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ORIGINAL ARTICLE

Phase I clinical and pharmacokinetic study of the glucose-conjugated cytotoxic agent D-19575 (glufosfamide) in patients with solid tumors

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Abstract
Purpose D-19575 (glufosfamide: β-D-glucosylisophosphoramide mustard) is an alkylating agent in which isophosphoramide mustard, the cytotoxic metabolite of ifosfamide, is covalently linked to β-D-glucose. We have performed a phase I study to determine the safety profile, pharmacokinetics, and antitumor activity of D-19575 in Japanese patients with advanced solid tumors
Methods Patients were treated with escalating doses of D-19575 administered by a two-step (fast–slow) intravenous infusion over 6 h every 3 weeks. Thirteen patients received 43 treatment cycles (median 3; range 1–11) at D-19575 doses of 3,200, 4,500, or 6,000 mg/m^2.
Results Hematologic toxicities and other side effects were generally mild. The maximum tolerated dose of D-19575 was 6,000 mg/m^2, at which two patients experienced dose-limiting toxicities (hypophosphatemia, hypokalemia, and metabolic acidosis each of grade 3). Pharmacokinetic analysis revealed a linear relation between the area under the concentration-versus-time curve (AUC) and dose. The AUC values for isophosphoramide mustard were substantially greater than those achieved by bolus administration or continuous infusion of ifosfamide in conventional therapy. One patient with gallbladder cancer previously treated with cisplatin and gemcitabine achieved a partial response lasting for >5 months, and eight patients achieved disease stabilization.
Conclusions Our results show that D-19575 can be safely administered by infusion over 6 h at 4,500 mg/m^2 every 3 weeks. The safety profile and potential antitumor activity of D-19575 show that phase II studies of this drug are warranted.

Keywords Glufosfamide · Isophosphoramide mustard · Glucose transporter · Pharmacokinetics

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Introduction

D-19575 (glufosfamide: β-D-glucosylisophosphoramide mustard) is a new-generation cytotoxic alkylating agent in which isophosphoramide mustard (IPM), the active metabolite of ifosfamide (IFO), is covalently linked to β-D-glucose (Fig. 1). The structure and metabolism of the D-19575 molecule are thought to be associated with two therapeutic advantages: a reduced generation of toxic metabolites compared with IFO, and targeting by the glucose moiety to rapidly proliferating tumor cells [1, 2].

Rapidly proliferating and energy-consuming cancer cells have been shown to overexpress certain glucose transporter proteins [3, 4]. D-19575 has the potential to target tumor

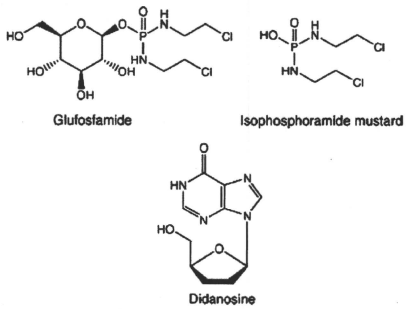

Fig. 1 Structures of D-19575 (glufosfamide), isophosphoramide mustard (the active cytotoxic metabolite of D-19575), and didanosine (internal standard)

cells by serving as a substrate for such glucose transporters in the plasma membrane [5–7]. Together with the increased metabolic rate and glucose consumption of tumor cells, this targeting mechanism may contribute to the relative selectivity of D-19575 for tumor cells.

Another characteristic of D-19575 is that, because of the absence of the oxazophosphorine ring in its structure, it does not release the urothelium irritant acrolein, which has been shown to induce hemorrhagic cystitis in individuals treated with IFO [2]. Moreover, the amount of toxic chloroacetaldehyde generated metabolically after administration of D-19575 is markedly reduced compared with that generated after IFO administration (unpublished data). Although the pathogenesis of IFO-induced nephrotoxicity is poorly understood, the reduced production of chloroacetaldehyde may minimize such toxicity of D-19575. Preclinical pharmacokinetic analysis of D-19575 has revealed that the drug is rapidly cleared by the kidneys and has favorable tissue-distribution and protein-binding profiles [8]. Toxicity studies in rodents have shown that D-19575 is more toxic when administered orally than intravenously, apparently because of a pronounced first-pass effect and increased production of toxic metabolites (data on file; ASTA Medica AG, Germany).

The aims of the present study were to determine the maximum tolerated dose (MTD) and dose-limiting toxicities (DLTs) of D-19575, to otherwise evaluate the safety profile of the drug, and to analyze its pharmacokinetics after administration by biphasic (fast–slow) intravenous infusion over 6 h every 3 weeks in Japanese patients with refractory advanced solid tumors. Furthermore, we performed a pharmacokinetic analysis of IPM generated from D-19575.

Patients and methods

Study objectives

This phase I study aimed to evaluate the safety (including DLTs and MTD) and pharmacokinetic profiles of D-19575 administered by intravenous infusion over 6 h in Japanese individuals with solid tumors who relapsed after adequate or standard chemotherapy or in those with advanced or metastatic solid tumors for whom no effective standard therapy was available. The study was fully supported by Medibic Pharma Co. Ltd (Tokyo, Japan) as a registration-directed clinical trial. D-19575 was synthesized in the Chemical Research Laboratories of ASTA Medica AG (Frankfurt, Germany). ASTA Medica AG's oncology division was acquired by Baxter International in 2001, but Baxter International itself terminated D-19575 development and shortly thereafter licensed their rights to Threshold Pharmaceuticals (Redwood City, CA, USA). In this study, D-19575 was supplied by Medibic Pharma Co. Ltd. Medibic Pharma and Threshold Pharmaceuticals agreed to co-develop D-19575 in Asia on December 2004.

Eligibility

Eligible patients were individuals aged 20–75 years who had solid tumors that were either refractory to conventional treatment or for which no standard treatment was available; had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; had adequate hematopoietic reserves [absolute neutrophil count (ANC) of $\geq$1,500/μL, platelet count of $\geq$100,000/μL, hemoglobin concentration of $\geq$9.0 g/dL]; had serum total bilirubin and creatinine concentrations of $\leq$1.5 times the upper limit of institutional normal (ULN); had serum aspartate aminotransferase and alanine aminotransferase activities of $\leq$2.5 times ULN; had not received chemotherapy or radiation therapy within the previous 4 weeks; had no exposure to nitrosoureas or mitomycin within the previous 6 weeks; and had given consent to be hospitalized during the first course of treatment with D-19575. Patients were ineligible if they had symptomatic brain metastasis; other nonmalignant systemic disease; an active, uncontrolled infection; preexisting nephrotoxicity of grade 3 or 4 [National Cancer Institute Common Toxicity Criteria (NCI-CTC)] resulting from previous therapy; or infection with human immunodeficiency virus or hepatitis B or C virus. They were also ineligible if they were pregnant or nursing, or if they required steroid therapy. All patients provided written informed consent before entering the study. The study was approved by the Institutional Review Board at each participating center and conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines.

A medical history was obtained from each patient, and physical examinations and routine laboratory evaluations were performed before treatment initiation and weekly thereafter. Chest and other relevant X-rays were obtained during screening and after alternate cycles of treatment. Adverse events were monitored and recorded throughout the study and were graded according to NCI-CTC, version 3.0. The tumor response was assessed for measurable target lesions according to Response Evaluation Criteria in Solid Tumors (RECIST).

Treatment administration

This open-label, dose-escalation phase I study of D-19575 was based on intravenous infusion of the drug in three cohorts of Japanese subjects with malignant solid tumors. D-19575 was administered intravenously over 6 h in a total volume of 1,000 mL of normal saline at doses of 3,200, 4,500, and 6,000 mg/m^2. One-quarter of the dose was administered during the first 30 min at a rate of 500 mL/h, with the remainder of the dose being administered over the subsequent 330 min at a rate of 136 mL/h. D-19575 was administered on day 1 once every 3 weeks. Antiemetic premedication was not mandatory in the protocol. Granulocyte colony-stimulating factor (G-CSF) was administered for febrile neutropenia, sepsis with neutropenia, or recurrent neutropenia of grade 4.

DLTs and MTD

The starting dose of D-19575 was 3,200 mg/m^2, which was increased to 4,500 and then to 6,000 mg/m^2 in subsequent cohorts of at least three patients. The following adverse events during cycle 1 were defined as DLTs: neutropenia of grade 4 (ANC of <500/μL) for >7 days; febrile neutropenia (fever of >38.0°C with an ANC of <1,000/μL); thrombocytopenia (platelet count of <25,000/μL); nausea or vomiting of grade ≥3 despite maximal antiemetic therapy; and any other nonhematologic toxicity of grade ≥3 considered related to D-19575. If any patient experienced a DLT during the first cycle, three additional patients were treated at the same dose. Patients who experienced a DLT could continue D-19575 therapy at the preceding dose level. Treatment in subsequent courses was reinitiated only after hematologic recovery (ANC of ≥1,500/μL, platelet count of ≥100,000/μL) and resolution of all other toxicities to grade ≤1 or baseline intensity. The MTD was defined as the dose at which two or more patients experienced a DLT in the first cycle. The recommended dose was defined as the dose level immediately below the MTD.

