were evaluated by cell survival with the colonogenic assay, the possibilities of other functional deviations, including apoptosis induction, cannot be excluded. In addition, it is entirely likely that other genes listed in the present study in Table I may very possibly have important roles in the cytotoxicity caused by cisplatin.

## Conclusion

cDNA microarray analysis of cells with cisplatin exposure for a relatively long duration, 5 days, yielded 38 up-regulated and 4 down-regulated gene expressions of more than 2-fold ratio alteration, after adjusting for the cell cycle distributions of the cisplatin-treated and untreated cells. These genes included those involved in apoptosis, cell cycle regulation and DNA metabolism/repair, suggesting their possible importance in the process of cytotoxicity of cisplatin. We envision that the present data will be of some use for future disclosures of molecular mechanisms of cisplatin cytotoxicity and resistance mechanism in a single-agent administration, or in combination with other drugs or radiation.

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REVIEW

# Amrubicin for non-small-cell lung cancer and small-cell lung cancer

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**Summary** Amrubicin is a totally synthetic anthracycline anticancer drug and a potent topoisomerase II inhibitor. Recently, amrubicin was approved in Japan for the treatment of small- and non-small-cell lung cancers (SCLC and NSCLC). Here, we review the efficacy and toxicities of amrubicin monotherapy and amrubicin in combination with cisplatin for extensive-disease SCLC (ED-SCLC), and of amrubicin monotherapy for advanced NSCLC, as observed in the clinical trials. Recommended dosage for previously untreated advanced NCSLC was 45 mg/m$^2$/day by intravenous administration for 3 days. Dose-limiting toxicities were leucopenia, thrombocytopenia, and gastrointestinal disturbance. Response rate was 27.9% for advanced NSCLC, and 75.8% for ED-SCLC with a median survival time (MST) of 11.7 months. Recommended dosage of amrubicin was 40 mg/m$^2$/day in combination with cisplatin at 60 mg/m$^2$/day, with MST of 13.6 months and 1-year survival rate of 56.1%. In sensitive or refractory relapsed SCLC, response rate was 52 and 50%, progression-free survival was 4.2 and 2.6 months, overall survival was 11.6 and 10.3 months, and 1-year survival rate was 46 and 40%, respectively. These results are promising for the treatment of both NSCLC and SCLC. Further clinical trials will clarify the status of amrubicin in the treatment of lung cancer.

**Keywords** Amrubicin · Anthracycline · Topoisomerase II inhibitor · Non-small-cell lung cancer · Small-cell lung cancer

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## Introduction

Amrubicin is a totally synthetic anthracycline anticancer drug based on doxorubicin, of which the hydroxyl group at position 9 has been replaced by an amino group in amrubicin to enhance the efficacy. This new derivative is believed to exhibit its antitumor effect through metabolic reduction in tumor cells and conversion to the active metabolite amrubicinol, which inhibits cell growth about 200 times as potently as the parent compound (Fig. 1),[1, 2] unlike other anthracycline anticancer drugs, such as doxorubicin, in which the metabolites are considered to have a weaker antitumor effect than the parent compound. In comparison with doxorubicin in vivo, amrubicin was shown to have a more potent antitumor effect and lower toxic effects on the heart, a site of delayed toxicity with doxorubicin, and on the liver and kidneys [3, 4]. In vivo comparison between single dose and repeated doses of amrubicin over five consecutive days in antitumor effects on several cell lines revealed superior antitumor effect for 5-day administration, demonstrating schedule dependence (Table 1) [5]. With respect to the mechanism of action, amrubicin seems to act on topoisomerase II, stabilizing a cleavable complex [6]. As for clinical trials, a single-dose phase I study was first performed in patients with various types of previously treated malignant tumors. Adverse events that were defined as dose-limiting toxicities (DLTs) were hematologic, including leukopenia, thrombocytopenia, and anemia; the maximum tolerated dose (MTD) was 130 mg/m$^2$, and the recommended dose for phase II clinical studies was set at 100 mg/m$^2$ [7]. Next, a phase I clinical study using five-consecutive-day administration was performed in patients with various types of previously treated malignant tumors. As expected, the DLT was myelosuppression, while the MTD was 25 mg/m$^2$ with a total dose of

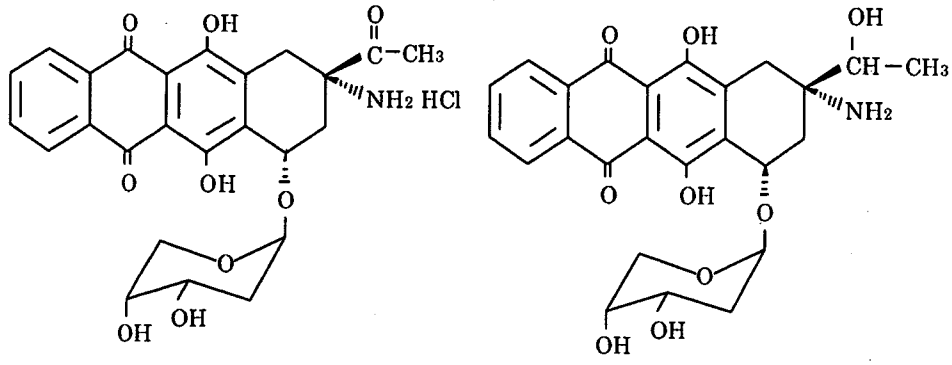

**Fig. 1** Chemical structures of amrubicin hydrochloride (*left*) and amrubicinol (*right*)

125 mg/m$^2$. However, because a clear tumor shrinking effect was not seen in any subject in this study,[8] subsequent repeated 5-day administration studies were not carried out. On the other hand, Feld et al. performed a clinical study of another anthracycline antitumor drug, epirubicin, for the treatment of non-small-cell lung cancer (NSCLC), and reported higher response rate in three-consecutive-day administration than in single-dose administration [9]. Based on these findings and in consideration of convenience in practical therapy, a regimen of repeated doses for three consecutive days came to be recommended for amrubicin as well. This article reviews the clinical studies of amrubicin for the treatment of NSCLC and small-cell lung cancer (SCLC) that have already been completed and suggests a course for investigations in the future.

## Non-small-cell lung cancer

Two single-dose phase II clinical studies of amrubicin for the treatment of NSCLC were conducted. First, an early phase II study targeted previously untreated NSCLC, starting with a dose of 100 mg/m$^2$ every 3 weeks. Adverse events in 16 subjects initially enrolled were mild, and the study was therefore continued in additional 26 subjects at an increased dose of 120 mg/m$^2$. Among 14 evaluable subjects of the initial 16, 1 subject (7.1%) had a partial response (PR), and among 20 evaluable subjects of the additional 26 after dose increase, 5 subjects (25%) had PR. Following these promising results, a late phase-II study was conducted for previously untreated NSCLC at a dose of 120 mg/m$^2$. A total of 62 patients were enrolled, but contrary to expectations only 6 subjects had PR, for an overall response rate of 9.7% [fn: New Drug Approval Package (in Japanese) http://www.info.pmda.go.jp/shinyaku/g020402/37009000_21400AMZ00465_x100_1.pdf, p501–510, p517–523, p524–532].

Prior to these studies, no phase I studies involving the recommended course of repeated administration over 3 days had been performed. Therefore, a phase I/II study on previously untreated NSCLC was conducted [10]. A dosage of 40 mg/m$^2$/day (total dose of 120 mg/m$^2$) was established for level 1, and was increased to 45 and 50 mg/m$^2$/day for levels 2 and 3, respectively. Four patients each were enrolled at dosage levels 1 and 2, and 5 patients at level 3 [10]. At level 3, grade 4 adverse events persisting 4 days or longer were leukopenia in two of five subjects and neutropenia in five of five subjects. Adverse events higher than grade 3 were thrombocytopenia in two of five subjects and anemia in two of five subjects. Non-hematologic grade 3 adverse events seen in one subject each were nausea/vomiting and melena. Grade 4 hematemesis was also seen in one subject. The DLTs were leukopenia, neutropenia, thrombocytopenia, and gastrointestinal disturbances, and so 50 mg/m$^2$ was considered to be the MTD [10]. The recommended dosage for phase II studies was considered to be 45 mg/m$^2$/day. Additional 15 evaluable patients were registered for the study at this dosage, and 7 of the total 28 subjects had PR, with an overall response rate of 25%. These results of amrubicin monotherapy for NSCLC were essentially as promising as the results for other novel

**Table 1** Effects of multiple administrations of amrubicin on the growth of human tumor xenografts

| Dose | Schedule | Minimum T/C (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Lung carcinoma | | Stomach carcinoma | | | | | |
| | | LX-1 | QG-56 | SC-2 | SC-7 | SC-9 | St-4 | St-15 | 4-1ST |
| 25 mg/kg | Once | 43 | 44 | 46 | 59 | 59 | 29 | 39 | 11 |
| 7.5 mg/kg | 5 qd | 31[a] | 38 | 36 | 37 | 37 | 29 | 24[a] | 13 |

[a] 7.5 mg/kg daily for 5 days shows significantly superior growth inhibition over single 25 mg/kg dose ($p<0.05$)

antitumor drugs, such as paclitaxel, launched in the 1990s [10]. Additional phase II studies were conducted to further ascertain efficacy and safety, at a dosage of 45 mg/m$^2$/day for three consecutive days every 3 weeks (Table 2) [11]. A total of 61 patients (45 males) were enrolled (median age, 65 years; range, 33 to 75 years), and the majority of subjects had a performance status (PS) of 0 to 1. All subjects were evaluable for both efficacy and safety. One subject had a complete response (CR) and 16 subjects had PR, with an overall response rate of 27.9%. Among toxicities, hematologic toxicities were observed frequently. Higher than grade 3 leukopenia and thrombocytopenia were seen in 52.5 and 14.8% of the subjects, respectively. Neutropenia was seen in 72.1%, and anemia in 23.0%. Non-hematologic adverse events were mild, including higher than grade 3 nausea/vomiting in 4.9% and anorexia in 4.9% (Table 3). In three subjects, interstitial pneumonitis that had developed before enrollment was exacerbated during the study, and two of these subjects died. The median survival time (MST) was 11.3 months and 1-year survival rate 47.7% (Table 4) [11]. These results of overall response rates and survival are comparable to those achieved with standard two-drug combination therapy containing a platinum agent for advanced NSCLC. At present, results of clinical trials of combination therapy using amrubicin plus other drugs to evaluate effects on NSCLC have not yet been reported. It is urgent that we explore combination therapy using amrubicin with other drugs that are known to be effective in the treatment of NSCLC, but it is also important that we clarify the position of amrubicin in the practical treatment of NSCLC.

