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PHASE I STUDIES

# Inhibition of EGFR, HER2 and HER3 signaling with AZD8931 alone and in combination with paclitaxel: Phase I study in Japanese patients with advanced solid malignancies and advanced breast cancer

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**Summary** *Background* AZD8931 is an equipotent, reversible inhibitor of signaling by epidermal growth factor receptor (EGFR), human EGFR 2 (HER2) and HER3. This two-part Japanese study (NCT01003158) assessed the safety/tolerability of AZD8931 monotherapy in patients with advanced solid tumors and in combination with paclitaxel in female patients with advanced breast cancer. *Methods* Monotherapy part: ascending doses of AZD8931 (40/60/80 mg twice daily [bid]) for 21 consecutive days. Combination part: AZD8931 40 mg bid and paclitaxel 90 mg/m$^2$ (on days 1, 8 and 15 of a 28-day cycle). *Results* Seventeen patients received AZD8931: 11 received AZD8931 monotherapy (40/60/80 mg [$n$=3/4/4]) and six AZD8931 40 mg bid plus paclitaxel. No dose-limiting toxicities were observed for AZD8931 alone or combined with paclitaxel. The most frequent adverse events (AEs) were diarrhea, paronychia, pustular rash and dry skin (each $n$=8) with AZD8931 monotherapy and diarrhea, stomatitis, rash, alopecia, epistaxis and neutropenia (each $n$=4) with combination therapy. Grade ≥3 AEs were reported for one, two and four patients in the 40 mg, 60 mg and combination groups, respectively. AZD8931 was rapidly absorbed with a half-life of 12 h. There was no evidence of pharmacokinetic interaction between AZD8931 and paclitaxel. Two patients (one in each part) had unconfirmed and confirmed partial responses, with a duration of 42 and 172 days, respectively. *Conclusion* Although maximum tolerated dose was not confirmed for AZD8931, based on overall incidence of rash and diarrhea AEs in the 80 mg group, doses up to 60 mg bid as monotherapy and 40 mg bid combined with paclitaxel are the feasible AZD8931 doses in Japanese patients.

**Keywords** AZD8931 · Paclitaxel · HER · Advanced solid tumors · Breast cancer

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## Introduction

The human epidermal growth factor receptor (HER/erbB) family of receptor tyrosine kinases comprises epidermal growth factor receptor EGFR (erbB1), HER2 (erbB2), HER3 (erbB3) and HER4 (erbB4). Homodimerization and/or heterodimerization of these receptors activates intracellular signaling pathways involved in cell proliferation and survival

during normal physiological processes [1–6]. However, aberrant signal transduction via EGFR, HER2 and HER3 has been identified as a common component of multiple cancer types and appears to promote solid tumor growth [7–12]. For example, EGFR activation is seen in tumor types such as non-small cell lung cancer, breast, colorectal, and head and neck cancer, and over-expression of EGFR is observed in a proportion of breast, ovarian, bladder and gastric malignancies [10].

Targeting HER family members with small molecular agents, such as gefitinib and erlotinib, has demonstrated efficacy in EGFR-mutation-positive non-small cell lung cancer [13–15]. Similarly, lapatinib has shown efficacy in the management of HER2 over-expressing metastatic breast cancer [16]. Results from preclinical studies have suggested that EGFR inhibition enhances the antitumor activity of chemotherapeutic agents [17, 18]. In one Phase III study of 86 patients with HER2-positive breast cancer, the combination of lapatinib and paclitaxel led to statistically significant improvements in time to progression, event-free survival, objective response rate and clinical benefit rate compared with paclitaxel and placebo [19].

To date, development of agents that specifically target the HER receptor pathway has focused on inhibition of EGFR and/or HER2. However, there is increasing evidence that HER3 plays an important role in human tumorigenesis [7] due to its effect on phosphatidylinositol 3-kinase (PI3K) signal transduction, a known mediator of cancer cell survival and acquired resistance [13, 20]. As such, more complex and equipotent inhibition of signaling by the HER receptor family may provide greater antitumor activity [15].

AZD8931 is an orally bioavailable, reversible, tyrosine kinase, equipotent inhibitor of EGFR, HER2 and HER3 signaling [15]. The combination of AZD8931 with paclitaxel has shown synergistic cytotoxicity in breast cancer cell lines and xenograft models [21]. This two-part study was conducted to assess the safety and tolerability of multiple ascending doses of AZD8931 monotherapy in Japanese patients with advanced solid tumors and in combination with paclitaxel in female Japanese patients with advanced breast cancer.

## Patients and methods

### Study design and patients

This was a two-part (monotherapy and combination therapy), single-center, Phase I open-label study (clinicaltrials.gov: NCT01003158). The monotherapy part enrolled male or female Japanese patients aged ≥20 years with histologically or cytologically confirmed advanced solid malignancies that were refractory to standard therapies or for which no standard therapy existed. The combination part enrolled female Japanese patients aged ≥20 years with histologically or cytologically confirmed locally advanced or metastatic breast cancer who were ineligible for hormonal or anthracycline therapy.

Inclusion criteria in both parts included: World Health Organization performance status of 0–2; life expectancy ≥12 weeks; absolute neutrophil count ≥1.5 × $10^9$/L or platelets ≥100 × $10^9$/L and hemoglobin >9 g/dL; serum bilirubin <1.5 times the upper limit of normal (ULN), alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase (ALT) <2.5 x ULN (except in patients with liver or bone metastases); serum creatinine ≤1.5 x ULN or creatinine clearance ≥50 mL/min; cardiac ejection fraction higher than the institution's lower limit of normal range. Patients were excluded from both parts if they had a history of cardiovascular disease; resting electrocardiogram (ECG) with measurable QTc interval >450 ms at ≥2 time points within 24 h; medical diagnosis of acne rosacea, psoriasis or severe atopic eczema; any ocular disease or condition that was active or likely to be aggravated during treatment; poorly controlled clinical disorders (eg diabetes mellitus, hypercalcemia or other systemic condition) or previous/current evidence of brain metastasis, interstitial lung disease or spinal cord compression; anticancer therapy within 4 weeks of the start of study treatment (6 weeks for nitrosurea or mitomycin C) or concomitant medication with potent inhibitors/inducers of CYP3A4 or CYP2D6; anti-seizure medication or corticosteroids; unresolved adverse events (AEs; Common Terminology Criteria for Adverse Events [CTCAE] grade ≥2) from previous anticancer therapy, as well as hypersensitivity to previous therapy with oral tyrosine kinase inhibitors; known hypersensitivity to paclitaxel or progression of disease during or within 6 months of receiving previous paclitaxel treatment (combination part only).

All patients provided written informed consent. The study was approved by the Institutional Review Board of Kinki University, Osakasayama, Japan, and was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice and the AstraZeneca policy on bioethics [22].

### Treatment

In the monotherapy part, patients in each dose cohort received a single oral dose of AZD8931 on day 1 (D1), followed by a 6-day observation period, and thereafter received AZD8931 twice daily (bid) for 21 consecutive days (R1–R21). The initial cohort received AZD8931 40 mg, followed by 60 mg and 80 mg in subsequent cohorts. Following review of AZD8931 data from a Caucasian Phase I study, 40 mg was considered the clinically feasible dose for long-term treatment; this was primarily based on the incidence of CTCAE grade 3 rash at doses of ≥80 mg, as well as CTCAE grade 3 diarrhea at doses ≥160 mg [23]. In the combination part, patients received AZD8931 bid (initial dose based on the maximum tolerated dose [MTD] determined in the monotherapy part) starting on

day 2 of a 28-day cycle, with paclitaxel 90 mg/m$^2$ administered on days 1, 8 and 15. Patients were able to continue treatment indefinitely if they did not meet a withdrawal criterion, were free from intolerable toxicity and were considered by the investigator to be receiving clinical benefit.