Pharmacokinetic analysis

Pharmacokinetic sampling was performed for the first and second cycles. Blood samples (2.0 mL) for analysis of the plasma concentrations of D-19575 and IPM were collected at 10 time points on the day of drug administration (total volume of 20 mL of blood): immediately before the start of drug infusion and at 0.5, 1, 3, 6 (immediately before the end of infusion), 6.5, 7, 8, 12, and 24 h after the start of infusion. Plasma samples were stored at −70°C until analysis. D-19575 and IPM in plasma samples were measured by high-performance liquid chromatography and tandem mass spectrometry at Covance Bioanalytical Services (Indianapolis, IN, USA).

The plasma concentration-versus-time data for D-19575 and IPM in cycles 1 and 2 were analyzed with a noncompartmental method. The pharmacokinetic parameters of D-19575 and IPM determined included the maximum observed plasma concentration (C_{max}), time to reach C_{max} (T_{max}), area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$), terminal elimination half-time ($t_{1/2}$), total body clearance (CL_{tot}), and volume of distribution at steady state (V_{ss}). The $AUC_{0-\infty}$ was determined by summing the area from time zero to the time of the last measured concentration (as calculated with the use of a log trapezoidal method) and the extrapolated area. The extrapolated area was determined by dividing the final concentration by the slope (k) of the terminal log linear phase. The absolute value of k was also used to estimate the apparent terminal elimination half-time: $t_{1/2} = \ln(2/k)$. The CL_{tot} was determined by dividing dose by $AUC_{0-\infty}$. The V_{ss} was calculated by multiplying CL_{tot} by the mean residence time, which was determined as the area under the moment curve to infinity divided by $AUC_{0-\infty}$. All pharmacokinetic parameters were calculated with the use of WinNonlin Professional 5.0 software (Pharsight Corporation, Mountain View, CA, USA), and all calculations were performed with the actual times recorded on the case report form and with zero substituted for concentrations below the quantification limit of the assay (5 ng/mL for both D-19575 and IPM). The plasma concentrations of D-19575 and IPM (day 1 of cycles 1 and 2) were listed by subject and summarized by dose (mean, standard deviation, coefficient of variation, minimum, maximum, number of observations). Dose-adjusted $AUC_{0-\infty}$ and C_{max} values were calculated for each subject by dividing $AUC_{0-\infty}$ and C_{max} by dose. Analysis of variance appropriate for a parallel, dose-ascending design was performed on the dose-adjusted parameters to assess dose proportionality. Individual and mean plasma concentrations of D-19575 and IPM versus time after administration of D-19575 were tabulated and presented graphically on both linear and logarithm scales. For the time course graphs,

values below the quantification limit of the assay were set to zero.

Results

Patient characteristics

Thirteen patients (nine men and four women; median age, 62 years) were enrolled in the study between January and August 2007. Patient characteristics are shown in Table 1. Most patients were heavily pretreated, with the median number of prior chemotherapy regimens being three.

Table 1 Patient characteristics

Characteristic	No. of patients (n = 13)
Sex (male/female)	9/4
Age (years)	
Median	62
Range	51–73
ECOG performance status	
0	3
1	10
Tumor type	
Colorectal cancer	7
Non-small cell lung cancer	1
Thymic cancer	1
Thymoma	1
Gallbladder cancer	1
Gastric cancer	1
Uterine corpus-endometrial cancer	1
Previous treatment	
Chemotherapy (no. of regimens)	
1	1
2	4
3	3
4	1
≥5	4
Radiation	3
Surgery	12

DLTs and MTD

Patient distribution by dose level is shown in Table 2.. No DLTs occurred in the patient cohorts treated with D-19575 at the doses of 3,200 mg/m^2 (n = 3) or 4,500 mg/m^2 (n = 7). At the dose of 6,000 mg/m^2, however, two of three treated patients experienced DLTs: One patient experienced metabolic acidosis and hypophosphatemia of grade 3, the other patient experienced hypophosphatemia and hypokalemia of grade 3. All DLTs were transient and reversible. The dose of 6,000 mg/m^2 was thus identified as the MTD, and enrollment of patients in the study was stopped. The recommended dose level for phase II evaluation was therefore determined to be 4,500 mg/m^2.

Safety

A total of 43 cycles of treatment was administered to the 13 patients, with a median of three cycles per patient and a range of 1–11 (Table 2). The incidence of hematologic toxicities by dose level is shown in Table 3. Clinically significant effects on ANC or platelet count were rare and only one patient, treated at the dose of 4,500 mg/m^2, experienced neutropenia of grade 4 without infection, which occurred during cycle 2 and was short-lived. Red blood cell transfusion was required in one patient treated at the dose of 4,500 mg/m^2 during cycle 3 because of the development of grade 4 anemia. Other hematologic toxicities were mostly of grade 1 or 2 and were reversible. The predominant nonhematologic toxicities were fatigue, nausea, a high urinary concentration of β_2-microglobulin, hypophosphatemia, hypokalemia, and metabolic acidosis (Table 4). Nonhematologic toxicities were also generally transient and reversible.

Pharmacokinetics

Plasma samples were obtained from all 13 patients during the first and second cycles of treatment. Plots of the mean plasma concentrations of D-19575 and IPM (the active metabolite of D-19575) versus time are shown in Fig. 2a. There was substantial interpatient variability in the pharmacokinetics of D-19575 after intravenous administration

Table 2 Dose-escalation scheme and summary of DLT incidence

Dose (mg/m^2)	No. of patients	No. of cycles	No. of patients with DLT	DLT (grade)
3,200	3	8	0	
4,500	7	28	0	
6,000	3	7	2	Hypophosphatemia (grade 3), hypokalemia (grade 3), metabolic acidosis (grade 3)
Total	13	43	2	

Table 3 No. of patients with hematologic toxicities (all cycles)

Dose (mg/m^2)	No. of patients	Neutropenia				Anemia				Thrombocytopenia			
		Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
3,200	3	1	0	1	0	1	0	0	0	0	0	0	0
4,500	7	1	0	1	1	2	1	0	1	2	0	1	0
6,000	3	0	1	0	0	0	0	0	0	1	0	0	0
Total	13	2	1	2	1	3	1	0	1	3	0	1	0

Table 4 Number of patients with nonhematologic toxicities (all cycles)

Dose (mg/m^2)	No. of patients	Fatigue/generalized weakness				Hypophosphatemia				Hypokalemia				Metabolic acidosis			
		Grade				Grade				Grade				Grade			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
3,200	3	3	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0
4,500	7	6	2	0	0	0	2	2	0	1	0	1	1	0	0	2	0
6,000	3	2	0	0	0	0	2	2[a]	0	0	0	1[a]	0	0	0	1[a]	0
Total	13	11	2	0	0	0	5	4	0	1	0	2	1	1	0	4	0

[a] DLT

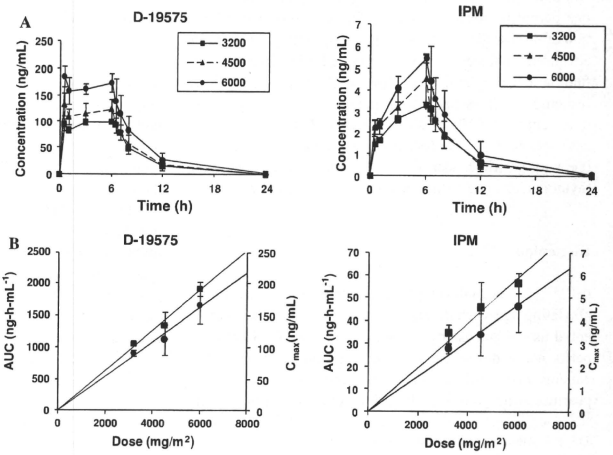

Fig. 2 Time course of the mean plasma concentrations of D-19575 and IPM after a single intravenous infusion of D-19575 (3,200, 4,500, or 6,000 mg/m^2) over 6 h (**a**) and the relations between the $AUC_{0-\infty}$ or C_{max} of D-19575 or IPM and the dose of D-19575 (**b**) for the first cycle of treatment. Data are means ± SD

of single doses of 3,200, 4,500, or 6,000 mg/m^2, but both D-19575 and IPM exhibited linear pharmacokinetics over the dose range studied (Fig. 2b). Pharmacokinetic parameters for D-19575 and IPM are summarized by dose of D-19575 in Tables 5 and 6, respectively. The mean C_{max} values of D-19575 were 107–192 ng/mL and were achieved at 2.46–3.33 h, with the mean $t_{1/2}$ values ranging from 2.30 to 2.53 h. D-19575 exhibited low CL_{tot} values [3.47–4.08 L/(h m^2)] as well as V_{ss} values (8.94–9.76 L/m^2) that were approximately equal to the volume of extracellular fluid. The plasma levels of IPM were smaller than those of D-19575 by a factor of ~25–30. The mean C_{max} values of IPM were 3.46–5.65 ng/mL and were achieved in 6.04–6.59 h, with mean $t_{1/2}$ values being similar to those for D-19575 and ranging between 2.38 and 2.66 h. There was no difference in pharmacokinetic data between cycles 1 and 2 for either D-19575 or IPM (data not shown).

