ANTICANCER RESEARCH

International Journal of Cancer Research and Treatment

ISSN: 0250-7005

February 16, 2011

Dr. Hirohito Kobayashi

Re: Your manuscript No. **12726-K** entitled **«A New Indicator of a Favorable...»**

Dear Dr

Referring to your above manuscript for publication in AR, please allow us to use this form letter in reply:

1. *Referee's recommendations:*
   - ■ Urgent to be published immediately.
   - □ Accepted in the presented form.
   - □ Accepted with minor changes.
   - ■ Accepted with grammatical or language corrections.
   - □ Remarks:

2. *Excess page charges.*
   - ■ Your article has approx. **5** printed pages and is in excess of the allotted number by approx. **1** printed pages. The charges are EURO € **210** per excess page, totalling EURO € **210**
     We ask you to confirm acceptance of these charges.
   - □ Your article includes pages with color figures. The charges are EURO € per color page, totalling EURO €
   - ■ Our invoice will be sent by e-mail to the corresponding author.

3. ■ Your article will appear in Volume **31**, Issue No. **3, 2011**

4. ■ Please order your reprints now. This will facilitate our prompt planning of future issues and rapid publication of your article. Reprints will be delivered by air mail within one month from publication.

We would appreciate your prompt reply.
With many thanks,
Yours sincerely,

John G. Delinassios

J.G. Delinassios
Managing Editor

EDITORIAL OFFICE: INTERNATIONAL INSTITUTE OF ANTICANCER RESEARCH
1st km Kapandritiou - Kalamou Rd., Kapandriti, P.O.B. 22, Attiki 19014, Greece. Tel.: 0030-22950-52945;
Tel & Fax:0030-22950-53389; e-mail: journals@iiar-anticancer.org

ANTICANCER RESEARCH
International Journal of Cancer Research and Treatment

Editorial Office: International Institute of Anticancer Research,
1st km Kapandritiou-Kalamou Rd., Kapandriti, P.O.B. 22, Attiki 19014, Greece
Fax: +30-22950-53389; Tel: +30-22950-52945; e-mail: journals@iiar-anticancer.org

ISSN: 0250-7005

Please type or print the requested information on the reprint order form and return it to the Editorial Office by fax or e-mail.
Fees for reprints, PDF file, or online open access must be paid for in advance.
If your paper is subject to charges for excess pages or color plates, please add these charges to the payment for reprints. The reprints are not to be sold.

PRICE LIST FOR REPRINTS WITHOUT COVER

Page length	Online Open Access Fee (Euro)	PDF File Fee (Euro)	Number of copies requested (prices are in Euro) 100	200	300	400	500	1000	1500	2000	3000	5000
1-4pp	1000	167	335	387	438	503	554	851	1135	1470	2038	3225
5-8	1200	219	438	503	580	645	722	1083	1445	1832	2554	4012
9-12	1400	277	554	619	709	787	877	1341	1780	2219	3096	4824
13-16	1600	354	709	787	890	993	1096	1625	2141	2657	3676	5715
17-20	1800	419	838	929	1032	1148	1277	1883	2451	3044	4244	6527

For reprints with cover: Please add EURO 140.00 per 100 copies.
Postage: Please add 5% on the above prices.

Reprint Order Form

Of my paper No. **12726-K** comprising **5** printed pages, entitled **«A New Indicator of a Favorable...»** accepted for publication in ANTICANCER RESEARCH Vol. **31** No. **3**

☐ I require a total of copies at EURO:
☐ I do not require reprints.
☐ Please send me a PDF file of the article at EURO:
☐ Please provide Online Open Access of the article at the IIAR website immediately upon publication at EURO:
☐ Please send me a copy of this issue containing my paper at EURO 45.00.
☐ Please enter my personal subscription to ANTICANCER RESEARCH at the special Author's price of EURO 417.00 (print or online); EURO 500.00 (print & online) (☐ Year: 2011).
☐ A check for the above amounts payable to J.G. Delinassios, Executive Publisher of Anticancer Research Journal, is enclosed.
☐ Please send an invoice to:

For EC countries: Please give your VAT number:
City and Date: Signature:
Exact postal address:
Tel:
Fax:

Cancer Immunol Immunother (2011) 60:1075–1084
DOI 10.1007/s00262-011-1021-7

ORIGINAL ARTICLE

Phase I/II study of adoptive transfer of γδ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma

Hirohito Kobayashi · Yoshimasa Tanaka · Junji Yagi · Nagahiro Minato · Kazunari Tanabe

Received: 8 February 2011 / Accepted: 7 April 2011 / Published online: 26 April 2011

Abstract Human Vγ2 Vδ2-bearing T cells have recently received much attention in cancer immunotherapy. In this study, we conducted a phase I/II clinical trial of the adoptive transfer of γδ T cells to patients with advanced renal cell carcinoma. Eleven patients who had undergone nephrectomy and had lung metastasis were enrolled. Peripheral blood γδ T cells obtained from the patients were stimulated ex vivo with 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), a synthetic pyrophosphomonoester antigen, and transferred in combination with zoledronic acid (Zol) and teceleukin (recombinant human interleukin-2). Expanded γδ T cells exhibited potent cytotoxic activity against tumor cells in vitro, and the proportion of peripheral blood γδ T cells among $CD3^+$ cells typically peaked three to 5 days after transfer. Tumor doubling time was prolonged in all 11 patients, and the best overall responses were 1 CR, 5 SD, and 5 PD, as defined based on Response Evaluation Criteria in Solid Tumors (RECIST). Although ten patients developed adverse reactions of grade ≥3, they were likely to have been the result of the concomitant infusion of Zol and IL-2, and most symptoms swiftly reverted to normal during the course of treatment. In conclusion, this clinical trial demonstrated that our regimen for the adoptive transfer of γδ T cells in combination with Zol and IL-2 was well tolerated and that objective clinical responses could be achieved in some patients with advanced renal cell carcinoma.

Keywords γδ T cell · Nitrogen-containing bisphosphonate · Pyrophosphomonoester · Isopentenyl pyrophosphate · Renal cell carcinoma · Cancer immunotherapy

Part of this study was previously published as a case report [43].

H. Kobayashi (✉) · K. Tanabe
Department of Urology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
e-mail: hirohitokobayashi-jua@umin.ac.jp

Y. Tanaka
Center for Innovation in Immunoregulative Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

Y. Tanaka · N. Minato
Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan

J. Yagi
Department of Microbiology and Immunology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

Introduction

Human γδ T cells that express Vγ2 Vδ2 (also termed Vγ9 Vδ2)-bearing TCR recognize nonpeptide antigens derived from microbial pathogens such as mycobacteria [1–4] and exhibit natural cytolytic activity against a wide array of tumor cells in vitro [5, 6]. It is worth noting that γδ T cells exert specific cytolysis in a TCR-dependent manner when they encounter human tumor cells pulsed with nitrogen-containing bisphosphonates (N-BPs), such as pamidronate and zoledronic acid (Zol) [7, 8]. It is demonstrated that the inhibition of farnesyl pyrophosphate synthase by N-BPs results in the accumulation of isopentenyl pyrophosphate (IPP), a prenyl pyrophosphate intermediate, in tumor cells, which leads to the activation of γδ T cells [9, 10]. Although the elevated level of IPP is

essential in the activation, it is not clear whether IPP per se is directly recognized by γδ T cells.

Based on these findings, a novel cancer immunotherapy using γδ T cells and N-BPs has been proposed [11–13]. It has recently been reported that the addition of Zol to first-line chemotherapy in the treatment of patients with multiple myeloma significantly improved disease-free survival [14]. Because Zol was administered every 3–4 weeks, the induction of tumor immunity, and especially the activation of γδ T cells, was considered to be one of the mechanisms by which Zol elicited beneficial effects on the clinical outcomes. In addition, we have previously observed that the proportion of renal cell carcinoma (RCC) patients whose peripheral blood γδ T cells were up-regulated increased as the patients progressed through the stages of the disease [15] and that the degree of the increase in γδ T cells in stage III patients correlated with their 10-year overall survival rate [16]. These clinical findings and in vitro observations encouraged us to develop a novel cancer immunotherapy.

We and others previously identified IPP-relating pyrophosphomonoesters and their nucleoside triphosphate γ-ester derivatives as antigens for human Vγ2 Vδ2-bearing T cells [2, 4]. Subsequently, it was demonstrated that (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, a microbial metabolite, was the most potent stimulant of natural origin [17]. Because of the structural similarity between pyrophosphomonoesters and bisphosphonates, several N-BPs were examined for antigenicity in stimulating γδ T cells, leading to the discovery that N-BPs of the second and third generations effectively induced the expansion of human γδ T cells both in vitro and in vivo [7]. There are several differences between pyrophosphomonoesters and N-BPs. Whereas pyrophosphomonoesters can stimulate both unprimed and primed γδ T cells without accessory cells, N-BPs require monocyte lineage cells for efficient stimulation in primary γδ T-cell responses and human tumor cells for primed γδ T cells [8]. In terms of stability, pyrophosphomonoesters can be readily hydrolyzed by serum alkaline phosphatases, but N-BPs are generally resistant to serum enzymes.

Taking these immunological and pharmacological properties of nonpeptide antigens into account, we employed a strategy consisting of the adoptive transfer of γδ T cells to yield effector cells, followed by N-BP infusion to sensitize tumor cells in the present phase I/II clinical trial. During the course of this study, we evaluated the safety of γδ T-cell transfer concomitant with Zol and IL-2 infusion, the kinetics of γδ T cells in the peripheral blood, the prolongation of tumor doubling time, and the clinical outcomes based on RECIST.

Materials and methods

Patients and patient eligibility

Patients with histologically confirmed renal cell carcinoma (any T, any N, and M1, stage IV), who had undergone nephrectomy, with PS of 0, who had evaluable lung metastasis on computed tomography (CT) 3 months before the start of treatment, whose tumor doubling time before treatment was evaluable, whose age ranged from 20 to 80 years old, whose life expectancy was at least 6 months, whose major organs maintained function, whose lung metastatic lesions progressed even after treatment with IFN-α for at least 3 months, who met the laboratory test standards of our institution, and who voluntarily provided written consent to participate in this trial after having been thoroughly briefed and informed of its nature, were eligible for enrollment. Major exclusion criteria were a history of cancer other than RCC within 2 years, treatment with anticancer drugs, treatment with steroids, and serious complications.

Study design

The protocol was designed and written by the authors, in collaboration with staff from the Translational Research Informatics Center (TRI), Kobe, Hyogo, Japan, and reviewed and approved by the Tokyo Women's Medical University Hospital Ethics Committee. This trial was a nonrandomized, uncontrolled, open-label, single-institutional study and is registered at http://www.clinicaltrials.gov as TRIC-CTR-GU-05-01. RCC patients who met the inclusion criteria were enrolled in this study.

Peripheral blood mononuclear cells were collected from each patient using an apheresis machine and then manually purified further with Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells were resuspended in 350 ml of the serum-free medium ALyS505 N (Cell Science & Technology Institute, Sendai, Miyagi, Japan) containing recombinant human interleukin-2 (rIL-2, Proleukin, Chiron, CA), 100 international units (IU)/ml, and stimulated with 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), a pyrophosphomonoester, prepared at our institution as previously described [2] at a final concentration of 100 μM in an air-permeable culture bag (Nipro Corp., Kita-ku, Osaka, Japan) for 11 days at 37°C in a humidified atmosphere containing 5% CO_2 at the Cell Processing Center of Tokyo Women's Medical University Hospital, which is based upon GMP. The expanded γδ T cells were transferred into a sterile infusion bag (Nipro Corp.). The patients were infused with 4 mg of Zol in 100 ml saline over a period of 30 min. Then, γδ T cells were administered for 5 min starting 2 h after the completion of Zol infusion.

