Sequence dependence of MEK inhibitor AZD6244 combined with gemcitabine for treatment of biliary cancer

Junyao Xu¹², Jennifer J. Knox³, Emin Ibrahimov¹, Eric Chen³, Stefano Serra⁴, Ming Tsao¹⁴, Pinjiang Cao¹, Douglass Vines⁵, David E.Green⁵, Mairead G. McNamara³, David W. Hedley¹³*  

¹Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, Canada  
²Department of Hepatobiliary Surgery, Sun yat-sen Memorial hospital, Sun yat-sen University, Guangzhou, China  
³Division of Medical Oncology and Hematology, Princess Margaret Hospital, 610 University Avenue, Toronto, Canada  
⁴Department of Pathology, University Health Network, Toronto General Hospital, Toronto, Canada  
⁵Radiation Medicine Program, Princess Margaret Hospital, 610 University Avenue, Toronto, Canada  

*Correspondence:  
David W Hedley, Division of Medical Oncology and Hematology, Princess Margaret Hospital, 610 University Ave, Toronto, Canada, M5G 2M9  
E-mail: David.Hedley@uhn.ca; TEL: (416) 946-2262  

Running title: Combination of AZD6244 and Gemcitabine in biliary cancer  

Keywords: ERK pathway, AZD6244, gemcitabine, biliary cancer, combination chemotherapy  

Statement of Conflicts of Interest:  
The authors state that they have no conflicts of interest regarding this work
**Translational relevance:**
Advanced biliary tract cancers have poor prognosis and emerging therapies focus on combining conventional chemotherapeutic agents with targeted compounds in the hope of obtaining more active and well tolerated combinations. The ERK pathway is frequently activated in biliary cancer and it has the potential to promote tumor growth and chemotherapy resistance. Our findings show that the antitumor effect of combined gemcitabine with the MEK inhibitor AZD6244 is strongly schedule-dependent. Concurrent administration of these two agents resulted in sub-additive or antagonistic effect, whereas enhanced effect over single agents was observed when gemcitabine was given following a 48hr interruption in AZD6244 dosing. Our study also provides a mechanistic explanation for this observation that delayed entry into S phase during MEK inhibition antagonizes the effects of the S-phase specific agent gemcitabine against biliary cancers. These observations have important implications regarding rational combination therapy schedules using these two agents.
Abstract

Purpose:
MEK inhibition has clinical activity against biliary cancers, and might therefore be successfully combined with gemcitabine; one of the most active chemotherapy agents for these cancers. As gemcitabine is active in S-phase, and the ERK pathway has a major role driving cell cycle progression, concurrent use of a MEK inhibitor could potentially antagonize the effect of gemcitabine. We therefore tested the sequence dependence of the combination of gemcitabine and the MEK inhibitor AZD6244 using a series of biliary cancer models.

Experimental Design:
Primary xenografts were established from patients with gallbladder and distal bile duct cancer, and grown in SCID mice at the subcutaneous site. Plasma and tumor drug levels, and the time course for recovery of ERK signaling and S-phase were measured in tumor-bearing mice treated for 48hr with AZD6244 and then monitored for 48hr off treatment. Based on these results, two different treatment schedules combining AZD6244 with gemcitabine were tested in four different biliary cancer models.

Results:
DNA synthesis was suppressed during treatment with AZD6244, and re-entry into S-phase was delayed by approximately 48hr post-treatment. Strong schedule dependence was seen in all four biliary cancer models tested, suggesting that combined treatment with AZD6244 plus gemcitabine would be more active in biliary cancer patients when gemcitabine is given following a 48hr interruption in AZD6244 dosing, rather than concurrently.

Conclusions:
The combination of AZD6244 plus gemcitabine is highly schedule dependent, and predicted to
be more effective in the clinic using sequential rather than simultaneous dosing protocols.