Table 5 Mean pharmacokinetic parameters of D-19575 in plasma after a 6 h intravenous infusion of the indicated doses of D-19575 during cycle 1

Dose (mg/m^2)	No. of patients	$AUC_{0-\infty}$ (ng h/mL)		C_{max} (ng/mL)		$t_{1/2}$ (h)		CL_{tot} [L/(h m^2)]		V_{ss} (L/m^2)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
3,200	3	914	43.6	107	2.65	2.38	0.07	3.47	0.18	8.94	0.74
4,500	7	1,130	239	135	21.0	2.30	0.34	4.08	0.86	9.76	1.37
6,000	3	1,659	276	192	11.0	2.53	0.34	3.65	0.70	9.71	1.43

Table 6 Mean pharmacokinetic parameters of IPM in plasma after a 6 h intravenous infusion of the indicated doses of D-19575 during cycle 1

Dose (mg/m^2)	No. of patients	$AUC_{0-\infty}$ (ng h/mL)		C_{max} (ng/mL)		$t_{1/2}$ (h)	
		Mean	SD	Mean	SD	Mean	SD
3,200	3	28.1	2:04	3.46	0.39	2.38	0.06
4,500	7	34.0	8.84	4.58	1.11	2.47	0.37
6,000	3	46.5	11.2	5.65	0.43	2.66	0.45

Antitumor activity

Among all 13 patients evaluable for response, evidence of antitumor activity was observed in nine individuals, with one partial response and eight subjects showing stabilization of disease (Table 7). A 65-year-old female patient with advanced gallbladder cancer who had been treated with fluorouracil, cisplatin, and gemcitabine achieved a partial response that persisted for >5 months after two cycles of treatment with D-19575 at 4,500 mg/m^2 (Fig. 3). Stable disease was confirmed in four patients with colorectal cancer, one with gastric cancer, one with thymic cancer, one with thymoma, and one with non-small cell lung cancer.

Discussion

D-19575 has been developed as a new-generation cytotoxic alkylating agent whose activity is due in part to the preferential use of glucose by malignant cells [9]. We have now performed a dose-escalation phase I study of D-19575 in patients with solid tumors for evaluation of the safety and pharmacokinetics of this drug. Previous clinical trials with D-19575 were conducted given as short 1 h infusion schedule [10, 11]. However, taking into account the cellular uptake and cleavage of D-19575, a more continuous infusion schedule seemed to be preferable. Indeed, a previous pharmacokinetic analysis found that biphasic (fast–slow) intravenous infusion schedule produced the desired profile of a rapidly achieved and sustained plasma concentration for more than 6 h [12]. Based on the data, we have also employed a two-step, fast/slow 6 h infusion of D-19575 to rapidly achieve steady-state concentrations and expose tumor cells to the study drug over a moderately prolonged time. The MTD was determined to be 6,000 mg/m^2, with the recommended dose for phase II studies being 4,500 mg/m^2. DLTs included hypophosphatemia, hypokalemia, and metabolic acidosis, all of which are presentations of nephrotoxicity. In animals treated with D-19575, nephrotoxicity was evidenced histologically by a focal vacuolization of proximal tubules and a diffuse dilation of distal tubules (data on file; ASTA Medica AG, Germany). A major advantage of D-19575 over IFO is that treatment with D-19575 does not require administration of sodium mercaptoethanesulfonate for protection against urothelial toxicity. Two recent phase II trials performed by the European Organization for Research and Treatment of Cancer (EORTC)—New Drug Development Group [10, 11], demonstrated that active hydration did not show any nephroprotective effect toward D-19575 by reducing the contact time of tubule cells with the drug. On the basis of these results, active hydration was not used routinely in our study. Despite concerns for potential renal dysfunction with D-19575, creatinine clearance did not undergo a substantial decline in any of the treated patients (data not shown).

Table 7 Antitumor activity of D-19575

Response	No of patients (%)
Partial response	1 (8)[a]
Stable disease	8 (62)[b]
Progressive disease	4 (30)
Not evaluable[c]	0 (0)

[a] Dose of 4,500 mg/m^2

[b] Two patients at a dose of 3,200 mg/m^2, four patients at 4,500 mg/m^2, and two patients at 6,000 mg/m^2

[c] Not assessed for response

The initial activation reaction in IFO metabolism that gives rise to antitumor activity is mediated predominantly by the cytochrome P450 enzyme CYP3A4. The oxidation

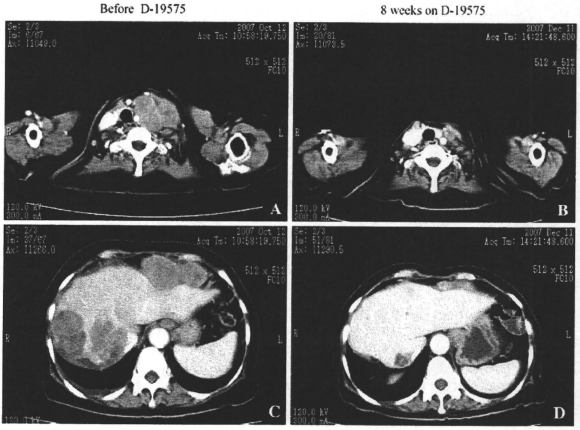

Fig. 3 Representative computed tomography images illustrating response in patient with gallbladder cancer: Tumor shrinkage in cervical lymph node metastasis (**a**, **b**) and multiple liver metastases (**c**, **d**)

of IFO occurs via two major routes: (1) at the cyclic C-4, resulting in the formation of 4-OH-IFO, which is then decomposed to the cytotoxic metabolite IPM [13] and acrolein; and (2) through side-chain dechlorethylation, resulting in the formation of chloroacetaldehyde [14]. We have now shown that the plasma levels of IPM were smaller than those of D-19575 by a factor of ~25–30 in patients treated with D-19575. The $AUC_{0-\infty}$ for IPM increased in a dose-proportional manner within the range of 28.1–46.5 ng h/mL, values that are markedly higher than those achieved for IPM generated from IFO after bolus administration (mean of 11.9 ng h/mL) or continuous infusion (mean of 17.8 ng h/mL) in conventional treatment of soft tissue sarcoma [15]. These pharmacokinetic data suggest that infusion of D-19575 allows the safe achievement of higher concentrations of IPM compared with those achieved with standard IFO therapy. We also obtained no evidence of a relation between pharmacokinetic parameters and the occurrence of renal toxicity in the present study.

The intracellular uptake of D-19575 is mediated by the facilitative glucose transporters GLUT1 to GLUT5, the sodium-dependent glucose transporters SGLT1 and SGLT2, and, possibly, other transporter proteins. Increased rates of glucose transport and glycolysis are characteristic features of malignant transformed cells that result in part from overexpression of glucose transporters [16–19]. Overexpression of GLUT1 to GLUT3 has been detected in a wide range of human cancers, most prominently in those of breast, colon, and liver, with the extent of overexpression generally being inversely correlated with prognosis [20–22]. Its uptake mechanism, coupled with the increased metabolic rate of tumor cells, may contribute the chemotherapeutic activity of D-19575. The level of GLUT1 expression in tumors has been shown to be positively correlated with 2-[^{18}F]fluoro-2-deoxy-D-glucose uptake, semi-quantified as standardized uptake value in positron emission tomography [23–26], suggesting that the latter parameter may be a suitable noninvasive biomarker for the sensitivity of cancers to D-19575.

In conclusion, the results of our phase I study suggest that D-19575 can be safely administered at a dose of 4,500 mg/m^2 by infusion over 6 h every 3 weeks to Japanese patients with advanced solid malignancies. They further suggest that this drug may prove clinically effective when administered as a single agent. The AUCs for IPM generated by D-19575 were found to be markedly higher than those achieved by administration of IFO, suggesting that infusion of D-19575 allows the safe achievement of higher concentrations of IPM compared with those generated by administration of IFO according to widespread clinical practice. The safety profile and the potential broad-spectrum efficacy of D-19575 thus warrant additional clinical evaluation of this new alkylating agent.