## Small-cell lung cancer

A single-dose phase II study of amrubicin in patients with SCLC, previously treated or untreated, was performed similarly to the NSCLC studies. The dose was started at 100 mg/m$^2$ and increased to 120 mg/m$^2$ during the study. Eleven patients were enrolled (7 at 100 mg/m$^2$), of whom ten had previously been treated. Two of the 6 evaluable subjects treated with 100 mg/m$^2$ had PR, but no response was seen in any of the 4 subjects treated with 120 mg/m$^2$. Overall, 2 of the ten subjects had PR, for a response rate of 20%. The main adverse event was myelotoxicity. Grade 4 thrombocytopenia was seen in 4 of the 11 subjects (3 treated with 100 mg/m$^2$). In order to ascertain the efficacy of amrubicin in SCLC more accurately, a late phase II study in previously untreated patients with advanced SCLC was conducted at a dosage of 45 mg/m$^2$/day for three consecutive days at 3-week intervals. From an ethical standpoint, this study was designed such that if a tumor shrinkage of 25% or more (measured bilaterally) after one course, or 50% or more after two courses of amrubicin was not obtained, the patient would immediately be switched to the standard therapeutic mode of a combination of cisplatin and etoposide. A total of 35 patients were enrolled, and among the 33 evaluable subjects 3 had CR and 22 had PR, for an overall response rate of 75.8% (CR rate 9.1%). The MST was 11.7 months, the 1-year survival rate 48.5%, and the 2-year survival rate 20.2% (Table 4) [12]. Because a promising result of monotherapy had been obtained, a phase I/II combination therapy clinical study for previously untreated advanced SCLC was performed using cisplatin, a drug that currently plays a central role in SCLC chemotherapy, and the results were reported by Ohe et al [13]. In level 1, the dosage of amrubicin was 40 mg/m$^2$/day for three consecutive days, and the dose of cisplatin was 60 mg/m$^2$ (day 1); in levels 2 and 3 the dosage of amrubicin was 45 mg/m$^2$/day and the doses of cisplatin were 60 mg/m$^2$ and 80 mg/m$^2$, respectively. The courses were administered at 3-week intervals. DLTs, consisting of febrile neutropenia, grade 4 neutropenia persisting 4 days or more, and constipation, were seen in all three subjects enrolled at level 2. Therefore, the dosages at level 2 were considered the MTD, and the recommended dosage for the phase II part of the study was determined to be 40 mg/m$^2$/day for amrubicin with 60 mg/m$^2$ cisplatin. Then the phase II study was conducted in 41 subjects at that recommended dosage.

**Table 2** Phase II studies of amrubicin in previously untreated advanced NSCLC: patient characteristics

| Characteristics | Value |
|---|---|
| No. of eligible patients | 61 |
| Sex (male/female) | 45/16 |
| Age, median years (range) | 65 (33–75) |
| Histology (adenocarcinoma/squamous/large cell) | 33/26/2 |
| Stage (IIIA/IIIB/IV) | 8/19/34 |
| PS (0/1/2) | 19/39/3 |
| No. of institutions | 16 |

**Table 3** Phase II studies of amrubicin in previously untreated advanced NSCLC: toxicities

| Toxicity | No. of patients | Frequency (%) | |
|---|---|---|---|
| | | >Gr. 1 | ≥Gr. 3 |
| Anemia | 61 | 78.7 | 23.0 |
| Leukopenia | 61 | 91.8 | 52.5 |
| Neutoropenia | 61 | 96.7 | 72.1 |
| Thrombocyopenia | 61 | 44.3 | 14.8 |
| Anorexia | 61 | 70.5 | 4.9 |
| Nausea/vomiting | 61 | 57.4 | 4.9 |
| Diarrhea | 61 | 9.8 | 0 |
| Alopecia | 60 | 71.7 | 1.7 |

**Table 4** Phase II study of amrubicin in previously untreated patients with lung cancer

| Study | No. of eligible patients | Response | MST | 1-yr survival | 2-yr survival |
|---|---|---|---|---|---|
| NSCLC | 61 | 27.9% | 11.3 months | 47.7% | 26.5% |
| ED-SCLC | 33 | 75.8% | 11.7 months | 48.5% | 20.2% |

*NSCLC*, non-small cell lung cancer
*ED-SCLC*, extensive disease-small cell lung cancer

The response rate was 87.8%, with a CR rate of 9.8%, and the MST and 1-year survival rate were reported to be 13.6 months and 56.1%, respectively [13]. With respect to the treatment status, 78% of the subjects were able to undergo 4 or more courses as scheduled, but there were nine subjects (22%) in whom treatment had to be terminated because no effect was seen in two patients and adverse events (gastric ulcer, neutropenia, thrombocytopenia, febrile neutropenia, hyponatremia, etc) occurred in seven patients. The dosage had to be decreased during treatment in 39 (23%) of the total 178 cycles. Almost all of the decreases involved a reduction in the dosage of amrubicin, to 30 mg/m$^2$/day in 12 (7%) of these cycles. Adverse events were higher than grade 3 leukopenia (65.9%), neutropenia (95.1%), thrombocytopenia (24.4%), and anemia (51.2%). Higher than grade 3 non-hematologic adverse events were anorexia (31.7%), nausea (19.5%), constipation (7.3%), vomiting (4.9%), and diarrhea (4.9%).

A recent Japanese study (Japan Clinical Oncology Group: JCOG 9511) comparing the combination of cisplatin and irinotecan hydrochloride (CPT-11) with the combination of cisplatin and etoposide in the treatment of ED-SCLC showed a significant advantage in overall survival favoring the combination of cisplatin/CPT-11 [14]. As the results obtained in this phase I/II study of the combination of cisplatin and amrubicin may be equal to or better than the results of cisplatin/CPT-11 combination therapy, JCOG is planning a randomized phase III study to compare the combinations of cisplatin/amrubicin and cisplatin/CPT-11 therapy for previously untreated ED-SCLC.

## Relapsed SCLC

While amrubicin monotherapy was highly effective for previously untreated SCLC, no study had been conducted to evaluate the efficacy in the treatment of relapsed SCLC. As such, a phase II study was conducted in patients with relapsed disease who had previously received one or two regimens including at least one regimen of platinum-based chemotherapy [15]. Sixty patients were enrolled in this multicenter study, comprising 44 sensitive cases in which CR or PR was observed with the previous chemotherapy and the disease was then shown to have progressed or relapsed at least 60 days after the final dosing in the previous chemotherapy, and 16 refractory cases in which the disease progressed within 60 days after the final dosing in the previous chemotherapy. In consideration of bone marrow exhaustion associated with the previous therapy, four or more courses of administration at the 40 mg/m$^2$ level for three consecutive days were repeated at 3-week intervals.

The response rate was 52% (95% CI: 38–65%). The progression-free survival, overall survival, and 1-year survival rate were 3.9 months, 11.2 months, and 44.1%, respectively. In sensitive cases, the response rate was 52% (95% CI: 37–67%), and the progression-free survival, overall survival, and 1-year survival rate were 4.2 months, 11.6 months, and 45.5%, respectively. In refractory cases, the response rate was 50% (95% CI: 25–75%), and the progression-free survival, overall survival, and 1-year survival rate were 2.6 months, 10.3 months, and 40.3%, respectively (Table 5) [15]. Common adverse events were hematologic toxicities, including grade 3–4 neutropenia (83.3%), leucopenia (70.0%), anemia (33.3%), thrombocytopenia (20.0%), and febrile neutropenia (5%). Non-hematologic adverse events included grade 3–4 anorexia (15%) and asthenia (15%) [15].

Based on the results of this study, the efficacy of monotherapy for relapsed SCLC was compared in the response rate. In sensitive cases, the response rate was highest 52% (23/44) with amrubicin, followed by 28% (18/63), 19% (9/47), 18% (30/168), and 17% (7/41) with irinotecan, docetaxel, topotecan, and vinorelbine, respectively: a promising result for amrubicin. In refractory cases, the response rate was highest 50% (8/16) with amrubicin, followed by 29% (7/24), 14% (5/38), 8% (6/75), 3% (1/28), and 0% (0/8) with paclitaxel, gemcitabine, topotecan, irinotecan, and vinorelbine, respectively (Table 6) [16]. The survival variables were compared with the results from a past study of topotecan [17]. The CR rate, PR rate, progression-free survival, and overall survival were 2.3%, 50%, 4.2 months, and 11.6 months in the amrubicin group, versus 0%, 24.3%, 3.3 months, and 6.3 months in the topotecan group, respectively, showing a favorable result of amrubicin.

Amrubicin showed a comparable response rate in sensitive and refractory cases; however, as the present study involved only Japanese patients, it is desirable to conduct clinical studies overseas to confirm the efficacy.

**Table 5** Phase II study of amrubicin in relapsed case or refractory case with small lung cancer: Response

| | Sensitive case | Refractory case | Total |
|---|---|---|---|
| No. of patients | 44 | 16 | 60 |
| CR | 1 | 1 | 2 |
| PR | 22 | 7 | 29 |
| SD | 10 | 2 | 12 |
| PD | 11 | 6 | 17 |
| Response rate (95% CI) | 52% (37–68%) | 50% (25–75%) | 52% (38–65%) |
| Progression-free survival (95% CI) | 4.2 months (3.6–5.3) | 2.9 months (1.4–4.6) | 3.9 months (3.4–4.6) |
| Median survival time (95% CI) | 11.6 months (10.0–15.8) | 10.3 months (4.8–∞) | 11. months (10.0–13.2) |
| 1-yr survival (95% CI) | 45.5% (29.9–59.8) | 40.3% (15.1–64.6) | 44.1% (30.6–56.8) |

∞: a symbol of infinite

## Future directions

As noted above, little evidence has been published concerning the efficacy of amrubicin in the treatment of NSCLC or SCLC. Only amrubicin monotherapy has been investigated for NSCLC, and only combination therapy with cisplatin has been investigated for SCLC.

At present, platinum-based doublet chemotherapy is considered the standard treatment as 1st line chemotherapy for advanced NSCLC. Therefore, combination therapy with cisplatin in previously untreated patients with advanced NSCLC should be tested. Combination therapy with carboplatin, an analog of cisplatin that is often used instead of cisplatin because of its milder toxicity profile, should also be evaluated. However, in combination with carboplatin, it is necessary to note that hematologic toxicities overlap, and therefore studies should start from phase I to determine a recommended dosage. Combination therapies with paclitaxel, docetaxel, gemcitabine, vinorelbine, and CPT-11, novel anticancer agents that became available in the 1990s, should also be topics of investigation as non-platinum regimens. However, it is already known that anthracycline anticancer agents and taxane agents interact: for example, in combination therapy using paclitaxel plus doxorubicin, it has been reported that if paclitaxel is administered first, not only do the pharmacokinetics of doxorubicin change, but its toxicity is increased [18]. Because amrubicin is also an anthracycline agent, any investigation of combination therapy with a taxane agent in particular should involve a pharmacokinetics study. Recently, Masuda et al. conducted a combination phase I study of CPT-11 and amrubicin, which led to a recommended dosage of 60 mg/m$^2$ of CPT-11 on days 1 and 8, and 25 mg/m$^2$ of amrubicin, days 1–3 every 3 weeks, the lowest dosage levels that had been tested in their study because of adverse events, including strong myelotoxicity [19]. Regardless of whether or not it is combined with a platinum drug, it is necessary to clarify whether amrubicin can become a viable first line chemotherapy candidate for advanced NSCLC in the future.

**Table 6** Responses of the "3rd generation drug" in sensitive relapse and refractory disease[a]

| | Responders/evaluable | |
|---|---|---|
| | Sensitive relapse | Refractory disease |
| Topotecan | 18% (30/168) | 8% (6/75) |
| Irinotecan | 28% (18/63) | 3% (1/28) |
| Docetaxel | 19% (9/47) | |
| Paclitaxel | | 29% (7/24) |
| Gemcitabine | | 14% (5/38) |
| Vinorelbine | 17% (7/41) | 0% (0/8) |
| Amrubicin | 52% (23/44) | 50% (8/16) |

[a] Glisson BS, Semin Oncol 30: 72–78, 2003

The second line treatment of NSCLC and 1st line treatment in elderly patients are in categories for which single-agent chemotherapy should be the recommended option. It is necessary to test amrubicin for these categories. To date, amrubicin has been approved and licensed for 3-day administration, but a phase I clinical study of this administration method has only been conducted in previously untreated patients, and there is still a problem concerning whether the recommended dosage of 45 mg/m$^2$/day is tolerable in previously treated patients, especially in light of its strong myelotoxicity. On this point, Okamoto et al. recently conducted a phase I study of amrubicin in previously treated patients with lung cancer, and reported a recommended phase II dosage of amrubicin at 35 mg/m$^2$/day for three consecutive days every 3 weeks [20].

