The 8th Annual Meeting of the Central European Society for Anticancer Drug Research (CESAR), was held in St. Gallen from the 1st to 3rd of July 2010. It attracted participants from Germany, Switzerland and Austria allowing face-to-face scientific exchange between clinical and pre-clinical scientists in a personal and stimulating atmosphere.

For more than 1000 years, up to the French Revolution, St. Gallen possessed a famous Benedictine monastery within its city walls. The monastery now houses an important library well known for its collection of early medieval manuscripts on medical plants and medical practice.

Today, St. Gallen’s Kantonsspital (= state hospital) harbours a highly active Clinical Oncology Department with Switzerland’s largest clinical trial unit in oncology, performing Phase I to III clinical trials. St. Gallen is also well-known for various local and international medical congresses, in particular the biannual International Breast Cancer Congress which usually concludes with an update of the St. Gallen Consensus regarding treatment of early breast cancer.

The 8th Annual Meeting of CESAR in St. Gallen was co-hosted locally by the Department of Oncology of the Kantonsspital St. Gallen and the Breast- and Tumorcenter ZeTuP, a private multidisciplinary oncology clinic. The Opening addresses were given by Prof. Dr. Ulrich Jaehde, President of the CESAR and representatives of the local organizers, Prof. Dr. Thomas Cerny, Department of Oncology of the Kantonsspital, and Prof. Dr. Hans-Jörg Senn and Dr. Rudolf Morant from the Breast- and Tumor Center, ZeTuP. Special emphasis was put on the chances and challenges of interdisciplinary oncological research performed by CESAR scientists and its working groups.

CESAR brings together researchers from preclinical disciplines, including molecular biology, chemistry, pharmacy and pharmacology as well as clinical investigators from a variety of specialized institutions, mostly located in Germany, Austria and Switzerland. The interdisciplinary nature of these groups provides a platform for innovative research and the creation and introduction of novel therapeutic concepts in the clinic.

The annual meeting focused in particular on novel therapeutic concepts in hemato-oncology. A distinctive example of such interdisciplinary research was initiated by Dr. Claudia Friesen from the University of Ulm and presented during the symposium on the first afternoon. Building on in vitro findings showing unexpected antitumor effects of the opioid methadone, current efforts of the group have been focused on exploring the underlying mechanisms and transferring this knowledge for use in clinical treatment concepts. Other symposia dedicated to new developments in the field of radio-immunotherapy, were presided by Prof. Dr. Jörg Kotzerke, President of the German Society of Nuclear Medicine and Personalized Medicine.
with or without PK/PD and chaired by Prof. Dr. Robert Mader, Vienna and Prof. Dr. Ulrich Jaehde, Bonn.

The interactive poster meeting was well attended and provided a means for young researchers to present their work to a wide audience. A selection of submitted abstracts was presented in an oral session. This year, and for the first time, CESAR granted a prize for the best submitted abstracts. Additionally, a mini-grant for the initiation of collaborative interdisciplinary research projects was granted. The introduction of mini-grants is part of an effort to stimulate collaborative research in oncology among CESAR members and scientific partners.

The extended abstracts published in this issue allow an insight into the wide spectrum of preclinical and clinical expertise put into the research efforts being done within CESAR and may also stimulate the readers to attend one of the next CESAR meetings. Preparations are already underway for the next annual meeting in 2011 to be held from 16 to 18 June in Greifswald, Germany.

_Rudolf Morant, St. Gallen, Switzerland_  
(Meeting President)  
Daniel Sehrt, Göttingen, Germany  
Ulrich Jaehde, Bonn, Germany
Cytotoxic effects of opioids on cancer cell lines**

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Introduction

Opioids are the most powerful analgesics and therefore the mainstay for treatment of cancer pain. The analgesic effect results from the binding of the opioid to specific receptors expressed by cells of the central nervous system. This leads to an inhibition of pain stimulation and transmission in the central nervous system [1].

Apart from the benchmark opioid, morphine, a number of synthetic and semi-synthetic agents belong to the clinically important opioids. The synthetic opioid D,L-methadone is an effective alternative to morphine in pain and substitution therapy. Additionally, it has been observed that D,L-methadone induces apoptosis in human lung cancer and leukemia cell lines [2, 3]. We have previously shown that the opioid D,L-methadone induces apoptosis in the acute myeloid leukemia cell line HL-60 and in the T-cell leukemia cell line CEM, as well as in chemo- and radio-resistant leukemia cells but not in healthy peripheral blood lymphocytes in vitro [3]. Since leukemia cells have been shown to express large numbers of opioid receptors on the surface [3], the question arises, to what extent do other opioids have the potential to act as anti-cancer drugs and how do different types of cancer respond to opioid treatment?

In this study, we first monitored the cytotoxic effect of various clinically relevant opioids on leukemia and glioblastoma cell lines in vitro over a wide range of concentrations alone and in combination with cytostatic drugs. Beside morphine and D,L-methadone, we also tested the semi-synthetic opioid buprenorphine, and the synthetic opioid fentanyl, which are also used in the treatment of cancer pain.

Material and methods

Drugs and reagents

D,L-Methadone hydrochloride (D,L-methadone, Sigma, Taufkirchen, Germany; lethal dose: 2 µg/ml D,L-methadone equivalent to 4 µg/ml D,L-methadone), buprenorphine hydrochloride (buprenorphine, Caesar & Lorenz GmbH, Hilden, Germany, lethal dose: 4 to 13 µg/ml), morphine hydrochloride trihydrate (morphine, Fagron GmbH & Co KG, Barsbüttel, Germany, lethal dose: 0.05 – 4 µg/ml), fentanyl citrate (fentanyl, Fagron GmbH & Co KG, lethal dose: 20 ng/ml) and doxorubicin (Sigma, plasma concentration: 0.1 µg/ml) were freshly dissolved in sterile distilled water prior to each experiment to ensure constant quality of the preparations.

Flow cytometric assay for the detection of cell surface opioid receptors

Cancer cells (5 × 10^5) were washed in PBS supplemented with 1% FCS, centrifuged...
and resuspended in 100 µl PBS/1% FCS containing naloxone fluorescein (0.1 mM, Invitrogen). After 30 min of incubation (at RT), the cells were washed twice with PBS/1% FCS, centrifuged and resuspended in ice-cold PBS/1% FCS [4]. Flow cytometry analysis was performed using FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Induction of apoptosis

Cancer cell lines were treated with various opioids alone, or in addition to different concentrations of doxorubicin. After different points in time, apoptosis was measured by flow cytometry as described [3]. To determine apoptosis, cells were lysed with Nicoletti-buffer containing sodium citrate (0.1%) plus Triton X-100 (0.1%) and propidium iodide (50 µg/ml) as described by Nicoletti et al. [5]. Propidium iodide (PI)-stained nuclei were analyzed by flow cytometry (FACSCalibur). The percentage of apoptotic cells was measured by hypodiploid DNA analysis as described in Material and Methods. Columns, mean of triplicates, bars, SD < 10%. Similar results were obtained in three independent experiments.

Results

Resistance to current cytostatics, such as the anthracycline doxorubicin, is a major concern in oncology and may be caused by deficiencies in the apoptotic pathways. Therefore, new options are needed to improve therapeutic success. D,L-methadone has been shown to overcome radio- and chemoresistance in leukemia cells at therapeutic concentrations (maximum tolerated dose in patients) and non-therapeutic toxic concentrations (lethal dose) by apoptosis induction [3]. To extend these studies, the question arose whether other opioids such as fentanyl, morphine and buprenorphine also have the potential to reduce the viability of leukemia cells expressing opioid receptors on their surface as assessed using flow cytometry analysis. Incubation of leukemia cell lines with buprenorphine, fentanyl or morphine demonstrated that only non-therapeutic concentrations induced apoptotic cell death. Thus, D,L-methadone seems to be the most potent cytotoxic opioid at therapeutic as well as at non-therapeutic concentrations.

We next focused on the co-treatment of different leukemic cell lines with one opioid (D,L-methadone, buprenorphine, fentanyl or morphine) in combination with the cytostatic drug doxorubicin. We found that therapeutic concentrations of D,L-methadone strongly increased doxorubicin-induced cell death in leukemia cells. In contrast, buprenorphine, fentanyl and morphine at therapeutic concentrations in combination with doxorubicin enhanced cell death only slightly in leukemia cells. Only non-therapeutic concentrations of buprenorphine, fentanyl and morphine in combination with doxorubicin strongly increased doxorubicin-induced cell death in leukemia cells. D,L-methadone in comparison to buprenorphine, and morphine always exhibited the strongest cytotoxic effects on the leukemia cells in combination treatment with doxorubicin using therapeutic concentrations as well as non-therapeutic concentrations. This indicates that specifically D,L-methadone increases therapeutic success in the treatment of leukemia, also at very low therapeutic concentrations.

To extend our studies in the field of the apoptosis-inducing potential of opioids in cancer cells, we also treated glioblastoma cells on the one hand with therapeutic and non-therapeutic concentrations of various opioids alone and on the other hand with various opioids in combination with the cytotoxic drug doxorubicin.

Firstly, we detected a marked opioid receptor expression on glioblastoma cells by using opioid receptor staining. This finding indicates that opioids can target glioblastoma cells. After 120 h incubation of the glioblastoma cell line A172 with various opioids (D,L-methadone, buprenorphine and morphine) at different concentrations (therapeu-
tic and non-therapeutic toxic) or doxorubicin (0.1 µg/ml) alone, we could not observe a strong induction of apoptosis. Co-treatment of A172 with doxorubicin and D,L-methadone (therapeutic and non-therapeutic toxic) induced a strong cell-kill up to 90%. However, buprenorphine (10 µg/ml) in combination with doxorubicin (0.1 µg/ml) stimulated an apoptotic cell death up to 55% only at non-therapeutic concentrations of buprenorphine (Figure 1). In contrast to D,L-methadone (10 µg/ml) and buprenorphine (10 µg/ml), morphine (10 µg/ml) did not enhance doxorubicin-induced apoptosis in glioblastoma cells at therapeutic as well as at non-therapeutic concentrations (Figure 1).

Therapeutic concentrations of D,L-methadone led to the highest levels of cell death induction in combination with doxorubicin. In contrast to these findings, morphine in therapeutic as well as in non-therapeutic concentrations demonstrated no potential to stimulate the doxorubicin-induced cell death whereas just non-therapeutic concentrations of buprenorphine sensitized glioblastoma cells for doxorubicin treatment but not concentrations in therapeutic ranges.

