and phospho-Akt expression was also observed in lymph node metastasis specimens.
**Conclusion:** Overall, our studies suggest that invasion in BMP2-induced EMT is mediated through down-regulation of PI-3 Kinase/Akt pathway.

**171** POSTER

**Integrin LFA-1 expression regulates angiogenesis-stimulating potential of colorectal carcinoma cells at premetastatic niches in the liver**

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The recruitment of vascular stromal and endothelial cells is an early pre-angiogenic event of premetastatic niches, but how the microenvironment created by avascular three-dimensional (3D) growth contributes to activation of the angiogenesis-stimulating potential in cancer cells is unclear. Herein, the proangiogenic profile of CT26 colon carcinoma cells was studied in seven-day cultured 3D-spheroids of <300 μm in diameter, produced by the hanging-drop method to mimic the microenvironment of premetastatic niches prior to hypoxia. Spheroid-derived CT26 cells increased VEGF secretion by 70%, which in turn increased in vitro endothelial cell migration by 2-fold. More importantly, spheroid-derived CT26 cells increased LFA-1-expressing cell fraction by 3-fold, and soluble ICAM-1, given to spheroid-cultured CT26 cells, further increased VEGF secretion by 90% via cyclooxygenase (COX)-2-dependent mechanism. Consistent with these findings, CT26 cancer cells also significantly increased LFA-1 expression at premetastatic niches within hepatic lobules. Angiogenesis also markedly increased in both subcutaneous tumors and hepatic metastases produced by spheroid-derived CT26 cells. Finally, two-dimensional electrophoresis plus mass spectrometry revealed that three-dimensional growth of CT26 cells led to the development of a VEGF-secreting cancer cell subset expressing a markedly proangiogenic protein profile, including 60S acidic ribosomal protein, ferritin heavy chain, phosphoglycerate kinase-1, estrogen-related receptor, vimentin and 14−3-3 epsilon alpha. Therefore, three-dimensional growth of cancer cells enriched the proangiogenic cancer cell phenotype needed for metastasis progression. The role of integrin LFA-1 and COX-2 in the microenvironmental activation of angiogenesis-stimulating potential of colorectal carcinoma cells potentially represents a new target combination for therapeutic strategies to block colorectal hepatic metastasis at premetastatic niches.

**172** POSTER

**Tumor-induced liver nerve growth factor (NGF): a new target for stromal cell inhibition during metastatic colorectal carcinoma growth**

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Besides its contribution to differentiation and survival of neurons, nerve growth factor (NGF) also plays a role in cancer progression. In the liver, expression of NGF is increased during tissue regeneration and hepatocellular carcinoma development, but its role during hepatic metastasis is not well understood. Herein, we investigated NGF and neurotrophin receptor expression by cancer and host cells in the hepatic metastasis microenvironment of murine and human systems. NGF immunostaining of metastatic colon cancer cells only occurred in 2 out of 24 patients with hepatic metastases, while around 80% of studied patients had hepatic metastases with NGF-expressing stromal cells. Not statistically significant correlation was demonstrated between NGF immunostaining of tumor-infiltrated stromal cells and cancer cell staining with anti-ki67 antibodies, suggesting that NGF was not involved in cancer cell proliferation. Hepatocytes and hepatic sinusoidal cells showed weak NGF immunostaining, while cholangiocytes had a high immunostaining in the hepatic tissue unaffected by cancer. Hepatic CT26 murine colorectal carcinoma metastases had an intense NGF immunostaining in those hepatocytes and myofibroblast-type stromal cells located at the invasion front of metastases. High NGF-expressing hepatocytes were preferentially located among cancer cells and had phenotypic features suggesting epithelial-to-mesenchymal transition. CT26 cancer cells did neither express in situ nor secrete in vitro NFG. p75-NTR had a low expression level in normal hepatic tissue, but it significantly increased in hepatocytes and HSCs located around and within hepatic metastases, while CT26 cancer cells were negative. Consistent with these in situ findings, NGF significantly increased by 3-fold in the hepatic blood obtained from livers affected by CT26 colorectal carcinoma metastases. NGF concentration was also 7 times higher in the supernatants from primary cultured HSCs than in those from hepatocytes, and it significantly increased in the supernatant of HSCs given C26 cancer cell-conditioned medium, and in those from cultured hepatocytes given tumor-activated HSC-conditioned medium. Recombinant murine NGF dose-dependent increased chemotactic migration, but not proliferation, of HSCs and some cancer cell lines in vitro. Moreover, HSC migration-stimulating activity induced by VEGF was NGF-dependent in vitro. Our results demonstrate for first time that hepatocytes and sinusoidal stellate cells express neutrotrophin receptor p75 and secrete NGF in response to specific stimulating factors released by cancer cell in the hepatic metastasis microenvironment of human and rodent colorectal carcinoma. Tumor-induced liver NGF contributed to intratumor stromal cell recruitment and potentially represents a promising target for tumor-activated stromal cells during metastatic colorectal carcinoma growth.

