Overview of presentations

The 17th meeting of the CESAR-APOH (Arbeitsgruppe Pharmakologie in der Onkologie und Hämatologie) was held at the Tumorbiology Center at the Albert Ludwigs University Freiburg, from 20 – 21 June, 2003. The interdisciplinary group APOH consists of clinical and pediatric oncologists, clinical pharmacologists, clinical pharmacists and chemists who meet once a year to discuss their latest scientific results. The APOH is part of the Central European Society of Anticancer Drug Research (CESAR).

The annual APOH meeting was embedded in the CESAR symposium “Novel Approaches for the Discovery of Anticancer Agents” which was organized by Prof. Dr. Heiner Fiebig, member of the scientific board of CESAR.

In the afternoon of June 20, the APOH meeting started with a workshop on population pharmacokinetics, moderated by Prof. Dr. Ulrich Jaehde (Bonn). The workshop consisted of 3 presentations given by Dr. Rüdiger Port (Heidelberg), Dr. Ralf A. Hilger (Essen) and Susanne Quellmann (Bonn). Three different software programs (NONMEM, Kinetica and WinNonMix) were used for analyzing the same set of data which had been sent from Prof. Jaehde to the presenters about 3 months before the workshop. The results as well as the handling of the 3 software programs were presented and the advantages and disadvantages of each program were discussed with the participants.

The second day started with the business meeting of the APOH. The present status of CESAR was described by Prof. Dr. Christian Dittrich (Vienna), the re-elected president of CESAR, reviewing the perspectives of this now established society within Europe. Furthermore, the chairman of CESAR-APOH, Prof. Jaehde, reviewed the first year of the group under his leadership. To get some closer relationship to the CESAR-AWO (Arbeitsgruppe Wirkstoffentwicklung in der Onkologie) it is planned to have a combined AWO-APOH meeting during the CESAR group meeting on February 20, 2004, in Frankfurt, with up to 4 presentations from both groups. The next annual APOH meeting is scheduled for June 18 – 19, 2004, in Rostock, organized by Prof. Dr. Gernot Hartung.

After the business meeting, new scientific results were presented in 15 oral presentations. A broad spectrum of topics was covered by the different speakers. Topics were the population pharmacokinetic project with doxorubicin and epirubicin, a new population pharmacokinetic project on oxaliplatin and recent results on drug monitoring, new drugs, surrogate markers, drug-drug interactions and multidrug resistance. The extended abstracts here summarize the content of 13 lectures.

The great success of the CESAR symposium with the embedded annual CESAR-APOH meeting will have a future. It is planned to organize such meetings on a regular basis every 2 years, the next in Vienna.

The APOH is open for all scientists working on pharmacological aspects in oncology and hematology. Further information regarding membership can be obtained from the chairman Prof. Dr. Ulrich Jaehde, Pharmazeutisches Institut der Universität Bonn, An der Immenburg 4, D-53121 Bonn, e-mail: u.jaehde@uni-bonn.de. Further information about CESAR can be seen in the internet: www.cesar-ewiv.org or obtained from the secretary of CESAR (CESARiat) e-mail: cesar@wienkav.at.
The influence of liver metastases on the pharmacokinetics of doxorubicin – a population-based pharmacokinetic project of the CESAR-APOH

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Introduction

Anticancer drugs are mainly metabolized by the liver, irrespective of the route of administration. Therefore, liver function, especially in patients with hepatic metastasis (filiae), has to be taken into account when drugs are administered which are primarily eliminated by the liver. Since damage to liver cells elevates concentrations of aspartate-amino-transferase (AST) and alanine-amino-transferase (ALT), and intrahepatic cholestase is associated with an increase in bilirubin, the serum concentrations of these parameters are used to estimate hepatic function. It is recommended that the dose of doxorubicin be reduced depending on the extent of serum AST and bilirubin elevation because an increase in toxicity of some anticancer drugs has been observed in cases of impaired liver function. There are no conclusive data, however, which can be used for calculating the required dose reduction, and recommendations which have been made so far are based on clinical experience.

The following study, carried out to investigate this problem, was a prospective, multicenter clinical trial in patients receiving doxorubicin for the treatment of various malignant diseases with and without hepatic metastasis. This was a project of the APOH, a working group of the Central European Society for Anticancer Drug Research (CESAR), held in Freiburg, Germany, June 18 – 21, 2003.

Patients and methods

A total of 203 patients (76 male, 123 female and 4 lacking information on gender) undergoing treatment with doxorubicin in a multidrug schedule in 3 different hospitals in Germany and Austria, were included in the study. The age range was 16.3 – 87.8 years (mean 52.9, median 53.9 years). The absolute dose of doxorubicin ranged from 18 – 150 mg (mean 73.6 mg, median 72.5 mg) and the infusion times ranged from 1 – 2,697 min (mean 54.2 min, median 12.5 min). Plasma concentrations of the parent drug and doxorubicinol were obtained at 3 different time points in each patient. The samples were taken between 0 and 30 min, 30 and 120 min, and 120 min and 48 h after the start of drug administration. Plasma concentrations were determined using HPLC with solid-phase extraction and fluorescence detection.

The Nonlinear Mixed-Effects Modelling program NONMEM, version V.1.1 [Beal and Sheiner 1999] was used for population pharmacokinetic analysis.
The initial exploration of the plasma concentration data revealed extremely high concentration measurements and excessive within-patient fluctuations which indicated that a relevant number of samples had been taken from the central line used for drug administration. Therefore, it was decided to eliminate all data from patients with a duration of infusion > 4 h, all samples taken during an infusion, and the first sample taken after the infusion in all patients. In addition, all patients with incomplete covariate data were also excluded. Because of the limited amount of data remaining, it was decided to include data from 2 previous clinical studies performed at institution 3 for which complete individual plasma concentration time profiles had been sampled. The final dataset analyzed included doxorubicin plasma concentrations from 181 patients (male = 69, female = 112) from the initial population pharmacokinetic study and serum concentrations from the 2 conventional pharmacokinetic studies involving 8 patients (male = 6, female = 2) in 1 case, and 6 patients (male = 2, female = 4) in the other. A further restriction in the data analysis was that only concentration data for the parent drug were used.

Results

The data were best fitted using a 3-compartment linear model. Despite the large interindividual variation, it was possible to show that clearance was related to AST, ALT, bilirubin in serum and the occurrence of filiae (Figure 1). Models based on the assumption of a linear dependence of clearance on the covariate were then fitted using each of the single variables AST, ALT, bilirubin and filiae. The goodness-of-fit for AST, bilirubin and filiae were about equivalent and superior to that for ALT in explaining at least part of the variation in clearance, with bilirubin slightly better than AST and filiae. The best model predicts a decrease in clearance of 6% for every 1 mg/dl increase in serum bilirubin. This is based on a mean clearance value of 54 l/h in a 70 kg patient and a serum bilirubin of 1 mg/dl.

Conclusions

Because patients with reduced liver function have only a limited capacity to clear drugs, administration of drugs metabolized and eliminated mainly by the liver is associated with an enhanced toxicity. Hence, dose reduction in accord with the degree of liver function impairment is recommended. This analysis shows that in the case of doxorubicin, elevated AST, bilirubin in serum and the presence of liver filiae are all associated with a decrease in clearance.

References

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Surrogate marker in clinical studies with anti-angiogenic drugs

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**Introduction**

Since the pioneering investigations of Folkman on the importance of neovascularization enabling tumor growth, an increasing number of anti-angiogenic agents targeting various mechanisms in tumor angiogenesis have entered clinical trials. These processes can be divided into pro- and antiangiogenic processes [Drevs et al. 2002].

Today, it is well-established that a solid malignant tumor relies on a blood supply in order to exceed the size of a few millimeters and to develop metastasis. This process involves deregulation of pro- and anti-angiogenic factors resulting in the stimulation of intracellular signaling pathways in surrounding endothelial cells and the final induction of vessel formation necessary for feeding the malignant cell clone [Folkman 1971].

New drugs targeting pathways involved in angiogenesis have shown promising results in preclinical studies where the anti-tumor activity is without side effects and the development of drug resistance. Despite this progress there is a need for a surrogate marker and this need first became apparent during the use of angiogenic agents in clinical phase I trials caused by the lack of activity even in high doses, missing toxicity as indicator for the optimal dose when testing cytotoxic agents or drug related toxicities at low doses raising the question of biological activity. Therefore, the development of a relevant surrogate marker has been delayed and most of those which have been developed have not yet been validated and remain at the experimental stage [Mross et al. 2002].