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Effects of Src inhibitors on cell growth and epidermal growth factor receptor and MET signaling in gefitinib-resistant non-small cell lung cancer cells with acquired *MET* amplification

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The efficacy of epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors such as gefitinib and erlotinib in non-small cell lung cancer (NSCLC) is often limited by the emergence of drug resistance conferred either by a secondary T790M mutation of *EGFR* or by acquired amplification of the *MET* gene. We now show that the extent of activation of the tyrosine kinase Src is markedly increased in gefitinib-resistant NSCLC (HCC827 GR) cells with *MET* amplification compared with that in the gefitinib-sensitive parental (HCC827) cells. In contrast, the extent of Src activation did not differ between gefitinib-resistant NSCLC (PC9/ZD) cells harboring the T790M mutation of *EGFR* and the corresponding gefitinib-sensitive parental (PC9) cells. This activation of Src in HCC827 GR cells was largely abolished by the MET-TKI PHA-665752 but was only partially inhibited by gefitinib, suggesting that Src activation is more dependent on MET signaling than on EGFR signaling in gefitinib-resistant NSCLC cells with *MET* amplification. Src inhibitors blocked Akt and Erk signaling pathways, resulting in both suppression of cell growth and induction of apoptosis, in HCC827 GR cells as effectively as did the combination of gefitinib and PHA-665752. Furthermore, Src inhibitor dasatinib inhibited tumor growth in HCC827 GR xenografts to a significantly greater extent than did treatment with gefitinib alone. These results provide a rationale for clinical targeting of Src in gefitinib-resistant NSCLC with *MET* amplification. (*Cancer Sci* 2010; 101: 167–172)

Upregulation of the EGFR occurs frequently and is negatively correlated with prognosis in many types of human malignancy.[1,2] Recognition of the role of EGFR in carcinogenesis has prompted the development of EGFR-targeted therapies.[3] TKI of EGFR, such as gefitinib and erlotinib, both of which compete with ATP for binding to the tyrosine kinase pocket of the receptor, have been extensively studied in patients with NSCLC.[4] Sensitivity to these drugs has been correlated with the presence of somatic mutations that affect the kinase domain of EGFR, such as deletions in exon 19 and the L858R mutation in exon 21 of the *EGFR* gene.[5–15] However, the acquisition of an additional mutation (T790M) in exon 20 of *EGFR* results in the development of resistance to EGFR-TKI.[16–19] Irreversible EGFR-TKI are thought to be a potential therapeutic option for overcoming such resistance.[20,21] Amplification of the gene for the receptor tyrosine kinase MET has also recently been identified as a mechanism of gefitinib resistance, being detected in 22% of tumor samples from NSCLC patients with *EGFR* mutations who acquired gefitinib resistance.[22,23] Exposure of gefitinib-resistant NSCLC cells with *MET* amplification to the MET-TKI PHA-665752 or to gefitinib alone did not inhibit cell growth or survival signaling, given that both EGFR and MET signaling were found to be activated and to be mediated by ErbB3 (also known as Her3) in these cells.[22,23] However, the combination of gefitinib and PHA-665752 overcame gefitinib resistance attributable to *MET* amplification.[22,23] No single agent that overcomes such resistance has been identified to date.

The proto-oncogene *SRC* has been implicated in the development and poor clinical prognosis of several types of solid tumor as a result of the mediation by its product of signaling between integrins or receptor tyrosine kinases and their downstream effectors.[24–26] We have examined the potential role of Src in EGFR or MET signaling and whether Src inhibitors might block these signaling pathways in gefitinib-resistant NSCLC cells with *MET* amplification. We also evaluated the potential antitumor effect of Src inhibitors in order to provide insight into the mechanism by which such inhibitors might overcome gefitinib resistance in NSCLC cells with *MET* amplification.

Materials and Methods

Cell lines and reagents. The human NSCLC cell lines H1299, H460, HCC827, HCC827 GR5, HCC827 GR6, and PC9 were obtained as described previously.[22,27] H1838 and H820 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). EBC-1 cells were obtained from the Health Science Research Resources Bank (Tokyo, Japan). PC9/ZD cells were established as a gefitinib-resistant clone from PC9 cells as previously described[28] and were shown to harbor the T790M mutation of *EGFR* by both PCR invader and PCR clamp assays carried out as previously described.[29,30] HCC827, PC9, and PC9/ZD cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% FBS. HCC827 GR5 and HCC827 GR6 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1 μM gefitinib. Dasatinib was kindly provided by Bristol-Myers Squibb (New York, NY, USA), gefitinib was obtained from AstraZeneca (Macclesfield, UK), PP1 was from Biomol Research Laboratories (Plymouth Meeting, PA, USA), and PHA-665752 was from Tocris Bioscience (Bristol, UK).

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Immunoblot analysis. Immunoblot analysis was carried out as described previously.[27] Antibodies to the Y845-phosphorylated form of EGFR, to EGFR, to phosphorylated Erk, to Erk, to phosphorylated Akt, to Akt, and to β-actin as well as HRP-conjugated goat antibodies to mouse or rabbit IgG were obtained as described previously.[27] Antibodies to the Y1234/Y1235-phosphorylated form of MET, to the Y1289-phosphorylated form of ErbB3, to the Y416-phosphorylated form of Src, to Src, and to PARP were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies to MET were from Zymed (South San Francisco, CA, USA) and those to ErbB3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoprecipitation assay. Total cell lysates (500 μg protein) were incubated overnight at 4°C with 5 μg of a mouse monoclonal antibody (H-12) to total Src (Santa Cruz Biotechnology) in a final volume of 200 μL. The immune complexes were precipitated by further incubation for 2 h at 4°C with a suspension of protein G- and protein A-conjugated agarose (Calbiochem, Darmstadt, Germany). The immunoprecipitates were resolved by SDS-PAGE on a 7.5% gel, and the separated proteins were subjected to immunoblot analysis as described previously,[27] with the exception that the incubation with primary antibodies was carried out for 48 h.

Cell growth inhibition assay. Cells were plated in 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of tested drugs for 72 h. TetraColor One (5 mM tetrazolium monosodium salt and 0.2 mM 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA, USA). Absorbance values were expressed as a percentage of that for untreated cells.

Assessment of tumor growth inhibition *in vivo*. Tumor cells (2×10^6) were injected s.c. into the right hind leg of 7-week-old female athymic nude mice. The mice were divided into three treatment groups of five animals: those treated over 28 days by oral gavage daily of vehicle, gefitinib (50 mg/kg), or dasatinib (15 mg/kg). Treatment was initiated when tumors in each group achieved an average volume of 200 mm^3, with tumor volume being determined twice weekly after the onset of treatment from caliper measurement of tumor length (L) and width (W) according to the formula $LW^2/2$.

Statistical analysis. Data are presented as means ± SE as indicated and were analyzed by Student's t-test. A P-value of <0.05 was considered statistically significant.

Results

Src is activated in gefitinib-resistant NSCLC cells with *MET* amplification. Amplification of *MET* is one mechanism for the acquisition of resistance to EGFR-TKI in NSCLC.[22,23] To explore approaches that might overcome such resistance, we examined the activation status of several signaling molecules in sublines of the gefitinib-sensitive, *EGFR* mutation-positive human NSCLC cell line HCC827 that have acquired *MET* amplification and gefitinib resistance. Immunoblot analysis revealed that the level of phosphorylation (activation) of both MET and ErbB3 was markedly increased in the HCC827 GR5 and GR6 sublines compared with the parental HCC827 cells (Fig. 1A), consistent with previous observations.[22,23] Furthermore, we found that the level of Src activation was also markedly increased in HCC827 GR cells compared with HCC827 cells (Fig. 1A). Such Src activation was not observed in PC9/ZD cells (Fig. 1A), a subline of the gefitinib-sensitive, *EGFR* mutation-positive human NSCLC cell line PC9 that has acquired a secondary T790M mutation of *EGFR* and consequent gefitinib resistance. These results thus suggested that Src might contribute to gefitinib resistance in NSCLC cells with *MET* amplification. We also found that H1838, EBC-1, and H820 NSCLC cells with *MET* amplification[31–33] have higher activation of Src than that in NSCLC cells without *MET* amplification (H1299 and H460) (Fig. 1B). These results suggested that Src activation is associated with *MET* amplification in NSCLC cells.

Src activation blocked by a MET inhibitor in gefitinib-resistant NSCLC cells with *MET* amplification. Src associates with many receptor tyrosine kinases including EGFR and MET and transduces signals to a variety of downstream effectors of these receptors.[24–26,34–36] To examine whether Src participates in MET or EGFR signaling in cells with *EGFR* mutations and with or without *MET* amplification, we examined the effects of the MET inhibitor PHA-665752 or the EGFR-TKI gefitinib on Src

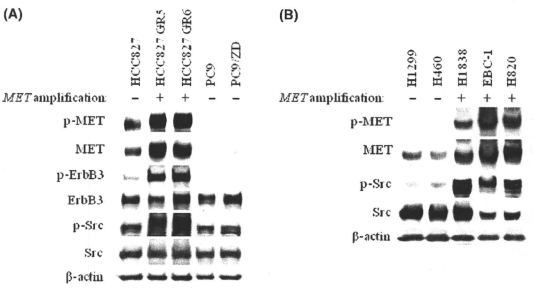

Fig. 1. Activation of Src in non-small cell lung cancer cells with or without *MET* amplification. (A) HCC827 cells, their gefitinib-resistant clones with *MET* amplification (HCC827 GR5 and GR6), PC9 cells, and their gefitinib-resistant clone with a secondary T790M mutation of epidermal growth factor receptor (PC9/ZD) were incubated for 24 h in medium containing 10% serum. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of MET, ErbB3, and Src as well as with those to β-actin (loading control). (B) H1299 and H460 cells without *MET* amplification, and H1838, EBC-1, and H820 cells with *MET* amplification were incubated for 24 h in medium containing 10% serum. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of MET and Src as well as with those to β-actin (loading control).

