Medicine did not start in Heidelberg immediately after the University Ruperta Carola was founded on 1st October 1386 by “Kurfürst Ruprecht Karl”, a date which will be celebrated in Heidelberg next year (2011) with the 625 year celebration of the University. Although a “facultas medicinae” was already planned at the beginning, it took almost 100 years more until it could be documented that two medical books had been bought by the faculty. Innovation was slow in those days and it took another century before medical sciences really started, marked by the establishment of a Botanical Garden in 1593, one of the oldest in the world. From then on, when the “hortus medicus” started to be a resource for drugs, pharmacotherapy of patients began and continued until today where research for new substances has reached an unexpected dimension and has become a driving force of human development. Following its mission, the scientific society CESAR contributes to the ongoing efforts in this field.

Today, the medical faculty of Heidelberg is one of largest in Germany and many of the 40,000 students at Heidelberg are educated in medicine. Their training and the start of their career in medicine or related disciplines often proceeds in close association with scientific work in research institutes such as the European Molecular Biology Lab (EMBL), the Max-Planck Institute for Medical Research, the Biochemistry Center, the Center for Molecular Biology and the German Cancer Research Center (DKFZ), which historically – as well as today – has strong connections and interactions with the Medical Faculty. It is of note that as early as 1906 the internist and surgeon Vincenz Czerny (1842 – 1916) founded the Institute of Experimental Cancer Research in Heidelberg, at the same time also planning a clinic for cancer patients with the explicit aim of investigating cancer “under one roof”. This institute, which has been regarded as precursor of the DKFZ, worked highly successfully until its science and function was destroyed in the 1930s by the NS-regime and its supporters. It took many years until Czerny’s idea of translational research on cancer could be reestablished by the foundation of the DKFZ in 1964, initiated and directed at the beginning by the surgeon K.H. Bauer (1890 – 1978). Since then the DKFZ has become a research institute well-known worldwide with more than 2,000 employees and about 1,000 junior and senior scientists working in basic and applied cancer research. In 2008, the former scientific director Harald zur Hausen (*1936) was awarded the Nobel-Prize for Medicine for the finding that cervical cancer is caused by an infection with the Papilloma virus. His research lead to the development of a vaccine against one of the most frequent cancers in women.

Based on the fact that the DKFZ in Heidelberg is home to the German section of the CESAR-EWIV, the Central European Society for Anticancer Drug Research Germany e.V. (CESAR-Deutschland) with the CESAR Biostatistics (CBS) and its treasurer Lutz Edler, the 10th Anniversary of the CESAR was celebrated here with the 7th CESAR Annual Meeting 2009 in recognition of the reputation of Heidelberg for oncology in Germany and worldwide. The meeting was organized by CESAR-Deutschland e.V., the German Cancer Research Center (DKFZ) and the National Center for Tumor Diseases (NCT), a joint institution of the DKFZ and the University of Heidelberg. It was organized locally by members of the Department of Biostatistics of the DKFZ, the Department of Preventive Oncology DKFZ/NCT, the NCT and the CESAR Central Office (CCO) in Vienna at-
tracting more than 100 participants from Austria, Germany, Switzerland and other European countries. The scientific program was shaped, reviewed and overseen by all representatives of the five CESAR working groups and by the clinical directors of the NCT.

The Central European Society for Anti-cancer Drug Development, CESAR-EWIV, was founded in 1999 by a group of preclinical and clinical scientists from Austria, Germany and Switzerland with the aim of creating a totally new organization aiming to foster research and development of novel antitumor agents, therapies and therapeutic strategies in oncology in these three countries and beyond.

The 7th CESAR Annual Meeting 2009 was dedicated to communicate and exchange cutting-edge preclinical and clinical data on the development of new compounds with antitumor potency. It provided a platform for basic and clinical investigators involved in cancer research to discuss novel therapeutic approaches regarding efficacy and safety ultimately leading to a better patient outcome in cancer treatment. Within the scope of the conference the topics focused on the development of new therapeutic compounds, pharmacokinetics and pharmacodynamics, dose-finding and early clinical drug development in clinical Phase I and Phase II trials. Most recent advances in preclinical and clinical drug development including treatment optimization as well as related fields in tumor biology, molecular biology, chemistry, pharmacology, toxicology, biometry and regulatory affairs were addressed. In 2009, besides the overview of the 10 year celebration, main topics were drug delivery systems, dose adaptation in the event of organ dysfunction and preclinical and translational research.

Novel for this CESAR Meeting were two tutorials attended by about 30 participants each. Tutorial I informed on methodological aspects of primary clinical endpoints and their use for assessing efficacy and safety in oncological trials. Tutorial II addressed investigator-initiated trials (IITs) and gave advice for handling a study from the therapeutic idea to the final study protocol. A highlight of the meeting was the Poster Session combined with the reception provided by the DKFZ which gave a total of 28 poster presenters an excellent opportunity, not only to explain their research to the official poster reviewers assigned from the Scientific Program Committee, but also to their colleagues and friends for more than three hours and to form valuable contacts for their future research and career. A total of 22 posters qualified for inclusion in this volume.

The 7th CESAR Annual Meeting 2009 was opened by the Meeting President Max E. Scheulen from Essen (DE), followed by three welcome addresses: Otmar Wiestler, Scientific Director of the DKFZ, Heidelberg (DE), gave a warm welcome to all participants and presented a comprehensive view on the enormous number of new findings in recent years and outlined his vision of future for successful cancer research. Since we now understand more and more of the fundamental causes and development processes in cancer, he asked for the use of this knowledge as swiftly and as directly as possible for the clinical care of cancer patients. Christof von Kalle, the Director of the NCT in Heidelberg (DE) explained the role of the NCT as a center devoting considerable efforts to the establishment of interdisciplinary structures for better treatment of cancer patients “under one roof”. The President of the CESAR-EWIV Ulrich Jaehde, Bonn (DE), welcomed all participants on behalf of the CESAR explaining the aims and structure of the CESAR in performing early drug development, pharmacodynamic/pharmacokinetic modeling in correlative as well as Phase I and Phase II studies and being a part thereof.

The first scientific session celebrating 10 Year CESAR Cancer Research started with a retrospective glance given by Max E. Scheulen, Essen (DE). He emphasized the success story of the CESAR in research for inventing, detecting, isolating and developing new antitumoral principles and anticancer agents, in the development of new therapies and therapeutic strategies, and in fostering the translation of basic research into clinical application. The integral drug development plan implemented by the CESAR and its working groups play therefore an important role. The look to the future was next made by Heiner Fiebig, Freiburg (DE), who highlighted the potential of gene signatures to predict effectiveness of targeted and cytotoxic drugs. In his laboratory human tumors were estab-
lished as xenografts by serial passage subcutaneously in nude mice, characterized for their sensitivity towards two targeted and ten standard cytotoxic anticancer agents and the tumor xenografts’ gene expression profiles were finally determined. He presented predictive gene signatures for twelve cytotoxic agents that have the potential to substantially increase tumor response rate compared to empirical drug treatment, but need to be further validated. Matthias Tacke from Dublin (EI) reported on the preclinical evaluation of anticancer efficacy of titanocene dichloride derivatives and the successful substitution of vanadium for titanium thus leading to new effective isostructural vanadocene dichloride compounds. Clinical developments within the CESAR were addressed by Dirk Strumbeg, Herne (DE), who reviewed the clinical development of sorafenib, an oral multikinase inhibitor (Raf, VEGFR-1, -2,-3,PDGFR-B, c-Kit, FLT-3 and RET). Sorafenib, now registered as Nexavar®, had been studied in the CESAR for pancreatic and prostate cancer and is now registered for renal and hepatocellular carcinoma. The broad spectrum of CESAR phase II-studies performed over 10 years was reviewed by Gernot Hartung, Oldenburg (DE). He demonstrated that a total of 15 multicenter studies could be performed in five to twelve oncology departments of the CESAR skillfully in a short period of time by rapid recruitment either sponsored by the CESAR as IITs or sponsored by the pharmaceutical industry with support from the CESAR Central Office (CCO) and the CESAR Biostatistics (CBS).

The first day ended with a session of submitted presentations. Rainer Schobert, Bayreuth (DE), characterized cancer specificity and antitumoral efficacy of three natural products when enhanced by structural modifications. Functional characterization and signal pathway analyses in the context of adhesion receptor activity of human tumor cells treated with specific phospholipids was described by Michael Alexander, Bonn (DE). Oksana Voloshenko, Heidelberg (DE), showed how detachment-induced sensitization to TRAIL-induced apoptosis explains the metastasis-specific role of the TRAIL/TRAIL-R system in epithelial tumor genesis. Marc-Steffen Raab, Heidelberg (DE), demonstrated the in vitro- and in vivo-antitumor efficacy of a prototype small molecule inhibitor of centrosomal clustering with specificity for tumor cells. With further evaluation and development this promises to be a lead compound of a new class of therapeutics for human malignancies. Daniela Schilling, Munich (DE), demonstrated that the addition of Hsp70 protein to hypoxic tumor cells significantly reduces clonogenic cell survival in normoxic tumor cells thus promoting tumor kill in potentially relevant radioresistant tumors.

The second day started with a session of invited contributions on drug delivery systems organized by the CESAR working group on New Drug Development in Oncology. Werner Lubitz, Vienna (A) provided an overview on bacterial ghosts (BGs), i.e., empty bacterial envelopes of Gram-negative bacteria produced by controlled expression of cloned gene forming a lysis-tunnel structure within the envelope of living bacteria. BGs function as advanced drug delivery systems which can target various histological types of cancer. However, defining specific conditions for selection and preparation of BG formulations require further work before they can be used clinically. The next presentation was on nanoparticles (NPs) and their property to release drugs only at the target sites. New insights into their therapeutic application were given by Paul Debagge, Innsbruck (A), who explained that a wide range of chemical formulations of NPs is presently being studied for clinical efficacy. A few, such as liposomes and protein-coated drug particles, have already entered clinical studies. He also indicated the need to study nanotoxicology. Also related to NPs was the presentation of Gert Storm, Utrecht (NL), who again focused on the impact of nanotechnology on targeted delivery of anticancer agents. He reported on selected research activities in his laboratory which are embedded in the EC-sponsored FP6-MEDITRANS project. Felix Kratz, Freiburg (DE), gave an overview on the various albumin-based drug delivery systems with a focus on systems that use endogenous albumin as a drug carrier. He distinguished between coupling of low molecular weight drugs to exogenous or endogenous albumin, conjugation with bioactive proteins and encapsulation of drugs into albumin NPs.
colleague Ulrich Massing, Freiburg (DE), posed the question “Why after 30 years of liposome research are there still only very few liposomal anticancer drugs on the market?”. He indicated the complexity of interactions between drugs and liposomal membranes and the problems arising when designing suitable liposomes, but he was optimistic with respect to their use in cancer treatment in the future, due to appearance of siRNA, peptides and antibodies, new water insoluble low molecular weight drugs and progress with respect to the manufacturing tools.

The invited presentations session of the CESAR working group Pharmacology in Oncology and Hematology dealt with dose adaptation in the presence of organ dysfunction. The academic point of view was addressed by Martin Czejka, Vienna (A), who distinguished between antineoplastic drugs which are pharmacologically active, such as anthracyclines, taxanes and 5-fluorouracil and compounds which have to be activated by metabolic processes such as capectabine, gemcitabine and irinotecan. With respect to dose reduction, he referred to the key role of pharmacokinetics for optimization of chemotherapeutic regimens, especially in elderly patients. Wolfgang Mück, Wuppertal (DE), presented the view of the pharmaceutical industry and Klaus Menges, Bonn (DE), that of the competent authority as scientist in the Federal Institute for Drugs and Medical Devices in Germany. According to him, product information on medicinal products in the form of the authorized “Summary of the Product Characteristics” (SmPC), package leaflet and labeling is the core information for deciding on market access of a product. He detailed the conditions how SmPC are created and what kind of information is available.

The third session of invited contributions dealt with Preclinical and Translational Research in Oncology which started with a presentation on targeting of blc-2 proteins in gastrointestinal cancer by Henning Schulze-Bergkamen, Heidelberg (DE). Proteins which exert either pro- or anti-apoptotic activity with the potential to augment antitumor activity of cancer therapeutics were examined. To target antiapoptotic bel-2 family proteins, various approaches, e.g. antisense oligonucleotides and non-peptidic small molecule inhibitors (SMI) are now under clinical investigation. Paolo Ceppi, Turin (Italy), addressed the significance of determining thymidylate synthase (TS) expression in thoracic cancers in the case of differential drug activity. Recent findings suggest the use of TS as a possible predictive marker of treatment efficacy. Carsten Grüllrich, Heidelberg (DE), presented the rationale of the recently started prospective randomized clinical trial HeiLivCa to determine if the combination of transarterial chemoembolization (TACE) with sorafenib is more effective than TACE plus placebo. The aim is to control tumor growth in patients with hepatocellular carcinoma eligible for liver transplantation. Hartmut Goldschmidt, head of the Section Multiple Myeloma in the University Clinic Heidelberg and at the same time leader of the German-Speaking Myeloma Multicenter Study Group (GMMG) for many years, gave a comprehensive overview on the drug bortezomib (VelcadeR) and its investigation in Phase II and Phase III studies in multiple myeloma. This proteasome inhibitor not only targets the myeloma cell, but also acts in the bone marrow micro-environment, inhibiting the binding of myeloma cells to bone marrow stromal cells.

Submitted presentations on the second day started with Michael Zepp, Heidelberg (DE), talking on rat monoclonal antibodies targeted against bone sialoprotein II and inhibiting tumor growth and osteolytic lesions induced by MDA-MB-231 breast cancer cells in nude rats. Gernot Hartung, Oldenburg (DE), reported on joint collaboration with researchers of the DKFZ on an in vivo-study of methotrexate and aminopterin bound to human serum albumin in Lewis-lung-carcinoma (LLC) bearing mice where neither MTX-HSA nor AMPT-HSA were active towards LLC. Gesina Wieker, Essen (DE), studied the association of genotypes of common functional CHK2 promoter polymorphisms and outcomes in cancer patients receiving radiotherapy. Artesunate, a well known and often used antimalaria drug, was investigated by S.A. Kabeer Rasheed, Heidelberg/Mannheim (DE), and colleagues who showed that this drug, which specifically targets transcription of u-PA, MMP-2 and MMP-7, markedly suppresses invasion and metastasis in NSCLC.
After the conference, all presenters of papers and posters were invited to submit an extended abstract for review and possible publication in the International Journal of Clinical Pharmacology and Therapeutics. A review process was implemented such that each submission was independently assessed by three members of the scientific program committee who were experts in the field. At the time of preparing this volume the CESAR is already preparing its 8th Annual Meeting for 2010 which will be held in St. Gallen, Switzerland, with promising new results on relevant and challenging topics of clinical and translational cancer research.

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New insights into nanoparticles – status in therapy*

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Introduction

An ideal pharmaceutical therapy concentrates an adequate amount of drug precisely at the lesion and retains it there – and nowhere else – for the period necessary for drug activity. Small molecule drugs permeate almost the entire body and enter many compartments, and to reach adequate concentrations within lesions high concentrations must be accepted at non-lesion sites. Nanomedicine aims to package drugs into nanoparticles 10 – 200 nm in size, potentially packaging many thousands of small drug molecules in each particle and to navigate these particles exclusively to the sites of disease [5], such as solid tumors, where they will release the drug load – ideally, in a controlled manner [2]. Nanoparticles not entering the lesion should be designed for rapid clearance without releasing drug at non-lesion sites. Navigation to the drug site involves “targeting” the nanoparticles. A wide range of chemical formulations of nanoparticles is presently being studied for clinical efficiency, including liposomes, dendrimers, and nanoparticles having matrices composed of polymers, protein, or a ceramic. Nanocages constructed of metals (gold) or carbon-based (fullerenes, nanotubes) are under development. A few, primarily liposomal nanoparticles and protein-coated drug nanoparticles, have already entered clinical use.

