QuSpez extracts (12.5 and 50 μg/ml), the cell growth and bi-dimensional organization were analyzed after 24h.
In vivo angiogenic assay: In vivo angiogeneic assay was performed in female Balb/C mice (6–8 weeks old) by analyzing the growth of blood vessels from subcutaneous tissue into a Matrigel plug. Matrigel was mixed with or without VA extracts and was injected into the abdominal subcutaneous tissue. The mice were also injected with VA preparations intraperitonially (IP) (20 μg/day). Mice were sacrificed after 7 days, and the Matrigel plugs were excised and processed for histological analysis.
Apoptosis assay: EA-hy926 cells were incubated for 24 hrs with varying concentrations of VA extracts (12.5 and 50 μg/ml). The induction of apoptosis by VA extracts was analysed by Annexin V labeling that recognizes exposed phosphotidyl serine on apoptotic cells and PI that binds to DNA.
Results: Treatment of the cells with VA Qu Spez was associated with a reduction in capillary network, in a dose dependant manner. VA Qu Spez at 50 μg/ml induced a nearly complete disruption of the capillary tube formation. The area of angiogenesis network was also reduced by 33%. In our in vivo studies, there was a dramatic reduction in the vascular density in the matrigel treated with VA Qu Spez at the time of the implantation (intra-matrigel treatment) and followed by systemic (IP) treatment as compared to control untreated mice. VA QuSpez also induced apoptosis (upto 60%) of EA-hy926 cells as analysed by Annexin V and PI staining.
Conclusions: Our results show that VA QuSpez reduces angiogenesis in vitro and in vivo and the induction of apoptosis is the one of the underlying mechanisms. The anti-angiogenic properties of VA extracts may explain at least in part to their efficacy as adjuvant therapy in cancer patients.

524 POSTER

Stimulatory effect of eucalyptus essential oil on macrophage/ granulocyte phagocytic activity: in vitro and in vivo evidences

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Background: many species of the genus *Eucalyptus* from the Myrtaceae family are used in folk medicine for a variety of pathologies. Monoterpenoid oil components of aromatic constituents are traditionally used as analgesic, anti-inflammatory, and antipyretic remedies and are commercially available for the treatment of the common cold and other symptoms of respiratory infections. Phytochemical analysis have shown, that the profile of the monoterpenoids changes among the *Eucalyptus* species with potential variations in medicinal properties. In *Eucalyptus globulus* the major monoterpenoid component is eucalyptol, constituting the 60–90%. Macrophages constitute one of the primary cellular mechanisms of the immune response playing a pivotal role in the detection and elimination of foreign body such as pathogenic microorganisms. To our knowledge, in literature actually there is no available data, concerning the influence of *Eucalyptus* essential oil in the cell components of the immune system, the only exception is for the effect of some cytokine production. In this study we investigated whether essential oil from *Eucalyptus globulus* (EO) is able to affect the phagocytic activity of human monocyte-derived macrophages (MDMs) *in vitro* and of rat peripheral blood monocytes/granulocytes *in vivo*.
Materials and Methods: analysis of morphological changes, characteristic of activated MDMs, was performed by scanning electron microscopy. The evaluation of phagocytic activity was carried out: a) in EO treated and untreated MDMs *in vitro* with confocal microscopy after fluorescent beads administration; b) in monocytes/granulocytes from peripheral blood of BDIX rats, after *in vivo* EO administration, with cytofluorimetric analysis using the phagotest kit from ORPEGEN Pharma. Immuno-suppression in BDIX rats was induced by administration of the chemotherapeutic agent 5-fluorouracil (5-FU).

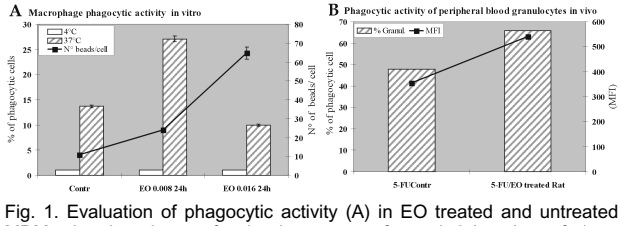

Fig. 1. Evaluation of phagocytic activity (A) in EO treated and untreated MDMs *in vitro*, by confocal microscopy, after administration of 1 μm fluorescent beads; and (B) in BDIX rat peripheral blood granulocytes, after *in vivo* EO treatment, in absence or in presence of 5-FU administration, by cytofluorimetric analysis.