**Imaging techniques**

Imaging techniques are used to identify directly or indirectly the influence of a compound on the targeted tumor vessel. Dynamic contrast enhanced MRI (dceMRI), a technique which indirectly reflects changes in the vascular bed of the tumor, has become a gold standard in phase I trials with anti-angiogenic agents. The method involves the use of gadolinium, a small molecular weight contrast agent that diffuses through vessels into the extracellular space. $K_{\text{trans}}$ describes the rate of rediffusion from the extracellular space back to the vessels and is mainly used to assess permeability and extravasation in various tissues. Although the above technique has been applied in many clinical trials, a relationship between $K_{\text{trans}}$ and dose or between $K_{\text{trans}}$ and response has only been seen in the case of a few drugs. Some treatment centers do not have access to dceMRI facilities. Furthermore the methods which are used tend to give highly variable data [Thomas et al. 2003a].

Another imaging method which is employed is the color Doppler imaging (CDI) technique which determines blood flow velocity in vessels of liver metastases as surrogate for drug-related effects on tumor vessels. Although positive correlations between CDI and vessel density have been described in animal trials, no significant data have been published so far from its use in phase I clinical trials with angiogenesis inhibitors. The results may be affected by differences in expertise between examiners and alterations in blood pressure [Drevs et al. 2000]. However, since this method is available in all treatment centers it deserves further evaluation.

**Soluble marker**

Monitoring a soluble marker present in peripheral blood allows the evaluation of drug effects at the systemic level. Investigations can focus on either the target itself and/or the molecules that are affected by inhibition at the target site or the course of the disease. Estimation of the blood levels of key substances in tumor angiogenesis can provide valuable information on drug effects even
When low doses with no or only minor toxic side effects are used. Evaluation of a soluble marker and concomitant pharmacokinetic profiling of the test drug may reveal physiologically relevant effects which correlate with the clinical data. A “response” in terms of change in the levels of soluble marker can serve as an early indication of a change in the course of tumor disease. Which combination of markers to use in a particular investigation will depend on basic knowledge on biological crosstalk associated with the targeted mechanism, subsets of factors relevant to tumor progression present in the bloodstream and the availability of analytical test systems. Because of the general acceptance of soluble factors closely associated with effective drug dosages and markers, an increasing number of clinical phase III trials include the measurement of tailored marker subsets. A detailed analysis of the significance of individual markers however, is necessary in order to streamline this process so that soluble marker data can be seen as a valuable addition to other data such as pharmacokinetic profiling, toxicity assessment, imaging techniques etc.

It is known, for example, that various molecules associated with endothelial cells are cleaved off and can be detected in the plasma of patients. sE-selectin, an endothelial cell adhesion molecule, mediates the initial step of leukocyte adhesion to activated vascular endothelium. sE-selectin may support tumor angiogenesis and the adhesion of tumor cells to endothelial cells at distant sites. The transmembrane tyrosine kinase TIE-2, the receptor for the angiopoietins-1 and -2, has been shown to be involved in angiogenic processes and is ubiquitously expressed on endothelial cells throughout the vasculature. In conditioned media of PMA-stimulated endothelial cells, a soluble form of this receptor comprising parts of the extracellular domain can be detected. The soluble form of TIE-2 (sTIE-2) could also be detected in human biological fluids such as sera and plasma from healthy controls. Similarly, the extracellular domain of VEGF-R1, an endothelial cellspecific transmembrane tyrosine receptor for the potent angiogenic factor, vascular endothelial growth factor (VEGF), can be cleaved from activated endothelial cells and is detectable in plasma from cancer patients. Thus, plasma levels of endothelial-associated proteins such as soluble E-selectin, TIE-2, and VEGF-R1 may give a measure of the amount of newly forming vessels in a patient's tumor. VEGF and basic fibroblast growth factor (bFGF) are potent endothelial cell-specific angiogenic factors with a key role in tumor angiogenesis. They are produced by tumor cells as well stromal cells and hypoxia is a potent stimulator of VEGF. The concentration of these angiogenic factors in the plasma may depend on the hypoxic status of a tumor.

**Future developments**

With increasing knowledge on the mechanisms of tumor angiogenesis new and potential useful methods are being used in clinical trials. The most promising developments are the detection of circulating endothelial cells and the wound angiogenesis assay.

Circulating endothelial cells can be detected in peripheral blood using specific antibodies and FACS analysis. Because of differences between the methods used results obtained are not always comparable. New antibodies will allow the detection of a larger number of cells in a smaller amount of blood [Thomas et al. 2003b].

The wound angiogenesis assay provides information on blood vessel changes before and during anti-angiogenic therapy and tissue can also be obtained for immunohistochemical analysis [Lockhart et al. 2003]. This is achieved using a punch biopsy of the skin of the lower arm and this is repeated after 14 days in order to obtain granulation tissue (“overpunch”). These methods, for the purpose of evaluation, have been included in ongoing clinical trials. A main disadvantage, however, is that the analysis involves healthy vessels and tissue and not tumor vessels and tumor tissue.

**Results from anti-angiogenic phase I drug trials**

Clinical phase I trials targeting the process of tumor angiogenesis substantiate the benefit of adding a surrogate marker to classical pharmacokinetics and the evaluation of toxic side effects.

When targeting the VEGF-VEGF-R-system using VEGF receptor tyrosine kinase inhibitor, serial VEGF and bFGF measurements demonstrated a correlation between prompt level changes and an improvement in the disease. The short-term effect after the (first) administration of the drug, in particular, was marked and to some extent, enabled early discrimination between responder and non-responder. These findings were supported by imaging techniques like dceMRI. Here, a decrease in $K_{trans}$ on days 2 and 21 after application of a VEGF-receptor tyrosine kinase inhibitor correlated with pharmacokinetic data as well as clinical outcome [Lockhart et al.]. Nevertheless, this kind of positive correlation involving a surrogate marker is not seen with all compounds.
Conclusions

Surrogate markers will remain secondary endpoints in clinical phase I trials with anti-angiogenic compounds as long as dose limiting factors like toxicity and pharmacokinetic parameters retain their importance. However, in cases of missed doses and early dose limitations, surrogate markers will be needed in order to make definitive decisions on the value of a drug undergoing development. In this regard, the optimal surrogate marker has to be defined for each individual compound.

In order to evaluate a relevant surrogate marker within a time frame short enough for the data to be used for dose adjustment and determination of the optimal dose is the challenge for future study designs.

References


DCE-MRI in clinical trials: data acquisition techniques and analysis methods

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Introduction

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is a method of imaging the physiology of the microcirculation [Mross et al. 2002]. A series of recent clinical studies have shown that DCE-MRI-based measures correlate well with tumor angiogenesis [Morgan et al. 2001]. The DCE-MRI technique is based on the continuous acquisition of 2D or 3D MR images during the distribution of an intravenously administered paramagnetic contrast agent bolus. The contrast agent is a gadolinium- (Gd) based chelate which is able to enter the extravascular extracellular space (EES) via the capillary bed. The pharmacokinetics of Gd distribution are modeled by a 2- or multi-compartment model and has been shown to be a useful predictor of the biological response of angiogenesis inhibitors [Drevs et al. 2001].

Data acquisition

The evaluation of Gd pharmacokinetics affords accurate data acquisition techniques depending on the tumor entities and the body region examined. Tumors of the breast and head have been most extensively examined so far and are characterized by contrast kinetics with a slow time constant and the lack of body motion during acquisition. Therefore, multislice 2D or 3D gradient-echo (GRE) MRI techniques with moderate temporal resolution are applicable where a linear dependency between tissue signal intensity and the Gd concentration is valid in the low flipangle regimen [Evelhoch 1999].

Tumors of the liver or lung require optimized protocols to meet the particular demands. Therefore, imaging of a single 2D slice with a fast data acquisition period to freeze breathing motion is advisable. Moreover, an increased image contrast between tumor and healthy tissue, for a more accurate tracing of the tumor during breathing, can be introduced by the use of inversion recovery techniques.

With the recent development of ultra-fast gradient systems by MR manufacturers, fast T1 quantitation techniques using the trueFISP sequence have become feasible [Scheffler 2001]. Due to saturation effects, T1 quantitation using GRE read-outs experience substantial underestimation when multiple images after the inversion pulse are read-out. This behavior cannot be observed for the trueFISP sequence where an analytical relationship between the signal intensity and successive excitations exists [Scheffler 2003]. This particular signal behavior is valid for a broad range of flipangles and therefore a high flipangle can be applied offering a high signal-to-noise-ratio in the single images and a more accurate T1 and hence concentration quantitation.

In the application of the IR trueFISP technique for liver and lung tumors, we use a high temporal resolution quantifying T1 every 3 s and acquiring up to 10 images at inversion times between 50 and 1,850 ms. A slice thickness of 10 mm is used and inplane resolution is 3.1 mm. The slice is positioned such that the tumor is cut in its biggest extension and the slice contains a piece of pathway of the descending aorta to measure the aortic signal. The assumption that through plane motion of the tumor can be neglected is fulfilled in the most cases where the slice is acquired in a coronal oriented view.

