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Downstream mechanistic markers of HDAC inhibition for drug discovery and clinical evaluation

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Post-translational modifications on histone proteins function as a 'histone code' to alter chromatin architecture and gene transcription. As the underlying cause of a large number of cancers is aberrant gene expression, one therapeutic tactic is to manipulate the histone code by developing small molecule inhibitors against chromatin modifying enzymes. Success in this field is exemplified by histone deacetylase inhibitors (HDACIs), e.g. the hydroxamate SAHA and the benzamide MS-275 which are undergoing clinical evaluation. Chromatin modifying enzymes also target non-histone proteins, including chaperone (e.g. HSP90), structural (e.g. α-tubulin) and transcription factor (e.g. p53, HIF-1α) proteins, A key requirement of mechanism-based drug discovery is to demonstrate that investigational compounds affect drug targets in the expected manner. We have evaluated ELISA methodologies (time-resolved fluorescence cell-based ELISAs (TRF-Cellisas and DELFIA assays) and the multiplexed assay platform, MesoScale Discovery (MSD), as tools for measuring biomarkers of HDAC inhibition. Histone H3 acetylation increased following exposure ($5\times$GI50 for 24h) of human tumour cells (HCT116) to SAHA (3-fold) and MS-275 respectively. These changes were similar to those measured by western blotting. A quantitative MSD assay was also suitable for measuring changes in H3 acetylation in peripheral mononuclear cells treated *ex vivo* with the same HDACIs for 4h. One key question for HDACI development is the need to define isoform selectivity. We have adapted the TRF-Cellisa to measure histone and α-tubulin acetylation, potential biomarkers of pan-HDAC and HDAC6 inhibition respectively. SAHA increased H3 and α-tubulin acetylation to similar extents whereas the non-HDAC6 inhibitor, sodium butyrate, only increased H3 acetylation. Eighteen hydroxamic acids (20 μM), identified from a focussed HDAC biochemical screen, increased acetylation of H3 (1.5−2.1-fold) and α-tubulin (1.6−2.5-fold) compared to control after 4h and 24h exposure. H3 and α-tubulin acetylation could also be quantified using the MSD in human prostate tumour cells (PC3LN3) grown as xenografts and treated with SAHA (50–100 mg/kg $\times$1 for 9 days). The MSD assay platform provides a sensitive, quantitative alternative to western blotting for measuring the downstream effects of HDACIs. We are presently validating assays for Good Clinical Laboratory Practice for use in future clinical trials.

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Modulation of estrogen and progesterone receptor signaling by selective HDAC inhibition

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Background: Modulation of estrogen signaling is one of the most successful modalities in the treatment of estrogen-receptor (ER) positive breast cancers. However, many ER-positive tumors show primary or acquired resistance to the commonly used anti-estrogens (e.g. tamoxifen, TAM) or aromatase inhibitors (AI). Particularly, tumors that express ER but not progesterone receptor (PR) may be more resistant to therapy.

Histone deacetylases (HDAC) play a central role in transcriptional regulation and may be involved in the pathogenesis of breast cancer. Several investigators have shown that HDAC inhibitors (HDACi) may restore sensitivity to anti-hormonal therapy. However, the mechanism and role of PR in this interaction and the relevance of individual HDAC enzymes remain unclear.

Here, we evaluated the roles of individual HDAC enzymes and the consequence of their inhibition on ER- and PR-mediated signaling in hormone-sensitive and hormone-resistant breast cancer cell lines. These findings are further evaluated in an ongoing clinical trial evaluating the HDACi, Vorinostat (SAHA) and tamoxifen after AI failure.

Methods: The roles of individual HDAC enzymes on ER and PR expression in pre-specified breast cancer cell lines were evaluated by selective inhibition of HDACs using siRNA and the use of various classes of HDACi.