Conclusion

Taken together, various opioids such as D,L-methadone, buprenorphine, and morphine used in therapeutic and non-therapeutic concentrations show differences in their ability to sensitize cancer cells to doxorubicin-induced apoptosis. D,L-methadone is the most promising opioid in cancer therapy. Our results have significant implications for the development of new treatment strategies, using D,L-methadone as an additional anticancer drug in cancer treatment to improve therapeutic success.

In general, our studies indicate that opioids, which are typically used in the treatment of cancer pain, show differences in their ability to induce apoptosis in various types of cancer. In contrast to buprenorphine, fentanyl and morphine, D,L-methadone is a promising therapeutic agent for enhancing doxorubicin-sensitivity in cancer cells at therapeutic concentrations. The clinical potential, especially of D,L-methadone, to enhance doxorubicin-induced apoptosis seems to be a novel treatment strategy using D,L-methadone as an additional anticancer drug to conventional treatment regimes, in particular with respect to overcoming resistances of cancer cells.

Acknowledgments

We thank the Deutsche Krebshilfe (http://www.krebshilfe.de, Grant 109035) for financially supporting our work. Furthermore, we are thankful to Gabriele Aggeler and Sabrina Nothdurft for their technical assistance and Andreas Alt for providing the opioids.

References

Integrating predictive biomarkers and classifiers into oncology clinical development programs: an adaptive, evidence-based approach*

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The future of oncology drug development lies in using predictive biomarkers to identify subsets of patients who will benefit from particular therapies. These technologies offer hope of enhancing the value of cancer medicines, and reducing size, cost, and failure rates in clinical trials. However, examples of failure of predictive biomarkers also exist. In these cases the use of biomarkers add cost and time, and increase complexity and narrow the treated population unnecessarily. A notable setback was the failure of using epidermal growth factor receptor (EGFR) expression to predict efficacy of EGFR directed antibodies. This anomaly may have been due to insufficient sensitivity, biased sampling, loss of antigen expression with storage, or tumor evolution between the time the biopsy was obtained and when the therapy was applied [1], but these issues affect all real world attempts to test a predictive biomarker hypothesis. Inconsistent results with predictive biomarkers have led to various attitudes from enthusiasm to skepticism [2, 3].

We have devised methods to adaptively integrate predictive biomarkers into clinical programs in a data driven manner whereby these biomarkers are emphasized in exact proportion to the evidence supporting their clinical predictive value. The program is built on 4 strategic principles: (1) adaptive decision making, (2) continuous integration of biomarker and clinical information, (3) validation of predictive biomarker hypotheses against clinical benefit, and (4) maximizing objective efficiency functions based on utility per resource unit expended.

First, efficiency of hypothesis testing is optimized in the proof of concept (PoC) trial, where efficiency is defined as the number of PoCs achieved per risk adjusted patient utilized in Phase 2 PoC trials and subsequent Phase 3 pivotal trials. A 30% increment in efficiency can be achieved compared to traditional trials by proper choice of false positive and false negative rates in the PoC trial [4, 5].

This enhanced efficiency then allows testing of two hypotheses simultaneously: (1) PoC for the therapy, and (2) a clinical benefit ID hypothesis (that a biomarker classifier can identify the subset of patients who will benefit). This “efficiency optimized biomarker stratified Phase 2 design” features 4 arms: biomarker positive (BM+) experimental, BM+ control, biomarker negative (BM-) experimental, and BM-control.

In a decision analysis guided Phase 2 – Phase 3 predictive biomarker transition, evidence supporting clinical benefit for BM+ and BM- patients contributes to a two dimensional decision rule, with 4 possible choices: (1) No Go, (2) Go to BM+ only Phase 3, (3) Go to traditional unsselected Phase 3, or (4) Go to a biomarker adaptive Phase 3. The team assigns utility values to possible outcomes resulting from these choices, and the data determines the choice which, risk adjusted, maximizes utility.

In the “Phase 2+ method”, tactics are devised for explicitly incorporating maturing overall survival data from Phase 2 into an interim analysis for the biomarker adaptive Phase 3. The biomarker adaptive Phase 3 enrolls the unslected population, but simultaneously tests efficacy hypotheses in the full population and the BM+ subset. The data from Phase 2 and from Phase 3 up to the interim analysis are used to allocate the false positive rate α between these two hypotheses and again to maximize an objective efficiency function [6]. The BM+ subset is emphasized in the analysis to a degree exactly equal to the extent to which the biomarker hypothesis has been effective in predicting clinical benefit, in contrast to other methods which utilize a fixed α split [7].

The resulting program demands a value from predictive biomarkers and harvests this
value optimally for oncology drug development.

References


MRI Molecular imaging with albumin nanoparticles: achievements and challenges*

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Introduction

Molecular imaging comprises an important tool in understanding biological processes and allows early detection of tumors and of small metastasis. However, until now detection of malignant cells hidden behind intact blood-tissue barriers still presents an almost invincible hurdle in nanomedicine. This is true for essentially all solid tumors. Since each organ maintains its own unique blood-tissue barrier and only one of these barriers (the blood-brain barrier) is partly understood, work should focus on solid tumors for which good models are available in vitro and in situ. A major challenge lies in the fact that tumors in the early stages do not yet release factors which increase the permeability of the blood-vessel-walls. The present state of the art in tumor therapy (Abraxane®) exploits such leaky blood-vessels in addition to the existing hypotension inside the tumor: this allows drugs to be taken up by the so-called “enhanced permeability and retention” effect (EPR-effect) [1]. At present one big challenge in nanomedicine is therefore the creation of particles which can cross tissue-barriers and which are directed by viable targeting groups, leading ideally to a 100% accumulation in the targeted destination. This would allow us to detect and treat in-situ tumors without causing severe side effects, thus increasing patient compliance and health. Interrelated to these research areas, the identification of suitable biomarkers and amplification of MR signals present further major challenges in targeted Molecular Imaging. This paper reports our iterative developmental studies in rats.

Materials and methods

We developed albumin-based nanoparticles bearing gadolinium in iterative cycles of design, synthesis and characterization [2, 3]. Introduction of in vitro analysis methods for size (Photon Correlation Spectroscopy, Transmission Electron Microscopy), purity (Thin Layer Chromatography), relaxivity (Molecular Resonance Imaging), gadolinium complexation (Arsenazo III), density (pyknometry), stability (Poly-Acrylamide-Gel-Electrophoresis) and electrical charge properties (Zeta potential) accompanying synthesis resulted in continuous improvement of the nanoparticles. As preliminary targeting group, we attached the tomato lectin Lycopersicon Esculentum Agglutinin (LEA) to the particles to mediate specific adhesion to oligolactosamines present on the inner surface of the vascular-wall. The LEA-bearing nanoparticles were tested in an ex vivo hemagglutination assay using freshly taken human blood. Subsequent to a positive agglutination result the nanoparticles were injected into adult Sprague-Dawley rats (either sex) and their pharmacokinetics were followed by MRI for up to 4 h. At 4 h, rats were sacrificed to eliminate movement- and other types of MRI artefacts, and the cadavers again visualized in MRI and the results evaluated. Additionally, organs were taken for immunohistochemistry of human serum albumin in light- and electron-microscopy; primary antibodies were used which do not cross-react with rat albumin. To explore the pharmacokinetics of these particles in human tissue, they were injected into cotyledons of term placentae, small samples of the cotyledons were then prepared for electron-microscopy. For flanking experiments carried out with human placentae, we adhered to guidelines issued by the local Ethics Committee.

Results

Our nanoparticles showed sizes of ~ 30 nm diameter in Transmission Electron Microscopy (TEM) as well as in Photon Correlation Spectroscopy (PCS), though PCS also revealed multipeak size distributions representing ag-
Discussion

Since the initial concept of a formulation with a targeted-drug-carrying-vehicle put forward by Paul Ehrlich in 1900 [4] the requirements for such particles have not changed and their advantages are obvious. We chose Human Serum Albumin (HSA) as the matrix for our particles because it is the most abundant protein in human plasma, where its major function is to transport endogenous (e.g.: fatty acids) as well as exogenous (e.g.: cyclosporine) substances. HSA is also a well studied protein [5] and has been used for nearly 70 years in treatment of hypovolemic shock [6]. The advantage of MRI as imaging method lies in the fact that the patient is not exposed to ionizing radiation; the disadvantage of MRI lies in its low signal amplification which can however be overcome by use of macromolecular probes [7]. Our iterative development of albumin nanoparticles has recently reached the stage at which we can demonstrate MR Molecular Imaging in a model system. Our LEA-targeted nanoparticles attached to the vascular wall and image it due either to its content of oligolactosamines or alternatively to its expression of albu min-receptors (SPARC) [2]. We used LEA only for preliminary exploration of the targeting abilities of our nanoparticles, but in future we will attach antibody (fragments) with specificities for appropriate biomarkers. In addition to successful positive targeting, our nanoparticles were also able to cross the basement membrane and enter the perivascular space. We are examining the quantitative aspects of this targeting in order to improve the nanoparticles. Even though we were able to achieve major milestones with this generation of nanoparticles, they cannot be produced on a routine or industrial basis. Their synthesis method, emulsification, lacks control of important factors determining size, leading to rather inhomogeneous particle populations having a PDI of about 1.7. This disparity in size is too high for any translational research or clinical application which would require a PDI of less than 1.1. In our recent developmental work we have been able to overcome this challenge.

Acknowledgment

The Austrian Nano-Initiative (Nanohealth Consortium), the Austrian Science Foundation (FWF) (Project N201-NAN) and the Austrian National Bank Jubilee Program supported this work (Projects 9273, 10844, 11574 and 13096).

References


Relevance of microRNA modulation in chemoresistant colon cancer in vitro*

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Background

Among small non-coding RNAs, micro-RNAs regulate a variety of physiological and pathological conditions such as cell cycle progression, oncogenic transformation and apoptosis [1]. Proposed recently as targets in oncology [2], we hypothesized that micro-RNAs may play a major role in cellular stress adaptation. Aim of this investigation was to identify differentially expressed micro-RNAs in a drug resistant colon cellular model and to assess their relevance for the maintenance of the resistance phenotype. In contrast to most of the investigations describing mechanisms of drug resistance, we were interested in the process of resistance development and therefore generated a multi-stage model with increasing degrees of chemoresistance to 5-fluorouracil (FU), i.e. low, intermediate and high-resistance phenotype.

