interaction with DNA. Although it is one of the most efficient chemotherapeutical agents, it has serious side effects: nausea, vomiting, hair spilling, cardiotoxic effect, etc. These could be limited by conjugation of Dau to polypeptide carriers (1). Oligoarginine is a de novo designed cell penetrating peptide, capable to translocate covalently attached cargo (2). For the comparative analysis of the effect of covalent linkage and of the length of the peptide chain on antitumor and cellular uptake properties we have prepared three groups of new Dau-oligoarginine conjugates containing different number of Arg residues. In these compounds we have insererted oxime-, hydrazone- or squaric acid linkage between Dau and oligoraginine. New conjugates were characterized by mass spectrometry and RP-HPLC. The antitumor activity of the conjugates was evaluated in vitro on HL-60 human leukemia and HepG2 human hepatoma cells by MTT assay. Cellular uptake properties under different conditions (e.g. concentration) was studied by flow cytometry on two cell lines. We found that Dau-conjugates were more effective and uptake was also higher on HL-60 cells. The type of the bond in the conjugates as well as the number of Arg residues influenced markedly both cytostatic effect and cellular uptake.

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POSTER SESSION

Experimental/Molecular therapeutics, pharmacogenomics 1

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Dietary flavonoid fisetin induces a forced exit from mitosis by targeting the spindle assembly checkpoint

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The spindle assembly checkpoint (SAC) regulates the fidelity of cell division by ensuring that chromosome segregation is delayed until all sister kinetochore pairs have achieved stable bipolar attachments with spindle microtubules. Interference with the SAC is a promising strategy for treatment of cancer as premature SAC inactivation causes chromosome mis-segregation leading to massive aneuploidy and subsequent cell death. To discover small molecules with anti-SAC activity, we performed a high-throughput screen (HTS) for compounds that cause a forced exit from mitotic arrest induced by the microtubule destabilizing drug nocodazole. In most human cell lines with a robust SAC nocodazole treatment leads to a cell cycle arrest at mitosis during which the mitotic cells round up and become loosely attached to the substrate. Our screening strategy was based on the different cell-to-substrate attachment properties of round loosely attached mitotic and well-adhered flat interphase HeLa cells. From a library consisting of 2000 biologically active and structurally diverse compounds we identified the flavonoid fisetin (3,3',4',7-tetrahydroxy-flavone) as a strong SAC inhibitor. Time lapse microscopy of H2B-GFP expressing HeLa cells confirmed that fisetin induces escape from nocodazole arrest in a proteasome-dependent manner. We also showed that fisetin can overcome taxol (a microtubule stabilizing drug) and monastrol (an Eg5 inhibitor) induced mitotic arrests. Also non-drug treated mitotic cells underwent premature mitotic exit accompanied by cytokinetic defects upon fisetin treatment. Next we investigated how fisetin interferes with SAC signaling by studying kinetochore accumulation of key SAC proteins in the presence of the drug. We showed that fisetin causes a significant reduction in kinetochore affinity of BubR1 and Bub1 proteins, and delocalization of Aurora B kinase from the inner centromere to the chromosome arms. Furthermore, fisetin inhibited Aurora B and Cdk1 kinase activities as indicated by reduced phosphorylation of CenpA, Cdc27, and nucleolin-1, known substrates of the two kinases. We speculate that inhibition of SAC by fisetin is mediated through interference with Cdk1 and/or Aurora B function. In conclusion, utilizing our novel HTS and subsequent biochemical assays we have identified the flavonoid fisetin as a potential SAC inhibitor, which provides a mechanism of action to explain the drugs' previously reported anti-carcinogenic activity.