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activation in HCC827 and HCC827 GR cells. In the parental HCC827 cells, Src activity (phosphorylation) was reduced by PHA-665752 and was abolished by gefitinib (Fig. 2A). In contrast, Src activation was partially reduced by PHA-665752 and was inhibited to a much lesser extent by gefitinib in HCC827 GR5 cells (Fig. 2A). Combined treatment with gefitinib and PHA-665752 resulted in complete suppression of Src activation in both the parental and GR cells (Fig. 2A). These results suggested that Src activation is dependent on MET signaling to a greater extent than on EGFR signaling in gefitinib-resistant cells with *MET* amplification, whereas the opposite is the case for cells without *MET* amplification.

Effects of Src inhibitors on EGFR, ErbB3, and MET activation in gefitinib-resistant NSCLC cells with *MET* amplification. Src activates EGFR by phosphorylating the Y845 residue of the receptor,[37] and it also interacts with MET.[35] We therefore examined the effects of the Src inhibitors PP1 and dasatinib on EGFR, ErbB3, and MET activation. Both PP1 and dasatinib abolished EGFR activation and inhibited ErbB3 and MET activation in parental HCC827 cells (Fig. 2A). In contrast, these Src inhibitors did not suppress ErbB3 or MET activation and induced only partial inhibition of EGFR activation in HCC827 GR5 cells (Fig. 2A), suggesting that *MET* amplification affects the interactions of EGFR, ErbB3, and MET with Src.

Increased association between MET and Src in gefitinib-resistant NSCLC cells with *MET* amplification. We examined the effects of *MET* amplification on the physical association of Src with EGFR, MET, and ErbB3. Src was immunoprecipitated from both HCC827 and HCC827 GR5 cell lysates, and the resulting precipitates were subjected to immunoblot analysis with antibodies to EGFR, MET, ErbB3, or Src. The amount of MET associated with Src was greater for HCC827 GR5 cells than for HCC827 cells, whereas the amount of EGFR associated with Src was greater for HCC827 cells than for HCC827 GR5 cells (Fig. 2B). No association of ErbB3 with Src was apparent for either cell type. These results suggested that *MET* amplification results in an increase in the association between MET and Src, as well as a concomitant decrease in that between EGFR and Src, in HCC827 GR cells.

Src inhibitors block Akt and Erk signaling in gefitinib-resistant NSCLC cells with *MET* amplification. We next examined the effects of the Src inhibitors PP1 and dasatinib on Akt and Erk signaling pathways, both of which are activated by EGFR and MET. Both PP1 and dasatinib induced complete inhibition of Akt and Erk activation, as did gefitinib, in the parental HCC827 cells (Fig. 2A). Consistent with previous observations,[22,23] the combination of gefitinib and PHA-665752 inhibited Akt and Erk activation in HCC827 GR5 cells, whereas neither agent alone had such an effect (Fig. 2A). Both PP1 and dasatinib inhibited Akt and Erk activation to similar extents as the combination of gefitinib and PHA-665752 in HCC827 GR5 cells (Fig. 2A). A single agent (Src inhibitor) was thus sufficient to block Akt and Erk signaling, which is important for cell survival and proliferation, respectively, in gefitinib-resistant NSCLC cells with *MET* amplification.

Src inhibitor dasatinib suppresses growth of gefitinib-resistant NSCLC cells with *MET* amplification. The combination of gefitinib and PHA-665752 was recently shown to inhibit the growth of, and to induce apoptosis in, HCC827 GR cells with *MET* amplification, whereas neither agent alone had such an effect.[22,23] Given that we found that Src inhibitors block Akt and Erk signaling pathways as effectively as the combination of gefitinib and PHA-665752 in such cells, we examined whether dasatinib might overcome gefitinib resistance in HCC827 GR cells. In the parental HCC827 cells, both gefitinib and dasatinib as well as the combination of gefitinib and PHA-665752 effectively inhibited cell growth, but PHA-665752 alone had less inhibitory effect (Fig. 3A). Dasatinib inhibited cell growth in a concentration-dependent manner by the same marked extent as the combination of gefitinib and PHA-665752, even in HCC827 GR5 cells, whereas neither gefitinib nor PHA-665752 alone had a substantial effect (Fig. 3B). We also examined the effect of dasatinib on apoptosis as assessed on the basis of cleavage of the enzyme PARP in both HCC827 and HCC827 GR5 cells. Dasatinib (but not gefitinib) induced apoptosis in HCC827 GR5 cells to the same marked extent as did the combination of gefitinib and PHA-665752, whereas dasatinib and gefitinib each induced apoptosis in the parental HCC827 cells (Fig. 3C). These results suggested that Src inhibitors efficiently induce growth inhibition and apoptosis in gefitinib-resistant NSCLC cells with *MET* amplification.

Src inhibitor dasatinib inhibits tumor growth in gefitinib-resistant NSCLC xenografts with *MET* amplification. To determine whether the efficacy of dasatinib in gefitinib-resistant NSCLC cells with *MET* amplification observed *in vitro* might also be apparent *in vivo*, we examined the antitumor effects of dasatinib in nude mice with solid tumors formed by HCC827 GR5 cells injected into the right hind leg. Gefitinib (50 mg/kg)

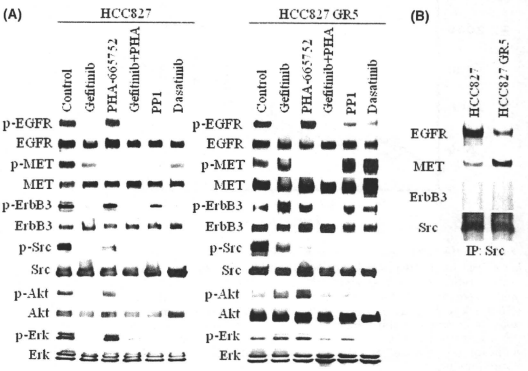

Fig. 2. Effects of various inhibitors on epidermal growth factor receptor (EGFR) and MET signaling in gefitinib-resistant non-small cell lung cancer cells with *MET* amplification. (A) HCC827 cells and a gefitinib-resistant clone with *MET* amplification (HCC827 GR5) were incubated for 12 h in the absence (control) or presence of gefitinib alone (1 μM), PHA-665752 alone (1 μM), gefitinib and PHA-665752 combined, PP1 (10 μM), or dasatinib (500 nM) in medium containing 10% serum. Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of EGFR, MET, ErbB3, Src, Akt, and Erk. (B) HCC827 and HCC827 GR5 cells were incubated for 24 h in medium containing 10% serum, lysed, and subjected to immunoprecipitation (IP) with an antibody to Src. The resulting precipitates were subjected to immunoblot analysis with antibodies to EGFR, MET, ErbB3, and Src.

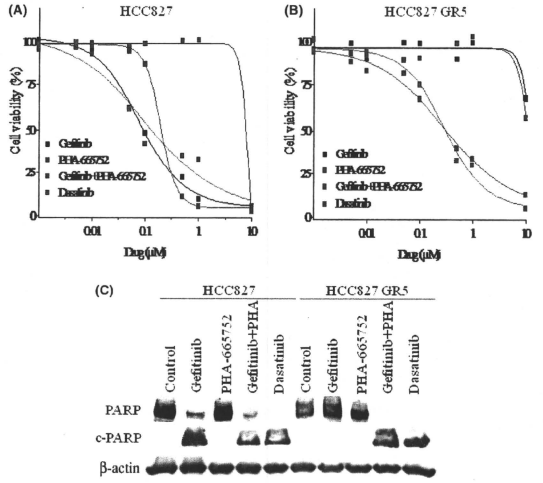

Fig. 3. Effects of dasatinib on growth and apoptosis in gefitinib-resistant non-small cell lung cancer cells with *MET* amplification. (A) HCC827 cells or (B) HCC827 GR5 cells were treated for 72 h with increasing concentrations of gefitinib alone, PHA-665752 alone, gefitinib and PHA-665752 in combination, or dasatinib alone in medium containing 10% serum, after which cell viability was assessed. Data are means of triplicates from a representative experiment and are expressed as a percentage of the value for untreated cells. (C) HCC827 and HCC827 GR5 cells were incubated for 72 h with gefitinib (1 μM) alone, PHA-665752 (1 μM) alone, gefitinib plus PHA-665752, or dasatinib (1 μM) in medium containing 10% serum. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to poly(ADP-ribose) polymerase (PARP) and to β-actin. The positions of intact PARP (116 kDa) and the 85-kDa cleavage fragment (c-PARP) are shown.

could not reduce tumor size compared with vehicle treatment (Fig. 4). In contrast, dasatinib (15 mg/kg) inhibited tumor growth in HCC827 GR5 xenografts to a significantly greater extent than did treatment with gefitinib or vehicle alone (Fig. 4). These results indicated that Src inhibitor effectively exerts anti-tumor effects in gefitinib-resistant NSCLC xenografts with *MET* amplification.