Subsequently, rIL-2, 1.4×10^6 JRU (Teceleukin, Shionogi & Co., Ltd., Japan, 1 JRU: Japan reference unit = 1 IU), was administered every day for 5 days. This procedure was repeated six times, once every 4 weeks. The target lung legions were measured through standard CT imaging at −3, 0, 3, and 7 months after the start of treatment. Changes in laboratory test values were monitored to assess patient safety throughout the study, and immunological properties of the expanded $\gamma\delta$ T cells and peripheral blood T cells were examined by means of flow cytometry. The standard cytotoxic assay was performed as described below.

The primary endpoints were the incidence of adverse events (AEs) and the increase in the proportion of peripheral blood $\gamma\delta$ T cells. All AEs occurred during and within 1 month after completion of the treatment and were classified according to the NCI-Common Terminology Criteria for Adverse Events (NCI-CTCAE) ver. 3.0. The proportion of Vδ2-bearing $\gamma\delta$ T cells among peripheral blood T cells was determined as described below.

The secondary endpoints were the prolongation of tumor doubling time and the best overall responses as defined by the RECIST criteria [18]. The target lesions were scanned using helical CT with a slice thickness of 5 mm. The tumor volume was calculated as $ab(a + b)\pi/12$, where a represents a major axis of the tumor ellipse and b a minor axis. The tumor doubling time was defined as $\log_2(T_1-T_0)/(\log V_1-\log V_0)$, where V_0 and V_1 represent the tumor volumes at time T_0 and T_1, respectively. When two or more lesions were present in the lungs, the tumor volume was defined as the sum of the individual tumor volumes.

Immunological monitoring

The profiling of peripheral lymphocytes and cultured cells was examined by means of flow cytometry. Cells were treated with PC5-conjugated anti-CD3 mAb (SK7, Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA), or phycoerythrin (PE)-conjugated anti-CD4, anti-CD8, or anti-CD25 mAbs (BD Pharmingen Inc., San Diego, CA, USA), and fluorescein isothiocyanate (FITC)-conjugated anti-Vδ2 mAb (Immunotech, Marseilles, France) at 2×10^5 cells/50 µl of phosphate buffered saline (PBS)/2% fetal calf serum (FCS) on ice for 30 min. After being washed three times with 200 µl of PBS/2% FCS, the cells were subjected to flow cytometry (EPICS XL, Coulter Electronics, Hialeah, FL, USA). The flow cytometric data were processed and analyzed using EXPO32 software (Coulter Electronics).

The serum cytokines, IL-2, IFN-γ, IL-4, IL-5, IL-10, and TNF-α, were measured using Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kits (Becton–Dickinson) according to the manufacturer's instructions.

To determine cytotoxic activity, the $\gamma\delta$ T cell–sensitive Daudi (Burkitt's lymphoma) line and the Caki-1 and VMRC-RCW (renal cell carcinomas) lines were treated with [^{51}Cr]-sodium chromate solution (3.7 MBq) at 37°C for 1 h. After being washed with medium, the tumor cells were resuspended in the medium and placed in a round-bottom 96-well plate. To the suspension was added the expanded $\gamma\delta$ T cells at the effector/target ratio of 40:1. The plate was briefly centrifuged and incubated at 37°C and 5% CO_2. After 4 h of incubation, the supernatants were examined for [^{51}Cr]-sodium chromate release using a γ-counter.

Data were collected by the investigators at Tokyo Women's Medical University Hospital and processed and analyzed by the staff of TRI.

Statistical analysis

Statistical analyses were conducted to test the differences between two items using the log-rank test, using the Stat View 5.0 J software package (Abacus Concepts, Inc, CA, USA).

Results

Patients' profiles

A total of 11 patients who were diagnosed with metastatic renal cell carcinoma were recruited between January 2006 and March 2008. All patients underwent nephrectomy for RCC before enrollment. The final outcomes of all patients were assessed in October 2008, and the data were compiled and analyzed in December 2008. The patients' profiles are presented in Table 1. Eight of the patients were men and three were women; at enrollment, the median age was 59.4 years and PS was 0. Memorial Sloan-Kettering Cancer Center (MSKCC) Risk status was assessed at the time of enrollment. Based on histological examination, 9 patients had been diagnosed with RCC of clear cell type, 1 with RCC of clear cell with sarcomatoid, and 1 with RCC of papillary cell type. All patients had lung metastasis and/or other site of distant metastasis and received IFN-α and/or IL-2 after the surgery.

After obtaining written informed consent, patients were treated for metastatic renal cell carcinoma in the Study Design section. Five patients completed the whole treatment schedule, whereas six eventually discontinued for various reasons: one patient due to a brain tumor after four cycles of the treatment, one patient due to a lack of efficacy after three cycles of treatment, two patients due to an investigator's decision in response to the patients' apprehension, and two patients through a withdrawal of informed consent.

Table 1 Patients' profile and clinical outcome

Patient	Age/sex	MSKCC[a] risk group	Type of cell[b]	Metastatic lesion	Previous treatment	Treatment cycles	Overall response	Clinical Outcome
TR1	68/M	Intermediate	Clear	Lung	IFN-α[c]	4	SD	Death
TR2	52/M	Poor	Clear with salcomatoid	Lung/bone/lymph node	IFN-α/IL-2[d]	2	PD	Death
TR3	65/M	Intermediate	Clear	Lung	IFN-α	6	SD	Survival
TR4	56/F	Intermediate	Clear	Lung/liver	IFN-α/IL-2/metastasectomy	1	PD	Survival
TR5	61/M	Intermediate	Clear	Lung	IFN-α	6	CR	Survival
TR6	63/M	Poor	Clear	Lung/bone/pleura	IFN-α/metastasectomy	1	PD	Survival
TR7	59/F	Good	Clear	Lung/pleura	IFN-α/metastasectomy	5	PD	Survival
TR8	39/M	Intermediate	Clear	Lung/pleura	IFN-α	3	PD	Survival
TR9	61/M	Intermediate	Clear	Lung	IFN-α	6	SD	Survival
TR10	66/F	Intermediate	Clear	Lung/lymph nodes/retro peritoneal cavity/muscle/ascending colon	IFN-α	6	SD	Survival
TR11	63/M	Intermediate	Papillary	Lung/lymph nodes	IFN-α	6	SD	Survival

[a] *MSKCC* Memorial Sloan-Kettering Cancer Center

[b] World Health Organization Classification of Tumors [44]

[c] *IFN-α* interferon-alpha

[d] *IL-2* interleukin-2

Immunological responses

PBMC were stimulated with 2M3B1PP, and the resulting $\gamma\delta$ T cells were collected and examined for immunological properties. As shown in Fig. 1a, there was no intrinsic difference in the proportion of $CD3^+$ cells (lozenge) in each cycle of expansion. By contrast, the proportions of $V\delta2^+$ cells among the $CD3^+$ cells (rectangle) decreased over the course of the treatment, whereas those of $CD4^+$ cells (cross) and $CD8^+$ cells (triangle) gradually increased. The absolute numbers of $V\delta2^+$ cells in transferred cells are measured in each cycle of the treatment in each patient. Whereas the number of $V\delta2^+$ cells in the sixth cycle was higher than that in the first cycle in patients who achieved SD/CR, the number significantly decreased as the cycle progressed in 3 of 5 PD patients as shown in Fig. 3b, c.

We then analyzed the proportion of Vδ2-bearing T cells among $CD3^+$ cells of peripheral blood before and after infusion of $\gamma\delta$ T cells. Peripheral blood samples were examined for the expression of CD3, CD4, CD8, CD25, and Vδ2-TCR. In the first cycle of treatment, the proportions of peripheral blood Vδ2-bearing $\gamma\delta$ T cells among $CD3^+$ T cells in all patients increased with time to different degrees. Typically, the proportion of $\gamma\delta$ T cells peaked 3–5 days after infusion, as shown in Fig. 2. On average, the $V\delta2^+/CD3^+$ ratio in the first infusion was 4.83% at the time of apheresis and 13.43% 1 week after infusion.

Serum cytokine contents before and after infusion in the first cycle of treatment were measured by Cell Beads Assay. Levels of serum IFN-γ, IL-5, and IL-10 concentrations are shown in Fig. 3a at different time points. IFN-γ peaked 1–2 days after infusion and gradually decreased to the levels that had existed before infusion in 7 days and IL-5 3–5 days after infusion and then swiftly decreased. There are, however, no statistically significant differences between the SD/CR patients (dark gray line) and the PD patients (bright gray line). We also measured IL-4, TNF-α, and IL-2, but no remarkable differences were observed between the two groups. In terms of cytotoxicity, SD/CR patients exhibited a slightly higher specific lysis against Daudi, Caki-1, and VMRC-RCW (dark gray bar) than PD patients (bright gray bar), though there was no statistically significant difference between the two groups, as shown in Fig. 3b. In SD/CR patients, tumoricidal activity in the sixth cycle appears to be lower than that in the first cycle, as depicted in Fig. 3c.

Clinical responses

In order to assess the secondary endpoints, tumor volumes were determined through CT −3 Mo, 0 Mo, +3 Mo, and +7 Mo after the start of treatment. No newly appearing lesions in the lungs were observed in any of the patients during the course of this study. As summarized in Fig. 4a, the tumor volumes of most metastatic lesions increased steeply in the