Methods

We evaluated differentially expressed micro-RNAs in a multi-stage colon cancer system with increasing degrees of resistance to the antimetabolite FU (resistant to 5 µM FU = low resistance phenotype, resistant to 25 µM FU = intermediate resistance phenotype, and resistant to 125 µM FU = high-resistance phenotype). We addressed the phenomenon of acquired drug resistance by generating different levels of chemoresistance described previously [3] with microRNA arrays and validated the candidate RNAs by quantitative PCR (qPCR, Taqman real-time PCR; Applied Biosystems, USA). To compare the different levels of drug resistance with the naïve cell line CCL227 (obtained from ATTC), a circular design was chosen, i.e. RNAs from two different subclones were labeled with Hy3 (green fluorescent dye) and Hy5 (red fluorescent dye) and hybridized together on one spotted array. This procedure was repeated for every combination of RNAs thus obtaining raw data for differentially expressed micro-RNAs for every possible combination of cell lines. Computational analysis of differentially expressed micro-RNAs was performed by using the software GeneSpring (normalization of raw data) and the software “R” in combination with Bioconductor (calculation of statistically significant differences among micro-RNAs). Among this pool of micro-RNAs, 10 were selected for modulation either by transfection of microRNA precursors or anti micro-RNAs, so-called antagomirs (Applied Biosystems; Exiqon, Denmark). Selection criteria for the candidate micro-RNAs were i) differentially regulated microRNA species associated with specific steps of the progression of resistance, ii) congruence of array profiling data and quantitative PCR and/or iii) microRNA species known to be associated with cellular stress tolerance.

Results

For every level of drug resistance (low, intermediate, and high) specific micro-RNAs were identified indicating an individual response to escalating cytotoxic stress at the molecular level. For all candidate genes further investigated in this study, there was a very good congruence between array data and the results from qPCR, performed as reverse transcriptase PCR with Taqman probes as this was one of the selection criteria (Figure 1). Although these studies are still ongoing, there was a massive influence of microRNA modulation with sensitizing effects observed for miR-10b, miR-141, and miR-200a. The effects after inhibition of the micro-RNAs ranged from mean factors between 2 up to 3.6 when assessed by cytotoxicity assays (read-out: IC50). Some peak values in these microRNA species went up to a factor of 5.9 in the high resistant phenotype (miR-10b), whereas other cytotoxic effects were more
evenly distributed across the levels of chemoresistance (miR-141 and miR-200a). Concerning the latter microRNAs, the sensibilization to 5-fluorouracil after silencing of the corresponding microRNA – as expected – was mainly observed in the positive cell line, i.e. the parent cell line CCL227.

Conclusions

Previous work from our team has demonstrated the orchestrated molecular response of cancer cells to 5-fluorouracil by global gene expression profiling. In our colon cancer model, stage dependent mRNA regulations affected vital cellular structures and functions. Likewise, microRNAs seem to follow a corresponding behavior with different microRNA patterns reflecting the step-wise development of resistance to 5-fluorouracil. In this study, we have at least individualized three microRNAs, which are very likely to play a decisive role in the cellular response to therapeutic stress, but via different cellular mechanisms. These mechanisms include a direct impact on cytotoxicity as well as probably a change in the biologic phenotype, e.g. associated with transdifferentiation thus possibly contributing to cellular migration and metastasis [4]. Their regulation might offer novel starting points to understand the regulatory aspect of drug resistance and to integrate rational approaches to circumvent chemoresistance.

Acknowledgment

This work has been supported by the “Initiative Krebsforschung”, Austria.
Suicide activation in a 5-fluorouracil resistant colon cancer model in vitro*

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Background

One of the limitations of all current therapies is a problem well-known to clinical oncologists: although an efficient agent for therapy is available, the specific distribution from the peripheral bloodstream to the malignant lesion is hardly selective. As a consequence, only a small percent of the administered dose distributes into the tumor, whereas the remaining drug causes unwanted side effects. One of the possible approaches to enhance the specificity of tumor therapy is gene therapy based on suicide genes exploiting tumor specific activation mechanisms [1]. Moreover, this approach may also be a valid alternative therapeutic strategy to overcome drug resistance by activation of atoxic compounds to cytotoxic agents.

Study aim

Aim of this project was the in vitro evaluation of tumor specific suicide systems exploiting tumor selective gene therapy techniques in a chemoresistant colon cancer model in vitro.

Methods

Adenoviral cosmids driving the suicide gene cytosine deaminase were generated to convert the prodrug 5-fluorocytosine (5-FC) to the toxic compound 5-fluorouracil (5-FU). To ensure specificity of drug activation, cytosine deaminase was selectively expressed and enhanced intracellularly under the control of the tumor associated promoters CEA and H19 [2, 3] after recombination via Cre/loxP-sequences in trans, thus preventing the unwanted expression of cytosine deaminase in normal cells. To achieve this, suicide plasmids were generated in E. coli using pIRESneo2 (Clontech) and subsequently transferred to the adenoviral cosmid pAxcw (RIKEN). After packing with λ-bacteriophages (Promega), cosmid DNA was isolated and transfected with the COS-TPC method in the cell line 293 as feeder cell line to obtain high-titre adenoviral cosmids for experimental purposes. After multiplication, isolation and control of the cosmids, the colon cells CCL227 and the resistant subclones were transfected using different viral titers and incubation periods. The activation of the Cre/loxP-system was then achieved in trans by co-infection of Cre recombinase (under the control of the promoters of CEA or H19) concomitantly with the suicide gene cytosine deaminase under control of the very strong CAG promoter [4]. Control plasmids were investigated in parallel as positive and negative controls.

To establish dose-response curves, transfected cells were incubated with 5-FC (4 – 7 days), which was converted intracellularly to 5-FU by selective expression of the enzyme cytosine deaminase. In these experiments, HeLa cells (CCL-2) devoid of CEA expression were used as negative controls.

The cytotoxic effect of 5-FC was assessed in a primary adenocarcinoma of the colon, its lymph node metastasis, and different chemoresistant subclones in vitro, which has previously been described in detail [5, 6].

Results

A prodrug activation of near 100%, was observed in vitro with constitutive suicide gene expression compared with the parent drug 5-FU. After cell specific conversion of 5-FC to 5-FU by CEA or H19-driven suicide systems, these results were attenuated indicating a strong dependence on the promoter activity in our cellular system. Chemoresistance was partially circumvented, particularly in the low and intermediate resistance phenotypes. Although H19 was expressed to a lesser degree in some normal cells, the efficiency obtained by H19-driven suicide systems was half of that of the parent drug 5-FU, but was remarkably higher when compared
with the constitutive activation of 5-FC via a control cosmid, where IC50 < 10,000 µM were often observed (Figure 1). These figures were further increased at higher viral titers (2 vs. 5 MOI) with the best results obtained in the low resistance phenotype (CCL227 continuously exposed to 5 µM FU). As all our resistant subclones were generated by continuous exposure to FU at increasing concentrations (acquired drug resistance phenotype).

Conclusion

Besides the feasibility of the chosen approach, these data indicate the constraints of the infection system. Due to the non-specific toxicity of adenoviruses, optimal virus titers around 200 – 300 MOI can hardly be achieved with the actual adenoviral systems, as titers above 50 MOI showed overt toxicity in our model. When available, gene delivery systems with a 10-fold lower intrinsic toxicity would be an excellent opportunity for further translation of tumor specific suicide systems for clinical applications. The data obtained with the H19 promoter clearly indicate that intracellular amplification of the signals generated via H19-driven suicide cosmids are superior to constitutive and linear activation of 5-FC by a factor up to 10, particularly in the low resistance phenotype.

Acknowledgment

This work was sponsored by a grant of the Jubiläumsfonds der Österreichischen Nationalbank (project nr. 11960).

References


Stable combretastatin A-4 analogues with sub-nanomolar efficacy against chemoresistant HT-29 cells*

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Introduction

Combretastatin A-4 (CA4), a cytotoxic cis-stilbene from the bark of the Cape Bushwillow (*Combretum caffrum*), binds to the colchicine binding site of β-tubulin and prevents polymerization of tubulin [1]. It is active both in multi-drug resistant tumors and in immature endothelial cells of the tumor blood vessels [2]. CA4 isomerizes in protic solvents to the inactive trans compound. The search for chemically more stable cis-configured CA4 analogues has led to imidazole- and oxazole-bridged derivatives [3]. A thioanisole of the oxazole series overcomes CA4 resistance in HT-29 colon carcinoma cells by a mechanism associated with an increase in p21cip1/waf1 levels [4]. Since HT-29 cells over-express MRP-1 and MRP-3 drug transporters [5], we investigated which substituents in the A- and B-rings render CA4 and its oxazole derivatives good or poor substrates for these efflux pumps. To this aim, the cytotoxicity of new oxazoles in HT-29 and other tumor cells was measured in the absence and presence of the selective MRP-1 inhibitor MK-571 and compared with that of CA4.

Materials and methods

The syntheses of the reagents 1 and of the oxazoles 2 are published elsewhere [3, 4].

Cells of human KB-V1 cervix carcinoma, HL-60 leukemia and MCF-7 breast carcinoma 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. HT-29 cells were a gift from the University Hospital Erlangen. Resistant KB-V1/Vbl (Pgp+) and MCF-7/Topo (BCRP+) cells were obtained by incubation of sensitive parent cells with 300 – 500 nM vinblastine or topotecan for ca. 90 d. All cells were cultivated in the media recommended by the suppliers at 37 °C and 10% CO₂.

Cytotoxicities were determined using a standard MTT (3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay.

Results

The oxazoles 2 were prepared from the isocyanides 1 and the corresponding aryl aldehydes following a procedure reported in the literature (Figure) [3]. Methylsulfanyl derivative 2b was oxidized to sulfone 2c.

The cytotoxicity of CA4 and of the oxazoles 2 were evaluated using the MTT assay against CA4-resistant HT-29 colon carcinoma and sensitive HL-60 leukemia, 518A2 melanoma, KB-V1/Vbl cervix and MCF-7/Topo breast carcinoma cells. Modifications of the A-ring such as halogenation of one meta-position or removal of the para-methoxy group had only a slight effect on the cytotoxicity, whereas modifications of the B-ring were more effective, in particular in the HT-29 cells. Except for the sulfone 2c, all compounds showed nanomolar to sub-nanomolar activity in the CA4-sensitive cancer cells. In the CA4-resistant HT-29 cells the phenols CA4, 2a, 2d, and 2f were all virtually inactive (Table). The replacement of the B-ring meta-hydroxy group (R₃ = OH) by H (as in 2b) or fluorine (as in 2e) or by amine (not shown) led to compounds of extraordinarily high cytotoxic activity in HT-29 cells (i.e. IC₅₀ < 1 nM). Co-incubation of HT-29 cells with the inactive compounds CA4, 2a, 2c, 2f and a non-toxic concentration of the MRP-1 inhibitor MK-571 markedly sensitized them to the oxazoles but not to CA4 itself.