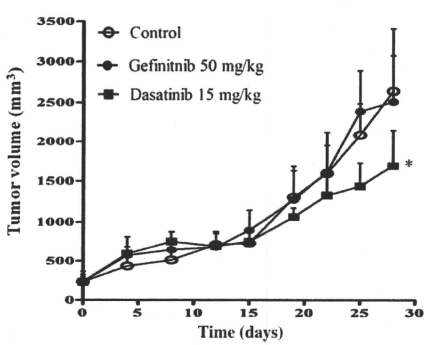

Fig. 4. Effects of dasatinib on the growth of gefitinib-resistant non-small cell lung cancer cells with *MET* amplification *in vivo*. Nude mice with tumor xenografts established by s.c. implantation of HCC827 GR5 cells were treated daily for 28 days with vehicle (control), gefitinib (50 mg/kg), or dasatinib (15 mg/kg) by oral gavage. Tumor volume was determined at the indicated times after the onset of treatment. Points indicate the mean of values from five mice per group; bars indicate SE. $*P < 0.05$ for dasatinib *versus* control or gefitinib alone (Student's *t*-test).

Discussion

The emergence of *MET* amplification induces ErbB3-dependent downstream signaling mediated by Akt and Erk that is important for cell survival and proliferation, ultimately leading to the development of gefitinib resistance, in NSCLC cells with *EGFR* mutations.[22,23] Although the combination of the specific MET inhibitor PHA-665752 and gefitinib is considered promising for overcoming gefitinib resistance due to *MET* amplification, a single-agent therapy to overcome such resistance would be more desirable.[22,23] We have shown that, in addition to MET activation, Src is markedly activated in NSCLC cells with *MET* amplification, including HCC827 GR cells. Forced expression of Src has previously been shown to result in gefitinib resistance in gallbladder adenocarcinoma cells[38] and to promote tumorigenesis in EGFR-overexpressing mammary epithelial cells.[39] In addition, MET and Src cooperate to mediate proliferation of breast cancer cells in the presence of EGFR-TKI.[34] Consistent with these previous observations, our results now suggest that Src contributes to gefitinib resistance in NSCLC cells with *MET* amplification and is a potential target molecule for overcoming such resistance.

To explore how Src activation affects MET or EGFR signaling in gefitinib-resistant NSCLC cells with *MET* amplification, we examined the effects of Src inhibitors on EGFR, ErbB3, and MET activation in both HCC827 and HCC827 GR5 cells. Gefitinib was previously shown to inhibit ErbB3 and MET activation as well as EGFR activation in the parental HCC827 cells,[22,23,40] suggestive of a functional interaction between EGFR and both ErbB3 and MET in *EGFR*-mutant NSCLC cells without *MET* amplification (Fig. 5A). In contrast, gefitinib did not inhibit ErbB3 or MET activation in HCC827 GR cells, with the combination of gefitinib and PHA-665752 being necessary to achieve inhibition of ErbB3 activation in these cells with *MET* amplification.[22,23] In addition, endogenous ErbB3 was co-immunoprecipitated with MET from HCC827 GR cells

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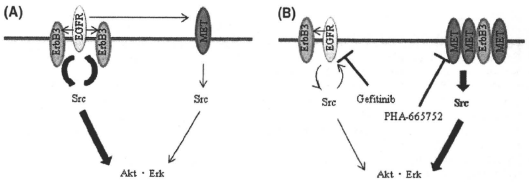

Fig. 5. Models for signaling pathways in gefitinib-sensitive non-small cell lung cancer (NSCLC) cells (A) and gefitinib-resistant NSCLC cells with acquired *MET* amplification (B). Src functions downstream of both epidermal growth factor receptor (EGFR) and MET as well as upstream of Akt and Erk signaling pathways and EGFR. However, the dependency of Src signaling is shifted from EGFR to MET and MET associates with ErbB3 after the acquisition of *MET* amplification. EGFR mediates, at least in part, activation of MET in gefitinib-sensitive NSCLC cells, whereas EGFR and MET function independently of each other in gefitinib-resistant NSCLC cells with acquired *MET* amplification. Pathways targeted by gefitinib or PHA-665752 are indicated, and the relative activities of signaling pathways are denoted by the width of the arrows.

but not from HCC827 cells.[22,23] These previous results thus suggested that ErbB3 signaling becomes more dependent on MET than on EGFR after emergence of *MET* amplification, and that the MET-ErbB3 signaling complex is largely independent of EGFR signaling (Fig. 5B).[22,23] We have shown that Src inhibitors reduced the extent of EGFR activation in both HCC827 and HCC827 GR5 cells, consistent with previous observations showing that Src mediates EGFR activation by phosphorylating its Y845 residue.[37,41] In HCC827 GR5 cells, however, Src inhibitors did not inhibit ErbB3 or MET activation, despite it doing so in the parental HCC827 cells. These results support the notion that MET signaling is independent of EGFR signaling as a result of the shift of the dependence of ErbB3 signaling from EGFR to MET in HCC827 GR cells (Fig. 5B).[22,23]

We examined whether *MET* amplification affects the physical association between Src and either EGFR, MET, or ErbB3 by immunoprecipitation. The association between MET and Src was increased in HCC827 GR5 cells compared with that in HCC827 cells, whereas the association between EGFR and Src was reduced in HCC827 GR5 cells. These findings are consistent with our results showing that PHA-665752 blocks Src activation to a greater extent in HCC827 GR5 cells than in HCC827 cells, a pattern opposite to that for the effects of gefitinib (Fig. 5). The mechanism of increased association between MET and Src induced by acquired *MET* amplification has remained unclear. It is possible that *MET* amplification alters the protein expression which mediates binding of Src to MET. On the basis of the notion that Src is activated downstream of MET signaling in HCC827 GR cells, we examined the effects of Src inhibitors in these cells on Akt and Erk signaling pathways, both of which are known to be activated by Src.[24–26,42] We have shown that Src inhibitors markedly inhibited Akt and Erk signaling pathways in gefitinib-resistant NSCLC cells with *MET* amplification. Previous studies found that neither gefitinib nor PHA-665752 alone blocked Akt or Erk pathways in HCC827 GR cells,[22,23] with the combination of both of these agents being necessary for such inhibition, consistent with the notion that Akt and Erk pathways are dependent on both EGFR and MET signaling in these cells (Fig. 5B). We observed that gefitinib and PHA-665752 each induced a slight increase in the phosphorylation levels of Akt in HCC827 GR5 cells (Fig. 2A), possibly because EGFR and MET pathways functionally compensate for each other when either is inhibited. Our results suggest that Src functions downstream of both EGFR and MET, but that it is mainly dependent on MET signaling in HCC827 GR cells. Together, our observations explain the ability of Src inhibitors to suppress Akt and Erk activation in gefitinib-resistant NSCLC cells with *MET* amplification (Fig. 5B).

Finally, we found that Src inhibitor dasatinib also inhibited the growth of HCC827 GR5 cells as well as did combined treatment with gefitinib and PHA-665752. HCC827 GR5 cells underwent apoptosis, as detected by PARP cleavage, after treatment with dasatinib. Furthermore, dasatinib inhibited tumor growth in HCC827 GR5 xenografts to a significantly greater extent than treatment with gefitinib alone. Our present data suggest that Src inhibitors might overcome gefitinib resistance in NSCLC patients with *MET* amplification. Our findings strengthen the rationale of the ongoing clinical trial of dasatinib for NSCLC patients who no longer respond to erlotinib or gefitinib (http://www.clinicaltrials.gov). The results of this clinical trial should provide insight into the relation between the efficacy of Src inhibitors and whether gefitinib resistance is attributable to the secondary T790M mutation of *EGFR* or to acquired *MET* amplification.

Abbreviations

EGFR	epidermal growth factor receptor
NSCLC	non-small cell lung cancer
PARP	poly(ADP-ribose) polymerase
TKI	tyrosine kinase inhibitor

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Cisplatin and Etoposide Chemotherapy Combined with Early Concurrent Twice-daily Thoracic Radiotherapy for Limited-disease Small Cell Lung Cancer in Elderly Patients

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Objective: The optimal management of elderly patients with limited-disease small cell lung cancer (LD-SCLC) has not been established.
Methods: The records of elderly (≥70 years of age) patients with LD-SCLC who had been treated with etoposide and cisplatin chemotherapy with early concurrent twice-daily thoracic radiotherapy (TRT) were reviewed retrospectively.
Results: Of the 25 elderly patients with LD-SCLC identified, 12 (48%) individuals received etoposide–cisplatin chemotherapy with early concurrent twice-daily TRT. The main toxicities of this treatment regimen were hematologic, with neutropenia of Grade 4 being observed in all patients and febrile neutropenia of Grade 3 in eight patients during the first cycle of chemoradiotherapy. The toxicity of TRT was acceptable, with all patients completing the planned radiotherapy within a median of 29 days (range, 19–33). No treatment-related deaths were observed. The median progression-free survival and overall survival times were 14.2 months (95% confidence interval, 4.3–18.2) and 24.1 months (95% confidence interval, 11.3–27.2), respectively.
Conclusions: Etoposide–cisplatin chemotherapy with early concurrent twice-daily TRT was highly myelotoxic in elderly patients with LD-SCLC, although no treatment-related deaths were observed in our cohort. Prospective studies are required to establish the optimal schedule and dose of chemotherapy and TRT in such patients.

