drug administration mode for PAC is a 1-hour weekly schedule. In a randomized study in breast cancer patients, it was shown that tumor response, progression-free survival and overall survival with a 1-hour weekly schedule was significantly better than a 3-hour 3-weekly schedule [Seidman et al. 2004]. The dose of the weekly PAC schedule was 80 mg/m² and the dose of the 3-week schedule was 175 mg/m². The weekly schedules most often used include doses in the range 80 – 100 mg/m². These dose levels are significantly higher (240 – 300 mg/m²) than the “classic” 175 mg/m² 3-hour infusion used in 3-weekly schedules and the 135 mg/m² 24-hour infusion used in 3-weekly schedules in former times.

In the case of most anticancer agents, the administered dose of paclitaxel is normalized to body surface area (BSA). However, the routine use of BSA as the only independent variable considered in drug dosing is questionable since the pharmacokinetic/pharmacodynamic variability with PAC remains large. The primary routes of PAC elimination consists of successive hydroxylation reactions mediated by the cytochrome P450 system [Sparreboom et al. 2005]. A further pathway of elimination is hepatobiliary and intestinal secretion of PAC by P-glycoprotein. It has been suggested that variability in these proteins accounts for most of the interindividual differences in drug clearance and therefore in the neutropenia, neurotoxicity, antitumor efficacy and indirectly in survival. In addition, the pharmacokinetics of PAC are age-dependent [Smorenburg et al. 2003].

The major focus of the present study was to determine the pharmacokinetics of a flat dose equal to 175 mg administered weekly. This dose corresponds to 100 mg/m² in a normal sized population with a mean BSA of 1.72 m².

Patients and methods

All patients received PAC 175 mg once weekly. 3/12 patients had slightly elevated bilirubin levels and 2 patients had elevated liver enzymes. Patients with advanced solid tumors where there was a possibility of response to PAC were included. Blood samples for the measurement of total PAC were taken during the first 48 h of the first drug administration within the first treatment cycle at pre-defined time points. PAC concentrations in plasma were analyzed by a HPLC technique described earlier. The study was approved by the local ethical committee. All patients gave informed consent.
Results

A total of 12 patients were included for pharmacokinetic evaluation after the first PAC dose administration and PK results were obtained. The PK evaluation showed a large interindividual variation in all parameters. The coefficient of variation of the PK parameters (mean values; terminal half-life 10.02 h, AUC 6,193 ng/ml × h, Clp 19.7 l/h/m², Cmax 3,161 ng/ml and Vss 121.6 l/m²) varied between 36% and 62%. The duration concentrations exceeded 0.05 µM was 32 h with a CV of 39%. Elevated bilirubin and liver enzyme contribute clearly to the large variation observed. The concentration-versus-time profile for PAC for all patients without bilirubin elevations is depicted in Figure 1.

Conclusions

In conclusion, the PAC pharmacokinetics after a flat dose of 175 mg shows a comparable PK profile similar to that known from 100 mg/m², albeit with a greater variation. Major factors with influence on these variations are liver impairment and the high variability of elimination pathways between different patients. Since age is also important, because of its influence on clearance, the variation in the PK parameters is consistent with previously published data. The patient population studied received a mean PAC dose of 98.3 mg/m² (with a fairly low CV of 9.3%). On the basis of these data, we suggest that a flat dose PAC is suitable in a palliative setting but further work on the optimization of PAC needs to be carried out and comparisons made with other treatment modalities.

Acknowledgment

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References

Enzastaurin and pemetrexed exert synergistic antitumor activity in thyroid cancer cell lines in vitro

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Background

The novel antifolate pemetrexed (Alimta®) has recently been approved for the treatment of NSCLC and, in combination with cisplatin, for mesothelioma. The agent acts primarily through inhibition of key enzymes of pyrimidine and purine neosynthesis. Its main targets comprise thymidylate synthase, dihydrofolate reductase, and glycaminide ribonucleotide formyltransferase [Zhao and Goldman 2004]. Enzastaurin (LY317615) has been developed as a selective inhibitor of the β-isofrom of protein kinase C (PKC-β). PKC-β is activated by growth factors, e.g. VEGF, and plays a critical role in malignancy-induced neoangiogenesis. In preclinical tumor models, enzastaurin inhibits VEGF phosphorylation by competing at the ATP-binding site and arrests ongoing development of new blood vessels [Keyes et al. 2004].