A minimum of three evaluable patients were to be dosed initially in each dose group in the monotherapy part. If no patient experienced a dose-limiting toxicity (DLT), dose escalation was permitted. If one patient experienced a DLT, additional patients were enrolled to a maximum of six; if no further patients experienced a DLT, enrollment into the next dose cohort was permitted. If ≥2 patients experienced a DLT, this dose was considered non-tolerated and the previous dose was defined as the MTD. The DLT evaluation period for the monotherapy part started from the first administration of AZD8931 and continued until R21 (within 28 days of the first dose); for the combination part, DLTs were evaluated for the first 28 days of cycle 1. In both parts, DLTs were defined as any of the following AEs or laboratory abnormalities considered related to AZD8931: clinically significant symptomatic ocular surface lesion; CTCAE grade 4 neutropenia or thrombocytopenia with a duration of ≥4 days; grade ≥3 neutropenia that was either associated with a body temperature of ≥38 °C and was unresponsive to antipyretics or required hospitalization; grade ≥3 thrombocytopenia associated with non-traumatic bleeding; grade ≥3 hyperkalemia or hyperglycemia; grade ≥3 events (that could not be attributable to other causes) of hypotension, urologic toxicity, clinically significant rash that despite optimal treatment remained grade ≥3, interstitial lung disease or pneumonitis, nausea, vomiting, or diarrhea; any other clinically significant grade ≥3 toxicity considered related to study drug; QTcF (Fridericia's correction) interval >500 ms or increased by >60 ms compared with baseline on two ECGs ≥30 min apart; symptomatic congestive cardiac failure associated with a decreased left ventricular ejection fraction (LVEF), or decrease in LVEF ≥20 % below the lower limit of the normal range; a delay of ≥7 days for paclitaxel administration on day 1 of cycle 2 as a consequence of AZD8931-induced toxicity (combination part only).

## Objectives and assessments

The primary objectives were to assess the safety and tolerability of multiple ascending doses of AZD8931 (monotherapy part) and of AZD8931 in combination with paclitaxel (combination part). Safety and tolerability were assessed throughout the study by evaluation of AEs using CTCAE version 3, laboratory findings, physical examinations, vital signs, cardiac monitoring and ophthalmic assessments. Full ophthalmic assessments were performed at screening and at R21; beyond R21, a full examination was only required in the case of a clinically significant ophthalmic abnormality. Cardiac monitoring was performed using a 12-lead ECG at screening, D1–D5, R1, R3, R7, R14, R21 and every 3 weeks thereafter for the monotherapy part, and at screening, D1–D4, D15 and every 4 weeks from cycle 2 onwards for the combination part. High-resolution computed tomography and arterial oxygen saturation were mandatory and had to be performed at baseline and throughout the study.

Key secondary objectives were to identify the MTD of continuous AZD8931 bid monotherapy and continuous AZD8931 bid in combination with paclitaxel; to characterize the pharmacokinetic (PK) profile of AZD8931 under both treatment regimens; and to characterize the PK profile of paclitaxel. During monotherapy, blood samples were taken for PK analysis on D1 and R14 pre-dose, 1, 2, 4, 6, 8, 10 (prior to the second dose of AZD8931 on R14), 24 (D1 only), 48 (D1 only) and 72 (D1 only) hours post-dose. Additional samples were taken pre-dose on days R3 and R7. During combination therapy: blood samples were taken for AZD8931 PK analysis pre-dose, 1, 2, 4, 6, 8 and 10 h post-dose on days 7 and 8; samples for AZD8931 combined with paclitaxel PK evaluations were taken pre-dose, 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 24 h (prior to AZD8931 dosing, day 1 only) post-dose on days 1 and 8. Plasma concentrations of AZD8931, *O*-desmethyl AZD8931 and paclitaxel were determined using high-performance liquid chromatography with mass spectrometry.

Preliminary efficacy of AZD8931 alone and in combination with paclitaxel was an exploratory objective. Tumor assessments were performed according to Response Evaluation Criteria in Solid Tumors (version 1.0) [24]. Baseline radiological tumor assessments were performed ≤4 weeks before the start of treatment, following 21 days of continuous multiple dosing and approximately every 6 weeks thereafter for AZD8931 monotherapy, or every 8 weeks for combination therapy, until withdrawal from the study. Optional exploratory objectives were to examine the relationship between exploratory biomarkers from blood and tumor tissue samples and clinical outcome, as well as pharmacogenetic analysis.

## Statistical analysis

No formal statistical analyses were performed; therefore, data are summarized descriptively. The safety analysis set comprised all patients who received at least one dose of AZD8931. For inclusion in the DLT set, evaluable patients were defined as those who had received ≥75 % of the planned dose of AZD8931 within 28 days of the first dose (both study parts), and for the combination part had completed at least one cycle of weekly paclitaxel and all safety assessments or experienced a DLT during the DLT evaluation period. Patients with evaluable PK data were included in the PK analysis set. Patients who had received at least one dose of AZD8931 and for whom tumor response data were available were included in the efficacy analysis.

## Results

### Patient characteristics and disposition

Between January 2010 and November 2011, 27 patients were enrolled. Seventeen patients (11 in the monotherapy part and six in the combination part) received at least one dose of AZD8931 and were included in the safety and PK analysis set (Table 1). Sixteen patients were evaluable for efficacy as one patient in the monotherapy part discontinued the study after the first dose of study drug and no efficacy data were recorded. Sixteen patients completed the 28-day evaluation period and 14 remained on treatment after this period.

### Safety and tolerability

There were no DLTs in either treatment part; therefore, the MTD of AZD8931 as monotherapy or in combination with paclitaxel could not be determined within the prespecified dose ranges. Dosing for the combination part (AZD8931 40 mg plus paclitaxel) was selected based on the incidence of rash and diarrhea AEs observed in the monotherapy part and that observed for patients receiving AZD8931 in combination with paclitaxel in a Western population (Clinicaltrials.gov NCT00900627) [25].

The median actual duration of AZD8931 treatment was 43.5 days (range 1–239) for the monotherapy part and 79.5 days (range 53–244) for the combination part. The most frequently reported AEs were diarrhea, paronychia, pustular rash and dry skin during AZD8931 monotherapy, and diarrhea, stomatitis, rash, alopecia, epistaxis and neutropenia during combination therapy (Table 2). Two ophthalmic AEs (eyelid edema and punctate keratitis) were reported in the present study, both in the combination part.