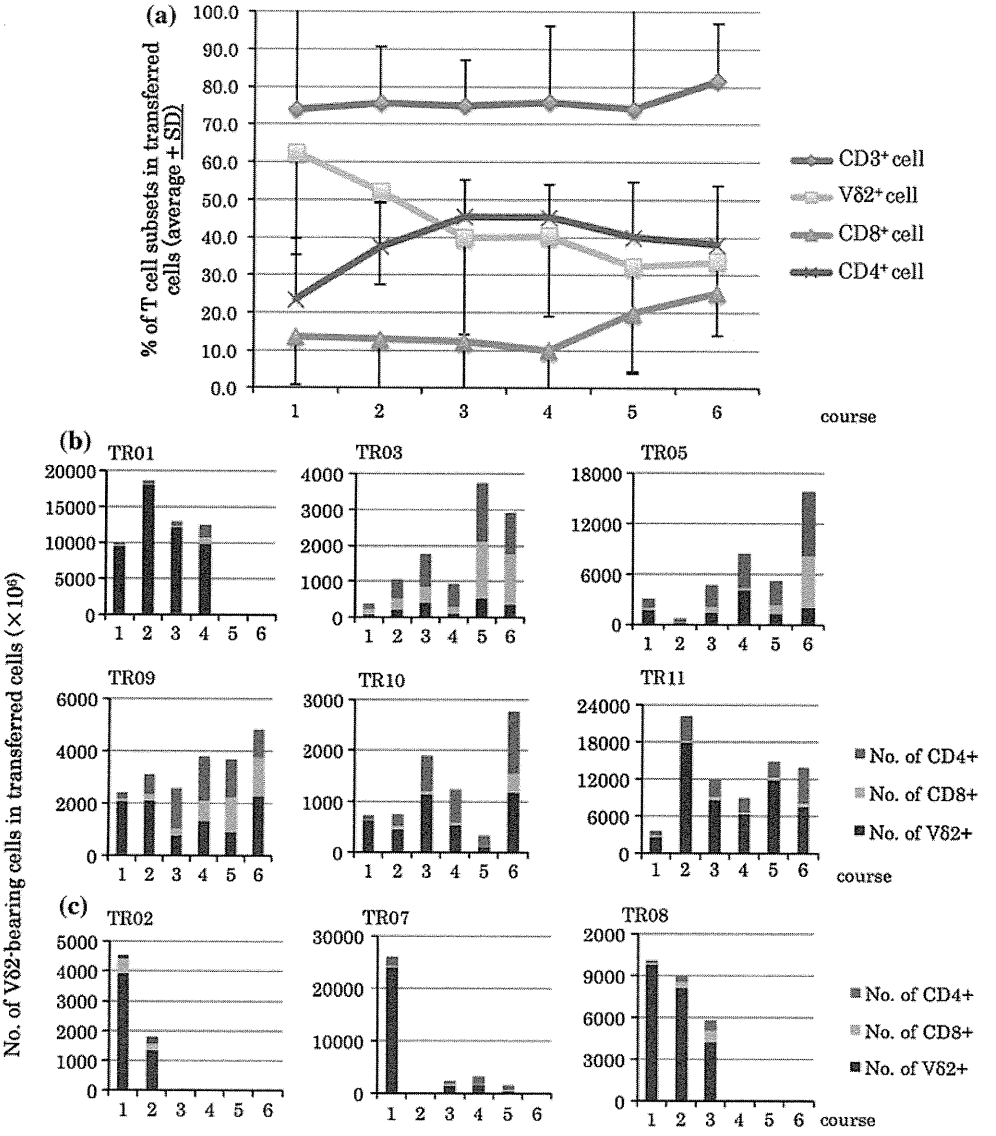

Fig. 1 **a** Relative proportions of T-cell subsets in transferred cells derived from patients who completed 6 cycles of the treatment. The proportions of $CD3^+$ cells (*lozenge*) and those of $V\delta2^+$ (*rectangle*), $CD4^+$ (*cross*), and $CD8^+$ cells (*triangle*) among $CD3^+$ cells were measured in each cycle of the treatment. **b/c** The absolute numbers of $V\delta2^+$, $CD4^+$, and $CD8^+$ cells among the transferred cells in each cycle of the treatment; SD/CR (**b**) and PD patients **c**

baseline period from −3 months to the beginning of treatment in all patients. After the start of treatment, the tumor volumes of some metastatic lesions began to decrease or continued to increase but at a more moderate rate. As depicted in the upper panels of Fig. 4b, the total tumor volumes in the lungs increased moderately in patients TR07 and TR08, whereas those in TR01, TR03, TR09, TR10, and TR11 did not change significantly. Remarkably, metastatic lesions disappeared within 7 months in TR05. Because the treatment was discontinued in TR02, TR04, and TR06, the total tumor volumes were not measured in this study. A moderate to significant increase in the tumor doubling time was observed in all patients as shown in the lower panels of Fig. 4b. Based on these results, one patient was considered to exhibit CR, five patients SD, and five patients PD (Table 1). Two patients had died as a result of malignant tumors by the end of the study in October 2008 (Table 1). It is encouraging to note that TR05, the patient who exhibited CR, has remained disease-free to date (more than 36 months after the completion of the treatment). Figure 4d demonstrates representative CT images of the metastatic lesions in TR05 at the beginning of the treatment and 3 months later; it was during this 3-month interval that the two lesions macroscopically disappeared.

Adverse events

In order to assess the safety of this protocol as the first primary endpoint, we monitored PS scores, laboratory test values, and adverse reactions throughout the study. Although the patient referred to as TR02 had a PS of 0 at pre-enrollment, his PS rose to 1 at the first apheresis through the second infusion of $\gamma\delta$ T cells, and he dropped out of the study after completing the second cycle. TR07, who had also had a PS of 0 at pre-enrollment, had developed a PS of 1 by the fifth $\gamma\delta$ T-cell transfer and dropped out of the study after

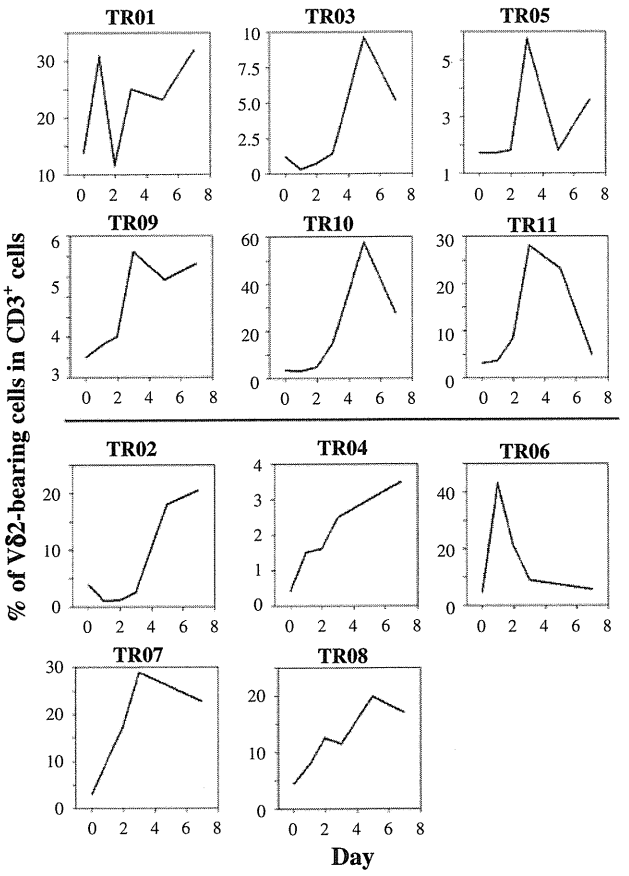

Fig. 2 Time course of Vδ2-bearing γδ T-cell circulation in the peripheral blood of patients with advanced RCC after infusion of 2M3B1PP-stimulated PBMC together with IL-2 and Zol. Before and after the infusion, PBMCs were examined for the expression of Vδ2 and CD3

completing the fifth cycle. The PS scores of the other patients were 0 at pre-enrollment and remained unchanged throughout the study. All patients developed adverse reactions of grade 1 or 2, including fatigue and fever, and ten patients developed adverse reactions of grade 3 or 4, as summarized in Table 2, including lymphopenia, hyponatremia, hypopotassemia, an increase in serum alanine aminotransferase, an increase in serum aspartate aminotransferase, an increase in serum creatinine, and a decrease in hemoglobin. All the deviated laboratory values reverted to normal during the course of the treatment. Patients, TR06 and TR10, whose serum creatinine increased should have been treated with rehydration therapy, but the symptom disappeared without treatment in a week. Almost all AEs occurred in the first cycle, and the frequency of these AEs decreased over the course of treatment.

Discussion

Kidney cancer is one of the leading cancer types among estimated new cancer cases and deaths [19]. Historically, metastatic RCC was relatively unresponsive to traditional chemotherapy, and biological response modifiers such as IL-2 and IFN-α were the favorable treatments for patients with advanced RCC [20]. The recent development of targeted agents has changed the management of metastatic RCC [21]. These agents have distinct modes of action and include multitargeted receptor tyrosine kinase inhibitors, mTOR inhibitors, and vascular endothelial growth factor signaling blockers [22–24]. Although the prognosis for patients with metastatic RCC has improved significantly because of advances in these treatments, it should be noted that the primary achievement of the target therapies is the induction of SD, not CR [21]. IL-2 remains the only agent known to produce durable complete responses [25], but its use is accompanied by severe side effects, including hypotension, capillary leak syndrome, renal insufficiency, and the like, and is limited to good responders to the lymphokines [20]. Notwithstanding the availability of a variety of therapeutics, no satisfactory regimen for advanced RCC has yet been defined.

It is well known that RCC sometimes evokes immune responses that lead to complete tumor remissions [20]. We recently conducted a pilot study investigating the safety and feasibility of the adoptive transfer of γδ T cells concomitantly with IL-2 [26]. γδ T cells exert tumoricidal activity via two distinct mechanisms, natural killer-like activity and γδ TCR-dependent cytotoxicity [27]. γδ TCR is involved in the antitumor activity that results when tumor cells are treated with N-BPs [5]. Whereas it is most likely that IPP per se or its derivatives, which are accumulated in the tumor cells via the inhibition of farnesyl pyrophosphate synthase by N-BPs, are presented to γδ T cells, the translocation or induction of antigenic proteinaceous entities to the cell surface is also plausible [28]. Generally, γδ TCR-dependent cytotoxicity is more potent than natural killer-like activity. We therefore examined the safety and clinical outcomes of the infusion of γδ T cells plus IL-2 and Zol in this study.

Regarding primary endpoints, we successfully observed an increase in the proportion of Vγ2 Vδ2-bearing γδ T cells in peripheral blood after the infusion of γδ T cells. There was a lag of several days between the infusion and the observed increase in the number of peripheral blood γδ T cells. This is probably because γδ T cells were trapped immediately after infusion in the reticuloendothelial system and the lungs. It is worth noting that the proportions of γδ T cells among the $CD3^+$ cells cultured with 2M3B1PP and IL-2 decreased as the number of treatment cycles increased, from 77.54% in the first cycle to 33.54% in the sixth cycle. Consequently, the tumoricidal activity of the cultured cells decreased, from 36.4 and 42.9% in the first cycle against Caki-1 and VMRC-RCW, respectively, to 15.2 and 21.0% in the sixth cycle. The lower proportion of γδ T cells in the cultured cells of the