**Introduction**

Cancers arising in the biliary tract, consisting of the intra- and extra-hepatic bile ducts and the gallbladder, remain a major challenge to surgical, medical, and radiation oncologists. The large majority of these tumors are not resectable at the time of initial diagnosis and the overall prognosis is poor, with less than 5% survival rate at 5 years(1, 2). Chemotherapy has limited activity, and gemcitabine appears to be one of the most active single agents(2). A phase III clinical trial in advanced biliary cancers reported a significant prolongation of median overall survival when gemcitabine was combined with cisplatin, compared to gemcitabine alone, establishing this combination as a global standard of care (3). However, the median overall survival of patients treated with the combination, despite an advancement was only 11.7 months, and so the discovery of active new agents is an urgent priority for patients with advanced biliary cancer.

The RAS/RAF/extracellular regulated kinase (ERK) signaling pathway plays a central role in the regulation of many cellular processes, including proliferation, survival, differentiation, apoptosis, motility, and metabolism(4, 5). This pathway is activated by a diverse group of extracellular signals, including growth factor receptors (e.g., epidermal growth factor receptor EGFR). Expression of EGFR has been validated to be enhanced in tumor samples from 39.2%–46.2% of patients with biliary cancers(6, 7). Moreover, activating mutations of KRAS and BRAF can occur in biliary cancers, with reported incidences of 8 to 58% (8) and 0 to 22%, respectively (9),
suggesting that activation of the RAF/MAPK signaling pathway may be a key event in a significant proportion of biliary cancers.

AZD6244 (Selumetinib, ARRY-142886; AstraZeneca) is a second-generation, potent, selective, orally available, and uncompetitive small molecule inhibitor of MEK1/2(10). Recent phase II data(11) using AZD6244 as monotherapy demonstrated a modest activity (12% RECIST response rate) and was well tolerated in patients with advanced biliary cancer, suggesting the feasibility of combination with other drugs.

Gemcitabine (2',2'-difluorodeoxycytidine) is a nucleoside analogue with activity against biliary cancer in several phase II studies(12) and has also been reviewed in another manuscript(13). Following uptake by nucleoside transporters, gemcitabine is phosphorylated to its active forms gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP). The latter competes with deoxycytidine triphosphate for incorporation into DNA, resulting in chain termination and DNA strand breaks(14). The cytotoxicity of gemcitabine may also stem from the inhibition of ribonucleotide reductase by dFdCDP (15). Both of these mechanisms probably contribute to its cytotoxic action, although it is not presently known to what extent.

Although gemcitabine is often combined with targeted agents, a recent preclinical study demonstrated that combining AZD6244 with gemcitabine did not show enhanced activity in a pancreatic cancer model(16). In a previous study we observed that treatment with AZD6244 depleted S phase cells in xenografts derived from pancreatic cancer cell lines(17). Therefore we reasoned that delayed entry into S-phase during MEK inhibition will antagonize the effects of
gemcitabine against biliary cancers, whereas treatment with MEK inhibitor following exposure to gemcitabine might enhance its effect through the inhibition of repopulation by surviving tumor cells. This was tested in the present paper using two recently-developed, patient-derived primary biliary cancer xenografts, supplemented by experiments using established cell lines.

Material and Methods

Cell lines. Human cholangiocarcinoma cell lines EGI-1 and TFK-1 were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Human and Animal Cell Lines, Braunschweig, Germany). EGI-1 cells were cultured in Dulbecco's MEM medium with 10% fetal bovine serum (FBS), TFK-1 cells were cultured in RPMI 1640 medium with 10% FBS. Cells were cultured at 37°C and 5% CO₂ atmosphere.