Conclusion

A series of new oxazole-bridged CA4 analogues with modified A- and/or B-rings was synthesized and evaluated for their cytotoxic activity in various tumour cell lines. The ac-
Activity profiles of derivatives modified in the B-ring in CA4-resistant HT-29 cells provided a hint as to a possible circumvention of this resistance. All analogues lacking the meta-OH group in the B-ring were highly active in HT-29 cells, whereas treatment with the selective MRP-1 inhibitor MK-571 sensitized these cells towards the inactive phenol derivatives. Apparently, CA4-resistance of HT-29 cells is, at least partially, mediated by the MRP-1 transporter, which preferentially expels glucuronated phenols [5]. The reason for the low degree of HT-29 cell sensitization for CA4 by MK-571 might lie in further deactivating mechanisms or in the low chemical stability of the cis-double bond of CA4. The recognition and transport of phenols by MRP transporters seems to be selective since the Pgp and BCRP transporters of KB-V1/Vbl and MCF-7/Topo cells do not pump CA4 and related compounds sufficiently to preclude drug induced cell death. Surprisingly, the sulfone 2c also gained in activity against HT-29 cells when MK-571 was added. Since 2c displayed no cytotoxic activity in the other CA4-sensitive cell lines used in this study, we doubt that MRP-1 inhibition is the reason for the activity enhancement by MK-571. As MK-571 is also known to be a potent antagonist of the leukotriene D4 receptor CysLT1, whose signaling facilitates survival of colon cancer cells, the activity of 2c in the presence of MK-571 might be due to a special mechanism of its action in HT-29 cells that is normally suppressed by this receptor [6, 7].

References


MRI molecular imaging with nanoparticles: a technical platform for early diagnosis of cancer*

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Introduction

Most contrast media used today are non-specific. They give signals at e.g. sites of high vascularization or raised permeability. An exception is for example the use of octreotide-labeled radioisotopes in nuclear medicine, which enable molecular imaging by binding of the molecules to somatostatin receptors [1]. For MRI, Molecular Imaging is only achievable by use of nanoparticles. Perfluorocarbon nanoparticles are in development for targeting and MR T1 imaging of vascular walls e.g. fibrins, integrins [2]. Clinically, the only nanoparticles used in MR are the superparamagnetic USPIOs (UltraSmall Particles of Iron Oxide) for T2 modality. They are used for imaging of lymph nodes and tumors [3].

In molecular imaging a lesion or illness is identified by the detection of a single molecule specific for this illness. Molecular detection is achieved by attaching antibodies or other targeting molecules to a signal-generating vector carrying signal moieties. In this way, the specificity of the signal is raised and background signals reduced. MI for clinical application is not always easy to establish. Low sensitivity, e.g. MRI, or low penetration depth, e.g. optical imaging, have hindered successful clinical use. The problem of low sensitivity can be overcome by use of adequate vectors. We aim to develop nanoparticles for Molecular Imaging in MRI, carrying hundreds of signal molecules [4]. Large numbers of signaling groups are needed because of the low sensitivity of the imaging technique. On the other hand, the use of MRI implies great advantages such as its high spatial resolution and the lack of ionizing radiation. Our final aim is to synthesize nanoparticles suitable for targeted imaging and therapy.

Methods

Albumin-based nanoparticles bearing gadolinium were developed in iterative cycles of design, synthesis, characterization and improvement [5, 6]. Tomato lectin was attached to the particles to mediate specific adhesion to oligolactosamines. Intravenous injection of the nanoparticles into rats, and MR imaging of the rats at 1.5 Tesla, was used to assess the molecular imaging capabilities of the nanoparticles. Pharmacokinetic studies examined the organs of the rats by MR T1 mapping, atomic absorption spectrometry for gadolinium, electron microscopy, and light microscopy using immunohistochemistry to localize human serum albumin.

Results

Lectin-loaded nanoparticles generated MR Molecular Imaging of the blood-vessel walls; light microscopy then demonstrated human serum albumin adhering to endothelial cells, passing as small granules through the smooth muscle cell layers, and accumulating in the adventitia of the large blood vessels of these rats. Non-derivatised nanoparticles provided blood-pool imaging, the blood signal fading after 1.5 hours; strong signals were also detected in liver and kidney. AAS data showed an accumulation of gadolinium predominantly in liver and kidney. Electron microscopy revealed electron dense material in the cytosol of liver parenchymal cells. Gadolinium was cleared rapidly in the first days and complete clearance required over 6 weeks.

Discussion

At present our nanoparticles are one or two particle generations of developmental
work away from translational optimization and regulatory certification [6]. Our development of the nanoparticles has reached the stage at which we can demonstrate MR molecular imaging in a model animal system. Our nanoparticles target the vascular wall and image it, due either to its content of oligolactosamines or alternatively, to its expression of albumin-receptors. We are examining the quantitative aspects of this targeting in order to improve the nanoparticles and to design the next generation of particles for imaging and/or drug delivery. For a future clinical approval issued by licensing agencies such as EMEA and FDA, it is important that the nanoparticles are non-toxic. Our nanoparticles contain albumin, the body’s major transport protein, and in vivo and in vitro testing revealed no toxic effects. Furthermore, albumin is available in large amounts and is inexpensive. Concerning cancer treatment, our nanoparticles can be loaded with anti-cancer drugs. Because of their large surface area, the amount of drug at the target site can be increased. On the other hand, success in this new area also depends on identification of relevant target molecules [7]. Targeting cancer cells with nanoparticles for imaging and targeted drug release promises great advantages. Side effects can be eliminated because the drug is mainly released at the target cells and nowhere else, and treatment is therefore safer and more efficient.

References


Lysophosphatidylcholine (LPC) as a pharmacological molecule for the reduction of tumor cell adhesion and metastasis*

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Reduced tumor cell adhesion via lysophosphatidylcholine

Introduction

The ex vivo exposure of murine B16.F10 melanoma cells with hydrogenated lysophosphatidylcholine (hLPC) showed impressive antimetastatic effects in mice [1]. In detail, a rapid uptake of hLPC by the melanoma cells and change in lipid membrane composition towards a higher level of saturated fatty acids resulted in reduced binding of the integrin VLA-4 to its ligand VCAM-1 or interaction with platelets via P-selectin. Since the hLPC-caused membrane alterations might influence lipid raft formation – an effect, which is well known for the hLPC-analogue hexadecylphosphocholine (Miltefosin) – lipid raft signaling is discussed to be affected by hLPC. Due to the fact, that both compounds exhibit a similar chemical structure, one can speculate about similar biophysical and biochemical properties in the cell which may lead to similar anti-cancer effects. Thus, morphological changes of the PM surface, identified by different microscopic techniques and the signaling mechanisms behind the antimetastatic effects of hLPC have to be elucidated.

To investigate the hLPC-effects on the molecular level, we used hLPC-exposed and non-exposed human MV3 tumor cells for gene expression analyses with microarray. The focus here lies on elucidating a functional link between cytoskeleton and cell adhesion proteins e.g. integrins and their connected signaling pathway level that explain the molecular mechanisms of hLPC anti-metastatic activity. Therefore, expression of membrane associated/integrated proteins – possibly located in raft microdomains – such as palmitoylated GTPases of the Ras-superfamily with importance for cellular adhesion and downstream signaling as well as structural/cytoskeletal reorganization were focused. The first set of proteins presented here, will help to explain the reduction of metastasis by hLPC and its potential as a pharmacological substance.

Material and methods

MV3 cell cultivation and hLPC-exposition experiments

MV3 melanoma cells are a stable cell line originating from a 76-year-old melanoma patient. MV3 cells are characterized by a high metastatic potential and can be splitted 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 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 × 10^5). After 24 h cells were used for hLPC exposition experiments. hLPC was solved in RPMI with 40 mg/ml BSA before incubation with MV3 cells.

For the experimental setup the six conditions arise from three treatments (none, 300 µM and 450 µM hLPC) and two 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.

Key words
lysophospholipids – lysophosphatidylcholine (LPC) – cellular adhesion molecules – signal transduction – tumor cell metastasis

*This extended abstract summarizes a poster presentation given by M. Alexander during the 8th Annual Meeting 2010 of the Central European Society for Anticancer Drug Research (CESAR) held in St. Gallen, Switzerland, July 01 – 03, 2010.

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International Journal of Clinical Pharmacology and Therapeutics, Vol. 49 – No. 1/2011 (75-77)
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) for processing on Illumina (San Diego, USA) human HT-12 microarrays to guarantee systematic global gene expression (GEX) analyses of untreated and hLPC-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**

The gene expression profiles of non-treated and hLPC treated MV3 cells were analyzed to identify altered expression of the cells by hLPC exposition. This strategy helps to obtain further insights into the antimetastatic effects of the phospholipid.

The data presented here show a first set of proteins which are altered in expression by hLPC exposition and which are involved in different biochemical functions of the cell (Figure 1). Deregulated proteins are known to play a role in the connection to the extracellular matrix (aminopeptidase N; APN), the regulation of guanine nucleotide composition in the cell (adenylate kinase isoenzyme 3/adenylate kinase isoenzyme 3 like-1; AK3/ AK3L1) and in the regulation of GEFs (guanine nucleotide exchange factors) by HERC6 (the protein contain RLD domain which can act as a GEF for small G-proteins). In addition, other identified genes – which are altered in expression by hLPC – are involved in the pathways of extracellular-regulated kinases (ERKs), MAP kinases, p38 and JNK (dual-specificity phosphatase1/MAP kinase phosphatase; DUSP1/MKP1) and in the TGFβ/SMAD pathway (serpinB7 and angiopoietin like 4; ANGPTL4).

This indicates that several essential signaling pathways and localizations in the melanoma cells are affected by hLPC treatment which are suggested to reduce cellular adhesion and metastasis of MV3 cells.

**Conclusion**

hLPC treatment is able to induce impressive antimetastatic effects in tumor cells and in mice [1].

We postulate that the incorporation of hLPC into the PM and maybe rafts lead to the alteration of the membrane-association and temporal localization of Ras-superfamily GTPases and their connected downstream signal transduction, e.g. the integrin signaling mechanisms which are not completely elucidated [2, 3]. The GTPase RhoA was found to be influenced in activation by hLPC in the context of cell invasiveness [4] and RhoA and RhoB were identified to be reduced in expression by hLPC-exposition to MV3 cells [5]. Our data provide evidence that hLPC induce alterations in transcription of genes – possibly via membrane associated GTPases – which are mediators in essential cellular pathways and functions e.g. cellular adhesion, GTPases, ERK and TGFβ/SMAD pathway.

