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.

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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.

[1] NA Colabufo et al. Small P-gp modulating molecules: SAR studies on tetrahydroisoquinoline derivatives. Bioorg Med Chem. 2008 Jan 1;16(1):362-73

[2] NA Colabufo et al. 4-Biphenyl and 2-naphthyl substituted 6,7-dimethoxytetrahydroisoquinoline derivatives as potent P-gp modulators.Bioorg Med Chem. 2008 Feb 2; [Epub ahead of print]

[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

stability in human serum for up to 24h. The stability of these aptamers in mouse serum, however, is significantly lower, with substantial degradation occurring within 20 min. Flow cytometry studies, have shown that these aptamers are able to bind to various cancer cell lines proposed to express the biomarker. Furthermore, the truncated versions of each aptamer displayed better binding than their full length versions. Finally, the shortened aptamers have demonstrated the ability to effect direct cell kill by inhibiting vital cellular pathways leading to cell apoptosis.

Our data demonstrates the therapeutic potential for aptamers targeting a cell surface biomarker involved in tumour progression and further studies are underway to characterise fully the anti-tumour efficacy of these reagents.

531 Poster

5T4-specific antibody responses are associated with survival in a phase II trial of renal cell carcinoma patients vaccinated with modified vaccinia Ankara delivering the tumour antigen 5T4 in combination with low-dose IL-2

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Background: The tumour antigen 5T4 is highly expressed in over 90% of renal cell carcinoma (RCC). Modified vaccinia Ankara (MVA) engineered to deliver 5T4 (TroVax) is being evaluated alongside low-dose IL-2 in an open label phase II trial in patients with metastatic RCC. The primary endpoints of this study are safety and immunological efficacy.

Materials and methods: Twenty five patients with locally advanced or metastatic RCC eligible for first or second line treatment with low dose IL-2 were recruited. IL-2 was given for up to 6 cycles with the following schedule: 250,000 U/kg/dose for 5 days in week 1 followed by 125,000 U/kg/dose for 5 days in each of weeks 2 though 6 inclusive, followed by a two week recovery. TroVax was administered by intra-muscular injection every 3-4 weeks for the first 4 injections and every 8 to 12 weeks thereafter. 5T4-specific cellular and humoral responses were monitored and clinical responses assessed by CT scan according to RECIST criteria.

Results: TroVax was well tolerated with no serious adverse events attributed to vaccination. 21 (84%) of 25 intent to treat patients mounted 5T4-specific antibody responses. Three patients showed complete responses (2 for 24+ and 1 for 12+ months), 6 patients had disease stabilization (6 to 21+ months) and the remainder had progressive disease. Median progression free survival (PFS) and overall survival (OS) was 3.4+ months (1.5-24.8+) and 12.9+ months (1.9-24.8+) respectively. A statistically significant correlation was detected between the magnitude of 5T4-specific antibody responses and PFS and OS (both $P<0.05$).

Conclusions: The primary endpoints of safety and immunological efficacy were met. TroVax was shown to be safe and well tolerated in all patients in combination with IL-2. The high frequency of 5T4-specific immune responses, number of complete clinical responses and correlation with clinical benefit are encouraging and warrant further investigation. A Phase III study with TroVax is ongoing in this indication.

532 Poster

Search for a breakthrough sensitizer in photodynamic therapy - contribution to expand a tidy technique

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Background: Photodymanic therapy (PDT) is presently a well established way for the treatment of oncological and non-oncological diseases. It is a minimal invasive procedure based on the destruction of malignant cells by action of singlet oxygen ($^1O_2$) generated through the combined action of a molecule (sensitizer) and light. The sensitizer which is not a therapeutic agent becomes active only when irradiated with low power light, developing a reaction cascade that produces apoptotic pathways leading to cell death. In absence of light the sensitizer is not harmful for cells. PDT has attracted a lot of interest due to the selectivity shown by malignant tumours for the molecules of porphyrins as sensitizers relatively to healthy tissues.Photofrin®, one of the most used sensitizers for cancer treatment actually approved by the FDA, is a β-substituted porphyrin. To become a more widely used technique, PDT needs the development of more specific an efficient sensitizers but, above all, get the sensibility and the motivation of the clinics to dominate the technique in a broader type of situations.