Primary xenografts. OCIP55 was established from a distal bile duct primary tumor resected by Whipple pancreatectomy, and GB1 was established from ascites fluid taken from a patient with advanced peritoneal metastases from a primary gallbladder cancer. On histological examination, these primary xenografts showed typical adenocarcinoma features (Supplementary Fig.S1). Mutational analysis by the Sequenom MassARRAY (San Diego, CA), using the laboratory-developed PMH Solid Tumor Panel (Molecular Diagnostics Laboratory, University Health Network), did not identify actionable mutations in these samples, although K-ras G12D mutations were identified in the EGI-1 and TFK-1 cell lines. Xenografts used in this study were 4~6 passages for OCIP55 and 2~4 passages for GB1. Drug treatment commenced after 4~6 weeks of tumor growth. Tumor models from human cancer cell lines were also set up to test the effect of drug treatments. 2 x 10⁶ EGI-1 or TFK-1 cell
suspension (0.5 ml) was injected subcutaneously into the right flank of 6-week-old male SCID mice. Drug treatment commenced after 2~4 weeks of tumor growth.

**Drug treatment.** AZD6244 was purchased from Selleck Chemicals (Houston, TX), and gemcitabine was obtained from the Princess Margaret Hospital pharmacy. To determine the effects of AZD6244 on cell cycle progression, five groups of five randomly assigned mice with OCIP55 xenografts were treated with 25mg/kg AZD6244 (12.5 mg/ml in DMSO) or vehicle control by oral gavage twice daily for 2 days. Then mice were sacrificed and the tumors removed at 4, 12, 24 and 48 hours after the final dose. Thirty min prior to sacrifice, tumor-bearing mice were given 50mg/kg of the thymidine analogue 5-ethynyl-2’-deoxyuridine (EdU; Molecular Probes, Invitrogen, OR, USA) by intraperitoneal(i.p.) injection. All harvested tumors were divided into three parts, which were disaggregated into single cell suspensions for flow cytometric analysis, snap-frozen in liquid nitrogen for immunofluorescence staining and LC-MS measurement of AZD6244, or formalin-fixed and then paraffin-embedded. Separate groups of tumor-bearing mice (n=6/group) treated using identical protocols were used for $^{18}$F-FLT PET imaging.

For tumor growth inhibition experiments, mice were treated with different treatment schedules incorporating gemcitabine 100mg/kg i.p. every four days, and AZD6244 or DMSO control. Caliper measurements of tumor volume (using the formula volume=$a*b^2$, where $a$ and $b$ are the longest and shortest diameters of the tumor, respectively) (18), animal body weight, and tumor condition were recorded thrice weekly for the duration of the study. Before and after each treatment cycle, complete blood counts were made in OCIP55-bearing mice. When the tumor
volume of the control group reached ~1200 mm$^3$, the experiments were ended and mice in all groups were sacrificed and the tumors were removed and weighed.

**Flow cytometric analysis.** Single-cell suspensions were prepared by an enzymatic technique as described previously (17), labeled with Alexa 488 azide obtained from Molecular Probes, Invitrogen, Eugene OR, by copper-catalyzed click chemistry and then stained with the DNA-specific dye DAPI at 1 μg/ml for 30 min. A Gallios flow cytometer (Beckman Coulter) was used for data acquisition. Cell cycle analysis was done using ModFit LT™ (Verity, Topsham, ME), and FCS3 Express (Denovo software) was used to analyze the EdU uptake.

