Phase I study of salazosulfapyridine in combination with cisplatin and pemetrexed for advanced non-small-cell lung cancer

Kohei Otsubo,1 Kaname Nosaki,2 Chiy o. K. Inamura,2 Hiroaki Ogata,1 Akitaka Fujita,1 Shinuya Sakata,1 Fumihiko Hira,2 Gouji Toyokawa,2 Eiji lwama,1 Taishi Harada,1 Takashi Seto,2 Mitsuhiro Takenoyama,2 Takeshi Ozeki,4 Taisei Mushiroda,4 Mieko Inada,2 Junji Kishimoto,5 Kenji Tsuchihashi,6 Kentaro Suina,6 Osamu Nagano,6 Hideyuki Saya,6 Yoichi Nakanishi1,5 and Isamu Okamoto1

1Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyush u University, Fukuoka; 2Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka; 3Department of Clinical Pharmacokinetics and Pharmacodynamics, Keio University School of Medicine, Tokyo; 4Laboratory for Pharmacogenomics, RIKEN Center for Integrative Medical Sciences, Yokohama City; 5Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka; 6Division of Gene Regulation, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan

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Correspondence
Isamu Okamoto, Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Tel: +81-92-642-5378; Fax: +81-92-642-5390; E-mail: okamotoi@kokyu.med.kyushu-u.ac.jp

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Cancer stem cells constitute a small population of cancer cells that are capable of self-renewal and cancer initiation.1 Cancer stem cells are also more resistant to cancer treatments, including chemotherapy and radiation therapy, than are other cancer cells.1,2,3 Treatment strategies that target CSCs are therefore being pursued in order to improve outcomes in cancer patients. Markers for CSCs are thought to include CD44, CD133, CD90, and aldehyde dehydrogenase, but no agents that specifically target CSCs have yet been established.4

CD44 is an adhesion molecule for the ECM and is implicated in various physiological and pathological processes, including tumor cell growth, invasion, and metastasis.5,6 It has also been identified as a cell surface marker for CSCs of solid tumors.5 Splice variant isoforms of CD44 are expressed in various tumors7–10 and have recently been found to bind to xCT, a subunit of a cystine-glutamate antiporter known as system xc(−). Such binding stabilizes expression of this transporter system at the cell surface and thereby promotes the intracellular synthesis of the antioxidant GSH from imported cystine. Expression of CD44v in tumor cells thus confers resistance to oxidative stress and thereby promotes tumor growth and treatment resistance.11 The stem cell-like characteristics of CD44v-positive cancer cells are dependent on xCT-mediated cystine transport and consequent upregulation of the GSH-dependent antioxidant system for maintenance of cellular redox homeostasis. Non-small-cell lung cancer is the leading cause of cancer-related mortality worldwide.12 The standard first-line treatment for advanced NSCLC has long been platinum-based combination chemotherapy, with the goal of prolongation of life and relief of symptoms.13,14 Recently, however, the identification of driver oncopgenes, such as those encoding mutant forms of EGFR and fusions of ALK, has led to the development of targeted agents such as EGFR-TKIs and ALK-TKIs that have proven superior to chemotherapy for first-line management of patients with advanced NSCLC positive for these.
that resulted in a treatment compliance rate of 70% during a
21-day treatment cycle. Objective tumor response was assessed
according to RECIST (version 1.1)(20) every 6 weeks for the first 6 months and every 9 weeks thereafter. Progression-free
survival was assessed for each patient who received at least
done dose of SASP.
Pharmacokinetic analysis. For pharmacokinetic analysis, the first individual dose of SASP on day 1 was administered orally
together with 200 mL water after the patients had fasted
overnight, with a meal being permitted after blood sampling at 4 h
after the drug was given. Blood samples were obtained before
and at 0.5, 1, 2, 3, 4, 6, 9, 12, and 24 h after SASP administra-
tion. The other two doses of SASP were not given on day 1.
Plasma was prepared from blood by centrifugation and was
stored at −20°C until analysis. The plasma concentration of
SASP was determined by a validated ultraperformance liquid
chromatography and tandem mass spectrometry method.(21)
The AUC0.24 for SASP was calculated according to the linear
trapezoidal rule.
Genotyping of ABCG2 and NAT2. The single nucleotide polymor-
morphism rs2231142 in ABCG2 (421C→A, Q141K) and NAT2
genotype (NAT2*4, *5B, *6A, *7B), both of which affect the
pharmacokinetics of SASP, were evaluated by direct sequenc-
ing of genomic DNA isolated from blood samples.
Measurement of free CD44v protein level in serum. Serum samples collected from 14 patients before treatment and on
day 21 of treatment cycle 1 were tested with ELISA for human
CD44v9 (Cosmo Bio, Tokyo, Japan). The amount of free
CD44v in serum was determined relative to the absorbance of
1 μL culture supernatant of the CD44v9-positive cell line OSC
19 as 1 unit.
Statistical analysis. The primary end-point of the study was
the percentage of patients who experienced DLT. Secondary
end-points included adverse events, pharmacokinetics of SASP,
response rate, and PFS. Changes in the serum level of free CD44v protein were assessed with the paired Student’s t-test as performed with SAS software version 9.3 (SAS Institute, Cary, NC, USA). A P-value of < 0.05 was considered statistically significant.