For ED-SCLC, based on the good results obtained from combination therapy with cisplatin, a randomized phase III study should be carried out involving a comparison with cisplatin–CPT-11 combination therapy. Other anticancer drugs that should be investigated for combination therapy include carboplatin, as well as the topoisomerase I inhibitors CPT-11 and topotecan, which have recently been playing major roles in the treatment of SCLC. Because no standard treatment has yet been established for SCLC that

recurs after the initial treatment, a possible target of amrubicin monotherapy is previously treated SCLC.

The clinical studies suggested above should be conducted for both NSCLC and SCLC; however, because amrubicin is strongly myelotoxic, special consideration should be taken if these drugs are used in combination. From this viewpoint, it is also important to examine the pharmacokinetic profile of amrubicin. There is only one report by Matsunaga et al. regarding the pharmacokinetics of amrubicin and its active metabolite amrubicinol in patients with lung cancer [21]. In this report, it was suggested that the area-under-the time curves of amrubicin and amrubicinol seemed to be associated with the hematologic toxicities, and interestingly interpatient variability in the enzymatic conversion of amrubicin to amrubicinol was small whereas a large interpatient variability in the clearance of amrubicin was observed [21].

## Conclusion

Clinical studies of the novel anticancer agent amrubicin have only begun, and we as yet have little evidence to evaluate. However, there are high expectations for this agent in the trial to improve outcome for both NSCLC and SCLC patients. Many issues remain to be resolved, such as how to position this drug in the actual treatment of lung cancer. In order to resolve this and other issues in the future, many high-quality clinical studies are needed.

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# Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor

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**Cetuximab (Erbitux, IMC-C225) is a monoclonal antibody targeted to the epidermal growth factor receptor (EGFR). To clarify the mode of antitumor action of cetuximab, we examined antibody-dependent cellular cytotoxicity (ADCC) activity against several tumor cell lines expressing wild-type or mutant EGFR. ADCC activity and complement-dependent cytolysis activity were analyzed using the CytoTox 96 assay. ADCC activities correlated with the EGFR expression value ($R = 0.924$). ADCC activities were detected against all tumor cell lines, except K562 cells in a manner dependent on the cellular EGFR expression level, whereas complement-dependent cytolysis activity was not detected in any of the cell lines. The ADCC activity mediated by cetuximab was examined in HEK293 cells transfected with wild-type EGFR (293W) and a deletional mutant of EGFR (293D) in comparison with the mock transfectant (293M). ADCC activity was detected in 293W and 293D cells, in a cetuximab dose-dependent manner, but not in 293M cells (<10%). These results indicate that ADCC-dependent antitumor activity results from the degree of affinity of cetuximab for the extracellular domain of EGFR, independent of EGFR mutation status. These results suggest ADCC activity to be one of the modes of therapeutic action of cetuximab and to depend on EGFR expression on the tumor cell surface. (*Cancer Sci* 2007)**

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors that is abnormally activated in many malignancies. EGFR is frequently overexpressed or abnormally activated in tumors. EGFR overexpression correlates with a worse outcome.[1,2] Early studies with anti-EGFR monoclonal antibodies (mAb) were shown to inhibit the growth of cancer cells bearing EGFR.[3]

Cetuximab (IMC-225, Erbitux) is a recombinant, human–murine chimeric mAb that is produced in mammalian (murine myeloma) cell culture and targeted specifically to EGFR. Cetuximab is composed of a murine Fv (EGFR-binding) lesion and a human IgG1 heavy and κ light chain Fc (constant) region. *In vitro* studies have shown that cetuximab competes with endogenous ligands to bind with the external domain of EGFR. Cetuximab binds to EGFR with 10-fold higher affinity than endogenous ligands (0.1–0.2 nM cetuximab *vs* 1 nM epidermal growth factor [EGF] or transforming growth factor (TGF)-α, respectively).[4] Cetuximab has shown promising preclinical and clinical activity in a variety of tumor types.[5]

The anti-tumor strategy is to direct mAb to the ligand-binding extracellular domain and to prevent ligand binding and ligand-dependent receptor inhibition. The use of humanized murine–human chimeric mAb of the IgG1 subtype is now well established for the treatment of human cancers. Treatment of advanced breast cancer with human epidermal growth factor receptor type 2 (HER-2)-specific trastuzumab (Herceptin) and of follicular non-Hodgkin B-cell lymphoma with CD20-specific rituximab (Mabthera, Rituxan) has been shown to increase overall survival. Human IgG1 is thought to eliminate tumor cells via complement-dependent cytolysis (CDC) and antibody-dependent cellular cytotoxicity (ADCC), depending on the target, and also by direct pro-apoptotic signaling or growth factor receptor antagonism. Clynes *et al.* suggested that ADCC is a major *in vivo* mechanism of IgG1 action.[6] Recently, several mAb, including trastuzumab, which act predominantly via ADCC and CDC have been approved for the treatment of cancer patients. These include chimeric IgG1 mAb rituximab binding to the B-cell differentiation antigen CD20 for the treatment of B-cell lymphomas,[7] humanized IgG1 mAb trastuzumab targeting HER-2 overexpressed in a subgroup of breast cancers,[8] and humanized IgG1 alemtuzumab (Campath) targeting the differentiation antigen CD52 for the treatment of B-cell chronic lymphocytic leukemia.[9]

We hypothesized that ADCC is a possible mode of action of cetuximab against EGFR-expressing tumors. The present study was designed to clarify the role of cetuximab in ADCC and CDC activity, and to evaluate the relationship between EGFR expression status and cetuximab-mediated ADCC and CDC activity.

## Methods

**Cell lines and cultures.** A human leukemia cell line (K562), a non-small cell lung cancer (NSCLC) cell line (A549) and a human embryonic kidney cell line (HEK293) were obtained from the American Type Culture Collection (Manassas, VA, USA). Human NSCLC cell lines A431, PC-9 and PC-14 were obtained from Tokyo Medical University (Tokyo, Japan). Human NSCLC cell lines Ma-1 and 11_18 were obtained from the National Cancer Center Research Institute (Tokyo, Japan). PC-9 and Ma-1 are known to contain E746_A750del, and 11_18 is known to contain L858R in tyrosine kinase domains of EGFR. The other cell lines are known to have wild-type EGFR. K562, HEK293, A431, PC-9, PC-14, Ma-1 and 11_18 cells were cultured in RPMI-1640 (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibico BRL, Grand Island, NY, USA). A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 10% heat-inactivated FBS.

**Plasmid construction and transfection.** Construction of the mock expression plasmid vector (empty vector) and of the wild-type EGFR and 15-bp deletional EGFR (E746-A750del type deletion)

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vectors, both of which possess the same deletion site as that observed in PC-9 cells, have been described elsewhere.[10] The plasmids were transfected into HEK293 cells and the transfectants were selected with Zeosin (Sigma). The stable transfectants (pooled cultures) of the empty vector, wild-type EGFR and its deletion mutant were designated 293M, 293W and 293D cells, respectively.

**Compound.** The mAb anti-EGFR cetuximab (IMC-225, Erbitux) was kindly provided by Bristol Myers Squibb (New York, NY, USA).

**Analysis of EGFR expression on the cell surface.** Cell surface expression of EGFR in tumor cell lines was quantified using a flow cytometric system (BD LSR; Becton-Dickinson, San Jose, CA, USA). The binding of cetuximab to tumor cell lines was titrated using FACS analysis. Cetuximab and another anti-EGFR mAb (R-1, sc-101; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies. Then, $1 \times 10^6$ tumor cells were incubated with 1 μg/mL cetuximab in 1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min at room temperature. After the first reactions, the cell surface was stained with 10 μg/mL fluorescein-conjugated antihuman IgG (Vector, Burlingame, CA, USA) for 45 min at room temperature in the dark. After the second reactions, the tumor cells were resuspended in 1 mL PBS. For analysis using the anti-EGFR mAb, 1 μg EGFR mAb per $1 \times 10^6$ tumor cells was used as the primary antibody. The secondary antibody was 10 μg/mL fluorescein-conjugated antimouse IgG (Vector). A minimum of $2 \times 10^4$ cells were analyzed by flow cytometry. Control experiments were carried out in the absence of primary antibodies. Data were analyzed with CellQuest software and the modifying program (Beckton Dickinson, CA, USA). The magnitude of surface expression of these proteins was indicated by the mean fluorescence intensity (MFI) of positively stained cells. The expression values were calculated as follows:

$$\text{Expression value} = (\text{MFI of positively stained cells})/(\text{MFI of control cells}).$$

The correlation between the expression of R-1-combined EGFR and that of cetuximab-combined EGFR were calculated using simple regression analysis.

**Cytotoxicity assays.** ADCC and CDC were examined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). For quantification of ADCC activity, peripheral blood mononuclear cells were isolated from healthy volunteers with Lymphocyte Separation Medium (Cappel, Aurora, OH, USA) and used as effector cells. The target cells were suspended in RPMI medium without FBS and plated in a 96-well U-bottom microtiter plate at $5 \times 10^3$ cells/well. Cetuximab was added in triplicate to the individual wells at various concentrations from 0.001 to 10 μg/mL and effector cells were added at an effector : target cell ratio of 10:1. For quantification of CDC activity, human serum from a healthy volunteer was obtained as a compliment source. To yield a 1:3 final dilution, 50 μL serum was added. The plates were incubated for 4 h at 37°C, and the absorbance of the supernatants at 490 nm was recorded to determine the release of lactate dehydrogenase. The average of absorbance values for the culture medium background was subtracted from experimental release ($A$), target cell spontaneous release ($B$), effector cell spontaneous release ($C$) and target cell maximum release ($D$). The specific cytolysis percentage was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = (A - B - C)/(D - B) \times 100.$$

The correlation between the expression of cetuximab-combined EGFR and ADCC activity was calculated using a simple regression analysis.

**Growth-inhibition assay.** We used the 3-(4,5-dimethylthiazol-2-$y$)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the cytotoxicity of various drug concentrations. Two hundred microliters of an exponentially growing cell suspension was seeded in a 96-well microtiter plate, and cetuximab-containing solution was added at various concentrations (from 0.001 to 100 μg/mL). Each experiment was carried out in triplicate for each drug concentration and independently three times.

**Growth inhibitory assay for the combination of gefitinib and cetuximab-mediated ADCC in the PC-9 cell line.** We analyzed the growth inhibitory effect of the combination of gefitinib and cetuximab-mediated ADCC in the PC-9 cell line using the MTT assay. Two hundred microliters containing 1000 PC-9 cells, and various concentrations of gefitinib, were seeded in a 96-well microtiter plate. Then, 10 μL of cetuximab-containing solutions of various concentrations (from 0.1 to 10 μg/mL) and 20 000 effector cells were added.

**Western blotting.** PC-9, PC-14 and A549 cell lines were seeded in cell culture plates at a density of $6.0 \times 10^5$ cells/plate and allowed to grow overnight in appropriate maintenance cell culture media for each cell line containing 10% heat-inactivated FBS. The media were then replaced with RPMI-1640 (Sigma) (PC-9 and PC-14) or DMEM without FBS, with or without cetuximab (10 and 100 μg/mL). The cells were incubated for a further 24 h and stimulated or not stimulated with EGF (100 ng/mL) under serum starvation conditions. Cells were washed with ice-cold PBS and scraped immediately after adding 50 μL of M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA). The protein extracts were separated by electrophoresis on 7.5% sodium dodecylsulfate–polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. The membranes were probed with a mouse monoclonal antibody against EGFR (Transduction Laboratory, San Diego, CA, USA), and phosphor-EGFR (specific for Tyr1068), Akt, phosphor-Akt, p44/42 MAPK and phosphor-p44/42 MAPK antibodies (Cell Signaling Technology, Beverly, MA, USA) as primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. The bands were visualized with an electrochemiluminescence reagent (ECL; Amersham, Piscataway, NJ, USA).