**Immunofluorescence staining.** Frozen sections cut from tumor tissue were fixed in 3.7% formaldehyde for 15 min, and then incubated with EdU reaction cocktail (Click-iT® EdU Alexa Fluor® 647 Imaging Kit, Molecular Probes, Invitrogen, OR, USA)) for 30 min, followed by incubation with primary rabbit anti-pERK1/2 antibody (1:50, Cell Signaling) and secondary FITC-conjugated anti-rabbit antibody. Control samples without EdU reaction and primary antibody exposure showed no specific staining. All sections were counterstained with DAPI to outline the nuclear area. Entire sections were imaged at 1 μm resolution using a laser scanning system (TISSUEscope; Biomedical Photometrics), and composite images of regions of interest were imaged at higher resolution (magnification×20), using a conventional fluorescence microscope and scanning stage (BX50; Olympus Corporation). Uncompressed TIFF images (8-bit) were acquired for analysis.
Analysis of plasma and tumor AZD6244 concentrations. AZD6244 concentrations in plasma and tumor samples were measured using a high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) technique, as described previously(19). Plasma samples were extracted by protein precipitation in acetonitrile. Homogenized tumor samples were obtained by liquid-liquid extraction with methyl tertbutylether (MTBE). Following centrifugation, the supernatants were evaporated to dryness using the Universal Vacuum System (Thermo Electron Corp., Milford, MA) and then dissolved again in HPLC mobile phase. Separation was carried out using a reversed-phase HyperClone BDS C18 column (5 μm, 50 x 2.0 mm, Phenomenex, Torrance, CA) and a gradient mobile phase containing water/acetonitrile/formic acid. Peaks were detected using API3200 tandem mass spectrometry (Applied Biosystem/MDS Sciex, Streetsville, Ontario, Canada).

18F-FLT PET imaging. Tumor-bearing mice were given 250 μCi of 18F-FLT intravenously. After 55 minutes (determined by preliminary dynamic 18F-FLT PET uptake experiments), the mice were anesthetized and underwent PET imaging for 10min using a microPET Focus 220 scanner (Siemens Medical Solutions), followed by a subsequent CT scan using a GE eXplore Locus Ultra microCT scanner (GE Healthcare) for anatomical reference. Reconstructed PET and CT data were co-registered and 2D regions of interest (ROI) were hand drawn to fit the primary tumor according to the CT and PET data sets. The standardized uptake value (SUV) was calculated using the following formula: Standardized uptake values (SUV= [Radioactivity/mL tissue] / [net injected radioactivity/body weight]). In the present research, 18F-FLT uptake in xenografts was reported as a ratio of SUV activity (tumor SUVmax/muscle SUVmean)(20),
obtained by using the SUV of the most intense pixel in the tumor regions and normalized with the corresponding muscle SUVmean value from the same mouse.

**Statistical methods.** The statistical significance of differences in numerical data between multiple groups was evaluated with a one-way ANOVA test with Tukey’s comparison post test, using Prism software (GraphPad). All statistical tests were 2-tailed. P < 0.05 was considered statistically significant.

**Results**

**Cell cycle effects of AZD6244 and their relation to gemcitabine sensitivity in vitro.**

Preliminary experiments were done to investigate the cell cycle effects of MEK inhibition, the recovery of S-phase entry following drug washout, and the effects on gemcitabine sensitivity in vitro, using EGI-1 and TFK-1 biliary cancer cell lines. Exposure to AZD6244 concentrations of 1μM and greater resulted in a decrease in ERK phosphorylation and increase in the expression of p27\textsuperscript{Kip1}, but not p21\textsuperscript{Cip1} (Fig. 1A). Cell cycle analysis following 24-hour exposure to AZD6244 showed a dose-dependent increase in the percentage of cells in G1, with a corresponding reduction in S-phase cells and in EdU labeling (Fig. 1B and supplementary Fig.S2). When cells treated with 1μM AZD6244 for 24h were placed in drug-free medium, re-entry into S-phase was delayed by approximately 15 hours, following which cell cycle distribution and EdU uptake returned to control values (Fig.2 and supplementary Fig.S3).

Next we determined the sensitivity of EGI-1 and TFK-1 cells to gemcitabine, using a commercial MTS assay (CellTiter 96® Aqueous One Solution Reagent; Promega, Madison, WI) and 96 well
plate reader. The IC50 of EGI-1 cells to gemcitabine was 0.051± 0.012μM and that of TFK-1 to gemcitabine was 0.45±0.35μM, which is consistent with previous reports for these cells(21-23).