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Tissue distribution and pharmacokinetics of an ATWLPPR-conjugated chlorine-type photosensitizer targeting neuropilin-1 in glioma-bearing nude mice

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Destruction of the neovasculature is essential for efficient tumor eradication by photodynamic therapy (PDT). The PDT anti-vascular effect can be promoted by developing addressed photosensitizers localized preferentially to the tumor vascular compartment. A new photosensitizer conjugated to an heptapeptide [H-Ala-Thr-Trp-Leu-Pro-Pro-Arg-OH (ATWLPPR)] targeting neuropilin-1, a Vascular Endothelial Growth Factor (VEGF) co-receptor, has been synthesized. It was administered intravenously for an easier access to endothelial cells lining the vasculature in human malignant glioma-bearing nude mice. Plasma pharmacokinetic parameters were derived from plasma concentration–time data using a non-compartmental analysis and validated a relatively rapid elimination from the blood compartment with an elimination rate constant of 0.062 h^{-1} and a biological half-life of 11.0 h. The photosensitizer was mainly concentrated in organs such as liver, spleen and kidneys, which are rich in reticuloendothelial cells. In these organs, the elimination profiles of the photosensitizer were comparable, with half-lives as short as 12.2, 15.1 and 19.7 h, respectively. The peptidic moiety of the conjugated photosensitizer was degraded to various rates depending on the organ considered, most of the degradation process occurred in organs of the reticuloendothelial system. A metabolic product resulting from the enzymatic cleavage of the peptide bond between Ala and Thr was detected in plasma at all the examined time points from 2 h post-injection. The conjugated photosensitizer accumulated rapidly and at high levels in the tumor, with 2.3% of injected dose per gram of tumor tissue at 1 h after injection. Taking into account the aspecific uptake of the degradation product, the tumor levels of total photoactivable compounds might exhibit an interesting photodynamic activity. On the contrary, levels of total photoactivable compounds remained low in the skin. This study provides essential information for the choice of the time interval not to exceed to activate the photosensitizer.

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Targeting of neuropilin-1 to improve the anti-vascular effect of photodynamic therapy in xenograft human malignant glioma

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The principle of photodynamic therapy (PDT) is based on the combined action of a photosensitizer (PS) localized in the tumor, light and oxygen. After light irradiation of the PS and in the presence of molecular oxygen, photo-oxydation reactions will lead to the production of reactive oxygen species, inducing a localized eradication of the tumor. However, PDT effects are mediated not only through direct killing of tumor cells but also through indirect effects, involving both initiation of an immune response and destruction of the neovasculature (anti-vascular effect).

The strategy developed in the laboratory aims to favour this anti-vascular effect by targeting tumor neovasculature. This approach was considered by coupling a PS (chlorin) to the heptapeptide ATWLPPR, targeting neuropilin-1 (NRP-1), a $VEGF_{165}$ (Vascular Endothelial Growth Factor, isoform 165) co-receptor. We previously confirmed molecular and cellular affinity for the conjugated PS and its in vitro photocytotoxicity (Tirand et al., J. Control Release, 2006). In vivo, we demonstrated that only the conjugated PS allowed a selective accumulation in endothelial cells lining tumor vessels (Thomas et al., Photochem. Photobiol. Sci., 2008). Metabolic profile and optimization of treatment conditions were performed in nude mice xenografted ectopically with U87 human malignant glioma cells (Tirand et al., Drug Metab. Dispos., 2007). The aim of this study was to validate and

to characterize in vivo the anti-vascular approach after PDT using the conjugated PS, compared to the non-conjugated PS.

The anti-vascular effect, for the conjugated PS, was characterized by a reduction in blood flow around 50% during treatment, a loss of the CD31 labelling integrity in endothelial cells from two hours post-PDT. In fine, 4 hours post-PDT, we observed for the conjugated-PS, microhemorrhages, vascular stasis and lumen trombosis confirmed by a decrease of the fibrinogen diffusion in tumor tissue. Following PDT, for this PS, endothelial cells became rounded but without change of morphological characteristics of the ultrastructures. In vivo, the photodynamic efficiency with the conjugated PS induced a statistically significant tumor growth delay compared to the non-coupled PS.

This targeting strategy of NRP-1 using a heptapeptide displays the potential of anti-vascular effect of PDT for glioblastomas treatment.