Data analysis

Parameters providing information about microcirculation can be derived from the
Results from an antiangiogenic Phase I drug trial

In our hospital, we have established a clinical Phase I unit with 2 MR scanners dedicated exclusively for research (www.mrdac.com). In collaboration with the tumor biology center (www.tumorbio.de), our trueFISP DCE-MRI protocol is currently applied in 4 clinical Phase I antiangiogenic trials. An example monitoring the therapeutic effect of the antiangiogenic drug is shown in Figure 1. The concentration curves are taken at 3 different early time points, prior to drug administration (d0), at day 2 (d2) and day 7 (d7), respectively. The curve values at day 7 are substantially decreased in comparison to the curves taken from earlier time points indicating an early tumor response to drug activity. The lower concentration values are concordantly reflected in significant lowered values for Ktrans (2.77 at d7 vs. 3.8 at d0 (1/min)) and iAUC (18.3 at d7 vs. 22.4 at d0 (mmol/l×s) for 60 s after onset of contrast agent uptake).

Conclusions

DCE-MRI is a valuable tool to assess microcirculation in tumors in clinical antiangiogenic trials. The inversion recovery trueFISP technique offers an accurate evaluation of pharmacokinetic parameters of lung and liver tumors. Compared to more conventional approaches [Evelhoch 1999] it offers higher sensitivity at a reasonable temporal resolution (3 s). Improved volume coverage offered by multislice- or 3D-acquisition schemes are applicable only for slowly perfused lesions such as in MR mammography. But this techniques may be problematic for abdominal metastasis, where the bolus passage time can be short.

With respect to deriving stable and robust surrogate parameters, the pharmacokinetic model used in the analysis of the signal time curves should be as simple as possible but still give an appropriate description of the local hemodynamics. This may be difficult in situations with complex regional blood supply, where data-driven analysis may yield more robust results.

References

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Cerebrospinal fluid pharmacokinetics after different dosage regimens of intraventricular etoposide

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Etoposide pharmacokinetics after intraventricular administration

Introduction

Etoposide, a semisynthetic podophyllotoxic derivative, exhibits significant cytotoxic activity against a variety of different tumor cells including medulloblastomas, primitive neuroectodermal tumors and neuroblastomas [Hill and Whelan 1981, Tomlinson et al. 1991]. However, even after high doses of systemic intravenous administration, rather low CSF levels (0.1 – 0.2 μg/ml) were reported in CSF [Postmus et al. 1984] which is mainly attributed to extensive plasma protein binding and rapid elimination of etoposide from the CSF. In vitro studies in human neuroblastoma cells revealed that a 24-h exposure to 0.08 μg/ml etoposide resulted in 70% growth inhibition [Hill and Whelan 1981].

Intraventricular administration of etoposide has been introduced to increase etoposide concentration in tumors which are located in or close to the CSF space by using a relatively small drug dose. Van der Gaast et al. [1992] reported the intra-CSF administration of etoposide once daily and every 12 h for 5 consecutive days in 2 patients. Later, our group showed that the injection of 0.5 mg etoposide every 12 h for 5 consecutive days, 7 patients (total number of cycles: 23) were treated with 0.5 mg etoposide every 24 h for 5 consecutive days and 2 patients (total number of cycles: 11) were treated with 1 mg etoposide every 24 h for 5 consecutive days. Serial CSF samples were drawn at the 1st day of treatment. In addition, samples were collected before and 0.25 h after each administration to determine peak and trough levels during the 5-day treatment. The samples were collected and kept at a temperature below –20 °C until analysis.

Etoposide levels in CSF were determined by reversed-phase high-performance liquid chromatography (HPLC) using a modification of an analytical method established in our laboratory for the analysis of plasma ultrafiltrate [Reif et al. 2001]. Briefly, a Hypersil ODS RP-18 column was used as stationary phase. The mobile phase consisted of methanol/0.01 M disodium hydrogenphosphate (Na2HPO4) in a ratio of 52 : 48 (v/v) and was adjusted with 85% phosphoric acid (H3PO4) to pH 6.0. Flow rate was set at 0.7 ml/min. Etoposide was quantified by electrochemical detection using a dual electrode at potentials of 100 mV and 500 mV. Between-run precision as coefficient of variation ranged from 1.7 – 12.1% and accuracy as relative error ranged from –9.97 to +3.10%. A 2-compartment model with zero-order input and first-order elimination from the central compartment was applied for parameter estima-
tion using the software program SipharmWin™ (release 1.14, Simed, France) and a Bayesian approach. The pharmacokinetic parameters such as the area under the curve (AUC), clearance (CL), the volume of distribution at steady state (Vss) and terminal half-life (t1/2z) were calculated based on the best-fitted curve.

**Results**

The pharmacokinetics of etoposide in CSF was best described by a linear 2-compartment model. Figure 1 is an example of a CSF concentration-time profile from a representative patient receiving the first cycle. The peak concentrations (4.90 ± 0.91 µg/ml, 11.09 ± 5.00 µg/ml and 19.97 ± 6.42 µg/ml) and the total AUC (98.86 ±18.24 mg × h/l, 131.98 ± 41.49 mg × h/l and 176.02 ± 45.16 mg × h/l) increased with the administered dose (0.25 mg q 12 h, 0.5 mg q 24 h and 1 mg q 24 h, respectively). Accordingly, pharmacokinetic parameters did not differ between the 3 different dosage regimens. Mean CL (0.56 ± 0.25 ml/min, 0.37 ± 0.22 ml/min and 0.51 ± 0.15 ml/min), Vss (0.14 ± 0.05 l, 0.11 ± 0.06 l and 0.14 ± 0.04 l) and t1/2z (7.20 ± 0.37 h, 7.02 ± 0.64 h and 7.06 ± 0.81 h) were in the same range for all groups of patients.

**Conclusions**

Etoposide exhibits linear pharmacokinetics which is best described by a 2-compartment model. The pharmacokinetic parameters do not seem to be dose-dependent in the range of doses investigated. All mean minimum concentrations are above the cytotoxic concentration of etoposide established in neuroblasticoma cells (0.08 µg/ml). Our data can be used to simulate concentration-time profiles for other dosage regimens. Clinical efficacy and toxicity of intraventricular etoposide will be investigated within a Phase II study.

**References**


**Figure 1.** Concentration-time profile in a representative patient (receiving 0.5 mg q 24 h) analyzed by using a 2-compartment model. The line represents the best curve fit.
Introduction

Despite the long-term clinical use of mesna as an uroprotective agent in oxazaphosphorine-containing chemotherapy protocols there are surprisingly few clinical studies addressing its influence on endogenous thiol-metabolism and the efficacy of alkylating drugs. The use of cysteine as a cyto- and uroprotective agent was soon abandoned on discovering that its tumor-protective action could not be demonstrated in preclinical studies. This was explained by the very low intracellular uptake of mesna which is due to its highly polar structure. However, low molecular weight thiols are highly interactive and capable of liberating reduced thiols from disulfides by generation of mixed disulfides. These effects could be demonstrated for mesna in preclinical and clinical studies which showed an increase in cysteine concentrations after application of mesna to cystine-containing media and plasma. The magnitude of this effect and its dependency on different application schedules of mesna was addressed in this study.

Mesna is able to induce cystinuria, and this is the most probable reason for the observed depletion in cysteine plasma levels. Whether the uroprotective effect of mesna is the sum of this cystinuria and mesna itself, has not yet been clarified. Therefore concentrations of mesna and cysteine in urine and plasma of these patients were measured.

Methods

Whole blood samples of mesna and cysteine and their disulfides were drawn at 0, 5, 10, 15, 30 minutes, 1, 1.5, 2, 4, 6, 8, 12, 24 hours on day 1 and 3 of the chemotherapy and immediately stored on ice. The samples were centrifuged at 6,000 rpm for 3 min. For total thiols, 100 ml of the supernatant plasma were incubated with 10 ml of 12 mmol dithiothreitol for 45 min at 37 °C, then for 20 min with 10 ml of 18.5 mmol thiolyte and deproteinized with 20 ml of 20% perchloric acid. After centrifuging at 6,000 rpm for 1.5 min, the supernatant was frozen immediately at –20 °C. Reduced thiols were processed as mentioned above, omitting the dithiothreitol incubation. Urine samples were collected as 12-h specimens on the day before the therapy started and as 4-h specimens on day 1 and 3 of the chemotherapy and frozen immediately at –20 °C. Further processing was executed as mentioned above after diluting 1 : 5 with distilled water. The fluorescent-labeled thiols were analyzed using high-performance liquid chromatography (HPLC). The disulfide forms of the thiols were calculated as [thiol]total [thiol]reduced.