Two combination protocols were evaluated in cell culture: sequential treatment consisting of 24 h exposure to 1 μM AZD6244 followed by incubation in drug-free medium for 24 h, then 24 h treatment with 0-10μM gemcitabine, or simultaneous exposure to 1μM AZD6244 and gemcitabine for 24 hr. Using the MTS assay, sequential treatment (IC50=0.09±0.04μM) but not simultaneous treatment (IC50=2.02±0.91μM) enhanced the inhibition of TFK-1 cell proliferation compared to gemcitabine alone. A similar result was observed with EGI-1 cells (Fig.3A). Since TFK-1 cells are relatively gemcitabine-resistant the sensitizing effect of pre-treatment with AZD6244 was investigated further, using a flow cytometric cell viability assay based on combined measurement of mitochondrial membrane potential and surface membrane integrity as previously described (24). Compared to gemcitabine alone, sequential treatment showed enhanced cytotoxic effect (P<0.05), whereas no significant difference was observed with the simultaneous combination (Fig.3B and supplementary FigS4). Similarly, using a clonogenic assay, we observed significant loss of viability in cells pre-exposed to 1μM AZD6244 prior to gemcitabine treatment (p<0.01), whereas there was no significant enhancement using simultaneous treatment. AZD6244 alone did not significantly affect clonogenic survival of TFK-1 cells (Fig.3C).

**Pharmacodynamic and pharmacokinetic analysis of AZD6244 in vivo**

To determine the optimal combination schedule in vivo, we first investigated the relationship between the pharmacokinetics of AZD6244 in OCIP 55 xenografts and the pharmacodynamic effects on ERK pathway inhibition and S-phase progression. As illustrated in Fig.4A and 4C,
dual fluorescence image analysis of phosphorylated ERK and EdU labeling in tissue sections showed that 48 h treatment with AZD6244 blocked the ERK pathway and inhibited EdU uptake. These effects persisted for 24h after the last dose of AZD6244, when phosphorylated ERK returned to pretreatment levels. The recovery of EdU uptake did not occur until the 48h time point, when it appeared to overshoot the value of the untreated control. A similar pattern was seen using flow cytometry to monitor EdU uptake during recovery from AZD6244 (Fig.4D).

Functional imaging of thymidine uptake using $^{18}$F-FLT PET would allow the recovery kinetics following MEK inhibition to be studied directly in biliary cancer patients, which is the goal of this research. Therefore, we applied this technique to a separate group of mice bearing OCIP55 xenografts, treated with AZD6244 according to the same protocol. As shown in Fig.5, tracer uptake was readily detected in the flanks of the tumor-bearing mice, and showed a significant decrease for 24hr after the last drug dose, recovering to untreated control values at 48hr, similar to the results obtained using EdU.

To explore the correlation between these pharmacodynamic effects and the drug levels, LC-MS was used to measure AZD6244 in plasma and tumor tissue. As shown in Fig.4E, a mean plasma concentration of 2403ng/ml was obtained 4 hours after the final dose of AZD6244 treatment, with a rapid decrease at the later time points that is consistent with the published data for this compound (10, 25). In contrast, although the concentration of AZD6244 in tumor tissue was lower than the plasma level at the 4hr time point, the drug was retained for longer in tumor tissue and showed the expected inverse relationship to ERK phosphorylation. Thus, the 48hr lag period between the last drug dose and re-entry into S-phase appears to be explained partly by the time needed for reactivation of the ERK pathway, and partly by a latent period in G1 following the

12
recovery of ERK signaling, similar to that seen following drug exposure in vitro (Fig.2). Based on these observations, we reasoned that gemcitabine would be relatively ineffective if given concurrently with AZD6244, or within 48hr of the last dose.

