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NMS-E628, a potent and orally available small molecule inhibitor of anaplastic lymphoma kinase, reduces tumor growth in an intracranial model of ALK-dependent NSCLC

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The ALK tyrosine kinase gene undergoes chromosomal rearrangements in the majority of Anaplastic Large Cell Lymphoma (ALCL) cases, and in a subset of Non Small Cell Lung Cancer (NSCLC), giving rise to various fusion proteins which bear a constitutively activated ALK kinase domain. Additionally, full length ALK is often found to be activated by gene amplification or by kinase domain point mutations in a significant fraction of neuroblastomas. In all three of these tumor types, there is strong evidence that activated ALK kinase is a driver oncogene, and that pharmacological intervention with small molecule inhibitors which target this kinase represents a promising therapeutic approach for affected patient populations.

We have previously presented the identification of NMS-E628, an orally available small-molecule ALK kinase inhibitor. Here, we describe further preclinical characterization of this molecule. In vitro, NMS-E628 very selectively inhibited proliferation of ALK-dependent cell lines with IC50s in the sub 100 nM range. Interestingly, short-term exposure of the Karpas-299 ALK+ ALCL cell lines to NMS-E628 induced long lasting and profound induction of cell cycle block and inhibition of proliferation which was maintained for several days following withdrawal of the drug. Concomitant with this effect, sustained inhibition of NPM-ALK autophosphorylation and downstream signaling was observed, despite persistent expression of NPM-ALK fusion protein.

In a murine subcutaneous xenograft model employing the H2228 human ALK+ NSCLC line, activity of NMS-E628 compared favorably with that of PF-02341066 (Crizotinib®), an agent which demonstrated a 57% response rate in ALK+ NSCLC patients in phase II clinical studies. Oral administration of NMS-E628 to H2228 tumor bearing mice yielded complete and durable regressions in all treated animals.

Since NMS-E628 is able to pass the blood–brain barrier, the compound was also tested for efficacy in an intracranial xenograft model employing H2228 tumors. MRI imaging demonstrated that NMS-E628 was able to effectively and dose-dependently control the growth of these intracranial tumors, leading to increased survival.

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Amuvatinib (MP-470), a multi-targeted tyrosine kinase inhibitor and DNA repair suppressor, synergizes with etoposide (VP-16) in small cell lung cancer (SCLC) cell lines and xenografts

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Background: MP-470 is an orally bioavailable multi-targeted tyrosine kinase inhibitor specifically designed to be a potent inhibitor of mutant c-Kit and PDGFRα. MP-470 also decreases Rad51-mediated homologous recombination DNA repair and increases cancer cells' chemosensitivity (Bristow et al., 2009). In a Phase 1b clinical study of MP-470 in combination with VP16+Carboplatin, several responses were observed in previously treated SCLC. To support a potential Phase 2 clinical study, we evaluated the effects of MP-470 as single agent and in combination with VP-16 and carboplatin in a panel of 5 SCLC cell lines. Efficacy of MP-470+VP-16 combination was studied also in SCLC NCI-H146 xenografts.

Methods: Viability of 5 SCLC cell lines (LB12-SCLC/OC2, LB13-SCLC/OC3, NCI-H146, NCI-H69 and NCI-H82) after treatment with MP-470, VP-16 and carboplatin as single agents or in combination was evaluated using the MTS assay and combination index (CI) was determined after simultaneous treatment for 72 hrs. Modulation of cell signaling pathways after MP-470 treatment was evaluated in these SCLC cell lines and xenografts by Reverse Phase Protein Array (RPPA) and Western blot. Tumor growth inhibition after administration of MP-470 and VP-16 was evaluated in NCI-H146 xenografts established in Swiss nude mice.

Results: All 5 SCLC cell lines tested were sensitive to MP-470 with LB12-SCLC/OC2 being the most sensitive (4.79 μM). When MP-470 and VP-16 were combined, effects produced were generally additive (on three of the five cell lines tested); synergism was observed in NCI-H146 (CI = 0.68±0.18). When MP-470 and VP-16 were combined to carboplatin, significant synergism was again evident in NCI-H146 (CI = 0.72±0.12) and additivity was observed in NCI-H69 and LB12-SCLC/OC2. RPPA analysis of cell extracts showed a significant dose and time dependent modulation of phospho-S6 and phospho-4EBP1 after MP-470 treatment. In vivo PO administration of MP-470 in combination with IV VP-16 in NCI-H146 tumor-bearing mice at well tolerated doses and regimens produced a sustained reduction in T/C ratio <39%. Modulation of Akt and 4EBP1 phosphorylation was observed in tumor extracts prepared from NCI-H146 xenografts after treatment with MP-470.

