Sequence Dependence of MEK Inhibitor AZD6244 Combined with Gemcitabine for the Treatment of Biliary Cancer

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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 extracellular signal-regulated kinase (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 severe combined immunodeficient (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 48 hours with AZD6244 and then monitored for 48 hours off treatment. On the basis of 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 reentry into S-phase was delayed by approximately 48 hours after 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 patients with biliary cancer when gemcitabine is given following a 48-hour 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. Clin Cancer Res; 1–10. ©2012 AACR.

Introduction

Cancers arising in the biliary tract, consisting of the intra- and extrahepatic bile ducts and the gallbladder, remain a major challenge to the surgical, medical, and radiation oncologists. A large majority of these tumors is 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 seems 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 with 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., EGF 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.
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 extracellular signal-regulated kinase (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 subadditive or antagonistic effect, whereas enhanced effect over single agents was observed when gemcitabine was given following a 48-hour interruption in AZD6244 dosing. Our study also provides a mechanistic explanation for this observation that delayed entry into S-phase during MAP–ERK kinase (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.

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 showed 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 has shown 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 the S-phase during MAP—ERK kinase (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 article using 2 recently developed, patient-derived primary biliary cancer xenografts, supplemented by experiments using established cell lines.

Materials 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). EGI-1 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS and 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 with Whipple pancreatectomy, and GB1 was established from ascites fluid taken from a patient with advanced peritoneal metastases from a primary gallbladder cancer. On histologic examination, these primary xenografts showed typical adenocarcinoma features (Supplementary Fig. S1). Mutational analysis by the Sequenom MassARRAY, 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-1and TFK-1 cell lines.

Xenografts used in this study were 4 to 6 passages for OCIP55 and 2 to 4 passages for GB1. Drug treatment commenced after 4 to 6 weeks of tumor growth. Tumor models from human cancer cell lines were also set up to test the effect of drug treatments. A total of 2 × 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 to 4 weeks of tumor growth.

Drug treatment

AZD6244 was purchased from Selleck Chemicals and gemcitabine was obtained from the Princess Margaret Hospital Pharmacy. To determine the effects of AZD6244 on cell-cycle progression, 5 groups of 5 randomly assigned mice with OCIP55 xenografts were treated with 25 mg/kg AZD6244 [12.5 mg/mL in dimethyl sulfoxide (DMSO)] or vehicle control by oral gavage twice daily for 2 days. The mice were then sacrificed and the tumors removed at 4, 12, 24, and 48 hours after the final dose. Thirty minutes before sacrifice, tumor-bearing mice were given 50 mg/kg of the thymidine analogue 5-ethyl-2′-deoxyuridine (EdU; Molecular Probes, Invitrogen) by intraperitoneal injection. All harvested tumors were divided into 3 parts, which were disaggregated into single-cell suspensions for flow cytometric analysis, snap-frozen in liquid nitrogen for immunofluorescence staining and liquid chromatography/mass spectrometry (LC/MS) measurement of AZD6244, or formalin-fixed and then paraffin-embedded. Separate groups

AZD6244 was well tolerated in patients with advanced biliary cancer, showing 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.

Recent phase II data (11) using AZD6244 as monotherapy was well tolerated in patients with advanced biliary cancer, suggesting the feasibility of combination with other drugs.
of tumor-bearing mice (n = 6/group) treated using identical protocols were used for \(^{18}F\)-fluorine-labelled thymidine (FLT) PET imaging.

For growth inhibition experiments, mice were treated with different treatment schedules incorporating gemcitabine 100 mg/kg intraperitoneally every 4 days, and AZD6244 or DMSO control. Caliper measurements of tumor volume (using the formula volume = \(a \times b^2\), where a and b are the longest and shortest diameters of the tumor, respectively; ref. 18), animal body weight, and tumor condition were recorded three times weekly for the duration of the study. Before and after each treatment cycle, complete blood counts were measured in OCIP55-bearing mice. When the tumor volume of the control group reached approximately 1,200 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 by copper-catalyzed click chemistry, and then stained with the DNA-specific dye 4’,6-diamidino-2-phenylindole (DAPI) at 1 μg/mL for 30 minutes. A Gallios flow cytometer (Beckman Coulter) was used for data acquisition. Cell-cycle analysis was done using ModFit LITTM (Verity), 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 minutes and then incubated with EdU reaction cocktail (Click-IT EdU Alexa Fluor 647 Imaging Kit, Molecular Probes, Invitrogen) for 30 minutes 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 (× 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. Following centrifugation, the supernatants were evaporated to dryness using the Universal Vacuum System (Thermo Electron Corp.) and then dissolved again in HPLC mobile phase. Separation was carried out using a reversed-phase HyperClone BDS C18 column (5 μm, 50 × 2.0 mm, Phenomenex) and a gradient mobile phase containing water/acetonitrile/formic acid. Peaks were detected using API3200 tandem mass spectrometry (Applied Biosystem/MDS Sciex).