In total, seven (41.2 %) patients had grade ≥3 AEs. Three patients receiving AZD8931 monotherapy had grade 3 AEs of anemia, intervertebral disc protrusion and cancer pain ($n$=1 each). Four patients receiving combination therapy had a total of six grade ≥3 AEs: grade 3 AEs were neutropenia (reported in two patients), leucopenia, peripheral sensory neuropathy and papular rash (reported in one patient each); grade 4 decreased neutrophil count was also reported in one patient. Only the papular rash event was considered related to AZD8931 treatment. Two patients had a serious AE (grade 3 intervertebral disc protrusion in the AZD8931 40 mg monotherapy cohort; grade 2 infectious pneumonia in the AZD8931 40 mg plus paclitaxel cohort); neither was considered to be related to study treatment. Only one AE, grade 1 pneumonia observed in a patient receiving AZD8931 40 mg bid in combination with paclitaxel, led to permanent treatment discontinuation; this event was considered by the investigator to be related to both AZD8931 and paclitaxel treatment. There were no findings of clinical concern for vital signs, ECGs, echocard-iogram or ophthalmological assessments, or for hematology or biochemical parameters.

**Table 1** Patient demographics and baseline characteristics (safety population)

| | AZD8931 monotherapy part | | | | AZD8931 combination part |
|---|---|---|---|---|---|
| | 40 mg bid ($n$=3) | 60 mg bid ($n$=4) | 80 mg bid ($n$=4) | Total ($n$=11) | 40 mg bid + paclitaxel ($n$=6) |
| Median age, years (range) | 58 (46–63) | 54 (37–77) | 64 (59–69) | 59 (37–77) | 54 (49–69) |
| Male/female, n | 2/1 | 2/2 | 3/1 | 7/4 | 0/6 |
| Primary tumor type, n (%) | | | | | |
| Breast | – | – | – | – | 6 (100) |
| Colorectal | 1 (33) | 1 (25) | – | 2 (18) | – |
| Lung | – | 1 (25) | – | 1 (9) | – |
| Skin/soft tissue | – | – | 1 (25) | 1 (9) | – |
| Stomach | – | 1 (25) | 1 (25) | 2 (18) | – |
| Thyroid | – | – | 1 (25) | 1 (9) | – |
| Other | 2 (67) | 1 (25) | 1 (25) | 4 (36) | – |
| Previous cancer treatment, n | | | | | |
| Chemotherapy | 3 (100) | 4 (100) | 4 (100) | 11 (100) | 6 (100) |
| Radiotherapy | 1 (33) | 1 (25) | 4 (100) | 6 (55) | 6 (100) |
| Immunotherapy | – | 1 (25) | – | 1 (9) | – |
| Hormonal therapy | – | – | – | – | 6 (100) |

**Table 2** AEs (all-causality) reported in >2 patients in the monotherapy and combination therapy parts overall (safety population)

| | Number of patients, n (%) | | | | |
|---|---|---|---|---|---|
| | AZD8931 monotherapy part | | | AZD8931 combination part | |
| Adverse event | 40 mg bid (*n*=3) | 60 mg bid (*n*=4) | 80 mg bid (*n*=4) | 40 mg bid + paclitaxel (*n*=6) | Total (*n*=17) |
| Any AE | 3 (100) | 4 (100) | 4 (100) | 6 (100) | 17 (100) |
| Diarrhea | 1 (33) | 3 (75) | 4 (100) | 4 (67) | 12 (71) |
| Paronychia | 2 (67) | 3 (75) | 3 (75) | 3 (50) | 11 (65) |
| Dry skin | 2 (67) | 3 (75) | 3 (75) | 3 (50) | 11 (65) |
| Pustular rash | 3 (100) | 2 (50) | 3 (75) | 2 (33) | 10 (59) |
| Stomatitis | 3 (100) | 1 (25) | 1 (25) | 4 (67) | 9 (53) |
| Epistaxis | 2 (67) | 1 (25) | 0 | 4 (67) | 7 (41) |
| Nausea | 0 | 3 (75) | 0 | 3 (50) | 6 (35) |
| Rash | 0 | 1 (25) | 1 (25) | 4 (67) | 6 (35) |
| Dysgeusia | 1 (33) | 1 (25) | 0 | 3 (50) | 5 (29) |
| Constipation | 1 (33) | 1 (25) | 1 (25) | 2 (33) | 5 (29) |
| Eczema | 1 (33) | 1 (25) | 1 (25) | 1 (17) | 4 (24) |
| Vomiting | 0 | 2 (50) | 0 | 2 (33) | 4 (24) |
| Pyrexia | 0 | 1 (25) | 1 (25) | 2 (33) | 4 (24) |
| Alopecia | 0 | 0 | 0 | 4 (67) | 4 (24) |
| Fatigue | 0 | 1 (25) | 0 | 3 (50) | 4 (24) |
| Neutropenia | 0 | 0 | 0 | 4 (67) | 4 (24) |
| Increased ALT | 2 (67) | 0 | 0 | 1 (17) | 3 (18) |
| Hypertension | 0 | 2 (50) | 1 (25) | 0 | 3 (18) |
| Decreased hemoglobin | 0 | 0 | 1 (25) | 2 (33) | 3 (18) |
| Peripheral sensory neuropathy | 0 | 0 | 0 | 3 (50) | 3 (18) |
| Leucopenia | 0 | 0 | 0 | 3 (50) | 3 (18) |

Patients with multiple occurrences of the same event were counted only once per event. Includes AEs with onset from the first dose to 30 days following the last dose of AZD8931

## Pharmacokinetic evaluation

In the monotherapy part following single oral dosing, quantifiable plasma concentrations of AZD8931 (Fig. 1) were present up to 72 h post-dose at all dose levels investigated, indicating that the drug was bioavailable following oral administration. AZD8931 exposure increased approximately dose proportionally after single and multiple dosing. AZD8931 was rapidly absorbed with maximum plasma concentrations ($C_{max}$) being achieved at a median of 1–2 h across doses (Table 3). Following $C_{max}$, the elimination was biphasic with a mean terminal elimination half-life of approximately 11.4–12.8 h, which appeared to be independent of dose. Plasma clearance of AZD8931 remained approximately constant across the dose range. The geometric mean steady-state (pre-dose) plasma concentration was achieved by day 3 of continuous dosing (R3). Consistent with single-dose data, the mean accumulation ratio observed for AZD8931 40–80 mg ranged from 1.62–to 1.83-fold. The formation of *O*-desmethyl AZD8931 was achieved with a median $t_{max}$ of 36–60 h post-dose; its elimination was slow, resulting in increased plasma accumulation compared with AZD8931 following bid dosing. Area under the concentration–time curve from zero to last quantifiable concentration and $C_{max}$ ratios of *O*-desmethyl AZD8931 to AZD8931 were 25.8–53.7 and 4.0–6.6 % after single dosing, and 57.8–69.7 and 33.4–42.5 % after multiple dosing, respectively. In the combination part, there was no apparent PK interaction between paclitaxel and AZD8931 (Tables 4 and 5).