**Inhibitory effect of sequential combination of AZD6244 and gemcitabine on tumor growth in vivo**

A preliminary study showed that long-term single agent treatment with AZD6244 (25mg/kg bid) or gemcitabine (100mg/kg biweekly) was well tolerated (Supplementary Fig.S5). AZD6244 monotherapy showed modest antitumor effects in all four models, whereas gemcitabine showed slight antitumor effect against TFK-1, but robust activity against EGI-1 xenografts, similar to the differential effects seen in vitro, and modest activity in the two primary xenograft models.

Two combination schedules were designed using the same total doses of gemcitabine and AZD6244 in four day treatment cycles (Fig. 6A). In schedule A, gemcitabine was given immediately after the final dose of 48hr treatment with AZD6244, followed by a 48hr treatment-free period prior to the next cycle (“simultaneous dosing”); in schedule-B, gemcitabine was given 48hours after the final dose of AZD6244, following which treatment with AZD6244 was restarted (“sequential dosing”). Both treatment schedules were well tolerated with no treatment-related deaths, and there were no significant differences in animal weight (Supplementary Fig.S6A). Weekly blood counts were done in the groups bearing OCIP55 xenografts (Supplementary Fig. S6B). Although there was a modest decrease in total white cell count in mice treated with each of the drug combination schedules, there was no significant difference between them. As shown in Fig. 6B and Supplementary Fig. S7, sequential treatment markedly
inhibited tumor growth in all four models, whereas simultaneous dosing was not significantly more effective than gemcitabine monotherapy with the exception of TFK-1, which was refractory to gemcitabine monotherapy but relatively sensitive to AZD6244.

**Discussion**

The ERK pathway is frequently activated in cancer due to genetic alterations in its regulation, and it has the potential to promote tumor growth and treatment resistance through multiple downstream effector pathways. Currently available MEK inhibitors have shown quite modest single agent activity against a range of tumor types, including biliary cancers, and it is therefore logical to consider incorporating these agents into combinations with standard chemotherapy drugs in the hope of obtaining more active and well tolerated combinations. However, because the ERK pathway plays a major role in cell cycle progression, there is a theoretical possibility that the sensitivity to S-phase specific agents like gemcitabine or 5-fluorouracil would be reduced during MEK inhibition. On the other hand, the administration of agents that inhibit tumor cell proliferation following treatment with chemotherapy might enhance its effect by suppressing repopulation by viable tumor cells during the interval between chemotherapy courses (26). This is addressed in the present paper.

We used two early passage primary biliary cancer xenografts, which are closer to the clinical goals of this project, supplemented with established biliary cancer cell lines that allowed preliminary *in vitro* testing of the cell cycle effects of AZD6244 and their impact on gemcitabine sensitivity. The percentage of cells incorporating EdU *in vitro* was decreased by 60-70% during treatment with AZD6244 at concentrations that blocked ERK signaling, indicating that this
pathway plays a dominant, but not critical role in S-phase progression of these cells. Recovery of cell cycle progression was delayed by 15 hr following removal of AZD6244, suggesting that the cell cycle was blocked at a checkpoint midway through G1, rather than at the G1/S boundary.

Three different assays were used to study the schedule dependence of AZD6244 plus gemcitabine drug combinations: a standard MTS assay, a flow cytometry assay combining measurement of mitochondrial membrane potential and surface membrane integrity, and a clonogenic survival assay. A similar result was obtained with all of these methods, with concurrent treatment having no significant effect compared to gemcitabine alone, whereas when gemcitabine treatment was delayed for 24 hr after AZD6244 removal, there was significant enhancement of its effect.