Results

Patient characteristics. Fifteen patients at two institutions were enrolled in the study between April 2015 and February 2016. The demographics and clinical characteristics of the study participants are shown in Table 1. The median age was 66 years (range, 42–74 years), 10 patients were men, and 14 individuals had stage IV disease. EGFR mutation status was evaluated in all patients, five of whom were found to harbor EGFR activating mutations (exon 19 deletions or L858R in exon 21) and had been previously treated with EGFR-TKIs. One patient was positive for the EML4-ALK fusion gene and had been previously treated with crizotinib and alectinib.

Maximum tolerated dose and DLT. Allocation of patients to treatment during the study is summarized in Figure 1, and DLTs apparent at each dose level are listed in Table 2. Dose-limiting toxicity was not observed in the first three patients treated at dose level 1. At dose level 2, the first patient experienced hives of grade 3 9 days after the onset of SASP treatment, and this condition was ameliorated immediately after discontinuation of SASP. The external Efficacy and Safety Review Committee recommended that the patient be excluded from DLT assessment because the hives were regarded as an incidental adverse event. The subsequent three patients at dose level 2 did not experience DLT. At dose level 3, two of three patients experienced DLT (anorexia of grade 3). According to the protocol, two additional patients were enrolled at dose level 2, and two of the total of five patients treated at this dose level experienced DLT (hypotension or pneumonitis, each of grade 3). To confirm the safety of dose level 1, three additional patients were enrolled, with one of the total of six patients treated at this dose level experiencing DLT (elevation of aspartate and alanine aminotransferase levels, each of grade 3). The MTD and RD of SASP, when given in combination with full-dose CDDP and PEM, were therefore determined to be 3 and 1.5 g/day, respectively. All DLTs were reversible after additional treatment or discontinuation of SASP.

Safety profile of combination therapy with CDDP, PEM, and SASP. Fifteen patients received combination therapy with CDDP, PEM, and SASP. The median number of treatment cycles was 2 (range, 1–17 cycles), and the median duration of SASP treatment was 45 days (range, 8–365 days). The most frequent drug-related adverse events (all CTCAE grades) during protocol treatment were anorexia (n = 14), fatigue (n = 14), nausea (n = 12), anemia (n = 10), vomiting (n = 9), constipation (n = 9), leukopenia (n = 9), and neutropenia (n = 9) (Table 3). Adverse events of grade 3 or 4 observed in ≥20% of patients included neutropenia (n = 8), hyponatremia.