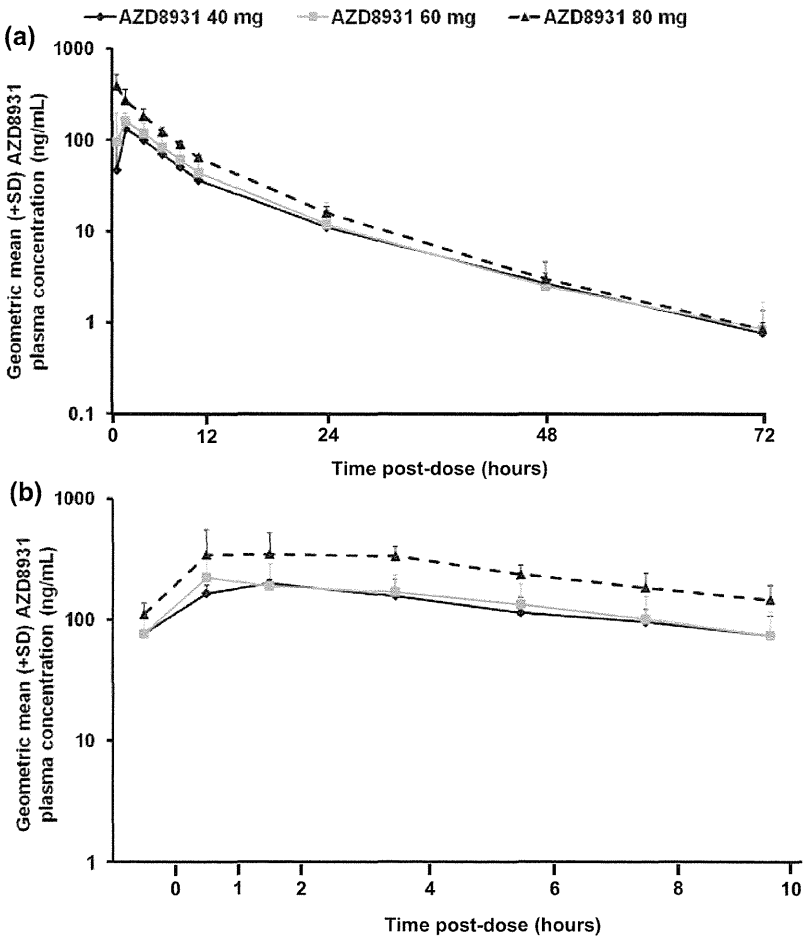


**Fig. 1** Geometric mean plasma concentration of AZD8931 following (**a**) single and (**b**) multiple doses in the monotherapy part (PK population)

**Table 3** Pharmacokinetic parameters of AZD8931 after single and multiple dosing in the monotherapy part (PK population)

| PK parameter | AZD8931 single dosing | | | AZD8931 multiple dosing | | |
|---|---|---|---|---|---|---|
| | 40 mg bid ($n$=3) | 60 mg bid ($n$=4) | 80 mg bid ($n$=4) | 40 mg bid ($n$=3) | 60 mg bid ($n$=4) | 80 mg bid ($n$=4) |
| AUC, ng.h/mL | 1,322 (18) | 1,620 (41) | 2,519 (16) | – | – | – |
| $AUC_{0-12,}$[a] ng.h/mL | 812 (3) | 1,035 (29) | 1,780 (21) | 1,466 (22) | 1,645 (41) | 2,953 (18) |
| $AUC_{0-t}$, ng.h/mL | 1,307 (17.2) | 1,599 (40) | 2,502 (15.8) | – | – | – |
| CL/F,[a,b] L/h | 30.6 (5.4) | 39.2 (15.5) | 32.1 (5.2) | 27.7 (5.5) | 38.5 (14.7) | 27.4 (4.7) |
| $C_{max,}$[a] ng/mL | 132 (14) | 189 (24) | 388 (36) | 207 (12) | 278 (41) | 474 (16) |
| $t_{½,}$[b] h | 12.6 (1.4) | 12.8 (1.7) | 11.4 (1.0) | – | – | – |
| $t_{max,}$[a,c] h | 2 (2–2) | 1.5 (1–2) | 1 (1–1) | 2 (2–4) | 1.5 (1–4) | 1 (1–4) |
| $V_{ss}/F$,[b] L | 375 (34) | 428 (109) | 303 (72) | – | – | – |
| Linearity factor[b] | – | – | – | 1.1 (0.1) | 1.0 (0.1) | 1.2 (0.5) |
| $R_{AC}$[b] | – | – | – | 1.8 (0.4) | 1.6 (0.3) | 1.7 (0.8) |

Values are presented as geometric mean (% coefficient of variation) unless otherwise stated; [a] at steady state for multiple dosing; [b] arithmetic mean (standard deviation); [c] median (range); AUC, area under the plasma concentration–time curve; $AUC_{0-12}$, area under the plasma concentration–time curve from time zero to 12 h; $AUC_{0-t}$, area under the plasma concentration–time curve from time zero to the time of the last quantifiable concentration; CL/F, total apparent drug clearance; $C_{max}$, maximum plasma concentration; $t_{½}$, terminal elimination half-life; $t_{max}$, time to reach maximum plasma concentration; $V_{ss}/F$, apparent volume of distribution at steady state; $R_{AC}$, accumulation ratio

**Table 4** Pharmacokinetic parameters of AZD8931 monotherapy and in combination with paclitaxel (PK population)

| PK parameter | AZD8931 ($n$=6) | AZD8931 + paclitaxel ($n$=6) |
|---|---|---|
| $AUC_{0-10}$, ng.h/mL | 1,047 (47) | 1,220 (58) |
| $C_{max}$, ng/mL | 202 (30) | 211 (60) |
| $t_{max}$,[a] h | 1 (1–2) | 2 (1–4) |

Values are presented as geometric mean (% coefficient of variation) unless otherwise stated; [a] median (range)

### Efficacy assessment

One patient receiving AZD8931 80 mg bid monotherapy had an unconfirmed partial response (PR); this 63-year-old male patient with metastatic gastric carcinoma had no prior surgery for gastric cancer and had received two previous chemotherapy regimens (plus prior radiotherapy for brain metastases). This patient had a HER2-negative tumor (IHC 1+); the retrospective use of this information, which was not part of the study protocol, was approved by the Institutional Review Board of Kinki University. At day 50, the patient had a 30.3 % reduction in target lesion size; by day 92, the target lesion size had increased by 52.2 % compared with the prior lowest size, and the overall response was therefore classified as progressive disease. A 49-year-old female patient with HER2-negative (IHC 0; information obtained as above) advanced breast cancer receiving AZD8931 plus paclitaxel had a confirmed PR with duration of response of 172 days. This patient had prior surgery for breast cancer and had received adjuvant radiotherapy followed by one chemotherapy (endoxan and farmorubicin) and two hormone therapy regimens. The patient had target lesions of skin/soft tissue and liver. A 48.7 % reduction in target lesion size was recorded at day 55, which reached a maximum reduction of 79.5 % at day 114; the patient was recorded as having progressive disease at day 255 with a 20 % increase in target lesion size.

**Table 5** Pharmacokinetic parameters of paclitaxel monotherapy and in combination with AZD8931 (PK population)

| PK parameters | Paclitaxel ($n$=6) | Paclitaxel + AZD8931 ($n$=6) |
|---|---|---|
| $AUC_{0-10}$, ng.h/mL | 7,022 (26) | 7,116 (25) |
| $C_{max}$, ng/mL | 6,367 (33) | 5,758 (44) |
| $t_{max}$,[a] h | 1 (1–1) | 1 (1–1) |

Values are presented as geometric mean (% coefficient of variation) unless otherwise stated; [a] median (range)

Seven patients had stable disease (≥6 weeks): one, two and four patients in the 40 mg bid (maxillary sinus cancer), 60 mg bid (colorectal cancer; stomach cancer) and combination therapy (all breast cancer) cohorts, respectively.