Results: The co-treatment of both ER-positive and ER-negative breast cancer cell lines with HDACi and TAM resulted in synergistic anti-tumor activity. Treatment of ER-positive cells with HDACi resulted in down-regulation of ER with minimal effects on PR. In contrast, HDACi treatment of ER-negative cells resulted in a down-regulation of PR. Selective depletion of HDAC1 resulted in down-regulation of ER, but did not affect PR expression, whereas the selective depletion of HDAC2 resulted in reduced expression of both, ER and PR. HDAC6 depletion had no effect on ER. Furthermore, the selective inhibition of HDAC1 and HDAC2, but not HDAC6 by siRNA depletion was associated with growth inhibition even in the absence of TAM.

Conclusions: The anti-tumor effects of TAM in breast cancer cell lines are enhanced by co-administration of HDACi irrespective of ER expression, and are mediated through selective inhibition of specific HDAC enzymes. The synergistic effects observed in ER-negative tumors are mediated through modulation of PR. These findings are currently being evaluated in a clinical trial.

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YM155, a novel small molecule survivin suppressant, exhibits curative antitumor activities in experimental human malignant lymphoma models in vivo

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Survivin is a member of the Inhibitor of Apoptosis (IAP) family of proteins, and is highly expressed in all primary tumor types. Given its preferential expression in tumor cells, and its ability to block apoptosis and regulate cancer cell proliferation, survivin appears to be a putative novel target for cancer therapy. YM155 is a novel, small molecule survivin suppressant (AACR-NCI-EORTC 2005, Abstract #B203). In a phase I, open-label study of YM155 with 7-day continuous i.v. infusion, partial response was observed in the patient with diffuse large B cell lymphoma (DLBCL) refractory to R-CHOP (ASCO Annual Meeting 2006, Abstract #3014). In this study, we evaluated pre-clinical efficacies of YM155 on experimental human DLBCL (RL) and Burkitt's lymphoma (Ramos) models. These two human lymphoma cell lines were either s.c. or i.v. inoculated to mice. Dose level and schedule of each drug was adjusted to clinical equivalent dose. Single administration of YM155 (7-day continuous s.c. infusion) at 3 mg/kg, vincristin (i.v.) at 0.5 mg/kg, or rituximab (i.p.) at 50 mg/kg was repeated for every three-week schedule in mice. We also conducted quantitative real time PCR and immunohistochemical analyses on survivin in the tumors obtained from the YM155-treated mice. In nude mice bearing RL and Ramos lymphoma xenografts, YM155 at 1 and 3 mg/kg induced statistically significant tumor regression with no decrease in body weight at day 21. YM155 suppressed survivin expression, and induced cell death in the tumors obtained from the YM155-treated mice. In addition, some of the YM155-treated animals cured and experienced complete regressions of tumors (4 of 6 animals in RL, 5 of 6 animals in Ramos). In SCID mice i.v. inoculated RL and Ramos, YM155 showed a significant survival benefit compared with control. Vincristine or Rituximab showed no superior antitumor activity or survival benefit to YM155 in all the evaluation models tested. These results clearly support the phase I clinical efficacies of YM155 observed in the patients with DLBCL. In conclusion, YM155 is expected to become a new chemotherapeutic option which can eradicate R-CHOP refractory and aggressive lymphomas in clinical setting.

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Sequence dependent anti-tumor activity of bortezomib and the KSP inhibitor, SB743921, in a solid tumor xenograft model

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SB-743921 is a potent specific kinesin spindle protein (KSP) inhibitor currently in Phase I/II clinical trials. KSP is a kinesin required for completion of mitosis, and KSP inhibitors cause mitotic arrest of tumor cells. Pre-clinical studies suggest that inhibiting exit from a KSP inhibitor induced mitotic arrest increases cancer cell toxicity. We therefore tested combinations of KSP and proteasome inhibitors in tissue culture. Exposure of cells to a KSP inhibitor followed by a proteasome inhibitor 24 hours later resulted in increased cancer cell death compared to the reverse order of addition.

Based upon sequence dependent activity *in vitro*, we initiated combination studies in nude mice, administering both drugs on q4dx3 schedule simultaneously and separated by 24 hours in both orders of addition. Studies to establish the maximum tolerated dose (MTD) of simultaneous and sequenced administration of SB-743921 and bortezomib identified a marked sequence dependence. Administration of bortezomib prior to SB-743921 was least well-tolerated, while on the reverse sequence both drugs could be administered at their respective single agent MTDs.