Conclusions: Overall, MP-470 is synergistic with DNA-damaging agents like VP-16 and improves VP-16 anti-cancer activity in SCLC xenografts. MP-470 warrants further testing in pre-clinical and clinical studies in combination with VP16-containing chemotherapy.

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Molecular mechanisms of the combination treatment of cetuximab and dasatinib in Kras mutant colorectal tumors

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Kras mutation is a predictive biomarker for resistance to cetuximab (Erbitux®) in metastatic colorectal cancer (mCRC). This study sought to determine if Kras mutant CRC lines could be sensitized to cetuximab *in vivo* using the FDA approved Src family kinase (SFK) inhibitor, dasatinib (BMS-354825, sprycel®). We analyzed 16 CRC lines for: (1) Kras mutation status, (2) dependence on mutant Kras signaling, (3) expression level of EGFR and SFKs. From these analyses, we selected three Kras mutant (LS180, LoVo, and HCT116) cell lines, and two Kras wild type cell lines (SW48 and CaCo2). *In vitro*, using PDL/laminin plates, Kras mutant cell lines were resistant to cetuximab whereas parental controls showed sensitive to cetuximab. Treatment with cetuximab and dasatinib showed a greater anti-proliferative effect on Kras mutant line as compared to either agent alone. To investigate a mechanism for this increased response in the combinatorial therapy we performed Human Phospho-kinase Antibody Array analysis (ARY003, R&D systems) measuring the relative phosphorylation levels of phosphorylation of 46 intracellular serine/threonine/tyrosine kinases in untreated, cetuximab, dasatinib or the combinatorial treatment in LS180, LoVo and HCT116 cells. The results of this experiment showed a compelling decrease in a broad spectrum of kinases when compared to the untreated or monotherapy treated controls. To strengthen our *in vitro* findings we analyzed tumor growth delay with cetuximab, dasatinib or the combination *in vivo*. Kras mutant xenografts showed resistance to cetuximab therapy, whereas Kras wild type demonstrated an anti-tumor response when treated with cetuximab. Kras mutant tumors exhibited minimal response to dasatinib in monotherapy. However, *as in vitro*, Kras mutant lines exhibited a response to the combination of cetuximab and dasatinib as compared to controls. Combinatorial treatment of Kras mutant xenografts resulted in decreased cell proliferation as measured by Ki67 and higher rates of apoptosis as measured by TUNEL compared to controls. The data presented herein indicate that dasatinib can sensitize Kras mutant CRC tumors to cetuximab and may do so by altering the activity of several key kinases. Further, these results suggest that signaling via the EGFR and SFKs may be necessary for cell proliferation and survival of Kras mutant CRC tumors. This data strengthen the rationale for clinical trials in this genetic setting combining cetuximab and dasatinib.

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Synergistic toxicity of tyrosine kinase inhibitors with Hsp90 inhibitor 17-AAG in lung cancer cell line

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Background: 17-allylamino demethoxygeldanamycin (17-AAG), a Hsp90 inhibitor is a synthetic derivative of geldanamycin which exhibits 100-fold higher binding affinity for tumor-cell derived Hsp90 compared to normal cells. 17-AAG blocks oncogene switching and inhibits multiple signaling pathways including ErbB and Src kinases. Gefitinib was shown to lose its ability to modulate ErbB pathways due to oncogene switching. Since 17-AAG blocks this oncogene switching, we examined effects of combining 17-AAG with tyrosine kinase inhibitors (TKIs) such as erlotinib (EGFR selective with no effect on A549 cells), gefitinib (EGFR selective), vandetanib (EGFR and VEGFR selective) and lapatinib (EGFR and Her-2 selective) in a lung cancer cell line A549. In addition we studied effects of 17-AAG on human nucleoside transporters because antagonistic effects of combination of 17-AAG with cytarabine were reported earlier in leukemic cells.

