MEK inhibitor for gastric cancer with MEK1 gene mutations

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**Running title:** MEK1 gene mutation and gastric cancer

**Key words:** gastric cancer; MEK1 gene mutation; MEK inhibitor.

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Abstract

The prognosis for patients with unresectable advanced or recurrent gastric cancer (GC) remains poor. The identification of additional oncogenes with influences similar to those of epidermal growth factor receptor gene mutations and upon which the growth of cancer cells is dependent is needed. In this study, we evaluated the sensitivity to MEK inhibitors (GSK1120212 and PD0325901) in several GC cell lines in vitro and found three poorly differentiated GC cell lines that were hypersensitive to the inhibitors. The sequence analyses in these three cell lines revealed that one cell line had a novel MEK1 mutation, while the other two had previously reported KRAS and MEK1 mutations, respectively; the gene statuses of the other resistant cell lines were all wild-type. Experiments using MEK1 expression vectors demonstrated that the MEK1 mutations induced the phosphorylation of ERK1/2 and had a transforming potential, enhancing the tumorigenicity. The MEK inhibitor dramatically reduced the phosphorylation of ERK1/2 and induced apoptosis in the cell lines with MEK1 mutations. In vivo, tumor growth was also dramatically decreased by an inhibitor. One of the 46 GC clinical samples that were examined had a MEK1 mutation; this tumor had a poorly differentiated histology. Considering the addiction of cancer cells to active MEK1 mutations for proliferation, GC with such oncogenic MEK1 mutations might be suitable for targeted therapy with MEK inhibitors.
Introduction

Gastric cancer is the third most common cause of death from malignant disease in men (fifth in women) worldwide (1). The prognosis of patients with unresectable advanced or recurrent gastric cancer remains poor, with a median survival time of less than 1 year in individuals receiving conventional therapy (2-5). The combination of trastuzumab, an antibody targeting human epidermal growth factor receptor (EGFR) type2 (HER2), with chemotherapy has yielded a survival benefit for patients with HER2-positive gastric or gastro-esophageal junction cancer (3); however, HER2-positive tumors only account for 7% to 17% of all gastric cancers (6-9). Fibroblast growth factor receptor 2 gene (FGFR2) or MET gene (MET) amplification has been also regarded as a potential target in GC, but the frequency is expected to be very low (10-12). Therefore, the identification of additional oncogenes with effects similar to those of EGFR mutations or anaplastic lymphoma kinase gene (ALK) rearrangements and upon which cancer cells are dependent is needed (13).

The mitogen-activated protein kinase (MAPK) pathway includes RAS, RAF, MEK, and ERK. Constitutive activation of this pathway can lead to uncontrolled cell growth and survival, ultimately resulting in oncogenic transformation and progression (14). Reflecting the central role of the MAPK pathway in cell proliferation, activated mutants of RAS family members (HRAS, KRAS, and NRAS) are among the oncoproteins most frequently detected in human malignancies (15). The
discovery of mutations of the *BRAF* gene in melanoma has further reinforced the substantial
collection of tremendous MAPK pathway involvement in carcinogenesis (16). However, very limited information is
available regarding somatic *MEK1* mutations in human malignancies (17). In the present study, we
tested the effects of MEK inhibitors in several GC cell lines *in vitro* and found three cell lines that
were hypersensitive to the inhibitors; one of these cell lines had a novel *MEK1 S72G* mutation.
Furthermore, the role of these mutations, a xenograft study using a MEK inhibitor, and the *MEK1* gene
statuses of clinical samples of GC were investigated.

**Materials and Methods**

**Cell cultures and reagents**

The HEK293 cell line (human embryonic kidney cell line) and the NIH-3T3 cell line (mouse
fibroblast cell line) were maintained in DMEM medium (Nissui Pharmaceutical, Tokyo, Japan)
supplemented with 10% FBS (GIBCO BRL, Grand Island, NY) in a humidified atmosphere of 5%
CO₂ at 37°C. All the GC cell lines used in this study were maintained in RPMI-1640 medium
(Sigma-Aldrich, St. Louis, MO, USA), except for IM95 (DMEM; Nissui Pharmaceutical, Tokyo,
Japan), supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The IM95 and
OCUM-1 cell lines were obtained from the Japanese Collection of Research Bioresources (Osaka,
Japan), while the other cell lines were provided by the National Cancer Center Research Institute
(Tokyo, Japan) in 2006. The OCUM-1, Okajima, SNU-16, and HEK293 cell lines were analyzed using a short tandem repeat (STR) method in July 2014, and the OCUM-1, SNU-16, and HEK293 cell lines were authenticated. The database did not include the STR pattern of the Okajima cell line, but the pattern did not match any of the other cell lines. GSK1120212 and PD0325901 (MEK inhibitors) were purchased from Selleck Chemicals (Houston, TX, USA) and Wako (Osaka, Japan), respectively (Fig. 1A).