## Results

**Binding properties of cetuximab to tumor cell lines expressing EGFR.** The A431 cells expressed a high level of EGFR on their surfaces. Cell surface EGFR expression values of the PC-9, PC-14, A549, Ma-1 and 11_18 cell lines were lower than that of A431. The MFI for the K562 cells was less than 10 (Table 1). A good

**Table 1. Epidermal growth factor receptor (EGFR) expression values and antibody-dependent cellular cytotoxicity (ADCC) activity**

| Cell line | EGFR expression (R-1) | EGFR expression (cetuximab) | ADCC (%) |
|---|---|---|---|
| A431 | 286.2 ± 13.7 | 318.9 ± 98.2 | 30.7 |
| PC-9 | 9.7 ± 6.2 | 20.1 ± 10.2 | 20.1 |
| PC-14 | 17.6 ± 1.5 | 42.2 ± 8.6 | 26.8 |
| A549 | 9.1 ± 1.9 | 19.1 ± 6.2 | 24.2 |
| Ma-1 | 13.8 ± 1.4 | 27.5 ± 2.9 | 22.3 |
| 11_18 | 6.1 ± 0.6 | 12.6 ± 1.1 | 15.5 |
| K562 | 1.1 ± 0.4 | 2.8 ± 1.6 | 7.0 |
| 293M | 3.7 ± 1.6 | 8.6 ± 3.2 | 8.2 |
| 293W | 40.19 ± 6.2 | 39.73 ± 6.2 | 16.3 |
| 293D | 55.21 ± 21.9 | 53.04 ± 8.2 | 18.9 |

Expression values and ADCC activity were calculated as described in the Materials and Methods section. The mean of expression values from three different experiments and standard deviations are shown. The values for cetuximab-combined EGFR expression are shown for a concentration of 1 μg/mL.



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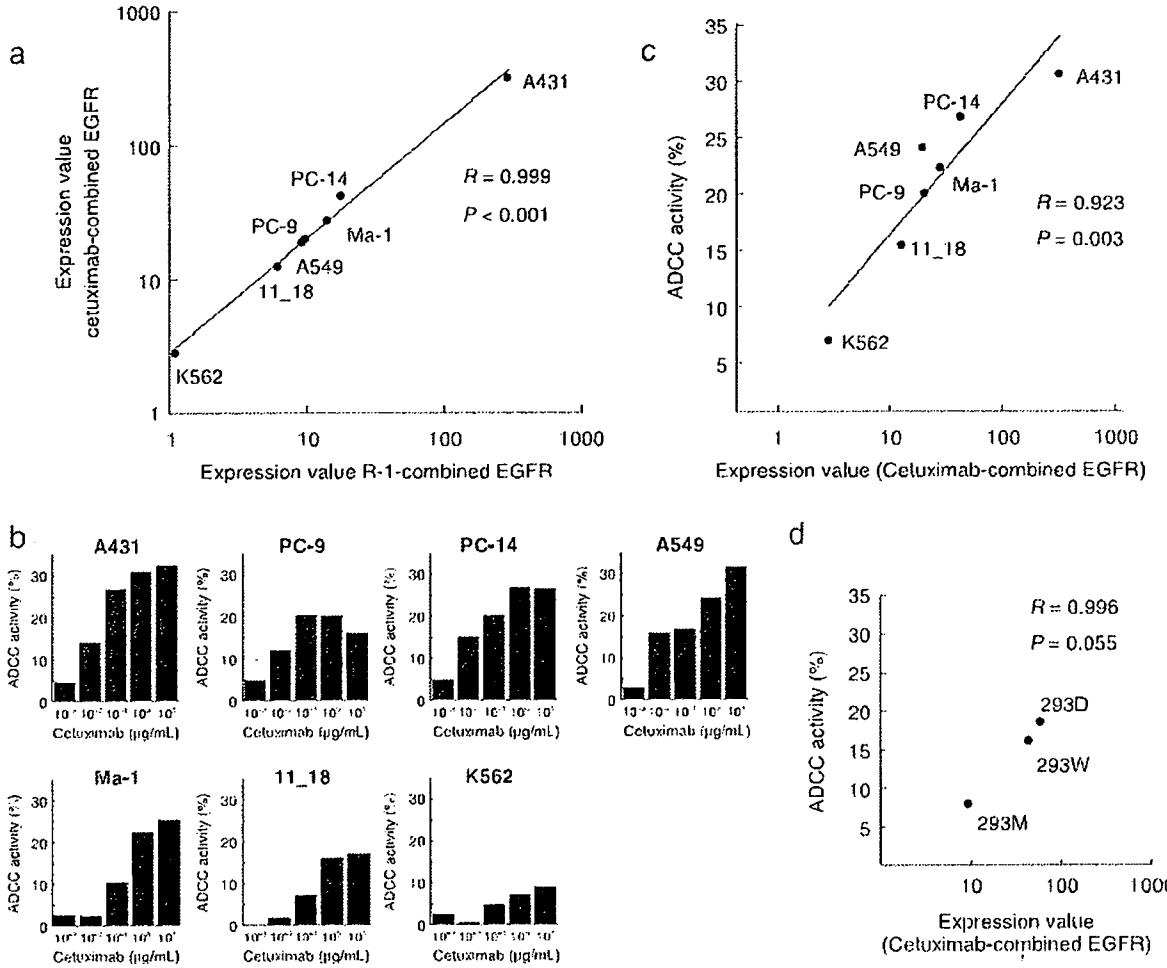


**Fig. 1.** Epidermal growth factor receptor (EGFR) expression and cetuximab-mediated antibody-dependent cellular cytotoxicity (ADCC) activity in the tumor cell lines. (a) Correlation between the expression of cetuximab-combined EGFR and R-1-combined EGFR. The values for cetuximab-combined EGFR expression are shown for a concentration of 1 μg/mL. The correlation coefficient between the results of these assays was 0.999. (b) Cetuximab-mediated ADCC activity in tumor cell lines at concentrations ranging from 0.001 to 10 μg/mL was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay. (c) Correlation between expression values of cetuximab-combined EGFR and ADCC activity in the seven tumor cell lines. The values for cetuximab-combined EGFR expression and cetuximab-mediated ADCC activity are shown for a concentration of 1 μg/mL. The correlation coefficient between the results of these assays was 0.924. (d) Correlation between expression values of cetuximab-combined EGFR and ADCC activity in transfected HEK293 cell lines. The correlation coefficient between the results of these assays was 0.952.

correlation was observed between the binding of cetuximab and R-1 antibody with a correlation coefficient of 0.999 ($P < 0.001$; Fig. 1a).

**ADCC and CDC activities in tumor cell lines.** ADCC activities of cetuximab were detected in all tumor cell lines except K562 (Table 1; Fig. 1b). In the K562 cells, % ADCC activities were lower than 10% at all concentrations of cetuximab examined (from 0.001 to 10 μg/mL). ADCC activity mediated by cetuximab was highly correlated with the binding values of cetuximab to cells expressing EGFR ($R = 0.924$, $P = 0.003$; Fig. 1c). CDC activity was not detected in any of the cell lines in the cetuximab concentration range from 0.001 to 10 μg/mL.

**Direct growth inhibitory effect of cetuximab on tumor cell lines.** Cetuximab showed no growth inhibitory effect in any of the cell lines examined, regardless of EGFR expression levels. Even the highest concentration of cetuximab (100 μg/mL) did not inhibit growth in any of the cell lines (Fig. 2).

**ADCC activities of cetuximab against the cells transfected with wild-type and mutant EGFR.** EGFR expression was detected in 293W and 293D cells, but not in 293M cells (Table 1). The ADCC activity mediated by cetuximab in 293W and 293D cells was dose dependent. In contrast, ADCC activities in 293M cells were <10% at all concentrations of cetuximab tested (0.001–10 μg/mL). There was a good correlation between the ADCC activities and the levels of cetuximab binding to EGFR in the cells ($R = 0.996$, $P = 0.055$; Fig. 1d). These results indicate that ADCC depends on the level of cetuximab binding to EGFR, but not the mutation status of the EGFR tyrosine kinase domains.

**Direct growth inhibitory effect of the combination of gefitinib and cetuximab-mediated ADCC in the PC-9 cell line.** The growth inhibitory effect in the PC-9 cell line was shown by effector cells at a gefitinib exposure exceeding 0.01 μM and was concentration dependent (Fig. 3). When cetuximab was added, growth was inhibited in a cetuximab concentration-dependent manner. An additive growth inhibitory effect was recognized between 0 and 0.01 μM of gefitinib. This additive growth inhibitory effect could not be evaluated at concentrations between 0.1 and 1.0 μM because of the strong inhibitory effect of gefitinib alone.

**Effect of cetuximab on phosphorylation of EGFR and its downstream signaling molecules in NSCLC cells.** Phosphorylation of EGFR

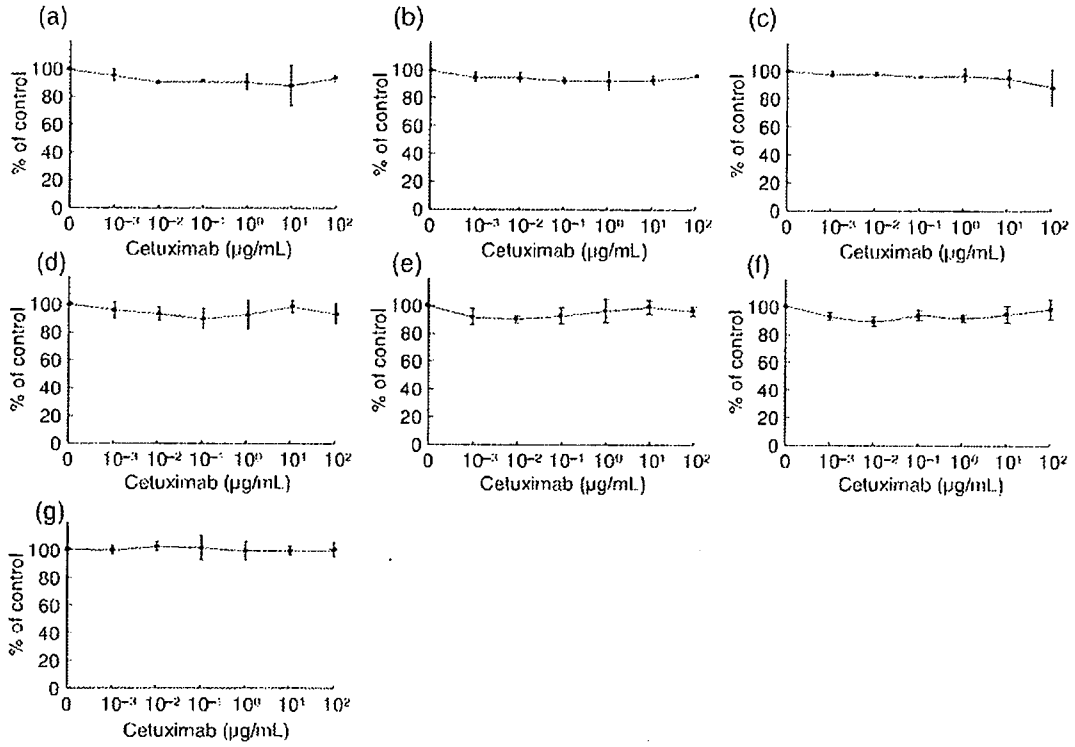


**Fig. 2.** Growth inhibitory effect of cetuximab on non-small cell lung cancer cell lines: (a) A431; (b) PC-9; (c) PC-14; (d) A549; (e) Ma-1; (f) 11_18; and (g) K562. Cell growth was not inhibited at any concentration, even a high concentration (10 μg/mL). The figure shows the dose-dependent growth inhibitory effect of gefitinib with various concentrations of cetuximab (0–10 μg/mL). Results are expressed as percentages of the untreated control value. The data shown are the mean + SD values from triplicate experiments.

