lation between paired tumor samples [Pearson correlation of percentage × intensity score, 0.922 ($p<.001$)]. While total EGFR staining was similar between tumor and normal tissues, cancer samples had markedly higher staining of pEGFR, Akt, pAkt, MAPK, and pMAPK. ACIS analysis of xenografts was poorly reproducible. There did not appear to be preferential activation of a particular EGFR signaling pathway.
Conclusions: ACIS IHC is quantitative, reproducible, and correlates with Western blots and ELISA in cell line pellets. A graphic microdissection technique appears to overcome the issue of tissue heterogeneity. Colorectal tumors show higher staining of pEGFR and downstream effectors compared to matched normal colorectal tissues.

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Development of HDAC Class I and II specific assays in order to identify novel small molecule inhibitors

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Histone deacetylase (HDAC) activity is associated with repression of gene expression. Aberrant gene expression is often observed in cancer, therefore, the enzymes involved in regulating gene expression are of particular interest as target proteins in oncology. HDACs are involved in deacetylating histone and non-histone proteins. e.g. HDAC6 functions as an α-tubulin deacetylase. Eleven members of the HDAC family have been identified in humans. Non-sirtuin HDACs can be divided into three distinct groups of Class **I** (HDAC1, 2, 3, 8), Class **II** (HDAC4, 5, 6, 7, 9, 10) and Class IV-proteins related to the human HDAC11 gene.
Topotarget has a novel HDAC inhibitor (HDACi), PXD101, currently undergoing Phase **I** clinical trials. Specific HDAC isotype *in vitro* biochemical assays have been developed and used to screen novel HDACi compounds. Details are given on the baculoviral expression and purification by affinity chromatography of a number of HDAC isotypes. Data on the optimization of the conditions for the Fleur de Lys™ HDAC assay is presented. A subset of small molecule HDACi compounds were screened, comprising 6 chemical classes – amides, sulphonamides, piperazine ketones, piperazine sulphones, heterocycles and ethers. The effect of these compounds on the activity of HDAC isotypes, representing both Class **I** and **II**, is described.
A cell-based assay was developed in order to study HDACi induced changes in α-tubulin and histone acetylation levels. These changes were detected using FACs and western blotting techniques. The kinetics of tubulin and histone acetylation was investigated following HDACi withdrawal. Data from these experiments are also presented.

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Effectiveness of a novel, selective inhibitor of the IGF-IR kinase against musculoskeletal tumors

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Background: The identification of new active agents against sarcoma is considered an important challenge in medical oncology. Several lines of evidence have indicated that Insulin-like Growth Factor-I (IGF-I) and its corresponding receptor (IGF-IR) were of major importance in deregulated sarcoma cell growth and pathogenesis, therefore representing a valuable therapeutic approach against these tumors. In this study, we analyzed the *in vitro* effects of the orally bioavailable, specific IGF-IR kinase inhibitor NVP-AEW541 in a panel of musculoskeletal tumor cell lines (5 human rhabdomyosarcoma; 10 human Ewing's sarcoma and 8 human osteosarcoma cell lines).
Methods and Results: A potent cell growth inhibitory activity of NVP-AEW541 was clearly observed either in monolayer and in anchorage-independent conditions. Ewing's sarcoma cells appeared to be particularly sensitive to the effects of this drug (IC_{50} ranging from 100 nM to 300 nM), whereas osteosarcoma cells were at least 10-fold more resistant to the drug, in agreement with previous observations obtained with the neutralizing anti-IGF-IR αIR3 antibody. The analysis of the effects of NVP-AEW541 on the cell cycle and apoptosis indicated a significant enhancement of the G1-phase rate and apoptotic rate in treated cells. In addition, NVP-AEW541 showed anti-angiogenetic activity since it significantly reduced the expression and secretion of VEGF-A by sarcoma cells, and supernatants of treated cells were unable to sustain the survival and proliferation of HUVEC endothelial cells. We also analyzed whether this agent is of value in being combined with conventional cytotoxic drugs for the design of more effective therapeutic regimens. Concurrent exposure of cells to NVP-AEW541 and other chemotherapeutic agents resulted in greater than additive interactions when vincristine and ifosfamide were used, whereas subadditive effects were observed with doxorubicin, cisplatin and actinomycin D.
Conclusions: All together, these results encourage future studies testing the *in vivo* therapeutic value and the general toxicity of this specific IGF-IR kinase inhibitor to be considered for innovative treatments of patients with sarcomas, particularly Ewing's sarcoma and rhabdomyosarcoma. A careful design of new regimens is required in order to identify the best therapeutic conditions and drug-drug interactions.