**Growth inhibition assay in vitro**

The growth-inhibitory effects of GSK1120212 and PD0325901 were examined using a 3, 4, 5-dimethyl-2H-tetrazolium bromide assay (MTT; Sigma-Aldrich), as described previously (18). The experiment was performed in triplicate.

**Antibody**

Rabbit antibodies specific for MEK1/2, ERK1/2, phospho-ERK1/2, caspase 3, cleaved caspase 3, PARP, cleaved PARP, and β-actin were obtained from Cell Signaling (Beverly, MA).

**Western blot analysis**

A western blot analysis was performed as described previously (18). Briefly, subconfluent cells
were washed with cold phosphate-buffered saline (PBS) and harvested with Lysis A buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH7.0), 5 mM EDTA, 50 mM sodium chloride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor mix, Complete™ (Roche Diagnostics). Whole-cell lysates were separated using SDS-PAGE and were blotted onto a polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin in a TBS buffer (pH8.0) with 0.1% Tween-20, the membrane was probed with the primary antibody. After rinsing twice with TBS buffer, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and washed, followed by visualization using an ECL detection system and LAS-4000 (GE Healthcare, Buckinghamshire, United Kingdom). When the phosphorylation levels of ERK1/2 and apoptosis-related molecules were examined after GSK1120212 exposure, the samples were collected 3 hours and 48 hours after stimulation, respectively.

**Mutational analysis for KRAS, BRAF, and MEK1 genes**

Genomic DNA samples from GC cell lines were screened for **KRAS** mutations (exon 2), **BRAF** mutations (exons 2, 4, 11, 12 and 15), and **MEK1** mutations (exons 2-11). The PCR reactions were performed using TaKaRa ExTaq (TaKaRa, Otsu, Japan). The primers are summarized in Supplementary Table S1. The PCR products were then directly sequenced using the BigDye
Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA). To confirm the results, the
PCR amplification was repeated, and the PCR products were subcloned using TOPO TA Cloning
kits (Invitrogen, Carlsbad, CA).

**Plasmid construction and transfectants**

PcDNA-MEK1 with myc-tag vector was obtained from Addgene. The MEK1 exon point
mutation Q56P or G72S was amplified using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa) and
primers (Supplementary Table S1). Vectors were transfected into HEK293 cells or NIH-3T3 cells
using FuGENE6 transfection reagent (Promega, Madison, WI). Hygromycin selection (100 μg/mL)
was performed on days 2-8 after transfection, and the cells were then cultured in normal medium.
The vectors and stable transfectant cell lines were designated as PcDNA-mock, PcDNA-MEK1 WT,
PcDNA-MEK1 Q56P, PcDNA-MEK1 S72G, HEK293-mock, HEK293-MEK1 WT, HEK293-MEK1
Q56P, HEK293-MEK1 S72G, 3T3-mock, 3T3-MEK1 WT, 3T3-MEK1 Q56P, and 3T3-MEK1
S72G.

**Focus formation assay**

The transfectant NIH-3T3 cell lines were then cultured for 2-3 weeks in DMEM medium
supplemented with 5% FBS. The focus formations were counted and photographed using a light
microscope. The experiment was performed in triplicate.

Annexin V binding apoptosis analysis

The Annexin V binding apoptosis analyses were performed as described previously (19). Briefly, the cells were exposed to GSK1120212 (1 nM) for 48 hours, and the binding of Annexin V and PI to the cells was then measured using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer’s instructions. The cells were stained with FITC Annexin V and PI at room temperature for 15 min and were analyzed using a flow cytometer and Cell Quest software (BD Biosciences). The experiment was performed in triplicate.