State of the art

Targeting of nano-agents is essential to overcome the numerous tissue barriers existing between the portal of entry (typically, intravascular application but also through the gut or the skin) and the target cells. The absence of a barrier which is normally present can be utilized, a good example being the enhanced permeability and retention (EPR) of nanoparticles found in many solid tumors. Coupled with the presence of albumin receptors such as the “Secreted Protein, Acidic and Rich in Cysteine” (SPARC) on endothelial cells and many tumor cells [6], EPR provides an effective means of accumulating albumin-coupled drugs such as Abraxane®, (Abraxis, Bioscience, Los Angeles, CA, USA) which carries taxanes into tumors. Albumin specific uptake is also being used to carry other drugs into tumors, including rapamycin, colchicines and heat shock protein 90 inhibitor. Specific uptake of nutrient molecules provides a further means of targeting tumor cells, for example uptake receptors for iron (transferring), lipoproteins or folate. If the target cells express specific proteins, generally present as a few thousand copies per square micrometer of cell surface, the nanoparticles can be targeted by attachment of groups recognizing those proteins specifically, for example nucleotides (aptamers), peptides, antibody molecules or their fragments (such as single-chain variable fragments (scFv). In this way a range of tumor-characteristic molecules can be targeted, including growth factor receptors such as the EGF receptor (erbB/HER/EGF-R), and cell surface adhesion molecules such as Ep-CAM. The design of nanoparticles for these applications requires the optimizing of several parameters, perhaps the most important of which is the drug load-to-targeting-group ratio, which can be as high as 20,000.

Challenges

Toxic effects of nanoparticles have been documented outside the nanomedical research area. However, nanoparticle therapeutics up to now has not led to significant health risks. However, the application to cells and tissues of agents which are of approximately the same size as many subcellular organelles and many tissue components such as the molecules of the extracellular matrix, may give rise to unexpected types of toxicity. Furthermore, the immense relative surface area of nanoparticles, which provides one of the great advantages for attachment of active and targeting groups, also has the potential of be-
Nanoparticles in therapy

ing involved in some toxic effects; the enhanced toxicity of “ultrafines” in industrial smokes as compared to microparticles in other smokes, has been well documented for over 50 years [3]. A multidisciplinary science of nanotoxicology is developing, matching the multidisciplinary nature of nanotechnology itself[4]. Nanotoxicology is a young science striving to minimize the novel toxic side-effects resulting from the nanoparticulate formulation of drugs which have often been used for long periods of time in other formulations.

One other major challenge is to design nanoparticles which release their drugs exclusively at the target site. The state of the art is far from achieving this, with the result that therapeutic applications are still limited to doses which the patient can tolerate, rather than the dose necessary to eliminate the tumor; in principle, this latter dose will be reached by a well-designed nanoparticulate formulation, and the formulation will exhibit no side effects.

Future directions

Both the FDA and EMEA have set up task forces in nanomedicine, and therefore take the future development of these formulations seriously [1]. Future developments should permit application of nano-agent therapeutics in the clinical setting of the treatment of cardiovascular diseases and cancers, at earlier stages than presently possible. Nanoparticles will be useful for delivering vaccines without requirement for additional adjuvants and with high efficacy. The targeted delivery of large genes to cells will allow specific delivery of programmable therapeutic factors, underpinning advanced forms of personalized medicine. The time scales involved are likely to match the development of liposome technology (1970 – 2005) or of immuno-targeting technology (1975 – 2005).

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References


Targeted doxorubicin-liposomes as a tool to circumvent P-gp-mediated resistance in ovarian carcinoma cells*


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Introduction

Resistance of tumor cells against chemotherapy is a therapy-limiting obstacle. Several molecular mechanisms of chemoresistance have been identified. Amongst these, an increased efflux of chemotherapeutics by special transporters is a dominant phenomenon. The upregulation of P-glycoprotein (P-gp) is well known for multidrug resistance (MDR) by mediating a cellular efflux of several cytostatics [3]. Doxorubicin is one of the most widely used drugs in chemotherapy. Furthermore, doxorubicin is successfully approved in a liposomal application form. The cytotoxic effect results from blockade of nuclear DNA replication, which finally leads to apoptosis of tumor cells. However, doxorubicin activity is therapeutically limited by chemoresistance which might, at least partly, be related to P-gp.

In order to investigate the impact of cellular transporters on the chemoresistance, we focused on the drug uptake of doxorubicin in A2780 ovarian cancer cells in comparison with the doxorubicin-resistant A2780adr cells and related this to the cytotoxicity. The free drug was compared to the commercial liposomal product (Caelyx®, Essex Pharma GmbH, Munich, Germany), which was further modified by coupling to holotransferrin to induce an endocytotic uptake of liposomes via the transferrin receptor (TfR) [7]. Although a recent study favored a caveolin-dependent endocytosis of cytostatic carriers [4], clathrin-mediated endocytosis via holotransferrin appeared promising since A2780 cells lack caveolin. The endocytotic pathway should circumvent the activity of resistance-related transporters and potentially modify the intracellular fate of the drugs compared to non-liposomal application. The cellular uptake of doxorubicin was determined in absence and presence of the P-gp inhibitor WK-X-24 [6].

Materials and methods

Caelyx® liposomes were obtained from commercial sources. The protein lipid anchor Cyanur-PEG-PE was synthesized as described [1]. Holotransferrin (Holo), doxorubicin, daunorubicin, and Sephadex G-50 were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). All other chemicals were obtained from Applichem (Darmstadt, Germany) unless otherwise stated.

Liposome preparation

The functionalization of the liposomes with holotransferrin was performed with a post-insertion method [5]. In a first step micelles of the anchor-lipid cyanur-PEG-PE were formed by hydration using borate-buffer (pH 8.8). These micelles were coupled to holotransferrin overnight at room temperature. Then, the micelles were coupled to holotransferrin overnight at room temperature. Therefore, the micelles were mixed with Caelyx® liposomes for 30 min at 56 °C followed by gel separation chromatography (Sephadex® G 50). Holotransferrin-coupled Caelyx® liposomes (Holo-Caelyx®) were used for the experiments within a time range of 48 hours (h) after functionalization.

Cell culture

The human ovarian carcinoma cell lines A2780 and A2780adr were obtained from ECACC, No. 93112519 (A2780) and No. 93112520 (A2780adr), UK. The cell lines were cultured in RPMI 1640 medium (PANTM-biotech, Passau, Germany) supplemented with 10% fetal calf serum (FCS) Sigma-Aldrich Chemie, 5% (50 µg/ml Streptomycin and 50 U/ml penicillin G) and 1.5% 365 µg/ml L-glutamine (Sigma-Aldrich Chemie) at 37 °C in a 5% CO₂ incubator.
Quantification of intracellular doxorubicin accumulation

For characterization of cellular doxorubicin uptake, 1 × 10^6 cells were seeded out in 6-well plates overnight. The medium was then changed and doxorubicin, Caelyx® or Holo-Caelyx® was added in a final concentration of 10 µM per well. At each incubation time point the medium was siphoned off quickly and the cells were washed once with 1 ml ice-cold PBS (4 °C). After removing PBS, cells were trypsinized for 2 min and resuspended in fresh cold medium (4 °C), put into a 2 ml Eppendorf tube and centrifuged for 1 min at 1,520 g. The supernatant was discarded and the cell pellet was resuspended in 1 ml ice-cold PBS. After centrifugation for 1 min at 24,100 g, the supernatant was removed, the pellet washed with 1 ml ice-cold PBS and centrifuged again. The pellet was subsequently stored in the dark in a refrigerator until further analysis. The cell pellet was lysed with 250 µl 2% Triton-X100 for 10 min, the volume made up to 2 ml with aqua millipore, and doxorubicin was quantified using a fluorescence spectrometer (excitation 488 nm, emission 555 nm) (LS 55; Perkin Elmer, Waltam, USA).

Results

Cytotoxicity of free doxorubicin was determined in both cell lines (MTT assay, after 24 h). The IC50 values (1.69 × 10^{-6} M in A2780 vs. 4.79 × 10^{-5} M in A2780adr) confirmed the doxorubicin resistance of A2780adr cells corresponding to a resistance factor of about 28. In contrast, the Caelyx® liposomes displayed nearly identical activity in both cell lines (5.89 × 10^{-6} M vs. 6.76 × 10^{-6} M, resistance factor 1.1), which indicate that liposomes are not affected by resistance. Furthermore, TIR-targeted liposomes (Holo-Caelyx®) were even more effective with an IC50 of 4.79 × 10^{-7} M in the resistant cells confirming that liposomes can overcome the chemoresistance.

To investigate whether this is solely related to a higher cellular uptake of liposomal doxorubicin, intracellular drug levels were determined after 1 h and 4 h in both cell lines (Figure 1A). It is evident that after 4 h the uptake of the free drug is lower in the A2780adr cells. However, these data did not reflect the marked differences in cytotoxic activity. Due to the faster cellular uptake kinetics of free daunorubicin, the effects of P-gp were much more marked. Therefore, the existence of further mechanisms of doxorubicin resistance, in addition to P-gp efflux, appear to be present.

All liposomes displayed relatively low, but comparable levels of doxorubicin indicating the independence of intracellular accumulation from resistance and a rapid saturation of the uptake. However, the relatively low benefit of targeting the TIR might be related to the peculiarities of the rapid endocytotic recycling of the TIR. Nevertheless, the low doxorubicin levels obtained by the liposomes alone cannot explain the high cytotoxicity and indicate that a different intracellular trafficking compared to the free drug [2].

To further focus on the impact of P-gp on resistance, cellular uptake was determined in the presence of the P-gp inhibitor WK-X-24 (Figure 1B). Daunorubicin displayed a total recovery of cellular uptake in A2780adr cells, whereas doxorubicin was only slightly affected. This finding supports the assumption that further resistance mechanisms are in-
volved. As expected, the liposomes were hardly influenced by blocking P-gp and the slight increase in the Holo-Caelyx® values could be related to an activated endocytosis, but this has no effect on the general conclusions.

Conclusion

With respect to the established benefit of passive targeting of cytostatic liposomes to tumor tissues, the present findings suggest that liposomes are promising tools for gaining an insight into the mechanisms of chemoresistance. Although the doxorubicin liposomes did not lead to higher intracellular drug levels, their much higher cytotoxic activity in resistant cells suggests alterations in intracellular trafficking and confirms the potential of liposomes to overcome chemoresistance.

References


Contribution of glutathione and MRP-mediated efflux to intracellular oxaliplatin accumulation

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Contribution of glutathione and MRP-mediated efflux to intracellular oxaliplatin accumulation

Introduction

Thirty years of experience using cisplatin in the treatment of cancer have resulted in a growing understanding of its mode of action and led to the successful development of second and third generation analogues. In contrast to the first clinically available compounds cisplatin and carboplatin which exhibit primary resistance against colorectal cancer, oxaliplatin is also effective in this tumor entity. However, some mechanisms associated with acquired resistance against oxaliplatin resemble those reported for cisplatin [1]. Detoxification of platinum complexes by intracellular formation of platinum-glutathione (GSH) adducts and their subsequent efflux via the ABC transporters MRP1 and MRP2 contribute to a decreased accumulation and have been repeatedly suggested as resistance mechanism for cisplatin [2] but have also been the subject of controversy [3]. The present study focuses on oxaliplatin and its detoxification via GSH and MRP-mediated efflux. Electrospray ionization mass spectrometry (ESI-MS) was applied to detect oxaliplatin-GSH adducts formed after incubation of oxaliplatin with GSH and Gü83 (Figure 1), a 4-aminobenzoic acid derivative recently shown to inhibit MRP1 (IC50 = 1.2 µM) and MRP2 (IC50 = 21.5 µM) [4], was used to investigate the contribution of the transporters to oxaliplatin efflux in oxaliplatin-sensitive and oxaliplatin-resistant ileum carcinoma cells.

Material and methods

Identification of platinum-GSH adducts

Oxaliplatin was incubated with GSH in a ratio of 1:10 (55 µM oxaliplatin and 550 µM GSH) in aqueous solution. A solution containing methanol (60%) and formic acid (1%) was added immediately or after a 12 h incubation time at 37 °C. Subsequently, electrospray ionization-mass spectrometry measurements were performed using an ESI-Q-TOF (QSTAR XL; Applied Biosystems, Darmstadt, Germany) equipped with a nanospray ion source.

Cell culture

The human ileocecal colorectal adenocarcinoma cell line HCT8 and its oxaliplatin-resistant variant HCT8ox were kindly provided by Dr. R.A. Hilger, University of Essen, Germany. Cells were cultivated in RPMI-1640™ medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin (37 °C, 5% CO2).

Cellular platinum accumulation

To investigate the cellular platinum accumulation, cells were allowed to attach in 6-well plates for 12 – 14 hours and incubated with oxaliplatin (100 µM) and in some experiments additionally with Gü83 (100 µM). At certain time points, cells were harvested and platinum was quantified using flameless atomic absorption spectrometry (SpectrAA™ Zeeman 220; Varian, Darmstadt, Germany). The platinum concentration was related to the protein content determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA).

Statistical analysis

Statistical analysis was performed using Mann-Whitney test. P values of < 0.05 were considered statistically significant.
Results and discussion

Following incubation of oxaliplatin with glutathione a 1:1 oxaliplatin-glutathione adduct ([M+H]⁺ = 614.28) was identified using ESI-MS. Most of this compound was bound to oxalic acid forming the molecule [M+H]⁺ = 704.29. Fragmentation of the product resulted in elimination of oxalic acid and appearance of [M+H]⁺ = 614.28. Formation of the adduct took place rapidly as it was found immediately after incubation of oxaliplatin with GSH. Since it was also detected after 12 h of incubation it can be considered as stable over this time period. The mass signal was identified in a cell-free environment but its presence is indicative of a potential intracellularly formed adduct representing a possible substrate for MRP efflux pumps.

To investigate whether oxaliplatin or its intracellularly formed adducts are actually pumped out of the cell via MRP1 or MRP2, oxaliplatin accumulation was determined in HCT8 and HCT8ox cells after treatment with oxaliplatin alone and in the presence of the MRP inhibitor Gü83. After incubation with oxaliplatin, a statistically significant lower platinum accumulation was found in the resistant variant at all time points (0.5, 1, 2 and 3 h) suggesting that influx and/or efflux were altered (Figure 2). Co-incubation of the cells with the MRP inhibitor Gü83 (100 µM) led to an increase in the cellular platinum accumulation in the sensitive and resistant cells. After 2 h the platinum accumulation expressed as platinum per gram protein (µmol/g) increased by 1.5-fold in the sensitive (p = 0.003, n = 3 in triplicate) and by twofold in the resistant cells (p = 0.003, n = 3 in triplicate) (Figure 2). These findings indicate that there is an efflux via MRP1 and/or MRP2 starting rapidly after oxaliplatin exposure.

Conclusions

The observed increase in platinum accumulation in the presence of the MRP inhibitor Gü83, seen with oxaliplatin-sensitive and -resistant cells, suggests that oxaliplatin and/or an oxaliplatin adduct are substrates of MRP1 and/or MRP2. Since the effect was comparable in sensitive and resistant cells no conclusions about the contribution of MRP-mediated efflux to oxaliplatin resistance can be drawn so far. A 1:1 oxaliplatin-GSH adduct was identified in a cell-free environment presenting a candidate MRP substrate. However, further experiments are required to confirm this finding in a cellular system.

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References

Contribution of glutathione and MRP-mediated efflux to intracellular oxaliplatin accumulation


Blocking of integrin-mediated human MV3 melanoma cell binding by commercial and modified heparins

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Heparin blocks integrin melanoma cell adhesion

Introduction

Tumor cell metastasis is the most serious complication in the course of malignant diseases. The molecular mechanisms of metastatic tumor spread are complex and not completely understood which complicates pharmacological antimetastatic approaches. Heparin, the anticoagulant drug of choice for more than 70 years, possesses antimetastatic effects, and low-molecular-weight heparins (LMWH) were able to prolong survival in cancer patients in a number of controlled clinical trials [4]. LMWH has no effect on the primary tumor but only on the spread of metastases.