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MEK1inhibition enhances arsenic trioxide (ato) induced apoptosis in acute leukemia

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According to recent laboratory studies, the blast cells of most acute myelogenous leukemias (AML) including acute promyelocytic leukemia (APL) show constitutive activation of extracellular signal-regulated kinases 1/2 (ERKs 1/2) as well as of the kinases immediately upstream of ERK, known as mitogen-activated protein (MAP)/ERK kinases (MEKs). Furthermore, we and others have demonstrated that down-modulation of MEK1 phosphorylation inhibits the proliferation and induces apoptosis of primary AML blasts. In this study, we firstly aimed at investigating whether the combination of Arsenic Trioxide (ATO) with agents that block the phosphorylation of MEK1 can potentiate the anti-leukemic action of ATO in APL. We then investigated whether this combination is capable to enhance apoptosis of non APL AML blasts. For our purposes we studied parental NB4 cell line, an arsenic-resistant NB4 subline (NB4-As^R) derived in our laboratory from the NB4 cell line, primary blast cells of typical hypergranular APL (M3) carrying PML/RARα fusion transcript, primary blast cells of AML (M1 or M2) carrying 47, XX, +8 or 46, XX inv (16), of acute monocytic leukemia (M5), of acute lymphocytic leukemia carrying 46, XX, del (11)(q23). Leukemic cells were pre-treated with PD98059 (Cell Signaling Technology, Beverly, MA) 10, 20 or 40 microM or PD184352 (kindly provided to us by Dr J. S. Sebolt-Leopold, Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI) 1 or 2 microM, and then treated with ATO 0.5–2microM. We found that leukemia cells exploit the Ras-MAPK activation pathway to phosphorylate at Ser112 and to inactivate the pro-apoptotic protein Bad, delaying arsenic trioxide (ATO)-induced apoptosis. Both in APL cell line NB4 and in primary blasts, the inhibition of ERK1/2 activity and of Bad phosphorylation by MEK1 inhibitors enhanced and accelerated apoptosis in ATO-treated cells. NB4-As^R showed stronger ERK1/2 activity (2.7 fold increase) and Bad phosphorylation (2.4 fold increase) compared to parental NB4 cells in response to ATO treatment. Upon ATO exposure, both NB4 and NB4-As^R cell lines doubled protein levels of the death antagonist Bcl-xL but the amount of free Bcl-xL that did not heterodimerize with Bad was 1.8 fold greater in NB4-As^R than in the parental line. MEK1 inhibitors dephosphorylated Bad and inhibited the ATO-induced increase of Bcl-xL, overcoming ATO resistance in NB4-As^R. Synergism, additive effects, and antagonism were assessed using the Chou-Talalay method and Calcusyn software (Biosoft, Ferguson, MO). PD + ATO combination appears to synergize for the induction of apoptosis primarily in arsenic resistant but also in parental NB4 cells. Furthermore, the combination PD + ATO significantly increased the ATO-induced apoptosis in primary acute leukemia blasts ($P<0.001$) These results may provide a rationale to develop combined MEK1 inhibitors plus ATO therapy in APL and in other types of acute leukemia.

