carcinomas capable of distinguishing samples according to clinical course and progression of the disease.

524 Poster
Effects of mycotoxins on apoptosis of human immune system

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Apoptosis is an important process in a wide variety of different biological systems and also in chemical-induced cell death.

The immune system is now recognized as a target organ for many xenobiotics such as drugs and chemicals, which are able to trigger unwanted apoptosis or to alter the regulation of programmed cell death. Reducing the number of immune-competent cells after xenobiotic treatment can lead to immunosuppressive effects, resulting in an increased susceptibility to tumors or infections diseases.

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing diseases and death in humans and animals at low concentrations. The immunotoxic effects can be mediated by direct toxin interactions with lymphocytes and other blood cells, provoking, among others effects, a rapid and strong apoptosis of human lymphoid cells.

Our objective was to explore the effects of deoxynivalenol (DON) and ochratoxin A (OTA) on apoptosis of immune-competent cells using cell lines as model.

The effects of both mycotoxins on apoptosis of lymphocytes at the cellular and molecular level were studied using flow cytometric analysis, western blotting or ELISA system. We found that toxin effects was origin-dependent and dose dependent and both mycotoxins caused inhibition of cell proliferation, mediated by activation of apoptosis pathway. In conclusion DON and OTA by apoptosis-induced in vitro on cells lines model may have some negative effects on human immunosystem that support further investigations

525 Poster
Lipoxygenase expression and intracellular localization in different types of cancer

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Lipoxygenases (LOX), particularly 5-LOX and 12-LOX, have been implicated in carcinogenesis and several LOX-inhibitory drugs and natural products have been tested in preclinical studies and early drug trials for anti-carcinogenic activity and clinical effects. Knowledge is still lacking on LOX and their products with respect to biological activities, particularly intracellularly. The expression and localization of 5- and 12-LOX was studied by immunohistochemistry in 10 samples from cancers and normal tissue of pancreas, breast, colon, stomach, prostate and lung. Staining intensity, localization and proportion of positive cells were scored. Furthermore, malignant cell lines from pancreas (PANC-1) and breast (T-47D) were synchronized by serum starvation before adding 10% FCS. Cells were stained for 5- and 12-LOX by immunoperoxidase or immunofluorescence after 0, 2 and 6 hours and analysed by light and confocal microscopy. The expression of 5-LOX was more marked in cancer compared with the normal counterpart for most tissues except colon (very little expression) and stomach (marked expression in normal and malignant tissue). The nuclear envelope was the most prominent localization. For 12-LOX the expression was generally more marked than that of 5-LOX; little or no difference was seen between cancer and normal tissue in breast, colon and stomach, but cancer of pancreas, prostate and lung showed increased expression. The nucleus and nuclear envelope stained strongly for 12-LOX. The in vitro experiments showed appearance of 5-LOX in the nuclear envelope following stimulation but 12-LOX was always seen at the nuclear envelope. Nuclear expression of 12-LOX changed following stimulation and showed different behaviour in the two cell lines; a transient increase in PANC-1 but a decrease in T-47D. In conclusion, changes in LOX expression in cancer are variable depending on tissue. Increased expression was seen for 5-LOX in cancer of breast and pancreas and for 12-LOX in cancer of pancreas, prostate and lung. As the activating protein of 5-LOX (FLAP) is located in the nuclear envelope, the shift in intracellular localization in the in vitro experiments might indicate a link to the cell cycle. The considerable differences between cancers of different origin, reflected also in the different behaviour of 12-LOX seen in the two cell lines, will have to be kept in mind when designing strategies for chemoprevention or treatment of cancer based on LOX inhibition.

526 Poster
Effect of stimuli treatment on proliferation and apoptosis of tumor cells

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The main obstacle against the success of therapy in many cancers seems to be the impossibility of eradication of all tumor cells. Increase of replicative capacity, loss of cell adhesion and angiogenesis process represent aggravating factors of clinical evolution for cancer patients. Breast and ovarian cancers represent some malignancies with high incidence and mortality throughout women, their ethiology involving many genetic, immunological and biochemical factors. Malignant evolution depends on the genetic profile of tumor, which dictates its reaction to citotoxic action exerted by chemotherapeutical agents or contributes to a resistant phenotype. Structural or gene expression alterations are responsible not only for the appearance of cancer, but also for the clinical responses of patients to chemotherapy. The present study focused on the potential influence of stimuli treatment (doxorubicin, cytokines, curcumin) on proliferation by cell cycle phases and apoptosis of breast and ovarian tumor cells. Experiments were performed on human breast and ovarian adenocarcinoma cell lines, levels of resistance being tested by measuring the expression of P-glycoprotein during cultivation of MCF-7, MDA-MB-231 and SK-OV-3 cells with stimuli. Sensibitility of tumor cells to stimuli treatment was evaluated by measuring the cytotoxicity induced by treatment using MTT or XTT colorimetric methods. We have also analyzed by flow-cytometry the influence of stimuli treatment on antigene expression of bcl-2, p53, Ki-67, Fas and P-glycoprotein, correlated with the modifications of apoptosis and cell cycle phases. Progression through cell cycle phases was evaluated by PI technique and flow-cytometry analysis, while percentages of apoptotic cells were detected by using Annexin V - FITC/PI coloration, followed by flow-cytometry. In addition, gene expression of molecules under study was analyzed by RT real-time PCR, and the results correlated with antigen expression detected by flow-cytometry. Data obtained could lead to a selection of patients who might benefit the most of antitumor immunotherapeutical strategies focused on diminishing the primary tumor and controlling/eliminating the metastases. Knowing the modifications induced by chemotherapeutical agents in human tumor cell lines will make possible the identification of new gene alterations associated with the resistant phenotype, which might be taken into account for future gene therapy of breast and ovarian cancers.