In addition to the anticoagulatory effects reducing metastasis, other activities of heparin are assumed to include inclusion of angiogenesis via the tissue factor pathway inhibitor, modulation of growth factor binding and inhibition of heparanase enzymatic activity. Most studies have focused on heparin interference with adhesion receptor P- and L-selectin inhibition. Most studies have focused on heparin interference with adhesion receptor P- and L-selectin activity and inhibition of cancer cell interaction with platelets, leukocytes and the endothelium [1]. Recently we could identify the integrin VLA-4 (very late activation antigen-4, CD29/CD49d), which is expressed on melanoma and myeloma cells, as a further target of heparin [2]. The interaction of VLA-4 positive MV3 melanoma cells with VCAM-1 (vascular cell adhesion molecule-1) was impaired by heparin. For interference with VLA-4 heparin had to exceed a critical threshold of saccharide units [7].

However, heparin as a glycosaminoglycan is a biopolymer with multiple biological activities. To utilize the advantages of heparin in tumor therapy it is important to separate the anticoagulant properties in order to avoid bleeding complications.

In the present study we elucidate the capacity of three different non-anticoagulant heparins to impair the VLA-4-mediated interaction between MV3 cells and VCAM-1. Two heparins with different degrees of N-acetylation (NA-heparin) and one glycol-split, reduced oxyheparin (RO-heparin) were applied.

We provide evidence that heparin binding to VLA-4 is strongly controlled by certain structural factors, which interestingly, differ partly from the structural requirements for P- and L-selectin inhibition.

Material and methods

Cell lines

The human melanoma cell line MV3 was grown in RPMI-1640 supplemented with 10% (v/v) FCS. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. For subcultivation, MV3 were detached at 80 – 90% confluency using a solution of EDTA (0.2 g/l EDTA × 4Na) for 3 min at 37 °C. All reagents were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Heparins and non-anticoagulant heparin derivatives

UFH (Heparin-Natrium 5000 ratiopharm®) was purchased from Ratiopharm GmbH (Ulm, Germany), the non-anticoagulant heparins (NA-heparins and RO-heparin) were prepared as recently described [6].

Cell-binding studies

The interaction of MV3 melanoma cells with VCAM-1 Fc chimera was detected under physiological flow conditions. For this
purpose glass slides were coated with human VCAM-1 Fc chimera and fitted into a parallel plate flow chamber, as described in detail previously [7]. The flow chamber was placed on an inverted microscope. PBS was used as flow medium at a shear rate of about 200 s\(^{-1}\). \(1 \times 10^6\) MV3 melanoma cells suspended in FCS-free RPMI medium were stimulated with 1 mM Mn\(^{2+}\) for 5 min at 37 °C. Cells were injected into the flow chamber and adhesion on the coated glass slide was determined in comparison to non-stimulated heparin-incubated cells (500 µg). In this way video sequences could be captured (CSC-795 camera, Pacific corporation, Tokyo, Japan) and analyzed with the Imagoquant MultiTrack-AVI-2 software (Mediquant GmbH, Lützen, Germany), which allows the adhesion and velocity of interacting cells to be evaluated.

Results

VLA-4 appears to be a promising target for heparin to interfere with metastasis. The relevance of VLA-4 in MV3 melanoma metastasis was initially investigated in an experimental metastasis study in NMRI nude mice (manuscript in preparation). The data clearly revealed the involvement of VLA-4 in hematogenous metastasis, since blockade of VLA-4 by pretreatment of MV3 cells with, or application of a VLA-4 blocking antibody significantly attenuated the number of metastatic foci in the lungs.

As a result of these findings, different heparins were used to investigate their capacity to interfere with VLA-4-mediated MV3 binding onto VCAM-1 in the microscopic flow chamber assay. The MV3 cells were preincubated with Mn\(^{2+}\), which is an experimentally accepted procedure to activate integrin binding and incubation with this agent induced a strong increase in MV3 binding.

A commercial unfractionated heparin (UFH) strongly attenuated VLA-4-mediated MV3 binding to VCAM-1, resulting in cell adhesion below the level of unstimulated cells.

To further focus on the structural requirements of heparin to interact with VLA-4 and to differentiate this from other activities of heparin, three different non-anticoagulant heparins were applied. The glycol split, RO-heparin also had a strong influence on MV3 cell adhesion to VCAM-1 and reduced the cell adhesion nearly to the level of unstimulated MV3 cells. After the first second of flow, only 54% of the cells adhered to the support-fixed VCAM-1 layer (Figure 1).

In contrast to RO-heparin, the two N-acetylated heparin derivatives (50% NA-heparin and 100% NA-heparin) impaired adhesion only slightly and the values did not differ significantly from stimulated MV3 cells. This indicates that N-desulfation and acetylation of heparin in the glucosamine units is a criterion to exclude the VLA-4 binding ability.

Conclusion

These results clearly demonstrate the dominant role of VLA-4 in MV3 melanoma cell metastasis and thus, justify the efforts to target VLA-4 with heparin. The data provide evidence that the anticoagulant activities of heparin are not required for VLA-4 blocking, since the non-anticoagulant RO-heparin is equally effective in blocking VLA-4 as a commercial UFH. Furthermore, the VLA-4 binding is critically controlled by certain structural parameters. N-acetylation of the glucosamine units is not compatible with VLA-4 binding. These findings differ to some extent from the requirements for P- and L-selectin binding [3]. A recent study has demonstrated that a certain chain length of heparin is essential for VLA-4 binding and that higher flexibility of the heparin chain induced by glycol-splitting of RO-heparin [5] augments inhibition. To obtain a deeper insight in the VLA-4 heparin interaction further heparin modifications, i.e. selective desulfations should be investigated.

Since tumors differ in adhesion receptor expression, a closer examination of the heparin interaction with different adhesion receptors or other molecules involved in metastasis...
could reveal applications of heparin derivatives for antimetastatic therapy.

References


Neoadjuvant treatment of adenocarcinomas of the gastroesophageal junction and stomach – a feasibility trial combining cisplatin and docetaxel with either 5-fluorouracil or capecitabine*

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Introduction

The prognosis of stomach cancer or carcinomas of the gastroesophageal junction still remains poor, despite attempts to improve the prognosis by multidisciplinary therapeutic approaches, including neoadjuvant systemic therapy. Even then, the results are daunting in terms of overall survival, especially in advanced stages of the disease [3]. Therefore, optimization of treatment regimens is urgently required. A pre- and postoperative systemic cytotoxic therapy with epirubicine, cisplatin and 5-fluorouracil (5-FU) in the context of the MAGIC trial [2] resulted in a significant improvement of 5-year progression-free and overall survival rates. This finding could be confirmed by the French FFCD trial [1]. However, only 42% (MAGIC trial) or 50% (FFCD trial) of patients underwent postoperative chemotherapy due to postoperative comorbidities, thus reducing the potential benefit of multimodal treatment. Systemic cytotoxic therapy therefore has to focus on the preoperative treatment schedules. The optimum neoadjuvant chemotherapy for stomach cancer is still the subject of clinical trials. Here we report the combination of two different fluoropyrimidines (5-FU or capecitabine) with taxanes and cisplatin in terms of treatment feasibility.

Patients and methods

21 patients (13 male, 8 female, median age 58 years (range 28 – 74)) with histologically proven locally advanced adenocarcinomas of the gastroesophageal junction (n = 11) or stomach (n = 10) were subjected to neoadjuvant chemotherapy containing cisplatin and taxanes, combined with conventional 5-FU/folinic acid (n = 5) or capecitabine (n = 16). All patients (minimum age for inclusion 18 years) had to have histologically confirmed metastasized or locally advanced adenocarcinoma of the stomach or gastroesophageal junction with no previous chemotherapy for advanced disease, no major organ dysfunction or impairment of general condition (ECOG performance score 0 – 1). Docetaxel and cisplatin were administered at 40 mg/m² every other week, combined with capecitabine at 625 mg/m² b.i.d. for a total of 64 days. Tumor assessments took place at initial staging, after 4 weeks of treatment and before surgery. Treatment was given until either the best response was achieved, disease progression occurred, intolerable toxicity appeared, withdrawal of informed consent occurred or death of the patient.

Results

No treatment-related deaths occurred at a median follow-up of 13 months (392 days, range 9 – 763), with 2 patients still being at a pre-operative stage. 17 out of the remaining 19 patients could be resected but the other 2 patients were not subjected to surgery due to metastatic disease. Ten patients (59%) showed pathological regression. Fifteen out of 17 operated patients were still alive, with 1 patient having died due to causes unrelated to cancer. Four patients relapsed (Days 70, 77, 158, 473 post surgery) and 1 of them subsequently succumbed to his...
malignant disease. Overall toxicity profiles were moderate, with 20 out of 21 patients up to now having completed the scheduled protocols. 1 patient stopped therapy due to a deteriorating general condition. However, in 5 out of 16 patients treated with capecitabine, this drug had to be interrupted due to hand-foot syndrome (n = 3), gastrointestinal symptoms (n = 1) or a deteriorating general condition (n = 1).

Conclusion

The benefit of neoadjuvant therapy relies on an early effect on occult metastases which may be present, an improvement in R0 resection rates by downsizing and potentially downstaging of the primary tumor as well as on the fact that general condition of the patient at the beginning of the treatment is better than after chemotherapy and tumor resection. This does not abolish the need for appropriate surgical procedures including D2 lymph node resections since in Western communities there is no role for adjuvant chemotheropy up to now.

The addition of docetaxel to the established schedule of cisplatin and 5-FU increased response rates [6]. The inconvenience of an infusion schedule can be overcome by giving capecitabine orally which has recently been compared to 5-FU within the context of a noninferiority trial. On the other hand, one has to consider the fact that this randomized Phase III trial has mainly been performed in an Asian population and the results may not always be transferable to patients of Caucasian ethnicity [4]. The combination of neoadjuvant docetaxel, cisplatin and capecitabine (called DXP in that trial) has also been shown feasible in advanced disease in a recent Korean trial [5].

The combination of taxanes with cisplatin and fluoropyrimidines is a feasible and active neoadjuvant regimen. No major differences in response between the two schedules could be observed. All toxicities were clinically manageable, with no treatment-related deaths and the results are therefore comparable to those previously reported within the context of the MAGIC trial [2]. The protocol containing cisplatin docetaxel and capecitabine (DCC protocol) will be further investigated within the context of a multicenter clinical trial currently being prepared.

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Albumin, a versatile carrier in oncology*

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Introduction

Albumin is becoming increasingly important as a drug carrier in cancer therapy since numerous preclinical studies have demonstrated an accumulation of albumin in experimental solid tumors [1]. This property is made use of in therapy regimens since anticancer prodrugs can be bound to exogenous or endogenous albumin and lipophilic anti-tumor agents, such as paclitaxel, can be encapsulated into albumin nanoparticles (Abraxane).

Albumin is the chief circulating protein in the blood and is a transport protein per se for a number of endogenous and exogenous compounds. Albumin uptake in malignant and inflamed tissue is mediated by the pathophysiology of tumor tissue, characterized by angiogenesis, hypervascularization, a defective vascular architecture and an impaired lymphatic drainage combined with the lack or the presence of a defective lymphatic drainage system (Enhanced Permeation and Retention EPR effect). In addition, as an explanation for the high albumin turnover in rodent tumors, Stehle et al. [2] have proposed that albumin is a major energy and nutrition source for tumor growth.

Drug delivery systems with albumin

Abraxane, an albumin nanoparticle

Clinically, one of the most advanced drug delivery systems is an albumin paclitaxel nanoparticle (Abraxane) that was approved in 2005 for treating metastatic breast cancer. American Bioscience, Inc. has developed an albumin-based nanoparticle technology (nab-technology) that is suitable for encapsulating lipophilic drugs into nanoparticles. Paclitaxel is mixed with human serum albumin in an aqueous solvent and passed under high pressure through a jet to form drug albumin nanoparticles, nab-paclitaxel, with a size in the range of approximately 130 nm. An obvious advantage of this formulation is that it avoids the use of polyethoxylated castor oil (Cremophor EL) and ethanol, and preclinically nab-paclitaxel has been shown to have superior antitumor efficacy to paclitaxel in a number of human tumor xenograft models based on the shift of the MTD of nab-paclitaxel (~2.2-fold) over the respective free drug in mice combined with ~1.3-fold increase in tumor accumulation [3]. An intriguing aspect of nab-paclitaxel is that nanoparticles disperse in the blood stream to form albumin molecules bearing physically adsorbed paclitaxel.

Besides tumor accumulation of nab-paclitaxel or the formed albumin-paclitaxel molecules due to the EPR effect, accumulation of the formed albumin-paclitaxel molecules is also due to transcytosis initiated by binding of albumin to a cell surface, 60-kDa glycoprotein (gp60) receptor (albumodin) as well as due to binding of albumin to SPARC (secreted protein acid and rich in cysteine) [3, 4].

Following Phase I and II studies, which, not surprisingly, showed no hypersensitivity reactions as observed with Cremophor EL formulated paclitaxel, a Phase III study in metastatic breast cancer was carried out successfully. This study paved the way for approval of Abraxane by the FDA for the treatment of patients with metastatic breast cancer in whom combination therapy had failed [5]. Following its approval in January 2005, Abraxane is being further evaluated for adjuvant, neoadjuvant, and first-line treatment in breast cancer as well as for other indications such as non-small cell cancer and ovarian cancer (www.americanbiosciences.com).

INNO-206, an albumin-binding prodrug of doxorubicin

An alternative to physically binding an anticancer drug to albumin is to attach the chemostatic agent covalently. Over the last few years we have developed a macro-molecular prodrug strategy based on two features: (a) in situ binding of the prodrug to the cysteine-34 position of endogenous albumin...
Liver tumor targeting with 

**L-HSA-DOXO**

A further application of DOXO-EMCH and albumin in drug delivery is liver tumor targeting using albumin conjugates containing galactose residues. These neoglyco-

protein conjugates were designed to selectively enter hepatocytes by binding to the asialoglycoprotein receptor which is over-expressed in approximately 80% of well differentiated and in approximately 20% of poorly differentiated forms of hepatocellular carcinoma. In collaboration with Fiume and coworkers, the (6-maleimidocaproyl) hydrazone derivative of doxorubicin (DOXO-EMCH) was coupled to a thiolated form of lactosaminated human albumin (L-HSA). The resulting conjugate L-HSA-DOXO achieved a very efficient targeting of the drug to the liver of treated mice with doxorubicin concentrations reaching levels 7 – 20 times higher than those in extrahepatic tissues. In further experiments against hepatocellular carcinoma induced in mice by N,N'-diethylnitrosamine, L-HSA-DOXO at a dose of 4 × 1 mg/kg doxorubicin equivalents significantly inhibited tumor growth without decreasing body weights [7]. In contrast, free doxorubicin administered at the same dose as the coupled drug had no effect on tumor growth and produced a significant decrease in the body weight of the treated animals. Experiments in healthy rats have shown that even a dose of 4 × 2 mg/kg L-HSA-DOXO, a dose twice that used in the therapeutic model, produces essentially no liver toxicity indicating an excellent therapeutic index for the novel conjugate and identifying it as a promising candidate for treating liver cancer.

**Conclusions**

The various uses of albumin as a drug carrier that have emerged in the past 10 years are of considerable interest and range from extending the half-life of therapeutically active proteins and peptides to drug targeting in oncology. The development and market approval of the paclitaxel albumin nanoparticle, Abraxane, can be viewed as a landmark, not just for albumin-based drug delivery technology but also for nanomedicine. Other drug formulations in which the drug is bound in vivo to albumin, such as the doxorubicin prodrug INNO-206, are advancing to Phase II trials. In addition, several pipeline products are under development with the platform technologies that are based on albumin as a drug carrier.

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References


Intracellular ATP depletion leads to reduced platinum accumulation in ovarian cancer cells

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Introduction

Cisplatin is one of the most effective drugs in the treatment of ovarian cancer. However, its therapeutic potential is limited by intrinsic or acquired resistance. Several molecular mechanisms of cisplatin resistance have been described including increased repair of DNA adducts, increased tolerance to DNA damage, elevated intracellular levels of glutathione or metallothioneines, increased efflux and decreased cellular uptake of platinum. The most frequently observed feature in cisplatin-resistant cell lines is reduced accumulation of the drug [1]. The exact mechanisms involved have not been completely identified yet. Although some findings suggest that passive diffusion across the membrane is a major mechanism of cisplatin uptake, other studies indicate that various energy-dependent cellular proteins, which utilize ATP hydrolysis as energy source, may be involved in intracellular trafficking of cisplatin [1].

