We treated mice bearing a moderately sensitive tumor with a clinically relevant schedule (120 mg/kg every 3 days), to develop *in vivo* resistance to gemcitabine. Mice bearing this tumor, Colon 26A (T/C=0.25) had to be treated for more than one year with gemcitabine. The most resistant tumor was used for transplantation, finally resulting in the completely resistant tumor Colon 26G (T/C=0.96). Initial studies focused on resistance mechanisms known from *in* vitro studies. However, in Colon 26G dCK activity was only 1.7-fold decreased, dCDA was not changed and DNA-polymerase activity was only marginally (1.7-fold) decreased. Although Colon 26A accumulated 1.5-fold more gemcitabine triphosphate 2 hr after a gemcitabine injection, these changes were considered insufficient to explain resistance. Therefore a microarray was performed on single stranded cDNA synthesized from mRNA of both Colon 26A and Colon 26G. A striking 1.996±0.095-log increase (mean±SD of 3 separate arrays) in expression of the RNR M1 subunit gene was found in Colon 26G, which was highly significant ($p<0.001$). The differences in expression of other genes involved in gemcitabine metabolism were smaller and not significant. The expression of both RNR M1 subunit protein and mRNA were >10-fold increased, as measured by western blotting and real time PCR.
In conclusion: this is the first model with *in vivo* induced resistance to gemcitabine. In contrast to most *in vitro* studies, dCK activity was not the most important determinant of gemcitabine resistance *in vivo*. This is also the first *in* vivo evidence for a key role for RNR in gemcitabine resistance. Thus, RNR should be included in selection of tumors for gemcitabine treatment.

485 POSTER

Rationale for combination of SDX-102 with Alimta in MTAP-negative tumors

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INTRODUCTION: SDX-102 (L-alanosine), a potent inhibitor of de novo purine biosynthesis, is being tested in Phase II clinical trials in selected patients with tumors that do not express methylthioadenosine phosphorylase (MTAP), a critical enzyme required for the purine salvage pathway. Alimta (pemetrexed) is a multitargeted antifolate that acts on a number of folate-dependent enzymes, including thymidylate synthase, dihydrofolate reductase, glycinamide ribonucleotidetide formyltranserase (GARFT), and aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT). GARFT and AICARFT are critical enzymes in de novo purine and pyrimidine biosynthesis. AIMS: The aims of this study were to: (1) measure the ATP-lowering activity of Alimta in cell lines from non-small cell lung cancer (NSCLC), mesothelioma and pancreatic cancer; (2) investigate the effect of engagement of the MTAP pathway on Alimta's activity; (3) test the anti-tumor efficacy of the combination of SDX-102 and Alimta in MTAP-negative tumor cells. RESULTS: Alimta was shown to lower intracellular ATP levels in several cell lines. Treatment with Alimta caused a 50% reduction of intracellular ATP after 72 hours incubation at concentrations ranging from 80 nM (NCI-H2452) to 5 μM (BXPC3). Activation of the purine salvage pathway, using an MTAP-substrate, was sufficient to block the Alimta-induced ATP depletion in the MTAP-expressing cells, but not in the MTAP-deleted cells. In MTAP-positive cells (HS-766T, A-427, and NCI-H226) treated with Alimta, ATP levels were restored to more than 85% of control cells by addition of an MTAP substrate. The MTAP substrate was able to fully rescue HS-766T cells from loss of viability induced by Alimta as measured by the MTT assay. Finally, low concentrations of SDX-102 was shown to enhance the cytotoxic activity of Alimta in several MTAP-deleted cell lines. In the mesothelioma cell line NCI-H2452, incubation with 200 nM SDX-102 shifted the IC75 of Alimta from 200 nM to 15 nM. CONCLUSIONS: These results suggest that Alimta treatment can lead to a reduction of the intracellular ATP levels in the cancer cell lines tested. In addition, engagement of the purine salvage pathway can protect MTAP-positive, but not MTAP-negative cells, from Alimta effect on ATP levels or cell viability. These results suggest that the combination of SDX-102 and Alimta in MTAP-negative tumors should be further pursued in pre-clinical experiments.