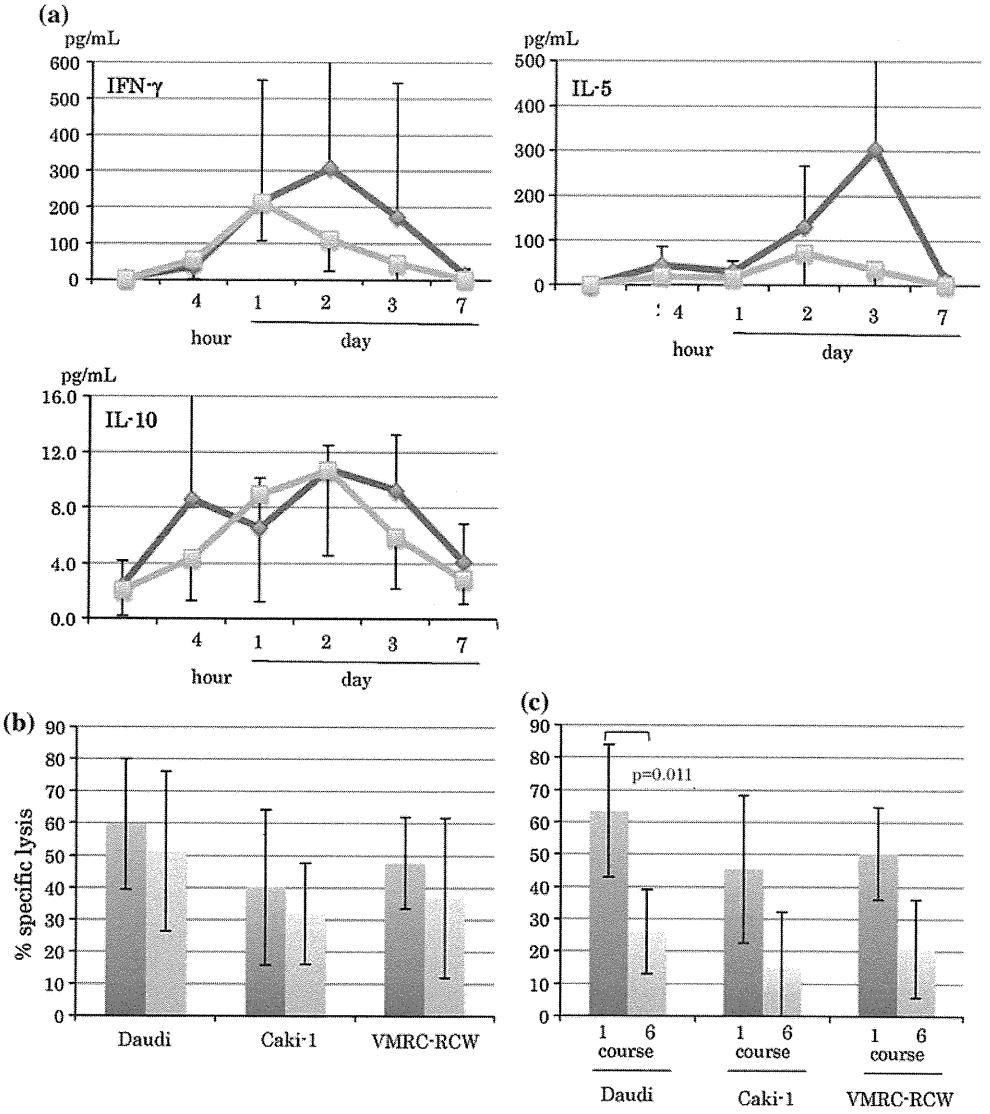

Fig. 3 **a** Serum cytokine concentrations at different time points of the first cycle of the treatment. *Dark gray line* indicates cytokine concentrations in sera obtained from SD/CR patients and *bright line* PD patients. The cytokine concentrations are shown as the average ±SDs. Cytotoxicity exhibited by cultured cells against Daudi, Caki-1, and VWRC-RCW was measured by the standard ^{51}Cr release assay. **b** Comparison of cytotoxic activity exhibited by cultured cells derived from SD/CR (*dark gray column*) and that from PD patients (*bright gray column*). **c** Comparison of cytotoxicity exhibited by cultured cells at the first and the sixth cycle of treatment; the first cycle (*dark gray column*) and the sixth cycle (*bright gray column*). The average and SDs of specific lysis were determined in SD/CR patients who had completed the six cycles of the treatment

sixth cycles compared to those of the first cycles may account for the reduced cytotoxicity. The proportion of $\gamma\delta$ T cells among the peripheral blood $CD3^+$ cells after infusion also decreased significantly. We did not observe such a decrease in the pilot study, in which expanded $\gamma\delta$ T cells and IL-2 were infused without Zol [26]. Thus, the induced unresponsiveness or hyporesponsiveness of $\gamma\delta$ T cells to 2M3B1PP is attributable to the prior infusion of Zol. This finding may lead to a future improvement in the current protocol. An improved regimen could consist of the collection of peripheral blood by apheresis, the storage of purified PMBC aliquots until use, and the expansion of cryopreserved $\gamma\delta$ T cells. Because our initial objective was to increase the number of $\gamma\delta$ T cells in peripheral blood by adoptive transfer of ex vivo expanded $\gamma\delta$ T cells, this new regimen would further our aims. In addition, we should determine the optimum dose of Zol in the future regimen because the current dose of Zol, 4 mg, was originally chosen for the treatment of patients with hypercalcimea, but not with malignant tumors.

Regarding another primary endpoint, we observed some AEs of grades 3 and 4. In our previous pilot study comprising $\gamma\delta$ T-cell transfer plus IL-2 infusion without Zol, AEs of these magnitudes did not occur during treatment [26]. Because Zol infusion itself is also well tolerated in clinical settings [29–37], the combination of Zol, IL-2, and $\gamma\delta$ T cells may have caused the AEs. Regardless, the symptoms were manageable and disappeared swiftly during the course of treatment, indicating that the regimen used in this study was well tolerated. In the pioneering study on N-BPs, AEs were induced after the initial infusion of N-BPs and essentially no AEs were observed in the subsequent infusions [38–40]. It is thus possible to reduce the dose of Zol in the initial infusion in order to reduce the severity of AEs. Furthermore, the interval between Zol administration and $\gamma\delta$ T-cell infusion should be optimized to minimize AEs in future regimens.

It is worth noting that tumor doubling time was successfully increased in all patients. In TR07, for instance, there

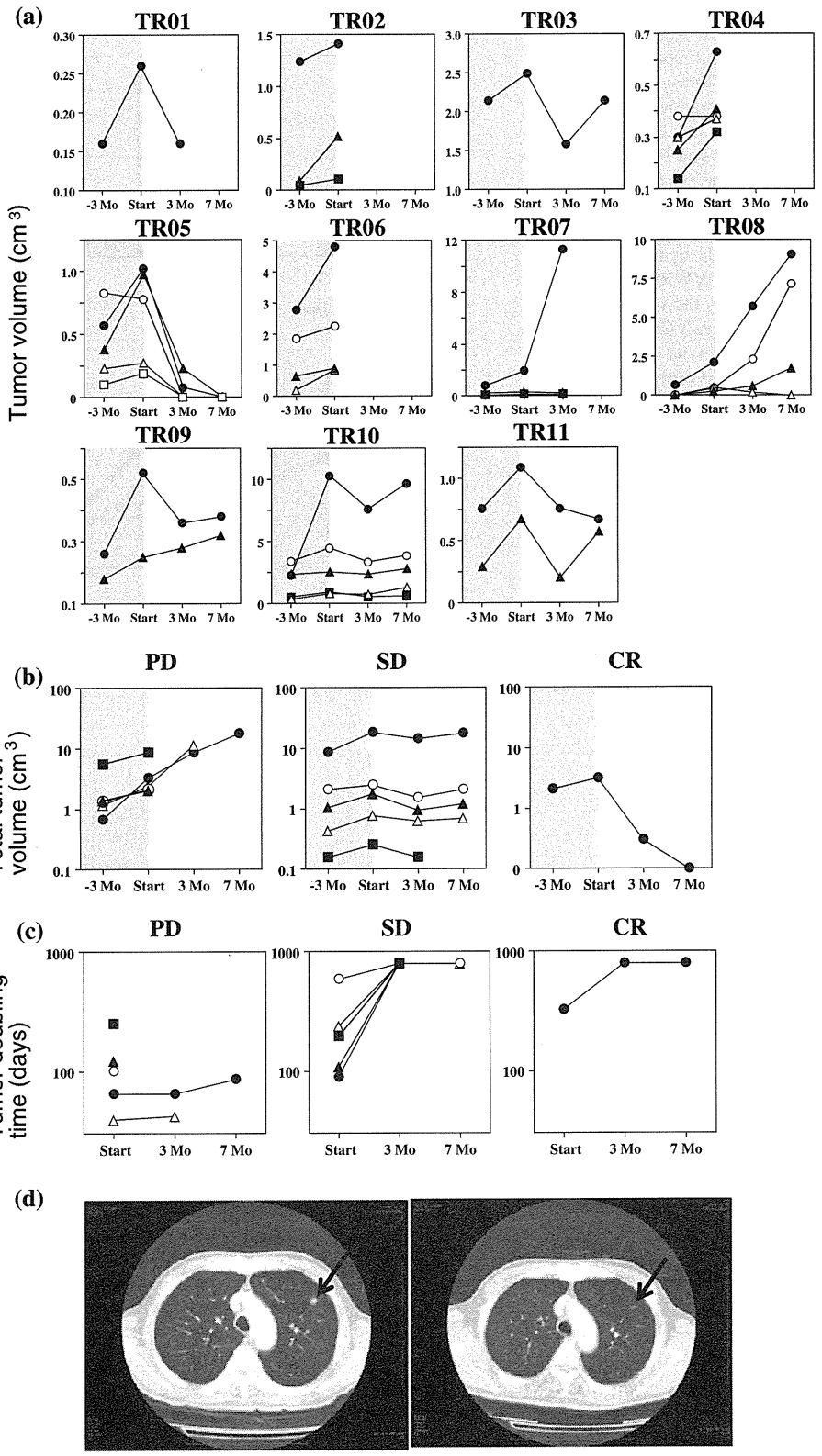

Fig. 4 **a** Time course of individual tumor volumes in the lungs of advanced RCC patients after infusion of 2M3B1PP-stimulated PBMC with IL-2 plus Zol. The tumor size of each lesion was measured by means of CT imaging −3 Mo, 0 Mo, 3 Mo, and 7 Mo after the start of the treatment. **b** Time course of total tumor volumes and **c** tumor doubling time. Total lung tumor volumes of each patient were calculated and plotted against time separately for three patient subgroups: PD (*closed triangle*: TR02, *open circle*: TR04, *closed square*: TR06, *open triangle*: TR07, and *closed circle*: TR08), SD (*closed square*: TR01, *open circle*: TR03, *open triangle*: TR09, *closed circle*, TR10, and *closed triangle*, TR11), and CR (*closed circle*: TR05). **d** CT images of metastatic lung tumors in an advanced RCC patient. Five metastatic lung lesions were observed through CT imaging in TR05; one metastatic site depicted here was measured at 1.26 × 1.08 cm (*not shown*), 1.33 × 0.97 cm (*left*), and 0.00 × 0.00 cm (*right*) at −3 Mo, 0 Mo, and 3 Mo after the start of treatment, respectively

were three metastatic tumors in the lungs, one of which grew consistently, while the other two remained stable. Summing the volumes of the individual tumors revealed that the tumor doubling time was prolonged even in this PD case. In TR05, there were five metastatic tumors, three of which disappeared within 3 months and two within

Table 2 Adverse events

Grade >3	Number of patients/frequency (%)					
	1 course ($n = 11$)	2 course ($n = 9$)	3 course ($n = 8$)	4 course ($n = 7$)	5 course ($n = 6$)	6 course ($n = 5$)
Lymphopenia	10 (91%)	3 (30%)	1 (13%)	1 (14%)	0 (0%)	0 (0%)
Neutropenia	0 (0%)	0 (0%)	0 (0%)	1 (14%)	1 (17%)	0 (0%)
Hyponatremia	2 (18%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)
Hypopotassemia	0 (0%)	1 (11%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)
AST	1 (9%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)	0 (0%)
ALT	1 (9%)	1 (11%)	2 (25%)	1 (14%)	0 (0%)	0 (0%)
Creatinine	1 (9%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)
Hemoglobin	0 (0%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)

7 months. To date, this patient has not developed any evidence of recurrent tumors during the nearly three-year period since the completion of the treatment.

In addition, several new lesions that had not been present in the CT images recorded 3 months before the start of the treatment were detected at the start of immunotherapy, but no new lesions were observed in the lungs after the start of immunotherapy. This finding suggests that the present regimen may prevent micrometastasis of renal cell carcinoma. It has recently been reported that the addition of Zol to the standard regimen for the treatment of breast cancer improved disease-free survival times [41]. It is therefore necessary to identify the mechanism by which Zol elicits its therapeutic effects [42].

Based on the present results and those of other clinical trials, it is most likely that $\gamma\delta$ T cells exert cytotoxic activity against malignant tumors in vivo. The effector roles of $\gamma\delta$ T cells in vivo are still controversial, however. Thus, it is imperative that the immunological properties of $\gamma\delta$ T cells in tumor-infiltrating lymphocytes be examined and the results applied to the development of efficacious regimens for malignant tumors.