Extracellular ATP is known to enhance cellular membrane permeability, which may lead to increased cellular accumulation of anticancer drugs due to enhanced passive diffusion. Therefore, extracellular ATP may be used to sensitize ovarian carcinoma cells [2]. However, the exposure to ATP does not increase the accumulation of cisplatin in A2780 and A2780cis cells [3]. One possible explanation is a simultaneous increase in cisplatin efflux by activating ATP-dependent efflux transporters (e.g., ATP7A, ATP7B and MRP2). Therefore, treatment of cancer cells with ATP may increase both cellular uptake and efflux of cisplatin at the same time resulting in unchanged net drug accumulation.

To further assess the role of ATP in cisplatin uptake and efflux, we investigated the accumulation of cisplatin under ATP-depleting conditions. ATP depletion can be achieved by using inhibitors of oxidative phosphorylation or mitochondrial electron transport chain (ETC) complex I – IV inhibitors. The latter are known to induce the production of reactive oxygen species leading to apoptosis [4]. For that reason, we chose an inhibitor of the oxidative phosphorylation, oligomycin, which depletes intracellular ATP levels by specific inhibition of the F₀F₁ ATP synthase.

Material and methods

Cell culture

The human ovarian carcinoma cell line A2780 and the cisplatin-resistant variant A2780cis were cultivated as monolayer in RPMI-1640™ medium with 10% fetal calf serum, 0.6 mM L-glutamine, 100 I.E./ml penicillin and 0.1 mg/ml streptomycin at 37 °C and 5% CO₂.

Measurement of intracellular ATP

Cellular ATP levels were determined using a bioluminescence-based assay [5]. 2 × 10⁴ cells were allowed to attach in white 96-well plates overnight. In some experiments the cells were subsequently treated with various concentrations of oligomycin in glucose-free medium for up to 60 minutes.

The solvent was then removed and the cells were lysed with 1% triton and incubated with D-luciferin and recombinant firefly luciferase in reaction buffer (ATP determination Kit™, Invitrogen, Germany) for 10 minutes. The luminescence signal was measured using the microtiter plate reader Lumistar™ (BMG-Lab Technologies, Offenburg, Germany) at 560 nm [5].

ATP concentrations were related to the protein content determined by the bicinchoninic acid protein assay (BCA™ Protein Assay Kit, Pierce, Rockford, USA). The between-day precision for the determination of ATP/protein was in the range of 18.9 – 24.2%.

Cytotoxicity

The cytotoxicity of cisplatin and oligomycin were determined using a MTT-based
Intracellular platinum accumulation

In order to characterize the influence of oligomycin on the uptake of cisplatin, 2 × 10^6 cells were allowed to attach in 6-well plates overnight and were co-incubated with oligomycin (1 µM) and cisplatin (100 µM) for up to 120 minutes after pretreatment with 1 µM oligomycin for 30 minutes. The cells were then trypsinized and centrifuged.

After lysis with concentrated nitric acid for 60 minutes at 80 °C, the intracellular platinum concentration was measured by flameless atomic absorption spectrometry (SpectrAA™ Zeeman 220 System, Varian, Darmstadt, Germany). Platinum concentrations were related to the protein content determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). All experiments were conducted in triplicate and were repeated 3 – 5 times.

Statistics

Since intracellular ATP levels, expressed as cellular ATP content related to cellular protein content, could not be assumed to have a Gaussian distribution, we used the median as a measure of central tendency. Consequently, the differences were analyzed using the non-parametric Mann-Whitney U-test.

Two-way ANOVA was used to analyze the effect of two independent variables (presence/absence of oligomycin and time) on intracellular cisplatin accumulation.

P values < 0.05 were considered significant.

Results and discussion

Firstly, we determined the baseline ATP levels (related to cellular protein content) in the A2780 human ovarian carcinoma cell line and its cisplatin-resistant variant A2780cis. Significant differences in ATP levels between sensitive (3.4 pmol ATP/µg protein) and resistant (4.1 pmol ATP/µg protein) cells were found (n = 28, p < 0.0001, Mann-Whitney U-test) suggesting that ATP may play a role in cisplatin resistance.

To investigate whether the accumulation of platinum is energy-dependent, we pre- and co-incubated the cells with oligomycin, an inhibitor of the ATP synthesis. The cytotoxicity tests showed that the EC50 values for oligomycin were 5.5 µM in the sensitive ovarian carcinoma cell line and 5.3 µM in the resistant cell line, respectively (n = 5).

In order to find the appropriate concentration for the reduction of intracellular ATP levels, we treated the cells with various concentrations of oligomycin (1.5 nM – 10 µM) in glucose-free medium. The lowest effective oligomycin concentration was determined as 1 µM reducing the intracellular ATP levels to 36.7 ± 5.6% (mean ± SEM, n = 3: conducted in triplicates) in the sensitive and to 41.4 ± 5.3% in the resistant cell line. The maximum effect was achieved within 30 minutes. Replacement of the oligomycin-containing medium with drug-free medium led to restoration of the initial ATP levels within 10 minutes. Therefore, we investigated the accumulation of cisplatin in our cell system in the presence of 1 µM oligomycin after 30 minutes pre-incubation with the same concentration of the inhibitor.

Earlier studies showed that on exposure to cisplatin for 2 hours, the cisplatin-resistant cell line accumulated 2.5-fold less platinum compared to the sensitive A2780 cells [7].
Under the conditions of ATP deficiency, the intracellular accumulation of cisplatin was reduced in A2780 cells compared to standard culture conditions (p < 0.0001) (Figure 1A). In the A2780cis cell line, cisplatin accumulation was also reduced, however, to a smaller extent (Figure 1B) (p = 0.02).

Conclusions

Our results indicate that cisplatin is taken up by energy-dependent processes in the sensitive A2780 cells, whereas cisplatin uptake in the resistant A2780cis cells is less dependent on intracellular energy sources. This may, however, be the result of the higher basal ATP levels in A2780cis cells compared to the sensitive A2780 cells. For that reason, further studies should focus on the contribution of increased intracellular ATP concentrations to cisplatin resistance in the A2780cis cell line.

References

**Tumor-selective amphiphilic para-quinones and tetramic acids**

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**Introduction**

Thymoquinone (TQ) is the active principle of the essential oil of black seed (*Nigella sativa*), and is responsible for the antioxidant, anti-inflammatory and antineoplastic effects of the seed. TQ is of low general toxicity and showed weak antitumor effects in numerous cancer cell and animal studies. In xenograft models it delayed tumor growth by induction of cell cycle arrest [5]. In in vitro experiments it induced apoptosis by both p53-dependent and p53-independent mechanisms [4]. The serine/threonine polo-like kinases (Plk), which are overexpressed in many human cancers, have recently been identified as targets for TQ. In HeLa cells TQ caused Plk mislocalization, chromosome congression defects, mitotic arrest and apoptosis [6]. Although black seed oil has customarily been used in folk medicine, clinical applications of pure TQ in man are hampered by its poor membrane permeability and chemical instability. We have now prepared amphiphilic “detergent-like” conjugates of TQ with fatty acid residues attached via non-hydrolysable C-C bonds [2]. These conjugates are expected to be readily accumulated by cancer cells, especially those with ω-3-fatty acid residues for which specific receptors exist. The new conjugates were tested for cytotoxicity against various cancer cells and for interference with Plk and other cancer-relevant targets.

Melophlins are antitumor metabolites of the marine sponge *melophlus sarasinorum*. Like the TQ-fatty acid conjugates, they feature an amphiphilic structure. Their polar 3-acyltetramic acid “head” can mimic an inorganic phosphate in the active site of kinases and can also sequester biologically indispensable metal cations by formation of chelate complexes [7]. The long alkyl “tail” of melophlins and similar 3-acyltetramic acids facilitates their uptake across cell membranes and their anchoring into suitably shaped crevices on the surface of target enzymes. We synthesized naturally occurring melophlins, non-natural analogues and also metal complexes thereof with side-chains of various lengths and degrees of branching [1]. We then elucidated the influence of these structural parameters on the cytotoxicity against various cancer cell lines, on the cellular actin framework and on hyaluronidases which are enzymes crucial to tumor growth, invasion and metastasis.

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**Material and methods**

Cells of human A-498 kidney cancer, KB-3-1 and KB-V1 cervix carcinomas, U-937 and HL-60 leukemia, MCF-7 breast carcinoma and murine L929 fibroblasts were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Cells of human 518A2 melanoma were a gift of the Department of Oncology and Hematology of the Martin-Luther-University Halle-Wittenberg. Resistant KB-V1/Vbl and MCF-7/Topo cells were obtained by incubation of sensitive parent cells with 300-500 nM vinblastine or topotecn for ca 90 d. All cells were cultivated in the media recommended by the suppliers at 37 °C and 10% CO₂. - Cytotoxicities were ascertained using the NBT assay. Cell staining: Rat kangaroo kidney epithelium cells were grown on glass coverslips in 4-well plates and incubated with melophlins for various periods of time. For labeling of the actin filaments, cells were fixed with formaldehyde (3.7%) for 10 min, made permeable by a 5 min treatment with Triton X-100 (0.1%) in PBS and stained with Alexa Fluor-488-conjugated phalloidin (1 µg/ml) for 30 min. Cells were rinsed with PBS, mounted in Prolong Antifade containing...
Results

6-alkyl derivatives of thymoquinone were prepared from TQ and the respective fatty acid via a coupling reaction induced by AgNO₃/(NH₄)₂S₂O₈. They were tested for growth inhibition of cells of human HL-60 leukemia, 518A2 melanoma, KB-V1/Vbl cervix and MCF-7/Topo breast carcinoma.

Unsaturated side-chains conferred greater activities than saturated side-chains of the same length. The conjugate derived from docosahexaenoic acid (DHA) was most active in all resistant tumor cells with IC₅₀ (72 h) values as low as 30 nM in MCF-7/Topo cells (Figure 1a). The mechanism of action of the conjugates is probably different from that of TQ. For instance, the DHA-conjugate induced distinct caspase-independent apoptosis in HL-60 and 518A2 cells concomitant with a loss of mitochondrial membrane potential and a subsequent rise in the levels of reactive oxygen species. Preliminary tests for interference with the polo-box domain (PBD) of Plk using a fluorescence polarization assay revealed that the DHA-conjugate clearly inhibits the function of the PBD of Plk1, Plk2 and Plk3 (apparent IC₅₀ < 10 µM) whereas the conjugate with α-linoleic acid was more selective and inhibited only the PBD of Plk2 (apparent IC₅₀ = 14 µM).

Within the series of natural melophlins, those bearing methyl-branched side-chains and methyl residues at the heterocyclic core, e.g., melophlin C, were most efficacious in MTT tests against various cancer cell lines. These melophlins were also effective in disrupting the actin filaments of rat kangaroo kidney epithelium cells (Figure 1b). In contrast, the human hyaluronidase PH-20, which is closely related to enzymes responsible for the decomposition of the extracellular hyaluronan matrix and which are over-expressed by many tumors in order to allow growth and invasion, was most strongly inhibited by melophlins with unbranched side-chains, i.e., by melophlin A (EC₅₀ = 2.6 ± 0.2 µM). Only these melophlins fit snugly into a crevice on the enzyme surface near the active site.

Conclusion

We found that the cell line specificity and the anticancer activity of the natural paraquinone TQ can be significantly increased by covalent attachment of fatty acyl residues of defined length and degree of unsaturation. In the case of the natural sponge-produced melophlins we identified actin and hyaluronidase activities were determined using a turbidimetric method [3] and cetyltrimethylammonium bromide to precipitate the residual high molecular weight hyaluronan after incubation with hyaluronidase with or without melophlins.
nidases as new cancer-relevant targets and also pinpointed structure-activity relationships that should allow a rational design of synthetic analogues with improved activity and cell line specificity.

References


Rat monoclonal antibodies against bone sialoprotein II inhibit tumor growth and osteolytic lesions in nude rats induced by MDA-MB-231 breast cancer cells

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Introduction

Breast cancer is the most frequent malignant disease in women living in Western, industrialized countries. In general, the primary carcinoma can be treated surgically, but the advent and growth of metastases is decisive for the subsequent prognosis. Besides lymph node metastases, metastases to the skeleton is most prevalent, followed by metastases to liver and lungs. The current treatment involves irradiation and various pharmacological agents in addition to surgery, but after the disease has started to metastasize to distant organs, there is no curative treatment available.

Recently, the use of antibodies has gained renewed interest because trastuzumab, an antibody against the antigen HER2, has shown activity in terms of prolonging the survival and improving the outcome in women with HER2-positive breast cancer after removal by surgery [1].

Besides the EGF-receptor family, other targets are being investigated because their expression is increased by breast cancer cells and they are assumed to contribute to cancer progression and metastases.

Bone sialoprotein II (BSP) is a phosphorylated glycoprotein and significant component of the bone extracellular matrix. Native BSP has an apparent molecular weight of 60 – 80 kDa based on SDS-PAGE. The function of BSP in mineralized bone tissues is probably that BSP acts as a nucleus for the formation of apatite crystals. Additional roles of BSP include MMP-2 activation, angiogenesis, and protection from complement-mediated cell lysis. Regulation of the BSP gene is important for bone matrix mineralization. Furthermore, it is thought to play a role in cancer metastases to bone [2]. This is based on the observation that increased serum levels of BSP are associated with an increased risk of skeletal metastases in breast cancer patients [3]. These considerations lead to the investigation reported here on whether BSP is a useful target for treating bone metastases in breast cancer [4, 5, 6, 7].

In the following we describe an animal model inducing lytic skeletal metastases. It is based on MDA-MB-231 human breast cancer cells growing in nude rats which induce lytic lesions following loco-regional implantation. We also describe the first results obtained in this model with a monoclonal antibody against BSP.

Material and methods

The in vivo-metastases model is based on the loco-regional growth of human tumor cells in nude rats which then induce lytic metastases in defined parts of the rat skeleton, only [3,4]. For that purpose and to facilitate their detection, breast cancer MDA-MB-231 cells were stably transduced with the firefly-luciferase- and red fluorescent protein (RFP) genes. The expression of luciferase allows the detection of the injected tumor cells as early as few minutes after cell implantation by intraperitoneal injection of luciferin.

Subconfluent MDA-MB-231 tumor cells were harvested using 2 mM EDTA in PBS (phosphate-buffered saline) without Ca²⁺ and Mg²⁺ but with 0.25% trypsin (Sigma, Taufkirchen, Germany). Cells were counted in a Neubauer’s chamber and suspended (5 × 10⁵ cells in 1 ml) in RPMI 1640.

Tumor cell implantation

For tumor cell inoculation, rats were anesthetized with isoflurane. The skin of the right hind leg was disinfected and shaved under anesthesia. A small incision was made (approximately 6 – 8 mm) close to the femoral artery.
for exposing the artery. After isolating the vessel, \(1 \times 10^5\) tumor cells (in 0.2 ml RPMI 1640) were injected into a side branch of the femoral artery. Surgical threads were used to temporarily close the various branches of the femoral artery to direct the tumor cells to the descending genicular and popliteal arteries, which supply the knee joint and the muscles of the leg (Figure 1A).

The appearance and development of bone metastases and lytic lesions were monitored by light emission released by luciferase-mediated metabolism of luciferin. The non-invasive photon emission was recorded at 14-day intervals using a Xenogen IVIS 100 imaging system. Additional examinations were carried out at longer time intervals using CT and MRI.

Treatment with the monoclonal rat anti-BSPII antibody (mra-BSP antibody) was started when tumor bearing rats had shown stable tumor growth, either after 2 weeks (early onset) or after 4 weeks (late onset) following tumor cell transplantation. The treatment was given by s.c. injection of the mra-BSP antibody and the dosage of 10 mg/kg/week was maintained for up to 6 weeks. The progressions in tumor size, as well as the resulting skeletal lesions were recorded over a period of 42 days after inoculation of tumor cells.

### Results

Metastases occurred exclusively in the femur, tibia and the fibula of the nude rats in the weeks after tumor cell inoculation.

The tumor take rate, determined in 30 nude rats, was high with 28 rats (93.3%) showing a clear luminescence signal. Accordingly, except for the two failures, tumor-bearing rats showed rapid tumor growth accompanied by lytic destruction of the femur and tibia of the respective hind leg.