## Discussion

This two-part, single-center, Phase I, open-label study demonstrated that AZD8931, both alone and in combination with paclitaxel, was generally well tolerated in Japanese patients with advanced solid tumors and advanced breast cancer, respectively. The MTD of AZD8931 could not be confirmed in this study, in either the monotherapy or the combination part, since no DLTs were observed up to the highest studied dose (80 mg bid). However, based on the overall incidence of rash and diarrhea AEs in the AZD8931 80 mg bid group, AZD8931 doses up to 60 mg bid in monotherapy and 40 mg bid in combination with paclitaxel were considered to be the highest tolerable doses. In a recently published Phase I study of Caucasian patients, AZD8931 monotherapy was generally well tolerated at doses up to and including 240 mg bid over a 21-day period in patients with advanced solid tumors [23]. Due to two DLTs in the 300 mg bid cohort, AZD8931 240 mg bid was declared the MTD. However, this was a small dose-escalation study of relatively short duration and the authors concluded that more long-term data are needed to confirm a dose suitable for chronic treatment. A lower MTD of 40 mg bid, which is comparable to that defined in Japanese patients, was determined for AZD8931 in combination with paclitaxel in a recent Phase I dose-finding study in Caucasian patients with refractory tumors who were exposed to AZD8931 for a longer duration (median 52 days) [25].

The safety profile of AZD8931 observed in this study of Japanese patients is consistent with that previously reported in the Caucasian population [25–28]. In both parts of the study, skin/subcutaneous and gastrointestinal disorders were amongst the most common AEs; this is consistent with the mechanisms and known safety profiles of agents that target HER signaling [26–28] and therefore suggests that relevant target inhibition is being achieved at the AZD8931 doses investigated in this study. Most observed AEs were mild or moderate in nature (CTCAE grade ≤2) and only one AE classified as CTCAE grade ≥3, which was observed in the combination therapy cohort, was considered to be related to AZD8931 treatment. Only two patients experienced severe AEs during the study, neither of which were considered to be related to AZD8931 treatment. There were some differences in the most commonly reported AEs between the monotherapy and combination therapy cohorts, although these differences

generally included a greater proportion of AEs known to be associated with paclitaxel treatment, such as neutropenia, leucopenia and alopecia [29]. It is interesting to note that around 50 % of Caucasian patients enrolled in the Phase I study experienced ophthalmic AEs that were considered to be related to treatment with AZD8931 [23]. In contrast, only two ophthalmic AEs related to AZD8931 treatment were reported in the present study. Furthermore, the rate of grade ≥3 AEs (41 % [$n$=7/17] versus 64 % [$n$=18/28]) and the discontinuation rate due to AEs (6 % [$n$=1/17] versus 21 % [$n$=6/28]) was lower in Japanese patients compared with the Caucasian study. These differences may reflect the relatively low dose ranges evaluated in the present study and suggest that a more conservative AZD8931 dosing regimen may lead to a better risk:benefit profile.

The steady-state PK profile of AZD8931 was supportive of bid oral dosing. Absorption was rapid after single and bid doses of AZD8931, with a median $t_{max}$ of between 1 and 2 h across the dose levels. Pre-dose plasma concentrations of AZD8931 achieved steady state by day R3 following bid dosing, and exposure increased in an approximately dose-proportional manner. This PK profile is consistent with that reported in the Caucasian population [23]. Importantly, co-administration with paclitaxel had no apparent effect on the PK of AZD8931, suggesting that the combination of these agents is feasible from a PK perspective.

AZD8931 is an equipotent inhibitor of EGFR, HER2 and HER3 signaling; amplification of HER2 is observed in ~20 % of patients with gastric cancer and ~25 % of patients with breast cancer [30, 31]. Unconfirmed and confirmed PRs were reported following AZD8931 80 mg bid monotherapy (in a patient with gastric cancer) and combination therapy (in a patient with breast cancer). Of interest, neither of these patients had HER2-amplified tumors. The PR in the monotherapy part suggests preliminary efficacy of AZD8931 treatment in gastric cancer, likely based on the inhibition of HER-family signaling by AZD8931. The PR in the patient with breast cancer in the combination part may have been attributable to the presence of paclitaxel. These PRs were reported on days 50 and 55, respectively, suggesting that the efficacy of AZD8931 is best observed over longer-term treatment. This is in line with the conclusions of the Phase I study in Caucasian patients, which suggested that the lack of objective tumor responses at day R21 may have been due to the short evaluation time frame and that a longer study period is necessary to observe responses beyond stable disease. However, further investigation is needed to establish the efficacy of AZD8931.

In conclusion, although no DLTs were observed, the safety profile of patients who received AZD8931 ≥60 mg bid suggests that AZD8931 doses up to 60 mg bid as monotherapy, and 40 mg bid combined with paclitaxel, are the feasible doses for evaluation in Japanese patients. AZD8931, is no longer in AstraZeneca-sponsored development following data from two Phase IIb trials in breast cancer that showed no evidence of a therapeutic benefit in patients receiving AZD8931 [32, 33]. Investigator-sponsored studies are continuing in other indications.

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**Ethical standards** All patients provided written informed consent. The study was approved by the Institutional Review Board of Kinki University, Osakasayama, Japan, and was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice and the AstraZeneca policy on bioethics [22]. The experiments comply with the current laws of the country in which they were performed.

**Conflict of interest** Takayasu Kurata has received lecturer's fees from AstraZeneca. Yasuhito Fujisaka has received research fees for clinical studies from AstraZeneca. Kazuhiko Nakagawa has received lecturer's fees and research fees for clinical studies from AstraZeneca. Eisei Shin and Nobuya Hayashi are employees of AstraZeneca. Junji Tsurutani, Wataru Okamoto, Hidetoshi Hayashi and Hisato Kawakami have no conflicts of interest to disclose.

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Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

# MEK Inhibitor for Gastric Cancer with *MEK1* Gene Mutations

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## Abstract

The prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor. The identification of additional oncogenes with influences similar to those of epidermal growth factor receptor gene mutations, upon which the growth of cancer cells is dependent, is needed. In this study, we evaluated sensitivity to MEK inhibitors (GSK1120212 and PD0325901) in several gastric cancer cell lines *in vitro* and found three poorly differentiated gastric cancer 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 gastric cancer 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, gastric cancer with such oncogenic *MEK1* mutations might be suitable for targeted therapy with MEK inhibitors. *Mol Cancer Ther; 13(12); 3098–106. ©2014 AACR.*

## 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) type 2 (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 gastric cancer, 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 (*ALK*) gene 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 contribution of the MAPK pathway to carcinogenesis (16). However, very limited information is available about somatic *MEK1* mutations in human malignancies (17). In this study, we tested the effects of MEK inhibitors in several gastric cancer 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 gastric cancer were investigated.

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**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

S. Sogabe and Y. Togashi contributed equally to this article.

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## 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) supplemented with 10% FBS (GIBCO BRL) in a humidified atmosphere of 5% $CO_2$ at 37°C. All the gastric cancer cell lines used in this study were maintained in RPMI-1640 medium (Sigma-Aldrich), except for IM95 (DMEM; Nissui Pharmaceutical), supplemented with 10% FBS in a humidified atmosphere of 5% $CO_2$ at 37°C. The IM95 and OCUM-1 cell lines were obtained from the Japanese Collection of Research Bioresources, 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 and Wako, respectively (Fig. 1A).