Table 1. Characteristics of patients with non-small-cell lung cancer treated with salazosulfapyridine in combination with cisplatin and pemetrexed (n = 15)

| Characteristics | No. of patients (n = 15) |
|----------------|-------------------------|
| Age, years     |                         |
| Median         | 66                      |
| Range          | 42–74                   |
| Sex, n (%)     |                         |
| Male           | 10 (67)                 |
| Female         | 5 (33)                  |
| ECOG performance status, n (%) |         |
| 0              | 6 (40)                  |
| 1              | 9 (60)                  |
| Clinical stage, n (%) |             |
| IIIB           | 1 (7)                   |
| IV             | 14 (93)                 |
| Histology, n (%) |                        |
| Adenocarcinoma | 15 (100)                |
| Smoking status, n (%) |                  |
| Never smoked   | 7 (47)                  |
| Ex-smoker      | 5 (33)                  |
| Current smoker | 3 (20)                  |
| Gene mutation status, n (%) |         |
| None           | 9 (60)                  |
| EGFR L858R     | 3 (20)                  |
| EGFR Ex19del   | 2 (13)                  |
| EML4-ALK       | 1 (7)                   |
| Prior treatment, n (%) |               |
| None           | 9 (60)                  |
| Gefitinib      | 4 (27)                  |
| Afatinib       | 1 (7)                   |
| Crizotinib and alectinib | 1 (7)       |

Table 2. Observed dose-limiting toxicities (DLTs) at each dose level of salazosulfapyridine (SASP) in patients with advanced non-small-cell lung cancer

| Dose level | SASP dose (g/day) | No. of DLTs/patients | DLTs |
|------------|-------------------|----------------------|------|
| 1          | 1.5               | 1/6                  | (1) ALT and AST elevation |
| 2          | 3.0               | 2/5†                 | (1) Hypotension; (2) Pneumonitis |
| 3          | 4.5               | 2/3                  | (1) Anorexia; (2) Anorexia |

†Patients eligible for evaluation of dose-limiting toxicity. ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Table 3. Frequency of drug-related adverse events in patients with advanced non-small-cell lung cancer during protocol treatment with salazosulfapyridine (SASP) in combination with cisplatin and pemetrexed

| Hematologic | All grades | Grade ≥3 |
|-------------|------------|----------|
| Neutropenia | 4 (67) | 3 (50) |
| Leukopenia  | 3 (50) | 2 (33) |
| Neutropenia | 4 (67) | 3 (50) |
| Thrombocytopenia | 2 (33) | 0 (0) |

Data are shown as n (%). ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, \(\gamma\)-glutamyl transpeptidase.

(n = 7), leukopenia (n = 6), and anorexia (n = 4). There were no treatment-related deaths.

Pharmacokinetics of SASP. The \(C_{\text{max}}\) of SASP after the first single dose on day 1 at levels 1, 2, and 3 ranged from 7.1 to 24.8 g/mL, from 8.5 to 39.2 g/mL, and from 6.5 to 13.9 g/mL, respectively (Table 4). The \(AUC_{0-24}\) after the first single dose on day 1 at levels 1, 2, and 3 ranged from 55.2 to 235.5 g/mL, from 87.2 to 477.4 g/mL, and from 49.1 to 158.4 g/mL, respectively. Both \(C_{\text{max}}\) and \(AUC_{0-24}\) thus varied markedly among individuals within each dose level. High \(AUC_{0-24}\) values for SASP were apparent in patients with two reduced-function alleles of \(ABCG2\) (A/A) or \(NAT2\) (*6A/*6A or *6A/*7B). In addition, exposure to SASP did not correspond to the occurrence of DLT.

Efficacy. Two of 15 patients were not evaluable for objective response according to RECIST because they had no post-treatment tumor measurement. Among the 13 assessable patients, four individuals showed a partial response (two at dose level 1, two at dose level 2), yielding an overall response rate of 26.7%. An additional seven patients (46.7%) experienced stable disease, yielding a disease control rate of 73.3%. Median PFS for all 15 patients was 11.7 months (Fig. 2a). At the time of data analysis, 11 of the 15 patients were alive, yielding a 1-year survival rate of 73%.

Analysis of free CD44v protein in serum. Serum was obtained from 14 patients both before treatment and on day 21 of cycle 1 for measurement of free CD44v protein level. The CD44v level after the first treatment cycle was significantly higher than that before treatment (Fig. 2b).