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Differential role of choline kinase alpha and beta isoforms in human carcinogenesis

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BACKGROUND: Overexpression of choline kinase alpha (ChoK alpha) has been related with cancer onset and progression. Two human genes, ChoK alpha and ChoK beta, code for enzymes with 65% homology and reported choline kinase activity. Here we compare the in vitro and in vivo characteristics of ChoK alpha1 and ChoK beta, and their potential role in carcinogenesis.

MATERIALS AND METHODS: in vitro choline kinase and ethanolamine kinase activity assays and the analysis of phosphocholine or phosphoethanolamine production in whole cells were carried using ^{14}C labelling of the proper substrates followed by a thin layer chromatography analysis. In order to compare the tumorigenic ability of each ChoK isoform, soft agar anchorage-independent growth and in vivo tumorigenic assays in athymic mice were carried out. ChoK alpha or beta mRNA levels in human tumor-derived cell lines were quantified by real-time reverse transcriptase PCR.

RESULTS: Both ChoK alpha1 and ChoK beta, showed choline and ethanolamine kinase activities in cell extracts assays. However they behave differentially when overexpressed in whole human embrionary kidney 293T cells, suggesting the involvement of each ChoK isoform in distinct biochemical pathways under in vivo conditions. In addition, while over-expression of ChoK alpha1 is fully oncogenic, ChoK beta over-expression is not sufficient to induce in vitro cell transformation nor in vivo tumor growth. Furthermore, ChoK beta is not a downstream target of Ras and Rho GTPases, as previously demonstrated for ChoK alpha1, which is activated by Ras and RhoA. We also investigated the mRNA levels of ChoK alpha and ChoK beta in a panel of breast cancer cells lines, finding a significant up-regulation of ChoK alpha mRNA levels in the tumoral cell lines but no changes were found in ChoK beta mRNA levels, compared with their normal counterpart. Finally, MN58b, a previously described potent inhibitor of ChoK alpha1 with in vivo antitumoral activity, shows more than 20-fold higher efficiency towards ChoK alpha1 than ChoK beta.

CONCLUSIONS: This study represents the first evidence of the distinct metabolic role of the alpha1 and beta isoforms of choline kinase, suggesting different physiological roles, and their implications in human carcinogenesis. These findings are very relevant for the screening of ChoK inhibitors, and for the design of novel anti-ChoK drugs with potential antitumoral use that should be focussed on ChoK alpha.

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Design of mechanisms of selective prodrug activation in the bone marrow microenvironment of experimental and human multiple myeloma