Results: Our results demonstrate that EO is able to activate MDMs and peripheral blood monocytes/granulocytes both *in vitro* (Fig. 1A) and *in vivo*, stimulating their phagocytic activity. EO is also able to induce a dramatic recovery of granulocyte phagocytic activity after bone marrow suppression induced by 5-FU (Fig. 1B).
Conclusion: Our results suggest that the components of essential oil extracts from eucalyptus represent a possible new class of immunoregulatory agents useful in chemotherapy.

Prodrugs

525 POSTER

Targeting Doxorubicin to LHRH-receptor positive tumors by the cytotoxic hybrid ZEN-008 (AN-152)

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ZEN-008 (AN-152) is a cytotoxic analog of the luteinizing hormone releasing hormone (LHRH) in which doxorubicin (DOX) is linked to [D-Lys^6]LHRH. ZEN-008 binds to LHRH-receptors, which are found on a variety of tumors including breast, prostate, ovarian and endometrial cancers. After binding, ZEN-008 is internalized and transported to the nucleus where it induces apoptosis upon release of DOX. The activity of this compound has been demonstrated in experimental models of a variety of human cancers. Here we report on the antitumor activity of ZEN-008 in experimental human endometrial cancers.
LHRH receptors were determined in the HEC-1A human endometrial cancer cell line. The efficacy of ZEN-008 was evaluated and compared to its cytoxic radical DOX in athymic nude mice bearing HEC-1A tumors. The safety and tolerability of ZEN-008 was evaluated in series of studies including safety pharmacology studies and acute and subchronic toxicity studies.
43 days after the injection of ZEN-008 HEC-1a tumor growth was significantly inhibited by 54.2%, while treatment with an equimolar dose of DOX only resulted in a nonsigificant tumor inhibition by 23.4%. WBC 8 days after application was signifcantly suppressed by DOX, but not by ZEN-008.
The good safety profile was confirmed in safety pharmacology studies evaluating the effects of ZEN-008 on respiratory and cardiovascular parameters in the dog as well as in the Irwin and Rotarod test. In the cardiovascular safety study in beagle dogs, no evidence of QT prolongation was seen at any dose administered. Superior tolerability of ZEN-008 as compared to DOX was confirmed in acute and subchronic toxicity studies in mice, rats and dogs, respectively. In contrast to DOX, where lymphohistiocytic myocarditis with intramuscular fibrosis was observed, ZEN-008 did not induce any cardiotoxicity.
Targeted chemotherapy with ZEN-008 is significantly more effective than DOX itself. ZEN-008 is less toxic than DOX as reflected by a consistently higher LD50 and reduced cardiotoxicity. Due to the attractive mechanism of action and the overall promising safety and toxicity profile, ZEN-008 was selected to be evaluated in clinical phase I trials. ZEN-008 is available as a red powder (50 mg) lyophilisate for i.v. application as a solution.

526 POSTER

A Prostate-Specific Antigen (PSA) activated channel-forming toxin as therapy for prostatic disease

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Background: While the exact physiologic function of the prostate is unknown, it is a gland associated with significant morbidity in the aging male. The prostate is the most common site of non-skin cancer diagnosed in American men, with one in six developing the disease during their lifetimes. In addition, approximately 80% of men will present with a symptomatic benign overgrowth of the prostate known as benign prostatic hyperplasia (BPH) by age 80. Prostate-specific antigen (PSA) is a serine protease that is secreted at high levels (micro to mg/ml) by the normal and diseased prostate. To develop effective prostate tissue-selective therapy for localized prostatic disease we modified proaerolysin (PA), the inactive precursor of a bacterial cytolytic pore-forming protein, to produce a PSA-activated protoxin (PRX302).
Materials and Methods: PRX302 was generated by replacing the wild type furin protease activation site within PA with a 6 amino acid PSA-selective activation site. PRX302 was tested for in vitro toxicity against PSA positive and negative prostate cancer cell lines. Intratumoral efficacy of PRX302 was evaluated in PSA-producing xenograft models. Since the PSA gene