Similar to the in vitro effects, the biliary cancer xenografts showed cell cycle arrest during treatment with AZD6244, with re-entry into S-phase delayed by approximately 48 hr following the last dose. This effect was readily detected using $^{18}$F-FLT PET imaging as well as by EdU pulse-labeling, suggesting the potential to use this technique to monitor the kinetics of S-phase recovery in biliary cancer patients treated with MEK inhibitors. Whereas the plasma concentrations of AZD6244 decreased rapidly following the last dose, AZD6244 was retained in tumor tissue at $\geq 1 \mu M$ concentrations for up to 24 hr, with the recovery of ERK phosphorylation showing the expected inverse correlation with tumor drug levels. Thus the delayed re-entry into S-phase seen in vivo is likely explained by pharmacodynamic effects of AZD6244 on ERK signaling, in addition to the lag in G1 exit that was observed in vitro. Based on these results, we designed two treatment protocols to test the schedule dependence of the gemcitabine + AZD6244 drug combination in biliary cancer.
Consistently in all four models tested, tumor growth control was improved when treatment with AZD6244 commenced immediately after gemcitabine and was discontinued 48hr prior to the next gemcitabine treatment, whereas when gemcitabine was given after AZD6244, the effect was sub-additive. We think it likely that this result can be translated into the clinic to treat biliary cancer patients, who are currently treated with gemcitabine plus low dose cisplatin. Preliminary results using our two primary biliary cancer models suggest that tumor growth control can be further enhanced when AZD6244 is given following cycles of gemcitabine plus low dose cisplatin (Supplementary Fig.S8).

In summary, the data presented in this paper strongly point to the importance of testing the sequence effects when combining MEK inhibitors with gemcitabine to treat biliary cancer patients (and potentially other tumor types); suggest the potential to incorporate MEK inhibitors into protocols combining gemcitabine, and they point to the utility of $^{18}$F-FLT PET imaging to establish the optimum period interrupting MEK inhibition prior to the next cycle of gemcitabine-based therapy. Our data suggests that planned or current trials that evaluate the combination of MEK inhibition concurrently with a chemotherapy that inhibits cell cycling may demonstrate no apparent advancement over single agent or other combinations and may possibly result in shut down of the further development of MEK inhibitors in biliary cancers in error. We are in the process of designing a clinical trial evaluating the sequential regimen of gemcitabine-based chemo-MEK inhibitor for patients with advanced biliary cancers based on the data in this paper.
Acknowledgements

We would like to thank all the members of David Hedley’s lab for helpful discussion. This work would not have been possible without the technical support and advice from Chaw Sue, May Cheung, Daniel Wu, Doug Vines, Trevor Do, and WenJiang Zhang.

Grant Support

The work was supported by the Marie Thompson Fund for Research in Biliary Cancer, the Princess Margaret Hospital, Toronto.

References


18
Figure legends

Fig. 1. Effect of AZD6244 on cell cycle phase distribution of human biliary cancer cell lines.

EGI-1 and TFK-1 cells were treated with indicated concentrations of AZD6244 for 24h, 48h or 72 h. (A) Proteins involved in ERK pathway were detected by immunoblotting. (B) Cell cycle profiles were detected by FACS. (C) Cells were pulsed with EdU for 30mins before harvest, and EdU incorporation was detected by FACS. Data were presented as mean ± SD of three separate experiments; representative images from one experiment were shown in FigS2.

Fig. 2. Time course of recovery of cell cycle phase distribution following AZD6244 treatment in vitro.

EGI-1 and TFK-1 cells were exposed to 1\mu{M} AZD6244 for 24 h, AZD6244 was withdrawn, and then cells incubated in drug-free medium for an additional indicated times. Cells exposed to equivalent volumes of DMSO served as controls. (A) Cell cycle phase distribution was determined by DNA flow cytometry. (B) Cells were pulsed with EdU for 30mins before harvest, and EdU incorporation was measured by flow cytometry. Data were presented as mean ± SD of three separate experiments, representative images from one experiment were shown in FigS3.

Fig. 3. Inhibitory effect of sequential treatments with AZD6244 and gemcitabine on cell proliferation in EGI-1 and TFK-1 cells.