The design of treating nude rats inoculated with MDA-MB-231 breast cancer cells with the mra-BSP antibody is shown in Table 1.

Sixteen nude rats were investigated in the late onset therapy group, 8 animals served as untreated control and 8 animals were treated with mra-BSP antibody.

Twelve nude rats were used in the early onset group – 6 for control and 6 for the

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<th>Table 1. Design and results of treating nude rats implanted with MDA-MB-231 breast cancer cells with the mra-BSP antibody.</th>
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Figure 1. The top of the figure (A) shows an aspect of the right hind leg after exposing and isolating the femoral artery. The vessel’s branches are indicated for better orientation. The bottom part of the figure (B) shows a comparison between the luciferin induced light emission from a tumor bearing control rat and a rat treated with the monoclonal rat anti-BSPII antibody.
mra-BSP antibody treated group. All 14 control rats showed a steady tumor growth. A typical course is shown in Figure 1B with the aspects recorded at 2, 4 and 6 weeks after tumor cell implantation. Due to the rapid tumor growth, eleven of the untreated control-rats had to be terminated for ethical reasons within the observation period. The growth of the lesions showed a tumor volume doubling time of 3 – 4 days in untreated rats.

The interim-analysis for the mra-BSP antibody indicated that treated rats did not show a significant increase in light emission nor a clinical deterioration. In fact, 6 of 8 rats receiving the late onset therapy with the mra-BSP antibody showed no light emission after 4 – 6 weeks (75%; p = 0.01 versus control), as shown in Figure 1B. Similarly, 5 of 6 rats receiving the early onset therapy with mra-BSP antibody showed no light emission after 4 to 6 weeks (83%; p < 0.01 versus control). Radiological and histological examinations confirmed that animals without light emission were free of tumor growth, corresponding to a complete remission.

Conclusions

In conclusion, the used monoclonal rat antibody directed against BSPII is a powerful tool in treating experimental skeletal metastases and warrants further development.

References


Web-based open source application for the randomization process in clinical trials: RANDI2*

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Introduction

The randomization process is pivotal for clinical trials with more than one treatment arm. This is because the same boundary conditions have to be used whenever new therapies are being compared with standard treatments or other experimental treatments. Kendall [3] describes “Chance” as a random error appearing to cause an association between an intervention and an outcome. The most important design strategy to minimize such random error is to have a large sample size. Randomization is based on four mathematical concepts: (i) complete randomization with probability $p = 1/n$ for the choice of one treatment out of $n$ with identically distributed Bernoulli random variables, (ii) restricted randomization with a variance-covariance matrix unequal $1/4 I$ (unique matrix), (iii) covariate adapted, and (iv) response adapted randomization. The random process itself is driven by a pseudo-random number generator using, e.g. the linear congruential generator $X_{n+1} = (aX_n + b) \mod m$, generating $m$ (modulus) random numbers. As stressed by Rosenberger and Lachin [4], randomization in a clinical trial is an issue in each of the three components design, conduct and analysis. The open source project RANDI2 provides a platform for the electronic randomization and possesses a much more reliable method than conventional telephone and fax procedures. It is flexible in use and can be customized to the demands of the study. However, other existing electronic systems are sometimes inflexible and not freely available. Thus, the development of RANDI2 was started.

Material and methods

The application RANDI2 [1, 6] is based on the programming language JAVA and realized in a 3-layer manner (divided into user interface, application/server logic including the randomization algorithms and persistence layer). The technology used in this application is (i) ICEfaces, a framework to form a dynamic and state of the art graphical web-based user interface; (ii) Spring, which organizes the configuration of the application components (dependency injection) and allows the implementation of cross-cutting routines (attributable to the use of aspect-orientated programming), i.e., security checks with access control lists and audit functionality; (iii) Hibernate, which enables independent usage of the system from the type and kind of data base. RANDI2 allows integration under different institutional circumstances independent of the database management system. Due to the architecture of the randomization model, dividing entities like study therapies, study centers, patient characteristics and others, the whole process is highly customized and open for further developments. An expert group was established with members of the German Cancer Research Center, the University of Heidelberg and the National Center for Tumor Disease to supervise the development of RANDI2 to a stage near to where it could be used and guaranteeing that the system meets the requirements of ongoing and planned trials. Given the great flexibility of the system, new biometrical technical expertise e.g. the new randomization algorithms can find their way into RANDI2. An example is minimization and some derivatives discussed recently [2].

Results

Some special functions could be achieved with the RANDI2 randomization system. With a single installation it was possible to manage several studies and centers thus alleviating the process of randomization, especially in bigger study centers. Use of the process is easy because of the implementation of the randomization configuration wizard which guides the user from general trial properties to
detailed algorithms and trial subject configuration. An audit functionality is built into the system ensuring that all user actions are logged in a database and accessible at the user interface. The data of configuration and randomization can be exported as a simple comma separated value (CSV) file or in the MS Excel format. There is also the possibility of visualizing the randomization results and the trial status using various charts such as the disposition of patients per arm or per trial site, progress of recruitment and discrepancy to the expected quota and disposition per stratum of the patients. A special function at the administration level is the management of roles and permissions with several predefined roles as administrator, principal investigator, investigator, statistician and monitor. There is also the possibility of creating and adapting new roles and of customizing single user rights. The configuration of patient data consists of dichotomous or ordinal, numeric data depending on the types of properties, calendar data, or text variables with the possibility of data validation, support of inclusion criteria and stratification. The process is divided in three parts: (i) choice, (ii) configuration and (iii) operation of the algorithm. The current version supports complete, biased coin, truncated randomization, block randomization and the Wei’s urn model randomization which includes the classical urn model as a special case [5]. In the stratification process of randomization the balancing of known covariates or prognostic factors should be derived. This is especially important for smaller trials [7]. Each influencing factor can be defined – nota bene – as a stratum and in the case of a simple stratification for each stratum an independent identical randomization process is started. The stratification procedure in RANDI2 is realized by generating a stratum identifier on the basis of the patient properties and an internal, separate randomization process is initialized.

There is great flexibility in applying RANDI2. Local conditions and study parameters can be varied over a wide range, as e.g., hardware equipment, database system, but also the number of study therapies, study centers and patient characteristics. Furthermore, the study parameters in RANDI2 are independent of a concrete realization or implementation of the randomization algorithm. In this frame a variable approach for available randomization algorithms is provided, in which – after a testing and simulation process – the procedure finally used can be chosen.

**Conclusion**

With the open source project RANDI2 the user can run standard randomization procedures and has the additional opportunity to use special and/or new methods quickly. RANDI2 supports the choice of the randomization procedure by testing and simulation resulting in a more reliable outcome of the study. Particular requirements can promptly be resolved in the system and with the necessary special knowledge ad-hoc solutions are possible. Moreover, RANDI2 offers a transparent randomization solution because of the open source nature of the project.
References


Successful sequential antiangiogenic therapy for alveolar soft part sarcoma – a case report*

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Key words
alveolar soft part sarcoma – antiangiogenic therapy – quality of life

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Introduction

Alveolar soft part sarcoma (ASPS) is a rare tumor entity accounting for <5% of childhood non-rhabdomyosarcoma soft tissue sarcomas. It mainly affects children and young adults and has a poor prognosis because of its resistance to conventional cytotoxic drugs. Randomized clinical trials concerning this tumor entity have not yet been carried out and reports on treatment are usually limited to a small number of cases [2]. The tumor is characterized by its high level of vascularization rendering it a potential target for novel treatment regimens with targeted drugs.

Patients and methods

We present a case involving a 23-year-old Caucasian male who was diagnosed 2 years previously with an alveolar soft part sarcoma of his right groin. The tumor had metastasized diffusely into both lungs as well as infiltrating the pelvic bone. As the tumor was initially classified as undifferentiated mesenchymal sarcoma, a neoadjuvant treatment concept with three cycles of ifosfamide and doxorubicin was commenced. However, since the disease continued to progress a second-line schedule with dacarbazine was started and further pursued, focusing on side effects due to side effects, an individual treatment approach with angiogenesis inhibitors was initiated and further pursued, focusing on sustained quality of life and physical mobility while aiming at disease control in this palliative situation with limited side effects.

Results

Combined treatment with an angiogenesis inhibitor (cediranib) and a Src-Abl kinase inhibitor (saracatinib) within the context of a Phase I trial could control tumor growth for a period of almost 1 year before the pulmonary metastases increased in size and painful local progression within the pelvic bone confined the patient to a wheelchair. Since the patient was still in good clinical condition we decided to switch the therapy to a combination of sorafenib (2 × 400 mg daily) and bevacizumab (escalating dose, up to 1.6 mg/kg weekly) in an individual approach aiming at tumor control and relief of local symptoms. So far, the patient has regained the ability to walk short distances, with side effects of treatment being tolerable, mainly in the form of hoarseness and mucositis not exceeding CTC Grade 2. Tumor manifestations overall indicate some slight regression, with a mixed response of largely regressing and slightly progressing local manifestations of the disease.

Conclusion

Especially in highly vascularized tumor entities, even after failure of first-line antiangiogenic strategies, a second line antiangiogenic approach may be beneficial in terms of tumor control to improve quality of life and reduce morbidity. Angiogenesis inhibitors have been studied in ASPS previously [1]. At the time, we had the possibility of including the patient in a Phase I trial with an angiogenesis inhibitor combined with a multi-kinase inhibitor (cediranib plus saracatinib) [6], two lines of conventional cytotoxic therapy having previously failed. When the patient experienced both pulmonary progression and increasing physical impairment due to local tumor growth after almost 1 year of disease stabilization, no further guidelines for this situation were available. Considering the remarkable stabilization of the patient’s disease with the antiangiogenic treatment men-
tioned above, we discussed the strategy of using other angiogenesis and multikinase inhibitors with the patient, namely sorafenib and bevacizumab, so far not known to show cross-resistance.

Having obtained informed consent for this individual approach, the second-line antiangiogenic treatment with bevacizumab and sorafenib was initiated and stabilization of the disease was obtained for more than 9 months. It was notable that the overall tumor load seemed to decrease slightly and there were some manifestations of visible shrinking, whereas others seemed to remain stable or increase marginally in size. We therefore hypothesized that the signal transduction pathways in alveolar soft part sarcoma deserve further evaluation since not only VEGF inhibition, but potentially a far more complex signal transduction network seems to trigger tumor growth, with possible heterogeneity or various tumor clones even within the same patient. Recently, gene expression profiling in ASPS has been performed, aiming at revealing potential novel targets for therapy [5], among these angiogenesis- or proliferation-related genes. However, large multicenter trials will be difficult to realize due to the small numbers of patients affected.

This paper emphasizes the need for further evaluation of signal transduction pathways in this disease, as second-line antiangiogenic therapy is still an option in such patients leading to a relief of symptoms and therefore a better quality of life, if not prolongation of overall survival.

References


First-line treatment of patients with metastatic pancreatic cancer: results of a Phase II trial with S-1 (CESAR-Study group)*

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Key words
pancreatic cancer – S-1 – first line – Phase II clinical trial – metastatic disease

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Introduction

Pancreatic cancer is the eighth most common cause of cancer-related death worldwide and is associated with a dismal prognosis and a 5-year survival rate of less than 4% [2]. Furthermore, intratumoral DPD mRNA expression level in pancreatic cancer is significantly higher than in other malignancies [3]. Although the susceptibility to chemotherapy of unresectable/advanced pancreatic cancer is very poor, the usefulness of new anticancer drugs, such as S-1, has been reported in recent years with unusually high response rates and meaningful survival benefit. S-1 is an oral anticancer drug containing tegafur (FT), a pro-drug of fluorouracil, combined with two modulators, 5-chloro-2,4-dihydroxypyridine (CDHP), which is an inhibitor of dihydropyrimidine dehydrogenase (DPD), and potassium oxonate (Oxo), at a molar ratio of 1 : 0.4 : 1. In a recent Phase II study in 40 patients with metastatic pancreatic cancer, single agent therapy with S-1 resulted in an overall response rate of 37.5% [1]. In another recent study in which S-1 was administered together with gemcitabine, objective responses were observed in 16 of 33 evaluable patients (48%), and median survival was 12.5 months (95% CI: 5.9 – 19.1) [9].

This study evaluated the antitumor effect and safety of S-1 in patients with metastatic pancreatic cancer.

Patients and methods

Chemo-naive patients with measurable metastatic lesions of pancreatic adenocarcinoma as defined by the Response Evaluation Criteria in Solid Tumors (RECIST) were enrolled and received S-1 orally after meals at a dose of 30 mg/m² twice daily (b.i.d.) for 14 days followed by a 1-week recovery period, repeated every 3 weeks. The primary endpoint was to evaluate the antitumor activity, as assessed by objective tumor response (overall response rate (ORR)), of single agent S-1 in chemotherapy-naive patients with locally advanced or metastatic pancreatic cancer. Secondary endpoints were (i) to evaluate the disease control rate (DCR), duration of response (DR), time to tumor progression (TTP) and survival rate, (ii) to investigate the effect of S-1 on Karnofsky Performance Status (KPS) and pain assessments, including pain intensity and analgesic consumption, and (iii) to evaluate the safety profile of S-1. Patients continued treatment until disease progression or unacceptable toxicity; thereafter, other chemotherapy (e.g., gemcitabine) was permitted.

The trial had a two-stage design with 22 patients evaluable for efficacy in stage 1 and an additional 18 patients in stage 2, if ≥ 3/22 pts ≥ (13.6%) had achieved a confirmed response at the first stage.

Results

A total of 27 chemo-naive patients with measurable lesions of pancreatic adenocarcinoma were enrolled in this Phase II study. The patients received a median of 5 cycles (1 – 19 cycles). The response (RECIST) could be evaluated in a total of 22 patients. Three patients showed confirmed partial remission (PR) of the target lesions. However, detection of clinically asymptomatic brain metastases
in 1 of these patients upon confirmation of PR prevented this study proceeding to the second stage. Overall, 15 pts (68.2%) had stable disease (SD) and 4 patients (18.2%) progressive disease (PD). The median time to progression (TTP) for all evaluable patients was 3.5 months (95% CI, 2.5 – 5.3 months). The overall survival (OS) for all patients (n = 27) was 9.1 months (95% CI, 4.7 – 11.2 months). The median duration of disease control for patients with SD or PR (n = 17) was 4.3 months (95% CI, 2.8 – 7.2 months).

S-1 was well tolerated. The most frequent grade 3/4 toxicities (occurring in > 5% of patients) were fatigue, vomiting, diarrhea, pain, weight loss and confusion state.

Reason for treatment discontinuation was progressive disease in 22 patients (81.5%), in 16 patients (59.3%) by imaging, in 6 patients (22.2%) by clinical progression.

Three patients were withdrawn from the study due to toxicity (hand-foot syndrome, sepsis and abdominal pain). Two additional patients were withdrawn due to withdrawal of consent and investigator’s judgment, respectively.

Most of the patients received gemcitabine as second line treatment. Current data suggest a lack of cross-resistance between S-1 and gemcitabine.

Conclusion

Although this study did not meet the predefined targets to proceed to the second stage, TTP and OS are at least comparable to gemcitabine as monotherapy [6], the current standard of care (Figure 1). A Phase II study of S-1 in gemcitabine-refractory metastatic pancreatic cancer showed marginal activity with a median progression free survival (PFS) of 2.0 months and a median survival time of 4.5 months [7]. Recently, two Phase II studies with S-1 in combination with gemcitabine for chemo-naïve patients with locally advanced or metastatic pancreatic cancer have been reported. The median time-to-progression as well as median overall survival was 4.9 months/7.9 months [4] and 5.4 months/8.4 months [10]. Experimental data suggest, that pretreatment with S-1 enhances gemcitabine effects in pancreatic cancer xenografts [8]. In conclusion, S-1 appears to be an active and well-tolerated drug in metastatic pancreatic cancer, the effectiveness of which should be further confirmed in a larger clinical trial.