Materials and Methods: The sensitizers 5,15-diarylporphyrins, (1-3) were sensitized as previously reported in Patent nº102721, WO 03/064427, PCT/EP03/00829.

For each experiment, cells were plated in 48 multiwells (Corning Costar Corp), in a concentration of 40 000 cells/mL and kept in the incubator overnight, in order to allow the attachment of the cells. The formulation of these sensitizers consisted in a 1 mg/mL solution in a ternary mixture of H_2O:PEG_{400}:EtOH (50/30/20, v/v/v), the desired concentrations being achieved by successive dilutions. The sensitizers were administered in several concentrations (50 nM, 250 nM, 500 nM, 1 μM, 5 μM, 10 μM) and cells were incubated for 24 hours. Cells were washed with PBS and new drug-free medium was added. Each plate was irradiated with a fluence rate of 7.5 mW/cm^2 until a total of 10 J or 5 J was reached. Cell viability was measured 24 hours after the photodynamic treatment.

Results: The IC_{50} values for dose/response curves for WiDr human colon adenocarcinoma cells and melanoma A375 irradiated with 10 J are reported in table 1 as well as the values obtained for Photofrin® as reference compound.

Table 1 (Poster 532)

Compound	IC50 - WiDr - 10J	IC50 - A375 - 10J
Photofrin®	666 nM	156 nM
Compound 1	38 nM	27 nM
Compound 2	27 nM	27 nM
Compound 3	88 nM	-

Using 5 J of energy the values of IC_{50} for the sensitizer 1 are 68 nM and 27 nM and for the sensitizer 2 are 32 nM and 27 nM for WiDr and A375 respectively.

Conclusion: In this study we determined the anti-tumoral activity of our new 5,15-diarylporphyrins (1-3) against WiDr and melanoma A-357 cancer cell lines. The IC_{50} values are compared with those for Photofrin® and show an activity of 20 times superior. The results for PDT action of compound 2 in the inhibition of tumor growth in implanted tumor in nude mice will be presented.

533 Poster

Evaluation of antiproliferative and molecular effects of vinorelbine and its active metabolite 4-O-deacetyl-vinorelbine on human endothelial cells in an in vitro simulation model of metronomic chemotherapy

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Background and Aim: Metronomic chemotherapy is a novel approach of cancer therapy, developed on the concept that activated vascular endothelial cells are selectively sensitive to protracted exposure to very low concentrations of cytotoxics. Microtubule-targeting drugs are most potent against endothelial cells. Thus availability of an oral formulation of vinorelbine (Navelbine®) prompted us to take into clinical investigation this antimitotic drug at a metronomic dosing schedule [NCT00278070]. In this context we investigated antiproliferative and molecular effects of vinorelbine (VRL) and its active metabolite 4-O-deacetyl-vinorelbine (DVRL) on proliferating endothelial cells in an in vitro simulation model of metronomic chemotherapy.

Methods: Human umbilical vein endothelial cells (HUVEC) were plated to sub-confluence in 96- or 6-well plates and treated with VRL and DVRL for 24 and 96h replacing medium every 24h. The effects of different concentrations of VRL and DVRL on cell proliferation and the expression of angiogenesis modulating molecules TSP-1, VEGF, VEGFr2 and IL8 were assessed. We employed cell proliferation (MTS) assay for growth inhibition and measured molecular biomarkers of angiogenesis at a transcript level (RT-PCR) and also as excreted proteins in cell medium (ELISA).

Results: The half-maximal inhibitory concentrations (IC50) obtained against HUVEC were four orders of magnitude lower at the 96h-exposure compared with the 24h-exposure (1.23 nM vs 32 μM for the VRL and 0.55 nM vs 78 μM for DVRL). Notably the IC50 observed at the 96h-exposure are close to the trough levels recorded in patients treated with metronomic oral vinorelbine (Briasoulis et al, 18th EORTC-NCI-AACR Symposium 2006). At molecular level concentrations of both compounds at the high nanomolar and low micromolar range, which are commonly achieved with the conventional dosing of vinorelbine, induced proangiogenic feedback effects on the exposed HUVEC: at concentrations above 100 nM we observed a dose-dependent increase of proangiogenic molecule IL-8 and a parallel decrease of antiangiogenic TSP-1 at mRNA and protein levels. Such molecular responses did not occur at low-range nanomolar