### Growth inhibition assay *in vitro*

The growth-inhibitory effects of GSK1120212 and PD0325901 were examined using the MTT assay (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 Technology.

### 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 mmol/L Tris–HCl (pH 7.0), 5 mmol/L EDTA, 50 mmol/L sodium chloride, 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and a protease inhibitor mix, Complete (Roche Diagnostics). Whole-cell lyses were separated using SDS-PAGE and were blotted onto a polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin in a TBS buffer (pH 8.0) 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 enhanced chemiluminescence

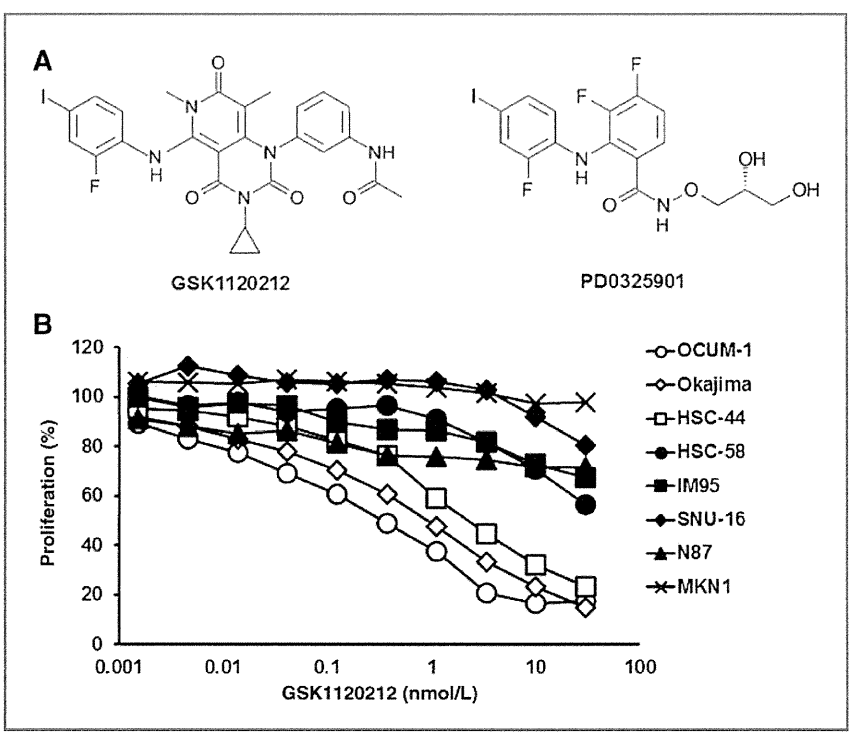


Figure 1. Structures of MEK inhibitors and sensitivities of several gastric cancer cell lines to these inhibitors. A, structures of GSK1120212 and PD0325901. B, sensitivities of several gastric cancer cell lines to MEK inhibitors. To examine the sensitivities of MEK inhibitors, we used the MTT assay. 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.

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(ECL) detection system and LAS-4000 (GE Healthcare). When the phosphorylation levels of ERK1/2 and apoptosis-related molecules were examined after GSK1120212 exposure, the samples were collected 3 and 48 hours after stimulation, respectively.

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

Genomic DNA samples from gastric cancer 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). 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). To confirm the results, the PCR amplification was repeated, and the PCR products were subcloned using TOPO TA Cloning kits (Invitrogen).

### 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). Hygromycin selection (100 μg/mL) was performed on days 2 to 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 to 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 nmol/L) for 48 hours, and the binding of Annexin V and propidium iodide (PI) to the cells was then measured using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's instructions. The cells were stained with FITC Annexin V and PI at room temperature for 15 minutes and were analyzed using a flow cytometer and CellQuest software (BD Biosciences). The experiment was performed in triplicate.

### Xenograft studies

Nude mice (6-week-old females; CLEA 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 (Osaka, Japan). The ethical procedures followed met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines. To evaluate tumorigenicity, a suspension of $1 \times 10^6$ 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 $1 \times 10^7$ cells (in 50 μL of PBS) with 50 μL of Matrigel (Okajima cell line) or $5 \times 10^6$ 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 gastric cancer who underwent surgical resection at Kinki University Hospital (Osaka, Japan) 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 (Osaka, Japan).

### Isolation of genomic DNA

Genomic DNA samples were extracted from surgical specimens preserved as formalin-fixed paraffin-embedded (FFPE) tissue using the QIAamp DNA Micro Kit (Qiagen), according to the manufacturer's instructions as described previously (10). Macrodissection 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 NanoDrop2000 (Thermo Fisher Scientific).

### 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). A *P* value of less than 0.05 was considered statistically significant.

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## 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 gastric cancer cell lines to MEK inhibitors, we used the MTT assay (Fig. 1B). The 50% inhibitory concentrations ($IC_{50}$) 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 *MEK1* mutations, which were associated with sensitivity to MEK inhibitors, we sequenced these genes using direct sequencing. The *MEK1* 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 cell lines that were not sensitive to MEK inhibitors did not have any mutations. The *MEK1* 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 *MEK1* S72G mutation in the Okajima cell line is a novel mutation. This novel mutation was confirmed using the TOPO TA Cloning Kit (Fig. 2B).

### MEK1 Q56P and S72G mutations increased the phosphorylation level of ERK1/2, had transformational abilities, and enhanced tumorigenicity

To address the role of the *MEK1* mutations, MEK1-overexpressed HEK293 and NIH-3T3 cell lines were created using each *MEK1* 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 *MEK1* mutations using a focus formation assay and a tumorigenicity assay with the NIH-3T3 cell lines and nude mice; the results showed that the *MEK1* 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 *MEK1* Q56P mutation and the novel *MEK1* S72G mutation have transformational abilities and enhance tumorigenicity by activating the MAPK pathway.

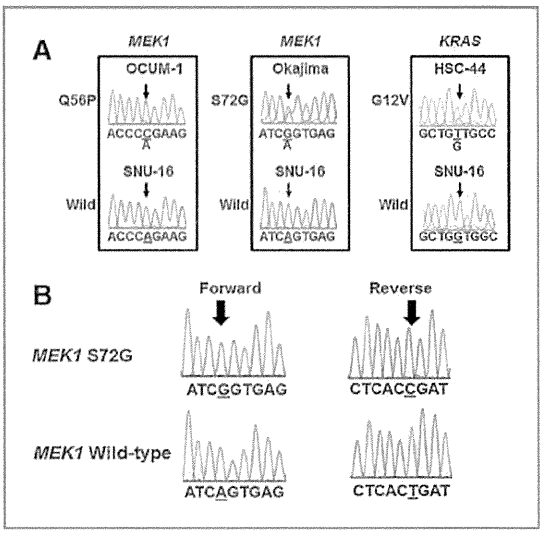


Figure 2. *MEK1* and *KRAS* mutations in each cell line. To search for *KRAS*, *BRAF*, and *MEK1* mutations, which were associated with the sensitivity to MEK inhibitors, we sequenced these genes using direct sequencing. A, *MEK1* Q56P mutation in the OCUM-1 cell line, *MEK1* S72G mutation in the Okajima 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 *MEK1* S72G–mutant allele and wild-type allele were both confirmed.

