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.

529 Poster

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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[3] Azzariti A et al. Cyclohexylpiperazine derivative PB28, a sigma2 agonist and sigma1 antagonist receptor, inhibits cell growth, modulates P-glycoprotein, and synergizes with anthracyclines in breast cancer.Mol Cancer Ther. 2006 Jul;5(7):1807-16.

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