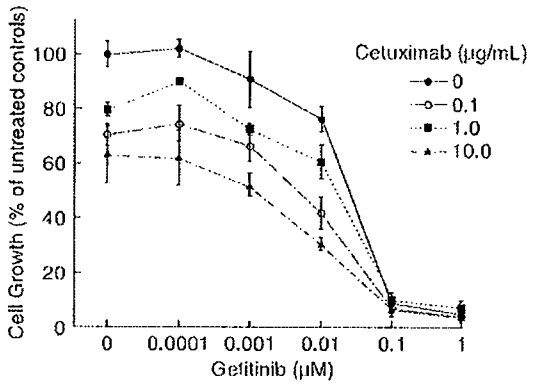


**Fig. 3.** Growth inhibitory effects of combining gefitinib and cetuximab-mediated antibody-dependent cellular cytotoxicity (ADCC). The figure shows dose-dependent growth inhibitory effects of gefitinib with various concentrations of cetuximab (solid circle, 0 μg/mL; solid square, 0.1 μg/mL; open circle, 1.0 μg/mL; solid triangle, 10 μg/mL). Results are expressed as a percentage of the untreated control value. The data shown represent the median values of triplicate experiments.

was strongly expressed in PC-9 regardless of EGF treatment, and the phosphorylation of EGFR continued the strong expression during cetuximab treatment. Phosphorylation of EGFR was slightly expressed in PC-14 and A549 without EGF treatment, but the phosphorylation of EGFR was enhanced by the EGF treatment. Although the enhancement of phosphorylation was inhibited dose dependently by cetuximab, the phosphorylation was not completely inhibited at the highest concentration (10 μg/mL) of cetuximab. Phosphorylation of 44/42 MAPK and Akt was increased in all cell lines compared with the absence of EGF treatment. Although the increase in phosphorylation was diminished by adding cetuximab, phosphorylation was not completely inhibited at the highest concentration (10 μg/mL) of cetuximab (Fig. 4).

## Discussion

Antibody therapies are a major approach in the treatment of various cancer types. Herein, we focused on the ADCC activity mediated by cetuximab against human lung cancer cells expressing wild-type or mutant EGFR. Neither CDC nor direct growth inhibition mediated by cetuximab was detectable in our experiments.

Direct growth inhibition, ADCC and CDC mediated by antibodies are the modes of action of antibody therapies. We previously demonstrated that ADCC is the major mode of action of trastuzumab in breast cancer cell lines, even when used in combination with cisplatin.[11] Cisplatin did not affect ADCC activity at the concentration for combined use *in vitro*. Clinical efficacies of cetuximab for various types of cancers have been demonstrated in many clinical studies using combinations with cytotoxic agents including cisplatin. Thus, ADCC is considered to be an important factor governing the efficacy of cetuximab.

Mukohara *et al.* reported that EGFR mutations in NSCLC cells are not associated with sensitivity to cetuximab *in vitro*.[12] They focused on the direct growth inhibitory effect of cetuximab against lung cancer cells. We previously demonstrated that PC-9 and 293 cells transfected with E746_A750del EGFR are



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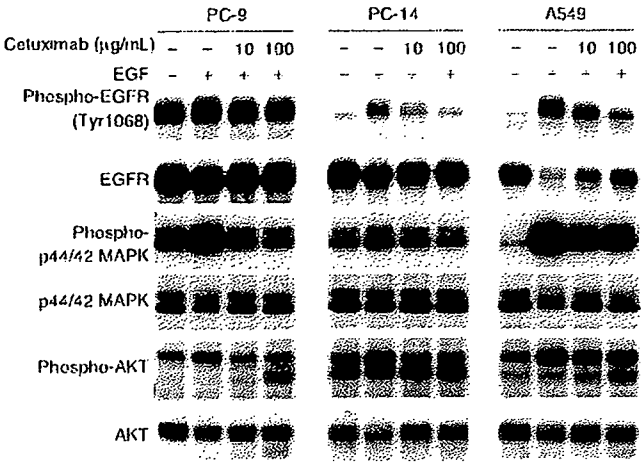


**Fig. 4.** Effects of cetuximab on phosphorylation of epidermal growth factor receptor (EGFR), Akt and p44/42 MAPK in non-small cell lung cancer cell lines. (a) EGFR mutant cell line PC-9 (with the E746_A750del mutation). (b) EGFR wild-type cell line PC-14. (c) EGFR wild-type cell line A549. Cells were treated with cetuximab at the indicated concentrations for 24 h. Immunoblots of cellular protein were analyzed for phosphorylated and total EGFR, p44/42 MAPK and Akt. The experiments were repeated at least twice.

hypersensitive to EGFR-tyrosine kinase inhibitors.[10] In contrast, we have demonstrated that ADCC activity mediated by cetuximab is not affected by EGFR mutation status in lung cancer cells or in 293 cells transfected with EGFR. Taken together, these observations indicate that cetuximab exerts its antitumor effects against human lung cancer cells independently of EGFR mutation status.

ADCC activity mediated by cetuximab has been demonstrated against 293 cells transfected with wild-type and mutated EGFR. Higher ADCC activity against 293D cells compared with 293W cells was observed with cetuximab exposure (Fig. 1d; Table 1). However, ADCC was correlated with EGFR expression levels in these transfectants. The activity appears to depend on expression levels but not mutation status.

Approximately 30 mutations of EGFR have been reported in lung cancer.[13–16] ADCC activity against PC-9 cells with E746_A750del in exon 19, one of the common mutations, has been demonstrated herein. We also examined ADCC activity against another human lung cancer cell line, 11_18,[17] with L858R in exon 21, which is another common mutation. Our results showed a strong positive correlation between ADCC activity and EGFR expression level, and that the impact on ADCC activity did not depend on the site of EGFR mutations.

Cetuximab is a chimeric antibody against the extracellular domain of EGFR. Other antitumor anti-EGFR antibodies currently under investigation clinically include humanized antibodies.[18] It remains unknown whether humanized and chimeric antibodies exert ADCC activity against lung cancer differentially, and this awaits future investigation.

Some investigators have reported on the predictive factor and enhancement of ADCC activity mediated by certain mAb other than cetuximab.[19–22] Important ADCC-mediating effector cells that express receptors against the Fc region of IgG include monocytes and macrophages (FcγRI, IIa and IIb), granulocytes (FcgRII) and natural killer cells (FcγRIII).[19] One group of researchers demonstrated single nucleotide polymorphisms of FcγRIII in individual patients correlating with rituximab-dependent ADCC activity and the clinical response to rituximab.[20] Carson *et al.* demonstrated that the natural killer cell-mediated ADCC activity of breast cancer cell lines expressing HER2/*neu*, in the presence of trastuzumab, was markedly enhanced following stimulation with interleukin 2 and proposed the concurrent use of trastuzumab and interleukin-2 therapy in patients with cancers expressing HER2/*neu*.[21] However, from the view point of mAb but not effector cells, lack of fucosylation of the antibodies affects ADCC enhancement.[22] Whether or not these factors enhance cetuximab-mediated ADCC activity warrants further examination.

We showed additional growth inhibition by gefitinib and cetuximab in PC-9 cells. PC-9 cells had a deletional mutation in exon 19 of *EGFR* and hyper-responsiveness to gefitinib. We think that cetuximab-mediated ADCC increased the growth inhibition-independent response to gefitinib. The ADCC activity could not be evaluated at higher concentrations of gefitinib (>0.1 μM) because PC-9 cells were sufficiently inhibited at the higher concentrations. Additionally, we showed that some phosphorylations downstream of EGFR in NSCLC cell lines were mediated by cetuximab, although cetuximab had no growth inhibitory effect on the cell lines. We think that cetuximab-combined EGFR inhibits binding of EGFR and its ligands, such as EGF, and that phosphorylation downstream of EGFR is inhibited as a consequence of the addition of cetuximab. We have shown that phosphorylation of 44/42 MAPK and Akt in NSCLC cell lines was increased by EGF treatment and decreased by then adding cetuximab. Phosphorylation of EGFR in PC-14 and A549 cells was decreased with the addition of cetuximab, as in the 44/42 MAPK and Akt cell lines. Phosphorylation of EGFR in PC-9 cells was strongly increased without ligands under serum starvation conditions and was not decreased by cetuximab. Phosphorylation that was independent of ligand binding to EGFR seem not to be controlled by cetuximab.

These results conclude that cetuximab has ADCC activity against tumor cells with EGFR expression, and ADCC activity depends on the degree of EGFR expression on tumor cell surfaces, additionally leading us to believe that cetuximab treatment has clinical activity in EGFR-expressing tumor cells via cetuximab-mediated ADCC.

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# AZD2171 Shows Potent Antitumor Activity Against Gastric Cancer Over-Expressing Fibroblast Growth Factor Receptor 2/Keratinocyte Growth Factor Receptor

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**Abstract**

**Purpose:** AZD2171 is an oral, highly potent, and selective vascular endothelial growth factor signaling inhibitor that inhibits all vascular endothelial growth factor receptor tyrosine kinases. The purpose of this study was to investigate the activity of AZD2171 in gastric cancer.

**Experimental Design:** We examined the antitumor effect of AZD2171 on the eight gastric cancer cell lines *in vitro* and *in vivo*.

**Results:** AZD2171 directly inhibited the growth of two gastric cancer cell lines (KATO-III and OCUM2M), with an $IC_{50}$ of 0.15 and 0.37 μmol/L, respectively, more potently than the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib. Reverse transcription-PCR experiments and immunoblotting revealed that sensitive cell lines dominantly expressed COOH terminus–truncated fibroblast growth factor receptor 2 (FGFR2) splicing variants that were constitutively phosphorylated and spontaneously dimerized. AZD2171 completely inhibited the phosphorylation of FGFR2 and downstream signaling proteins (FRS2, AKT, and mitogen-activated protein kinase) in sensitive cell lines at a 10-fold lower concentration (0.1 μmol/L) than in the other cell lines. An *in vitro* kinase assay showed that AZD2171 inhibited kinase activity of immunoprecipitated FGFR2 with submicromolar $K_i$ values (~0.05 μmol/L). Finally, we assessed the antitumor activity of AZD2171 in human gastric tumor xenograft models in mice. Oral administration of AZD2171 (1.5 or 6 mg/kg/d) significantly and dose-dependently inhibited tumor growth in mice bearing KATO-III and OCUM2M tumor xenografts.

**Conclusions:** AZD2171 exerted potent antitumor activity against gastric cancer xenografts over-expressing FGFR2. The results of these preclinical studies indicate that AZD2171 may provide clinical benefit in patients with certain types of gastric cancer.

Various anticancer therapies for gastric cancer have been investigated over the past two decades. Despite intensive studies, the prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor (1, 2), and new therapeutic modalities are needed.

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Fibroblast growth factors (FGF) and their signaling receptors have been found to be associated with multiple biological activities, including proliferation, differentiation, motility, and transforming activities (3–5). The *K-sam* gene was first identified as an amplified gene in human gastric cancer cell line KATO-III (6, 7), and its product was later found to be identical to the bacteria-expressed kinase, or keratinocyte growth factor receptor (KGFR), and FGF receptor 2 (FGFR2). FGFR2/KGFR/K-sam is preferentially amplified in poorly differentiated types of gastric cancers with a malignant phenotype, and its protein expression was detected by immunohistochemical staining from 20 of 38 cases of the undifferentiated type of advanced stomach cancer (8, 9). Thus, FGFR2 signaling may be as a promising molecular target for gastric cancer.