**Results**

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

Preliminary experiments were carried out 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 1 μmol/L and greater resulted in a decrease in ERK phosphorylation and increase in the expression of p27\(^{kip1}\), but not p21\(^{cip1}\) (Fig. 1A). Cell-cycle analysis following 24-hour exposure to AZD6244 showed a dose-dependent increase in the percentage of cells in G\(_1\), with a corresponding reduction in S-phase cells and in EdU labeling (Fig. 1B and Supplementary Fig. S2). When cells treated with 1 μmol/L AZD6244 for 24 hours were placed in drug-free medium, reentry into the 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 (Cell-Titer 96 Aqueous One Solution Reagent; Promega) and...
96-well plate reader. The IC_{50} of EGI-1 cells to gemcitabine was 0.051 ± 0.012 μmol/L and that of TFK-1 to gemcitabine was 0.45 ± 0.35 μmol/L, which is consistent with previous reports for these cells (21–23). Two combination protocols were evaluated in cell culture: sequential treatment consisting of 24-hour exposure to 1 μmol/L AZD6244 followed by incubation in drug-free medium for 24 hours, then 24-hour treatment with 0 to 10 μmol/L gemcitabine, or simultaneous exposure to 1 μmol/L AZD6244 and gemcitabine for 24 hours. Using the MTS assay, sequential treatment (IC_{50} = 0.09 ± 0.04 μmol/L), but not simultaneous treatment (IC_{50} = 2.02 ± 0.91 μmol/L), enhanced the inhibition of TFK-1 cell proliferation compared with gemcitabine alone. A similar result was observed with EGI-1 cells (Fig. 3A). Because TFK-1 cells are relatively gemcitabine-resistant, the sensitizing effect of pretreatment 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 with 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 Fig. S4). Similarly, using a clonogenic assay, we observed significant loss of viability in cells preexposed to 1 μmol/L AZD6244 before 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-hour treatment with AZD6244 blocked the ERK pathway and inhibited EdU uptake. These effects persisted for 24 hours after the last dose of AZD6244, when phosphorylated ERK returned to pretreatment levels. The recovery of EdU uptake did not occur until the 48-hour time point, when it seemed 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 18F-FLT PET would allow the recovery kinetics following MEK inhibition to be studied directly in patients with biliary cancer, 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 24 hours after the last drug dose, recovering to untreated control values at 48 hours, similar to the results obtained using EdU.

To explore the correlation between these pharmacodynamic effects and the drug levels, liquid chromatography/mass spectrometry (LC/MS) was used to measure AZD6244 in plasma and tumor tissue. As shown in Fig. 4E, a mean plasma concentration of 2,403 ng/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 4-hour time point, the drug was retained for longer in tumor tissue and showed the expected inverse relationship to ERK phosphorylation. Thus, the 48-hour lag period between the last drug dose and reentry into the S-phase seems to be explained partly by the time needed for reactivation of the ERK pathway and partly by a latent period in G1 following the recovery of ERK signaling, similar to that seen following drug exposure in vitro (Fig. 2). On the basis of these observations, we reasoned that gemcitabine would be relatively ineffective if given concurrently with AZD6244, or within 48 hours 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 (25 mg/kg twice daily) or gemcitabine (100 mg/kg biweekly) was well tolerated (Supplementary Fig. S5). AZD6244 monotherapy showed modest antitumor effects in all 4 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 2 primary xenograft models.

Two combination schedules were designed using the same total doses of gemcitabine and AZD6244 in 4-day treatment cycles (Fig. 6A). In schedule A, gemcitabine was given immediately after the final dose of 48-hour treatment with AZD6244, followed by a 48-hour treatment-free period before the next cycle ("simultaneous dosing"); in schedule B, gemcitabine was given 48 hours 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 OCP55 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 4 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 article.

We used 2 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% to 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 hours 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 with gemcitabine alone, whereas when gemcitabine treatment was delayed for 24 hours 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 reentry into S-phase delayed by approximately 48 hours following the last dose. This effect was readily detected using 18F-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 patients with biliary cancer treated with MEK inhibitors. Although the plasma concentrations of AZD6244 decreased rapidly following the last dose, AZD6244 was retained in tumor tissue at 1 μmol/L or more concentrations for up to 24 hours with the recovery of ERK phosphorylation showing the expected inverse correlation with tumor drug levels. Thus, the delayed reentry 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. On the basis of these results, we designed 2 treatment protocols to test the schedule dependence of the gemcitabine + AZD6244 drug combination in biliary cancer.

Consistently in all 4 models tested, tumor growth control was improved when treatment with AZD6244 commenced immediately after gemcitabine and was discontinued 48 hours before the next gemcitabine treatment, whereas when gemcitabine was given after AZD6244, the effect was subadditive. We think that this result can be translated into the
clinic to treat biliary patients with cancer, who are currently treated with gemcitabine plus low-dose cisplatin. Preliminary results using our 2 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 article strongly point to the importance of testing the sequence effects when combining MEK inhibitors with gemcitabine to treat patients with biliary cancer (and potentially other tumor types); suggest the potential to incorporate MEK inhibitors into protocols combining gemcitabine, and they point to the use of 18F-FLT PET imaging to establish the optimum period interrupting MEK inhibition before the next cycle of gemcitabine-based therapy. Our data suggest that planned or current trials that evaluate the combination of MEK inhibition concurrently with a chemotherapy that inhibits cell cycling may show 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 article.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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