Objectives

The specific aim of this study was to determine in vitro growth inhibition of thyroid cancer cell lines exposed to clinically achievable concentrations of pemetrexed and enzastaurin. In addition, we have examined whether pemetrexed and enzastaurin exhibit synergistic antiproliferative effects on thyroid cancer cell lines in vitro.

Methods

Soft-agar cloning experiments [Hanauske and von Hoff 1993, Hanauske et al. 2004] were performed using three thyroid cancer cell lines (papillary subtype: BHT-101; follicular subtype: ML-1 and CGTH-W-1). Cells were exposed to 1 ×, 0.1 ×, and 0.01 × peak plasma concentration (ppc) of pemetrexed (1 × ppc = 100 µg/ml) or enzastaurin (1 × ppc = 1,400 nM) or the combination of both drugs (0.1 × ppc pemetrexed plus 1 ×, 0.1 ×, or 0.01 × ppc enzastaurin) for 1 hour or 7 days (continuous exposure). Subsequently, clonogenic tumor growth was evaluated after 1 week. In negative controls, the antitumor compound was substituted by 0.9% NaCl. Positive controls contained 10⁻³ mol/l ammonium vanadate to inhibit cell growth. Colony formation was evaluated with an inverted microscope after an incubation period of 7 days at 37 °C, 5% CO₂ and 100% humidity. Experiments were considered to be evaluable if positive controls demonstrated ≤ 30% colony formation compared to NaCl control. Cell lines were defined as resistant if cell survival was > 50% of untreated control.

Synergistic antitumor activity was determined using the median effect analysis method [Chou and Talalay 1984].

Results

All thyroid cancer cell lines studied in 1-h- and continuous exposure experiments demonstrated sensitivity to pemetrexed at 1 × ppc. At 0.1 × ppc, ML-1 but not CGTH-W-1 or BHT-101 was sensitive to pemetrexed after 1 h exposure. However, all cell lines retained drug sensitivity in continuous exposure experiments. Of interest, all cell lines demonstrated sensitivity to enzastaurin at 1 × ppc in 1-h- and continuous exposure experiments. For all three thyroid cancer cell lines, considerable sensitivity was observed in combination experiments including 10 µg/ml pemetrexed and various concentrations of enzastaurin (1 × ppc; 0.1 × ppc; 0.01 × ppc) (Figure 1).

Synergistic effects were observed for ML-1 and CGTH-W1 but not for BHT-101 after 1 h exposure. In continuous exposure experiments, synergism was noted for all cell lines investigated.
Conclusions

In addition to its well-documented antiangiogenic effects on endothelial cells, the specific PKC-β inhibitor enzastaurin exerts a direct, concentration-dependent, antitumor activity on colony-forming units in human thyroid cancer cell lines in vitro. This observation is the basis of the hypothesis that enzastaurin is an antitumor compound with multiple targets. The combination of enzastaurin and pemetrexed has synergistic antitumor activity under most of the experimental conditions examined. These results provide a rationale for investigating the combination of enzastaurin and pemetrexed in the treatment of patients with thyroid cancer.