Sub-confluent monolayers of cells were treated with indicated concentration of gemcitabine 24h alone, or concomitantly with 1\mu{M} AZD6244 for 24h or pretreated with 1\mu{M} AZD6244 24h and another 24h drug free interval in fresh medium. Cell viability and proliferation were detected by (A) MTS assay. (B) Flow cytometry analysis of PI/MMP. (C) Clonogenic assay as described in
Material and Methods. In the clonogenic assay, the survival fraction was normalized by the control group. Data were presented as mean ± SD of at least three independent experiments. Representative images shown in FigS4.

**Fig. 4. Time course of recovery of MEK pathway and cell cycle phase distribution following AZD6244 treatment in vivo.**

OCIP55 subcutaneously xenografted mice (n=5/group) were acutely treated with 48h AZD6244, then after indicated time post final dose, mice were scarified. Mice treated with DMSO served as controls. (A) Representative fluorescence images (20 × single fields) of tissue stained for phosphorylated ERK (red), EdU (green) and counterstained for DAPI (blue). (B) Mice were pulsed with EdU injection (i.p.) 30mins before sacrificed. EdU incorporation was measured by flow cytometry. (C and D) Quantative analysis of (A) and (B). (E) AZD6244 concentration in tumor and plasma were detected by LC-MS. *indicates P<0.05, ** indicated P<0.01; upper: vs control group, bottom: vs 48h group.

**Fig. 5. Time course of recovery of proliferation following AZD6244 treatment in vivo detected by 18F-FLT PET-CT scan.**

Mice bearing subcutaneously-grown OCIP55 xenografted (n=6/group) were treated with 48h AZD6244, then after indicated time post final dose, mice were scanned. Mice treated with DMSO served as controls. *indicates P<0.05, ** indicated P<0.01; upper: vs control group, bottom: vs 48h group.

**Fig. 6. Inhibitory effect of sequential treatments with AZD6244 and gemcitabine on tumor**
growth in vivo.

(A) Schematic schedule of treatments. Schedule-A represents concomitant combination of AZD6244 and gemcitabine. Schedule-B represents a 48h interval of AZD6244 followed by next gemcitabine. (B) Tumor growth curve of OCIP55, GB1, EGI-1 and TFK-1 xenografted mice.
Figure 1

A

<table>
<thead>
<tr>
<th>AZD6244(μM)</th>
<th>EGI-1</th>
<th>TFK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

B

C

AZD6244 24h(μM)
**Figure 2**

**A**

- **AZD6244 1μM 24h**
  - EGI-1
    - % Total cells vs. Time points since washout (h)
  - TFK-1
    - % Total cells vs. Time points since washout (h)

- **DMSO 24h**
  - % Total cells vs. Time points since washout (h)

**B**

- **EGI-1**
  - % EdU positive cells vs. Time points since washout (h)
  - **TFK-1**
Figure 3

(A) 
TFK-1

Proportion of proliferation (%)

0 20 40 60 80 100 120

Gem(μM)

0 0.01 0.02 0.05 0.1 1 10

AZD6244+Gem
AZD6244-24h-Gem

Gem

(B) 

Viable cell percentage (%)

Control AZD6244 Gem AZD6244+Gem AZD6244-24h-Gem

(C) 

Survival fraction (%)

Control AZD6244 Gem AZD6244+Gem AZD6244-24h-Gem
Figure 4

A

B

C

D

E

Author Manuscript Published OnlineFirst on October 22, 2012; DOI: 10.1158/1078-0432.CCR-12-2557
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5

[Image of PET scans and bar graph showing SUVmax values over time after final dose]
Figure 6

A

Schedule A

Schedule B

B

OCIP55

GB1

EGI-1

TFK-1
Sequence dependence of MEK inhibitor AZD6244 combined with gemcitabine for treatment of biliary cancer

Junyao Xu, Jennifer Knox, Emin Ibrahimov, et al.

Clin Cancer Res  Published OnlineFirst October 22, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-2557

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/10/22/1078-0432.CCR-12-2557.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.