Results

Eighteen patients entered the study protocol and 8 of them received the scheduled 2 cycles of chemotherapy. Reasons for drop-out were progression of the disease, drug toxicity and patient refusal to participate.
All patients showed a rapid rise in plasma cysteine levels after mesna application was started. The magnitude of this effect differed depending on the mesna application schedule used. Peak concentrations on bolus infusion of mesna were up to 4 times those of baseline and concentrations were up to twice those of baseline after 5 min on day 1 following continuous infusion. On day 3, peak concentrations up to 3 times those of baseline were only observed on bolus infusion, whereas there was no rise in cysteine levels on continuous infusion. (Figure 1). The rise in cysteine concentration was seen at every bolus application of mesna but the peak levels showed a constant decline (data not shown).

Plasma cystine levels changed in the opposite way. On bolus application of mesna there was a stepwise decline on day 1 to about 50% of baseline levels 10 min after the first bolus. In contrast, on continuous infusion of mesna there was a steady decline in plasma cystine levels to 50% of baseline at 4 h. On day 3 cystine concentrations were lowest in both arms of the study with values falling to about 25% of initial concentrations (Figure 2).

Mesna concentrations after bolus application always rose quickly to 175 μmol/l after 5 min. On continuous infusion, a steady state of 10 μmol/l was reached after 1 h (data not shown).

Cysteine concentrations in urine on bolus infusion of mesna rose to 500 μmol on day 1 and day 3. The mesna concentrations in urine after bolus infusion on day 1 were up to 1,000 μmol and on day 3 up to 2,000 μmol. On continuous infusion we observed the same concentrations of mesna in urine but cysteine concentrations increased up to 650 μmol (data not shown).

Conclusions

The results of our presented study confirm previous preclinical and clinical findings showing that mesna influences endogenous thiol-metabolism. We could demonstrate a considerable increase in cysteine plasma levels despite the fact that cystine plasma levels fell continuously. The most probable explanation for both effects is the interaction of mesna and cystine resulting in the liberation of cysteine while generating a mixed disulfide of mesna-cysteine. This effect was dependent on the application schedule of mesna where there were more marked changes on bolus application (most probably related to the resulting higher concentration of mesna-) and the baseline cystine levels showing a smaller rise at day 3 when cystine levels were lowest due to the foregoing cystinuria.

Although not tested in this study, the rise in the cysteine concentration is in a range where an effect on the anti-tumor efficacy of oxazaphosphorines cannot be ruled out completely. In contrast, the plasma cystine depletion by prolonged mesna application could enhance cytotoxic effects of alkylating drugs. On the basis of these results we conclude that the continuous infusion of mesna is a superior therapeutic schedule.

The findings from the urinary measurements indicate that cysteine has a confounding effect on the uroprotective action of mesna.
The role of hepatic Mrp2 in the interaction of flavopiridol and bilirubin: impact on therapy

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Interaction of flavopiridol with bilirubin

Introduction

Flavopiridol (FLAP; NSC 649890, L86-8275, HMR 1275) is a selective inhibitor of cyclin-dependent kinases (cdk1, cdk2, cdk4, cdk7) presently undergoing clinical phase II trials as an anticancer agent. FLAP is extensively metabolized in the rat liver to FLAP-5 glucuronide (M1) and FLAP-7 glucuronide (M2), which are mainly excreted into bile [Jäger et al. 1998]. Pronounced glucuronidation followed by biliary elimination could also be observed in cancer patients as indicated by high glucuronide levels in plasma and enterohepatic circulation [Lush et al. 1997]. Moreover, the main side effect, diarrhoea, is also linked to biliary excretion of FLAP glucuronides [Innocenti et al. 2000]. Another toxicity of note during FLAP treatment is the induction of reversible conjugated hyperbilirubinemia (grade 2 or greater). It was observed in up to 22% of patients at doses of 78 mg/m²/day × 3 and lasted less than 48 hours post infusion [Senderowicz et al. 1998]. Conjugated bilirubin excretion into bile is mediated with high affinity by the ATP-dependent transporter Mrp2. Hence, our hypothesis was that FLAP glucuronides may also be actively transported across the canalicular membrane into bile via Mrp2. This hypothesis is supported by previous studies from our lab showing a clear preference of Mrp2 for the biliary excretion of glucuronides from a structurally similar flavonoid, genistein [Jäger et al. 1997]. FLAP glucuronides may also be competitively inhibited by Mrp2. Therefore, our hypothesis was investigated using an isolated perfused rat liver model. In Mrp2-deficient TR-rats, the biliary excretion of FLAP glucuronides into bile was significantly reduced compared to wild-type rats (Figure 1). This indicates that in control rats, M1 and M2 are almost exclusively eliminated into bile by Mrp2.

Results

Using an isolated perfused rat liver model of Mrp2-deficient TR-rats, we found that the biliary excretion of the FLAP glucuronides metabolites M1 and M2 was reduced to 4.3% and 5.4% compared to Wistar rats (Figure 1). This indicates that in control rats, M1 and M2 are almost exclusively eliminated into bile by Mrp2.

Conclusion

FLAP glucuronides may compete with Mrp2-specific substrates, resulting in a reduced biliary secretion of bilirubin and a consequent decrease in the clearance of FLAP glucuronides.

Key words
flavopiridol – metabolism – Mrp2 – bilirubin

This extended abstract summarizes a lecture given by W. Jäger during the Annual Symposium of the Working Group for Pharmacology in Oncology and Hematology (APOH) of the Central European Society for Anticancer Drug Research (CESAR), held in Freiburg, Germany, June 20–21, 2003.

Received August 19, 2003; accepted August 21, 2003.

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elimination of bilirubin glucuronides by 54.23% (from 2.45 ± 0.32 to 1.12 ± 0.39 nmol/g liver/min (Figure 2). After withdrawal of FLAP from the perfusion medium, the biliary excretion of glucuronidated bilirubin rapidly recovered within about 10 min to reach levels before FLAP administration. To determine whether FLAP also inhibits biliary excretion of bilirubin at a concentration of 5 μM, similar experiments were performed. Fifteen min after the addition of FLAP, bilirubin excretion declined by 29.8% (from 1.97 ± 0.41 to 1.38 ± nmol/g liver/min). Later, when the FLAP addition was stopped, bilirubin excretion recovered in about 20 min to control (pre-FLAP) levels. Thus, the inhibitory effect of FLAP was again completely reversible (data not shown).

Conclusions

In conclusion, biliary excretion of FLAP glucuronides is mediated almost exclusively by Mrp2 in normal rats. This can explain the decrease in conjugated bilirubin excretion during FLAP perfusion and may also apply to human liver. Therefore, conjugated serum bilirubin should be monitored under FLAP therapy.

Acknowledgments

This project was supported by grants from the Jubiläumsfonds der Österreichischen Nationalbank (7935 and 9894). S.A. thanks the Austrian Academy of Science for a scholarship.

References


Figure 1. Cumulative secretion of FLAP, M1 and M2 into bile of Wistar and TR- rats; * = p < 0.05 significantly different from control.

Figure 2. Effect of 5 μM and 30 μM FLAP on the biliary excretion of 5 μM bilirubin in the isolated perfused rat liver of Wistar rats. After achieving a constant biliary excretion of bilirubin (t = 20 min), 5 μM or 30 μM FLAP was applied for 15 min.
Assessment of platinum sensitivity in human tumor cells

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Introduction

Cisplatin is a widely used platinum complex with high cytotoxic activity against several tumors. A cisplatin-based chemotherapy can be impaired by existing or acquired resistance [Akiyama et al. 1999]. Therefore, it is of interest to investigate mechanisms leading to cisplatin resistance and to assess parameters influencing platinum sensitivity of human tumor cells. The cytotoxic effect of cisplatin is based on the formation of platinum-DNA adducts which can be influenced by cisplatin uptake, efflux and intracellular glutathione concentration. Moreover, platinum-DNA adduct levels strongly depend on DNA repair rate [Jordan and Carmo-Fonseca 2000]. The aim of this study is to characterize cisplatin sensitivity of tumor cells by means of the above-mentioned parameters using two cisplatin-sensitive and -resistant human tumor cell lines.

