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Poster Sessions

Antimetabolites

468 POSTER

The role of thymidine kinase and thymidylate synthase in the response of tumor cells to the suicide prodrug 2′-F-ara-deoxyuridine

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Prodrug approaches in cancer drug development are aimed to enhance the tumor selectivity of anticancer agents. 2′-F-ara-deoxyuridine (FAU) is a suicide prodrug which has to be phosphorylated by thymidine kinase (TK1), and methylated by thymidylate synthase (TS) in tumor cells before it can be incorporated into their DNA causing cancer cell-specific death. Therapeutic approaches exist which target the enzyme TS such as 5-fluorouracil (5-FU). Resistance to 5-FU stems from an up-regulation in TS. FAU could offer a targeted treatment to patients with high levels of TS. Studies of TS protein and mRNA revealed an inverse relationship between TS expression and a cell's responsiveness to 5-FU.

To assess whether sensitivity of cancer cells to FAU is related to TK1 or TS levels, we analyzed protein expression by immunohistochemistry and mRNA expression by RT-PCR in a panel of eight human tumor cell lines including breast (MCF-7, MDA-MB-231, Hs578T), colon (HT29, HCT116), lung (H23), leukemia (K562) and ovarian (AG6000) cancers. TK1 and TS activity data were extracted from the National Cancer Institute's Developmental Therapeutics Program target database. mRNA expression of TK1 and TS was quantified in relation to the housekeeping gene hypoxantine phosphoribosyl-transferase and relative expression intensities (RI) were determined. Protein expression was analyzed by assigning an intensity score (0 to 3+). TK1, TS expression and enzyme activity were compared to FAU's cytotoxic activity as determined by 5 day methyltetrazolium (MTT) assays.

We found that those cells which had low TS levels were resistant to FAU, while those with high amounts proved sensitive. E.g. H23 cells that strongly express TS mRNA (RI 2.6) and protein (3+), they also exhibit exquisitely high TS activity and are very sensitive to FAU. The inhibitory concentration (IC) 50% of FAU in H23 was 800 nM, whereas all lines with low TS protein or mRNA such as Hs578T (RI 1.6) and MCF-7 (1+) were insensitive (IC50 >100 μM).

In contrast, TK1 levels appear not to be a major rate limiting determinant of FAU cytotoxicity because FAU sensitive H23 cells exhibit low TK1 (RI 0.5) activity, whereas cell lines with higher TK1 activity such as HCT116 (RI 3) cells were not responsive to FAU. However, K562 leukemia cells with the highest TK activity (RI 5.8), high TS protein levels (2+) and intermediate TS activity were also partially responsive to FAU (IC50 = 10 μM). These results indicate that low TK1 expression is sufficient for effective phosphorylation of FAU, but that high TK1 levels can increase FAU cytotoxicity.

Our data suggest that FAU may be useful as an alternative therapy for cancer patients over expressing TS (e.g. when resistant to 5-FU) and that both TK and TS at the protein and mRNA level should be evaluated as markers of response in early clinical trials aimed to investigating the therapeutic potential of FAU.

469 POSTER

The nucleoside analogue sapacitabine (CYC682) synergises with histone deacetylase inhibitors in multiple tumour types

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Background: CNDAC (1-(2-C-cyano-2-deoxy-b-D-arabino-pentofuranosyl)-cytosine) is a 2′-deoxycytidine analogue that hinders DNA synthesis by inhibiting DNA polymerase and strand elongation. In contrast to gemcitabine and cytarabine which block cells in S-phase, CNDAC induces an S-phase delay, followed by arrest in G2. This is a function of the cyano group incorporated into the ribose moiety of CNDAC, which promotes the induction of single strand DNA breaks after it has been integrated into DNA, making CNDAC unique among this class of agents. Sapacitabine (CYC682) is a palmitoyl-derivative of CNDAC that is orally administered to patients and undergoing phase II clinical trials. Addition of the fatty-acid group to CNDAC reduces inactivation of the nucleoside analogue by deamination, and enhances activity as shown in preclinical studies.