is only found in primates and humans, PRX302 was subsequently injected into monkey prostates and toxicity evaluated.
Results: PA is cytotoxic to prostate cancer cells in vitro but no therapeutic index could be achieved by intratumoral injection into prostate cancer xenografts. Substitution of the PSA-selective activation site for the furin site neutralized furin activation. PRX302 is activated by purified PSA and produces PSA-dependent cell killing at picomolar concentrations in vitro. PRX302 was stable to activation in human plasma containing high levels of PSA (10,000 ng/ml). Single dose intratumoral injections of 1–10 micrograms of PRX302 produced significant and often complete tumor and biochemical (i.e. no measurable serum PSA) regression of PSA-secreting human prostate cancer xenografts. Injection of a single 25 μl dose of PRX302 into non-PSA producing dog prostates produced no effect at doses up to 400 μg of PRX302. In contrast, injection of 0.35 μg and 4.1 μg of PRX302 into PSA-producing prostates of cynomolgus monkeys resulted in destruction of ~25% and 50% of the prostate gland respectively. This extensive damage was confined to the prostate with no toxicity observed in any other normal tissue including those tissues adjacent to the prostate (e.g. urinary bladder, urethra, rectum, seminal vesicles).
Conclusions: Our observations demonstrate the potential for intraprostatic application of this engineered PSA-activated protoxin for the treatment of locally recurrent or advanced prostate cancer and for benign prostatic hyperplasia (BPH). A phase I clinical trial for men with locally recurrent prostate cancer after definitive radiation therapy is currently in progress.

527 POSTER

Fibroblast Activation Protein (FAP) activated anti-stromal prodrug therapy for cancer

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Background: The tumor stroma compartment represents a major component of the mass of most carcinomas. Reactive tumor stromal fibroblasts differ from fibroblasts of normal adult tissues in regards to morphology, gene expression and production of important biological mediators such as growth factors and proteases. A highly consistent trait of tumor stromal myofibroblasts is the induction of fibroblast-activation protein (FAP), a membrane-bound serine protease that has dipeptidase and gelatinase/collagenase activity. FAP is not expressed in normal adult tissues, but has been shown to be selectively expressed by stromal myofibroblasts in >90% of epithelial cancers examined in one study with minimal expression in either cancerous epithelial or adjacent normal tissues. Therefore, a FAP-activated prodrug strategy is proposed that takes advantage of FAP's enzymatic activity to selectively activate a highly potent cytotoxin thapsigargin (TG) in the peritumoral fluid leading to death of FAP positive tumor stromal cells while at the same time generating a bystander effect that also results in death of tumor and endothelial cells.
Material and Methods: A map of FAP cleavage sites within recombinant gelatin was generated using LC-MS-MS and from this map a series of peptides were tested as FAP substrates. The best FAP substrate was coupled to a TG analog to create a FAP-activatable prodrug that was screened for plasma stability and for in vitro toxicity against FAP-positive fibroblast and negative cancer cell lines. The FAP prodrug was then tested for efficacy against human breast cancer xenografts.
Results: Analysis of the gelatin cleavage sites following FAP digestion showed preferred cleavage after proline, but also cleavage after alanine and glycine. Glycine was the preferred in the P2 position, while alanine was preferred in P'1. Seven amino acid fluorescence quenched peptides from the cleavage map were readily cleaved by FAP with Km values of 5–50 micromolar. A consensus peptide was also readily cleaved by FAP. This peptide was coupled to a potent 8-O-12 aminododecanoyl analog of TG (12ADT) to produce the FAP activated prodrug. This prodrug was stable to hydrolysis in human and mouse plasma and was selectively toxic to FAP positive cells in vitro. Preliminary in vivo studies demonstrated significant antitumor activity against MDA-MB-231 human breast cancer xenografts with no significant toxicity to treated animals. Further studies are underway to evaluate in vivo toxicity, pharmacokinetics, biodistribution and efficacy against a panel of breast cancer xenografts producing varying amounts of stroma.
Conclusions: Advantage can be taken of FAP's selective expression by reactive fibroblasts within human tumors and its unique enzymatic activity to generate prodrugs that are selectively activated within human cancers with minimal side effects to normal host tissues.