Methods

Two pairs of parental and cisplatin-resistant human tumor cell lines were used: the testicular carcinoma cell line 2102/2102KlB and the ovarian carcinoma cell line A2780/A2780cis. The respective resistant cell lines were generated by repeated cisplatin exposure. Cells were cultured in RPMI medium (supplemented with 10% fetal calf serum, 3 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin) at 37 °C in humidified air with 5% CO₂. For the experiments, 10⁵ – 10⁷ cells were incubated with cisplatin (10 – 125 μM) up to 4 h and subsequently incubated up to 4 h with drug-free medium to study DNA repair. Cytotoxicity (IC₅₀ values) was ascertained by using the MTT assay [Mosmann 1983]. DNA platination was measured by using a previously published method [Kloft et al. 1999] adapted to tumor cells: DNA concentration was measured by UV photometry after isolation with solid phase extraction (QIAamp™, Qiagen, Hilden, Germany) and platinum concentration was measured by flameless atomic absorption spectrometry (FAAS). Based on these 2 concentrations, the platinum-nucleotide ratio (Pt atoms: 10⁴ nucleotides) was calculated in order to characterize DNA platination. The intracellular glutathione concentration was quantified by capillary zone electrophoresis (CZE) and laser-induced fluorescence (LIF) detection [Parmentier et al. 1999].

Results

For the testicular carcinoma cell line 2102, IC₅₀ values were 2.39 ± 0.31 μM in the resistant and 1.22 ± 0.10 μM in the parental cell line. According to this, the IC₅₀ ratio resistant/sensitive was 1.9. For the ovarian carcinoma cell line A2780, IC₅₀ values were 13.20 ± 5.75 μM and 2.46 ± 0.49 μM with an IC₅₀ ratio resistant/sensitive of 5.4. The DNA platination in tumor cells was concentration-dependent (Figure 1) and time-dependent over the investigated range. The platinum-DNA adduct formation differed between sensitive and resistant cells (Figure 1). The platinum-nucleotide ratio in 2102 was in average 1.7-fold higher and in A2780 5.4-fold higher in sensitive cells compared to their respective resistant sublines. These ratios correspond to the ratios of IC₅₀ values. The CZE method to quantify reduced glutathione in cultured cell lines was validated and applied to our cell lines. Intracellular glutathione concentrations were up to 3 times higher and – as first results let assume – DNA repair rate was faster in resistant cells.

Conclusions

All parameters studied were obviously different in cisplatin-resistant cell lines. In the investigated cell lines, the average plati-
num-DNA adduct ratio reflects the IC50 ratio (resistant/sensitive). This result indicates the importance of DNA platination for the characterization of platinum sensitivity of tumor cells. Beside the DNA platination, other parameters seem to be related to cytotoxicity. Future studies will reveal which parameters are most predictive for cytotoxic activity and can be used for a quantitative assessment of platinum sensitivity of human tumor cells.

Acknowledgment

Supported by the Deutsche Forschungsgemeinschaft (GRK 677).

References

Introduction

More than 1,000 different kinases are involved in the transduction of outer cellular signals to the nuclei, regulating growth, differentiation and death. The mitogen-activated protein kinases (MAPKs) are the heart of this signal transductional network. Although activated in the cytosol, the MAPKs translocate to the nucleus and phosphorylate a large number of nuclear proteins. With the investigations on Ras-transmitted extracellular growth signaling, the MAPK pathway has emerged as the crucial route between membrane-bound Ras and the nucleus.

Ras initiates this pathway by recruiting Raf kinase to the plasma membrane, where it is further modified for full activation. Mutations of the Ras oncogene have been linked to tumor progression and correlated to increased risk of cancer recurrence and death. The frequencies of Ras mutations in solid human tumors are given in Table 1. Antisense and dominantly negative experiments have demonstrated that eliminating Raf kinase function reverses the Ras-transformed phenotype.

The MAPK pathway represents a cascade of phosphorylation events including 3 pivotal kinases, namely Raf, MEK (MAP kinase kinase), and ERK (MAP kinase). These kinases offer new opportunities for the development of novel target-specific anticancer drugs, probably with a spectrum of lower toxicity compared to conventional chemotherapeutic agents.

A number of drugs inhibiting Ras, Raf or MEK, are currently under clinical investigation. Among these, BAY 43-9006, a novel potent and orally active inhibitor of Raf kinase and thus directed toward a specific molecular target misregulated in many tumors, has now reached phase II clinical studies. It was the purpose of the presented study to develop a method for the quantification of the inhibitory potency of this new compound, measuring the phosphorylated (activated) ERK as a biomarker.

Methods and results

Evidence of Raf kinase activation was measured by phorbol myristate acetate (PMA) stimulated ERK phosphorylation at 2 different dosages, 80 and 400 nM, respectively. Therefore, blood was withdrawn into EDTA container, lymphocytes were isolated using a density gradient centrifugation method (d = 1.077) and aliquots of either the whole blood or isolated lymphocytes were stimulated in the presence or absence of the Raf kinase inhibitor (RKI). For validation experiments, the tosylated form of the inhibitor was used, BAY 54-9085. The stimulated cells were divided for FACS and Western blot analyses, respectively.
FACS was performed using dual color expression of CD7 (PE) and goat anti-rabbit IgG (FITC) against phospho-p44/42 MAP kinase (ERK1/2). For validation of the obtained results, the same antibody was used for corresponding Western blot analyses. Specifically, about 300 blood samples from 30 volunteers were analyzed in the absence and presence of PMA for the development and validation of the biomarker assay. We detected a circadian rhythm in phosphorylation of ERK1/2 proteins after PMA stimulation. Analyzing the measured values, we were able to describe a sinus-like distribution of the maximum inducible ERK1/2 phosphorylation at 12 o’clock (midday), with a coefficient of correlation $r^2 = 0.70$. The unstimulated controls demonstrated no circadian rhythm with a mean and standard deviation of 5 ± 5% of activated (positive for phosphorylated ERK1/2) CD7-positive lymphocytes. Comparing different time windows, there was a significantly (p < 0.001) higher PMA-inducible ERK1/2 phosphorylation between 11 and 13 o’clock, compared to the time windows 8 – 10 and 15 – 17 o’clock (81 ± 28 and 46 ± 21), respectively.

Conclusions

We could demonstrate that biomarker measurements could be complicated by circadian variability of the specific molecular target. Extracellular signal-regulated kinase (ERK) and p38 are members of the mitogen-activated protein kinase (MAPK) family. Their closely related cellular kinase functions are probably identified only in part. In a wide range of animal clock structures, ERK plays an important role in the circadian time-keeping mechanism. This function seems to be highly conserved during the evolutionary process among animal species. Considering the observed circadian rhythm in this study, phosphorylated ERK1/2 may serve as a biomarker for drugs targeting the MAP kinase cascade. However, the demonstrated circadian regulation demands strict protocols for the realization of biomarker analyses.

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Williams JA, Su HS, Bernards A, Field J, Sehgal A 2001 A circadian output in Drosophila mediated by neurofibromatosis-1 and Ras/MAPK. Science 293: 2251-2256
Antitumor effect and potentiation or reduction in cytotoxic drug activity in human colon carcinoma cells by the Raf kinase inhibitor (RKI) BAY 43-9006

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Introduction

Proliferation, differentiation, survival and apoptosis of all eukaryotic cells are controlled by a highly interactive network of protein kinases and other signal messengers. Many receptor tyrosine kinases and cytokine receptors in association with G proteins are known to activate intracellular protein serine/threonine kinases termed mitogen-activated protein kinases (MAPks). Raf kinase plays a central role in oncogenic signaling and acts as a downstream effector of Ras in the MAPks pathway. Experimental evidence supports a direct role for Raf kinase in the development and maintenance of human malignancies. For instance, the Raf kinases are the direct downstream mediators of the Ras proteins, whose oncogenic version is associated with approximately 30% of human solid tumor types. Raf mutations, especially mutations of the B-Raf gene, were detected in a wide range of human tumors and in 66% of malignant melanomas. Independent of its mutation status, Raf is also activated in tumor cells containing enhanced growth factor signaling pathways, such as those induced by mutant or constitutively expressed EGF receptor family members. In addition, analysis of the transcriptional program induced by Raf in epithelial cells revealed autocrine activation of the EGF receptor to be responsible for the ability of Raf activation to protect transformed cells from apoptosis. Therefore, the collective evidence suggests that Raf is a viable anticancer drug target.

The drug BAY 43-9006 is a novel potent and selective small-molecule inhibitor of Raf kinase and the first compound in this class to undergo clinical testing. Anticancer activity has been shown in numerous tumor models with K-Ras mutations and in tumor models that express wild type Ras, but overexpress growth factor receptors.

The present study evaluates the effects of combining BAY 43-9006 and cytotoxic drugs (paclitaxel, 5-FU, oxaliplatin, and SN-38, the most active metabolite of the clinically used CPT-11) on human cancer cells (HCT8, HCT116, HT29 and H1299) using 4 sequencing protocols (cytotoxic drug first, then RKI, RKI first, then cytotoxic drug, RKI first, then cytotoxic drug, then RKI, and RKI and cytotoxic drug together throughout). Furthermore, we analyzed the effect of RKI on colorectal cancer cells showing marked resistance against SN-38.