Further analyses will give insight into the reduction of metastasis by hLPC and its potential as a novel compound for mediating therapeutic antimetastatic effects.
References


Chemosensitivity of conjunctival melanoma cell lines to chemotherapeutic agents*

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Introduction

Conjunctival melanoma is a melanocytic tumor of the ocular surface with an incidence of 0.02 to 0.08 cases in 100,000 inhabitants in the industrial world. Therapy is based on complete excision of the tumor, supported by adjuvant therapy. Chemotherapy plays a role as an adjuvant tool in local therapy and as systemic therapy in metastatic disease. The overall rate for local recurrence in conjunctival melanoma is about 43 to 52% and metastatic disease occurs in about 26% after 10 years. Unlike other ocular melanomas, conjunctival melanoma metastases most often affect the regional lymph nodes and parotid gland. Hematogenous spread occurs without organ preference. Radical surgery (i.e. orbital exenteration) has not proved to be more beneficial than conservative treatment that maintains the eye.

Methods

The two conjunctival cell lines used in this study were CRMM-1 and CRMM-2 that are generated from locally recurrent tumours (C = conjunctival; R = recurrent; MM = malignant melanoma). For details on the characteristics, see the published data in Reference 1 and Table 1. The growth curves of each line are shown in Figure 1 and 2. The cell lines were generated stable under culture conditions and mycoplasm-negative in repeated testing. In the case of the CRMM-1-line, cells of various passages between passage 5 and 61 were used for the experiments. In the case of the CRMM-2-line, cells of various passages between passage 33 and 57 were used for the experiments. Ham’s F12, 10% FCS (fetal calf serum) with 100 U/ml penicillin and 100 µg/ml streptomycin was used as culture media. Change of media was performed twice weekly. For both cell lines, the splitting ratio was 1:5 – 1:7 and the cell-doubling time was about 75 h.

Chemotherapeutic agents

The following substances were used to test the sensitivity of the cell lines: All-trans-retinoic-acid (ATRA), cisplatin, fotemustine, imatinib (Gleevec®), mitomycin C.

Cytotoxicity assays

The sensitivity to the drug was assessed using the sulforhodamine B (SRB) test system, a proliferation assay described by Skehan et al. [2]. Adherent cells in appropriate culture media and concentrations per well were sown in 96-well plates (Falcon, USA) and allowed to attach overnight. Incubation with increasing concentrations of the drugs was done for 24 h.

Statistical analysis:

The IC50 (inhibitory concentration) describes the concentration of the substance that leads to an inhibition of cell growth in 50% of the treated cells. It was determined by n = 4 experiments on different days and varying passages of the cell lines. In each experiment, n = 8 measurement-repetitions were performed. The mean IC50 was calculated and is presented with the standard deviation. Statistical analysis was performed with Excel 2000 for Windows XP.

Results

Table 2 and Figure 3 show the results of the mean IC50 for all substances in the tested cell lines. For an example of the IC50 estima-
It was observed that cisplatin and mitomycin C had an inhibiting effect in conjunctival melanoma cells. All-trans-retinoic acid (ATRA), fotemustine and imatinib demonstrated only weak antitumoral activity in vitro. The results in detail were as follows:

<table>
<thead>
<tr>
<th>Substance</th>
<th>IC50 (µM) ± SD (% SD)</th>
<th>CRMM-1</th>
<th>CRMM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA</td>
<td>94.9 ± 7.8 (8.2)</td>
<td>67.3 ± 3.1 (4.5)</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>6.2 ± 1.1 (17.6)</td>
<td>2.95 ± 0.4 (11.9)</td>
<td></td>
</tr>
<tr>
<td>Fotemustin</td>
<td>&gt; 333</td>
<td>102.6 ± 20.8 (20.3)</td>
<td></td>
</tr>
<tr>
<td>Imatinib</td>
<td>56.4 ± 1.5 (2.7)</td>
<td>56.2 ± 2.9 (6.2)</td>
<td></td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>4.9 ± 0.4 (7.2)</td>
<td>6.7 ± 0.3 (4.6)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Growth kinetic of the cell line CRMM-1 (passage 43) determined with a SRB-assay (sulforhodamine B). A density of 2500 cells/well was chosen for the experiments.

Figure 2. Growth kinetic of the cell line CRMM-2 (passage 44) determined with a SRB-assay (sulforhodamine B). A density of 3000 cells/well was chosen for the experiments.

Figure 3. Logarithmic function of the IC50 results of the tested substances on CRMM-1 and CRMM-2. Cisplatin and mitomycin C reached IC50 values below or around 10 µM.
Mitomycin C: The cell lines were incubated with mitomycin C for 24 h and the substance inhibited cell growth with an IC50 below 10 µM in both cell lines (CRMM-1: 4.9 ± 0.4 µM; CRMM-2: 6.7 ± 0.3 µM).

Conclusions

These are the first successful studies on the chemosensitivity of two conjunctival melanoma cell lines. We confirmed the efficacy of a drug that is used in the local chemotherapy of the tumor (i.e. mitomycin C) and identified other agents that may play a role in the therapy of the malignant melanoma of the conjunctiva in future strategies. The investigations should improve local tumor control as well as the treatment of metastatic disease.

Acknowledgment

The study was supported by the Dr.-Werner-Jackstädt-Stiftung, Wuppertal, Germany

References


Cross-resistance of 5-fluorouracil-resistant colon carcinoma in vitro


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Introduction

Therapy failure due to drug resistance is a major problem in cancer therapy, as progression of the disease after an initial response is a common event. Insensitivity to a single agent may have far-reaching consequences as this process is not necessarily limited to the administered drug, but may decrease the activity of other anticancer drugs acting via different pharmacologic mechanisms. In spite of a variety of investigations on this subject, little is known about the effects of 5-fluorouracil (FU) resistance in colon carcinoma concerning cross-resistance to other therapeutic anticancer agents. The aim of this study was to evaluate whether resistance to FU is directly associated with resistance to other chemotherapeutics with different pharmacological points of attack, or if a change in therapy is adequate to overcome resistance. Different degrees of resistance in colon cancer in vitro have been tested to assess whether the extent of FU resistance correlates with loss of effectiveness of therapeutic options.

Material and Methods

Cross-resistance to FU-resistant colon carcinoma cell lines was evaluated using standard cytotoxic drugs (oxaliplatin, vinorelbine, the active metabolite of irinotecan termed SN-38), and targeted therapies (cetuximab, bevacizumab, wortmannin, sirtinol, AKT IV-inhibitor, rapamycin), alone or in combination by growth inhibition experiments. Thus, the selected panel includes compounds indicated for the therapy of advanced colorectal cancer as well as investigational agents with a focus on the survival pathway PI3K-Akt-mTOR. Cytotoxicity was determined using CellTiter 96 Non Radioactive Cell Proliferation Assay®, Promega (MTT-assay, which uses the conversion of (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide to assess mitochondrial activity). Dose-response curves and IC50 were obtained by non-linear fitting to a sigmoid model. Cytotoxicity of these substances – either alone or in combination – was investigated in the human colon cancer cell lines CCL228 (primary adenocarcinoma, also termed SW480 in the literature), CCL227 (lymph node metastasis, also termed SW620; both cell lines were obtained from the American Type Culture Collection, ATCC), and in FU-resistant subclones of CCL227 (low-, intermediate-, and high-resistant phenotypes); this model has been described previously [1].

Results and discussion

SN-38 (7-Ethyl-10-hydroxy-camptothecin), the active metabolite of irinotecan, overcame resistance in the low-resistance phenotype, but was less effective in the high-resistance subclones by a factor of 10. Present data indicate that resistance to FU is accompanied by impaired activity of SN-38 (Figure 1).

In contrast, acquired FU-resistance was entirely circumvented by oxaliplatin. It was of interest that sequential exposure to oxaliplatin or SN-38 followed by FU was associated with an antagonistic effect. Loss of sensitivity in the sequential exposure schedule resulted in a 2-fold increase in IC50 values for oxaliplatin and a 10-fold increase for SN-38. It has been demonstrated that oxaliplatin and irinotecan alter transport and/or metabolism of the fluoropyrimidines and decrease the incorporation of fluoropyrimidines into nucleic acids [2]. Our data indicate a sequence-dependent effect of FU when combined with oxaliplatin/SN-38, which may even be antagonistic. This antagonistic effect of FU to
SN-38 can be explained, at least in part, by the fact that high concentrations of FU suppress topoisomerase I synthesis and attenuate SN-38 activity, which directly targets this enzyme [3].

The monoclonal antibodies (cetuximab, bevacizumab) had no influence on the cell viability, either alone or in combination with SN-38 or oxaliplatin. The absence of response of our cellular systems to cetuximab could be attributed to the lack of EGF-receptor expression of all the cell lines investigated, but also to the in vitro system, selected, which is devoid of effector cells for ADCC-dependent cytotoxicity. In this context, it is worth noting that in other studies, EGFR expression levels do not necessarily correlate with response to cetuximab treatment [4]. In contrast to the lack of EGF receptors, our cell lines have previously been shown to be VEGFR positive and to produce VEGF depending on the degree of resistance [5]. Since inhibition of VEGF receptor does not result in a marked growth-inhibiting effect and most notably that there is no difference between the abundantly secreting cell lines and those which produce VEGF to a lesser extent, VEGF production apparently plays no pivotal role in our in vitro setting. Potentially, the different VEGF-production profiles of our cell lines are more of systemic relevance, than of direct relevance for the survival of the tumor cell model investigated.

Vinorelbine exhibited a biphasic dose-response curve and completely overcame resistance to FU. A possible explanation for the biphasic shape of the curve is that antimicrotubule agents exert additional cytotoxic effects at very high concentrations. These include in particular the activation of TNF-receptor as well as the release of cytokines and interleukins and result in apoptosis independent of microtubule interaction [6].

Regarding kinase inhibitors, cell lines were completely insensitive to wortmannin and sirtinol. In contrast, rapamycin and AKT IV-inhibitor were very active regardless of FU-resistance levels. It is worth noting that PI3-kinase and AKT/mTOR build a signaling network that is finely balanced in normal tissues. A possible explanation could be a mutation of PI3-kinase, which is often observed in colorectal cancer cells [7]. Additional investigations to clarify the status of this intracellular signaling pathway of our cellular systems are therefore required.

Conclusion

Although this study provides evidence that even highly chemo-resistant subclones retain their susceptibility to growth inhibition as shown for a variety of cytotoxic agents, several anticancer drugs lose their efficacy entirely or partially. In FU-resistant colon cells in vitro, the complete absence of activity (cetuximab, bevacizumab, wortmannin, and sirtinol) and partial cross-resistance to the active metabolite of irinotecan, SN-38, was observed. In contrast, other compounds such as the classical cytotoxic agents (oxaliplatin, vinorelbine) were successful in overcoming resistance entirely. The same observation was also made for targeted approaches (rapamycin and AKT IV-inhibitor) suggesting that resistance to FU is not necessarily associated with a loss of therapeutic options in advanced colorectal cancer.

