TAC-101 (4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid) Inhibits Spontaneous Mediastinal Lymph Node Metastasis Produced by Orthotopic Implantation of Lewis Lung Carcinoma

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The anti-tumor and anti-metastatic effects of 4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid (TAC-101) were investigated using our established lung cancer model. Orthotopic implantation of Lewis lung carcinoma (LLC) cells into the lung parenchyma produced a solitary tumor nodule in the lung followed by mediastinal lymph node metastasis. Daily oral administration of TAC-101 at doses ranging from 4 to 16 mg/kg resulted in a significant inhibition of lymphatic metastasis (inhibition rate=57 to 76%), while only the dose of 16 mg/kg significantly inhibited tumor growth at the implanted sites (inhibition rate=46%). Combined treatment with *cis*-diamminedichloroplatinum (CDDP) and TAC-101 (8 mg/kg, p.o., daily) enhanced the anti-tumor effect of CDDP (7 mg/kg, i.v., bolus) against both the growth of implanted tumor and lymphatic metastasis. In addition, this combined treatment significantly prolonged the survival time of LLC tumor-bearing mice as compared to treatment with each agent alone. The anti-activating protein-1 (AP-1) activity of TAC-101 caused inhibition of LLC cell invasion through the repression of expression of urokinase-type plasminogen activator and its receptor. The anti-invasive activity of TAC-101 may be involved in its *in vivo* anti-metastatic activity. These findings suggest that TAC-101 is a novel anti-cancer agent that may improve the therapeutic modalities for lung cancer patients with metastatic disease.

Key words: LLC — Mediastinal lymph node metastasis — AP-1 — u-PA — TAC-101

Lung neoplasia is a major cause of death in cancer patients throughout the world. Diagnostic techniques and therapeutic modalities for lung cancer patients have been improved over the past decade; however, the overall death rate is still high.[1] Despite undergoing curative surgical treatment, many patients develop recurrent disease and most of such recurrent disease includes distant metastasis.[2, 3] Therefore, controlling metastatic disease is one of the important problems that must be addressed in order to conquer lung neoplasia.

Among the various clinicopathological factors, lymphatic metastasis is one of the most critical factors for the prognosis of lung cancer patients.[4–6] Although experimental models for lung cancer have been reported by several investigators, these models have included some important drawbacks, such as ectopic implantations and complicated procedures.[7–9] Recently, we established a model of spontaneous lymphatic metastasis produced by orthotopic implantation of lung cancer cells.[10] Direct implantation of Lewis lung carcinoma (LLC) admixed with "MATRIGEL" into the left lobe of the lung caused the formation of a solitary tumor followed by metastasis to the mediastinal lymph node. This model should be useful for investigating therapeutic approaches for lung cancer disease in preclinical studies.

We have reported that 4-[3,5-bis(trimethylsilyl)benzamido]benzoic acid, TAC-101, abolished activating protein-1 (AP-1) binding to consensus DNA and inhibited the experimental liver metastasis of gastrointestinal tract cancer in animal models.[11] AP-1 has been reported to be a major transcriptional enhancer for the expression of urokinase-type plasminogen activator, overexpression of which has been reported to be correlated with lymphatic metastasis of lung cancer.[12–16] In the present study, we investigated the effects of TAC-101 on the growth at the implantation site and the spontaneous lymphatic metastasis caused by the orthotopic implantation of LLC, and we examined its anti-metastatic mechanism of action *in vitro*. Since platinum agents have been used as standard treatment modalities for lung cancer,[1, 17, 18] combination therapy of TAC-101 and CDDP in the LLC model was also examined.

MATERIALS AND METHODS

Chemicals TAC-101 was synthesized by Taiho Pharmaceutical Co., Ltd. (Saitama). *cis*-Diamminedichloroplatinum

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(CDDP) was purchased from Nippon Kayaku Co., Ltd. (Tokyo). For *in vivo* experiments, TAC-101 was suspended in 0.5% hydroxypropoxyl methyl cellulose. For *in vitro* experiments, TAC-101 was dissolved in dimethyl sulfoxide at a concentration of 20 mM for the stock solutions, and kept at $-20°C$ until use.

Cell line LLC cell line was kindly provided by Dr. K. Takeda (Tohoku University, Miyagi) and was maintained in Dulbecco's modified MEM (DMEM) supplemented with 10% fetal calf serum (FCS) and L-glutamine in a humidified atmosphere of 5% CO_2 at 37°C.