Xenograft studies

Nude mice (6-week-old females; CLEA Japan, Tokyo, Japan) and NOD/SCID mice (6-week-old females; CLEA Japan) were used for the in vivo studies and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines. To evaluate tumorigenicity, a suspension of 1 × 10⁶ NIH-3T3 transfectant cells (in 100 μL of PBS) was subcutaneously inoculated into the right
flank of each nude mouse (n = 5), and tumor formation was examined after 2 weeks based on a previous report (17). To evaluate the effects of GSK1120212, a suspension of 1 × 10^7 cells (in 50 μL of PBS) with 50 μL of Matrigel (Okajima cell line) or 5 × 10^6 cells (in 50 μL of PBS) with 50 μL of Matrigel (SNU-16 cell line) was subcutaneously inoculated into the right flank of each NOD/SCID mouse (n = 5); treatment was then initiated when the tumors in each group achieved an average volume of approximately 150 mm^3. In the treatment groups, GSK1120212 (0.5 or 1.0 mg/kg) was administered by oral gavage daily for 7 days based on the results of a previous study (20); the control animals received 0.5% methylcellulose as a vehicle. The tumor volume was calculated as the length × width^2 × 0.5. The tumor formation and volume were assessed every 2 to 3 days. This method has been previously described (21).

**Patients**

Patients with advanced GC who underwent surgical resection at Kinki University Hospital between April 2009 and March 2012 were enrolled. This study was retrospectively performed and was approved by the institutional review board of the Kinki University Faculty of Medicine.

**Isolation of genomic DNA**

Genomic DNA samples were extracted from surgical specimens preserved as FFPE tissue using
the QIAamp DNA Micro kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions as described previously (10). Macro-dissection of the FFPE samples was performed to select a cancer region, which was marked by a pathologist after deparaffinization. The DNA concentration was determined using the NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA).

**Statistical analysis**

Continuous variables were analyzed using the Student t-test, and the results were expressed as the average and standard deviation (SD). The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA). A P-value of less than 0.05 was considered statistically significant.

**Results**

OCUM-1, Okajima, and HSC-44 cell lines were hypersensitive to MEK inhibitors, and these cell lines had MEK1 mutations or a KRAS mutation

To examine the sensitivities of several GC cell lines to MEK inhibitors, we used an MTT assay (Fig. 1B). The 50% inhibitory concentrations (IC50) of the two MEK inhibitors (GSK1120212 and PD0325901) are summarized in Table 1. The OCUM-1, Okajima, and HSC-44 cell lines were hypersensitive to both MEK inhibitors; all three of these cell lines exhibited a poorly differentiated
histology.

Next, to search for *KRAS*, *BRAF*, and *MEKI* mutations, which were associated with a sensitivity to MEK inhibitors, we sequenced these genes using direct sequencing. The *MEKI* Q56P and S72G mutations were found in the OCUM-1 and Okajima cell lines, respectively, and a *KRAS* G12V mutation was also found in the HSC-44 cell line (Fig. 2A). In contrast, all the cell lines that were not sensitive to MEK inhibitors did not have any mutations. The *MEKI* Q56P mutation in the OCUM-1 cell line and the *KRAS* G12V mutation in the HSC-44 cell line have been previously reported (17), whereas the *MEKI* S72G mutation in the Okajima cell line is a novel mutation. This novel mutation was confirmed using the TOPO TA Cloning kit (Fig. 2B).

**MEKI Q56P and S72G mutations increased the phosphorylation level of ERK1/2, had transformational abilities, and enhanced the tumorigenicity**

To address the role of the *MEKI* mutations, MEK1-overexpressed HEK293 and NIH-3T3 cell lines were created using each *MEKI* expression vector (wild-type, Q56P, or S72G). ERK1/2 was phosphorylated in the HEK293-MEK1 Q56P, HEK293-MEK1 S72G, 3T3-MEK1-Q56P, and 3T3-MEK1-S72G cell lines, compared with the controls (Fig. 3A and B). We then investigated the transformational abilities and tumorigenicities of the *MEKI* mutations using a focus formation assay and a tumorigenicity assay with the NIH-3T3 cell lines and nude mice; the results showed that the
MEKI Q56P and S72G mutations had transformational abilities and enhanced the tumorigenicity, compared with the controls. Foci or tumors were not formed in the controls (mock and wild-type) (Fig. 3C and D). These findings suggest that both the MEKI Q56P mutation and the novel MEKI S72G mutation have transformational abilities and enhance tumorigenicity by activating the MAPK pathway.