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Anti-tumor activity, pharmacokinetic and pharmacodynamic effects of the MEK inhibitor ARRY-142886 (AZD6244) in a BxPC3 pancreatic tumor xenograft model

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Background: ARRY-142886(AZD6244), a potent, selective MEK1,2 inhibitor currently in Phase I trials, has demonstrated efficacy in numerous tumor models, including HT29, BxPC3, MIA PaCa2, A549, Colon26, PANC-1, LoVo, Calu6, HCT116, MDA-MB-231, ZR-75–1 and LOX. The

current report extends these findings by examining the relationships between *in vivo* activity, plasma drug concentrations, and inhibition of the pharmacodynamic marker, phospho-ERK (pERK), in both tumor and whole blood samples.
Methods: Nude mice with established BxPC3 tumors were treated with ARRY-142886(AZD6244) at 1, 3 and 10 mg/kg, po, bid for 14 days. Effects on tumor growth were assessed. Mice were euthanized at 1, 3, 6 and 9 hours after the last dose. Tumors were analyzed for pERK by Western blot analysis. Whole blood was analyzed for drug concentrations by mass spectrometry and TPA-induced pERK by flow cytometry.
Results: ARRY-142886(AZD6244) inhibited tumor growth by 50% at the 1 mg/kg dose and >90% at the higher doses. Mean plasma concentrations of ARRY-142886(AZD6244) at the last time point (9 hours) were 0.18, 0.34 and 0.95 ug/ml for the 1, 3 and 10 mg/kg doses, respectively. Inhibition of pERK in tumors from both the 3 and 10 mg/kg groups was marked and also sustained throughout the sampling period, whereas the effects at the 1 mg/kg dose were not sustained at the later time point. Inhibition of pERK in *ex vivo* blood was weak but consistent with the EC_{50} (~3 ug/ml) for exogenously added drug. This value in mouse blood is higher than that seen in human blood (EC_{50} ~0.5ug/ml).
Conclusions: Whole blood pERK is not a predictive biomarker in mice, although, due to increased sensitivity, it may be better in humans. These results demonstrate that near complete inhibition of BxPC3 tumor growth corresponds to C_{min} drug concentrations of greater than 0.3ug/ml and sustained inhibition of pERK in tumors.

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Novel role of fumarate in antagonizing VHL function

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The fumarate hydratase (FH) gene product plays an essential role in the Krebs cycle by catalyzing the conversion of fumarate to malate. Germline mutations in FH predispose to dominantly inherited uterine fibroids and papillary renal cell cancer, although the pathway contributing to tumorigenesis is unknown. Hypoxia-inducible factors HIF-1 and HIF-2 promote survival and are required for tumorigenesis in many types of primary tumors and metastases. Under normal oxygen tension, the HIFs are unstable due to the activity of VHL, a protein that targets HIF for proteasome-dependent degradation. The stabilization of HIF proteins under hypoxia results from inactivation of HIF prolyl hydoxylase (HPH) enzymes that hydroxylate HIF, thus preventing an essential prerequisite for VHL recognition of HIF. In this study, we examined whether FH inactivation influences HIF expression. We demonstrate that inhibition of FH, either by siRNA or pharmacologic means, is correlated with an upregulation of HIF-1 and HIF-2 proteins, which reflects the upregulation of these proteins in FH renal tumors. Treatment of cells with fumarate also increased HIF expression. The most potent HIF upregulation was elicited by the combination of a pharmacologic FH inhibitor and fumarate, the latter exhibiting a dose-dependent effect upon HIF. Elevated HIF levels correlated with increased transcription and expression of VEGF, which was corroborated by CD31 staining of FH renal tumors. The mechanism for increased HIF expression was posttranscriptional, due to protein stabilization in a VHL-dependent manner. In vitro binding assays revealed that fumarate prevented the association of VHL with HIF in a dose-dependent fashion that was reversed by exogenous addition of 2-ketoglutarate. Collectively, these data suggest that the mechanism for FH-mediated HIF upregulation depends upon the accumulation of fumarate, which acts as a competitive inhibitor of 2-ketoglutarate, an essential cofactor for HPH. Thus, fumarate impairs the activity of HPH and prevents VHL from recognizing HIF, culminating in elevated levels of transcriptionally active HIF protein. Our results delineate a novel fumarate-dependent pathway for regulation of HIF expression and they highlight a previously unrecognized relationship between dysregulated metabolic pathway intermediates and tumorigenesis. Our data highlight FH as an important molecular target whose function is compromised in a subset of cancers.