Histone deacetylase (HDAC) inhibitors are a relatively new class of anti-cancer therapeutics that induce apoptosis by modulating gene expression of proteins in multiple pathways. In this study we investigated whether HDAC inhibitors could enhance the pro-apoptotic effect of CNDAC.

Methods: In vitro synergy was assessed using the Chou and Talalay median effect model and determined for both sequential and concomitant treatments. The molecular basis for these synergistic interactions was explored using flow cytometry-based techniques and western blot analysis.

Results: Synergistic cell killing was observed in a variety of tumour types, including acute myeloid leukaemia (AML), non-hodgkins lymphoma, cutaneous T-cell lymphoma and non-small cell lung cancer. Mode of action analysis in AML cells demonstrated that combining CNDAC with suberoylanilide hydroxamic acid (SAHA) or valproate induced a synergistic time- and dose-dependent increase in apoptotic cells. The schedule of administration was not critical, since different treatment regimes produced synergistic increases in apoptosis. Western blotting analysis identified changes in the levels of select pro- and anti-apoptotic proteins that are consistent with increased apoptosis.

Conclusions: CNDAC and HDAC inhibitors demonstrated synergy in several cell lines derived from diverse tumour types. CNDAC-induced apoptosis was enhanced by HDAC inhibitors due to selective modulation of proteins that regulate apoptosis. These agents have non-overlapping toxicities and represent a combination with significant promise that is worthy of investigation in the clinic.

470 POSTER

Target identification permits rational development of the prodrug GMX1777 for the treatment of melanoma

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GMX1777 is a prodrug of the cyanoguanidinopyridine GMX1778 and demonstrates strong anti-tumor activity *in vitro* and *in vivo* across a variety of human tumor cell lines and in mouse xenograft models. Based on pre-clinical data GMX1777 was tested in a Phase I trial as a 24 hour infusion every three weeks in patients with solid tumors. Recently, we identified that GMX1778 is a potent and specific inhibitor of nicotinamide phosphoribosyltransferase (NAMPRT), the rate limiting enzyme in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+), and results in NAD^+ depletion in tumor cells. Because DNA damage repair pathways are extremely dependent on NAD^+ levels, we tested a wide spectrum of DNA-damaging agents for synergistic cell killing in combination with GMX1778 *in vitro*. The DNA-alkylating agent temozolomide exhibited the greatest synergy with GMX1778 treatment. The clinical development of GMX1777 in combination with temozolomide is currently being explored for the treatment of melanoma patients. To this end, pre-clinical development of a new dosing schedule that would accommodate a combination treatment with temozolomide resulted in a dosing schedule of five daily 3 hour infusions of GMX1777. This dosing schedule was as effective in mouse xenograft models as the previous 24 hour infusion schedule. Additional pre-clinical results indicate that cells with high melanin content exhibit increased retention of GMX1778. *In vitro* data suggests that this characteristic can be exploited in order to provide increased sensitization of melanin-containing melanoma cells to temozolomide treatment. Overall, these results illustrate the impact that rational drug combinations utilizing targeted agents can have on the clinical development of novel cancer therapeutics.

471 POSTER

Plasma pharmacokinetics of CP-4055 in patients with acute myeloid leukaemia at the recommended phase II dose

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Background: CP-4055 (ELACYT™, cytarabine 5′-elaidic acid ester) is a novel cytotoxic nucleoside analogue. While CP-4055 has similar mechanisms of action to cytarabine, it is, unlike cytarabine (ara-C) independent of nucleoside transporters for cellular uptake. The study was conducted as part of a phase I dose escalation study. The aim was to determine the plasma levels of CP-4055 and its metabolites, ara-C and