AZD2171 is a potent, ATP-competitive small molecule that inhibits all vascular endothelial growth factor receptors [VEGFR-1, VEGFR-2 (also known as KDR), and VEGFR-3]. *In vitro* studies have shown that recombinant VEGFR-2 tyrosine kinase activity was potently inhibited by AZD2171 ($IC_{50}$ <1 nmol/L; ref. 10). AZD2171 also showed potent activity versus VEGFR-1 and VEGFR-3 ($IC_{50}$, 5 and ≤3 nmol/L, respectively). VEGF-stimulated proliferation and VEGFR-2 phosphorylation of human umbilical vascular endothelial cells

was inhibited by AZD2171 ($IC_{50}$, 0.4 and 0.5 nmol/L, respectively). In *in vivo* studies, inhibition of VEGFR-2 signaling by AZD2171 reduced microvessel density and dose-dependently inhibited the growth of various human tumor xenografts (colon, lung, prostate, breast, and ovary; ref. 10). These data are consistent with potent inhibition of VEGF signaling, angiogenesis, neovascular survival, and tumor growth. On the other hand, because it was known that AZD2171 also possesses additional activity against FGFR1 ($IC_{50}$, 26 nmol/L; ref. 10), we hypothesized that AZD2171 may exhibit the additional anticancer activity against FGFR-overexpressing gastric cancer cells.

Our previous studies showed significant activities of the dual VEGFR-2 and epidermal growth factor receptor inhibitor ZD6474 against poorly differentiated gastric cancer (11) and non-small-cell lung cancer with epidermal growth factor receptor mutations (12, 13), both *in vitro* and *in vivo*. Based on these findings, we proceeded to investigate the anticancer activity of AZD2171 in preclinical models (gastric cell lines and xenografts).

## Materials and Methods

*Anticancer agents.* AZD2171 and gefitinib (Iressa) were provided by AstraZeneca. AZD2171 and gefitinib were dissolved in DMSO for the *in vitro* experiments, and AZD2171 was suspended in 1% (w/v) aqueous polysorbate 80 and administered in a dose of 0.1 mL/10 g per body weight in the *in vivo* experiments.

*Cell culture.* Human gastric cancer cell lines 44As3, 58As1, OKAJIMA, OCUM2M, KATO-III, MKN-1, MKN-28, and MKN-74 were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and penicillin-streptomycin.

*Established highly tumorigenic cell line.* Signet ring cell gastric carcinoma cell line KATO-III was gift from Dr. M. Sekiguchi (University of Tokyo, Tokyo, Japan). All of the presented *in vitro* experiments were done using the KATOIII cell line. We conducted a preliminary experiment to compare the cellular characteristics of TU-KATO-III cells and KATOIII cells, and the results revealed that a high expression level of FGFR2 and high sensitivity to AZD2171 were still maintained in the TU-KATO-III cells (data not shown). KATO-III did not show tumorigenicity following repeated implantation of the cultured cells into BALB/c nude mice. Following s.c. inoculation into nonobese diabetic/severe combined immunodeficient mice, 80% to 100% of the KATO-III cells caused the formation of tumor. Following this result, we cultured the cancer cells isolated from the tumor of mice that developed 2 to 3 months following the implantation of KATO-III cells and attempted s.c. injection into nude mice, in turn, of the incubated cells. This sequence of manipulations was repeated for seven cycles in an attempt to reliably isolate cell lines that would have higher potential to undergo tumor formation over short periods of time. In this way, we obtained a cell line (TU-kato-III) from KATO-III cells that possessed a high tumorigenic potential.

In vitro *growth inhibition assay.* The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate the growth-inhibitory effect of AZD2171. Cell suspensions (180 μL) were seeded into each well of 96-well microculture plate and incubated in 10% fetal bovine serum medium for 24 h. The cells were exposed to AZD2171 or gefitinib at concentrations ranging from 4 nmol/L to 80 μmol/L and cultured at 37°C in a humidified atmosphere for 72 h. After the culture period, 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added, and the plates were incubated for 4 h. After centrifugation, the culture medium was discarded, and wells were filled with DMSO. The absorbance of the cultures at 562 nmol/L was measured using Delta-soft on a Macintosh computer (Apple) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMatellics). This experiment was done in triplicate.

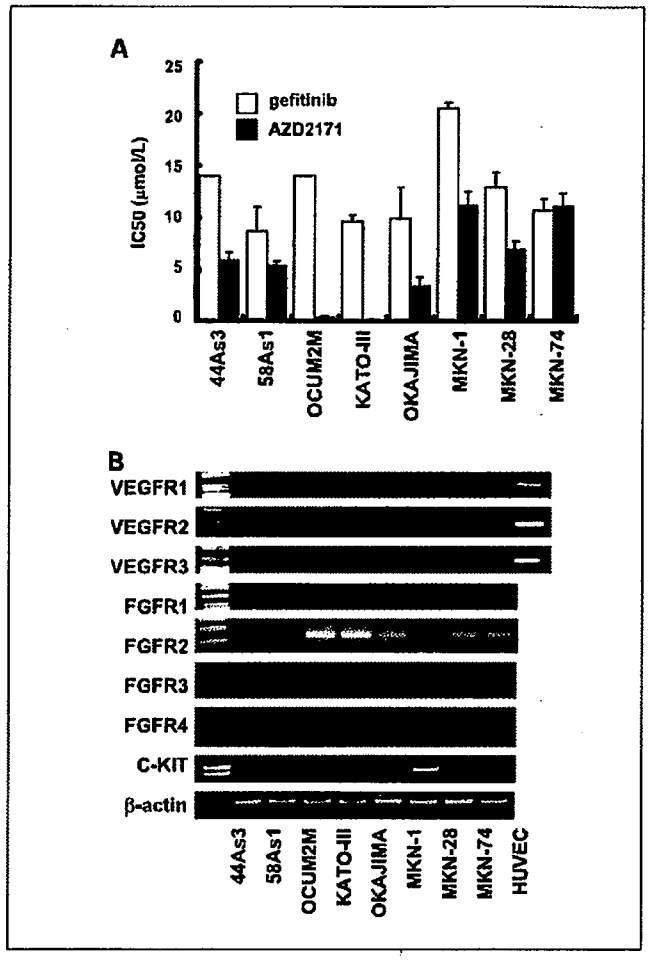


Fig. 1. *A, in vitro* growth-inhibitory effect of AZD2171 and gefitinib on eight gastric cancer cell lines. AZD2171 had a growth-inhibitory effect on KATO-III cells and OCUM2M cells ($IC_{50}$, 0.15 and 0.37 μmol/L, respectively). Columns, mean $IC_{50}$ of each compound from three independent experiments; bars, SD. □, $IC_{50}$ of gefitinib; ■, $IC_{50}$ of AZD2171. *B*, the mRNA expression levels of VEGFRs, FGFRs, and c-KIT in gastric cancer cell lines were determined by reverse transcription-PCR. Human umbilical vascular endothelial cells were used as the positive control for the VEGFRs. No mRNA expression of VEGFRs or c-KIT was detected by reverse transcription-PCR in both sensitive cell lines, but FGFR2 was strongly detected; however, little faint or none was detected in the other cell lines.

*Reverse-transcription PCR.* Using a GeneAmp RNA-PCR kit (Applied Biosystems), 5 μg of total RNA from each cultured cell line was converted to cDNA. The PCR amplification procedure consisted of 28 to 35 cycles (95°C for 45 s, 62°C for 45 s, and 72°C for 60 s) followed by incubation at 72°C for 7 min, and the bands were visualized by ethidium bromide staining. The following primers were used for the PCR: human-specific β-actin, forward 5-GGAAATCGTGCGTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGGACAG-3; VEGFR-1, forward 5-TAGCGTCACCAGCAGCGAAAGC-3 and reverse 5-CCTTTCTTTTGGGTCTCTGTGC-3; VEGFR-2, forward 5-CAGACGGACAGTGGTATGGTTC-3 and reverse 5-ACCTGCTGGTGGAAAGAACAAC-3; VEGFR-3, forward 5-AGCCATTCATCAACAAGCCT-3 and reverse 5-GGCAACAGCTGGATGTCATA-3; c-KIT, forward 5-GCCCACAATAGATTGGTATTT-3 and reverse 5-AGCATCTTTACAGCGACAGTC-3; FGFR1, forward 5-GGAGGATCGAGCTCACTCGTGG-3 and reverse

5-CGGAGAAGTAGGTGGTGTCAC-3; FGFR2, forward 5-CAGTAGGACTGTAGACAGTGAA-3 and reverse 5-CCGGTGAGGCGATCGCTCCACA-3; FGFR3, forward 5-GGTCAAGGATGGCACAGGGCTG-3 and reverse 5-AGCAGCTTCTTGTCCATCCGCT-3; and FGFR4, forward 5-CCGCCTAGAGATTGCCAGCTTC-3 and reverse 5-AGGCCTGTCCATCCTTAAGCCA-3.

*Real-time reverse transcription-PCR.* Real-time reverse transcription-PCR amplification was done by using a Premix Ex Taq and Smart Cycler system (Takara Bio, Inc.) according to the manufacturer's instructions. The following primers were used: FGFR2 (IIIb), forward 5-GATAAATAGTTCCAATGCAGAAGTGCT-3 and reverse 5-TGCCCTATATAATTGGAGACCTTACA-3 (7); FGFR2 (COOH-terminal), forward 5-GAATACTTGGACCTCAGCCAA-3 and reverse 5-AACACTGCCGTTTATGTGTGG-3; and human-specific β-actin, forward 5-GGAAATCGTGCGTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGGACAG-3. The experiment was independently done in triplicate using β-actin as a reference to normalize the data.

*Western blotting.* Cells were cultured overnight in 10% serum-containing medium or serum-starved medium and exposed to 0.1 to 10 μmol/L of AZD2171 for 3 h before addition of KGF (100 ng/mL) for 15 min. Immunoblotting was done as described previously (14). In brief, after lysing the cells in radioimmunoprecipitation buffer, the lysate was electrophoresed through 10% (w/v) polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes and reacted with the following antibodies: anti-FGFR2 (H-80) and anti-FGFR2 (C-17) antibody (Santa Cruz Biotechnology, Inc.); anti-phosphotyrosine antibody PY20 (BD Transduction Laboratories); anti-phosphorylated FGFR (Tyr653/654), anti-mitogen-activated protein kinase, anti-phosphorylated mitogen-activated protein kinase antibody, anti-AKT, anti-phosphorylated AKT, and anti-rabbit horseradish peroxidase-conjugated antibody (Cell Signaling Technology); and anti-β-actin antibody (Sigma). Visualization was achieved with an enhanced chemiluminescent detection reagent (Amersham Bioscience).

*FGFR2 kinase assay.* FGFR2/KGFR kinase activity was quantified by using a Universal Tyrosine Kinase Assay kit (Takara) according to manufacturer's instructions. FGFR2/KGFR proteins were collected from the KATO-III, OCUM2M, and OKAJIMA cell lysates by overnight immunoprecipitation with an anti-FGFR2 antibody. The FGFR2/KGFR immune complexes were washed thrice with radioimmunoprecipitation assay buffer and diluted kinase reaction buffer. Immobilized tyrosine kinase substrate (poly[Glu-Tyr]) was incubated for 30 min at 37°C with each sample in the presence of kinase-reacting solution and ATP. Samples were washed four times, blocked with blocking solution, and incubated with anti-phosphotyrosine antibody (PY20) conjugated to horseradish peroxidase. The absorbance of the phosphorylated substrate was measured at 450 nm.