Materials and methods

Cell lines: HCT8 WT, HCT8 (SN-38-resistant), HT29 WT, HCT116 (p53+/+ and p53–/–), H1299.

SRB assay and MTT assay were performed according to standard protocols.

Cell cycle analysis was performed by flow cytometry. Cells were cultured in specific medium. After 24 h, the medium was replaced with fresh medium containing oxaliplatin (26.8 µM) or cisplatin (10.4 µM) ± RKI (range 4.5 – 25.6 µM), or with RKI alone (range 4.5 – 71.1 µM) for 24 h. Cells were washed and cell cycle analysis was assessed after additional incubation with either RKI or control medium for 4, 8, 11 and 16 h. A total of 1 × 10⁶ cells were lysated with DNA-Prep (< 0.1% potassium cyanide, < 0.1% NaN₃, nonionic detergents, saline and stabilizers) and stained with DNA-Prep stain (50 g/ml propidium iodid, RNAse (Type II-A, bovine pancreas (4 KU/ml)) < 0.1% NaN₃, saline and stabilizers) and stained with DNA-Prep stain (50 µg/ml propidium iodid, RNAse (Type II-A, bovine pancreas (4 KU/ml)) < 0.1% NaN₃, saline and stabilizers). Samples were analyzed by FACS within 30 min after preparation.

Results

Occasionally, we found antagonism using schedules in which RKI and cytotoxic com-
pounds (paclitaxel and 5-FU) were present in tandem throughout. Particularly, the combination of oxaliplatin and RKI led to marked antagonism in all sequencing protocols used. In contrast, weak or moderate synergy could be demonstrated in colon cancer cells using sequential protocols, i.e. initial treatment with cytotoxic drugs and subsequent RKI incubation for 48 hours. These cytotoxic drugs were paclitaxel and SN-38. Moreover, protracted schedules with initial RKI treatment (day 1), subsequent combination of RKI with SN-38 for 24 h (day 2) and thereafter RKI treatment for another 48 h (days 3 and 4), resulted in additive antitumor activity. Anti-cancer activity of RKI in cell lines HCT8 and HT29 was comparable to derivative cell lines showing marked resistance against SN-38 reflecting an apparent lack of cross-resistance between SN-38 and RKI in colorectal cancer cells.

RKI itself does not affect cell cycle, but overcomes G2 arrest due to cisplatin and G1 arrest due to oxaliplatin. BAY 43-9006 down-regulates cyclin kinase inhibitor p21<sup>CIP1</sup> and the G1-acting cyclin D1 whereas the expression of cyclin-dependent kinases (cdk4/cdk6) were not changed. Furthermore, RKI inhibits the expression of the G2-acting CDC2 (cdk1), which in association with cyclin B is known to be necessary for entering mitosis.

### Conclusions

Our data suggest I. additive action or moderate synergy using RKI in combination with numerous cytotoxic agents (e.g. paclitaxel, 5-FU, SN-38); II. marked reduction of oxaliplatin activity by RKI in human carcinoma cells. The results indicate that Raf kinase activity might be important for oxaliplatin-induced cytotoxicity. Furthermore, lacking cross-resistance between SN-38 and RKI might provide a rationale for designing clinical trials using CPT-11 in combination with BAY 43-9006 in patients with colorectal cancer.

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Drug-drug interaction pharmacokinetic study with the Raf kinase inhibitor (RKI) BAY 43-9006 administered in combination with irinotecan (CPT-11) in patients with solid tumors

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Introduction

Growth receptors and mitogenic stimuli play a critical role in the development of cancer. During its life-time each cancer cell expresses several distinct growth receptors and the expression of these can be stimulated by growth factors/mitogens. When a receptor at the cell surface is stimulated by its agonist, a signal cascade runs to the nucleus from the extracellular region of the receptor through the cell membrane via the intracellular region of the receptor. This signalling pathway is long and has several steps with check points which can be blocked by suitable blocking agents. One important pathway includes the Raf, Ras, MEK 1/2 and Erk 1/2 pathway which operates when the mitogen-activated protein kinase (MAPK) is activated in response to an extracellular stimuli and this pathway transduces signals from the cell surface (from the growth receptor) to the nucleus [Hilger et al. 2002, Cobb 2000]. A total of 12 MAPKs have been identified so far and these can be subdivided into 3 families. The extracellular signal-regulated kinases (Erk) are mainly activated by growth factors and mitogenic stimuli and have been linked to cell survival. One common feature of the MAPKs (Erk is a MAPK) is that they can be activated by phosphorylation within a protein kinase cascade. Once activated, MAPKs phosphorylate their substrates at specific serine/threonine residues. Since MAPK targets include several transcription factors they are involved in the regulation of gene expression.

One major focus of recent research has been to assess the importance of these signalling pathways as potential targets for anticancer drugs with new modes of action [Hilger et al. 2002]. A Raf-kinase inhibitor (RKI) has been developed by BAYER AG which has passed an extended phase I study program involving a variety of treatment schedules [Strumberg et al. 2003].

Classical cytotoxic anticancer drugs generally have specific actions (e.g. inhibition of topoisomerase I or II) but also interfere with signalling pathways although such actions are not well understood e.g. c-Jun N-terminal kinases (JNK; also referred to as stress-activated protein kinases, SAPK) are activated by cellular stress including heat shock, irradiation and agents which damage DNA and are involved in the induction of apoptosis. A logical approach is therefore to combine the RKI with classical cytotoxic agents since recent work has shown that the RKI BAY 43-9006 and CPT-11 have additive or synergistic actions [Heim et al. 2003]. Because a pharmacological drug-drug interaction cannot be ruled out, interaction studies were started using the RKI BAY 43-9006 in combination with the most important anticancer drugs such as docetaxel, gemcitabine, paclitaxel/carboplatin, 5-FU, capecitabine, oxaliplatin, doxorubicin and irinotecan. Preliminary results on the RKI + DOX study and the RKI + CPT-11 study are reported [Richly et al. 2003].

Patients and methods

The study protocol included three groups of 6 patients given different RKI doses. Group 1 received 2 × 100 mg/d po, Group 2 2 × 200 mg/d and Group 3 2 × 400 mg/d whereas the dose of CPT-11, 125 mg/m²iv on days 1, 8, 15, 22 and 43 did not differ. One treatment cycle was defined as four CPT-11 applications at weekly intervals followed by a 2-week rest period. Patients with advanced solid tumors in whom a therapeutic response
to CPT-11 could be anticipated were eligible for inclusion.

Blood samples for measurement of CPT-11 and SN-38 (the major metabolite of CPT-11) were obtained both during and in the absence of RKI treatment during the first 48 h of the first (without RKI) and the second treatment cycle (with RKI). The RKI treatment started on day 4 and was administered daily. Blood samples for determination of RKI were obtained during CPT-11 treatment and in the absence of CPT-11 treatment on day 42 (last day of the treatment cycle 1) and day 43 (first day of the treatment cycle 2).

Results

Estimates of toxicity, response and pharmacokinetics during the first RKI dose level (2x100 mg) could be made in a total of 9/18 patients (6 patients in the 1st cohort, 3 patients in the 2nd cohort). All symptoms of toxicity were considered to be due to CPT-11 or RKI. In this small group of patients there were 2 cases with grade 3 diarrhoea and 1 case with grade 3 leucopenia. All other toxicities such as nausea, vomiting, fatigue, anemia, arthralgia were grade 2 or 1 when judged as drug-related. No skin rashes were seen.

The PK evaluation of the three parameters, Cmax, AUC and terminal half-life in the 1st and 2nd treatment cycle showed no significant differences for CPT-11 and SN-38, with or without RKI, nor were there differences in the pharmacokinetics of RKI with or without CPT-11 and SN-38. Representative concentration-versus-time profiles for CPT-11, SN-38 and RKI are depicted in Figure 1.

Conclusions

In conclusion, BAY 43-9006, a Raf kinase inhibitor, is currently under clinical investigation in extensive pharmacological studies involving combination treatment strategies with most of the commonly used cytotoxic agents. The toxicity profile of the combination CPT-11 and BAY 43-9006 was that expected at the dose levels tested. In particular, there was no additive or super-additive diarrhoea problems (both drugs have exhibited this adverse effect in phase I studies). The combination therapy was satisfactory and with it stabilization and regression of the tumor in the current study was observed. Initial pharmacokinetic results show no significant drug-drug interaction in contrast to that observed with RKI + DOX [Richly et al. 2003]. This preliminary report therefore indicates that the combination CPT-11 and SN-38 PK is not significantly influenced by the addition of the RKI. In addition, there is no indication that the PK of RKI are influenced significantly by CPT-11 and SN-38.