486 POSTER

A phase I study of pemetrexed supplemented with folic acid (FA) and vitamin b12 (VB12) in Japanese patients with solid tumors

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LY231514 (ALIMTA®; pemetrexed, N-[4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid) is a novel multitargeted antifolate antimetabolite. The antitumor activity of this agent likely derives from inhibition of several key folate-requiring enzymes, including thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). In initial phase I study d1, q21d without vitamin supplementation, maximum tolerated dose (MTD) was determined to be 600 mg/m^2. However, life threatening toxicities were observed in the subsequent phase I or II studies, in which a multivariate analysis suggested baseline homocysteine plasma concentration was a statistically significant predictor for the toxicities. In addition, in a phase III trial (CDDP+/−LY231514 for malignant pleural mesothelioma: MPM), the results indicated that supplementation of FA and VB_{12} reduced the incidence of neutropenic fever and other side effects. LY231514 has recently been approved for MPM in combination with CDDP in US and is also under development for a wide range of human cancers. Based on those results mainly obtained from pts in Western population, we planned another phase I trial of LY231514 with FA and VB_{12} in advanced cancers for Japanese pts. Primary endpoints were determination of MTD and recommended dose (RD). The starting dose was 300 mg/m^2 and the dose level has escalated up to 1200 mg/m^2.
In total, 31 pts (NSCLC:19, MPM:7, thymoma:2, rectal cancer:1, alveolar soft part sarcoma:1, unknouwn:1) were administrated. Dose limiting toxicities (DLT) included ALT elevation at 700 mg/m^2, infection and skin rash at 1200 mg/m^2 (each one patient). As consequence of the observation that the incidence of DLT was for 2/6 at 1200 mg/m^2, the MTD and RD were assessed at 1200 mg/m ^{2} and 1000 mg/m^2, respectively. Hematological toxicities (G3 leukopenia: 3 pts, G3 neutropenia: 4 pts, G3 lymphopenia: 1 pts) and non-hematological toxicities (G3 ALT elevation: 2 pts, G2 AST elevation: 2 pts, G3 skin rash: 1 pts, G2 mucositis: 2 pts) were observed in cycle 1. Dose-normalized plasma LY231514 concentrations in Japanese pts following administration of LY231514 with FA and VB_{12} were similar to those in Western pts without vitamin supplementation. LY231514 pharmacokinetics in Japanese pts did not appear to be overtly different from those in Western pts. 27 of 31 enrolled were evaluable for antitumor activity to date and 5pts achieved PR (NSCLC: 4 pts, thymoma: 1 pts). 4PR/16 evaluable pts were observed in previously treated advanced NSCLC. 4 pts are too early to evaluate.
As a result of this study, the chemotherapy of LY231514 with FA and VB_{12} supplementation expressed tolerable toxicity profile and the MTD/RD of LY231514 was assessed at 1200 mg/m^2/1000 mg/m^2. This study also showed potent anti-tumor activity of LY231514 against advanced NSCLC.

487 POSTER

Correlations of in vitro chemosensitivity of solid tumors to Pemetrexed (P, ALIMTA®) and target gene expression

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Background: The new antifolate ALIMTA® (Pemetrexed, P) is clinically active in various solid tumors and has recently been approved for the treatment of mesothelioma. Main targets include key enzymes in purine and pyrimidine neosynthesis including thymidilate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). P is one of the best substrates presently known for folyl polyglutamate synthetase (FPGS). Polyglutamted P has a substantially higher potency for inhibition of GARFT and TS. The specific aim of the present study was to correlate FPGS gene expression with in vitro chemosensitivity of freshly explanted human tumor specimens.
Methods: Freshly biopsied tumor cells (solid tumors, pleural effusions, or ascites) were used for soft-agar cell cloning experiments. Cells were exposed to several concentrations of P and clonogenic tumor growth

was evaluated after three weeks of cultivation. An aliquot of the same tumor specimens was shock-frozen immediately after removal from the patient. Subsequently, total RNA was isolated and reversely transcribed to cDNA followed by real-time multiplex PCR experiments based on Taqman technology. Results from these gene expression experiments were normalized against β-actin transcripts.
Results: A total of 29 tumor samples was collected from a variety of solid tumors. Samples were investigated for gene expression of FPGS and GARFT. No clear difference in FPGS gene expression was observed between P-sensitive and P-resistant specimens (average±SD: 428±138 versus 884±994; R=0.15, n=25). In contrast, GARFT transcripts were expressed at low levels in P-sensitive specimens (117±52 versus 318±235; n = 13). Our data indicate that GARFT expression levels and clonogenic survival after ALIMTA exposure are correlated (R=0.7810). This correlation may help identify potentially clinically sensitive tumors and supports the design of subsequent clinical trials.
Supported in part by Eli Lilly and Company