*Chemical cross-link analysis.* The chemical cross-link analysis was carried out as described previously (15). In brief, KATO-III cells and OKAJIMA cells were cultured under serum-starved conditions for 24 h, and after stimulation with KGF (100 ng/mL) for 15 min, they were collected and washed with PBS and incubated for 30 min in PBS

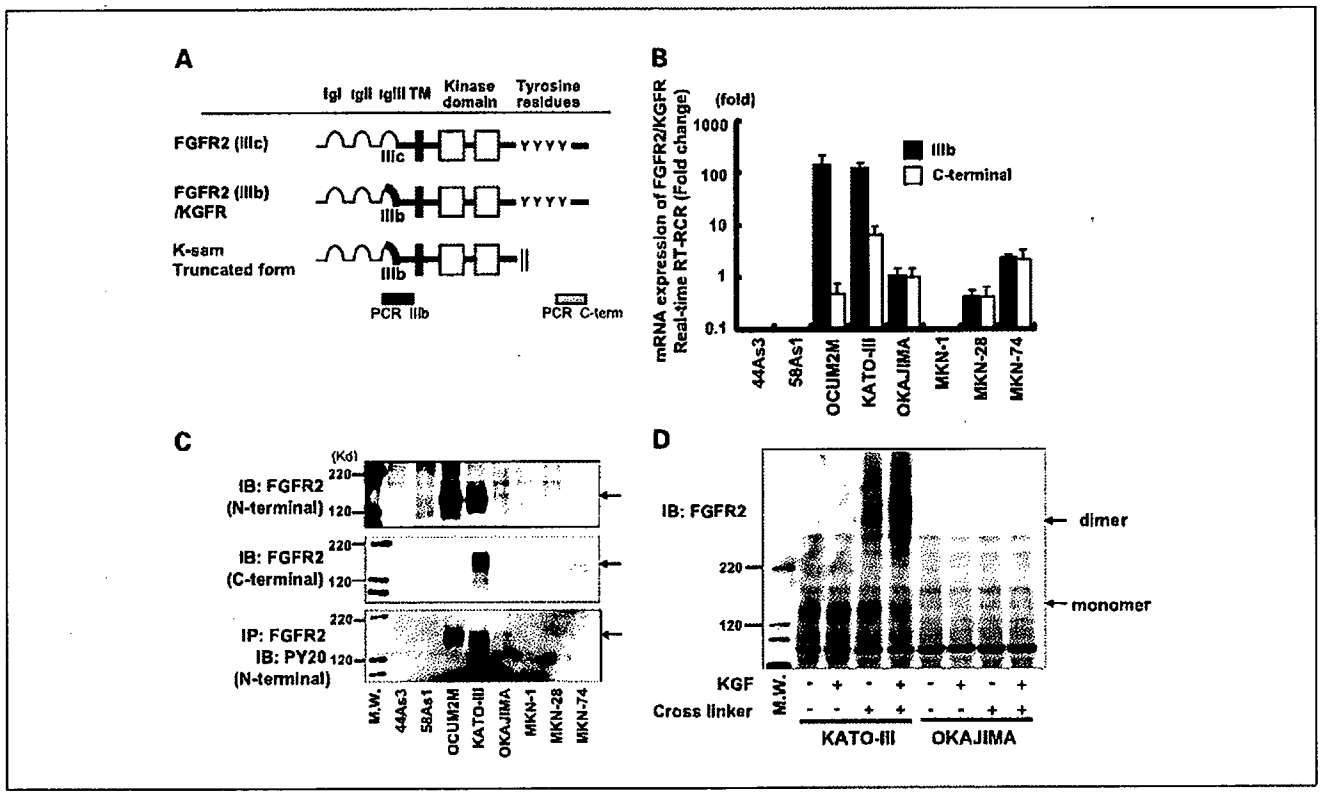


Fig. 2. *A*, schematic representation of FGFR2 and regions amplified by PCR. *B*, mRNA expression levels of FGFR2 were quantified by detecting the extracellular domain or COOH-terminal region by real-time reverse transcription-PCR. Expression in the cells is shown as a ratio to expression in OKAJIMA cells. FGFR2 was overexpressed in KATO-III cells and OCUM2M cells by about 100-fold compared with the other cell lines. The majority of the FGFR2 in the sensitive cell lines KATO-III and OCUM2M had no COOH-terminal region. *C*, protein expression levels of FGFR2 were determined by Western blotting with antibodies to the $NH_2$ or COOH termini. Both AZD2171-sensitive cell lines overexpressed FGFR2, and the phosphorylation levels were markedly higher. *D*, chemical cross-linking analysis. Cells were cultured under serum-starved conditions for 24 h and then stimulated with KGF (100 ng/mL) for 15 min. After collecting and washing them with PBS, they were incubated for 30 min in PBS containing cross-linker substrate. The reaction was terminated by adding 250 mmol/L glycine for 5 min. In spite of the serum-starved conditions, high levels of expression of the dimerized form were observed in KATO-III cells in the absence of ligand stimulation. This phenomenon was not observed in the control undifferentiated OKAJIMA cell line. Ligand stimulation resulted in a mild increase in the dimerized form in KATO-III cells. Arrows indicate monomer or dimer formation.

containing 1.5 mmol/L of the non-permeable cross-linker bis-(sulfosuccinimidyl) substrate (Pierce). The reaction was terminated by adding 250 mmol/L glycine for 5 min, and the cells were analyzed by immunoblotting with FGFR2 antibody (Sigma).

*FGFR2/KGFR gene silencing with small interfering RNA.* Predesigned small interfering RNA (siRNA) targeting FGFR2 was purchased from Ambion. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. The cells were then transfected with the FGFR2 targeting siRNA or non-silencing siRNA using RNAiFect Transfection Reagent (Qiagen) according to the manufacturer's protocol and incubated another 72 h. Cell growth was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, $2 \times 10^5$ cells per well were plated on a six-well plate for 24 h and transfected with siRNA under the same conditions.

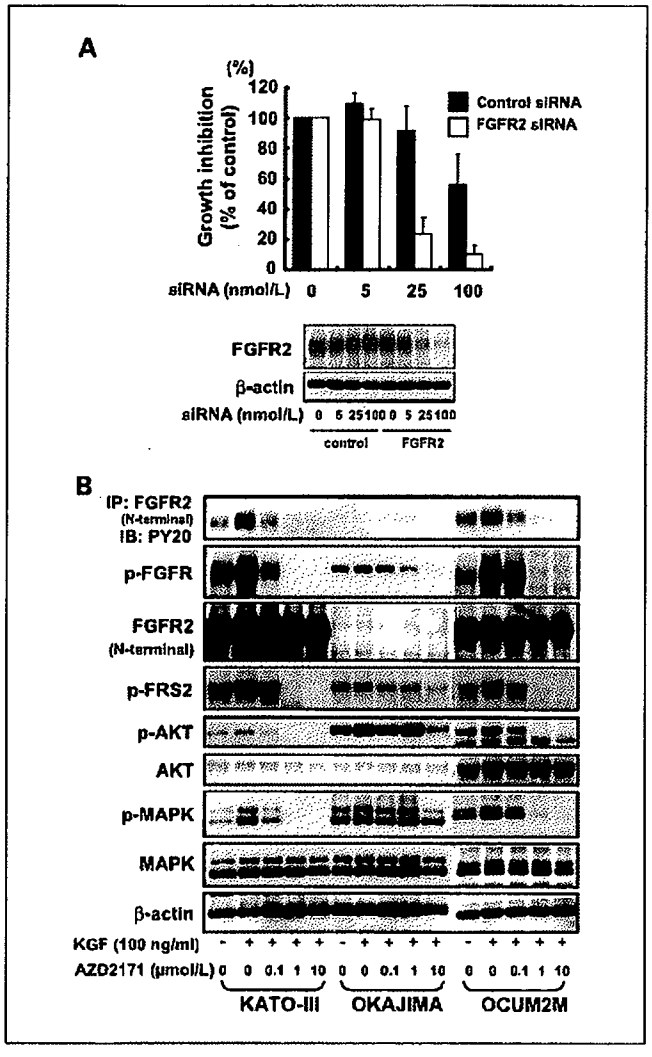


Fig. 3. *A*, FGFR2 targeting siRNA and cellular growth-inhibitory effect. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. After incubation, the cells were transfected with FGFR2-targeting or non-silencing siRNA and incubated for another 72 h. Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, $2 \times 10^5$ cells per well were plated on a six-well plate and treated similarly. Marked inhibition of cell growth (~80%) was observed by FGFR2 targeting siRNA compared with control siRNA (*top*). Reduction of FGFR2 protein expression in KATO-III cells was confirmed by immunoblotting (*bottom*). Columns, % control absorbance in three independent experiments; bars, SD. *B*, Western blotting for downstream molecules of FGFR2 signaling. Cells were cultured overnight under serum-starved conditions and exposed to 0.1 to 10 μmol/L AZD2171 for 3 h before adding 100 ng/mL KGF for 15 min. AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2 at 1 μmol/L in the sensitive cell lines, compared with 10 μmol/L in the control cell line OKAJIMA. Similar results were observed for FRS-2, AKT, and mitogen-activated protein kinase (*MAPK*).

In vivo *experiments.* Tumorigenic TU-kato-III cells were derived from the gastric cancer cell line KATO-III. Four-week-old female BALB/c nude mice were purchased from CLEA Japan, Inc. and maintained under specific-pathogen-free conditions; $5 \times 10^6$ TU-kato-III cells or OCUM2M cells were s.c. injected into both flanks of each mouse. When the tumors had reached a volume of 0.1-0.3 $cm^3$, the mice were randomized into three groups (three per group) and given AZD2171, 1.5 or 6.0 mg/kg/d, or vehicle once daily by oral gavage for 3 weeks. Tumor volume was calculated using the formula: (length × width) × $\sqrt{}$(length × width) × ($\pi$/6), where length is the longest diameter across the tumor, and width is the corresponding perpendicular. All mice were sacrificed on day 21, and the tumors were collected. The protocol of the experiment was approved by the Committee for Ethics in Animal Experimentation and conducted in accordance with the Guidelines for Animal Experiments of National Cancer Center.

## Results

*AZD2171 showed growth-inhibitory activity* in vitro. To evaluate the growth-inhibitory activity of AZD2171 *in vitro*, we did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays on eight gastric cancer cell lines. The epidermal growth factor receptor–specific tyrosine kinase inhibitor gefitinib was used as a reference. The $IC_{50}$ of gefitinib for all cell lines was between 7 and 20 μmol/L. AZD2171 inhibited the growth of KATO-III cells and OCUM2M cells ($IC_{50}$, 0.15 and 0.37 μmol/L, respectively) more potently than the other cell lines (Fig. 1A).

*Expression levels of tyrosine kinase receptors.* To elucidate the mechanism of action of AZD2171 in the two sensitive cell lines, we measured mRNA expression levels of VEGFRs, FGFRs, and c-KIT, whose kinase activity have been reported to be inhibited by AZD2171 (10). No mRNA expression of VEGFRs or c-KIT was detected by reverse transcription-PCR in either sensitive cell lines. FGFR2 transcripts, however, were strongly expressed in both sensitive cell lines but not strongly in the other cell lines (Fig. 1B). Since we previously found that FGFR2/KGFR/K-sam with a deletion of COOH-terminal exons was amplified in both sensitive cell lines (9), we speculated that amplified FGFR2/KGFR might be associated with sensitivity to AZD2171.

*Sensitive cells expressed constitutively active and spontaneously dimerized FGFR2/KGFR.* We quantified mRNA expression levels of FGFR2 by real-time reverse transcription-PCR with primers that detect the extracellular domain (IIIb region, see Fig. 2A) and COOH-terminal region. The results show that KATO-III cells and OCUM2M cells expressed FGFR2 100-fold higher than the other cells tested. The COOH-terminal region of FGFR2 was deleted in the KATO-III cells and OCUM2M cells (Fig. 2B). Overexpression and markedly increased phosphorylation of FGFR2 was observed in the AZD2171-sensitive cell lines (Fig. 2C).