### 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 nmol/L) in each gastric cancer 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 nonsensitive 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

Table 1. $IC_{50}$ of MEK inhibitors

| | OCUM-1 | Okajima | HSC-44 | HSC-58 | IM95 | SNU-16 | N87 | MKN1 |
|---|---|---|---|---|---|---|---|---|
| GSK (nmol/L) | 0.33 | 0.92 | 2.23 | 43.6 | 1,485 | 8,140 | >10,000 | >10,000 |
| PD (nmol/L) | 3.4 | 33 | 38 | 453 | 6,570 | >10,000 | >10,000 | >10,000 |

Abbreviations: GSK, GSK1120212; PD, PD0325901.

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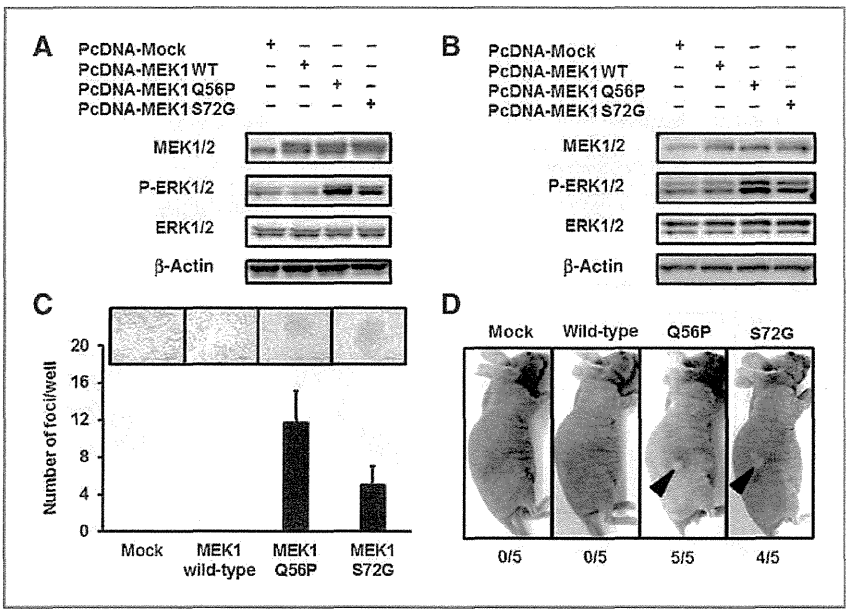


Figure 3. Transformational ability of each *MEK1* mutation. A, MEK1 expression and phosphorylation of ERK1/2 in transfectant HEK293 cell lines. To address the role of the *MEK1* mutations, expression vectors and *MEK1*-overexpressed HEK293 cell lines were created. Each *MEK1* gene was equally introduced 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. β-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. β-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 to 3 weeks and photographed. Both *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 μm. 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 ($1 \times 10^6$) 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.

GSK1120212 exposure (1 nmol/L; 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].

### Clinicopathologic features of patients with MEK1-mutated gastric cancer

A total of 46 patients with advanced gastric cancer 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 gastric cancer with a *MEK1* Q56P mutation; this patient was a 64-year-old male whose gastric cancer had been diagnosed as

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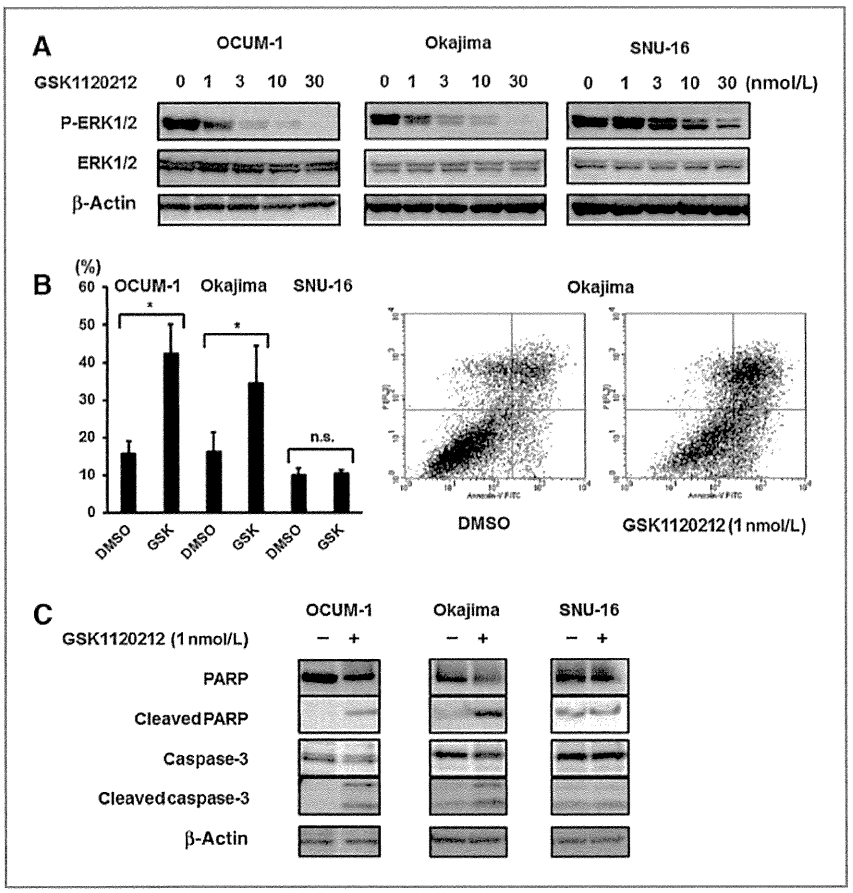


Figure 4. Phosphorylation level of ERK1/2 and apoptosis after GSK1120212 exposure. A, phosphorylation levels of ERK1/2 after GSK1120212 exposure. When the phosphorylation levels were examined after GSK1120212 exposure (0, 1, 3, 10, and 30 nmol/L), the samples were collected 3 hours after stimulation. 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 nonsensitive 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 nmol/L) 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 as a 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 nmol/L), 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. β-Actin was used as an internal control.

a poorly differentiated scirrhous adenocarcinoma stage IV located in corpus.

## Discussion

The Cancer Genome Atlas (TCGA) dataset for gastric cancer has shown that the gastric cancer populations with nonsynonymous *KRAS*, *BRAF*, or *MEK1* mutations were relatively small (28 of 289, 24 of 289, and 7 of 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 gastric cancer 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 and colleagues (17). In addition, a *MEK1* Q56P mutation was identified in a clinical sample of a poorly differentiated gastric cancer; to the best of our knowledge, this is the first study in which a clinical sample of a *MEK1* Q56P-mutated gastric cancer has been identified.