Reduction in the phosphorylation level of ERK1/2 and induction of apoptosis in response to GSK1120212 in the OCUM-1 and Okajima cell lines

Next, we examined the phosphorylation levels of ERK1/2 after GSK1120212 exposure (0, 1, 3, 10, and 30 nM) in each GC cell line. Three hours of exposure to GSK1120212 induced a significant decrease in the phosphorylation levels of ERK1/2 in the hypersensitive cell lines (OCUM-1 and Okajima), compared with the level in a non-sensitive cell line (SNU-16) (Fig. 4A). We then analyzed the Annexin V binding apoptosis of the cell lines after exposure to GSK1120212 using a flow cytometer. The number of apoptotic cells in the OCUM-1 and Okajima cell lines, but not the SNU-16 cell line, increased greatly after GSK1120212 exposure (1 nM) (Fig. 4B). Western blot analyses for apoptosis-related molecules revealed that 48 hours of exposure to the reagent also greatly increased the levels of cleaved PARP and cleaved caspase 3 in the OCUM-1 and Okajima cell lines, compared with the SNU-16 cell line (Fig. 4C).
**In vivo efficacy of GSK1120212 in the Okajima cell line**

To perform a xenograft study, we used the Okajima (MEK1 Q72S) and the SNU-16 cell line (MEK1 wild-type). To evaluate the effects of GSK1120212, a suspension of $1 \times 10^7$ cells (in 50 μL PBS) with 50 μL of Matrigel (Okajima cell line) or $5 \times 10^6$ cells (in 50 μL PBS) with 50 μL of Matrigel (SNU-16 cell line) was subcutaneously inoculated into the right flank of each NOD/SCID mouse (n = 5). In the treatment groups, GSK1120212 (0.5 or 1.0 mg/kg) was administered by oral gavage daily for 7 days; the control animals received 0.5% methylcellulose as a vehicle. The tumors from the Okajima cell line were dramatically reduced by treatment with GSK1120212 (vehicle: 179.86 ± 44.88 mm$^3$ vs. GSK1120212 [0.5 mg/kg]: 89.4 ± 22.84 mm$^3$, $P = 0.0039^*$, or vs. GSK1120212 [1.0 mg/kg]: 27.04 ± 26.7 mm$^3$, $P = 0.00018^*$; GSK1120212 [0.5 mg/kg] vs. GSK1120212 [1.0 mg/kg], $P = 0.0041^*$) (Fig. 5A and B). The phosphorylation of ERK1/2 in the tumors was inhibited by GSK1120212 (Fig. 5B). In contrast, the tumors from the SNU-16 cell line were not reduced by the drug (vehicle: 335.62 ± 131.36 mm$^3$ vs. GSK1120212 [0.5 mg/kg]: 346.5 ± 182.31 mm$^3$, $P = 0.92$, or vs. GSK1120212 [1.0 mg/kg]: 307.68 ± 106.03, $P = 0.72$; GSK1120212 [0.5 mg/kg] vs. GSK1120212 [1.0 mg/kg], $P = 0.69$) (Fig. 5A).

**Clinicopathological features of patients with MEK1-mutated GC**
A total of 46 patients with advanced GC participated in this study. We evaluated the patient characteristics according to their MEK1 gene status. The isolated genomic DNA samples were directly sequenced. The clinical features of all the patients are summarized in Supplementary Table S2. One of the patients had GC with a MEK1 Q56P mutation; this patient was a 64-year-old male whose GC had been diagnosed as a poorly differentiated scirrhus adenocarcinoma stage IV located in Corpus.

Discussion

The Cancer Genome Atlas (TCGA) dataset for GC has shown that the GC populations with non-synonymous KRAS, BRAF, or MEK1 mutations were relatively small (28/289, 24/289, and 7/289, respectively), and MEK1 Q56P or S72G mutations have not been identified in the TCGA dataset. In this study, we identified MEK1 mutations in poorly differentiated GC cell lines that were hypersensitive to MEK inhibitors and showed that these mutations have transformational abilities and that the growth of the cancer cells is dependent on these mutations. Specifically, the MEK1 S72Q mutation in the Okajima cell line is a novel activating mutation, whereas the MEK1 Q56P mutation in the OCUM-1 cell line has been previously reported by Choi et al. (17). In addition, a MEK1 Q56P mutation was identified in a clinical sample of a poorly differentiated GC; to the best of our knowledge, this is the first study in which a clinical sample of a MEK1 Q56P-mutated GC has
been identified.