Immunoblotting with antibodies for the COOH and $NH_2$ termini revealed that almost all the FGFR2 expressed by OCUM2M cells, and about half of FGFR2 expressed by KATO-III cells, were truncated (Fig. 2C). Although the KATO-III cells expressed wild-type receptor to some extent, the

**Table 1.** *In vitro* kinase assay of AZD2171 against FGFR2

| Cell line | $K_m$ | $K_i$ (μmol/L) |
|---|---|---|
| KATO-III | 8.3 ± 3.3 | 0.067 ± 0.017 |
| OCUM2M | 7.1 ± 1.4 | 0.072 ± 0.022 |
| OKAJIMA | 11.0 ± 5.0 | 0.049 ± 0.041 |

COOH-terminal truncated type was dominantly expressed in AZD2171-sensitive cell lines.

A chemical cross-linking analysis was done to evaluate the dimerization of FGFR2. High dimerization of FGFR2 was observed in the KATO-III cells even in the absence of ligand stimulation (Fig. 2D), but no such phenomenon was observed in the control undifferentiated OKAJIMA cell line. Ligand stimulation increased the level of the dimerized-form in KATO-III cells. Taken together, these findings show that the sensitive cell lines expressed high levels of FGFR2 that was highly phosphorylated and spontaneously dimerized without ligand stimulation, suggesting that FGFR2 signaling is constitutively activated in these cells. This evidence is consistent with the widely recognized findings that cancer cells sensitive to other tyrosine kinase inhibitors, such as gefitinib and imatinib, overexpress the highly phosphorylated target receptor with an increased level of dimerization in a ligand-independent manner (12, 16, 17).

***FGFR2 targeting siRNA showed a potent growth-inhibitory effect on KATO-III cells.*** To investigate the dependency of cell growth through activated FGFR2 signaling in the AZD2171-sensitive KATO-III cell line, we evaluated the growth-inhibitory effect of siRNA targeted to FGFR2 in KATO-III cells. Targeted siRNA (5-100 nmol/L) decreased FGFR2 and inhibited cell growth (>80%) in a dose-dependent manner (Fig. 3A). The results show that most of the growth of KATO-III cells is dependent on activated FGFR2 signaling, suggesting that the FGFR signaling dependency may be responsible for the higher growth-inhibitory effect of AZD2171 on KATO-III cells.

***AZD2171 inhibited FGFR2 signaling.*** Next, we examined the effect of AZD2171 on FGFR2 downstream phosphorylation signals (i.e., FRS-2, AKT, and mitogen-activated protein kinase). AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2, FRS-2, AKT, and mitogen-activated protein kinase at 1 μmol/L in KATO-III cells, compared with 10 μmol/L in OKAJIMA cells. These results clearly show that AZD2171 possesses inhibitory activity against FGFR2 in cell-based studies and significantly inhibits the phosphorylation of FGFR2 at 1 μmol/L in sensitive cells.

***FGFR2 kinase inhibition of AZD2171.*** To quantify the inhibitory activity of AZD2171 on FGFR2 kinase under cell-free conditions, we calculated the $K_i$ values for immunoprecipitated FGFR2 derived from KATO-III, OCUM2M, and OKAJIMA cells. The $K_i$ values of AZD2171 for FGFR2 in each of these cell lines were 0.067 ± 0.017, 0.072 ± 0.022, and 0.049 ± 0.041 μmol/L, respectively (Table 1). In contrast, the $K_i$ value of AZD2171 for recombinant VEGFR-2 was 0.0009 μmol/L (data not shown) and was consistent with previous reports (10). At the cellular level, phosphorylation of

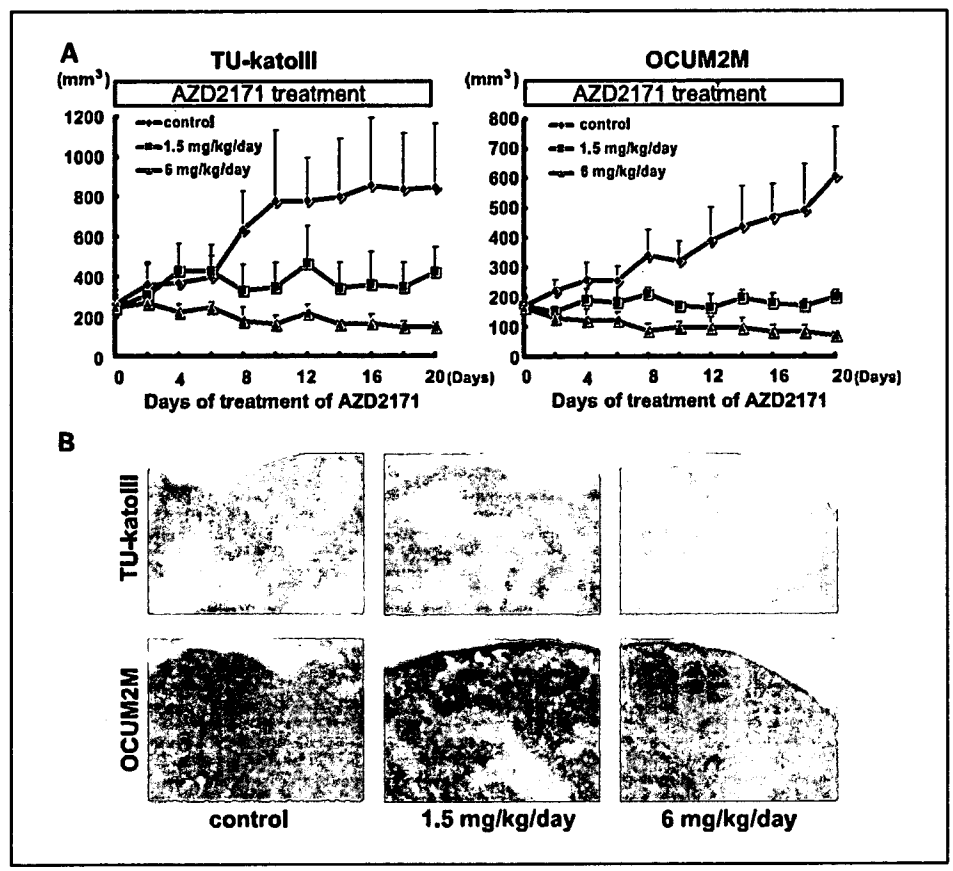


Fig. 4. *A, in vivo* growth-inhibitory effect of AZD2171 in a tumor xenograft model. After s.c. injecting $5 \times 10^6$ TU-kato-III or OCUM2M cells into both flanks of the mice, AZD2171 (1.5 or 6.0 mg/kg/d), or vehicle, was administered orally once daily for 3 wks. A marked tumor growth-inhibitory effect was observed at the low dose (1.5 mg/kg/d) of AZD2171 in both the TU-kato-III tumors and OCUM2M tumors, and the high dose (6.0 mg/kg/d) of AZD2171 completely inhibited the growth of both cell lines. *B*, representative H&E staining of tumor tissue from mice treated with AZD2171. Broad dose-dependent necrosis was observed. Original magnification, ×40.

FGFR2 was inhibited at 10-fold lower concentrations of AZD2171 in the sensitive cell lines (Fig. 3B), but there were no marked differences between the kinase-inhibitory effects among the proteins derived from the cell lines in this cell-free assay. This discrepancy is discussed in the Discussion.

*In vivo antitumor activity of AZD2171 against FGFR2-overexpressing gastric cancer.* To elucidate the *in vivo* antitumor activity of AZD2171 in mice bearing gastric cancer tumor xenografts, we used the newly established tumorigenic subline TU-kato-III (derived from KATO-III) and OCCUM2M. We attempted to perform control experiments using OKAJIMA cells *in vivo* as suggested by the reviewer. Unfortunately, however, the cell lines grew slowly in the mice, and we could not precisely evaluate the antitumor activity of AZD2171 in the model. However, the results of preliminarily experiments showed that AZD2171 seemed to be less effective against OKAJIMA cells than against KatoIII and OCUM2M cell *in vivo*. Mice implanted the TU-kato-III and OCUM2M tumors were given a low or high dose of AZD2171 (i.e., 1.5 or 6.0 mg/kg/d), or vehicle, orally for 3 weeks. AZD2171 (1.5 mg/kg/d) significantly inhibited tumor growth in the mice bearing TU-kato-III and OCUM2M tumors, and the higher dose (6.0 mg/kg/d) completely inhibited the growth of both tumor models (Fig. 4A). H&E staining showed broad dose-dependent necrosis of core tumor tissue in mice treated with AZD2171 (Fig. 4B). Thus, AZD2171 showed marked antitumor activity *in vivo* against both human gastric tumor xenografts.

## Discussion

Recent studies have shown that FGFRs and their ligands are promising therapeutic target molecules for various malignant diseases, such as prostate cancer (18), breast cancer (5, 19), endometrial carcinoma (20), synovial sarcomas (21), thyroid carcinoma (22, 23), and hematopoietic malignancies (24–27). These findings are based on the biological properties of malignant cells expressing activated FGFR, like FGFR fusion tyrosine kinase, involved in chromosomal translocations, gene amplification of FGFRs, or overexpression of FGFs (5, 18–27). In the case of gastric cancer, the results of immunohistochemical analysis of clinical samples revealed that 20 of 38 cases of advanced undifferentiated type of gastric cancer were FGFR2/K-sam positive, whereas none of the 11 cases with the differentiated or intestinal type of cancer showed positive staining for K-sam (8). The results suggest that FGFR2/K-sam overexpression is associated with the undifferentiated type of stomach cancers. The results of fluorescence *in situ* hybridization analysis of the gastric cancer specimens showed gene amplification of FGFR2/K-sam in 2.9% (28). The clinical implication of FGFR2 overexpression/amplification in gastric cancers remains to be fully clarified, and further investigation is needed.

AZD2171 has the most potent kinase-inhibitory activity against VEGFR-2 ($IC_{50}$ < 1 nmol/L); it also possesses additional activity against VEGFR-1, VEGFR-3, and c-Kit ($IC_{50}$, 5, ≤3, and 2 nmol/L, respectively; ref. 10). AZD2171 showed antiangiogenic activity and broad antitumor activity consistent with potent inhibition of VEGF-induced angiogenesis. We showed kinase-inhibitory activity of AZD2171 against FGFR2 in the present study. When cancer cells are dependent on FGFR2 signaling, AZD2171 can be expected to give additional therapeutic benefit in addition to its antiangiogenic effects.

A cell-based Western blotting analysis showed that phosphorylation of FGFR2 in KATO-III cells and OCUM2M cells was inhibited by AZD2171 at 10-fold lower dose than in OKAJIMA cells (Fig. 3B). However, there was no significant difference in the $K_i$ values of AZD2171 between the FGFR2 derived from KATO-III, OCUM2M, and OKAJIMA in an *in vitro* kinase assay. This may be attributable to the different conditions between the cell-based and cell-free assays. For example, undefined intrinsic intracellular factors may influence kinase activity: (*a*) differences in baseline intracellular FGFR2 phosphatase activity in each cell line, (*b*) differences in intracellular concentration of (|transporters, such as ATP-binding cassette transporters, may be involved in this phenomenon refs. 29, 30), and (*c*) undefined intrinsic inhibitory factors that bind the compounds directly may also be involved (e.g., Brehmer D, et al. have identified various gefitinib binding proteins by affinity chromatography; ref. 31).

In conclusion, AZD2171, a potent inhibitor of all VEGFRs (VEGFR-1, VEGFR-2, and VEGFR-3), was found to have antitumor effect against gastric cancer xenografts in line with previous findings in colon, lung, prostate, breast, and ovarian tumor xenografts (10). The results of this study suggest that activation of the FGFR2 pathway may be a promising target for gastric cancer therapy. AZD2171 may provide a clinical benefit to gastric cancer patients.

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