Materials and Methods: The human lung cancer cell line A549 was obtained from American Type Culture Collection (Manassas,VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 10% glucose. All cultures were kept at 37ºC in 5% CO_2/95% air and sub-cultured every 2−3 days to

maintain exponential growth. All experiments were conducted with cells in exponential growth phase. Cells were free of mycoplasma contamination.
Results: 17-AAG was toxic to A549 cells and synergistic toxicity was observed during simultaneous exposures with each of the four TKIs. Flow cytometry analysis of cell surface expression of EGFR using monoclonal anti-EGFR antibodies showed 50% decrease upon pre-treatment with 17-AAG for 24 h. Uridine transport in A549 cells mediated by human equilibrative nucleoside transporter 1 (hENT1) was inhibited by 17-AAG with an IC_{50} value of 15 μM.
Conclusions: Combination therapy with 17-AAG and several clinically used TKIs looks promising and should lead to the design of future trials based on these combinations. The inhibition by 17-AAG of hENT-1 mediated uridine transport suggests an explanation for the observed antagonism between cytarabine with 17-AAG in leukemic cells since cellular uptake of cytarabine is mediated primarily by hENT1. These results caution against combination of nucleoside analogs with 17-AAG in patients.

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Investigating the role of Hedgehog signaling in tumor models

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Background: Hedgehog (Hh) signaling is activated in medulloblastoma (MB) and basal cell carcinoma, either through loss of the inhibitory protein, Patched (Ptch), or through genetic activation of Smoothened (SMO), a protein that transduces Hh signals. In some colon and pancreatic tumor xenograft models, antagonism of Hh signaling has been reported to attenuate tumor growth, and inhibition of growth correlated with reduced stromal expression of Gli1, a marker of Hh signaling. Here we describe the effects of antagonizing Hh signaling a Ptch-deficient model of MB. We also describe the effects of antagonizing Hh signaling in models of pancreatic, prostate, lung, and bile duct cancers, including models with expression of Hh in the tumor and Gli1 in the stroma.
Materials and Methods: Tumor bearing nude mice were treated with an antagonistic anti-Hh antibody or with specific SMO small molecule antagonists. Effects of these treatments on Gli1 RNA expression and tumor growth rate were assessed.
Results: Mice bearing tumor allografts derived from a Ptch+/− p53−/− mouse model of MB were treated with SMO antagonists. These treatments caused regression of the allografts and robust reduction in tumor Gli1 expression. Mice bearing tumors of ten different xenograft models were also treated with Smoothened antagonists or with anti-Hh antibody. The xenograft models were patient-derived pancreatic or lung tumors or were cell line models of pancreatic, prostate, or bile duct cancers. In these models, antibody and compound treatment significantly reduced Gli1 expression in the stroma, but neither treatment affected tumor growth. All treatments were well tolerated, and no significant weight loss was observed.
Conclusions: The SMO antagonists used in these studies are efficacious in a preclinical model of MB and are ineffective in other tumor models. Although antagonism of Hh signaling in the stroma has previously been correlated with attenuated growth of some tumor models, our data indicate antagonism of Hh signaling in the stroma is insufficient to inhibit growth of all tumor models.

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The treatment of breast cancer tumor growth and metastasis with an anti-MMP9 deoxyribozyme