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A phase I clinical and pharmacokinetic study of the Raf kinase inhibitor (RKI) BAY 43-9006 administered in combination with doxorubicin in patients with solid tumors

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Introduction

Molecular pathways, especially those involving alterations in signal transduction from extracellular signals to intracellular effector structures and molecules, play a key role in most malignant diseases. These pathways include signal transduction to the nucleus by changing gene expression, the cell cycle control pathway, the apoptotic pathway and the angiogenesis-metastasis route. A number of studies have examined various methods of selectively inhibiting intracellular signal transduction. Ras proteins play a pivotal role in controlling normal changes in cell growth. Mutated Ras remains in an active state and relays uncontrolled proliferative signals. In addition, many growth factor receptors signal through Ras. Raf kinase is a protein involved in the Ras signal transduction pathway. Thus, activation of Raf kinase, via activation of Ras, is considered an important mechanism by which human cancer develops. Ras mutations occur in approximately 30% of human cancers. In addition, the Raf/Mek/Erk pathway may be important in maintaining growth of tumors that do not have Ras mutations, thus expanding the applicability of this therapeutic target.

The drug BAY 43-9006 is a novel, potent and selective small-molecule inhibitor of Raf kinase and the first compound in this class to undergo clinical testing. Activity was observed in cancers having Ras mutations, as well as in a cancer in which Ras was activated through overexpression of growth factor receptors. These experiments suggest a potential use of this compound in a large spectrum of cancer types.

Several mechanistic reasons have been put forward to support the strategy of combining BAY 43-9006 with cytotoxic chemotherapeutic agents. Combining agents with differing mechanisms of action is known to enhance anti-cancer activity. The use of a cell growth inhibitor like RKI may be most beneficial during the off-period between pulses of cytotoxic chemotherapy because inhibiting the Ras pathway can prevent a malignant clone from avoiding chemotherapy-induced apoptosis. In vitro studies show that BAY 43-9006 causes redistribution of cells into S and M phases of the cell cycle, which may make them more sensitive to chemotherapeutic agents that target these cell cycle phases.

Indeed, preclinical experiments in an in vitro setting indicate that combination of BAY 43-9006 with a variety of chemotherapeutic agents results in additive cytotoxic activity. Doxorubicin, a chemotherapeutic agent widely used alone and in combination, in a variety of cancers having a high percentage of Ras mutations, was selected to be combined with BAY 43-9006 in this study from a number of candidate chemotherapeutic agents. The primary objective of this phase I study was to define the safety profile of BAY 43-9006 administered in combination with doxorubicin.

Patients and methods

To date, 29 patients with advanced, refractory solid tumors who had not received any prior anthracycline treatment were treated with doxorubicin (60 mg/m²) every 3 weeks for a maximum of 6 consecutive cycles.

BAY 43-9006 in combination with doxorubicin chemotherapy was administered at 3 dose levels (DL): DL1, 100 mg bid continuous dosing (cd), DL2, 200 mg bid cd, and DL3, 400 mg bid cd. After cycle 6, the treatment was continued with BAY 43-9006 only, if applicable. Patients who had received anthracycline-containing regimens prior to the study had to have an appropriate total cu-
Cumulative dose reduction so as not to exceed the lifetime maximal total dosage of 450 mg/m² doxorubicin or equivalent anthracycline compound. Cohort evaluation was done at each dose level, at the end of cycle 2 (i.e. 6 weeks after the start of treatment).

The schedule for BAY 43-9006 PK sampling was as follows: cycle 1: day 21 pre-dose 0.5 h, 1 h, 2 h, 4 h, 8 h, 10 h and 12 h and day 22; cycle 2, day 1: pre-dose, 0.5 h (post-infusion of doxorubicin), 1 h, 2 h, 4 h, 8 h, 10 h, 12 h.

The sampling schedule for doxorubicin/doxorubicinol PK sampling was: cycle 1: day 1 (no BAY 43-9006) and day 22 (with BAY 43-9006): predose, post infusion (0.5h), 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h.

PK parameters were calculated using Kinetica and Topfit programs and a 3-compartment model.

Results

Toxicity and response were evaluable in a total of 24 out of 29 enrolled patients.

Dose-limiting toxicity (DLT) was observed at various dose levels (DL): DL 100 mg: DLT seen in 1 of 6 patients (Gr. III leucopenia and fever); DL 200 mg: no DLT seen in 6 patients; DL 400 mg: out of a group of 12 patients 3 patients had DLT (2 patients with temporary rash, palmar-plantar erythema Gr. III and 1 patient with Gr. IV thrombocytopenia and leucopenia). A summary of adverse events is shown in Table 1. One confirmed partial response was observed at DL 400 mg bid continuous treatment (patient with mesothelioma). A total of 10 additional patients had stable disease (SD) > 3 months.

Doxorubicin plasma Cmax/AUC values increased on escalating the dose of BAY 43-9006. Pharmacokinetic profiles for the dose interval (0-τ) were obtained at the start of treatment, within the first 72 h and thereafter weekly. In the 4 of 6 evaluable patients treated with 400 mg bid, the geometric mean of doxorubicin AUC(0-τ) after the third cycle (3,193 ng*h/ml) was significantly higher than the corresponding data for the first cycle (2,366 ng*h/ml). Furthermore, patients with liver metastases and elevated values of AST and conjugated bilirubin, when compared to patients with normal hepatic function, showed a higher AUC(0-τ) for doxorubicin at all dose levels. The present data do not suggest any relationship between adverse events, especially skin toxicities, and AUC(0-τ) for doxorubicin.

Conclusions

BAY 43-9006 is a new Raf kinase inhibitor that is generally well-tolerated using continuous oral dosing. Our data suggest a pharmacological interaction of BAY 43-9006 at DL 400 mg bid with doxorubicin resulting in significantly increased AUC(0-τ) for doxorubicin. This effect appears to be pronounced in patients with liver metastases and elevated values for AST and conjugated bilirubin. In further phase II studies we recommend a dose level of 400 mg bid (continuous dosing) for the RKI BAY 43-9006 when given in combination with doxorubicin (60 mg/m², 3-weekly for a maximum of 6 consecutive cycles).

References

Novel antitumoral compound isolated from Clusia rosea

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Introduction

The explosive growth of phytotherapy has created a new awareness of the potential value of natural products as anticancer agents. Derivatives of natural products belong to the clinically most important anticancer drugs, e.g. the epipodophyllotoxins, vinca alkaloids, the taxanes and camptothecins. In view of the great diversity of flora throughout the world, this source is certain to remain an important source of new cytostatics in oncology.

There is no clear evidence showing that Clusia spp was used therapeutically in folk medicine, although an ethanol extract of the resins collected directly from the flowers of this plant is commonly used to treat leg ulcers by some farmers in Great Antilles, in particular the Cubans, in order to prevent local infections. In screening for natural compound with antitumor activity, we detected in vitro activity of Clusia spp on several tumor cell lines. These promising results with the crude extract led us to characterize the structural and functional aspects of the most active species collected by our group.

The first investigations of Clusia floral resins were carried out by De Oliveira et al. [1996, 1999] and resulted in the isolation, as O-methyl derivatives, of 4 polyisoprenylated benzophenones, clusianone, grandone, nemorosone and hydroxy-nemorosone. The isolation of 2 additional O-methyl derivatives of polyisoprenylated benzophenones possessing the bicyclo[3.3.1]-nonenetrione moiety (7-epi-nemorosone and nemorosone II) was subsequently reported as were the investigations on the plant compounds isolated from the neotropical genus Clusia L. (Clusiaceae or Guttiferae). The antitumor activities of these agents, investigated using chromeno-coumarin calanoid A, an agent isolated from the genus Clusiacea and used in clinical trials, involve nonnucleoside inhibition of HIV-reverse transcriptase. Antitumor activity is also associated with the occurrence of a family of structurally complex and bioactive polycyclic prenylated acyl phloroglucinol derivatives, isoprenylated benzophenones, biflavonoids and triterpenes.

Methods and results

Preparation of the plant extracts

The collection of Clusia spp was done in Florida, USA. The resin was carefully separated from the plant tissue and pooled. A methanolic extraction was carried out, followed by centrifugation at 5,000 × g. The supernatant was taken and dried in a SpeedVac concentrator, Thermo-Savant, New York, USA.

Isolation and purification of the active compounds

Active substances present in crude extracts were separated using a 250 mm × 10 mm column, packed with Nucleosil C-18 (Macherey-Nagel, Düren, Germany), a RP-HPLC separation module connected to a PDA 996 detector (Waters, Milford, USA) and a gradient system composed of ammonium formate 0.01 M, methanol and acetonitrile. The amount of separated substance was determined and resuspended in methanol to give a stock solution of 50 mg/ml which was then stored and protected from light. The purity and structure of the isolated polyisoprenylated [3.3.1]-non-enetrione moiety was determined using NMR-, UV- and MS-spectroscopic analysis prior to confirmation using crystal structure analysis.