Discussion

The efficacy of cancer therapies including cytotoxic chemotherapy and radiation therapy is attributable in part to the production of reactive oxygen species and the consequent induction of oxidative stress in cancer cells. Variant isoforms of CD44 have recently been found to stabilize the cystine transporter subunit xCT and thereby to promote intracellular formation of the antioxidant GSH and consequent chemoresistance in cancer cells. Given that CD44 is a CSC marker, the CD44v–xCT complex is thought to play an important role in chemoresistance in CSCs. This complex is

Table 4. Pharmacokinetic parameters of salazosulfapyridine and genotypes of \(ABCG2\) and \(NAT2\) in patients with advanced non-small-cell lung carcinoma

| Dose level | Patient | \(C_{\text{max}}\) (μg/mL) | \(AUC_{0-24}\) (g·h/mL) | \(ABCG2\) | \(NAT2\) |
|------------|---------|------------------------|-------------------------|----------|----------|
| 1          | 1       | 9.5                    | 86.8                    | C/C      | *6A/*4   |
|            | 2       | 7.1                    | 55.2                    | C/C      | *6A/*4   |
|            | 3       | 10.5                   | 119.3                   | C/C      | *6A/*7B  |
|            | 13†     | 10.6                   | 96.6                    | C/C      | *6A/*6A  |
|            | 14      | 7.9                    | 87.2                    | G/A      | *6A/*4   |
|            | 15      | 24.8                   | 235.5                   | A/A      | *6A/*6A  |
| 2          | 4       | 17.9                   | 157.3                   | C/A      | *6A/*7B  |
|            | 5       | 8.5                    | 102.8                   | C/A      | *6A/*4   |
|            | 6       | 39.2                   | 477.4                   | A/A      | *6A/*7B  |
|            | 7       | 17.1                   | 225.7                   | C/A      | *6A/*4   |
|            | 11†     | 13.1                   | 132.8                   | C/C      | *6A/*4   |
|            | 12†     | 21.4                   | 247.4                   | C/C      | *6A/*6A  |
| 3          | 8†      | 10.0                   | 158.4                   | C/C      | *6A/*6A  |
|            | 9†      | 13.9                   | 146.7                   | C/C      | *6A/*4   |
|            | 10      | 6.5                    | 49.1                    | C/C      | *6A/*4   |

†Patients with dose-limiting toxicities. \(AUC_{0-24}\), area under the concentration–time curve from 0 to 24 h; \(C_{\text{max}}\), maximum plasma concentration.
therefore considered a potential novel target for therapy directed against CSCs. Salazosulfapyridine, a drug commonly used to treat ulcerative colitis and rheumatoid arthritis, is a well-characterized specific inhibitor of xCT. Given that SASP has shown efficacy for treatment of CD44v-positive tumors in animal models, it is a potential anticancer drug for targeting of CSCs. This was a phase I trial of SASP in combination with standard chemotherapy for non-squamous NSCLC. The primary objective of our trial was to determine the RD of SASP.

Our results show that SASP combined with standard-dose CDDP and PEM has a manageable safety profile when given at dose level 1 (1.5 g/day). Two of three patients experienced anorexia of grade 3 as a DLT at dose level 3 (4.5 g/day), despite adequate prophylactic treatment with anti-emetic agents. The common adverse events of SASP are gastrointestinal toxicity (nausea, vomiting, gastrointestinal upset, diarrhea, and stomach cramps), fatigue, and headache. It is possible that the observed anorexia reflected synergistic toxicity for the combination of SASP and CDDP.  

One patient treated at dose level 2 (3 g/day) experienced pneumonitis of grade 3 as a DLT. Although respiratory disorders are not common adverse events of SASP treatment, SASP has previously been implicated in the development of pneumonitis. Hypotension of grade 3, which has previously been reported as a severe side-effect of SASP, was observed as a DLT in one patient at dose level 2. The occurrence of DLT for this combination therapy was independent of SASP exposure as reflected by AUC0-24 and Cmax.

