

and is an inhibitor of PIM kinases. The aim of this study was to explore further its mechanism of action *in vitro*, and to determine its cytotoxicity both *in vitro* and *in vivo*.

**Methods:** *In vitro* cytotoxicity of CXR1002 was determined using an ATP depletion assay, alone and in combination with 8 other drugs in a panel of tumor cell lines. The mode of action was examined using microarray analysis (followed by analysis using the Ingenuity system) and western blots. Five CXR1002 xenograft studies (25 mg/kg *p.o.* 3x per week) were performed in nu/nu mice.

**Results:** CXR1002 was cytotoxic to a wide range of human tumor cells, including pancreatic and ovarian carcinoma and sarcoma. Cell lines derived from hematological malignancies were the most sensitive to CXR1002. The IC50 value was lower after 7 days vs 2 days exposure (range 100–590  $\mu$ M vs 175–>1000  $\mu$ M), suggesting potency was linked to duration of exposure. IC50 values for the most sensitive cell lines were substantially lower than the plasma concentrations achieved with a non-toxic dose in an on-going phase I trial. Drug combination studies with 8 anti-cancer drugs in 11 human cancer cell lines indicated that CXR1002 was synergistic with other anti-tumor drugs, particularly gemcitabine. Microarray studies in the pancreatic cell line PANC-1 treated with an IC15 dose of CXR1002 showed 4996 gene changes. Representation analysis of the 4996 signature list identified a number of pathways that were over-represented, in particular, genes in the ER stress pathway, including the ATF family of transcription factors. Western blot analysis of PANC-1 cells using PCNA, cleaved PARP and caspase antibodies showed that PCNA was reduced and cleaved PARP and cleaved caspases 3 & 7 were increased 24 hr after treatment with 300  $\mu$ M CXR1002, suggesting a pro-apoptotic/anti-proliferative outcome. CXR1002 was active in all 5 human xenograft (*i.e.* pancreatic, liver, prostate, lung and colon) models examined with best absolute tumor volume as a % of control of 50.13%, 77.05%, 19.14%, 75.04% and 49.22% respectively.

**Conclusions:** CXR1002 is a unique potential anti-cancer therapy that exhibits unique pharmacokinetics and a wide spectrum of biological activities. CXR1002 appears to act in part by its ability to induce ER stress. A phase I human trial is on-going.

124

POSTER

#### Mechanisms of action of histone deacetylase inhibitors (HDACi)

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**Background:** Histone deacetylases (HDACs) are promising targets for anti-cancer drug development as evidenced by the rapid development of HDAC inhibitors (HDACi) as chemotherapeutics. While HDACi are now approved agents for the treatment of certain haematological malignancies, their mechanisms of action are not fully understood. New isoform-specific HDACi are being designed to target specific HDACs in the hope that a more tailored, less toxic approach to cancer therapy will be achieved. The purpose of this study is to determine whether an HDAC 1/2-specific HDACi will have more potent anti-cancer activities than an HDACi specific for HDACs 1/2/3 and 6.

**Material and Methods:** Two structurally different HDACi were used: vorinostat, an HDACi that inhibits class I and II HDACs (HDAC1/2/3 and 6) and MRLB223, a recently developed HDACi specific for HDAC1 and HDAC2 (class I). E $\mu$ myc lymphomas were used to assess the biological activities of the two HDACi both *in vitro* and *in vivo*. Apoptosis readouts used were: propidium iodide staining to assess cell membrane permeabilisation; TMRE staining to assess mitochondrial function; histone acetylation and; TUNEL staining. C57BL/6 mice bearing E $\mu$ -myc lymphomas were used for therapy studies. Anti-tumor efficacy was determined by assessing the tumor-free and overall survival of tumor-bearing, HDACi-treated mice.

**Results:** Both vorinostat and MRLB-223 killed E $\mu$ myc lymphoma cells *in vitro* and engaged the same apoptotic pathways. Both HDACi induced histone hyperacetylation prior to cell death. The kinetics of apoptosis induced by MRLB-223 was slower than vorinostat and required significantly higher concentrations. *In vivo*, the survival of vorinostat- and MRLB-223-treated mice was significantly extended compared to vehicle-treated mice. However, while MRLB-223 still provided a therapeutic benefit to the mice, the effect was not nearly as robust as that provided by vorinostat.

**Conclusions:** We have shown the HDAC1/2 inhibitor MRLB-223 can achieve similar anti-tumor activities as vorinostat *in vitro* and *in vivo*. However, MRLB223 was limited in its efficacy as higher concentrations were required to achieve the same effects as vorinostat. Interestingly, MRLB223 displayed increased toxicity *in vivo* compared to vorinostat. Therefore, while targeting the enzymes HDAC1 and HDAC2 may be sufficient to cause apoptosis in E $\mu$ myc lymphomas, vorinostat was found to be the superior therapeutic agent.