References

Measurement of 5-FU plasma levels in patients with advanced cancer: correct approach to practical procedures is essential**


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**Key words**

5-Fluorouracil (5-FU) – chemotherapy – advanced colorectal cancer

*Both contributed equally to generate the data of this work.

*This extended abstract summarizes a poster presentation given by M. Blaschke during the 8th Annual Meeting 2010 of the Central European Society for Anticancer Drug Research (CESAR) held in St. Gallen, Switzerland, July 01 – 03, 2010.

Introduction

5-Fluorouracil (5-FU) is one of the oldest and still most used chemotherapeutics. Over the last decade a variety of intravenous infusion schedules for 5-FU have been developed. In the US mainly the bolus regimen is in use (MAYO regimen: 600 mg/m² Bolus) [1]. In France the Folfox4 regimen is preferred, which consists of 400 mg/m² Bolus + 600 mg/m² continuous infusion over 48 h [2]. In Germany the high dose AIO (Arbeitsgemeinschaft Internistische Onkologie) schedule is preferentially applied in a dose of 2,000 – 2,600 mg/m² over 24 h [3] in the treatment of several gastrointestinal tumors.

The pharmacokinetics of 5-FU have been studied intensively: 5-FU disappears rapidly from the plasma with a half-life of 10 – 20 minutes. Its total body clearance varies depending on the administration schedule: 0.5 – 1.5 l/min for bolus application versus 5 – 58 l/min for continuous infusions [4]. The key degrading enzyme is DPD (Dihydropyrimidine-dehydrogenase) which is mainly expressed in the liver. This enzyme is mutated in 5 – 8% of patients (exon14 skipping), which can cause severe (even lethal) side effects in patients receiving 5-FU therapy [5].

In the course of time it became obvious that there is a fine line defining the optimal dose of 5-FU. High levels will induce severe side effects (diarrhea, neutropenia, hand/foot syndrome) whereas low levels will have no therapeutic effect. Moreover there is an individual therapeutic balance between benefit and side effects.

So far the 5-FU dose adjustment has been calculated using body surface area, but it became clear, that individual variations concerning adsorption, distribution, metabolism and excretion of 5-FU require an individual 5-FU dose adjustment.

Recent studies [6, 7, 8] demonstrated that the optimal AUC (area under the curve) range for all 5-FU regimens is 20 – 25 mg/h/l (in our system 18 – 25 mg/h/l). For a 24 h continuous infusion the corresponding steady state levels would be 830 – 1,040 ng/ml. These findings are consistent with an earlier study which obtained steady state concentrations between 6 – 8 µM over a period of 24 h.

Methods

5-FU plasma levels (n = 108) were measured in 11 consecutive patients (9 mCRCa (1,500; 2,000 mg/m²/d); 1 stomachCa (2,000; 2,600 mg/m²/d), 1 esophagusCa (1,500; 2,000 mg/m²/d) at 0h, 2–3h and 21–23h after the start of the infusion. AUC values were calculated according to Gamelin et al. 2008 (J.Clin.Oncol.).

Figure 1 shows the typical pump (Baxter Folfusor SV4) which is used routinely for 24 h continuous infusion of 5-FU solutions in Germany. The pump has a maximal volume of 130 ml and the flow is driven by pressure evoked by filling the elastomeric pump balloon with the solution infused. The 5-FU reservoir is connected through a 0.5 m tubing filled with saline for safety reasons with the connecting piece. The white connecting piece contains a glass capillary which regulates the flow rate (33 °C; 4 ml/h) and has to be fixed to the patient’s skin.

Blood samples were collected under the standardized conditions a) before (n = 54), b) 2 – 3 h (n = 9) and c) 21 – 23 h (n = 45) after starting the 24 h continuous infusions. Samples were immediately placed on ice, centrifuged within 30 min and stored at –20 °C. 5-FU concentrations were measured once weekly using a Saladax 5-FU PCMTM-Immunoassay.

Results

In 6 patients, 5-FU levels were measured after 2 – 3 h and compared to those measured...
21 – 23 h after start of the infusion. The 5-FU levels and the resulting AUC-values were 50% higher in plasma samples obtained at the later time points. The plasma levels taken at the later time points resulted in constant AUC-values within one individual. The 5-FU concentrations in blood obtained were proportional to the 5-FU doses applied.

Two different 5-FU doses (1,500 mg/m²/d; 2,000 mg/m²/d) were compared. The variation in AUC values is higher with the lower dose than for the higher dose, with no single measurement within an AUC range of 18 – 25 mg/h/l. For the 5-FU dose of 2,000 mg/m²/d 48% of the measurements were within an AUC range of 18 – 25 mg/h/l (25 measurements in 7 different patients), 5 measurements resulted in AUC values below 18 mg/h/l and 8 measurements in 3 patients were above 25 mg/h/l.

Discussion

Our experience over 12 months demonstrated that the measurement of 5-FU concentration in patient plasma is possible but that several factors have to be carefully controlled: 1. The communication between the department administering 5-FU and the lab, has to be very close. 2. The patients have to be asked to come back 21 – 23h after the 5-FU infusion has been started. 3. The samples must be kept on ice from the moment they are collected. Shortly after sample collection (< 1h) the plasma has to be centrifuged and stored at –20 °C.

Experience showed that the 5-FU Baxter Folfusor pump is empty after 21 – 24 h. If the pump is empty at the time point of blood sampling, the 5-FU level is no longer at steady state level and the AUC value cannot be calculated. If the reservoir contains 5-FU we decided to calculate the AUC values by multiplying by 23 h instead of the theoretical 24 h to obtain a more accurate AUC value. Theoretically the 5-FU steady state level is reached 2 – 3 h after the start of the infusion, but our data showed that the values obtained after 2 – 3 h were 50% lower than values obtained after 21 – 23 h. All AUC-values obtained from samples taken after 2 – 3 h were low (AUC23h ~ 10 mg/h/l). AUC23h values are calculated using values from samples obtained at the later time point. Stable values were obtained with low intra-individual variation. 48% of all measurements (2,000 mg/m²/d) were within the proposed optimal range of AUC23h = 18 – 25 mg/h/l. We observed a close correlation between the 5-FU concentrations which showed high intra-individual constancy with the constant AUC-values and were dose-dependent. At the same time a high inter-individual variation especially with low 5-FU doses could be observed. The determination of the AUC-value still needs to be optimized, as the pumps deplete even faster during the summertime, the overall flow is not linear and the flow increases with the infusion time.

As more and more cancer cases come to light and develop into a “chronic” disease, 5-FU measurements represent a further step towards individualized anticancer therapy. This is of special importance if we consider that the average patient is over 70 years of age.

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Comparison of seminal vesicle, non-malignant and malignant prostate tissues with gene expression patterns using quantitative real-time PCR*


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Introduction

Advances in the fields of molecular biology and genetics have increased our understanding of biological events and biomarkers related to the initiation and progression of prostate cancer (PCA) [1, 2, 3]. Taking advantage of this knowledge, we selected 85 genes representative for cancer hallmarks [4] and investigated their expression patterns concomitantly in several cancer tissue types and various stages of aggressiveness in order to identify molecular subclasses of PCA.

Patients and methods

The gene expression profiles were assessed using quantitative real time PCR (Qrt-PCR) in a total of 170 samples obtained from 11 patients with benign prostatic hyperplasia and from 122 patients with PCA. All 122 patients underwent primary radical prostatectomy with pelvic lymphnode sampling at the Stephanshorn Clinic in St. Gallen. The study protocol was approved by the ethic committee of St. Gallen. The resected specimens of the prostate were immediately transferred on ice to the Institute for Pathology of the Kantonsspital, St.Gallen for examination. Small tissue samples from macroscopically visible tumor and non-tumor prostate tissue or from the seminal vesicle were dissected, snap frozen in liquid nitrogen and cryo-preserved at –80 °C. These samples were cut in a cryo-microtome and a slide of each probe was stained with hematoxylin-eosin for histological verification. After reassessment, the samples selected for molecular analysis using Qrt-PCR had the following distribution: 116 PCA, 6 BPH, 13 non-malignant prostatic and 24 seminal vesicle tissues. Data were analyzed by means of unsupervised hierarchical clustering based on Kendal correlation matrix and tree view program [5]. Other statistical analyses were carried out at the 5% level of significance and performed with S-Plus software (Version 6.1, Insightful Corporation, Seattle, WA, USA).

Results

Unsupervised hierarchical clustering analysis [5] clearly discriminated the samples into 7 classes. As shown in Figure 1, there was distinct discrimination between the several tissue types investigated. One cluster identified almost exclusively seminal vesicles, another BPH tissues and non-malignant prostate tissues. The third one grouped tissues of patients with PCA but representing only the benign hyperplasia or the focal tumors with less than 30% tumor cells. In particular, the values for cyclooxygenase 2 (COX2) [6] transcripts were 100 times higher in the seminal vesicle specimens as compared to all PCA samples. The 116 PCA samples were classified in the remaining four groups with a specific grade of aggressiveness. Markers of proliferation, cell membrane degradation and normal-like tissues were very important for discriminating the non-malignant samples and identification of the stages of malignancy. Genes related to proliferation were positively correlated with the Gleason grade and discriminated a group with a high likelihood of PSA recurrence after curative prostatectomy from a group with a low likelihood of recurrence. This prediction, if validated, may help to decide to which patients adjuvant therapy should be offered. An analy-
sis was made of the 116 PCA samples exclusively (results not shown). The sequence of the single specimen changed but the intrinsic biology of the four groups remained consistent with the overall model obtained using all 170 samples.

**Discussion**

Gene expression patterns reveal the biological background of the intrinsic tissue types, supporting a rational understanding of PCA development and progression. In this study we analyzed the transcriptional expression levels of known genes and also used non-malignant tissues to verify the expected changes in distribution of their expression. The developed Qrt-PCR method is robust, reproducible, cost-effective and easily applicable in routine medical practice and in core biopsies [7]. In conclusion, the concomitant assessment of the selected genes allows the further classification of PCA specimens, correlates with pathological categories such as the Gleason score and offers additional information on the likelihood of tumor recurrence with therapeutic implications.

**Acknowledgment**

This study was financially supported by the STIFTUP and OSKK.