Anti-tumor activity of sequenced and simultaneous administration was explored in HT29 tumor xenografts. There were two objectives of this study, 1) to compare single agent activity to the combination of SB-743921 administered prior to bortezomib at their respective MTDs, and

2) to evaluate the effects of sequence of administration on efficacy. The mean time to endpoint (TTE), defined as tumor volume =1000 mm^3, of untreated control animals was 25.4 days. The mean TTE of bortezomib (33.9 days) or SB-743921 (48.6 days) administered as a single agent at MTD did not differ significantly from untreated controls. Administration of SB-743921 24 hours prior to bortezomib resulted in a TTE of 58 days, which was superior to untreated control ($p = 0.004$) or SB743921 alone ($p = 0.038$) and comparable to activity of paclitaxel (TTE=59 days). In order to evaluate the effects of sequence of administration of bortezomib and SB-743921, the dose of SB743921 was lowered to accommodate all schedules. The combination had activity that was superior to bortezomib monotherapy when dosed simultaneously or with SB743921 first. Dosing of bortezomib prior to SB743921 was not significantly different from either monotherapy. The anti-tumor activity of the combination suggests that exploration is warranted in disease settings where bortezomib is approved.

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The role of Src-family kinases in the activation of the EGFR following chemotherapy

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Human cancer cells may respond to chemotherapy by activating the EGFR and survival pathways such as Akt. We have shown recently that colorectal cancer (CRC) and non-small cell lung carcinoma (NSCLC) cells that respond to chemotherapy by up-regulating phospho-EGFR, are sensitized to EGFR-targeted tyrosine kinase inhibition. We investigated the mechanism of EGFR activation following chemotherapy treatment in LoVo and HCT116-$p53^{+/+}$ CRC and H460 NSCLC cells. Following treatment with chemotherapy (antimetabolites/Topoisomerase-1 inhibitors/platinum compounds/taxanes), we found that increased phospho-EGFR was associated with increased Src-family kinase (SFK) activity. Using the SFK inhibitor PP2 and the broad-spectrum (MMP) matrix-metalloprotease and ADAM (a desintegrin and metalloprotease) inhibitor GM6001, we showed that chemotherapy-activated EGFR phosphorylation was abolished. Furthermore, we found that GM6001 had no effect on SFK activity, indicating that SFK's act upstream of metalloproteases. In addition, when PP2 or GM6001 were combined with chemotherapy, we found an additive or synergistic interaction in these cell lines. Further studies indicated that ADAM-17 and TGF-α were important regulators of chemotherapy-induced EGFR activity in the CRC and NSCLC cells. Our findings indicate that increased EGFR activity following chemotherapy is induced via SFK-mediated activation of ADAM-17, which induces shedding of TGF-α. Moreover, targeting SFK or metalloproteases in combination with existing chemotherapies may have therapeutic potential for the treatment of CRC and NSCLC tumours.

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Glut1 antibodies decrease proliferation and enhance the induction of apoptosis in human non small cell lung cancer (NSCLC) and breast cancer (BC) cell lines