GSK1120212 is an inhibitor of MEK1/2 that exhibits a high potency, selectivity, and long circulating half-life (22). The results of a phase III study have demonstrated that GSK1120212 is associated with a significant improvement in progression-free survival and overall survival, compared with chemotherapy, in patients with V600E or V600K BRAF-mutated advanced melanoma (23). Several studies have shown that KRAS and/or BRAF mutations are associated with the sensitivity to MEK inhibitors in melanoma, thyroid cancer, colon cancer, and ovarian cancer (23-27). However, very limited information is available regarding somatic MEK1 mutations in human malignancies. Similar to our present study, Choi et al. has reported that the MEK1 Q56P mutation identified in the OCUM-1 cell line has a transformational ability (17), and somatic mutations in the MEK1 gene have been reported in several other cancers including lung cancer, ovarian cancer, colon cancer, and melanoma (28-32). Our present study demonstrated that both the MEK1 G56P and the novel MEK1 S72G mutation in poorly differentiated GC cell lines that were hypersensitive to MEK inhibitors have transformational abilities and that the growth of the cancer cells was dependent on these mutations. In both in vitro and in vivo studies, the GC cell lines with MEK1 mutations dramatically responded to the MEK inhibitor. Therefore, MEK inhibitors can be effective for patients with MEK1 mutations in a manner similar to the effect of EGFR-tyrosine kinase inhibitors in patients with EGFR mutations and the effect of ALK inhibitors in patients with
ALK rearrangements (33-36). Then, not only KRAS and BRAF mutations, but also MEK1 mutations should be recognized as predictive biomarkers for the efficacy of MEK inhibitors.

In general, patients with a poorly differentiated GC histology have a poor prognosis and their treatment is challenging (37). FGFR2 or MET amplification seems to be predominant in poorly differentiated GC (10-12). Similarly, in the present study, the 2 GC cell lines with MEK1 mutations both had a poorly differentiated histology, and the one GC clinical sample with a MEK1 mutation was a poorly differentiated adenocarcinoma. Choi et al. previously reported that 1 of 86 GC samples had a MEK1 mutation and that the sample was a poorly differentiated adenocarcinoma (well-differentiated, 0/40 and poorly differentiated, 1/46) (17). Despite the relatively small number of samples, these results suggest that GC with MEK1 mutations might be likely to have a poorly differentiated adenocarcinoma histology, similar to that resulting from FGFR2 or MET amplification, and treatment with a MEK inhibitor might be a promising option for such GC patients. To confirm these findings, larger studies are needed.

In conclusion, we have identified MEK1 mutations in poorly differentiated GC cell lines and a poorly differentiated GC clinical sample and have shown that the mutations have transformational abilities and that the growth of the cancer cells is dependent on these mutations. In particular, the MEK1 S72Q mutation in the Okajima cell line is a novel activating mutation. Our results warrant strong consideration of the development of MEK inhibitors for the treatment of GC with MEK1
mutations.

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Disclosure Statement

The authors have no conflicts of interest to disclose.
References

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<th>IM95</th>
<th>SNU-16</th>
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Abbreviations: IC<sub>50</sub>, 50% inhibitory concentration; GSK, GSK1120212; PD, PD0325901.
**Figure legends**

**Figure 1.** Structures of MEK inhibitors and sensitivities of several GC cell lines to these inhibitors.

A. Structures of GSK1120212 and PD0325901. **B.** Sensitivities of several GC cell lines to MEK inhibitors. To examine the sensitivities of MEK inhibitors, we used an MTT assay. The experiment was performed in triplicate. The OCUM-1, Okajima, and HSC-44 cell lines were hypersensitive to the inhibitor. All of these cell lines had a poorly differentiated histology. Line, mean of independent triplicate experiments.