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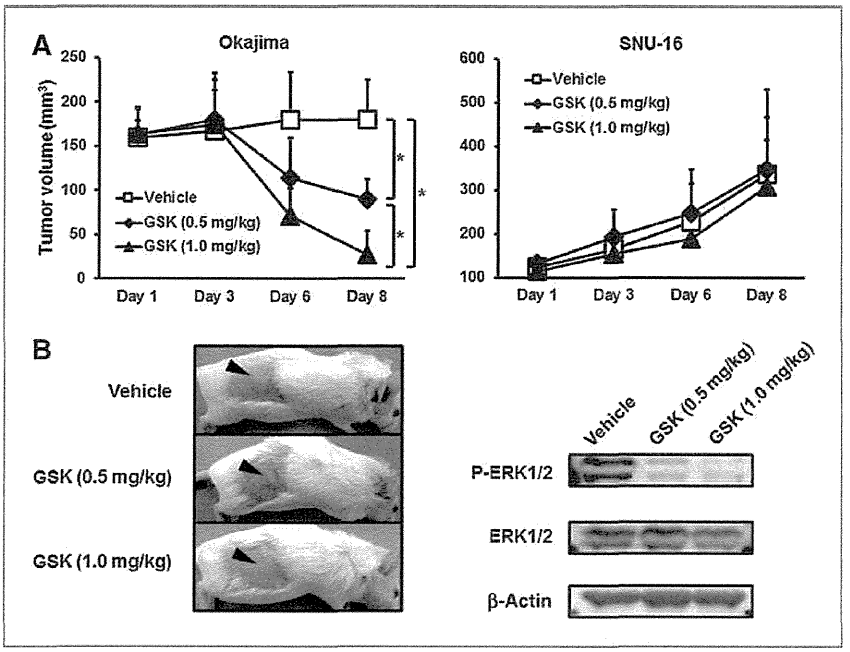


Figure 5. Xenograft study. We used the Okajima (*MEK1* Q72S) and the SNU-16 (*MEK1* wild-type) cell lines in the xenograft study. A suspension of $1 \times 10^7$ cells (in 50 μL of PBS) with 50 μL of Matrigel (Okajima cell line) or $5 \times 10^6$ cells (in 50 μL of PBS) with 50 μL of Matrigel was subcutaneously 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 a 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.

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 about the somatic *MEK1* mutations in human malignancies. Similar to our present study, Choi and colleagues (17) have reported that the *MEK1* Q56P mutation identified in the OCUM-1 cell line has a transformational ability, 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 gastric cancer 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 gastric cancer 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 gastric cancer histology have a poor prognosis and their treatment is challenging (37). *FGFR2* or *MET* amplification seems to be predominant in poorly differentiated gastric cancer (10–12). Similarly, in this study, the two gastric cancer cell lines with *MEK1* mutations had a poorly differentiated histology, and the one gastric cancer clinical

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sample with a *MEK1* mutation was a poorly differentiated adenocarcinoma. Choi and colleagues (17) previously reported that 1 of 86 gastric cancer samples had a *MEK1* mutation and that the sample was a poorly differentiated adenocarcinoma (well-differentiated, 0 of 40 and poorly differentiated, 1 of 46). Despite the relatively small number of samples, these results suggest that gastric cancer 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 patients with gastric cancer. To confirm these findings, larger studies are needed.

In conclusion, we have identified *MEK1* mutations in poorly differentiated gastric cancer cell lines and a poorly differentiated gastric cancer 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 in the development of MEK inhibitors for the treatment of gastric cancer with *MEK1* mutations.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** S. Sogabe, Y. Togashi, H. Kato, E. Banno, Y. Takeyama, K. Nishio

**Development of methodology:** S. Sogabe, H. Kato, Y. Takeyama

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Sogabe, Y. Togashi, H. Kato, A. Kogita, T. Yasuda

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Sogabe, Y. Togashi, H. Kato, S. Tomida, T. Yasuda

**Writing, review, and/or revision of the manuscript:** S. Sogabe, Y. Togashi, H. Hayashi, M.A. de Velasco, K. Nishio

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** T. Mizukami, Y. Sakamoto, M. Terashima, K. Sakai, Y. Fujita

**Study supervision:** K. Okuno, K. Nishio

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Molecular Cancer Therapeutics

# MEK Inhibitor for Gastric Cancer with *MEK1* Gene Mutations

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# Phase II study of pemetrexed and carboplatin plus bevacizumab, followed by maintenance pemetrexed and bevacizumab in Japanese patients with non-squamous non-small cell lung cancer

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**Abstract.** The present study evaluated the efficacy and safety of pemetrexed, carboplatin and bevacizumab, followed by maintenance pemetrexed and bevacizumab, in chemotherapy-naïve patients with stage IIIB/IV non-squamous non-small cell lung cancer (NSCLC). The patients were administered pemetrexed (500 mg/m$^2$), carboplatin (area under the concentration-time curve, 6.0 mg/ml x min) and bevacizumab (15 mg/kg) intravenously every three weeks for up to six cycles. Patients who did not experience tumor progression remained on maintenance pemetrexed and bevacizumab until disease progression or unacceptable toxicity occurred. The primary endpoint was the overall response rate. Of the 26 patients enrolled between March 2010 and April 2011, three were excluded due to brain metastases, therefore the intention-to-treat (ITT) population consisted of 23 patients. The median age was 64 years (range, 40-74 years) and 15 patients were male. In total, six patients had a performance status of 0, and 20 had stage IV tumors. The response rate was 69.6% [95% confidence interval (CI), 47.1-86.8], the disease control rate was 100% and the time to response was 1.2 months (95% CI, 0.72-1.93). The median progression-free survival time was 8.6 months (95% CI, 5.9-10.9) and the median overall survival time was 18.6 months (95% CI, 12.9-24.8). There were no grade 3 or worse hemorrhagic events and the feasibility was modest. Overall, pemetrexed and carboplatin plus bevacizumab, followed by maintenance pemetrexed and bevacizumab, was effective and tolerable in the patients with non-squamous NSCLC, and the time to response was relatively short.

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*Key words:* non-squamous non-small cell lung cancer, chemotherapy, pemetrexed, bevacizumab, Japanese

## Introduction

The leading global cause of cancer-related mortality is lung cancer (1). Overall, ~85% of patients with lung cancer have non-small cell lung cancer (NSCLC), with the majority being diagnosed with advanced-stage disease. Although NSCLC is histologically heterogeneous and can be classified into several subtypes, treatment strategies have been determined solely by the disease stage (2). Platinum-based doublet chemotherapy regimens are the standard first-line treatment for patients with advanced (stage IIIB/IV) disease, regardless of histology, as they have demonstrated a survival benefit greater than that of the best supportive care (3-5).

In phase III trials comparing the efficacy of cisplatin/pemetrexed and cisplatin/gemcitabine, overall survival (OS) was greater with cisplatin/pemetrexed in patients with adenocarcinoma and large cell carcinoma, but not in patients with squamous cell carcinoma, indicating that survival following treatment with cytotoxic agents was dependent on the histological type of the NSCLC (6). Furthermore, a large, randomized, phase III trial found that maintenance therapy with pemetrexed was effective and well-tolerated in patients with advanced non-squamous NSCLC who did not progress following induction therapy with pemetrexed plus cisplatin (7).

Molecularly-targeted agents, including those targeting epidermal growth factor, vascular endothelial growth factor (VEGF), platelet-derived growth factor and insulin-like growth factor I signaling, have been developed due to our increased understanding of the pathogenesis of NSCLC. A rationale for histology-based treatment approaches has been provided by clinical trials of targeted and more novel chemotherapy drugs, which have demonstrated that outcomes are dependent on the histological subgroup (8-11). Angiogenesis is particularly critical to tumor growth and metastatic dissemination, and the overexpression of VEGF has been associated with a poor prognosis in patients with NSCLC (12,13).

Bevacizumab is an anti-VEGF monoclonal antibody that has been revealed to inhibit tumor-associated angiogenesis in preclinical and clinical studies (14,15).