**173** POSTER

**Arf6-AMAP1 pathway in invasion of lung cancer and malignant mesothelioma cell lines**

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**Backgrounds:** Distant metastses are the major problem in cancer therapeutics. For most carcinomas, metastases begin with invasion of cancer cells into the basement membrane or the stromal environment. We have shown that a small GTPase Arf6 and its effector AMAP1 play pivotal roles in invasion and metastasis of significant populations of breast cancers. It has been well documented that lung cancers show even more invasive and metastatic tendency clinically than breast cancers.
Tumor cells, including those of epithelial origin, exhibit two distinct phenotypes for their invasion, namely mesenchymal type and amoeboid-like type. The former requires activities of matrix proteases and calpain, while the latter ROCK, a Rho-dependent kinase. Here we examine whether Arf6 and AMAP1 are involved in invasive activities of lung cancer cells and mesothelioma cells, together with analysis on what types of invasiveness each of these cells exhibits.
**Materials and Methods:** Non-small cell lung cancer cell lines (H1299, Lu99, H460, A549, PC9, PC14, H1650, H441, H522, H1975 and H520 (and malignant mesothelioma cell lines (211H, H2052 and H28) were used.
To examine types of the invasiveness, we used ALLN (a calpain inhibitor) and Y27632 (a ROCK inhibitor). We also used a cocktail of protease inhibitors, which contains GM6001 (a multi metalloprotease inhibitor), E-64 (a cystein inhibitor), pepstatin A, leupeptin, and aprotinin. We performed a matrigel chemoinvasion assay to measure invasive activities, using Biocoat Matrigel chambers (Becton Dickinson). Protein knock-down was done by the siRNA technique using RNAiMAX (Invitrogen). Cell viability was measured using Cell Countin Kit-8 (Dojindo Molecular Technologies).
**Results:** Six of 14 cell lines we examined (H1299, Lu99, PC9, PC14, 211H and H2052) showed appreciable matrigel invasive activities in vitro, while other three cell lines (H460, A549 and H1650) also exhibit less but detectable levels of invasive activities. Among the former 6 cell lines, we found that siRNA-mediated knockdown of Arf6 and AMAP1 both significantly inhibits invasion of H1299, Lu99, PC14 and 211H. On the other hand, AMAP1 knockdown, but not Arf6 knockdown, inhibited invasion of PC9 and H2052. We moreover found that H1299, Lu99, PC9 and H2052 exhibit the typical mesenchymal-type of invasion, while PC14 and 211H are not.
**Conclusions:** Consistent with previous studies, our results also suggest that invasive phenotypes are highly diversified among different lung cancer cells and mesothelioma cells. Still then, one can consider Arf6 and/or AMAP1 as molecular targets for the adjuvant therapy of some lung cancers and mesotheliomas.