Key words: elderly – small cell lung cancer – chemoradiotherapy – cisplatin – etoposide – concurrent thoracic radiotherapy

INTRODUCTION

Small cell lung cancer (SCLC) accounts for 10–15% of all lung cancer cases, with individuals aged 70 years or older constituting up to 25–40% of the SCLC patients (1,2). Limited-disease (LD) SCLC is a disease that is confined to one hemithorax and its regional lymph nodes and which can be encompassed by a single radiation therapy port. About 30–40% of all SCLC patients present with LD-SCLC (1,2). The proportion of elderly SCLC patients continues to increase with the growing geriatric population (1,3).

The combination of radiotherapy and chemotherapy, specifically etoposide and cisplatin chemotherapy with early concurrent twice-daily thoracic radiotherapy (TRT), is now regarded as the standard treatment for LD-SCLC (4). However, many clinical trials of potential new treatments for LD-SCLC have excluded elderly patients for various reasons, such as the presence of concomitant chronic illness, a decline in organ function that may interfere with drug clearance and possible decreased bone marrow tolerance to myelosuppressive agents (5). The optimal management of elderly patients with LD-SCLC has therefore not been defined to date.

We have now performed a retrospective analysis to evaluate patient characteristics as well as treatment delivery, toxicity and antitumor efficacy for elderly individuals (70 years or

older) with LD-SCLC who were treated with etoposide and cisplatin chemotherapy and early concurrent twice-daily TRT.

PATIENTS AND METHODS

We retrospectively evaluated the records of elderly ($\geq$70 years) patients with LD-SCLC who were treated at Kinki University School of Medicine from January 2003 to December 2008. All patients had a pathological diagnosis of SCLC. LD-SCLC was defined as cancer that is confined to one hemithorax including contralateral mediastinal and hilar lymph nodes as well as ipsilateral or bilateral supraclavicular lymph nodes, but excluding malignant pleural effusion. Response evaluation was assessed after completion of treatment on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST). Laboratory testing and toxicities were graded weekly during the whole treatment according to the National Cancer Institute—Common Terminology Criteria for Adverse Events (NCI-CTCAE, version 3). Progression-free survival time was measured from the date of initiation of treatment to the date of disease progression. Overall survival time was measured from the date of initiation of treatment to death or to the time that the patient was last known to be alive. After completion of all treatment, patients were followed up at 1- to 2-month intervals until the time of progression or death. Median progression-free survival time and overall survival time were estimated by the Kaplan–Meier method.

RESULTS

Patient Characteristics

Of the 170 SCLC patients treated between 2003 and 2008, 48 individuals were diagnosed with LD-SCLC and 25 of these individuals were 70 years of age or older. Among these 25 patients, 12 (48%) elderly patients with LD-SCLC received etoposide and cisplatin chemotherapy with early concurrent twice-daily TRT. The characteristics of these 12 patients are shown in Table 1. They included eight men and four women as well as seven individuals aged between 70 and 74 years and five aged 75 years or older. All the patients were in good general condition, although they had some complications. The remaining 13 patients' characteristics are shown in Table 2. Two of the 13 elderly patients with LD-SCLC were treated with chemotherapy and sequential TRT, and 1 patient was treated with etoposide–carboplatin and concurrent TRT. Chemotherapy alone was administered in 4 of the 13 patients. Two patients were subjected to surgery followed by chemotherapy. Four patients did not receive intensive therapy.

Treatment Delivery

The treatment plan consisted of an initial cycle of concurrent chemoradiotherapy followed by three cycles of consolidation chemotherapy (Table 3). All patients received the same chemotherapy regimen of cisplatin at 40–80 mg/m^2 on day 1 combined with etoposide at 80–100 mg/m^2 on days 1–3. Twice-daily TRT was performed with X-rays at 6–10 MV and with an interval of at least 6 h and a total dose of 45 Gy (1.5 Gy bid) over 3 weeks. TRT was initiated on day 1 of the first cycle of chemotherapy. All patients completed the TRT protocol, with the days of irradiation ranging from 19 to 33 (median of 29). Reasons for a delay in TRT included febrile neutropenia of Grade 3 in eight patients and leukopenia of Grade 4 in three patients. All patients proceeded to consolidation chemotherapy. However, five patients (42%) did not complete the planned three cycles of consolidation

Table 1. Characteristics of the study cohort

Patient	Age/sex	TNM stage	PS	Complications	Smoking history
1	70/M	T2N1M0	1	HT	20/day × 50 years
2	70/M	T3N1M0	0	Berger disease, old TB	40/day × 50 years
3	71/M	T3N2M0	0	DM, bladder cancer	20/day × 50 years
4	71/M	T1N2M0	1	Harada disease	20/day × 50 years
5	72/F	T2N2M0	1	HT, old TB, asthma, one kidney	20/day × 35 years
6	72/M	T1N2M0	0	HT, hyperlithuria	10/day × 50 years
7	73/M	T1N2M0	1	HT	25/day × 60 years
8	76/M	T2N1M0	0	None	20/day × 50 years
9	77/F	T3N0M0	1	Deafness	15/day × 57 years
10	78/M	T3N0M0	0	DM, ASO, old TB	20/day × 58 years
11	79/F	T2N2M0	1	None	None
12	79/F	T1N2M0	0	HT	5/day × 50 years

PS, Eastern Cooperative Oncology Group performance status; HT, hypertension; TB, tuberculosis; DM, type 2 diabetes mellitus; ASO, arteriosclerosis obliterans.

Table 2. Characteristics of patients who did not received EP with concurrent TRT

Patient	Age/sex	TNM stage	PS	Complications	Treatment	Reason[a]
1	70/M	T4N2M0	1	HT, renal dysfunction	CE and sequential TRT	Complication
2	70/M	T1N0M0	1	HT, DM	Surgery	Physician's decision
3	71/M	T3N2M0	1	HT	Best supportive care	Patient's refusal
4	72/M	T2N1M0	1	DM, renal dysfunction	CE and concurrent TRT	Complication
5	74/M	T3N2M0	1	HT, renal dysfunction	CE and sequential TRT	Complication
6	74/M	T2N1M0	2	DM, IP, chronic renal failure, dialysis, old TB	Chemotherapy	Complication
7	75/M	T3N2M0	3	HCC, chronic HCV	Best supportive care	Complication
8	77/M	T2N1M0	2	renal dysfunction, dementia	Chemotherapy	Complication
9	78/M	T1N1M0	1	SSS, HT, DM	Chemotherapy	Physician's decision
10	81/M	T2N2M0	1	renal dysfunction	Chemotherapy	Patient's refusal
11	82/M	T1N2M0	1	HT	Surgery	Physician's decision
12	84/M	T2N0M0	2	HT	Best supportive care	Patient's refusal
13	84/M	T2N0M0	2	HT, asthma, heart failure, cerebral infarction	Best supportive care	Complication

EP, etoposide and cisplatin; TRT, thoracic radiotherapy; CE, carboplatin and etoposide; IP, interstitial pneumonia; HCC, hepatic cancer; HCV, hepatitis C virus; SSS, sick sinus syndrome.
[a]The reason for not to select the combination therapy of etoposide and cisplatin with early concurrent TRT.

chemotherapy because of the development of pneumonitis of Grade 3 in one patient, a decline in renal function in one patient, suspected invasive aspergillosis in one patient and refusal by two patients. A dose reduction was necessary in seven patients because of the development of febrile neutropenia of Grade 3 in three patients, leukopenia of Grade 4 in two patients and nausea–vomiting of Grade 3 in two patients. The actual dose intensities of cisplatin and etoposide were 13.7 mg/m^2/week (68.7% of the planned dose intensity) and 52.4 mg/m^2/week (69.9% of the planned dose intensity), respectively.

Toxicities

Reported toxicities during the concurrent chemoradiotherapy are listed in Table 4. Leukopenia and neutropenia of Grade 3 or 4 were observed in all patients (100%), and eight patients (67%) had febrile neutropenia of Grade 3. Thrombocytopenia of Grade 3 or 4 was apparent in three patients (25%), with one patient requiring platelet transfusion. Reported toxicities during the consolidation chemotherapy are listed in Table 5. Leukopenia and neutropenia of Grade 3 or 4 were observed in 8 (67%) and 11 (92%) patients, respectively, and 4 patients (33%) developed febrile neutropenia of Grade 3. Anemia and thrombocytopenia of Grade 3 or 4 were each observed in four patients (33%). The major non-hematologic toxicity observed during the entire treatment period was nausea–vomiting. None of the patients developed esophagitis of Grade 3 or 4, but one patient manifested radiation pneumonitis of Grade 3 during consolidation chemotherapy. There were no treatment-related deaths.

Response and Survival

All 12 patients were evaluated for progression-free survival and overall survival. With a median follow-up time of 23.1 months (ranged, 7.2–45.0 months), six patients were still alive. An objective tumor response was observed in all patients: a complete response (CR) in five patients and a partial response in seven patients (Table 3). Prophylactic cranial irradiation was not routinely administered and delivered to three patients who achieved CR after completion of the planned treatment. The median progression-free survival time was 14.2 months, and the median overall survival time was 24.1 months.