ara-U in patients receiving the recommended phase II dose (RP2D) by continuous infusion (CIV).
Material and Methods: Eight patients with relapsed/refractory acute myeloid leukaemia (AML) receiving 2000 mg/m^2/day CP-4055 CIV (24 hours) in a day 1−5 q3w schedule, were included. Blood samples for plasma pharmacokinetics were taken at time intervals on day 1 through day 6.
Results: Steady state concentrations (Css) of ara-C and ara-U were reached within day 1, while Css for CP-4055 apparently was not reached until 96 h after start of infusion. A threefold variation was seen in area under the curve (AUC) of CP-4055 and a five fold variation in AUC of ara-U. Smaller variation was seen in AUC of ara-C. There seemed to be an inverse relationship between AUC of CP-4055 and ara-U. CP-4055 ($t0.5 = 2 \pm 0.58$ h) was detected in plasma up to 24 hours after end of infusion in three out of seven patients. CP-4055 was well tolerated at RP2D. Complete remission was attained in a 24 year old female patient receiving CP-4055. The induction course was discontinued after for only 3 days due to elevation of liver function tests.
Conclusions: CP-4055 is well tolerated and activity has been reported in patients with acute myeloid leukaemia (AML) at the RP2D. Variations were seen in AUC of CP-4055 (three fold) and ara-U (five fold). Smaller variations were seen in AUC of ara-C. The study has continued into the phase II part.

472 POSTER

The IMPDH inhibitor AVN944 synergizes with Clofarabine to induce cell death in myeloid cancer cell lines

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AVN944 is an orally bioavailable inhibitor of inosine monophosphate dehydrogenase 1 and 2 (IMPDH). IMPDH2 is highly upregulated, and required for the de novo production of guanine nucleotides in many malignancies, including hematologic cancers. AVN944 is currently in a repeat dose escalation trial in patients with advanced hematologic cancers as well as a Phase II trial in pancreatic cancer in combination with gemcitabine. Over the last several years molecularly targeted agents have gained approval from the Food and Drug Administration as standard of care for specific tumor types. These agents, however, are not curative as single agents, as even the best example, imatinib, does not eradicate BCR-ABL positive cells in most patients. In order to identify drugs that would synergize with AVN944 we designed combination treatments with drugs approved for treatment of leukemia and lymphoma that would complement the nucleotide metabolism and energy pathway inhibition activity of AVN944. Compounds tested included Ara-c, 5FU, Clofarabine and hydroxyurea. One agent, Clofarabine, inhibits DNA synthesis by a direct inhibition of ribonucleotide reductase, DNA polymerase and induction of DNA strand breaks whereas AVN944 reduces the pool of available GTP nucleotides required for synthesis of DNA and progression through S phase of the cell cycle. To test the activity of these agents in combination, we treated HL-60 and KG-1 cells for 48 and 72 hours with a course of 4 doses each agent alone, and in combination. Apoptotic cell death, using flow cytometry on Annexin V and staining for propidium iodide. Calculations of synergy were carried out using the method developed by Chou and Talay, (1998) whereby combination indices (C.I) are calculated and values <1.0 is indicative of a synergistic combination. The C.I. for HL-60 and KG-1 at 72 hours treatment using 50% cell death as the endpoint was 0.20 and 0.5 respectively. These values clearly indicate the combination of AVN944 with Clofarabine is strongly synergistic. Upon confirmation of these data in in vivo xenograft experiments a clinical trial would be designed to test the combined activity of these agents in man.

Bioreductive agents

473 POSTER

The bioreductive prodrug PR-104 is activated under aerobic conditions by human aldo-keto reductase 1C3 (prostaglandin F synthase)