**174** POSTER

**Epigenetic changes of tumor suppressor genes and therapeutic implications in glioblastoma**

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**Background:** Glioblastoma is the most common and aggressive type of primary brain tumor and there have been little improvements of its poor survival rate during the last decades. Aberrant DNA methylation, including

hypermethylation of tumour suppressor genes, is a hallmark of cancer. Information about which methylation events are disease specific has a great potential in diagnostics and drug development. The aim of this study was to investigate the methylation status of the tumour suppressor genes SHP1, SOCS1 and STAT1, their counterparts SOCS3 and SHP2, as well as the drug resistance gene MGMT, and their effect on protein expression and cytotoxic drug sensitivity in glioblastoma cell lines. A further aim was to investigate the possibility to increase cytotoxic drug sensitivity in the glioblastoma lines by demethylation treatment.
**Methods:** To study methylation patterns, bisulfite treatment of total DNA followed by PCR amplification and Pyrosequencing® analysis was employed. Protein expression of total lysates was evaluated by Western blot analysis. Cytotoxic drug sensitivity was analysed by the fluorometric microculture cytotoxicity assay. Demethylation was obtained by treatment with the drug decitabine. Six glioblastoma cell lines were used in the studies.
**Results:** MGMT, SHP1 and SOCS1 were methylated at varying levels in the analyzed gene regions, whereas SHP2, SOCS3 and STAT1 were not methylated. The observed methylation levels in MGMT and SHP1 were associated with a reduction of protein expression. In addition, a low degree of methylation and a high protein level of MGMT were related to a decreased sensitivity to the cytotoxic drugs 5-fluorouracil, 17-AAG, bortezomib, and picropodophyllin. Finally, it was possible to increase the sensitivity in the glioblastoma cells lines to several cytotoxic drugs by demethylation treatment with the drug decitabine.
**Conclusions:** Epigenetic regulation of MGMT and SHP1 appear to affect tumor phenotype in glioblastoma. The correlation between a low degree of methylation of MGMT and SHP1, a high protein level and low sensitivity to several cytotoxic drugs constitutes a potential predictive marker for chemotherapy of glioblastoma. Finally, the possibility to increase cytotoxic drug sensitivity by demethylation treatment points to novel therapeutic strategies in combination drug therapy of glioblastoma.

# Natural products and marine compounds

**175** POSTER

**Role of ERK activation in triptolide-induced apoptosis in MDA-MB-231 human breast cancer cells**

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**Background:** Triptolide (PG490), a compound isolated from *Trypterygium wilfordii*, has been shown to have potent activity in a variety of xenograft tumor models. However, very little is known about the molecular mechanism by which triptolide acts in cancer cells. Therefore, the aim of this study was to investigate the role of extracellular signal-regulated protein kinase (ERK), a member of the mitogen-activated protein kinase family, in triptolide-induced cell death using the human breast cancer cell line MDA-MB-231.
**Materials and Methods:** MTT assay was used to determine cell viability upon treatment with 0−40 ng/mL triptolide. Apoptosis was assessed by annexin-V/7AAD staining, and caspase 3/7 activity was measured by a fluorescence-based assay kit. To assess the involvement of ERK and caspases, phosphorylated ERK and cleaved PARP were probed by western blot, respectively, as well as by the use of a MEK inhibitor, U0126, and the pan-caspase inhibitor, Z-VAD-FMK. Expression of phosphorylated eIF2α was determined by western blot.
**Results:** Dose-dependent reduction in MDA-MB-231 cell viability was observed upon a 72-hour exposure to triptolide, with an IC50 value of 1.9 ng/mL. A 3.2-fold increase in annexin-V+/7AAD− cells was observed when cells were treated with 4 ng/mL triptolide for 48 hours, indicating induction of apoptosis. Triptolide-induced apoptosis was caspase-dependent, as supported by significant increases in caspase 3/7 activity, PARP cleavage and cell viability in the presence of caspase inhibitor Z-VAD-FMK. ERK was activated as early as 2 hour post triptolide treatment, and remained activated for 48 hours. eIF2α was also activated in a time-dependent manner in triptolide-treated cells. The concomitant use of MEK inhibitor, U0126, attenuated triptolide-induced caspase 3/7 activation, PARP cleavage, and significantly increased cell viability from 49% to 98%, indicating that ERK activation acts upstream of caspase activation.
**Conclusion:** Our data demonstrated for the first time that ERK activation played an important role in triptolide-induced apoptosis, in contrast to the general view that ERK activation contributes to cancer cell survival and proliferation. Furthermore, the sustained activation of ERK, together with eIF2α activation, suggested a possible link of triptolide-induced apoptosis to endoplasmic reticulum stress which warrants further characterization.