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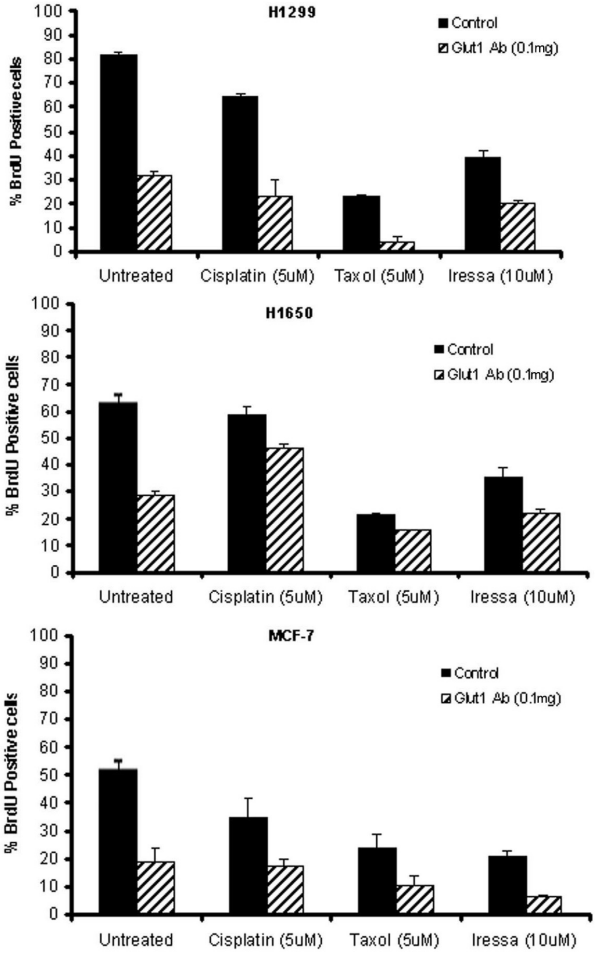
Background: A tumors' malignant potential is determined by its ability to generate energy and survive in hypoxic and acidotic environments by switching to anaerobic glycolysis. Anerobic glycolysis is an inefficient method of breaking down glucose for energy necessitating increased glucose uptake, which is facilitated by the membranous translocation of a high affinity Glucose Transporter, Glut-1. Glut-1 belongs to a family of trans-membrane glucose transporters, Glut-1−12, and is preferentially up regulated by tumors, presumably because it is a high affinity glucose transporter. We therefore hypothesized that blocking membranous Glut-1 by an anti-Glut-1 antibody would abrogate tumor growth.
Materials and Methods: BC and NSCLC cell lines were first assessed for increased Glut-1 expression. NSCLC cell lines H1260 and H1650 and BC cell line MCF7 were plated on chamber slides and incubated in presence of 5 μM cisplatin, 5 μM paclitaxel or 10 μM geftinib in absence or presence of 0.1 mg/ml anti-Glut-1 antibody. Proliferation was assessed by BrdU. Apoptosis was assessed by TUNEL Assay and PARP cleavage.
Results: In the H1299 NSCLC line treatment with the anti-Glut-1 antibody alone, inhibited proliferation by 62%, when added to cisplatin, paclitaxel and gefitinib it enhanced inhibition of proliferation by 62%, 74% and 42%, respectively. Similarly in the H1650 NSCLC cell line, treatment with anti-Glut-1 antibody alone inhibited proliferation by 55%; when added to cisplatin, paclitaxel and gefitinib it enhanced inhibition of proliferation by 18%; 23% and 46%, respectively. In the MCF7 NSCLC cell line, anti-Glut-1 antibody alone inhibited proliferation by 59%, when added to cisplatin, paclitaxel and gefitinib it enhanced inhibition of proliferation by 40%, 47% and 59%, respectively. Please see attached figure. Apoptosis was assessed after treatment with 5 μM cisplatin, 5 μM paclitaxel or 10 μM geftinib alone, and with anti-Glut-1 antibodies. Glut-1 antibodies enhanced the apoptotic effects of cisplatin, paclitaxel and gefitinib in H1650 cell line by 43%, 62% and 111%; in H1299 by 111%, 30% and 71% and in MCF7 cell line by 37%, 91% and 133%, respectively.



Conclusions: Our results indicate that anti-Glut-1 antibodies inhibit proliferation and enhance the inhibition of proliferation and apoptosis induced by cisplatin, paclitaxel and gefitinib in the evaluated NSCLC and BC cell lines, providing evidence that the use of antibodies to Glut-1 may be a viable therapeutic strategy in tumors that over express Glut-1.

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HSP27 as a novel target in Non-small cell lung cancer with particular implications for migration and metastasis

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Purpose: The role of HSP27 in non-small cell lung cancer (NSCLC) was evaluated with a focus on expression in metastases versus primary tumor and its role in-vitro in cell motility and migration.
Methods: Tumor tissue microarrays (TMA) were stained for HSP27 and Phospho-HSP27 (IHC) and results correlated with outcome and stage. Expression in 27 metastases was compared with the respective matched primary tumors. Cell motility in HSP27 lentivirus transfected A549 NSCLC