Pattern of Relapse

Seven of the 12 patients relapsed, 3 with local regional failure inside the radiation field and 4 with distant failure. Among the latter four patients, three individuals manifested metastases in the brain as the sole site and the remaining individual had both local and distant failure including the liver.

DISCUSSION

Two meta-analyses have shown that the combined modality of chemotherapy and TRT improves the survival of individuals with LD-SCLC in comparison with chemotherapy alone (6,7). The schedule, dose and fractionation of TRT have been extensively investigated in patients with LD-SCLC in several randomized controlled trials (8,9). On the basis of two pivotal Phase III trials (10,11), etoposide and cisplatin

Table 3. Treatment details and outcome for the study cohort

Patient	Regimen (mg/m^2)	Total no. of cycles	Dose reduction in consolidation chemotherapy	DI of P (mg/m^2/week)	RDI of P (%)	DI of E (mg/m^2/week)	RDI of E (%)	Duration of TRT (days)	V20 (%)	Response	PFS (months)	Survival time (months)
1	E(100) + P(40)[a] + TRT	2	No	5.0	25.0	37.5	50.0	23	21	CR	14.0+	14.0+
2	E(100) + P(80) + TRT	4	Yes	17.2	86.0	73.7	98.2	19	25	CR	7.3+	7.3+
3	E(100) + P(80) + TRT	4	Yes	17.8	89.1	68.7	91.6	27	35	CR	10.7+	10.7+
4	E(100) + P(80) + TRT	4	Yes	15.7	78.4	61.6	82.1	30	13	PR	9.3	22.2
5	E(100) + P(80) + TRT	2	No	9.5	47.5	35.6	47.5	29	20	PR	4.3	11.4
6	E(100) + P(80) + TRT	4	No	19.6	98.2	73.7	98.2	29	30	PR	18.2	48.1+
7	E(100) + P(80) + TRT	4	No	17.2	86.2	64.6	86.2	26	27	PR	13.1	26.1+
8	E(80)[b] + P(80) + TRT	3	Yes	11.4	56.9	39.4	52.5	30	21	PR	8.3	17.1
9	E(100) + P(60)[a] + TRT	4	Yes	14.4	71.8	61.0	81.4	30	25	CR	20.6+	20.6+
10	E(100) + P(80) + TRT	4	Yes	13.1	65.5	49.1	65.5	28	NA	PR	14.4	16.5
11	E(100) + P(80) + TRT	2	Yes	7.9	39.5	30.5	40.6	33	26	PR	3.9	14.8
12	E(100) + P(80) + TRT	2	No	9.9	49.6	37.2	49.6	29	22	CR	14.1	27.2

DI, dose intensity; P, cisplatin; RDI, relative dose intensity; E, etoposide; V20, the percentage of lung volume receiving >20 Gy; PFS, progression-free survival; CR, complete response; +, without event; PR, partial response; NA, not available.
[a]Dose reduction because of a decline in renal function.
[b]Dose reduction because of physician's decision.

Table 4. Toxicities during concurrent chemoradiotherapy

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Grade 3 or 4 (%)
Leukopenia	0	0	2	10	100
Neutropenia	0	0	0	12	100
Anemia	2	1	0	0	0
Thrombocytopenia	0	2	2	1	25
Febrile neutropenia	—	—	8	0	67
Nausea–vomiting	2	2	2	0	17
Esophagitis	1	3	0	0	0
Appetite loss	5	2	2	0	17

Table 5. Toxicities during consolidation chemotherapy

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Grade 3 or 4 (%)
Leukopenia	0	2	4	4	67
Neutropenia	0	0	2	9	92
Anemia	2	4	3	1	33
Thrombocytopenia	2	2	2	2	33
Febrile neutropenia	—	—	4	0	33
Nausea–vomiting	2	5	2	0	17
Appetite loss	4	1	1	0	8
Radiation pneumonitis	3	0	1	0	8

chemotherapy with early concurrent twice-daily TRT is currently considered the standard treatment for patients with LD-SCLC. An age-specific subset analysis of one of these Phase III trials (11), in which patients received etoposide–cisplatin with early concurrent TRT, showed that the survival outcomes for individuals aged 70 years or older were similar to those of their younger counterparts, although the elderly patients experienced greater toxicity, in particular hematologic toxicity (12). However, given that the patients in this subgroup analysis were assigned either once- or twice-daily TRT, the significance of early concurrent twice-daily TRT in the management of elderly patients with LD-SCLC has remained undefined. No specific Phase III trial of elderly patients with LD-SCLC has been reported. We therefore retrospectively analyzed the feasibility and antitumor efficacy of etoposide–cisplatin chemotherapy with early concurrent twice-daily TRT for treatment of LD-SCLC in patients aged 70 years or older.

The median overall survival time of 24.1 months in our cohort is similar to that described for non-elderly patients with LD-SCLC in previous studies (10,11). This favorable survival outcome may be attributable to the strict selection of elderly patients in good general condition; all 12 patients in the present study had normal organ function, an Eastern

Cooperative Oncology Group performance status of 0 or 1 and no severe co-morbidity. Given that the elderly are more likely to have reduced organ function as well as concomitant morbidities or medications, the general condition of elderly SCLC patients is worse than that of younger patients (1). Among LD-SCLC patients, increasing age was found to be significantly associated with a lower likelihood of receiving combined chemoradiotherapy (7). Indeed, in the present study, only 12 (48%) of the 25 identified elderly patients with LD-SCLC were treated with etoposide–cisplatin and early concurrent twice-daily TRT.

Despite the strict selection of patients, highly treatment-related toxicity was observed in our cohort. The major adverse events were hematologic toxicities, with neutropenia of Grade 4 being apparent in all patients (100%) and febrile neutropenia of Grade 3 in eight patients (67%) during the first cycle of concurrent chemoradiotherapy. The previous analysis of the outcome of elderly patients in the Phase III study in which individuals received etoposide–cisplatin chemotherapy with early concurrent once- or twice-daily TRT found statistically significant differences not only in the incidence of hematologic toxicity (Grade 4 or 5: 61% in younger patients vs. 84% in patients aged 70 years or older, $P < 0.01$) but also in that of treatment-related deaths (1% vs. 10%, respectively, $P = 0.01$) (12). Although no treatment-related deaths were observed in the present study, severe hematologic toxicity was consistent with that in this foregoing analysis (12). In addition, maintenance of the optimal dose intensity of chemotherapy was difficult in our cohort because of frequent dose reductions or treatment delays due to hematologic or infection-related toxicities. Indeed, the actual dose intensity was <70% of the planned dose intensity for both etoposide and cisplatin in the present study, a value much smaller than that for non-elderly patients in a previous Phase III study (>90% for both agents) (10). On the other hand, the toxicity of radiotherapy was acceptable in our study, with all patients completing TRT within a median of 29 days (range, 19–33). None of our patients developed radiation esophagitis of Grade 3 or higher. With regard to pulmonary complications, one patient developed radiation pneumonitis of Grade 3. A recent meta-analysis of randomized trials in which patients with LD-SCLC were treated with chemoradiotherapy reported that the time between the first day of chemotherapy and the last day of radiotherapy was an important prognostic factor for LD-SCLC, with the survival advantage being more pronounced if the TRT was completed in <30 days (13). In the present study, a shorter time to completion of TRT may also be associated with our favorable survival outcome. However, elderly patients with LD-SCLC must be carefully selected and monitored during treatment because of the increased potential for the development of treatment-related morbidity and mortality.

The optimal therapeutic strategy for elderly patients with LD-SCLC remains a matter of debate. Despite the highly treatment-related toxicity, patients in our cohort derived a survival benefit with no treatment-related deaths, suggesting that the full-dose chemoradiotherapy may represent a valid option for 'fit' elderly patients with adequate organ function. Since the general condition of elderly patients varies widely from patients to patients, prospective evaluation and definition of 'fit' elderly patients who are candidates for full-dose chemoradiotherapy are important. Research is also needed to develop modified chemoradiotherapy regimens that are less toxic for the elderly. A modified chemotherapy schedule designed to reduce toxicity for elderly patients with LD-SCLC was evaluated in a Phase II trial, with two cycles of a chemotherapy regimen (oral etoposide and carboplatin) combined with early concurrent twice-daily TRT being found to have acceptable toxicity and to produce promising results, with a 5-year survival rate of 13% (14). A recent Phase III trial specifically designed for elderly or poor-risk patients with extensive-disease SCLC found that split doses of cisplatin plus etoposide (cisplatin at 25 mg/m^2 and etoposide at 80 mg/m^2 on days 1–3) could be safely administered and were effective (15). Such split-dose chemotherapy might also be suitable for the treatment of patients with LD-SCLC. We are currently conducting a clinical trial to evaluate the feasibility of etoposide at 80 mg/m^2 and cisplatin at 25 mg/m^2 on days 1–3 with early concurrent twice-daily TRT for elderly patients with LD-SCLC.

The overall findings of the present study suggest that administration of full-dose chemotherapy and early concurrent twice-daily TRT is highly myelotoxic for elderly patients with LD-SCLC. Development and assessment of modified treatment regimens with reduced toxicity are thus warranted for such patients.

Conflict of interest statement

None declared.

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