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Background: Despite continued improvements in diagnosis, surgical techniques, and chemotherapy, breast cancer patients are still overcome by cancer metastasis. Tumor cell proliferation, invasion and metastasis are known to be mediated, at least in part, through degradation of basement membrane by neutral MMPs produced by tumor and stromal cells. Evidence suggests that MMP-9 plays a significant role in breast tumor cell proliferation, invasion and metastasis. Our novel catalytic DNA molecule (DNAzyme) based strategy is capable of specifically down regulating MMP9 expression without affecting other MMPs.
Materials & methods: DNAzymes are catalytic enzymes that bind to and cleave specific mRNA, resulting in a decreased protein expression. The application of anti-MMP-9 DNAzyme (AM9D) for the treatment of metastatic breast cancer was evaluated *in vitro* and *in vivo* using MDA-MB-231 human breast cancer cells and a MMTV-PyMT transgenic breast cancer mouse model, respectively. AM9D was intratumorally injected into the mammary tumors of the transgenic mice, once a week for 4 weeks. Tumor sizes were monitored bi-weekly and final tumor size was measured by weighing tumors. The role of AM9D on MMP-9 protein production and blood vessel formation were determined by immunohistochemistry. To determine safety and efficacy of AM9D systemically delivered to animals, AM9D was labeled with ^{35}S and injected intravenously into the tail vein of mice. Distribution and clearance rates were determined by excising tissues and quantizing the amount of radioactivity in each tissue.
Results: The DNAzyme studied in these experiments represent a novel application of this nucleic acid. Treatment with AM9D in vitro lead to reduced expression of MMP-9 mRNA and in vivo resulted in delayed rate of tumor growth, retarded final tumor volume by up to 70%, and a reduction in lung macrometastases. This decrease in tumor growth and lung metastasis was correlated with decreased MMP-9 protein production within the treated tumor tissues. Tumors treated with AM9D were also less vascular compared to control and untreated tumors. Furthermore, DNAzyme is distributed to major organs including lung through intravenous injection, thus, breaking ground for a clinical treatment strategy.
Conclusion: These results show that targeting and down regulation of MMP-9 by a novel DNAzyme molecule could prove useful as a therapy against breast carcinoma tumor growth and invasion.

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N3-Substituted temozolomide analogs overcome methylguanine DNA methyltransferase and mismatch repair

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Glioblastoma multiforme (GBM) is the most prevalent and aggressive malignant adult CNS tumor. Treatment includes radiotherapy and temozolomide (TMZ) alkylating agent chemotherapy. TMZ methylates purine residues of DNA: *N7*-guanine, *N3*-adenine and *O6*-guanine. *O6*-methylguanine, the primary cytotoxic lesion, is a substrate for direct repair by methylguanine DNA methyltransferase (MGMT). Response to TMZ requires low MGMT levels and functional DNA mismatch repair (MMR). Resistance to TMZ (inherent or acquired), conferred by MGMT expression or MMR deficiency (a consequence of mutation(s) in MMR proteins), represents a huge barrier to successful treatment of GBM.
To address this problem, analogs of TMZ have been synthesized, substituting the N3 methyl moiety with substituents which may lead to DNA lesions able to evade MGMT and DNA MMR.
MTT assays were conducted to compare *in vitro* antitumor activity of TMZ and novel analogs in SNB19 and U373 isogenic glioma cell line pairs: (V = vector control; M = MGMT transfected). TMZ potency reduced 13- and 5.4-fold in SNB19 and U373 cells expressing MGMT; in contrast SNB19M and U373M cells were equi-sensitive as SNB19V and U373V cells to analogs **1** and **2** (Table).

N3-substituent	Analog	GI_{50} (μM)			
		SNB19V	SNB19M	U373V	U373M
CH_3	TMZ	35.7	469.9	68.0	368.7
$CH_2C{=}CH$	**1**	35.6	37.8	37.6	36.1
CH_2SOCH_3	**2**	28.9	14.3	8.2	7.3

In addition, analogs **1** and **2** inhibit growth of vector control glioma cells generated to possess acquired resistance to TMZ. GI_{50} values <50 μM were observed in SNB19VR (MMR deficient; hMSH6 loss) and U373VR (MGMT up-regulation) following analog **1** or **2** challenge.
Analogs **1** and **2** cause G2/M cell cycle arrest in glioma cells irrespective of MGMT status and MMR deficient HCT116 colorectal carcinoma cells. Comet assays demonstrate DNA single strand breaks following SNB19V cell treatment with TMZ and novel analogs, formation of γH2AX foci infer conversion to DNA double strand breaks preceding death by autophagy and apoptosis.
Taq polymerase stop assays reveal that N3 propargyl imidazotetrazine **1** and ring opened N3 propargyl imidazotriazene preferentially alkylate guanine rich DNA sequences. *N-7* guanine alkylation by analog **1** was detected by piperidine cleavage assay.
We conclude that novel imidazotetrazines **1** and **2** elicit *in vitro* antitumor activity irrespective of MGMT and MMR status. Such molecules may offer