References


Safety, efficacy and pharmacokinetics of nimotuzumab, a humanized monoclonal anti-epidermal growth factor receptor (EGFR) antibody, in patients with locally advanced or metastatic pancreatic cancer*

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Introduction

Pancreatic cancer is a devastating health problem, with an estimated 28,900 deaths in Germany alone in 2001 and a 5-year survival rate of 4% [3]. The disease is characterized by early locoregional spread and distant metastases. As a consequence, the majority of patients present with advanced, non-resectable disease. For these patients, systemic chemotherapy is largely ineffective [6] although gemcitabine has demonstrated a modest clinical benefit and has become standard chemotherapy for advanced pancreatic cancer [2]. The median survival of patients with advanced disease continues to be less than 6 months, and evaluation of novel therapeutic targets is needed to improve the outcome for these patients.

The epidermal growth factor receptor (EGFR) seems to play a particularly important role in carcinogenesis of human cancers, including pancreatic cancer. Increased expression of EGFR and its ligand has been detected in human pancreatic cancer tissue [4]. Moreover, coexpression of EGFR and its ligand has been proven to predict poor prognosis in pancreatic cancer [4] and it has been proposed that coexpression of EGFR and its ligand functions as an autocrine loop to constantly stimulate cell proliferation. Therefore, blockade of EGFR activity would interrupt EGFR-mediated signal transduction pathways and result in suppression of tumor growth.

Nimotuzumab (OSAG101, Theraloc®) is a humanized monoclonal antibody (mAb) that binds to the EGFR. In preclinical studies the antibody has shown potent antitumor activity. Based on Phase I data, the recommended dose has been established at 200 mg weekly [1]. A previous Phase II study in children with high grade brain tumors showed activity of nimotuzumab as a monotherapeutic agent, even in prognostically very unfavorable pontine glioma. No clinically relevant drug-related side effects were reported [5].

We report here the results of a multicenter Phase II trial of nimotuzumab monotherapy in patients with advanced pancreatic cancer. The objectives of the trial were to determine the objective tumor response rate, time to disease progression (PFS), overall survival (OS) and safety profile.

Patients and methods

Patients who failed standard chemotherapy with gemcitabine or another first line regimen for advanced disease and had at least one measurable lesion as defined by Response Evaluation Criteria in Solid Tumors (RECIST) criteria were eligible for the study. Nimotuzumab was given i.v. as induction therapy at 200 mg once weekly for 6 weeks. Follow-up by CT was performed after 8 weeks. Patients continued receiving treatment every 3 weeks until disease progression or unacceptable toxicity occurred. Endpoints
included tumor response (RECIST), time to disease progression and safety. Blood samples were collected prior to first dose, at end of infusion, and 3 h, 6 h, 48 h, as well as before each subsequent nimotuzumab dose was administered. Nimotuzumab concentrations in serum were measured by cellular ELISA and calculations performed using the Kinetica program.

**Results**

In total, 54 patients were treated (28 women/26 men; ECOG status of 1 (n = 40) or 0 (n = 14), median age 63.6 y (range 46 – 83 y)). A total of 7 patients had locally advanced disease only, the majority (47 patients) presented with metastatic disease. All patients received prior cytotoxic treatment, 1 regimen (28 patients), 2 regimens (20 patients) or 3 or more different regimens (6 patients).

Treatment-related adverse events with reported possible relation to the study drug were CTC Grade 3 for a total of 3 patients: a vascular event (deep vein thrombosis), 1 patient with hemorrhage/bleeding and complete recovery, and 1 patient with gastrointestinal obstruction. Other adverse events with a possible relation to nimotuzumab (CTC Grade 1/CTC Grade 2) were constitutional (fever, chills) in 7 patients (5/2), skin toxicity (rash) in 5 patients (CTC Grade 1 only), fatigue in 5 patients (4/1), nausea in 4 patients (2/2), pain in 2 patients (CTC Grade 1 only), allergic reactions in 2 patients (CTC Grade 2 only), anorexia CTC Grade 2 in 1 patient, and cardiac arrhythmia CTC Grade 2 in 1 patient.

The objective response was evaluable in a total of 35 patients. In summary, no objective response was seen but 6 patients showed sustained stabilization of previous progressive disease with a median TTP of 19.2 weeks in these patients (range: 14.1 – 26.1 weeks). A correlation between the occurrence of skin toxicity and response cannot be established so far, most likely due to the small numbers of patients studied within the context of a Phase II trial. However, if rash was associated with successful treatment, even higher response rates might be postulated in those patients experiencing a rash more than CTC Grade I.

A total of 54 patients were evaluable for PFS and OS. The median PFS for all patients was 6.7 weeks. PFS after 1 year was 10.3%. Furthermore, median OS was 18.1 weeks.

After 200 mg single dose, the mean value of Cmax was 141 ± 33 µg/ml. The t1/2 was 45 h and volume of distribution 1.46 ± 0.3 l. The total clearance was 23 ± 6 ml/h and trough values after 168 h were 6.2 ± 6.3 µg/ml.

**Conclusion**

This study in patients with locally advanced or metastatic pancreatic cancer was initiated to evaluate the activity and safety of nimotuzumab (OSAG 101, Theraloc®) as second-line therapy. However, nearly half of enrolled patients received at least 2 prior treatment regimens for advanced disease.

A total of 3 patients experienced CTC Grade 3 adverse events, which are more likely to be disease-related than causally linked to the applied study-drug. Other adverse events were generally mild, tolerable, and clinically easy to manage. It is noteworthy that skin toxicity, a frequent and typically adverse event with all anti-EGFR directed drug strategies, occurred in a minority of patients with CTC Grade 1 only. Therefore, nimotuzumab is clinically much better tolerable compared to other EGFR-directed drugs currently in use. In conclusion, these data confirm that nimotuzumab is safe and very well tolerated.

Primary endpoint of the study was the remission rate after administration of at least 6 doses of study drug. Considering that all patients had been treated previously, and nearly half of them with two or more regimens, an objective tumor response with nimotuzumab was not be expected. However, sustained stabilization of previous progressive disease with a median TTP of 19.2 weeks in 6 out of 54 patients indicates antitumor activity.

Currently the EGFR status of tumors as a predictive factor for anti-EGFR directed drugs is under discussion. In this study, immunohistochemical analysis of EGFR expression in the tumors was not performed. More recent analyses in lung cancer have suggested that the presence of EGFR mutations or EGFR amplification measured by fluorescence in situ hybridization may be more useful [7]. However, studies in more than 200
pancreatic tumors did not identify any EGFR mutations [8].
To improve efficacy, a randomized, placebo-controlled multi-center trial in combination with gemcitabine was started.

References

Continuous monitoring of toxicity in clinical trials – simulating the risk of stopping prematurely*

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Introduction

In early-phase clinical trials new compounds with anti-tumor potency carry the risk of provoking toxic reactions in patients. Therefore we have developed a simulation program (ToxCrit) to determine the probability of premature stopping of a trial due to an unacceptable toxicity rate. When a clinical trial protocol is developed in order to investigate the safety and efficacy of a new compound, an unacceptable toxicity rate should also be defined. If the number of subjects with toxic reactions in the trial is higher than this predefined unacceptable toxicity rate, the investigation should be stopped or interrupted and modified (adapted). With ToxCrit, the conduct of a trial can be simulated repetitively on the computer. One can see how often a trial must be stopped in the simulation because of a too high toxicity rate. In other words, the risk of prematurely stopping a trial due to an unacceptable toxicity rate is calculated. This risk can be used to analyze if a statistical trial design is appropriate for the investigation or whether it should be modified because of the too high risk of being stopped.

Material and methods

ToxCrit offers two ways of simulating a trial based on different methodological approaches. Both approaches have supporters in the biometrical community. The Bayesian approach [1, 6] uses stopping criteria that rely on the a posteriori distribution of the true toxicity rate (beta-distribution). The frequentist approach uses the border of one-sided confidence intervals for the true toxicity rate (Pearson-Clopper confidence limits) [2]. Both the Bayesian and the frequentist approach are programmed, and in ToxCrit it is possible to choose one or the other approach.

To eliminate programming errors ToxCrit was reimplemented by a second software developer in another programming language (validation). ToxCrit was then implemented by a third person in a third programming language. The reason for the third implementation was the intention to offer ToxCrit as a web application.

Results

ToxCrit has been implemented in the three different programming languages R, SAS® and Java®. The Java® implementation is deployed in the institutional intranet as a web application and can be accessed with a web browser.

ToxCrit requires six input parameters (npat, ptotxicrit, ptox, pabbruch, nsims, seed). The parameters nsims and seed are more of a technical nature and can be disregarded for an initial understanding of the simulation program. The parameters npat, ptotxicrit, ptox and pabbruch mean the following: npat is the total number of patients to be enrolled in the trial; ptotxicrit is the unacceptable toxicity rate for the trial (its value should be chosen based on ethical considerations); ptox is the true toxicity rate for the compound being investigated. As the true toxicity rate (ptox) is not known, we have to use an assumed true toxicity rate (this rate should be defined with the help of preclinical data and other applicable medical experience or data). The parameter pabbruch defines the confidence level for the unacceptable toxicity rate (ptotxicrit). A commonly accepted value for pabbruch is 95%; in general it is chosen by a biostatistician.

Figure 1 shows a comparison between the results of the Bayesian and the frequentist approach. Five parameters for ToxCrit are fixed (npat = 20, ptotxicrit = 0.2, pabbruch = 95%, nsims = 1,000,000, seed = 123,456), while the true toxicity rate (pox) is outlined on the x-axis and the resulting risk of premature

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Key words
continuous monitoring – toxicity – clinical trials

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stopping on the y-axis. As ptoxcrit, the unacceptable toxicity rate is fixed at 0.2, it is interesting to see how the risk of premature stopping increases or decreases with variations in the true toxicity rate (ptoxt). It is obvious that the Bayesian approach is in general the more conservative and yields a higher risk of premature stopping than the frequentist approach. This can be explained by the fact that the Bayesian approach takes the a posteriori information of the true toxicity into account, while the frequentist approach does not.

The risk of premature stopping shown in Figure 1 is an example with a given parameter constellation and does not represent the risk of premature stopping for any trial. However the Bayesian approach will always have more conservative results than the frequentistic one and therefore should be used for reasons of safety.

The number of simulation runs (nsim) has an influence on the precision of the calculation. Following the law of large numbers, the value for nsim should be chosen as high as possible. That does have its limitations in calculation time, which increases polynomially with the number of simulation runs. For a very precise simulation result we recommend using nsim = 1,000,000. But already 100,000 runs are sufficient if a maximal error of less than half a percent is acceptable. The parameter seed is a randomly chosen number that makes the simulation reproducible and therefore can be freely chosen.

Conclusion

As a result of various implementations of the applied pseudorandom number generator [3], the three implementations of ToxCrit supply different results. Despite this discrepancy the results converge with a simulation number chosen adequately high (<1,000,000), which attests to its correctness.

ToxCrit has been validated according to the standardized operating procedures of the Patient and Trials Center at the NCT Heidelberg. The web-based implementation is already in use for the planning of early clinical phase trials.

For clinical investigations toxicity-related interruption criteria should be defined in the trial protocol [4]. For the ethical assessment of a clinical trial a precise specification of when to stop in case of unacceptable toxicity is an important consideration. In this regard this program has proved its worth. The web application with its user-friendly interface and its short calculation times has been shown to be applicable for routine usage.

References

Reduction in human melanoma cell adhesion receptor activity by lysophosphatidylcholine (LPC) treatment – functional characterization and signal pathway analyses*

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Introduction

Tumor cell metastasis is the most fatal complication of cancer. Cell adhesion receptors, such as P- and L-selectin, and the integrin VLA-4 are closely involved in the course of hematogenous metastasis, mediating manifold interactions of tumor cells with platelets and leukocytes. Recent data on the antimetastatic effects of empty liposomes of saturated phosphatidylcholine have been reported [2]. It might be assumed that lysophosphatidylcholine (LPC) is the active agent for this effect since, (i) we could detect a rapid uptake of LPC by different tumor cells, and (ii) this is in line with clinical findings on reduced levels of LPC in tumor patients suffering from cachexia. However, the mechanisms remain to be elucidated.

Recent experiments confirmed that incubation with 450 µM, but not 300 µM LPC of murine B16.F10 melanoma cells significantly reduced the cell- and matrix-binding activity of the integrin VLA-4, and this was clearly reflected in the reduced rate of metastasis in mice [5]. It was of interest that the scanning electron microscopy showed the presence of marked morphological changes, i.e. increased numbers of protrusions on the surface of the cells exposed to LPC. Similar findings were obtained using the human melanoma cells line MV3. It remains open whether this is solely a membrane effect of LPC or induced by a complex interference with intracellular signaling.

The latter case would open a novel therapeutic approach to interfere with tumor cell metastasis using specific phospholipids. To obtain an insight into the mechanisms behind these effects, the present study focused on the signaling pathway and the influence of LPC using gene expression analyses.

Materials and methods

MV3 cell cultivation and LPC exposition experiments

MV3 melanoma cells is a stable cell line originating from a 76-years-old melanoma patient. MV3 cells are characterized by a high metastatic potential and can be split and cultivated in an unlimited ratio for in vitro experiments. Each expression result is based on one individual well with cultivated MV3 cells.

MV3 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2 and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS). Cell suspensions were seeded in 6-
well plates ($5 \times 10^5$). After 24 h cells were used for LPC exposition experiments. LPC was solved in RPMI with 40 mg/ml BSA before incubation with MV3 cells.

In the experimental setup the six conditions arise from the three treatments (none, 300 µM and 450 µM LPC) and 2 incubation times (3 and 6 days).

**RNA isolation and quality control (QC)**

MV3 cells in 6-well plates were directly lysed with RLT buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol (Sigma-Aldrich, Deisenhofen, Germany). Total RNA of each lysed individual MV3 cell culture was isolated (RNAeasy mini kit, Qiagen, Hilden, Germany) including a DNAse digestion step, according to the manufacturer’s instructions.

Quality of total RNA was controlled via gel electrophoresis using a BioAnalyzer 2100 (Agilent Technologies, Waldbronn, Germany) with RNA 6000 nano lab chips following the instructions of the manufacturer’s protocol. All RNA samples showed intact 28S and 18S ribosomal RNA signals. Only RNA with measured RNA integrity numbers (RIN) of > 9.7 or better were used for Chip-based gene expression (GEX) analyses on the illumina platform.

**Gene expression analyses**

500 ng of total RNA were used for reverse transcription into biotin-labelled cRNA (Illumina® TotalPrep™-96 RNA Amplification Kit, Ambion/Applied Biosystems, Darmstadt, Germany) for processing on illumina (San Diego, CA, USA) human HT-12 microarrays to guarantee systematic global gene expres-
sion (GEX) analyses of untreated and LPC-treated MV3 cells. Human HT-12 microarrays interrogate for more than 99.99% of all known human genes (approx. 25,000 annotated RefSeq and UniGene genes) containing more than 48,000 probes. All expression profiles were extracted and average normalized using GenomeStudio software (Illumina, San Diego, CA, USA).

Results

To obtain further insights into the antimetastatic effects of LPC based on a reduction in adhesion receptor activity, the gene expression profiles of non-treated and LPC treated MV3 cells were analyzed. With respect to the in vitro findings showing that a long LPC cell incubation time and high concentrations accelerated the reduction in adhesion, two LPC concentrations in two incubation time frames (3 and 6 days) were compared. We focused on the α- and β-subunits of integrins to reflect the reduced cell binding by VLA-4 (very late activation antigen-4) [1] and matrix (fibronectin) interaction via VLA-2 and two of the best studied Rho-GTPases, RhoA and RhoB. Heatmap analyses identified a clear trend for reduced gene expression of the GTPases RhoA and RhoB (Figure 1A) by treatment with both, 300 µM and 450 µM LPC. Furthermore, lower fluorescence signal intensity for the integrin subunits α4, α2 and β1 (ITGA4, ITGA2 and ITGB1) were also detected (Figure 1B), indicating a decreased transcription of these integrins caused by LPC in a concentration dependent manner.

Furthermore, the gene expression of RhoA and RhoB is higher for untreated MV3 cells after 6 days compared to 3 days of cultivation and is more efficiently decreased by LPC than after 3 days.

In contrast, the expression of integrin subunits α4, α2 and β1 in both untreated and LPC-treated samples is lower after 6 days compared to 3 days of cultivation. Nevertheless, for both time frames a decreased expression could be observed with both LPC concentrations.

Conclusion

LPC possesses antimetastatic effects which have been shown to be mediated by reduced integrin activity. Integrin signaling is related to the activity of several GTPases by complex, and not yet fully elucidated mechanisms [6].