Only approximately one-third of an oral dose of SASP is absorbed by the intestine. The remaining drug is metabolized by intestinal bacteria to 5-aminosalicylic acid and sulfapyridine, which have no effect on xCT. Sulfapyridine is relatively well absorbed from the intestine and further metabolized by NAT2 in the liver, whereas 5-aminosalicylic acid is absorbed to a much lesser extent. Breast cancer resistance protein, which is an ATP-binding cassette transporter encoded by ABCG2, restricts intestinal SASP absorption. Similar to the results of a previous phase I study of SASP monotherapy, exposure to SASP after oral doses of 1.5–4.5 g/day in the present study was not dose-dependent, likely reflecting genetic polymorphism of ABCG2 and NAT2.

A previous phase I/II study of SASP in patients with recurrent or progressive glioma found no tumor shrinkage after treatment at a dose of 1.5–6 g/day. A more recent phase I study of SASP at a dose of 8–12 g/day in previously treated patients with advanced gastric cancer also detected no tumor shrinkage. In our present phase I study, the response rate (26.7%) according to RECIST did not differ from that previously observed for chemotherapy with CDDP and PEM in patients advanced NSCLC. However, the median PFS of 11.7 months in our study was substantially longer than that (4.0–5.3 months) reported in previous studies of CDDP and PEM for non-squamous NSCLC. Given that tumor relapse after chemotherapy is thought to be driven by CSCs, a reduction in the number of CSCs induced by SASP treatment may prolong time to relapse. Indeed, immunohistochemical analysis of tumor tissue obtained before treatment in the present study revealed that the median PFS of patients (n = 5) with a high proportion of CD44v-positive cells was longer than that of those (n = 8) with a low proportion (>12 vs. 7.9 months), despite the lack of a confirmed partial response in the former patients. Although similar evaluation with a larger sample size is warranted, these data suggest that the proportion of CD44v-positive cells is a potential predictive biomarker for SASP treatment.

The precise mechanism of CD44v release and the origin of circulating CD44v are not known, although CD44v may be released in at least two distinct types of vesicles, apoptotic blebs and exosomes. The adhesion molecule L1 has been shown to be cleaved in exosomes and apoptotic membrane vesicles released from ovarian cancer cells undergoing apoptosis. Cleavage of CD44 also occurs in exosomes and might be induced by apoptotic stimuli. Together, these observations suggest that CD44v might be cleaved in and enter plasma by way of apoptotic blebs and exosomes released from CD44v-positive stem-like cancer cells undergoing apoptosis. Consistent with our data, SASP treatment was previously associated with a decrease in the number of CD44v-positive cells in post-treatment tumor tissue of patients with advanced gastric cancer. It is thus possible that the prolonged PFS observed in the present study was due to a SASP-induced reduction in the number of CD44v-positive CSCs that are the origin of disease recurrence.

In conclusion, we found that SASP was safe at the RD of 1.5 g/day in combination with CDDP and PEM. Our results suggest that this triplet regimen may prolong PFS compared with that achieved with CDDP and PEM alone. Given that only approximately one-third of SASP given orally is absorbed by the intestine, and that the remaining drug induces gastrointestinal toxicity, an i.v. injectable, water-soluble form of the drug is now under development in order to reduce such toxicity and to facilitate maintenance of an effective blood concentration of SASP. Further development of additional agents that target the CD44v–xCT complex is also warranted to evaluate the efficacy of CSC-targeted therapy as a novel treatment strategy.

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Fig. 2. (a) Progression-free survival (PFS) for all study patients with advanced non-small-cell lung cancer treated with salazosulfapyridine in combination with cisplatin and pemetrexed (n = 15). (b) Serum level of free CD44 variant (CD44v) before treatment and at day 21 of treatment cycle 1 for 14 of the study patients. **P < 0.01 (paired Student’s t-test).
Disclosure Statement
The authors have no conflict of interest.

Abbreviations
ALK
anaplastic lymphoma kinase

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Time course of the volume of tumors formed by HCT116 p53−/− cells in nude mice treated with CDDP (2 mg/kg) plus SASP (50, 100, 200, or 400 mg/kg). CDDP was injected intraperitoneally 5, 8, and 11 days after subcutaneous injection of $2 \times 10^6$ cells. SASP or saline was injected intraperitoneally once daily from days 5 to 10. Data are means ± SEM for 5 mice per group.