Cytotoxicity assays

The drug sensitivity was assessed using a battery of 25 tumor and non-tumor cell lines established and validated in our laboratory. The proliferation assay involves a sulforhodamine-B test system is described by Skehan et al. [1990].

The determination of cytotoxicity of cells growing in suspension was determined with the MTT assay. The cell lines used originated from gastrointestinal tumors, such as stomach, colon and ileocecal carcinoma, cells...
from breast and ovarian carcinoma, large-cell carcinoma of the lung, neuroblastoma and leukemia cell lines. Because resistance is a major reason for treatment failure in malignant diseases, we included mainly biochemically, well-characterized cell lines resistant to clinically important cytostatics. Two non-tumoral cell lines, a human embryonic diploid lung fibroblast cell line and a mouse fibroblast cell line, were also investigated. The mean IC\textsubscript{50} concentration for the induction of cytotoxicity with the isolated clusianone derivative in the tumor cell lines used was 2.6 \mu g/ml. Comparing the calculated IC\textsubscript{50} concentrations for resistant cell lines to the parental wild-type showed no detectable cross-resistance between 5 FU, raltitrexed, cis-Pt, doxorubicin, SN38 and vinblastine. The cytotoxic concentration of the clusianone derivative was 4-fold and more than 16-fold higher than that in the human lung fibroblast and the mouse fibroblast cell lines, respectively.

**Topoisomerase I relaxation assay**

The DNA relaxation assay was used in order to determine the effect of extracted clusianone derivative on topoisomerase I activity. This method is based on the ATP-independent unwinding of supercoiled pBR322 plasmid DNA dimers by topoisomerase I. Two sources of topoisomerase I were employed in these experiments: nuclear extracts from tumor cell lines and an isolated human Topo I, purchased from Topogen, Columbus, OH, USA.

The nuclear extracts used in this assay were prepared from the H460 NSCLC cell line according to the methods modified by Danks et al. [1988]. Complete inhibition of enzymatic activity was observed at 100 \mu g/ml of clusianone derivative, which is approximately 38-fold greater than that necessary for a 50\% inhibition of cellular growth in our battery of tumoral cell lines.

**Topoisomerase II-catalyzed decatenation assay**

The DNA topoisomerase II catalyzes the DNA decatenation of kinetoplast DNA in the presence of ATP. Eukaryotic topoisomerase II activity (Topogen, Columbus, OH, USA) was assayed by decatenation of KDNA and the appearance of 2.5 kb DNA was monitored. The products were separated on 1% agarose gel containing 0.1 \mu g/ml of ethidium bromide and 9 V/cm for 0.5 hours. Complete inhibition of topoisomerase II decatenation of KDNA occurred at a clusianone derivative concentration of 50 \mu g/ml. This concentration is 19-fold greater than the calculated IC\textsubscript{50} for the tested tumor cell lines.

**Measurement of the telomerase activity by TRAP**

In order to examine if the new clusianone derivative influences telomerase activity, the telomeric repeat amplification assay (TRAP), a TRAP-ELISA kit (Oncogene, San Diego, CA, USA) based on the original method described by Kim et al. [1994] was performed. Inhibition of telomerase activity obtained in these assays was reached at a concentration approximately 10-fold greater than that necessary for a 50\% inhibition of cellular growth.

**Conclusions**

A novel and potentially useful antitumoral compound was isolated by us from Clusia rosea, and studies in 1996 involved the first isolation and characterization, without further derivatization, of a polyisoprenylated benzophenone. In the present study, we demonstrated that the new clusianone derivative is a small molecule that targets DNA topoisomerases and telomerase.

**References**


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Gene expression profiling of colon cancer reveals a broad molecular repertoire in 5-fluorouracil resistance

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Introduction

Resistance to anticancer drugs is a major obstacle to chemotherapy of human cancer. In patients with metastasized colorectal carcinoma, resistance to the widely used antimetabolite 5-fluorouracil seriously limits therapeutic success. A variety of potential mechanisms of 5-fluorouracil resistance have been described in the literature focusing on the expression and the activity of 5-fluorouracil-converting enzymes as key players in (fluoro)pyrimidine metabolism. The predictive value of enzymes such as thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase, has been documented in primary [Edler et al. 2002], in locally advanced [Allegra et al. 2002], and in advanced colon cancer [Ladner et al. 2000, Salonga et al. 2000]. These studies have also shown the limitations of univariate approaches to putatively complex phenomena such as sensitivity or resistance. Moreover, little is known about the molecular events responsible for the progression from sensitive to resistant or even highly resistant metastatic disease.

Methods

We applied expression analysis of more than 12,000 genes using the gene array technology to study progressive stages of resistance to 5-fluorouracil in vitro. After stepwise selection for resistance in a cell-line derived from a colon cancer lymph node metastasis, we compared the mRNA expression profile of parent cells with that of cells resistant to low (5 μM), intermediate (25 μM), and high (125 μM) concentrations of 5-fluorouracil. As a prerequisite for this investigation, we excluded the influence of cell growth kinetics as confounding factor by selection of subclones with similar population doubling times.

Results

Resistance to 5-fluorouracil was associated with significant changes (2-fold and more) in the expression of 370 genes, only 13 with marked variation (4-fold and more). We focused on 238 genes with unique change events that were affected by a significant up- or down-regulation either in the early, intermediate or late stage during progression of FU resistance. Remarkably, we found that 87% of changes (207 of 238) occurred either in the early (115) or intermediate stage (92), during progression from sensitive to low (5 μM) or intermediate resistant cells (25 μM).

In order to learn more about the nature of gene expression changes involved in early, intermediate and late stages, we next analyzed the function of affected genes. Acquired drug resistance affected a broad spectrum of essential cellular functions including apoptosis, signal transduction and cell cycle control. We detected a number of genes known to be associated with resistance to anticancer agents, such as the pleiotropic drug resistance mediating P-glycoprotein MDR (ABCB1) and the ABCC6 gene (ATP-binding cassette sub-family 6 member 1). We also noticed other important functions like drug metabolism (NAD(P)H quinone dehydrogenase 1, NQO1; cytochrome P450 IIIB6, CYP2B6), programmed cell death (BCL2-interacting killer, BIK; Caspase 4,
CASP4) and increased DNA repair (RAD51-interacting protein, PIR51; meiotic recombination 11 homologue A, MRE11A).

Interestingly, the majority of affected genes during the early stage is represented by 3 functional groups: cytoskeleton, signal transduction and cell adhesion, whereas changes during the intermediate stage involved genes with functions in DNA repair, replication and mitosis. Among the early events in response to 5-fluorouracil, we observed genes with function in the cytoskeleton (intermediate filament-associated keratins, e.g. KRTHB1, KRTHB6, KRT5, KRT7, KRT19 and components of the actin cytoskeleton, e.g. profilin 2, PFN2; myristoylated alanine-rich protein kinase C substrate, MARCKS; capping protein, CAPG), cell adhesion (intercellular adhesion molecule 2, ICAM2; L1 cell adhesion molecule, L1CAM; activated leukocyte cell adhesion molecule, ALCAM), cell-cell contact (cadherin 1, CDH1) and cell-matrix interaction (integrin β4, ITGB4; collagen type IV α2, COL4A2; collagen type IX α3, COL9A3).

Discussion

Unexpectedly, the majority of unique changes occurred early during development of resistance to low and intermediate doses of 5-fluorouracil. Response to high concentrations of 5-fluorouracil involved considerably less dramatic alterations in gene expression, although the degree of resistance still increased remarkably (14-fold increase in the IC50). These findings suggest an important role for the cytoskeleton and cell adhesion-mediated resistance in the early response of drug-naive cancer cells. On the contrary, well-known 5-fluorouracil targets such as thymidylate synthase were up-regulated in the late stage only, explaining the moderately predictive value of this parameter for response to chemotherapy.

Conclusions

Though focused on mRNA expression, our findings emphasize that the key steps towards resistance occur at an early stage after drug exposure. Our data support the intriguing view that, amongst an extensive repertoire, specific changes in tumor microenvironment may play a major role in intrinsic and/or acquired drug resistance. Since complex biological systems are notoriously difficult to modulate, the numerous observed interactions among tumor cells or between the tumor and the surrounding matrix may seriously limit therapeutic options to overcome resistance to 5-fluorouracil. As a consequence, acquired drug resistance to 5-fluorouracil – and probably also to its peroral prodrugs – should be faced by exploiting novel targets rather than modulation of resistance.

Acknowledgment

This work was supported by a grant from the Austrian Science Foundation (FWF), project P12816-MOB.

References