Our data provide evidence that LPC induce a reduced transcription of the GTPases RhoA and RhoB and the integrins VLA-4 and VLA-2. It can be hypothesized that altered membrane association, dissociation and a modified time frame of membrane bound Rho and/or other prenylated GTPases are responsible for these effects. In the next step we will focus on whether LPC induce Rho-GTPases indirectly by membrane effects or by LPC receptors.

Overall, these data suggest that phospholipids such as saturated LPC are novel compounds for mediating antimetastatic effects via complex signaling pathways. The findings are consistent with the breaking of new ground for therapeutic antimetastatic approaches.

References

Emerging role of thymidylate synthase for the pharmacogenomic selection of patients with thoracic cancer*

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Key words

*This extended abstract summarizes a poster presentation given by P. Ceppi during the Annual Meeting 2009 of the Central European Society for Anticancer Drug Research (CESAR) in Heidelberg, Germany, October 29 – 31, 2009

Introduction

The use of antifolate drugs, and particularly of pemetrexed, is emerging as a relevant therapeutic strategy for the treatment of some specific thoracic cancers.

Pemetrexed is a novel multitargeted antifolate which has been shown in vitro to inhibit at least three different enzymes in the folate pathway: thymidylate synthase (TS), dihydrofolate reductase (DHFR) and glycaminamide ribonucleotide formyl transferase (GARFT) of which TS is its main target [1]. These enzymes are involved in the synthesis of nucleotides and interference with their function will ultimately hinder the synthesis of RNA and DNA. Pre-clinical studies have demonstrated the cytotoxic activity of this agent in a broad range of tumor types including non-small cell lung cancer (NSCLC). In vitro studies indicated that tumor cell lines expressing high levels of TS or DHFR have reduced sensitivity to pemetrexed, suggesting that increased expression levels might correlate with reduced clinical efficacy [2].

In NSCLC, which accounts for more than 80% of all lung cancer, two recent independent Phase III trials showed that pemetrexed alone or in combination with cisplatin demonstrated a statistically superior activity in patients with non-squamous histotypes [3, 4]. Although histology has not consistently been associated with clinical outcomes in almost every trial testing the role of platinum-based chemotherapy in advanced NSCLC, it has now emerged as a potential predictive factor due to the evidence of the differential expression of TS between the different tumor histotypes, which seems to play an important role [3]. Preliminary molecular evidence, in fact, has previously shown that TS expression is significantly higher in squamous cell carcinoma compared with adenocarcinoma by means of both Real-Time PCR gene quantification and of immunohistochemistry for the detection of the protein levels [5].

In undifferentiated large cell carcinoma, a subsequent analysis consistently reported significantly higher TS levels in desmocollin 3 (DSC-3)-positive as compared to DSC-3-negative tumors (indicating a squamous and adenocarcinoma origin of the tumors, respectively), reporting that lack of DSC-3 immunoreactivity in this histologic subtype is associated with lower TS expression [8]. In the same experimental setting, TS expression levels were tested in 22 cases having a cytological diagnosis of NSCLC with no clear histological definition, and in the corresponding histological samples obtained at the time of subsequent surgical resection. As a result, a significantly high degree of concordance in terms of TS protein expression in matched specimens was detected. This result raises the possibility of using the protein levels, evaluated by immunohistochemistry on cytological specimens, to select the patients with unresectable advanced disease to be treated with TS-inhibiting agents.

On the other hand, the small cell carcinoma (SCLC) group of patients, comprising approximately 13% of all lung cancers, has shown minimal response to pemetrexed in several studies including a large Phase III study [6]. Again, the explanation for these results may be found at the molecular level, in that SCLC has been shown to have very high TS expression when compared with either well-differentiated pulmonary neuroendocrine tumors or NSCLC [7]. These data once again support the hypothesis that elevated TS expression decreases the response to pemetrexed, and that the determination of TS levels can be used to exclude those patients who are most likely to be unaffected from the treatment.

A clear example of this is found in a very recent retrospective study from our group carried out on malignant pleural mesothelioma (MPM), a disease in which pemetrexed has been previously shown to be clinically active. We showed that TS protein and gene expression levels are significantly correlated with the clinical outcome in a consecutive series of 60 patients with advanced MPM treated with pemetrexed, with or without plat-
Conclusion

Taken together all these recent findings point to the use of TS as a possible predictive marker of treatment efficacy in patients with thoracic tumors who are to be treated with anti-metabolite agents, and indicate that both real-time PCR and immunohistochemistry may be used to assess TS expression levels.

In conclusion, while waiting for the results of currently ongoing prospective studies investigating the efficacy of TS determination in larger set of patients, TS can be added to the list of the candidate biomarkers useful for the pharmacogenomic stratification of the patients.

References


Evidence for the conversion of docetaxel into 7’-epidocetaxel in patients receiving Taxotere®-based conventional chemotherapy*

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Introduction

Epimerization at the C7-position of the baccatin moiety (axial or equatorial hydrox group) is a common in-vitro pathway for taxanes, including the natural precursor 10-deacetyl baccatin III and the antineoplastic drugs paclitaxel [1] and docetaxel [2]. In contrast to paclitaxel [3], to date the epimerization of docetaxel during chemotherapy has not been described in patients and only in-vitro and animal data are available [4]. The purpose of this investigation was to investigate the presence and (if possible) to quantitate 7’-epidocetaxel in plasma and urine samples of patients treated with Taxotere®.

Patients and methods

Twelve patients suffering from mammary carcinoma (n = 9), bronchial carcinoma (n = 1) and prostate cancer (n = 2) were included in this investigation (registration number E 943, top4 of the ethics committee Salzburg).

Patients received various conventional Taxotere®-based schedules as a mono- or combination chemotherapy. Mammary carcinoma: monotherapy (n = 7), docetaxel + epirubicin (n = 2); prostate cancer: docetaxel + bicalutamid (n = 2); bronchial carcinoma: monotherapy (n = 1).

Blood samples of 5 ml were drawn from each patient before infusion (0 min), at the end of the infusion (EOI) and 20 min after the infusion (EOI + 20 min). After centrifuging blood samples for 5 min, 1.0 ml aliquots of the supernatant plasma were frozen immediately at −80 °C. Urine was also collected for each subject from the start of the Taxotere® infusion until 6 hours later, pooled, mixed and 6 ml aliquots were frozen at −80 °C until analyzed. Docetaxel and 7’-epidocetaxel were separated from matrix compounds by solid phase extraction (Oasis HLB® cartridges) and quantified using a validated reversed phase HPLC method as described recently [5]. External standard calibration was performed using pure chemical standard substances supplied by the manufacturer. The limit of quantitation (LOQ) for docetaxel and 7’-epidocetaxel were identical (LOQ < 0.05 µg/ml) because of the very similar physicochemical properties of the two compounds.

Results

Docetaxel was not detectable in plasma samples collected before start of infusion and therefore accumulation of the drug from previous cycles of chemotherapy can be excluded.

Plasma concentrations of docetaxel at the end of the infusion (EOI) could be measured in each patient (range from 0.6 to 3.43 µg/ml, n = 12). Twenty minutes after EOI docetaxel plasma concentrations ranged from 0.10 µg/ml to 0.81 µg/ml (n = 11).

In 8 of 12 patients 7’-epidocetaxel could be quantified in plasma at EOI with concentrations ranging from 0.05 µg/ml to 0.54 µg/ml. 20 minutes later 7’-epidocetaxel concentrations were below LOQ in all plasma samples because of rapid distribution of docetaxel from blood into tissue.

Docetaxel concentrations in pooled urine ranged from 3.21 µg/ml to 66.37 µg/ml and could be measured in each sample (n = 12). In contrast, 7’-epidocetaxel concentrations were much lower ranging from 0.1 µg/ml to 0.5 µg/ml (n = 7, below LOQ in 5 patients). Figure 1 depicts the individual plasma and urine concentrations as a scattergram (lines represent mean values ± standard deviation).

The amount of 7’-epidocetaxel at EOI in plasma as a mean percentage compared to...
Plasma concentrations of docetaxel at EOI were similar in prostate, lung and mammary carcinoma patients. It was of interest that the amounts of 7'-epidocetaxel at EOI were clearly lower in the 2 prostate cancer patients.

To our knowledge this is the first time that detection and quantification of 7'-epidocetaxel in blood and urine of chemotherapy patients has been reported. Our results are in accordance with existing in-vitro and animal findings: 7'-epidocetaxel is a significant docetaxel metabolite in man with amounts of about 14% in blood and 2% in urine.

It has been shown [6] that all docetaxel metabolites have much weaker cytotoxic and myelotoxic properties compared with the parent compound. For 7'-epidocetaxel it remains open, whether this metabolite shows identical pharmacological activity as the parent compound or not. This question should be answered by a further clinical investigation currently in progress involving assessment of the pharmacokinetic data of 7'-epidocetaxel.

**References**


New doxorubicin N-acyl hydrazones with improved efficacy and cell line specificity show modes of action different from the parent drug

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Introduction

Doxorubicin (1) – a metabolite from Streptomyces – is one of the most used drugs for the treatment of hematological cancer, solid tumors, lymphomas and soft tissue sarcomas although its clinical use is limited because of cardiac toxicity and multi-drug resistance [5]. Many analogues of doxorubicin have been developed to circumvent these drawbacks while retaining the desired therapeutic effect [3, 7]. As part of a project to improve the efficacy of anti-cancer drugs in resistant cancer cells we have investigated such derivatives based on the attachment of doxorubicin to fatty acid or terpene carriers [2, 6]. Doxorubicin N-acyl hydrazones 2 derived from saturated, unsaturated and menthyl-terminated fatty acids were tested in vitro using the MTT assay against HL-60, 518A2, HT-29/Colc (62.5 nM colchicine), KB-V1/Vbl (340 nM vinblastine) and MCF-7/Topo (550 nM topotecan) cells. The most potent derivatives were also tested in HT-29, KB-V1 and MCF-7 cells desensitized to 1 by repeated treatment with clinically relevant doses (HT-29/Dox: 75 nM, KB-V1/Dox: 200 nM, MCF-7/Dox: 55 nM) as well as in HT-29/Colc, KB-V1/Vbl and MCF-7/Topo cells in the presence of selective inhibitors of the respective ABC-transporters (10 µM MK-571 added to the MRP-1 overexpressing HT-29/Colc cells, 24 µM verapamil hydrochloride added to the P-gp overexpressing KB-V1/Vbl cells and 1.2 µM fumitremorgin C added to the BCRP overexpressing MCF-7/Topo cells). The apoptosis caused by 1 and 2 was analyzed by DNA fragmentation (Gel electrophoretic analysis), the involvement of caspases as assessed using the Caspase-Glo Assay and changes in the mitochondrial membrane potential by JC-1 staining. The generation of ROS was assessed with the NBT assay (HL-60, 518A2) and via cyclic voltammetry in air-saturated N,N-dimethylformamide. The pharmacokinetic and tissue distribution in NMRI nu/nu mice was studied using the drug specific intrinsic fluorescence (2.8 mg/kg, Exc.: 490 nm, Em.: 594 nm).

Results

All tested compounds gave different cytotoxic activity profiles: hydrazone 2a was more active than its C₁₈-fatty acyl analogue with three C = C bonds in all five cell lines. Compared with 1, it was three times more efficacious in the multi-drug resistant KB-V1/Vbl cells and it was the most unsuitable substrate for the P-gp drug transporters in these cells. The menthol derivative 2d was twice as active as 1 in the KB-V1/Vbl and 518A2 cells and also more efficacious in HT-29, KB-V1...
and MCF-7 cells that had been desensitized with clinical relevant doses of 1. Doxorubicin and its derivatives 2a, b and 2d induced apoptosis via caspase-8 activation in the leukemia cells, but via caspase-9 activation in the melanoma cells. In contrast, hydrazone 2c acted via caspase-8 activation in both cell lines. Those compounds with greater cytotoxic activity also caused the greatest increase in ROS levels, exceeding even that of 1. This is in line with their cyclic voltammograms: 2a, 2b, 2c and 2d led to ROS formation with better reversibility and regeneration of the 5,12-quinone core than 1. Finally, doxorubicin and its derivatives 2a, b and 2d induced apoptosis via caspase-8 activation in the leukemia cells, but via caspase-9 activation in the melanoma cells. In contrast, hydrazone 2c acted via caspase-8 activation in both cell lines. Those compounds with greater cytotoxic activity also caused the greatest increase in ROS levels, exceeding even that of 1. This is in line with their cyclic voltammograms: 2a, b, c and d led to ROS formation with better reversibility and regeneration of the 5,12-quinone core than 1. Finally, doxorubicin and its derivatives 2a, b and c was determined relative to an untreated control: hydrazone 2a was the only compound found in the lungs as early as 0.5 min (Figure 2).

**Conclusion**

The saturated heptadecanoyl derivative 2a was the only fatty acid hydrazone to show distinct activity against various cancer cells. In the multi-drug resistant cell line KB-V1/Vbl, it was three times more efficacious than doxorubicin, partly because it is less well pumped out by the ABC-transporter, P-gp. The long-spacered menthol derivative 2d performed best among the terpenyl hydrazones, even in cells desensitized with 1. The mode of cytotoxic action of 1 and 2 was mainly apoptotic, while the precise mechanism and the involvement of caspases varied for cell lines and test compounds. Moreover, we found a relationship between the efficacy of the compounds and their ability to generate ROS, especially in the 518A2 cells. The degree of ROS generation in turn seems to be dependent on the reversibility and regeneration of the 5,12-quinone core, which is more the case for the hydrazones 2. Major differences between the test compounds were also noted in regard to their pharmacokinetics and tissue distributions. Only 2a could be detected in the lung.

In summary, we showed that the efficacy and selectivity of N-acyl hydrazones with suitably chosen terminal groups and spacers can surpass that of the parent drug.

**References**

Plasma disposition of capecitabine (CCB) and its metabolites 5'-deoxy-5-fluorocytidine (5'-DFCR) and 5'-deoxy-5-fluorouracil (5'-DFUR) with two different capecitabine/oxaliplatin dosage regimens*

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Introduction

Capecitabine is a thymidine phosphorylase (TP) activated oral fluoropyrimidine, designed to generate 5-fluorouracil via a three-step enzymatic cascade preferentially within tumors. This tumor selectivity is achieved by the significantly higher activity of TP in tumors compared to healthy tissue [1].

Capecitabine is given either as a single agent or as part of combination regimens with irinotecan or oxaliplatin (OxPt) using their different mechanisms of action to increase therapeutic efficacy without toxic overlap [2].

The combination of capecitabine and OxPt (Xelox) has demonstrated promising synergistic activity in advanced colorectal cancer therapy [3, 4].

This pharmacokinetic (PK) study was designed to evaluate whether capecitabine is metabolized to the same extent when administered in two different capecitabine doses together with OxPt.

Material and methods

14 patients suffering from advanced colorectal cancer were included in this pharmacokinetic trial.

They received two different capecitabine/OxPt schedules (Xelox I+II).

In the Xelox I schedule, 130 mg/m² i.v. OxPt were infused and this was followed by 1250 mg/m² capecitabine p.o. twice daily for 2 weeks. After a pause of 1 week, the Xelox II schedule was administered by giving an 85 mg/m² OxPt i.v. infusion and 1,750 mg/m² capecitabine p.o. twice daily for 1 week [3].

Separation of capecitabine, 5'-DFCR and 5'-DFUR, from matrix compounds was performed using a highly selective solid-phase extraction procedure. Quantification of compounds in plasma samples was achieved by two sensitive HPLC/UV methods.

A non-compartment model has been used for pharmacokinetic calculations (model 202; WinNonlin® Professional V 5.1). Statistical analysis was performed using the 2-sided Student’s t-test for paired data. Descriptive statistics were accomplished by using GraphPad Prism®.

Results

The table presents the mean (±SD) of the key PK parameters for capecitabine and its metabolites showing no statistically significant differences.