**Figure 2.** MEK1 and KRAS mutations in each cell line. To search for KRAS, BRAF, and MEK1 mutations, which were associated with the sensitivity to MEK inhibitors, we sequenced these genes using direct sequencing. **A.** MEK1 Q56P mutation in the OCUM-1 cell line, MEK1 S72G mutation in the Okajima cell line, and KRAS G12V mutation in the HSC-44 cell line. MEK1 gene exon 2 sequencing revealed a MEK1 Q56P mutation (A > C) in the OCUM-1 cell line and a MEK1 S72G mutation (A > G) in the Okajima cell line. KRAS gene exon 2 sequencing revealed KRAS G12V (G > T) in the HSC-44 cell line. All the cell lines that were not sensitive to MEK inhibitors did not have any mutations. We used the SNU-16 cell line as a wild-type control. **B.** MEK1 gene sequence of the Okajima cell line after the insertion of a TOPO cloning vector. To confirm the results, the PCR
amplification was repeated and the PCR products were subcloned using TOPO TA Cloning kits. The

\textit{MEK1} S72G mutant allele and wild-type allele were both confirmed.

**Figure 3.** Transformational ability of each \textit{MEK1} mutation. 

A. MEK1 expression and phosphorylation of ERK1/2 in transfectant HEK293 cell lines. To address the role of the \textit{MEK1} mutations, expression vectors and \textit{MEK1}-overexpressed HEK293 cell lines were created. Each \textit{MEK1} gene was equally introduced into the cell lines, and ERK1/2 was phosphorylated in the HEK293-MEK1 Q56P and HEK293-MEK1 S72G cell lines, compared with HEK293-mock or HEK293-MEK1 WT. \(\beta\)-actin was used as an internal control. B. MEK1 expression and phosphorylation of ERK1/2 in transfectant NIH-3T3 cell lines. The expressions of MEK1 in the transfectant cell lines were confirmed using western blot analyses. Similar to the HEK293 cell lines, ERK1/2 was phosphorylated in the 3T3-MEK1 Q56P and 3T3-MEK1 S72G cell lines. \(\beta\)-actin was used as an internal control. C. Transformational ability. To investigate the transformational abilities of these mutations, we used a focus formation assay to examine NIH-3T3 cell lines. Transfectant NIH-3T3 cell lines were cultured for 2-3 weeks and photographed. Both \textit{MEK1} mutations had transformational abilities (mock, 0; wild-type, 0; Q56P, 11.7 ± 3.5; S72G, 5 ± 2). The controls (mock and wild-type) did not exhibit the formation of any foci. Columns, mean of independent triplicate experiments; error bars, SD; scale bar, 20 \(\mu\)m. D. Tumorigenicity. To investigate the tumorigenicities
of these mutations, we used a tumorigenicity assay to examine NIH-3T3 cell lines and nude mice. Transfectant NIH-3T3 cells (1 × 10^6) were injected subcutaneously into the right flank of nude mice; tumor formation was then examined 2 weeks after injection based on the results of a previous report (17). Both MEK1 mutations enhanced the tumorigenicity (mock, 0/5; wild-type, 0/5; Q56P, 5/5; S72G, 4/5). The controls (mock and wild-type) did not exhibit the formation of any tumors.

Figure 4. Phosphorylation level of ERK1/2 and apoptosis after GSK1120212 exposure. A.

Phosphorylation levels of ERK1/2 after GSK1120212 exposure. When the phosphorylation levels were examined after GSK1120212 exposure (0, 1, 3, 10, and 30 nM), the samples were collected 3 hours after the stimulation. GSK1120212 induced a significant decrease in the phosphorylation levels of ERK1/2 in the hypersensitive cell lines (OCUM-1 and Okajima), compared with that in the non-sensitive cell line (SNU-16). β-actin was used as an internal control. B. Annexin V binding apoptosis analyses. The cells were exposed to GSK1120212 (1 nM) for 48 hours and were then harvested and stained with FITC Annexin V and PI. The cells were analyzed using a flow cytometer. The experiment was performed in triplicate, and DMSO was used for the control. The number of apoptotic cells in the OCUM-1 and Okajima cell lines increased greatly after GSK1120212 exposure (DMSO: 15.7 ± 3.3% vs. GSK1120212: 42.3 ± 8.0%; P = 0.017*, and DMSO: 16.2 ± 5.1% vs. GSK1120212: 34.4 ± 10.0%; P = 0.025*, respectively), but not in the SNU-16 cell line (DMSO: 9.9
± 2.0% vs. GSK1120212: 10.4 ± 1.1%; \( P = 0.54 \). Columns, mean of independent triplicate experiments; error bars, SD; GSK, GSK1120212; *\( P < 0.05 \); n.s., not significant. C. Western blot analyses for apoptosis-related molecules. When apoptosis-related molecules were examined after GSK1120212 exposure (1 nM), the samples were collected 48 hours after the stimulation.