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Multiple myeloma (MM) is an incurable B-cell cancer characterized by monoclonal proliferation of tumour cells in the bone marrow. MM cells secrete MMP-9, a matrix metalloproteinase (MMP) implicated in tumour invasion, migration and angiogenesis. MMPs have conventionally been targeted with inhibitors but without success in the clinic. In an alternative approach, we have sought to subvert the proteolytic activity of overexpressed MMPs to activate prodrug substrates selectively in the tumour microenvironment. We have earlier reported [Van Valckenborgh et al (2005) Leukemia 19: 1628 - 1633] that prototype oligopeptide substrates of MMP-9 are activated by the bone marrow (BM) cells of the 5T33MM murine model of multiple myeloma (MM). Towards control of the mechanism of prodrug activation in the BM microenvironment, we now report the design (by controlling binding to the S1' position in the active site of MMP-9) of novel latently fluorescent prodrugs of cytotoxic and vascular disrupting agents and demonstrate that selective cellular activation translates from the murine model to human MM cells. Novel cytotoxic DNA-topoisomerase inhibitors incorporating the oligopeptide sequences: D-ala-ala-ala-leu-gly~nva-pro (EV1) and D-ala-ala-ala-leu-gly~ile (EV2) when modified at their N-terminus with FITC, afford prodrugs EV1-FITC and EV2-FITC, respectively, which are efficiently activated by MMP-9 rich BM cells or homogenates from the 5T33MM model to release the active agent (as shown by self-reporting fluorescence release and HPLC-MS in vitro metabolism data). EV2-FITC at 20μM in 5T33MM-diseased BM homogenate (500 μg protein/ml) was metabolized to the active agent (complete at 6h) whereas essentially no metabolism (at 24h) occurred in BM homogenate from naïve animals; nor was the prodrug degraded in homogenates from the non-tumour bearing organs, including liver, of the 5T33MM mice. Furthermore, EV1-FITC and EV2-FITC were incubated with human CD138+ (MMP-9-expressing) MM cells immunomagnetically isolated from BM samples of MM patients. Addition of either prodrug (20 μM) in the absence of MMP inhibitors resulted in a high release of fluorescence. The MMP-2/9 specific inhibitor peptide CTT (50μM) inhibited fluorescence release when the MM cells were pre-incubated (1h) whereas the control peptide STT and the serine proteinase inhibitor aprotinin had no effect upon fluorescence release, consistent with MMP-9 mediated prodrug activation. We further show that the optimized oligopeptide sequences can be used to conjugate experimental vascular disrupting agents that are similarly selectively activated in the BM microenvironment. The data indicates that exploiting endoproteolytic activity and tumour phenotype is feasible and that controlling mechanisms of selective prodrug activation in the BM microenvironment has the potential to improve the therapeutic index for MM patients.

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Artesunate mediates growth inhibitory effects in human pancreatic cancer cells through modulation of multiple signalling pathways

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Introduction: Pancreatic cancer is one of the most aggressive human malignancies, with an extremely poor prognosis. Systemic chemotherapy with gemcitabine is regarded as the standard chemotherapeutic approach. However, the median survival is still only around 6 months.

Artesunate, ART, a semisynthetic derivative of artemisinin, the active principle of Artemisia annua L., initially described as an anti-malarial drug, revealed remarkable antineoplastic activity against tumor cells. In the present study, we evaluated the effect of Artesunate on BxPc-3 and Miapaca-2 pancreatic cancer cell lines and the mechanisms by which Artesunate affects tumor growth of the 2 human pancreatic cancer cell lines.

Methods: BxPc-3 (moderately differentiated) and Miapaca-2 (poorly differentiated) pancreatic cancer cell lines were treated with varying concentrations of ART and the effect was monitored by MTS assay for evaluating of cell proliferation and by flow cytometry and detection of cytoplasmic histon-associated DNA fragments for apoptosis evaluation. In addition cDNA array contained 7000 genes is then fabricated and used as a tool to identify differentially expressed genes upon treatment with ART 20μM for 48 h. The microarray data were validated by positive correlation with quantitative real-time RT-PCR in a subset of genes from BxPc-3 cell line (GDF15, GAAD45a, COX-2, VEGF, PCNA, FOS and DDIT3); Moreover pathways associated with these expression changes were identified using the Ingenuity Pathway Analysis tool (IPA).

Results: ART induces growth arrest and apoptosis in BxPc-3 and Miapaca-2 pancreatic cancer cell lines in a time and dose dependant manner, and the effect was more prominent with the poorly differentiated Miapaca-2 cells. The expression analysis identified a common set of genes that were regulated by ART in the two pancreatic cell lines. Association of modulated genes with biological functional groups identified several pathways affected by ART including cell signalling, cell cycle, cell differentiation and apoptosis. In addition we identified GDF15, GAAD45a, PCNA, FOS and DDIT3 to be novel candidate genes involved in NS-398 mechanism of action on human pancreatic cancer cell lines.

Conclusion: The molecular mechanisms of ART-induced growth inhibition in human pancreatic cancer cells depend upon the differentiation stage of the cell lines examined. Moreover, we introduced GDF15, GAAD45a, PCNA, FOS and DDIT3 to be the novel molecular targets of ART and a new insight for possible combination therapy with chemotherapeutic agents to decrease their side effects and improve their efficiencies.