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PR-104, a hypoxia-activated prodrug currently in clinical trial, is a water-soluble phosphate ester which is converted *in vivo* to the corresponding alcohol, PR-104A. This 3,5-dinitrobenzamide-2-nitrogen mustard is activated by reduction to the corresponding 5-hydroxylamine (PR-104H) and 5-amine (PR-104M) (Patterson et al., Clin Can Res 2007, 13:3922). We have observed that certain neoplastic cell lines display atypical aerobic cell sensitivity that correlates with NQO1 expression in vitro but NQO1 cDNA expression did not increase aerobic cytotoxicity or PR-104A metabolism (Guise et al., Biochem Pharmacol, 2007, 74:810). This suggested the hypothesis that the aerobic PR-104A reductase is co-ordinately regulated with NQO-1. To identify the reductase, a panel of 12 cell lines were evaluated for aerobic PR-104A sensitivity and were shown to cover a 78-fold range (IC50 = 2–157 uM). In parallel, aerobic PR-104A metabolism was monitored using LC/MS/MS and compared with the gene expression profile (Affymetrix U122Plus2.0 chips) by unsupervised hierarchical clustering analysis (Cluster 3.0). Probes associated with gene ontology term 'oxidoreductase activity' and descendents separated the 12 cell lines into three distinguishable groups, indicating that the resulting expression profiles were cell-specific. Two-way ANOVA and false discovery rate correction (FDR, Limma's empirical Bayes adjusted p value <0.1) identified 260 probes that were positively correlated with the rate of PR-104H & M formation across the 12 cell lines. Intriguingly, a cluster of four aldo-keto reductases genes (AKR 1B10, 1C1, 1C2 and 1C3) featured in the seven most highly up-regulated messages in PR-104A metabolism-proficient cells (65, 41, 31 and 42-fold, respectively; adj. p values <0.02). The NQO1 transcript was also co-ordinately expressed (9.3-fold range; adj. p = 0.054), independently supporting the relationship we have previously observed.
We expressed these candidate aldo-keto reductases in aerobic metabolism-null HCT116 cells and showed by LC/MS/MS that only AKR1C3 (NM_003739) expression results in activation of PR-104A to its active metabolites. Cytotoxicity assays confirmed AKR1C3 as a major determinant of aerobic PR-104A sensitivity, and aerobic metabolism of PR-104A was shown to be highly correlated with AKR1C3 expression by western blot. To our knowledge this is the first report that AKR1C3 is capable of nitroreduction. The clinical implications of this finding will be discussed.

474 POSTER

Relationships between reductive metabolism, DNA crosslinks and antitumor activity of the hypoxia-activated prodrug PR-104 in preclinical models

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Background: PR-104, currently in clinical trial, is a phosphate ester that is converted systemically to the dinitrobenzamide nitrogen mustard PR-104A which is reduced selectively in hypoxic cells to DNA inter-strand crosslinking (ICL) hydroxylamine (PR-104H) and amine (PR-104M) metabolites (Patterson et al., Clin Cancer Res 2007; 13: 3922–3932). Here we evaluate the role of ICL in the hypoxic and aerobic cytotoxicity of PR-104, and the utility of ICL as a surrogate marker for clonogenic cell killing.
Materials and Methods: Using a panel of 9 human tumor cell lines, cytotoxicity was determined by clonogenic assay following a 2 hr exposure to PR-104A, at a range of concentrations, under aerobic or hypoxic conditions. PR-104H and PR-104M concentrations were determined by HPLC/mass spectrometry (LC/MS/MS) during this exposure and ICL by the alkaline comet assay after growth for a further 24 hr in fresh medium under aerobic conditions. ICL and clonogenic cell killing were also assessed in the corresponding human tumor xenografts following i.p. dosing of CD-1 nude mice with PR-104 (0.6 mmol/kg).
Results and Conclusions: PR-104A was selectively toxic to all cell lines under hypoxia, but with widely differing hypoxic cytotoxicity ratios (from 6.6 for H460 to 53 for HCT116 cells), primarily reflecting large differences in aerobic cytotoxicity. Under hypoxia, the relationship between ICL and cell killing was indistinguishable across the cell line panel, suggesting that ICL are responsible for hypoxic cytotoxicity in all cases. Under aerobic conditions there was a similar relationship between ICL and cytotoxicity in most cell lines, but cells with very low rates of PR-104H and PR-104M formation (A2780, C33A, H1299) did not show ICL even after highly cytotoxic PR-104A exposures. These same 3 cell lines have low levels of AKR1C3 expression, which is the major enzyme responsible for aerobic PR-104A reduction in human tumor cell lines (Guise et al., this meeting). Thus there is an ICL-unrelated mechanism of PR-104A cytotoxicity in cells with very low reductase activity. Despite this, in xenografts ICL frequency was highly correlated with clonogenic cell killing ($r^2 = 0.743$), and A2780, C33A and H1299 followed the same pattern. Therefore the ICL-independent cytotoxicity observed in culture is not significant in the pharmacologically relevant dose range in mice, and ICL frequency appears to be a broadly applicable pharmacodynamic biomarker for tumor cell killing by PR-104.