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TSA regulates P-glycoprotein and multidrug resistance associated protein expression in cancer cells

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Multidrug resistance (MDR) constitutes a major obstacle for success of cancer treatment. Although several mechanisms could be involved in the acquisition of this phenotype, the role of two different membrane proteins, P-glycoprotein (Pgp) and multidrug resistance associated protein (MRP) has been well established. Both proteins are members of the same ATP-binding cassette superfamily of transport proteins.

We have studied the effects of histone deacetylase inhibitors, such as TSA and SAHA on Pgp and MRP expression in different cancer cell models, including HT-29 and HCT-15 human colon carcinoma cell lines; IMIM-PC-1, RWP-1 and IMIM-PC-2 human pancreatic adenocarcinoma cell lines; MCF-7 and MCF-7/Adr human breast carcinoma; HL-60, HL-60R, K-562 and K562/Adr human leukaemia cell lines. In all these cell lines we

have studied the effects of TSA treatment on MDR1 and MRP-1 mRNAs expression by real time RT-PCR as well as the levels and activity of Pgp and MRP1 by Western blot and flow citometry based on daunomicyn accumulation.

Our results demonstrate that TSA regulates differentially MDR 1 mRNA expression. TSA treatment induced an increase in MDR1 mRNA in HT-29, IMIM-PC-1, IMIM-PC-2, RWP-1, MCF-7 and K-562 cell lines. However TSA treatment induced a decrease in MDR1 mRNA in HCT-15, MCF-7/Adr and K562/Adr. Interestingly no Pgp protein and activity was detected in HT-29, IMIM-PC-1, IMIM-PC-2, RWP-1, MCF-7 and K-562, despite the TSA-induced increase in MDR1 mRNA. In HCT-15, MCF-7/Adr and K562/Adr cells, high levels of Pgp expression and activity were founded and TSA produced a very significant decrease both in Pgp levels and activity. We have previously shown that the MDR1 mRNA expressed in HT-29, IMIM-PC-1, IMIM-PC-2, RWP-1, MCF-7 and K-562 cell lines was different in its 5´ UTR than the MDR1 mRNA present in HTC-15, MCF-7/Adr and K562/Adr cell lines, being the reason for such differences the alternative use of two promoters in the MDR1 gene. Our data demonstrate that TSA regulates both MDR1 promoters in opposite ways. These results are quite important, since TSA inhibits the promoter that is related to the expression of an active Pgp protein and it activates the proximal promoter that does not produce active Pgp protein due to a translational blockade as we have previously shown.

TSA and SAHA also regulate MRP1 expression. In fact TSA treatment produced a decrease of MRP-1 mRNA in HT-29, IMIM-PC-1, IMIM-PC-2, RWP-1 cells and more important, a decrease in MDR1 mRNA and MDR1 protein in HL-60R, a cell line that overexpress MRP 1 protein.

Taken together our results demonstrate that iHDACS such as TSA and SAHA that are not substrate of Pgp or MRP1, show besides to their effects on cell proliferation and apoptosis, an additional clinical benefit down regulating the active forms of Pgp and MRP-1 in different cell models.

528 Poster

Synergistic effects of the PKCβII inhibitor enzastaurin and the antifolate pemetrexed in chemoresistant ovarian cancer cell lines

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The impact of enzastaurin, a selective inhibitor of protein kinase C beta (PKCβ) and the AKT pathway, and the combination treatment with pemetrexed, a multitarget inhibitor of folate pathways, were analyzed when applied to the ovarian cell line HEY and subclones with selective resistance against cisplatin, etoposide, docetaxel, paclitaxel, gemcitabine, pemetrexed and enzastaurin.

After exposition to enzastaurin (5 - 10 μM) immunoblot analyses were performed determining the expression of the enzastaurin targets PKCβII and glycogen synthase kinase 3 β (GSK3β) and the extracellular-signal regulated kinase (ERK1/2). The cytotoxic activity of enzastaurin (0,63 – 40 μM) was assessed by MTT assay and induction of apoptosis was verified by Elisa and DAPI staining. In addition, we looked for synergistic effects on proliferation inhibition by combination treatment of enzastaurin and pemetrexed.