The figure depicts the mean plasma concentration time curves of the investigated compounds. In order to provide a clearer overview, the individual times when blood samples were taken (0, 30, 60, 90 and 120 minutes) are shown on the x-axis in the consecutive order of the days on which samples were taken. The time between the sampling days is excluded. Day 1 = first sampling day, Monday (first week), 0 – 120 minutes. Day 2 = second sampling day, Friday (first week), is shown as 200 – 320 minutes. Day 3 = third sampling day, Monday (second week), 400 – 520 minutes. Day 4 = fourth and final sampling day, Friday (second week), 600 – 720 minutes.

The mean plasma concentration time curves of capecitabine, 5'-DFCR and 5'-DFUR in the Xelox I regimen are in accordance with previous findings concerning the PK of capecitabine. As can be seen from the figure, the mean plasma concentration of
capecitabine after a high dose of capecitabine (Xelox II) is clearly increased.

These higher plasma levels in the Xelox II schedule lead to correspondingly higher AUCs. As a prodrug, it is essential to know the amount of metabolic conversion and activation of capecitabine doses. The percentage distribution of capecitabine : 5'-DFCR : 5'-DFUR AUCs was nearly identical in both Xelox regimens (26% : 26% : 48% versus 27% : 29% : 44%).

By comparing the key PK parameters for capecitabine and its metabolites with the data from a previous capecitabine-monotherapy study, we were able to address the question regarding a possible influence of oxaliplatin on the pharmacokinetics and metabolism of capecitabine. No statistically significant differences have been found.

These results confirm the theoretical assumption that oxaliplatin has no influence on the three step metabolic pathway of capecitabine because of the non-enzymatic conversion.

### Conclusion

The outcome of this PK trial demonstrates that high doses of capecitabine are metabolized nearly dose-dependently (Xelox II: increase of Σ AUCinf ~ 30%) compared to the standard dose.

For all compounds, there was no evidence of saturation of the metabolizing processes or any significant delay in elimination rate. The PK indicate that both schedules are safe and efficient.

### References


Introduction

Neuroblastoma is the most common extracranial solid tumor in early childhood arising from precursor cells of the neural crest. During the last two decades advances have been made in predicting outcome in the patient and improving therapeutic options [5]. However, high-risk neuroblastoma patients still have a 5-year-survival rate less than 35% [2, 3, 7]. This is due to drug resistance of primary tumors or metastases after relapse. One important criterion in the classification of high-risk neuroblastoma is amplified MYCN oncogene that is still the most crucial prognostic factor in neuroblastoma. Acting as an oncoprotein, the transcription factor MYCN supports tumor proliferation, angiogenesis and metastasis leading to unrestricted tumor progression and poor outcome. In contrast, MYCN activates also tumor suppressing pathways, such as apoptosis. Recently, it was shown that MYCN deregulates important members of the cell cycle machinery especially in advanced neuroblastoma [6]. Furthermore, MYCN-amplified neuroblastoma cells fail to respond with a G1 cell cycle arrest following DNA damage [1]. This is at least partly due to transcriptional suppression of CDKN1A (p21) by deregulated MYCN. However, the precise mechanisms by which MYCN mediates drug resistance in neuroblastoma are poorly understood.

To further investigate the DNA damage response in neuroblastoma and the role of MYCN in this regard, we assessed cellular phenotypes of a broad range of neuroblastoma cell lines after treatment with doxorubicin using fluorescence activated cell sorting (FACS). Moreover, we used the small molecule inhibitor nutlin-3a to test whether displacement of the p53-MDM2 interaction sensitizes neuroblastoma cells to DNA damage.

Material and methods

Cell cycle arrest and apoptosis of 13 neuroblastoma cell lines (MYCN single-copy: SH-SY5Y, SK-N-AS, SH-EP, NB-7; MYCN amplified: SK-N-BE(2)C, LA-N-5, Kelly, IMR32, IMR5/75; MYCN/CDK4/MDM2 co-amplified: NGP, LS, TR14; c-MYC amplified: SJ-NB-12) and one c-MYC amplified Ewing sarcoma cell line (SK-N-MC) were analyzed after treatment with 0.1 µg/ml doxorubicin and 1 µM nutlin-3a (1004372, Biozol) using fluorescence activated cell sorting (FACS). After treatment with the named compounds viability assays were performed and anchorage independent growth was tested in a soft agar assay.

All neuroblastoma cell lines were analyzed using DNA fingerprinting by the “Deutsche Sammlung von Mikroorganismen und Zellkulturen” (DSMZ) in September 2008.

Results

Analyses of cell cycle and apoptosis of different neuroblastoma cell lines following DNA damage revealed an impaired G1 cell cycle arrest in MYCN amplified cells. After treatment with doxorubicin, MYCN amplified cells tended to arrest in the G2/M phase and this was correlated in most cases with a decreased specific apoptosis (SH-SY5Y, MYCN single-copy: untreated control G1 55.4 ± 0.35, S 34.09 ± 0.73, G2/M 10.51 ± 0.41, subG1 16.12 ± 0.32; doxorubicin G1 5.24 ± 2.18, S 0.42 ± 0.73, G2/M 94.33 ± 1.55, subG1 21.79 ± 6.26 (cells in %)).
MYCN plays a role in DNA damage response we treated MYCN single-copy and MYCN amplified neuroblastoma cell lines with the small molecule inhibitor nutlin-3a, which leads to p53-MDM2 disruption. FACS analyses revealed that treatment with nutlin-3a only slightly increased G1 cell cycle arrest following DNA damage despite p21 induction (LS: untreated control 51.12 ± 0.37, nutlin-3a 64.72 ± 1.07, doxorubicin 5.24 ± 0.62; p14-induced doxorubicin 39.09 ± 2.18 vs. p14-uninduced doxorubicin 39.43 ± 1.97 (% cells in G1)), but increased apoptosis following DNA damage in MYCN amplified neuroblastoma cells, similar to nutlin-3a (IMR5/75-p14-clone35: p14-induced untreated control 14.87 ± 0.28 vs. p14-uninduced untreated control 8.19 ± 2.14; p14-induced + doxorubicin 39.47 ± 0.06 vs. p14-uninduced + doxorubicin 32.56 ± 0.48 (% cells in subG1)).

Conclusion

The transcription factor MYCN deregulates core DNA damage response pathways. Upregulation of MDM2 and CDK2 (via suppression of p21CDKN1A) leads to p53 function loss and thereby to inhibition of cell cycle arrest and apoptosis upon DNA damage [4], despite transcriptional activation of TP53 by MYCN [6].

Our results suggest that future studies should concentrate on a combined approach to restore p53 functions in MYCN amplified neuroblastoma cells.

References


Radiation induced stress proteins*

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Introduction

The major heat-inducible member of the stress proteins with a molecular weight of about 70 kDa (Heat shock protein 70, Hsp70) is a ubiquitous protein contributing to a number of cellular processes including the protection of cells from apoptosis induced by environmental stress. Recently, we and others could show that a variety of tumor cells, in contrast to normal tissues, have the unique feature of expressing Hsp70 on their plasma membrane [1]. Monitoring the clinical outcome (up to 5 years) demonstrated a negative correlation between the Hsp70 membrane status and the overall survival in patients with lower rectal and squamous cell carcinomas of the lung [2].

In a recent study we were able to track the expression of Hsp70 located in the membrane of tumor cells before and after stress [3]. In non-stressed tumor cells Hsp70 is associated with Bag-4 [4] and the lipid-raft compound globotriaosylceramide (Gb3) in the plasma membrane [5].

Hypoxia is known to limit the therapeutic efficacy of radiation therapy. Therefore, we addressed the question whether hypoxia might affect the Hsp70 membrane expression. In hypoxic tumor cells the Hsp70 membrane expression is elevated and Hsp70 is co-located with phosphatidylserine (PS) in the plasma membrane.

Material and methods

The tumor cell subline CX-, derived from the parental human colon carcinoma cell line CX2 (Tumorzellbank, DKFZ, Heidelberg, Germany), was generated by fluorescence-activated cell sorting using the FITC-conjugated Hsp70-specific mAb cmHsp70.1 (mult-immune, Munich, Germany). CX- tumor cells have a low Hsp70 membrane expression and are PS membrane negative. Hypoxic conditions were achieved by incubating cells in a N2 rich atmosphere in a vacuum chamber (final O2 concentration below 0.66%, 5 mmHg) for 48 h. Radiation experiments took place with an irradiation dose of 4 Gy.

Following normoxia or hypoxia treatment and/or incubation with Hsp70 protein or BSA, cells were incubated with Annexin V-FITC (Roche Diagnostics, Mannheim, Germany). For double-staining experiments, Annexin V-PE pre-stained cells were incubated with the membrane-Hsp70-specific mouse monoclonal IgG1 antibody cmHsp70.1-FITC (mult-immune GmbH). Viable cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Unilamellar vesicles were extracted with an extruder delivered by Avanti Polar Lipids (Alabaster, AL, U.S.A.). To test the interaction of Hsp70 with unilamellar vesicles, Hsp70 and liposomes were incubated for 30 min at room temperature (RT). Measurement took place by applying the pellet and supernatant fractions to Western blots followed by densitometrical determination of band volumes.

Tests for apoptosis were performed with a Caspase-3 apoptosis assay (BD Biosciences; 550480) or by checking the nuclei for fragmentation by DAPI staining.

Bovine serum albumin (BSA) or His-tagged Hsp70 protein were used either unlabeled or labeled with fluorescein isothiocyanate (FITC). Proliferation was measured using the colorimetric alamarBlue assay (Biosource, Camarillo, CA, USA). For clonogenic cell survival assays, CX-tumor cell colonies were fixed in methanol on d6 after treatment, stained with crystal violet and counted automatically with a Bioreader (Bio-Sys GmbH, Karben, Germany).

Results

Using confocal microscopy and FACS analysis we could show that Hsp70 and phosphatidylserine (PS) are co-located on the surface of hypoxic tumor cells. These data were confirmed by adding FITC-labeled Hsp70 protein to hypoxic tumor cells showing an interaction of extracellular Hsp70 with PS. Moreover, studies with artificial unilamellar liposomes composed of phosphatidylcholine (PC) and phosphatidylserine (PS) at physiological molar ratios of 8/2 confirmed this in-
teraction. Further experiments have shown that increasing the PS proportion increases the binding of Hsp70 to PS. On the other hand, vesicles composed of PC and phosphatidylglycerol (PG) show only a weak binding of Hsp70. These data exclude a non-specific charge-related interaction of Hsp70 with lipids. The interaction of Hsp70 with surface PS reduces the clonogenic cell survival in normoxic (EC50 of Hsp70 = 85 μg/ml) and hypoxic (EC50 of Hsp70 = 55 μg/ml) tumor cells. The radiation-induced tumor cell killing was significantly enhanced by the addition of Hsp70 protein (50 μg/ml). Since apoptosis was not significantly enhanced in normoxic and hypoxic tumor cells by the addition of extracellular Hsp70, we hypothesize that the Hsp70 protein-induced reduction in clonogenic cell survival might be mediated by necrosis rather than apoptosis.

Conclusion

Taken together, our findings might have future clinical relevance in that hypoxic tumors could be rendered more sensitive to radiotherapy by the addition of extracellular Hsp70 protein prior to radiotherapy (Figure 1). One approach could be to include Hsp90 inhibitors as part of the radiotherapy protocol. Apart from the inhibition of the interaction of the molecular chaperone Hsp90 with their oncogenic client proteins, Hsp90 inhibitors are known to strongly up-regulate the synthesis of Hsp70 in tumor cells. Given that these newly synthesized Hsp70 proteins are secreted by tumor cells, the extracellular localized Hsp70 molecules could improve the outcome of radiotherapy. Furthermore, we could show that tumor cells with a high Hsp70 membrane expression are susceptible to the kill mediated by activated natural killer (NK) cells [6]. Consequently, the inhibition of extracellular Hsp70 would block this immunological effect.

In summary, we can state that, depending on the localization of Hsp70, this molecule exerts dual effects. High cytosolic Hsp70 lev-

![Figure 1. Hypoxic tumors marked by increased PS-expression on the outer leaflet (blue) and increased membrane expression of cellular Hsp70 (red) might be rendered more sensitive to radiation therapy by prior treatment with Hsp70 (green).](image)

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References

Experiences with bortezomib in multiple myeloma – from Phase II studies to daily practice

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Introduction

Bortezomib (VELCADE) is a proteasome inhibitor. It not only targets the myeloma cell, but also acts in the bone marrow micro-environment, inhibiting the binding of myeloma cells to bone marrow stromal cells [1]. Two open-label Phase II trials (SUMMIT (n = 202) and CREST (n = 54)) established the efficacy of bortezomib in a dosage between 1.0 and 1.3 mg/m² administered by intravenous bolus on Days 1, 4, 8 and 11 of a 21-day cycle for a maximum of 8 cycles in heavily pre-treated patients suffering from relapsed/refractory multiple myeloma [3, 4]. The randomized open-label Phase III APEX trial which recruited 669 patients in North America and Europe with relapsed multiple myeloma after up to three previous therapies demonstrated the superiority of a bortezomib 1.3 mg/m² regimen over a high-dose dexamethasone regimen [5]. In parallel to this trial, heavily pre-treated patients were included in a “Bortezomib access program” in Europe that was initiated by Millenium Pharmaceuticals. As a next step bortezomib was evaluated in newly diagnosed myeloma patients. A regimen of bortezomib plus melphalan and prednisone was significantly more effective than a regimen of melphalan plus prednisone alone with respect to the time to progression and response rates, according to data from the Phase III VISTA trial in 682 elderly patients with newly diagnosed multiple myeloma who were not eligible for stem cell transplantation [7].

Results

As previously shown a fast reduction of tumor burden can result from a single agent bortezomib treatment, and similar therapeutic effects could be seen when the agent was administered in combination with the old gold standard melphalan/prednisone where the latter regimen was superior with regard to overall response, progression free survival and overall survival time. We also learned that the addition of dexamethasone to bortezomib increases the response rates. In a subgroup assessment we found that myeloma patients with kidney involvement benefitted in partic-
ular from treatment with bortezomib. In summary the treatment with bortezomib versus dexamethasone improved the overall response rate, the progression free time and the overall survival in myeloma patients suffering from up to 3 relapses.

At the 50th meeting of the American Society of Hematology in 2008 first results in terms of responses from the joint trial of the Haemato Oncology Foundation for Adults in the Netherlands study group (HOVON) and the German-Speaking Myeloma Multicenter Study Group (GMMG) were presented. In concordance with three other large prospective transplant trials we found higher response rates with bortezomib compared to vincristine during induction therapy (VAD versus PAD) and after stem cell transplantation. Furthermore, first results of a French and an Italian study showed a prolongation of progression free survival in this context.

The incidence of herpes zoster manifestations during bortezomib treatment was increased. Therefore we implemented a regular antiviral prophylaxis in our HOVON/GMMG study. As a result of this prophylaxis during induction treatment, the incidence of herpes zoster manifestations could be reduced to 3%.

Besides these adverse events, most of the subjects treated with bortezomib tolerated this therapy well. Side effects with a prevalence of > 10% in the PAD group compared to subjects treated with VAD were gastrointestinal symptoms (38% versus 30%), fatigue (29% versus 26%), peripheral neuropathy (CTC Grade 3 and 4) (16% versus 6%), rash (13% versus 11%) and pneumonia (11% versus 10%).

In summary, at least 80% of patients treated with PAD completed 3 cycles of induction chemotherapy without dose reduction in the HOVON 65/GMMG-HD4 trial. Additionally, stem cell collection was successful and sufficient in the PAD group, and 87% of patients achieved ABSCT.

Conclusion

The proteasome inhibitor bortezomib has shown impressive therapeutic potency in relapsed and newly diagnosed myeloma patients. It has recently been shown that specific bortezomib dosage modifications improve the management of sensory neuropathy and neuralgias with consecutive amelioration of the patient’s well-being up to a resolution of neurological symptoms [6].

A future therapeutic strategy might be a development towards dose reductions and towards a decrease in application frequency of bortezomib. Since not all myeloma patients achieve sufficient response after a treatment with bortezomib, a future aim should be to find characteristics of responding patients. First approaches have been realized in this context by the analysis of biomolecular features [2].

According to the many impressive findings regarding the therapeutic impact of bortezomib as part of the induction chemotherapy in myeloma patients the “Medizinische Dienst der Krankenkassen” in Germany approved the use of bortezomib prior to autologous stem cell transplantation.

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