Mice Female C57BL/6CrSlc mice (5–6 weeks old) were purchased from Japan SLC Inc. (Shizuoka). They were maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, under laminar air-flow conditions. This study was conducted in accordance with the standards established by the Guideline for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Intrapulmonary implantation of LLC and evaluation of anti-tumor activity Orthotopic implantation of LLC cells into the lung was performed as described previously with some modification.[10] The left chests of anesthetized mice were incised (approximately 5 mm in length) and 20-μl aliquots of LLC cell suspension (2×10^3 cells) admixed with "MATRIGEL" (Collaborative Biochemical Products Inc., MA; 20 μg) were injected into the left lung parenchyma through the intercostal space. The skin incision was closed with "AUTOCRIP" (Becton Dickinson Co., MD). The anti-tumor effect was evaluated by measuring the weight of orthotopic tumor in the lung and the metastasized tumor at mediastinal lymph nodes on day 17 after the implantation. The survival time (days) of mice after the implantation was determined to evaluate the life-prolonging effect.

***In vivo* treatment with TAC-101 and CDDP** TAC-101 was administered p.o. daily for 14 days, starting on day 1 after tumor implantation. CDDP (7 mg/kg), a clinically equivalent dose in mice,[19] was given i.v. on day 1. In the experiments with combination treatment, CDDP was given i.v. on day 1 and TAC-101 was given p.o. for 14 days starting on day 1.

Invasion assay The invasive ability of tumor cells was assessed in a Chemotaxicell chamber (Kurabo Inc., Osaka; 8-μm pores) as described previously.[20] Filters of the chamber were coated with 1 μg/10 μl of fibronectin (Iwaki Glass Co., Ltd., Tokyo) on the lower surface and 10 μg/50 μl of "MATRIGEL" on the upper surface and dried. LLC cells suspended in DMEM/F-12 with 0.1% bovine serum albumin (BSA) (1×10^5/chamber) were added to the upper compartment of the chamber, and incubated in the presence of various concentrations of TAC-101 in both upper and lower compartments. After 24 h of incubation, the

invaded cells on the lower surface of the chamber were determined by crystal violet staining. Each condition was tested in quadruplicate.

Assay for *in vitro* cytotoxic activity LLC cells were resuspended in DMEM/F-12 supplemented with 10% FCS, and seeded into 96-well culture plates (2×10^3/well). After 48 h of preincubation, the medium was replaced with DMEM/F-12 containing 0.1% BSA and various concentrations of TAC-101, and the cultures were incubated for a further 24 h. Crystal violet staining was performed to evaluate the activity.

Zymographic assay for urokinase-type plasminogen activator (u-PA) u-PA activity was detected using zymography as previously described.[21] LLC cells (5×10^4/well) suspended in DMEM/F-12 supplemented with 10% FCS were seeded into 24-well culture plates. After a 24-h preincubation, the medium was replaced with DMEM/F-12 containing 0.1% BSA plus various concentrations of TAC-101, and the cultures were incubated for a further 24 h. Aliquots of conditioned medium (20 μg of protein) were electrophoresed on fibrinogen (Nacalai Tesque, Osaka)-containing gels and incubated for 48 h at 37°C. Fibrinolytic activities were quantified using a Master Scan Gel Analysis System (Scanalytics, Billerica, MA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) LLC cells (2×10^5/well) suspended in DMEM/F-12 supplemented with 10% FCS were seeded into 6-well culture plates and preincubated for 48 h. After preincubation, the medium was replaced with fresh DMEM/F-12 containing 0.1% BSA and TAC-101, and then incubation was continued for 3, 6, 12 or 24 h. Total RNA was isolated using "ISOGEN" (Nippon Gene Inc., Tokyo). Five micrograms of isolated RNA was reverse-transcribed using a First Strand cDNA Synthesis Kit (Life Science Inc., FL) at 42°C (45 min) to maximize cDNA synthesis, and terminated by heating at 99°C (5 min). PCR was performed using cDNA templates (contamination of genomic DNA was not observed in a preliminary experiment; data not shown) and specific oligonucleotide primers for u-PA, u-PAR, PAI-1, PAI-2[22] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[23] as described previously. The relative abundances of u-PA, u-PAR and PAI-1 were expressed as density ratios relative to GAPDH, as determined by using the Master Scan Gel Analysis System (Scanalytics, Billerica, MA).

Dual reporter assay for evaluation of AP-1 activity LLC cells (2×10^5/well) suspended in DMEM/F-12 with 10% FCS were seeded into 6-well culture plates. After 48 h of preincubation, pAP-1 Luc plasmid (Stratagene, Funakoshi, Tokyo, 1 μg/well) and pRL-TK vector (Promega Co.,WI; 0.1 μg/well) were cotransfected using FUGENE 6 reagent (Roche Diagnostics, GmbH, Mannheim, Germany) and incubation was continued for a further 20 h. The medium of transfected cells was replaced with DMEM/F-

12 containing 0.1% BSA plus various concentrations of TAC-101, and the culture was incubated for a further 6 or 24 h. At the end of the incubation, cell lysates were prepared and AP-1 luciferase activity was determined by using the "Dual-Luciferase" Reporter Assay System (Promega Co., MA). The relative AP-1 activity was determined using the pRL-TK luciferase activity as an internal control.