All resistant cell lines have a significantly stronger expression of phosphorylated GSK3β with highest level detected in the cisplatin-resistant compared to the parental HEY cell line. A decrease of phosphorylation occurred after 30 min of enzastaurin (5 μM) exposure, most remarkable in the parental HEY cell line and its cisplatin and gemcitabine resistant counterpart. Stimulation with enzastaurin also caused a decline of activated ERK1/2 in the parental HEY cell line, which was absent in the enzastaurin resistant subclone. Proliferation and apoptosis analyses displayed the docetaxel-resistant with the highest resistance to enzastaurin treatment, whereas the cisplatin-resistant HEYs exhibited the strongest sensitivity. Costimulation with pemetrexed showed a synergistic proliferation inhibition with the strongest effect in the docetaxel and gemcitabine resistant subclones.

The results indicate that ovarian cancer cell lines with high expression levels of phosphorylated PKCβII and GSK3β exhibit also strong dephosphorylation of GSK3β in response to enzastaurin stimulation lacking a correlation to the responsiveness to enzastaurin. As well, an inhibitory effect of the ERK1/2 pathway was proven by enzastaurin stimulation. Finally, treatment with enzastaurin alone shows nearly identical effects on proliferation inhibition among the various chemoresistant subclones, but the combination of pemetrexed and enzastaurin exhibits synergistic inhibitory effects on proliferation with the most promising activity in the docetaxel and gemcitabine resistant cell lines.

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Newly synthesised PgP modulators display anticancer activity

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Starting from our preliminary results on the activity of the new sigma-2 receptor agonist, PB28, as anticancer drug and PgP modulator, a new class of drugs was synthesized and characterized. They are pure PgP inhibitors and among them, MC18 and MC70 seemed to be the more active [1,2]. All three compound, PB28, MC18 and MC70, showed to strongly increase doxorubicin effectiveness in a PgP overexpressing breast cancer cell line. The high synergism between each newly synthesized drug and the anthracycline suggested to deeply investigate PgP inhibitors anticancer activity.

The characterization was carried out in MCF7 ADR breast cancer cell line, overexpressing PgP. To identify the mode of action of these three drugs, microarray, cell cycle and main cellular signaling pathways analysis were performed. For microarray analysis, cells were exposed to 25nM PB28 and 20microM MC18 and MC70 for 2 days, mRNA was extracted and processed on Affymetrix GeneChip Human Gene 1.0 ST. The capability of each compound to modulate cell cycle was determined by flow cytometry and western blotting analysis allowed to discriminate cellular targets involved in their mechanism of action.

Preliminary evaluation of microarray data suggested that these agents did not modulate mRNA expression and probably they could act at a post-transcriptional step. This hypothesis was also supported by the evidence that PB28 decreased PgP protein expression [3] but not the mRNA expression level.

Flow cytometry analysis of cell cycle showed that PB28 and MC18 induced only a slight increase in G0/G1 phase conversely, MC70 increased cells accumulation in G2/M phase. These preliminary results evidenced how biological effects are strictly related to drug chemical structure. Moreover, western blotting analysis demonstrated that only MC18 and MC70 stimulated Akt activation without affecting p-ERK1/2 phosphorylation.

These evidences enlighten that, even if these agents has been designed and synthesized as pure PgP inhibitors, a complete analysis of their mechanism of action could optimize their pharmacological utilization.

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530 Poster

Development and characterisation of aptamers for cancer therapy

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The development of aptamer technology has opened up the number of tractable drug targets by offering novel means of interacting with DNA and proteins. We have utilised aptamer technology to generate inhibitors of a specific tumour marker involved in tumour progression.

Using a modified version of the traditional SELEX procedure we have generated three aptamer species towards a designed peptide of our target marker. To characterise the affinity of our aptamers for the target, we have explored the use of fluorescent spectroscopy, utilising a dye displacement method and a fluorescence resonance energy transfer (FRET) assay. As aptamers are short oligonucleotides they are intrinsically susceptible to nuclease degradation, which may limit their further study in animal models and subsequently their therapeutic application. Thus, the stability of these aptamers in mouse and human serum was investigated. To increase the potential therapeutic utility of aptamers, it is also necessary to truncate the aptamers from their 75 b length to typically 25 b. Consequently, two shorter versions of each aptamer, based on software predictions of their secondary structure and the initial design of the aptamer library used in the selection process. The binding of the full length and shorter aptamers were subsequently assessed, in vitro, using flow cytometry. Furthermore, the cell toxicity potential of these aptamers has also been measured in sulforhodamine B (SRB) assays.

Selection and affinity characterisation have provided three aptamer species showing specific binding to our target. Gel electrophoresis analysis of aptamer stability assays indicated that our aptamers posses remarkable