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports, and Technology of Japan (MEXT) and by Special Coordination Funds for Promoting Science and Technology from MEXT and Astellas Pharma Inc. as part of the "Formation of Innovation Center for Fusion of Advanced Technologies" program. The authors wish to acknowledge the support provided by the staff from the Translational Research Informatics Center (TRI), Kobe, Hyogo, Japan, and would like to thank Ms Clare Dover for providing English correction prior to paper submission.

References

1. Hayday AC (2009) $\gamma\delta$ T cells and the lymphoid stress-surveillance response. Immunity 31:184–196
2. Tanaka Y, Morita CT, Nieves E, Brenner MB, Bloom BR (1995) Natural and synthetic non-peptide antigens recognized by human $\gamma\delta$ T cells. Nature 375:155–158
3. Tanaka Y, Sano S, Nieves E, De Libero G, Rosa D, Modlin RL, Brenner MB, Bloom BR, Morita CT (1994) Nonpeptide ligands for human $\gamma\delta$ T cells. Proc Natl Acad Sci U S A 91:8175–8179
4. Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, Bonneville M, Fournie JJ (1994) Stimulation of human $\gamma\delta$ T cells by nonpeptidic mycobacterial ligands. Science 264:267–270
5. Kato Y, Tanaka Y, Miyagawa F, Yamashita S, Minato N (2001) Targeting of tumor cells for human $\gamma\delta$ T cells by nonpeptide antigens. J Immunol 167:5092–5098
6. Bukowski JF, Morita CT, Tanaka Y, Bloom BR, Brenner MB, Band H (1995) Vγ2 Vδ2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. J Immunol 154:998–1006
7. Kunzmann V, Bauer E, Wilhelm M (1999) γ/δ T-cell stimulation by pamidronate. N Engl J Med 340:737–738
8. Miyagawa F, Tanaka Y, Yamashita S, Minato N (2001) Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human $\gamma\delta$ T cells by aminobisphosphonate antigen. J Immunol 166:5508–5514
9. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G (2003) Human T cell receptor $\gamma\delta$ cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 197:163–168
10. Jauhiainen M, Monkkonen H, Raikkonen J, Monkkonen J, Auriola S (2009) Analysis of endogenous ATP analogs and mevalonate pathway metabolites in cancer cell cultures using liquid chromatography-electrospray ionization mass spectrometry. J Chromatography B 877: 2967–2975
11. Bonneville M, Scotet E (2006) Human Vγ9 Vδ2 T cells: promising new leads for immunotherapy of infections and tumors. Curr Opin Immunol 18:539–546
12. Kabelitz D, Wesch D, He W (2007) Perspectives of $\gamma\delta$ T cells in tumor immunology. Cancer Res 67:5–8
13. Kabelitz D, Wesch D, Pitters E, Zoller M (2004) Potential of human $\gamma\delta$ T lymphocytes for immunotherapy of cancer. Int J Cancer 112:727–732
14. Morgan GJ, Davies FE, Gregory WM, Cocks K, Bell SE, Szubert AJ, Navarro-Coy N, Drayson MT, Owen RG, Feyler S, Ashcroft AJ, Ross F, Byrne J, Roddie H, Rudin C, Cook G, Jackson GH, Child JA, On behalf of the National Cancer Research Institute Haematological Oncology Clinical Study Group (2010) First-line treatment with zoledronic acid as compared with clodronic acid in multiple myeloma (MRC Myeloma IX): a randomised controlled trial. Lancet 376:1989–1999
15. Kobayashi H, Tanaka Y, Yagi J, Toma H, Uchiyama T (2001) γ/δ T cells provide innate immunity against renal cell carcinoma. Cancer Immunol Immunother 50:115–124
16. Kobayashi H, Tanaka Y, Nakazawa H, Yagi J, Minato N, Tanabe K (2011) A new indicator of a favorable progress in locally

advanced renal cell carcinomas: γδ T-Cells in peripheral blood. Anticancer Res (in press)
17. Hintz M, Reichenberg A, Altincicek B, Bahr U, Gschwind RM, Kollas AK, Beck E, Wiesner J, Eberl M, Jomaa H (2001) Identification of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human γδ T cells in *Escherichia coli*. FEBS Lett 509:317–322
18. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205–216
19. Reeves DJ, Liu CY (2009) Treatment of metastatic renal cell carcinoma. Cancer Chemother Pharmacol 64:11–25
20. Atkins MB (2009) Treatment selection for patients with metastatic renal cell carcinoma: identification of features favoring upfront IL-2-based immunotherapy. Med Oncol 26(Suppl 1):18–22
21. Bellmunt J (2009) Future developments in renal cell carcinoma. Ann Oncol 20(Suppl 1):113–117
22. Feldman DR, Baum MS, Ginsberg MS, Hassoun H, Flombaum CD, Velasco S, Fischer P, Ronnen E, Ishill N, Patil S, Motzer RJ (2009) Phase I trial of bevacizumab plus escalated doses of sunitinib in patients with metastatic renal cell carcinoma. J Clin Oncol 27:1432–1439
23. Escudier B, Szczylik C, Hutson TF, Demkow T, Staehler M, Rolland F, Negrier S, Laferriere N, Scheuring UJ, Cella D, Shah S, Bukowski RM (2009) Randomized phase II trial of first-line treatment with sorafenib versus interferon α-2a in patients with metastatic renal cell carcinoma. J Clin Oncol 27:1280–1289
24. Motzer RJ, Hudes GR, Curti BD, McDermott DF, Escudier BJ, Negrier S, Duclos B, Moore L, O'Toole T, Boni JP, Dutcher JP (2007) Phase I/II trial of temsirolimus combined with interferon α for advanced renal cell carcinoma. J Clin Oncol 25:3958–3964
25. McDermott DF (2009) The application of high-dose interleukin-2 for metastatic renal cell carcinoma. Med Oncol 26(Suppl 1):13–17
26. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, Minato N, Toma H (2007) Safety profile and anti-tumor effects of adoptive immunotherapy using γδ T cells against advanced renal cell carcinoma: a pilot study. Cancer Immunol Immunother 56:469–476
27. Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, Golan DE, Brenner MB (1995) Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. Immunity 3:495–507
28. Morita CT, Jin C, Sarikonda G, Wang H (2007) Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev 215:59–76
29. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M (2000) Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 96:384–394
30. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP (2003) γδ T cells for immune therapy of patients with lymphoid malignancies Blood 102:200–206
31. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N, Salerno A, Eberl M, Hayday AC (2007) Targeting human γδ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res 67:7450–7457
32. Kunzmann V, Wilhelm M (2005) Anti-lymphoma effect of γδ T cells. Leuk Lymphoma 46:671–680
33. Santini D, Martini F, Fratto ME, Galluzzo S, Vincenzi B, Agrati C, Turchi F, Piacentini P, Rocci L, Manavalan JS, Tonini G, Poccia F (2009) In vivo effects of zoledronic acid on peripheral γδ T lymphocytes in early breast cancer patients. Cancer Immunol Immunother 58:31–38
34. Lamb LS Jr (2009) γδ T cells as immune effectors against high-grade gliomas. Immunol Res 45:85–95
35. Laggner U, Lopez JS, Perera G, Warbey VS, Sita-Lumsden A, O'Doherty MJ, Hayday A, Harries M, Nestle FO (2009) Regression of melanoma metastases following treatment with the n-bisphosphonate zoledronate and localised radiotherapy. Clin Immunol 131:367–373
36. Viey E, Fromont G, Escudier B, Morel Y, Da Rocha S, Chouaib S, Caignard A (2005) Phosphostim-activated γδ T cells kill autologous metastatic renal cell carcinoma. J Immunol 174:1338–1347
37. Sicard H, Ingoure S, Luciani B, Serraz C, Fournie JJ, Bonneville M, Tiollier J, Romagne F (2005) In vivo immunomanipulation of Vγ9 Vδ2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model. J Immunol 175:5471–5480
38. Buckler HM, Mercer SJ, Davison CE, Hollis S, Richardson PC, Anderson DG (1998) Evaluation of adverse experiences related to pamidronate infusion in Paget's disease of bone. Ann Rheum Dis 57:572
39. Adami S, Bhalla AK, Dorizzi R, Montesanti F, Rosini S, Salvagno G, Lo Cascio V (1987) The acute-phase response after bisphosphonate administration. Calcif Tissue Int 41:326–331
40. Purohit OP, Anthony C, Radstone CR, Owen J, Coleman RE (1994) High-dose intravenous pamidronate for metastatic bone pain. Br J Cancer 70:554–558
41. Gnant M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Postlberger S, Menzel C, Jakesz R et al (2009) Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N Engl J Med 360:679–691
42. Bivi N, Romanello M, Harrison R, Clarke I, Hoyle DC, Moro L, Ortolani F, Bonetti A, Quadrifoglio F, Tell G, Delneri D (2009) Identification of secondary targets of N-containing bisphosphonates in mammalian cells via parallel competition analysis of the barcoded yeast deletion collection. Genome Biol 10:R93.1–R93.11
43. Kobayashi H, Tanaka Y, Shimmura H, Minato N, Tanabe K (2010) Complete remission of lung metastasis following adoptive immunotherapy using activated autologous γδ T-cells in a patient with renal cell carcinoma. Anticancer Res 30:575–580
44. Eble JN, Sauter G, Epstein JI, Sesterhenn IA (eds) (2004) World Health Organization Classification of Tumors. Pathology and Genetics of Tumors of the Urinary System and Male Genital Organs, IRAC Press, Lyon

Adrenomedullin: A Novel Therapy for Intractable Ulcerative Colitis

To the Editor:

In May 2010, a 68-year-old woman undergoing treatment for diabetes presented with a 3-year history of ulcerative colitis (UC). The patient was steroid-dependent, so a regimen of mesalazine (5-aminosalicylic acid [5-ASA]), prednisolone (PSL), and azathioprine (AZA) was prescribed. Despite this therapy the patient experienced a rapid deterioration, with severe abdominal pain and bloody stool (>10 times/day). Further evaluation revealed deep ulceration and erosion throughout the large intestine, which gave an Ulcerative Colitis Disease Activity Index (UCDAI) score of 12. Higher doses of PSL and AZA in combination with leukocytapheresis failed to induce remission (UCDAI score: 7). Addition of immunosuppressants or biologics was deemed unfeasible due to the patient's age, impaired glucose tolerance, and old tuberculosis, as well as the significant risk of concomitant infectious disease. After ruling out the presence of ischemic heart disease, cerebrovascular disorder, or other cardiovascular or malignant disease, adrenomedullin (AM; 1.5 pmol/kg/min) was intravenously administered for 8 hours per day for 12 days. A few days after starting the AM treatment the patient's abdominal pain and bloody stool appeared to go into remission. No adverse events were observed apart from a slight decline in blood pressure. Endoscopy at 2 weeks revealed significant mucosal regeneration (Fig. 1) and spider web-like scarring in some ulcers