528 POSTER

Gemcitabine prodrug has efficacy when dosed orally in a human colon tumor xenograft model

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Clinically, Gemzar® (gemcitabine hydrochloride) shows good efficacy as an anticancer drug used in the treatment of pancreatic, NSLC, and breast cancer when dosed intravenously. When gemcitabine is dosed orally to mice, the drug causes gastrointestinal toxicity. In an effort to improve oral delivery of gemcitabine, the current study examines a gemcitabine prodrug (LY) for its properties in the treatment of a human colon tumor xenograft model. LY is a prodrug that has little to no cytotoxic activity in 3-day proliferation assay using two cell lines, the acute promyelocytic leukemia HL-60 cells and the human colon carcinoma HCT-116 cells. LY was stable in mouse and human small intestinal epithelial homogenates, but was susceptible to hepatic hydrolysis in vitro. When dosed orally to mice, high plasma concentrations of intact prodrug were detected in the portal vein. The prodrug was then hydrolyzed systemically to release gemcitabine. LY demonstrated significant anti-tumor activity in the HCT-116 colon tumor xenograft model when administered on three dose schedules (qd × 14, q2d × 7, q3d × 4). A dose response was observed in all studies and a margin of safety of 2 on at least one dose schedule. LY may be clinically efficacious after oral administration and may provide important new applications for this drug.

529 POSTER

An albumin-binding prodrug of doxorubicin that is cleaved by prostate-specific antigen: development and biological evaluation in an orthotopic mouse model

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Metastatic prostate cancer is difficult to treat, and to develop better compounds, good models mimicking the characteristics of human disease are required. Such a model was developed using the PSA-positive prostate cell line LnCAP implanted orthotopically into SCID mice. Transduction of the cell line with a retrovirus encoding a luciferase-neomycin resistance fusion protein, generating LNCaP LN, was used to monitor the growth of the tumors in the mouse prostate via in vivo bioluminescence. Luciferase assays from organ homogenates allowed the detection of metastasizing tumor cells, identifying the lung as a prime target.
Prostate-specific antigen (PSA), a serine protease that is over-expressed in prostate carcinoma, represents a molecular target for selectively releasing an anticancer agent from a prodrug formulation. An albumin-binding prodrug, EMC-Arg-Arg-Ser-Ser-Tyr-Tyr–Ser-Gly-DOXO [EMC: ε-maleimidocaproic acid; DOXO: doxorubicin], was efficiently cleaved by PSA at the P_1–P'_1 scissile bond releasing the doxorubicin dipeptide Ser-Gly-DOXO. Albumin is a suitable carrier of cytostatic agents due to passive accumulation in solid tumors. EMC-Arg-Arg-Ser-Ser-Tyr-Tyr–Ser-Gly-DOXO showed no in vivo activity in the PSA-negative PC-3 model, but good activity in the CWR22 PSA-positive model [1]. However, cleavage with PSA released the less active doxorubicin dipeptide DOXO-Gly-Ser, and not doxorubicin. Thus, to improve the compound, a spectrum of amino acids was tested that allow free doxorubicin to be released. An arginine residue proved effective and the doxorubicin dipeptide Ser-Arg-DOXO was degraded to doxorubicin in PSA positive xenografts (CWR22, LNCAP). The new prodrug EMC-Arg-Arg-Ser-Ser-Tyr-Tyr–Ser-Arg-DOXO bound rapidly to the cysteine-34 position of albumin, and was efficiently cleaved by PSA, releasing Ser-Arg-DOXO.
We thus used our orthotopic LNCaP model to test this novel targeted compound. SCID mice were injected orthotopically with the LNCaP LN cell line. Mice bearing tumors were readily randomized according to the in vivo luciferase signal, and treated with the novel formulation. Results from the treatment, analysed in vivo using bioluminescence, and at necropsy via primary tumor size, as well as metastatic burden, will be presented.

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