125

POSTER

#### Targeting different conformations of BRAF kinase: efficacy of Omni-Raf inhibitors in NRAS and BRAF mutant tumors

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**Background:** The RAS-RAF-ERK cascade is aberrantly activated in many types of cancer. RAF inhibitors such as PLX4032 have demonstrated clinical efficacy in melanoma patients carrying the oncogenic mutant form of BRAF. Tumors driven by RAS or other elevated upstream signaling can bypass mutant BRAF-targeted compounds by mechanisms that are currently under investigation. In particular, the induced or autonomous conformational changes of the RAF kinases potentially can dislodge a conformation-specific inhibitor and even turn an inhibitor-bound RAF molecule into an activator of other RAF molecules. By applying the scaffold-based discovery method, we have identified a new generation of RAF inhibitors that show potent inhibition of all RAF isoforms, including mutant BRAF. As revealed by X-ray co-crystallography, these compounds have the unique ability to bind in both the active and inactive states of the RAF kinases, thereby avoiding the conformational restraints of the activation loop. We call this class of compounds the Omni-Raf inhibitors (ORIs).

**Material and Methods:** This study used BRAF<sup>V600E</sup> cell lines and NRAS mutant cell lines from commercial sources. The sensitivities of these cell lines to ORIs and BRAF<sup>V600E</sup>-specific inhibitor PLX4720 were determined by both growth and MTT assays. Balb/C nude, female mice were used for the xenograft studies. The treatment was started when mean tumor size reaches approximately 100 mm<sup>3</sup>. On the last day of the efficacy studies, the blood samples were collected at different time points after dosing to determine the plasma exposures of the compounds.

**Results:** In cell culture, the ORIs not only showed improved activity against BRAF mutant cell lines, including some previously known to be resistant to BRAF<sup>V600E</sup>-specific inhibitors, but also potentially inhibit melanoma cell lines driven by mutated NRAS. In xenograft models using BRAF<sup>V600E</sup> and NRAS-driven cells, the ORIs demonstrated over 90% tumor growth inhibition, including significant tumor regression, whereas the BRAF<sup>V600E</sup>-specific inhibitors showed no effect on NRAS-driven tumors.

**Conclusion:** These results show that RAF inhibitors structurally designed to target multiple conformations of the enzymes can prevent upstream signals from bypassing RAF inhibition.

126

POSTER

#### Impairment of S-nitrosothiol homeostasis and nitrosative stress modulate proliferation of breast cancer cells

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**Background:** Augmented nitric oxide (NO) levels in tumors have been usually detected compared to surrounding healthy tissue, and protein modifications induced by NO may constitute a significant regulating factor affecting both tumor progression and antitumoral treatment. S-nitrosothiol (SNO) formation is a cysteine modification, also referred to as S-nitrosation or S-nitrosylation, that controls the function of proteins in a manner similar to phosphorylation. One of the specific mechanisms governing protein de-nitrosylation is the system thioredoxin/thioredoxin reductase (Trx/TrxR). Manipulation or alteration of this enzymatic system may alter SNO homeostasis in tumor cells, providing new insights into the role of NO in cancer and its therapeutic significance.

**Materials and Methods:** Human breast cancer cells (MCF-7, MDA-MB-231 and BT-474) were pretreated or not with the specific TrxR inhibitor auranofin and exposed to different doses of S-nitroso-L-Cysteine (CSNO). Cell proliferation was measured using the XTT assay, and phosphorylation of Akt and Erk1/2 and cyclin D1 levels were determined by western blot using the corresponding specific antibodies.

**Results:** Treatment with auranofin and 100 nM CSNO enhanced cell proliferation of MCF-7 (ER+), but not of MDA-MB-231 (ER-, mut p53), or BT-474 (ER+, mut p53) cells. The augmented rate of cell growth was associated with Akt and Erk1/2 phosphorylation and higher expression of cyclin D1. Significantly, this pro-proliferative effect was abolished by the estrogen receptor (ER) antagonist fulvestrant or the p53 specific inhibitor pythiphrin- $\alpha$ . In contrast, in all the three cell lines, a high CSNO dose (500  $\mu$ M) reduced cell proliferation and this effect was potentiated by pretreatment with auranofin.

**Conclusions:** Impairment of SNO homeostasis modulated tumour cell growth depending on the grade of the subsequent nitrosative stress. A