References


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Figure 1. A–C: Cell survival of the thyroid cancer cell lines BHT-101 (A), ML-1 (B), and CGTH-W1 (C) exposed 7 days to pemetrexed (1 ×, 0.1 × and 0.01 × ppc; 1 × ppc = 100 μg/ml), enzastaurin (1 ×, 0.1 × and 0.01 × ppc; 1 × ppc = 1,400 nM) or drug combination (10 μg/ml pemetrexed + 1 ×, 0.1 × or 0.01 × ppc enzastaurin). Each data point represents the mean of six replicates with the respective standard deviation. Synergistic antitumor activity as determined by the median effect analysis method was observed in each cell line at each concentration studied. ppc = peak plasma concentration.
Contrast-enhanced MR imaging in the indirect monitoring of drug release from an interstitial depot

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Introduction

Interstitial administration of cytostatic depot preparations is most common in brain tumor patients [Guerin et al. 2004, Menei and Benoit 2003]. Little is known about the kinetics of drug release in this setting except results from a few invasive animal experiments. Thus, the monitoring of drug release in individual patients is desirable, especially in those cases where the therapeutic result is unsatisfactory. Such studies would help to assess whether failure is due to inadequate drug delivery or lack of drug efficacy in the patient.

The localization of drug depot preparations in tissue has been visualized using radiolabeling [Harrington et al. 2001] and by encapsulating contrast agents [Chen et al. 2005, Rubesova et al. 2002, Saito et al. 2004]. An instantaneous release of a drug-contrast agent complex from thermolabile liposomes on application of hyperthermia could be monitored in vivo by magnetic resonance (MR) imaging [Viglianti et al. 2004]. Sustained drug release should, in principle, also be accessible to non-invasive monitoring if it could be made sure that the contrast agent is released simultaneously with the drug.

Methods

The hydrophilic antimetabolite fludarabine monophosphate (Fludara®) was encapsulated in multivesicular liposomes along with the even more hydrophilic MR contrast agent Gd-DTPA (Magnevist®) to test the hypothesis that both agents are released simultaneously on disintegration of the liposomal walls. The preparation was injected subcutaneously in rats and the amounts of drug and contrast agent remaining at the injection site were monitored for 4–6 weeks by repeated in vivo 19F MR spectroscopy and 1H MR imaging (T1w, 3D FLASH).

Results

In three animals, the amount of drug stayed constant for about 9 days and then decreased exponentially towards zero or a plateau above zero with a half-life of 6 days (Figure 1). The lag time was shorter in another animal where the depot was located at a different site and split into subdepots (Figure 1).

MR images showed that the drug depots in Animals 1–3 were shaped like hollow caps on Day 1 and that these then expanded to become ellipsoid-shaped and more homogeneous within about one week. Distinct voids within the depots were seen on Day 1 (most clearly visible in rotating 3D projections) but not at later times. Depot volume increased from 50–80% to up to 160% of the injected volume during the lag time of drug release and then decreased at the same rate as the amount of drug, with the same fraction remaining at the end of the observation period (Figure 1, plots on right hand side).

In a control experiment, the drug and the contrast agent were injected as a solution, without encapsulation to verify that both become undetectable within a few hours.

Conclusions

Sustained drug release from an interstitial depot can be indirectly monitored by MR imaging if a co-encapsulated contrast agent is released simultaneously with the drug. This can be achieved by encapsulating a hydrophilic monophosphate ester prodrug in the aqueous space of liposomes along with an even more hydrophilic contrast agent.
Figure 1. Depot volume (open squares, left y-axis) and FLAMP-reference signal intensity ratio (cross symbols, right y-axis) versus time after injection (Animals 1 – 4). Left column: absolute data. Solid and dotted curves: FLAMP/reference and volume predictions of pharmacokinetic model. Right column: MRI volumes as percentage of maximum volume, FLAMP/reference as percentage of the fitted initial value (S₀). Point in parentheses (bottom row): excluded from model fit. A mixed-effects model was fitted to the data of Animals 1 – 3 while the data of Animal 4 were analyzed individually.