GSK1120212 greatly increased the expression of cleaved PARP and cleaved caspase 3 in the OCUM-1 and Okajima cell lines, compared with the SNU-16 cell line. \( \beta \)-actin was used as an internal control.

Figure 5. Xenograft study. We used the Okajima (\( MEK1 \) Q72S) and the SNU-16 (\( MEK1 \) wild-type) cell lines in the xenograft study. A suspension of \( 1 \times 10^7 \) cells (in 50 \( \mu \)L of PBS) with 50 \( \mu \)L of Matrigel (Okajima cell line) or \( 5 \times 10^6 \) cells (in 50 \( \mu \)L of PBS) with 50 \( \mu \)L of Matrigel was subcutaneously inoculated into the right flank of each NOD/SCID mouse (\( n = 5 \)). In the treatment groups, GSK1120212 (0.5 or 1.0 mg/kg) was administered by oral gavage daily for 7 days; the control animals received 0.5% methylcellulose as the vehicle. A. Tumor volumes of Okajima and SNU-16 cell line. The tumors from the Okajima cell line dramatically decreased in size after GSK1120212 exposure (vehicle: 179.86 ± 44.88 mm\(^3\) vs. GSK1120212 [0.5 mg/kg]: 89.4 ± 22.84 mm\(^3\), \( P = 0.0039^* \), or vs. GSK1120212 [1.0 mg/kg]: 27.04 ± 26.7, \( P = 0.00018^* \); GSK1120212 [0.5 mg/kg] vs. GSK1120212 [1.0 mg/kg], \( P = 0.0041^* \)). Because the tumor growth of the Okajima cell
line was very slow, only a small change was observed in the vehicle group. In contrast, the tumors from the SNU-16 cell line did not decrease in size after drug exposure (vehicle: 335.62 ± 131.36 mm$^3$ vs. GSK1120212 [0.5 mg/kg]: 346.5 ± 182.31 mm$^3$, $P = 0.92$, or vs. GSK1120212 [1.0 mg/kg]: 307.68 ± 106.03, $P = 0.72$; GSK1120212 [0.5 mg/kg] vs. GSK1120212 [1.0 mg/kg], $P = 0.69$). Lines, mean of 5 mice; error bars, SD; GSK, GSK1120212 *$P<0.05$. B. Photographs of tumors in the Okajima cell line and western blot analyses of Okajima in vivo samples. The tumors from the Okajima cell line decreased in size in a dose-dependent manner. The phosphorylation of ERK1/2 was inhibited by GSK1120212. β-actin was used as an internal control. GSK, GSK1120212.
Figure 1

A

![Chemical structures of GSK1120212 and PD0325901](image)

B

![Cell proliferation graph](image)

Proliferation (%) vs. GSK1120212 concentration (nM) for different cell lines:
- OCUM-1
- Okajima
- HSC-44
- HSC-58
- IM95
- SNU-16
- N87
- MKN1
Figure 2

A

**MEK1**

- **OCUM-1**
  - Q56P
  - ACCCCGAAG
  - Wild

- **SNU-16**
  - S72G
  - ACCCAGAAG
  - Wild

**MEK1**

- **Okajima**
  - ATCGGTGAG
  - Wild

- **SNU-16**
  - G12V
  - GCTGGTGCC
  - Wild

**KRAS**

- **HSC-44**
  - GCTGGTGCC
  - Wild

B

**MEK1 S72G**

- **Forward**
  - ATCGGTGAG

- **Reverse**
  - CTCACCGGAT

**MEK1 Wild-type**

- **Forward**
  - ATCAGTGAG

- **Reverse**
  - CTCACTGAT
Figure 4

A

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C

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Figure 5

A

Tumor volume (mm$^3$)

- Vehicle
- GSK (0.5 mg/kg)
- GSK (1.0 mg/kg)

Day 1  Day 3  Day 6  Day 8

Okajima

SNU-16

B

Vehicle

GSK (0.5 mg/kg)

GSK (1.0 mg/kg)

P-ERK1/2

ERK1/2

β-actin
Molecular Cancer Therapeutics

MEK inhibitor for gastric cancer with MEK1 gene mutations

Mol Cancer Ther Published OnlineFirst September 24, 2014.

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