488 POSTER

VX-944: an inosine monophosphate dehydrogenase inhibitor with unique anti-cancer activity

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Inosine monophosphate dehydrogenase (IMPDH) is an essential rate-limiting enzyme in the de novo guanine nucleotide biosynthetic pathway required for cell proliferation, and hence an attractive anti-cancer target. VX-944 is an orally bioavailable, uncompetitive and non-nucleoside IMPDH inhibitor. We have previously shown that VX-944 is the most potent cellular IMPDH inhibitor described thus far, with IC50 values ranging from 20–200 nM in AML patient samples and immortalized cell lines derived from hematological malignancies. We have also shown that VX-944 induces apoptosis synergistically with Fludarabine and Doxorubicin. Notably, VX-944 potency is not affected by MDR pumps (Jain et al, Blood 2002: 100, ibid 2003: 243). In studies reported here, we demonstrate that VX-944 also inhibits proliferation of cell lines derived from human colon, breast, lung, pancreatic, melanoma and other solid tumors with an IC50 value range of 25–250 nM. VX-944 was 3–40-fold more potent than mycophenolic acid, another IMPDH inhibitor. Complete inhibition of anchorage-independent colony formation was observed at 200–800 nM VX-944 in many of these cell lines. Activating mutations in BRAF, Ras or p53 oncogenes did not appear to alter the sensitivity to VX-944. VX-944 induced apoptosis, correlating with caspase activation, PARP cleavage and a decrease in cell viability. The induction of caspases and apoptosis was blocked by guanosine addition, consistent with the specificity of VX-944 for IMPDH. The anti-proliferative activity of VX-944 was confirmed using surgical explants derived from colon, melanoma and pancreatic cancer patients. VX-944 demonstrated dose-dependent growth inhibition using the Extreme Drug Resistance (EDR®) Assay, with median IC50 values of 250 nM for pancreatic (n=14), 330 nM for melanoma (n=21), and 500 nM for colon cancer specimens (n=15). The pancreatic, melanoma and colon specimens were tested with standard chemotherapeutics to establish their sensitivity to gemcitabine, temozolomide or 5FU combined with leucovorin respectively. VX-944 was equally active in tumor samples sensitive or resistant to these agents. Many colon specimens sensitive to VX-944 were observed to be resistant to Irinotecan, Mitomycin C, Carmustine or Topotecan. Our results, demonstrating the potent anti-cancer activity of VX-944, combined with its desirable drug-like properties, indicate that VX-944 may be an attractive new agent for the treatment of patients with aggressive cancers, particularly with refractory cancers.

489 POSTER

5,10-methylenetetrahydrofolate decreases 5-fluorouracil systemic toxicity without concomitant loss of antitumor activity