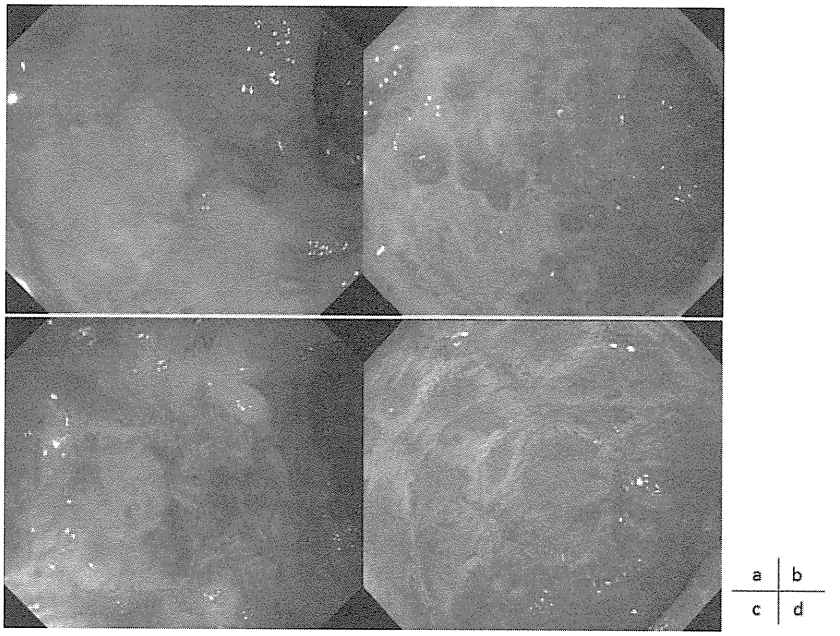

FIGURE 1. Colonoscopic findings. Wide and deep ulcers were observed in the transverse colon (a) and the sigmoid colon (b) before AM therapy. Two weeks after AM therapy (c,d), significant neovascularization and mucosal regeneration were observed at the margin and base of deep ulcers in the transverse colon (c). In the sigmoid colon, fibrosis (scarring) and vasodilation were seen in relatively shallow ulcers. Scarred regions had a reticulated, spider web-like appearance (d). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

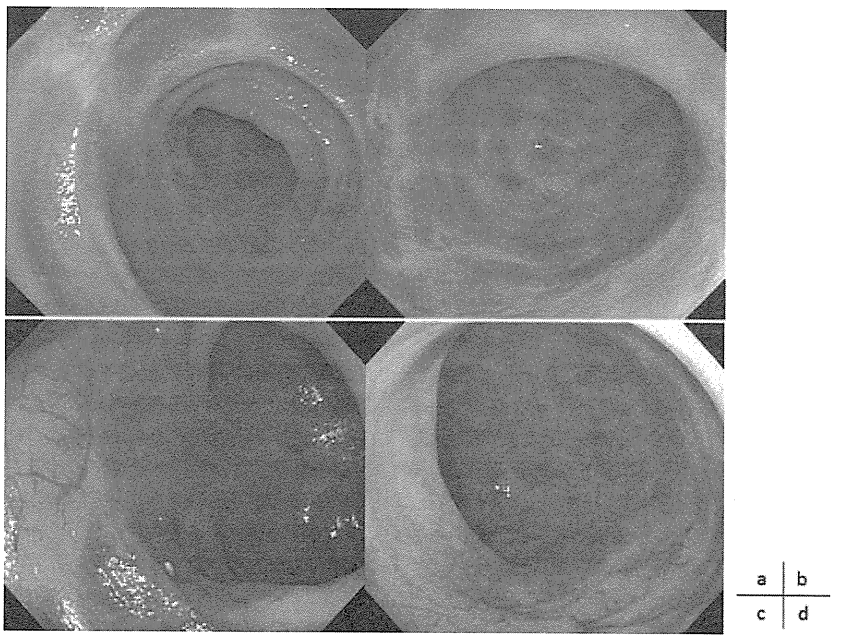

FIGURE 2. Colonoscopic findings. Three months after treatment with AM the ulcers had disappeared and ulcer scars were observed in the transverse colon (a) and the sigmoid colon (b). One year after the treatment with AM the mucosa of the transverse colon (c) and sigmoid colon (d) remained in remission without steroid therapy. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Supported in part by a Translational Research grant (09156271) from the Ministry of Health, Labour and Welfare of Japan.

DOI 10.1002/ibd.22891
Published online in Wiley Online Library (wileyonlinelibrary.com).

(Fig. 2), and the patient's UCDAI score had declined to 2. After 3 months, all of the patient's colonic lesions had healed with scarring and her UCDAI score had reached 0, so the PSL was discontinued.

AM was first identified as a biologically active peptide with potent vasodilating action,[1] but is now known to exert a wide range of physiological effects, including cardiovascular protection,[2] neovascularization, and suppression of inflammation and apoptosis. We previously reported that AM therapy was effective in an animal colitis model,[3] and that AM's mechanism of action is likely attributable to its suppression of inflammatory cytokines and activation of regulatory cytokines in intestinal intraepithelial lymphocytes, as well as to its protection of intercellular junctions and its antibacterial activity.[4] In addition, AM reportedly suppresses cytokine production in trinitrobenzene sulfonic acid (TNBS)-induced colitis,[5] and exerts beneficial effects on microvascular function[6] and the reepithelialization[7] of ulcers in an experimental model of colitis.

Although AM has potent hypotensive activity, we observed only minor hemodynamic effects after administering a dose of 1.5 pmol/kg/min, which we considered safe based on human dose–response data in our possession.

Conventional treatment of active UC focuses on steroids, immunosuppressants, and biologics, but the use of these drugs is restricted in geriatric and immunocompromised patients.[8] AM, on the other hand, is a physiological peptide and is therefore anticipated to have excellent safety. Here we present the first reported case in which AM was used to treat a patient with intractable UC. AM treatment produced mucosal regeneration accompanied by marked neovascularization and vasodilation visible on endoscopic examination. These findings are suggestive of AM's potential to be a ground-breaking modality with a novel mechanism of action that differs from existing immunomodulation therapy.

Shinya Ashizuka, MD, PhD
Toshihiro Kita, MD, PhD
Haruhiko Inatsu, MD
Kazuo Kitamura, MD, PhD
Division of Circulation and Body Fluid Regulation, Faculty of Medicine
University of Miyazaki
Miyazaki, Japan

REFERENCES

1. Kitamura K, Kangawa K, Kawamoto M, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromacytoma. *Biochem Biophys Res Commun.* 1993; 192:553–560.
2. Kataoka Y, Miyazaki S, Yasuda S, et al. The first clinical pilot study of intravenous adrenomedullin administration in patients with acute myocardial infarction. *J Cardiovasc Pharmacol.* 2010;56:413–419.
3. Ashizuka S, Ishikawa N, Kato J, et al. Effect of adrenomedullin administration on acetic acid-induced colitis in rats. *Peptides.* 2005;26: 2610–2615.
4. Ashizuka S, Inagaki-Ohara K, Kuwasako K, et al. Adrenomedullin treatment reduces intestinal inflammation and maintains epithelial barrier function in mice administered dextran sulphate sodium. *Microbiol Immunol.* 2009; 53:573–581.
5. Gonzales-Rey E, Fernandez-Martin A, Chorny A, et al. Therapeutic effect of urocortin and adrenomedullin in a murine model of Crohn's disease. *Gut.* 2006;55:824–832.
6. Talero E, Alvarez de Sotomayor M, Sánchez-Fidalgo S, et al. Vascular contribution of adrenomedullin to microcirculatory improvement in experimental colitis. *Eur J Pharmacol.* 2011;30:601–607.
7. Hayashi Y, Narumi K, Tsuji S, et al. Impact of adrenomedullin on dextran sulfate sodium-induced inflammatory colitis in mice: insights from in vitro and in vivo experimental studies. *Int J Colorectal Dis.* 2011;26:1453–1462.
8. Burger D, Travis S. Conventional medical management of inflammatory bowel disease. *Gastroenterology.* 2011;140:1827–1837.

Japanese Journal of Clinical Oncology Advance Access published February 27, 2012

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2012
doi:10.1093/jjco/hys012

Original Article

Safety, Efficacy and Pharmacokinetics of Neratinib (HKI-272) in Japanese Patients with Advanced Solid Tumors: A Phase 1 Dose-escalation Study

Yoshinori Ito[1,*], Mitsukuni Suenaga[1], Kiyohiko Hatake[1], Shunji Takahashi[1], Masahiro Yokoyama[1], Yusuke Onozawa[2], Kentaro Yamazaki[2], Shuichi Hironaka[2], Kiyoshi Hashigami[3], Hirotaka Hasegawa[3], Nobuko Takenaka[4] and Narikazu Boku[2]

[1]Department of Medical Oncology, The Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, [2]Division of Gastrointestinal Oncology, Shizuoka Cancer Center, Shizuoka, [3]Department of Oncology, Clinical R&D, Pfizer Inc, Tokyo and [4]Department of Clinical Pharmacology, Clinical R&D, Pfizer Inc, Tokyo, Japan

*For reprints and all correspondence: Yoshinori Ito, Breast Cancer Division, Department of Medical Oncology, The Cancer Institute of the Japanese Foundation for Cancer Research, 3-8-31 Ariake Koto-ku, Tokyo 135-8550, Japan. E-mail: yito@jfcr.or.jp

Received November 11, 2011; accepted January 26, 2012

Objective: Neratinib (HKI-272), a potent, irreversible, small-molecule, orally administered, pan-ErbB inhibitor that blocks signal transduction via inhibition of three epidermal growth factor receptors [ErbB1, ErbB2 (Her2) and ErbB4], is being developed for the treatment of solid tumors, including breast cancer. This Phase 1 dose-escalation study assessed the safety, tolerability, maximum-tolerated dose, antitumor activity and pharmacokinetics of neratinib in Japanese patients with advanced solid tumors.

Methods: Patients received neratinib 80, 160, 240 or 320 mg orally; each patient enrolled in only one dose cohort. Patients received a single dose in week 1, followed by daily continuous doses. Blood samples collected were on days 1 and 21 for pharmacokinetic analyses.

Results: Twenty-one patients were enrolled (3 breast cancer; 17 colorectal cancer; 1 gastric cancer). Neratinib-related adverse events (all grades) included diarrhea (20 patients), fatigue (14 patients), nausea and abdominal pain (9 patients each) and anorexia (8 patients). Grade $\geq$3 neratinib-related adverse events in two or more patients were diarrhea and anorexia (two patients each). Dose-limiting toxicities were diarrhea and anorexia (two patients, 320 mg dose). The maximum-tolerated dose and recommended dose was neratinib 240 mg once daily. Of 21 evaluable patients, 2 with breast cancer had partial response, 3 had stable disease $\geq$24 weeks, 7 had stable disease $\geq$16 weeks and 9 had progressive disease. Pharmacokinetic analyses indicated that neratinib exposures increased with dose.

Conclusions: The safety, efficacy and pharmacokinetic profiles of neratinib are consistent with those reported for non-Japanese patients and warrant further investigation of neratinib in Japanese patients with solid tumors.

Key words: ErbB2 – maximum-tolerated dose – neratinib – Phase I clinical trial – treatment efficacy

INTRODUCTION

Dysregulation of growth factor signaling due to hyperactivation of the epidermal growth factor receptor (EGFR/ErbB) family of tyrosine kinase receptors has been observed in several cancer types (1) and is associated with increased proliferation, angiogenesis, metastasis and decreased apoptosis (2). Due to its implication in tumorigenesis, inhibition of

this family of kinase receptors may be a novel and viable treatment option for patients who are intolerant to chemotherapy or those refractory to the current standard of care.