**176** POSTER

**Outcome of three Phase I trials of the marine compound ES-285 (3 hour infusion) in patients with refractory solid tumors**

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**Background:** ES-285 is a marine compound originating from the mollusc *Spisula polynyma*, an edible clam, which is also known as the Stimpson or Atlantic surf clam. The drug has cytotoxic properties by disrupting actin fibers and interacting with the ceramide pathway. The agent has a broad antitumor spectrum in vitro, in vivo and in xenograft models. ES-285 was subject to 4 parallel Phase I studies in patients (pts) with solid tumors, three of them reported here, all of them using 3-hour infusions of the compound.
**Material and Methods:** Pts had advanced malignancies, good performance status (ECOG PS 0−2) and adequate organ function. The following intravenous schedules of ES-285 were tested: (A) 3 h d1 qwk, (B 3 h d1−5 q3wk, and (C) 3 h d1 q3wk.
**Results:** The dose of ES-285 per administration was ranging from 2–256 mg/m$^2$, depending on the study. 117 pts were entered (25−61 per trial), their median age was ranging from 52−59 yrs per trial, and there was a male predominance. The most common tumor types were colorectal, renal and prostate cancer and melanoma. Pts received a median of 2 cycles of treatment in all studies, ranging from 1−18 per patient. Less than 15% of treatment cycles were delayed. More than 80% of pts went off study due to disease progression (83−88%). Only 8.0–13.4% of patients discontinued due to toxicity. The most common clinical adverse events were nausea, vomiting, asthenia, pyrexia (all schedules) and injection site reactions. Anemia, lymphocytopenia and increases of serum liver enzymes were frequently seen, independent of treatment scheme. Ten dose-limiting events were observed, mainly consisting of grade 3/4 (CTC version 2.0) reversible increases in serum ALT, AST and reversible neurotoxicity. Only in schedule C the maximum tolerated dose (200 mg/m$^2$) and recommended dose (160 mg/m$^2$) could be established. Among 117 pts, one melanoma patient had a non-confirmed partial response (RECIST) and 29 pts had disease stabilization as best response.
**Conclusions:** After thorough review of the risk/benefit outcomes of the Phase I program the clinical studies with ES-285 were discontinued.

**177** POSTER

**Antiproliferative effects of fluoro-chalcone derivatives in human melanoma A375 cells and peripheral blood mononuclear cells**

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**Background:** At present, no treatment options are available for patients with advanced melanoma providing either sufficient response rates or a significant prolongation of overall survival. Chalcones are included in fruits and vegetables, and are suggested to be cancer-preventive. In this study, we reported the effects of synthetic chalcone derivatives on proliferation of human melanoma cells and peripheral blood mononuclear cells (PBMCs).
**Material and Methods:** Twelve synthetic derivatives of methoxy-and/or fluoro-chalcones were included in this study. To measure the effect of chalcone derivatives on cells of a human melanoma A375 cell line, we used the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay procedures. Effects of chalcones on the proliferation of PBMCs in response to a T cell mitogen concanavalin A was assessed by [$^3$H] thymidine incorporation. PBMCs were isolated from seven healthy subjects. Cell cycle and apoptosis was detected by TUNEL assay and PI staining of the cells, using flow cytometric analysis.
**Results:** Four out of the 12 chalcone derivatives: 4-trifluoromethyl-4′-methoxychalcone (CH-1), 4-trifluoromethyl-2′-methoxychalcone (CH-3), 3-trifluoromethyl-2′, 4′-dimethoxychalcone (CH-4) and 3-trifluoromethyl-4′-methoxychalcone (CH-7) exhibited the strongest antiproliferative effects on the melanoma cells with $IC_{50}$ values of 9.6, 5.7, 5.8 and 7.2 μM, respectively. Then, we studied the effects of CH-1, CH-3 and CH-4 on apoptosis and cell cycle of A375 cells. 10 μM CH-3 induced apoptosis in 0.15, 15.3 and 54.05% of A375 cells at 24, 48, and 72 hr of culture, respectively. Percent of G2/M phase cells in control wells was 31.2, whereas CH-3and CH-4 caused accumulation of cells in the G2/M phase to be 70.1% and 90.55%, respectively. On the other hand, CH-1 reduced the G1 phase cells, as