**References**

Biomarker response on exposure to sunitinib and its primary metabolite (SU12662) in metastatic colorectal cancer patients*

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Objectives

Sunitinib is a multi-tyrosine kinase inhibitor and approved for metastatic renal cell carcinoma (mRCC) and imatinib-resistant gastrointestinal stromal tumors (GIST). Currently it is also being tested in metastatic colorectal cancer (mCRC) [1]. For sunitinib and other anti-angiogenic drugs, the potential of individualized dose adaptation based on pharmacokinetic parameters and biomarker measurements has not been explored yet since the understanding of the dose-exposure-effect relationship is still limited. It has been shown for healthy volunteers [2] as well as for cancer patients that sunitinib influences the plasma concentrations of various circulating proteins which can serve as biomarkers for its anti-angiogenic properties [3]. In this investigation plasma concentrations of the drug and its main metabolite, SU12662, and three biomarkers (VEGF-A, soluble VEGFR-2, and soluble VEGFR-3) were determined in mCRC patients within the CESAR C-II-005 study [see Mross et al., this issue, p. 96]. Based on these data, the changes in biomarker concentrations on exposure to sunitinib and its active metabolite SU12662 were investigated.

Patients and methods

23 patients with mCRC were enrolled into this prospective study. A daily dose of 37.5 mg of sunitinib was administered on a 4-week on/2-week off treatment schedule in addition to folinate, fluorouracil, and irinotecan (FOLFIRI) as first-line therapy. The dose was reduced to 25 mg/day in case of toxicity. Samples were drawn at baseline and at seven predefined time-points during the Weeks 1, 3, 5, 7, 9, 11, and 13. Blood samples were taken prior to sunitinib administration to obtain drug trough concentrations. Plasma levels of sunitinib and SU12662 were determined by LC-MS/MS and those of VEGF-A, sVEGFR-2, and sVEGFR-3 using commercially available (R&D Systems, Minneapolis, USA) and previously validated immunoassays (unpublished data). Six patients were excluded from the analysis due to missing data. Plasma concentrations of the active drug and biomarker response were compared to a dataset previously obtained in healthy volunteers (n = 12) [2]. Furthermore, data were compared with corresponding predictions of appropriate pharmacokinetic/pharmacodynamic models previously developed for this healthy population. The two populations differed mainly in median age and BMI with 40.5 (range 27 – 54) years vs. 60 (range 33 – 75) years and 22.7 (range 20 – 26.7) kg/m² vs. 26.7 (range 19.3 – 39.9) kg/m², respectively. The ratio of male/female was 6/6 and 10/7 for healthy volunteers and mCRC patients, respectively.

Results and discussion

A total of 17 patients received 37.5 mg sunitinib daily during the first cycle. Only in one patient the dose reduced to 25 mg/day due to toxicity. Assuming a linear dose-concentration relationship [4] the drug concentrations were normalized to the dose given to
compare the results with those observed in healthy volunteers who received a daily dose of 50 mg sunitinib for 3 (n = 4) and 5 consecutive days (n = 8) [2]. After 1 day ‘on treatment’ median plasma concentrations of 12.40 (range 7.96 – 21.18) ng/ml and 3.14 (range 1.60 – 6.74) ng/ml were observed for sunitinib and SU12662 (n = 14), respectively, which were described satisfactorily by the predictions of the pharmacokinetic model, developed previously for healthy volunteers [2]. Maximum median concentrations were observed after 4 weeks for sunitinib and after 2 weeks for SU12662 with 45.74 (range 18.83 – 52.67) ng/ml (n = 16) and 18.56 (range 7.18 – 40.29) ng/ml (n = 10), respectively. Median concentrations and their variability were comparable to the model predictions at all measured time-points. This is in contrast to a recently published meta-analysis on patients with other types of cancer (mainly metastatic renal cell carcinoma and GIST) where the tumor type was identified as a covariate for the CL/F of sunitinib and SU12662 [4].

VEGF-A concentrations exhibited large inter-individual variability with no significant changes in the median from baseline level (Figure 1A). The lack of a change in the median is in contrast to previous studies conducted in healthy volunteers [2] and cancer patients [3] where plasma concentrations of VEGF-A increased after drug administration. The reason for this difference remains unclear. Sample processing has been shown to have a large influence on the measured VEGF-A concentrations [5] and therefore the differences in handling of the samples in the four participating centers may have masked a potential pharmacological effect.

Maximum response of sVEGFR-2 and sVEGFR-3 was observed at the end of each cycle with concentrations of 52 – 94% and 32 – 96% of the corresponding baseline value, respectively (Figure 1B, C). All biomarker concentrations returned to baseline after two weeks “off treatment”. These observations were comparable to data previously published for various tumor types (mRCC, GIST, metastatic breast cancer, neuroendocrine tumor, mCRC) (1; 3; 6; 7). In comparison with the predictions of the PK/PD model developed for healthy volunteers, changes in the median of the soluble receptors relative to the baseline value tended to be greater in mCRC patients. Possible reasons for these observations, e.g. differences in treatment, treatment duration, and/or the influence of the tumor on biomarker response, as well as the effect of specific patient covariates (gender, age, weight etc.) on PK and PD will be further investigated.

Conclusions

Plasma concentrations of sunitinib and SU12662 seem to be comparable in mCRC patients and healthy volunteers when the differences in administered dose and treatment duration are considered. After one day “on treatment” the prediction interval of the pharmacokinetic model previously developed for healthy volunteers describes the concentrations in mCRC patients satisfactor-
rily. With the exception of VEGF-A, changes in biomarker levels relative to the corresponding baseline value were comparable to data previously observed in cancer patients with other tumor types. The available PK/PD models will be further developed to provide the basis for a PK/biomarker-guided dosing strategy.

References


**Choice and simulation of the randomization procedure for clinical trials**

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**Introduction**

If treatment arms are to be compared, an idealized clinical study should be randomized. The randomization process is the random allocation of patients to treatment arms and many different methods exist to carry out this task [1]. Each of these algorithms has its advantages and disadvantages depending on the study design. For example, some frequently used methods such as the block randomization and the urn model are used to obtain treatment arms with an equal number of patients. Other algorithms are rarely used and are more complex such as the minimization algorithm. Because of the diversity in the different randomization methods, it is sometimes difficult to choose a convenient randomization process for a given study design. A simulation procedure would therefore offer the opportunity to compare various randomization algorithms in advance and help to choose the best available method.

**Material and methods**

The web application RANDI2 [2, 3] – an online randomization tool – provides a flexible platform for diverse clinical studies, supporting common randomization algorithms. The methods offered are complete, truncated, block and biased coin randomization, urn model and biased coin minimization model [4]. Because of the different algorithms, the adaptable configuration of the study design – like freely configurable patient properties and stratification factors – and the open source nature of the project, RANDI2 is predestined to introduce a simulation component. Another reason to use RANDI2 is the application architecture which provides all necessary elements to integrate a new module. The design includes the programming language, JAVA, and a 3-layer application design. The layers are user interface, application logic and the persistence layer. A more detailed explanation of the architecture and the frameworks used can be taken from [5]. The fundamentals of the simulation are based on Monte Carlo methods. These are a class of algorithms which run many times to generate random samplings to execute subsequent statistical analyses. Examples for measures of statistical analyses are the mean and median of the patient size in the treatment arms after the random allocation.

**Results**

The simulation tool for the randomization system RANDI2 was developed to give an overview of possible patient distribution at the end of the study recruitment period applying various randomization and design assumptions. The determinants of the study design define the main parameters for the simulation of the randomization process including the variables taken into consideration such as patient properties, number of treatment arms, and the number of patients for each treatment arm. The other parameters are simulation specific such as the number of simulation runs and the maximum duration of the simulation as stop criteria. It is also possible to define a seed value to obtain repeatable results. Given the determinants, the simulation can be run using different randomization algorithms or the same algorithms with different parameters. For example it is feasible to choose block randomization with different block sizes and an urn model for a comparison. The choice of parameters can be changed to define several strata, and to assign special probability distributions – at the moment uniform and concrete (ratio) distribution are realized – and afterwards the simulation process has to run again. The simulation measures are deviation from minimum, maximum, mean, and median and marginal balance. The marginal balance is the key measure. It shows the balance between actual and planned subject size between the treatment arms. The minimum, mean and maximum marginal balances of the simulation runs are shown. “Better” re-
Results for the marginal balance are close to zero. Other measures concern the treatment arms and the subgroups, such as the minimum, maximum, mean and median of patients per treatment arm or per subgroup and treatment arm. The simulation results can be visualized and exported into HTML format. For small simulation sample sizes, it is also possible to collect raw data, including the patient properties and allocation results. To perform further statistical analysis, the results can be exported into comma separated value (CSV) format. The resulting simulation data are the basis for a comparison of several possible simulation settings, e.g. for parameters and stratifications as well as the algorithms used. For illustration a simple example will show the results of the simulation component. The sample trial has the following properties: (i) two treatment arms, (ii) 100 patients per arm, (iii) two trial sites and (iv) two patient’s properties (sex and fitness level 1 – 3), resulting in six subgroups. Uniform distributed patient properties and 10,000 runs are assumed as simulation parameters. The complete randomization, block randomization (block size = 8) and the biased coin minimization (p = 0.9) are compared. Complete randomization was selected as reference because it is the ideal randomization method. Table 1 shows the results of this simulation example. It can be recognized that the minimization causes the smallest marginal balance and the least difference in patient size per treatment arm, followed by the block randomization. In this example, the best algorithms of those selected are the minimization and the block randomization.

### Table 1. Results of a simulation randomization example with 10,000 runs for 100 patients for each of the 2 treatment arms with 2 internal patient properties resulting in 6 subgroups.

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>Min MB</th>
<th>Mean MB</th>
<th>Max MB</th>
</tr>
</thead>
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<td>0.25</td>
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<tr>
<td>Block randomization</td>
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</tr>
<tr>
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<td>0.03</td>
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<table>
<thead>
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<th>Arm name</th>
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<th>Max per cent</th>
<th>Mean PAT</th>
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<td></td>
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<td></td>
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<td>–25.00%</td>
<td>124</td>
<td>24.00%</td>
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<tr>
<td>a2</td>
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<td>–24.00%</td>
<td>125</td>
<td>25.00%</td>
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<tr>
<td>Block randomization</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>a1</td>
<td>94</td>
<td>–6.00%</td>
<td>105</td>
<td>5.00%</td>
<td>100.02</td>
</tr>
</tbody>
</table>

MB = marginal balance; PAT = patients.

## Conclusions

With the newly introduced simulation tool, integrated into RANDI2, there is now the possibility to test the randomization outcome of various study designs and randomization algorithms to be used. The simulation tool supports the choice of a suitable randomization procedure and its fine tuning and can help in understanding the behavior of various algorithms for a given study design. Furthermore, all the results can be visualized, compared, and exported for subsequent use.

## References


Anti-inflammatory and anti-cancer activities of essential oils and their biological constituents

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Introduction

Pancreatic cancer has two main problems: early diagnosis is difficult and there is a lack of effective treatments [1]. The cancer is resistant to most forms of treatment, such as chemotherapy, radiation, and combination therapies effective in other tumors [2, 3].