Several drugs have been developed and marketed that selectively inhibit the ErbB receptor kinases, such as the small-molecule, reversible, adenosine triphosphate-competitive inhibitors erlotinib and gefitinib, which target ErbB1 (3,4), and lapatinib, which targets both ErbB1 and ErbB2 (Her2) (5). Monoclonal antibodies have also demonstrated antitumor activity, such as trastuzumab, which binds to ErbB2 (6), and panitumumab and cetuximab, which bind to ErbB1 (7,8). There are, however, various limitations to the safety and efficacy of these drugs. For example, gefitinib and erlotinib provide progression-free survival (PFS) times of only 9–13 months in patients with non-small cell lung cancer showing EGFR mutations. Likewise, trastuzumab is associated with response rates of only 15–26% when given as monotherapy (9,10) and 42% in combination with paclitaxel for the treatment of patients with metastatic breast cancer (11). Trastuzumab is also associated with cardiac toxicity, particularly in patients previously treated with anthracyclines (12,13).

Neratinib (HKI-272) is a potent, orally administered, small-molecule, pan-ErbB inhibitor that irreversibly blocks signal transduction via inhibition of ErbB1, ErbB2 and ErbB4 (14–16). Neratinib has shown promising antitumor activity in a variety of solid tumors, including breast cancer and non-small cell lung cancer (17,18). In addition, neratinib can potentially overcome the acquired resistance of the EGFR 'gatekeeper' T790M mutation. This mutation typically develops in the tumors of lung cancer patients that harbor the EGFR kinase domain-sensitizing mutation after treatment with reversible inhibitors such as gefitinib or erlotinib and subsequent disease progression (19–22).

In the Phase 1, first-in-human dose-escalation study of neratinib in patients with solid tumors that was conducted in the USA, the maximum-tolerated dose (MTD) of neratinib was found to be 320 mg once daily (17). In addition, neratinib exposure was dose-dependent and the pharmacokinetic (PK) results favored a once-daily dosing regimen (17). Neratinib was also clinically active in patients with advanced and/or metastatic ErbB2-positive breast cancer, even under conditions of trastuzumab resistance, and was well tolerated as a once-daily orally dosed agent (17). However, due to the primary dose-limiting toxicity (DLT) of diarrhea, the therapeutic dose was limited to 240 mg once daily in later Phase 2 studies. In patients with advanced ErbB2-positive breast cancer, the 16-week PFS rates were 59 and 78% for patients with prior trastuzumab and no prior trastuzumab treatments, respectively, and the objective response rates (ORRs) were 24 and 56%, respectively (18).

Because the efficacy and safety of drugs, such as gefitinib and sunitinib, can vary between Western and Asian populations (23), we assessed the safety and tolerability, and determined the MTD of oral neratinib in Japanese patients with solid tumors in this Phase 1 study. The preliminary antitumor activity and the PK profile of neratinib in the same patient population were also evaluated.

PATIENTS AND METHODS

Study Design

This was a multicenter, open-label, Phase 1, ascending single and multiple oral dose study conducted in Japan to determine the safety, tolerability, MTD, antitumor activity and PK of neratinib in Japanese patients with advanced solid tumors. Each patient participated in only one dose cohort (three to six patients) and received a single dose of neratinib. After a 1-week observation period, patients received neratinib as a continual oral daily dose for up to 6 months (six cycles), or longer at the same dose level if neratinib was well tolerated and the patient showed no evidence of progressive disease (PD).

This study was conducted in accordance with the International Conference on Harmonisation Guideline for Good Clinical Practice and the ethical principles that have origins in the Declaration of Helsinki. The study protocol was approved by an Institutional Review Board and written informed consent was obtained from all patients before their enrollment in this study.

Patient Eligibility

Patients were eligible for enrollment if they were $\geq$20 years of age and had a histologic/cytologic diagnosis of metastatic or advanced cancer that had failed to respond to standard effective therapy or for which no standard effective treatment was available, a life expectancy of $\geq$12 weeks and a measurable lesion as defined by modified Response Evaluation Criteria in Solid Tumors guidelines. Other key inclusion criteria were a performance status of 0–2 on the Eastern Cooperative Oncology Group (ECOG) scale, an absolute neutrophil count of $\geq 1.5 \times 10^9$/l, platelet count $\geq 100 \times 10^9$/l, creatinine level $\leq 1.5\times$ the upper limit of normal and total bilirubin $\leq 1.5\times$ the upper limit of normal.

The main exclusion criteria were the following: anticancer chemotherapy, radiotherapy, immunotherapy or investigational agents within 4 weeks before treatment day 1; prior treatment with anthracyclines with a cumulative dose of doxorubicin or equivalent >400 mg/m^2; automatic electrocardiogram (ECG)-corrected QT (QTc) interval reading at screening >470 ms; left ventricular ejection fraction (LVEF) below the institutional range of normal as measured by echocardiogram; significant gastrointestinal disorders with diarrhea as a major symptom; and a history of clinically significant cardiac disease, including congestive heart failure, myocardial infarction and significant arrhythmia.

Dose Escalation

Neratinib was administered orally once daily with food, preferably in the morning. After administration of the single dose and a 1-week observation period, patients were treated at the same dose level with continual oral daily doses in 28-day cycles. Dose cohorts consisted of neratinib 80, 160, 240 and 320 mg. The starting dose was based on the results

of the Phase 1, first-in-human study of neratinib in patients with solid tumors that was conducted in the USA, in which neratinib-related Grade 3 adverse events (AEs) were not reported at doses $\leq$80 mg (17). The decision to proceed to the next dose level was made after the last patient in a cohort had been evaluated through ~14 days of continuous daily administration. Enrollment at the next dose level occurred according to the following criteria: if no patients experienced a DLT, then three–six patients were enrolled at the next dose level; if one patient experienced a DLT, then an additional six patients were treated at the same dose level and the dose escalated if no more than one of those patients had a DLT. If two or more patients at a dose level experienced a DLT by day 14 of continuous daily dosing, dose escalation stopped and the previous dose level was considered the MTD. If a patient in any dose cohort had a toxicity that met the definition of DLT, then the patient's dose was reduced by one dose level, and if the patient experienced a second DLT, then the dose was further decreased by one dose level. No more than two dose reductions were allowed for any patient.

A DLT was defined as any neratinib-related non-hematologic Grade 3 or any Grade 4 AE according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0, with the exception of Grade 3 nausea, vomiting, diarrhea or rash, unless the patient was receiving appropriate medical therapy. Additional DLTs included Grade 2 or 3 diarrhea lasting >2 days, for which the patient was receiving appropriate medical therapy or for that which was associated with fever or dehydration. DLTs were assessed during the first 21 days following the administration of the first dose in the continual single-dose period.

Evaluation of Patients

Safety evaluations were based on the incidence and severity of AEs, the DLTs at each dose level and changes in clinical laboratory test results over time. AEs were monitored and recorded continuously during the study, while laboratory evaluations were conducted at screening; on day 1 of the single-dose period; on days 1, 7, 14 and 21 of cycle 1 of the continuous dosing period; on days 1 and 14 of cycles 2 through 6; and at the final evaluation (30 days after the last dose). Other safety assessments included vital signs, interim history, radiographs, cardiac evaluations, echocardiogram and ECGs. The efficacy population included all patients who received $\geq$2 weeks of neratinib therapy and underwent $\geq$1 tumor assessment ~8 weeks after starting continual daily neratinib administration. In addition, patients with disease progression prior to receiving 14 days of neratinib therapy were considered evaluable for efficacy.

PK Analyses

Timed blood samples for PK analyses of neratinib were collected on day 1 and on day 14 (study day 21) of continual daily dose administration. Samples were collected at 0 h (pre-dose) and at 1, 2, 4, 6, 8 and 24 h after dose administration. Samples were also collected at 48 h after dose administration on day 1 of the single-dose period. Plasma neratinib concentrations were measured using a validated liquid chromatography/tandem mass spectrometry method. PK analyses were performed for each patient using non-compartmental methods (24) with WinNonLin® Enterprise application, version 5.1 (Pharsight Corporation, CA, USA). The parameters determined included the following: observed maximum concentration (C_{max}), area under the concentration–time curve (AUC) from time zero extrapolated to infinite time ($AUC_{0-\infty}$), AUC at steady state (AUC_{ss}), AUC from time 0–24 h (AUC_{0-24h}), time of maximum concentration (t_{max}), terminal-phase elimination half-life ($t_{1/2}$), the apparent volume of distribution for the terminal disposition phase (V_z/F) and the apparent oral clearance (CL/F).

The preliminary assessment of dose proportionality was evaluated by the following power model:

$$C_{max}, \quad AUC_{0-\infty} \quad \text{or} \quad AUC_{ss} = \alpha \times \text{dose}^{\beta} \qquad (1)$$

where α is the coefficient and β is the exponent of the linear-regression model on log-transformed parameters, C_{max}, $AUC_{0-\infty}$, AUC_{ss} and dose. The 95% confidence intervals (CIs) for the exponents were also calculated. The validity of the power model was evaluated by performing a lack-of-fit test. A P-value for the lack-of-fit test of <0.05 would imply that there was a significant lack of fit in the power model and that the point estimate derived from the power model was not valid.

Determination of Sample Size

Approximately 28 patients were to be enrolled in this study. This estimate was based on a maximum of 6 patients per dose cohort over approximately four dose levels and enrolling 4–7 additional patients (total 10 patients) at the recommended dose. The actual number of patients enrolled was dependent on the tolerability of neratinib and the number of dose levels required to attain the MTD.

The sample size for this study was determined by clinical rather than statistical considerations. With cohort sizes of three to six patients, if the true underlying rates of DLT were 0.1, 0.2, 0.3, 0.4 and 0.5, there would be 0.91, 0.71, 0.49, 0.31 and 0.17 chances, respectively, of escalating to the next higher dose level. If the frequencies of AEs of Grade $\geq$3 were 0.1, 0.25 and 0.5, the probabilities of detecting one or more such events in six patients receiving neratinib would be 0.469, 0.822 and 0.984, respectively, and the probabilities of detecting one or more such events in 10 patients would be 0.651, 0.944 and 0.999, respectively.

Table 1. Patients' demographics and baseline characteristics

Characteristic	Dose cohorts, mg neratinib				
	80 ($n = 3$)	160 ($n = 3$)	240 ($n = 10$)	320 ($n = 5$)	Total ($n = 21$)
Median age, years (range)	54 (44–63)	47 (44–54)	64.5 (39–78)	61 (47–66)	61 (39–78)
Primary diagnosis, *n* (%)					
Breast cancer	0	1	1	1	3 (14)
Colorectal cancer	3	1	9	4	17 (81)
Gastric cancer	0	1	0	0	1 (5)
ECOG performance status, *n* (%)					
0	3	3	9	4	19 (91)
1	0	0	1	1	2 (10)
Prior chemotherapy, immunotherapy or hormonal therapy, *n* (%)					
Yes	3	3	10	5	21 (100)
Prior chemotherapy, *n* (%)					
2	2	1	4	1	8 (38)
3	0	0	2	2	4 (19)
4	0	0	2	1	3 (14)
5	0	1	0	0	1 (5)
6	1	0	1	1	3 (14)
9	0	0	1	0	1 (5)
12	0	1	0	0	1 (5)
Prior radiotherapy, *n* (%)					
Yes	0	1	3	0	4 (19)
No	3	2	7	5	17 (81)
Prior surgical therapy/cancer biopsy, *n* (%)					
Yes	3	3	10	5	21 (100)

ECOG, Eastern Cooperative Oncology Group.