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Background: The antimetabolite 5-fluorouracil (5-FU) is the standard treatment for numerous cancer types, in particular colorectal cancer. Despite its antitumor activity, 5-FU can cause dose-limiting side effects, including decreased white blood cell and platelet counts, which can impair its efficacy. To increase 5-FU antitumor activity, it is commonly used in combination with folinic acid (leucovorin). However, leucovorin can also increase the severity of 5-FU side effects. Furthermore, leucovorin must be intracellularly converted into its active metabolite 5,10-methylenetetrahydofolate (CoFactor™), potentially limiting the full anti-tumor activity of this drug combination. In contrast, CoFactor supplies 5,10-methylenetrahydrofolate directly and has demonstrated antitumor activity in combination with 5-FU in phase I/II clinical trials for colon and breast cancer. To further investigate the activity of CoFactor in comparison to leucovorin, we examined both the systemic toxicity and antitumor activity of these drugs in combination with 5-FU using *in vivo* mouse models.
Methods: For tumor studies, nude mice were inoculated subcutaneously with HT-29 colorectal tumor cells. After tumors reached approximately 50mm^3 in volume, mice were treated with combinations of 5-FU, CoFactor, and leucovorin by intraperitoneal injection for 7 consecutive days (0.6mg/mouse/drug). Tumor volumes were calculated every 2 to 3 days. For toxicity analysis, BALB/c mice were injected with the same schedule and dosage of drugs. Complete blood cell counts were analyzed pre-treatment and eight days after treatment initiation. Simultaneously, survival was followed for 15 days.
Results: In BALB/c mice, treatment with either 5-FU alone or combination 5-FU/leucovorin caused 100% mortality within 12 days of treatment initiation. In contrast, significantly more ($p<0.05$, Logrank test) CoFactor/5-FU treated mice survived (38%) beyond this time. Blood analysis revealed significantly more white blood cells in 5-FU/CoFactor treated mice than 5-FU/leucovorin treated mice ($p<0.05$, Student's t test). Specifically, we observed significantly more platelets and neutrophils in the 5-FU/CoFactor treated group. In contrast to the lower systemic toxicity profile of CoFactor/5-FU, this drug combination still maintained its antitumor activity in the HT-29 nude mouse model. Compared to the mean tumor volume in mice treated with only 5-FU (368.5±63.7, mean±SEM, n=10), we observed significant inhibition ($p<0.05$, Student's t test) of tumor growth with combination CoFactor/5-FU (225.4±32.0, n=12). Furthermore, CoFactor/5-FU treated mice had smaller tumor volumes than leucovorin/5-FU treated mice (262.0±36.5, n=11).
Conclusions: This study suggests CoFactor can reduce 5-FU mediated hematological toxicity while simultaneously increasing its antitumor activity in comparison to leucovorin.

490 POSTER

Synergistic combination of SDX-102 with docetaxol or 5-fluorouracil in pre-clinical models of lung and pancreatic cancer

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INTRODUCTION. SDX-102 (L-alanosine), a selective inhibitor of de-novo purine biosynthesis, is being tested in clinical trials in patients with tumors defective in the purine salvage pathway. These tumors do not express methylthioadenosine phosphorylase (MTAP), a critical enzyme of purine salvage from the polyamine catabolic pathway. Docetaxol is a microtubule-stabilizer anti-neoplastic agent, structurally related to paclitaxel, widely used in several indications, including non-small cell lung cancer (NSCLC). Fluorouracil (5-FU) is an anti-metabolite also frequently used in several cancer indications, including pancreatic cancer and NSCLC. AIMS. The aims of this study were to: (1) compare the activity of docetaxol and 5-FU in MTAP-positive and MTAP-negative NSCLC, pancreatic cancer and mesotheliomas cancer cell lines; (2) test the anti-neoplastic efficacy of the combination of SDX-102 with docetaxol or 5-FU in in-vitro and in in-vivo human xenograft models. RESULTS. The effect of docetaxol or 5-FU alone or in combination with SDX-102, on proliferation and survival in MTAP-negative cells was measured by MTT assay 72 hours post-treatment. Docetaxol and 5-FU, used alone, displayed a range of activity in several MTAP-negative cell lines (IC50 range 0.2–4.63 μM for 5-FU; 1–5nM for docetaxol) comparable to the IC50s values reported in the literature for MTAP-positive cells from the same tumor type. In vitro combinations of SDX-102 with 5-FU or docetaxol in MTAP-negative cells demonstrate additive to synergistic interactions when analyzed using the combinatorial index (CI) analysis method of Chou and Talalay (CI range: 1.1–0.5). In vivo, the combination of docetaxol (10 mg/kg) and SDX-102 (50 mg/kg) was superior to either single agent in a mesothelioma xenograft model (H-Meso-1) in SCID mice. Treatment was initiated at a tumor volume of 100 mm3. Thirty-one days following treatment the mean tumor volume of the combination group was 217 mm3 compared to 655 mm3 and 1035 mm3 for taxotere and SDX-102-treated groups, respectively, while control tumors were 1272 mm3. CONCLUSIONS. These results suggest that studies of SDX-102 in combination with either docetaxol or 5-FU should be further explored in preclinical models.