Natural products have been a continuous source of novel compounds for the treatment of numerous diseases, with natural products and their synthetic derivatives comprising over 60% of the approved anticancer drug candidates developed between 1981 and 2002. The pharmaceutical properties of aromatic plants are partially attributed to essential oils. Essential oils are natural, complex, multi-component systems composed mainly of terpenes [4].

In order to find essential oils with a potential anti-cancer or anti-inflammatory effect, we screened three pancreatic cell lines with several essential oils.

Material and methods

Essential oils

One example of an essential oil from several plants (Myrtaceae, Schisandraceae, Lauraceae, Asteraceae, Pinaceae, etc.) was included in the analysis. The essential oils are complex aromatic-smelling volatile mixtures of compounds having low molecular weights and diverse chemical structures. Predominant compounds are monoterpene hydrocarbons, sesquiterpene hydrocarbons, their corresponding oxidized products, phenylpropanoids, as well as minor amounts of diterpenoids and miscellaneous volatile organic compounds. They were isolated, identified, and characterized as described in Schitzeler et al. [5] and Mulyaningsih et al. [6].

Cell culture maintenance

Humans pancreatic cancer cell lines (BxPC-3, MiaPaCa-2) were cultivated in RPMI-1640 or in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen) and 100 Units penicillin/ml and 100 mg streptomycin/ml (Gibco). Primary human pancreatic ductal epithelial cells (HPDE-c7) immortalized by E6/E7 genes of human papilloma virus (HPV)-16, virus (HPDE-E6E7c7), were maintained in keratinocyte-SFM medium supplemented with bovine pituitary extract and epidermal growth factor (Gibco-BRL). The cell lines were maintained at 37 ºC in a 5% CO2 atmosphere and 95% humidity.

Cell viability test

The essential oils were dissolved on DMSO. Approximately 5 × 104 growing cells were placed in each well of a 96-well plate (Greiner, Frickenhausen, Germany) with 200 µl of medium and were allowed to adhere overnight. Cells were treated with each essential oil at a concentration of 100 µg/ml for 24 h. The viability assay was carried out using sulforhodamine B (SRB). The absorbance at 570 nm was measured in an ELISA microplate reader Tecan Infinite M200.

Those essential oils which produce an SRB lower than 50% in the cancer cell line and no effect or up 50% in the normal cell line were considered to be cytotoxic.

Reactive Oxygen Species (ROS) production

The antioxidant or ROS activity was measured using the dichlorofluorescein (DCFH2, Cell Biolabs) according to the manufacturer’s instructions for the kinetic assays. The fluo-
Table 1. Anti-cancer and anti-inflammatory effect of 50 different essential oils.

<table>
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<tr>
<th>HDPE-E6E7</th>
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<th>BxCP-3</th>
<th>MiaPaCa-2</th>
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</thead>
<tbody>
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<td>Viability*</td>
<td>Viability*</td>
<td>ROSS**</td>
</tr>
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</table>

*(% Control), NT* = No Test, ROS** = Reactive oxygen species, NOS*** = nitric oxide.
rescence production was read with a fluorometric plate reader Tecan Infinite M200 at 480 nm.

**Nitric oxide (NO) production**

The nitrate in the culture supernatants was measured by adding Gries reagent (AXORRA) according to manufacturer’s instructions. Incubation was for 24 h with 250 mg/ml of each essential oil.

**Measurement of caspase activities**

Cells were plated in 96-well plates at 5 × 10^3 growing cells. The essential oils at 250 mg/ml were incubated for 3 h and total caspase activities detected using the Caspase-Glo 3/7 assay kit (Promega) according to the supplier’s instructions.

**Statistical analysis**

The Student’s t-test and one-way ANOVA were used to determine the statistical significance of the difference between values of the various experimental and control groups. Data expressed as means ± standard error (SE) are taken from at least three-independent experiments performed with six replicates of each.

**Results**

**Cell viability test**

Of the essential oils examined, 5 showed cytotoxic activity in the MIAPaCa-2 cell line and 7 essential oils exhibited cytotoxic activity in the BxCP-3 cell line. All essential oils showed activities up to 50 % of viability in the HPDE-E6E7c7 cell line (Table 1).

**Reactive oxygen species (ROS) production**

The essential oils number 39, 38, 1, and 12, produced the highest amounts of ROS, while the essential oil number 25, 28, and 30 exhibited low activity with respect to control (Table 1).

**Nitric oxide (NO) production**

All the essential oils produced higher concentration of NO (Table 1).

**Measurement of caspase activities**

Essential oil number 22 was associated with the highest production of caspases, followed by the essential oils number 23, 49, and 15. The remaining 23 essential oils showed median caspase activity (Table 1).

**Conclusion**

The initial screening to investigate the anti-cancer activity in the 50 essential oils resulted in 10 potential candidates. The anti-inflammatory activity was investigated by measuring the production of ROS and NO. ROS are implicated in the cellular activity of a variety of inflammatory responses. Seven of the essential oils increased the level of ROS. The ROS activity is also implicated in the mediation of cell death.

On the other hand, all the essential oils tested produced high levels of NOS activity. These findings need to be corroborated with the activation of the iNOS pathway.

The classical apoptosis pathway, mediated by caspase activity, was positively activated by three of the 50 essential oils.

Further analyses are required to establish the mechanisms giving rise to these effects.

**References**

A preliminary report of a Phase II study of folinic acid, 5-fluorouracil, irinotecan (FOLFIRI) plus sunitinib with toxicity, efficacy, pharmacokinetics, biomarker, imaging data in patients with colorectal cancer with liver metastases as 1st line treatment – a study of the CESAR central european society for anticancer drug research – EWIV**

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**This extended abstract summarizes four lectures given by B. Moritz, M. Büchert, J. Arends and U. Jaehde in the Working Group Session “Phase I – III studies” during the 8th Annual Meeting 2010 of the Central European Society for Anticancer Drug Research (CESAR) held in St. Gallen, Switzerland, July 01 – 03, 2010.

Introduction

This investigator (KM) initiated Phase II trial (IIT) started in 2008. Inhibition of angiogenesis with bevacizumab has become standard treatment in combination with FOLFOX or FOLFIRI in patients with mCRC [1]. The rationale for this Phase II study came from a Phase I study demonstrating, that patients with mCRC can be treated very effectively and with acceptable toxicity with FOLFIRI plus sunitinib at 37.5 mg (instead of 50 mg) [2]. FOLFIRI was given in the conventional 2 weeks schedule (2qw) whereas sunitinib was given for 4 weeks with 2 weeks off. Therefore, one treatment cycle was defined as 3 times FOLFIRI given every 2 weeks (totally 6 weeks) with 4 week treatment with sunitinib followed by 2 weeks drug holiday. Nearly in parallel a large international multicenter randomized Phase III trial in patients with CRC was initiated comparing FOLFIRI ± sunitinib. It was our aim to elucidate the anti-angiogenic activity of sunitinib via Dynamic-Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI) and Dynamic-Contrast-Enhanced Ultrasound Imaging (DCE-USI; CEUS). Initially we set the DCE-MRI and DCE-USI measurements over the whole period of 2 cycles (= 12 weeks). Due to these rather sophisticated imaging techniques it was defined that only patients with at least one liver metastasis > 2 cm in diameter were included to be sure that with a very limited number of patients a maximum of relevant data would be produced. During treatment of the first 4 patients it was recognized that the target lesion in the liver became too small for evaluation by DCE-MRI and DCE-USI due to the highly effective treatment. The protocol was amended to evaluate these imaging parameters earlier than originally planned to ensure that the size of the liver metastases was not too small to be quantified. In Figure 1 the intense research program during the first 2 cycles is depicted.

Methods

No final data can be shown as the full biometric evaluation and report have not yet
been completed. Instead some important preliminary findings will be shown. 23 patients were included. From nearly all patients the toxicity data were documented. From most of the patient the concentrations of sunitinib, its main metabolite SU12662 and various biomarkers (VEGF-A, sVEGFR-2, sVEGFR-3) were measured in plasma. In the majority of the patients DCE-MRI data were taken, but not always four times as it was originally planned. Up to now, it was possible to evaluate the DCE-USI data in one sub-group of patients only. Three different sonographic machines in 3 different study sites were used. In order to perform a central evaluation of the raw data from the 4 participating centers with the software package Qontrast® from Bracco Inc., transformation of the video sequence in different formats (DICOM, AVI 1 and AVI 2) will need to be successfully completed.

### Results

23 patients received up to 8 treatment cycles (median 4 treatment cycles). The toxicity was significant. Neutropenia, leukopenia, thrombocytopenia, infection, hand & foot syndrome, rash, diarrhea, stomatitis and anal fissure were observed and led to dose reduction of the drugs applied. In 18 patients DCE-MRI data were generated. iAUC60 and Ktrans were decreasing in the majority of the patients but in some patients just the opposite was seen. In 18 patients the sum of the target lesion sizes were calculated by MRI. Here again the majority showed a decrease while in some patients an increase was observed (Figure 2). The final analysis will include the complete staging data (with CT scans). The DCE-USI (CEUS) data are currently not ready for analysis.

The pharmacokinetic data fit well to the PK evaluated in healthy volunteers [3]. Plasma concentrations of VEGF-A increased immediately after administration exhibiting a large inter-individual variability. Maximum...
response of the soluble receptor levels was observed at the end of each cycle with concentrations of 52 – 94% (sVEGFR-2) and 32 – 96% (sVEFGR-3) of the respective baseline values. All biomarker levels returned to baseline after 2 weeks “off treatment” [4]. Correlation analyses between PK/PD and DCE-MRI, response or toxicity are ongoing.

Conclusions

This IIT was challenging for logistical reasons and included a broad translational research program. Thus far, the analysis showed that standardization of each step within the study and at each center is essential for success. The drug combination of FOLFIRI and sunitinib is a very effective regimen (2 patients underwent potentially curative hepatic surgery), nonetheless, it is more toxic than initially thought and known from literature. In the meantime, the Phase III study was stopped based on a recommendation of the Drug Safety Monitoring Board (DSMB), as no advantage in antitumor efficacy could be shown. It seems that toxicity was remarkable, similarly as in our study.

Although anti-angiogenic effects could clearly be seen, this drug combination is associated with remarkable toxicity. Addition of an inhibitor of VEGFR-TK either to FOLFOX or FOLFIRI in treatment of patients with mCRC is obviously a difficult task [5] as some prominent drugs failed in similar situations.

Acknowledgment

The trial was supported by an IIT grant of Pfizer Inc.

References