RESULTS

Patient Characteristics

A total of 21 patients (median age: 61 years; range: 39–78 years) were enrolled in this study from March 2007 to March 2009. The baseline characteristics of the 21 patients are presented in Table 1. Seventeen patients had a primary diagnosis of colorectal cancer, three had a diagnosis of breast cancer and one had a diagnosis of gastric cancer. All 21 (100%) patients had an ECOG performance status of 0 or 1. All patients had received prior cancer-related surgery and chemotherapy and four had received prior radiotherapy.

Dose Escalation of Neratinib

Diarrhea and anorexia were the only reported DLTs for two (40%) patients in the 320 mg dose cohort in this study; one patient had Grade 3 diarrhea and Grade 3 anorexia, and the other patient had Grade 2 diarrhea and Grade 3 anorexia. Neratinib 240 mg was determined to be the MTD and was thus used for the expanded MTD cohort. Therefore, the 240 mg cohort was expanded to include an additional seven patients to confirm the safety and tolerability of the MTD of neratinib.

Safety

All 21 (100%) patients completed the single-dose period and then started the continual daily dose period. The median duration of treatment in the continual daily dose period was 14.9 weeks (range: 2.1–39.9 weeks). The median relative dose intensity was 1.00 for each dose level (range in 240 mg: 0.75–1.00), indicating that patients received close to the initial scheduled daily dose.

All 21 patients experienced AEs that were considered neratinib-related (Table 2). The most common neratinib-related AEs were: diarrhea (20 patients, 95%); fatigue (14, 67%); nausea and abdominal pain (9, 43%

Table 2. Neratinib-related adverse events of all grades that occurred in ≥15% of patients and of Grade ≥3 that occurred in one or more patients from screening visit until 30 days after last dose of neratinib

Adverse event	Dose cohorts, mg neratinib				
	80 ($n = 3$)	160 ($n = 3$)	240 ($n = 10$)	320 ($n = 5$)	Total ($n = 21$)
Diarrhea	2	3	10	5	20 (95)
Grade ≥3	0	1	0	1	2 (10)
Fatigue	1	3	6	4	14 (67)
Abdominal pain	1	2	4	2	9 (43)
Nausea	0	3	3	3	9 (43)
Anorexia	0	1	5	2	8 (38)
Grade ≥3	0	0	0	2	2 (10)
Aspartate aminotransferase increased	1	1	4	2	8 (38)
Grade ≥3	0	0	1	0	1 (5)
Blood alkaline phosphatase increased	0	1	6	1	8 (38)
Hemoglobin decreased	0	1	4	3	8 (38)
Alanine aminotransferase increased	1	0	4	2	7 (33)
Grade ≥3	0	0	1	0	1 (5)
Blood albumin decreased	2	1	2	2	7 (33)
Weight decreased	0	1	3	3	7 (33)
Blood triglycerides increased	1	2	2	1	6 (29)
Rash	2	0	3	1	6 (29)
Blood creatine phosphokinase increased	0	0	2	3	5 (24)
Hyperglycemia	1	0	3	1	5 (24)
Pyrexia	0	0	3	2	5 (24)
Vomiting	0	1	3	1	5 (24)
Blood creatinine increased	0	1	1	2	4 (19)
Stomatitis	1	0	2	1	4 (19)
Neutropenia	0	1	0	1	2 (10)
Grade ≥3	0	0	0	1	1 (5)
Esophageal varices	0	0	1	0	1 (5)
Grade ≥3	0	0	1	0	1 (5)

each); increased aspartate aminotransferase levels, increased blood alkaline phosphatase levels, decreased hemoglobin levels and anorexia (8, 38% each); and increased alanine aminotransferase levels, decreased blood albumin levels and decreased weight (7, 33% each). Anorexia and diarrhea were the most common Grade ≥3 neratinib-related AEs (2, 10% each; Table 2). The median onset of diarrhea was 10.0 days

Table 3. Best overall response in the evaluable population

Response	Dose cohorts, mg neratinib				
	80 ($n = 3$)	160 ($n = 3$)	240 ($n = 10$)	320 ($n = 5$)	Total ($n = 21$)
CR, *n*	0	0	0	0	0
PR, *n*	0	0	1	1	2
ORR, %	0	0	10.0	20.0	9.5
SD, *n*					
≥16 weeks	1	1	4	1	7
≥24 weeks	0	0	2	1	3
CBR, %	0	0	30.0	40.0	23.8
PD, *n*	2	2	3	2	9

CR, complete response; PR, partial response; ORR, objective response rate (CR + PR); SD, stable disease; CBR, clinical benefit rate (CR + PR + SD ≥24 weeks); PD, progressive disease.

and the median duration was 2.0 days. Even though all diarrhea AEs were considered neratinib-related, no patient had a Grade ≥4 event. Diarrhea was managed by dose interruption, dose reduction and appropriate medication and resolved in 90% of the patients. Cardiovascular AEs were reported for one patient who had an LVEF that decreased from normal at baseline to <50%. However, the decrease in LVEF was related to the patient's underlying disease of sinus bradycardia and was considered not related to neratinib therapy by the treating investigator.

Serious AEs were reported for six patients; anorexia and fatigue (two patients each); hydronephrosis, nausea, dysphagia, esophageal varices and dyspnea (one patient each). One neratinib-related serious AE, esophageal varices, was reported for a patient in the 240 mg cohort. No patient discontinued treatment and was withdrawn from this study due to an AE, and no deaths were reported during the study or within 30 days after the last dose was administered.

A total of three (14%) patients had dose reductions due to AEs; two patients in the 320 mg cohort had diarrhea and anorexia, and one patient in the 240 mg cohort had diarrhea. All AEs that led to dose reductions were considered neratinib-related.

Antitumor Activity

All 21 patients were considered evaluable for efficacy (Table 3). Two of the three patients with primary diagnoses of breast cancer had a partial response (PR). ErbB2 status was positive for one of these two patients but unknown for the other patient; both patients had received a prior trastuzumab-containing regimen. Three patients had stable disease (SD) ≥24 weeks, seven patients had SD ≥16 weeks and nine patients had PD. The ORR [complete response (CR) + PR] for all patients was 9.5% (95% CI: 1.2–30.4)

Table 4. Pharmacokinetic parameters of neratinib in Japanese patients with advanced solid tumors

Dose cohort, mg (*n*)	Parameter, mean (CV%)						
	C_{max} (ng/ml)	t_{max}[a] (h)	$t_{1/2}$ (h)	AUC[b] (ng×h/ml)	CL/F (l/h/kg)	V_z/F (l/kg)	*R*
Study day 1							
80 (3)	33.3 (43)	4.0 (2.0–8.0)	NC	NC	NC	NC	NA
160 (3)	51.4 (43)	3.9 (3.9–4.0)	11.1 (28)	638 (66)	5.8 (61)	84 (45)	NA
240 (10)	76.3 (41)	5.9 (2.0–8.0)	14.3 (19)[c]	1640 (48)[c]	3.7 (97)[c]	65 (63)[c]	NA
320 (5)	93.2 (40)	4.0 (3.9–7.9)	16.0 (13)	2290 (46)	3.0 (61)	71 (69)	NA
Study day 21							
80 (3)	41.9 (62)	4.0 (2.0–6.0)	17.6 (50)	581 (46)	2.7 (35)	76 (81)	1.5 (49)[d]
160 (3)	57.4 (80)	3.9 (2.0–4.0)	12.7 (27)	688 (79)	12.0 (129)	192 (122)	1.3 (60)
240 (10)	81.5 (56)	4.0 (2.0-7.9)	22.7 (88)[e]	1110 (59)[e]	5.4 (74)[e]	149 (74)[e]	1.2 (43)[e]
320 (3)	143.0 (34)	3.9 (0.0-5.9)	22.1 (12)[d]	2040 (10)[d]	2.5 (6)[d]	80 (18)[d]	1.3 (4)[d]

CV%, percent coefficient of variation; C_{max}, peak concentration; t_{max}, time to peak concentration; $t_{1/2}$, terminal phase elimination half-life; AUC, area under the concentration–time curve; CL/F, apparent oral dose clearance; V_z/F, apparent volume of distribution; *R*, accumulation ratio (quotient of AUC_{ss} on day 1 to AUC_{0-24h} on study day 21); NC, not calculated; NA, not applicable.
[a]t_{max} reported as median (range: minimum–maximum).
[b]Reported as AUC from time zero extrapolated to infinite time ($AUC_{0-\infty}$) for study day 1, and the steady-state AUC (AUC_{ss}) for study day 21.
[c]$n = 8$.
[d]$n = 2$.
[e]$n = 9$.

and the clinical benefit rate (CR + PR + SD ≥24 weeks) was 23.8% (95% CI: 8.2–47.2). Durations of response for the two patients with PR were 16.1 and 32.3 weeks, respectively. The median duration of SD was 16.7 weeks (95% CI: 16.3–24.1) among 10 patients with SD. The median time to progression was 16.1 weeks (95% CI: 8.4–17.0) for all patients.

Pharmacokinetics

Plasma samples for PK analyses were available for all 21 patients who received neratinib doses ranging from 80 to 320 mg. Samples collected within 5 days after dose reduction were not included in the PK analysis. The PK parameters are summarized in Table 4. Following single doses of neratinib from 80 to 320 mg on study day 1, the absorption of neratinib was relatively slow with a median t_{max} of 4–6 h and mean $t_{1/2}$ for the 160–320 mg dose cohorts ranged from 11 to 16 h (percent coefficient of variation, 13–28%). Multiple-dose exposure was 1.2- to 1.5-fold greater than single-dose exposure across the entire dose range, as assessed by the mean accumulation ratio (*R*, AUC_{ss} on study day 21 to AUC_{0-24h} on study day 1; Table 4). These results suggest that there is no major accumulation of neratinib after repeated daily administration to patients with solid tumors.

After single and multiple oral doses of neratinib, C_{max}, $AUC_{0-\infty}$ and AUC_{ss} appeared to increase with the increasing dose (Fig. 1). The power-model assessment confirmed the dose proportionality of neratinib administration. For day 1, exponents for C_{max} and $AUC_{0-\infty}$ were 0.73 and 1.28, respectively, and the corresponding 95% CIs of the exponents were 0.23–1.24 and 0.32–2.24. For day 21, the exponents for C_{max} and AUC_{ss} were 0.88 and 0.78, respectively, and the corresponding 95% CIs of the exponents were 0.03–1.73 and −0.17–1.73. For all of the parameters on days 1 and 21, the CIs contained one, suggesting that there is no lack of dose proportionality. In addition, the lack-of-fit tests for the models were not statistically significant, thus suggesting a linear relationship for C_{max}, $AUC_{0-\infty}$ and AUC_{ss} versus dose.

A comparison of our PK results in our Japanese patients versus patients in the neratinib study that was conducted in the USA (17), using our in-house data, is presented in Fig. 2. Although the variability in C_{max}, $AUC_{0-\infty}$ and AUC_{ss} is large, there is overlap of the PK exposures between the Japanese and US studies. This comparison suggests that there are no relevant differences in the PK between Japanese patients and those patients (92% white) in the US study.

DISCUSSION

In this Phase 1 study, neratinib as a single agent was administered to Japanese patients with advanced solid tumors. The reported DLTs were Grades 2 and 3 diarrhea and Grade 3 anorexia for two patients in the 320 mg dose cohorts; therefore, the MTD of neratinib for Japanese patients was determined to be 240 mg once daily. In comparison, the MTD was found to be 320 mg once daily in the Phase 1