References


Searching for cellular targets of novel pteridines downstream of cAMP PDE inhibition

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Introduction

The second messenger cAMP is known to be involved in cell growth and differentiation. The intracellular level of cAMP is regulated by adenylate cyclase and 3',5'-cyclic nucleotide phosphodiesterase (PDE). Many tumor cells exhibit significantly decreased cAMP levels resulting from increased cAMP PDE activity. Alkylamino substituted pteridines have been reported to act as potent PDE inhibitors, showing efficient cell growth inhibition and induction of apoptosis in a panel of tumor cell lines [Marko et al. 1998, Merz et al. 1998]. Alkylamino-pteridines bearing identical substituents in 4- and 7-position are accessible by a markedly simplified synthesis and show no loss of growth inhibitory properties, as compared to first generation compounds. The synthesis of these pteridines is achieved by successive nucleophilic substitution of the chlorine atoms of 2,4,6,7-tetrachloropteridine. Novel analogues bearing 4,7-dipyrolidino- and 4,7-dithiazolidino-substituents with various further substituents in 6-position have been found to display potent growth inhibitory effectiveness in the human colon cancer cell line HCT-116. We therefore searched for their potential influence on various tumor-related target proteins in these cells. As there is a cross-talk of the cAMP signaling cascade and the mitogen-activated protein kinase (MAPK) cascade via protein kinase A (PKA)-mediated inhibition of Raf-1, we also tested the effects on the MAPK cascade downstream of Raf-1 at the level of extra-cellular regulated kinase 1 and 2 (ERK1/ERK2).

Sulforhodamine B assay

HCT-116 cells were seeded in 24-well tissue culture plates at a density of 4,000 cells per well. After 24 h, the medium was removed, and the cells were incubated with the test compounds for 72 h. The assay was performed as described previously [Merz et al. 1998].

cAMP/PDE assay

Cells were seeded (1 – 1.2 × 10^6 cells per 10 cm dish) and cultivated as described above. Cells were harvested in Tris buffer (50 mM Tris/Cl, pH 7.4, 10 mM MgCl_2, 0.1 mM EDTA, 5 mM benzamidine, 0.5 mM β-mercaptoethanol, mix of protease inhibitors), and PDE assays were performed according to a modified method of Pöch [1971].

Western blot

Cells were seeded (1.2 × 10^6 cells per 10 cm dish) and cultivated as described above. Cells were serum-depleted on the third day. After additional 24 h, cells were preincubated for 30 min in serum-free medium with the respective compounds before being treated with EGF (15 min). Cells were harvested in lysis buffer (25 mM Tris/Cl, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 0.27 M saccharose, several protease inhibitors and stabilizers). Cell lysate was subjected to SDS-PAGE and Western blot analysis (phospho p44/p42 MAP kinase antibody, NEB/Cell Signaling) [Kern et al. 2005].

Determination of protein concentration

Protein concentration was determined according to the method of Bradford using bovine serum albumin (BSA) as standard.
Results

All novel pteridine derivatives (Figure 1) potently inhibit the growth and cAMP-specific PDE activity isolated from the human colon cancer cell line HCT-116, showing nanomolar IC_{50} values (Figure 1). Subtypes of the cAMP-specific PDE4 family were found earlier by us to be aberrantly expressed in many human tumor cells [Marko et al. 2000]. Inhibition of these PDEs results in an elevation of the intracellular cAMP level, with the consequence of PKA activation. One way by which PKA activation might contribute to cell growth inhibition is through a cross-talk with the MAPK cascade via PKA-mediated inhibition of Raf-1 [Cook and McCormick 1993]. We therefore tested the effect of the novel pteridines on the MAPK cascade downstream of Raf-1 at the ERK1/2 (p42/p44) level. Only minor modulating effects on ERK1/2 phosphorylation were observed in this cell system by Western blot analysis (Figure 2). According to these first results, the strong growth inhibitory effects of these compounds do not appear to be primarily mediated via interference with the MAPK cascade in HCT-116 cells.

Conclusion

The novel pteridine derivatives effectively inhibit cAMP PDE activity in the human colon carcinoma cell line HCT-116 in the low micromolar range.

The strong growth inhibitory effects of the alkylamino-pteridines used in this study do not appear to be mediated via interference with the MAPK cascade in this cell line.

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