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JANEX-1, a novel anti-cancer agent with anti-thrombotic properties

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Background: Here we provide experimental evidence that identifies JAK3 as one of the regulators of platelet function.
Methods: To study the effects of JANEX-1 on platelet function, platelets were subjected to aggregation and functionality assays, immunoprecipitation and western blot analysis, cytoskeletal fractionation, high-resolution low-voltage scanning electron microscopy and transmission electron microscopy. In vivo anticoagulant activity was assessed by measuring bleeding and clotting times in mice as well as improved event-free survival in a mouse model of thromboplastin-induced generalized and invariably fatal thromboembolism.
Results: Treatment of platelets with thrombin induced tyrosine phosphorylation of the JAK3 target substrates STAT1 and STAT3. Platelets from JAK3-deficient mice displayed a decrease in tyrosine phosphorylation of STAT1 and STAT3. In accordance with these data, pretreatment of human platelets with the JAK3 inhibitor JANEX-1 markedly decreased the base-line enzymatic activity of constitutively active JAK3 and abolished the thrombin-induced tyrosine phosphorylation of STAT1 and STAT3. Following thrombin stimulation, JANEX-1-treated platelets did not undergo shape changes indicative of activation such as pseudopod formation. JANEX-1 inhibited thrombin-induced degranulation/serotonin release as well as platelet aggregation. Highly effective platelet inhibitory plasma concentrations of JANEX-1 were achieved in mice without toxicity. JANEX-1 prolonged the bleeding time of mice in a dose-dependent manner and improved event-free survival in a mouse model of thromboplastin-induced generalized and invariably fatal thromboembolism.
Conclusion: To our knowledge, JANEX-1 is the first anti-cancer agent with anti-thrombotic properties that prevents platelet aggregation by inhibiting JAK3.

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The HIV protease inhibitor Amprenavir as a radiation sensitizers

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Background: We have shownt that PI3K activation both *in vitro* and *in vivo* is a critical step regulating tumor cell radiosensitivity in multiple human tumors. There are, however, currently no clinically useful inhibitors of PI3K. Akt is an immediate downstream target of PI3K. There are a number of reports documenting insulin resistance and diabetes in patients on HIV protease inhibitors (HPIs). Since we know that Akt signaling plays a role in insulin signaling, we speculated that these side effects of HPIs might be due to interference with Akt signaling.
Material and Methods: We obtained the HIV protease inhibitor amprenavir from the patient pharmacy. We tested our panel of cell lines with increased signaling thru PI3K that is either Ras or EGFR dependent. The concentration and time course required to inhibit Akt phosphorylation was determined by Western blot analysis. Clonogenic survival curves were carried out.
Results: Akt is a serine/threonine kinase that is phosphorylated at two sites, Thr 308 (kinase domain) and Ser 473 (C-terminal regulatory region). It is the Ser 473 site that appears to be necessary for maximal activation of Akt. We initially tested the human head and neck cancer cell line SQ20B with a constitutively active EGFR receptor and thus increased signaling through PI3K. We found that concentrations of 20 μM completely down-regulated Akt phosphorylation at Ser 473. There was no change in phosphorylation at the Thr 308 site. Clonogenic assays in SQ20B cells showed radiosensitization after treatment with 20 μM amprenavir with the surviving fraction after 2 Gy going from 70% to 51% after 2 Gy and amprenavir. Similar Western blot and survival data was also obtained in the human bladder cancer cell line T24 which has a mutated H-Ras and thus increased signaling thru the PI3K pathway.
Conclusions: HPIs may be useful as radiosensitizers in cells with activation of Akt. Because Akt is constitutively active only in tumors, this approach may be specific for tumors. Further, since HPIs can be given chronically with tolerable to minimal side-effects, this approach could be clinically applicable.