Statistics   Welch's *t*-test for continuous data[24] and Wilcoxon's exact test for discrete data[25] were performed as two-sided tests. Dunnett's *t*-test was performed as a two-sided test in order to decrease the multiplicity in comparisons of drug-treated groups with controls.[26] To analyze the statistical significance of combination therapy, one-sided Welch's *t*-test for tumor weight and one-sided Wilcoxon's exact test for survival time were performed using the all rejecting rule against an *a-priori* ordered hypothesis, comparing TAC-101 alone and CDDP alone with combined drug treatment.[27]

RESULTS

Effect of TAC-101 on the growth of the inoculated tumor and lymph node metastasis after orthotopic implantation of LLC   Daily oral administration of TAC-101 slightly inhibited the growth of LLC tumors at the implantation site (Fig. 1A). The inhibitory effect of 16 mg/kg TAC-101 was significant (inhibition rate=46%). On the other hand, metastasis to the mediastinal lymph nodes was significantly inhibited by TAC-101 in a dose-dependent manner in the dose range from 4 to 16 mg/kg (Fig. 1B: inhibition rate=57 to 76%). Mice treated with vehicle, 2 and 16 mg/kg of TAC-101 showed a reduction of body

weight from day 10 after implantation, whereas the administration of 4 and 8 mg/kg TAC-101 prevented the loss of body weight in tumor-bearing mice (Fig. 1C). Thus, appropriate concentrations of TAC-101 can prevent the loss of body weight in tumor-bearing mice. Since 16 mg/kg of TAC-101 showed anti-tumor and anti-metastatic effects without preventing the reduction of body weight, lack of a positive effect on body weight may have been due to drug-induced toxicity. Therefore, in the following experiments, we used 8 mg/kg TAC-101, since it appeared to be the optimal dose in this model, as was previously reported.[11]

Combined effect of CDDP and TAC-101 in the lung cancer model   As shown in Fig. 2A, combined treatment with TAC-101 (8 mg/kg) and CDDP (7 mg/kg) significantly inhibited tumor growth in the lung as compared to treatment with each drug alone. TAC-101 also produced a significant inhibition of lymph node metastasis, while CDDP alone did not inhibit metastasis (Fig. 2B). However, the anti-metastatic activity of CDDP was enhanced by the combination with TAC-101. Combination with TAC-101 did not enhance the loss of body weight caused by CDDP treatment (Fig. 2C). We next examined the life-prolonging effect achieved with the combined treatment modality (Fig. 3). All the untreated control mice died of tumor burden within 26 days after the implantation. Similar enhanced survival rates were observed in the group of mice which had received either TAC-101 or CDDP alone. The group which received CDDP and TAC-101 showed a significantly prolonged survival time. The T/C values of survival time in TAC-101, CDDP and the combined treatment groups were 124, 118 and 180%, respectively. These findings clearly indicate that the combination of TAC-101
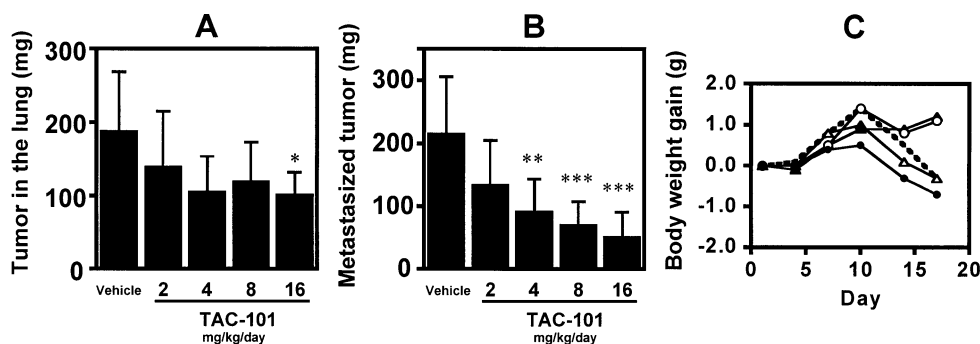

Fig. 1.   Dose-escalating experiment with TAC-101. LLC cell suspension admixed with "MATRIGEL" was orthotopically implanted into the left lung of mice. TAC-101 was administered p.o. for 14 days, starting on day 1. The weight of tumor in the lung for orthotopic tumor (A) and at the mediastinal lymph node for metastasized tumor (B) was measured on day 17 after the implantation. Body weight of each group was measured twice a week during the experiment (C). Mean body weight gain of the vehicle-treated group (dotted, no symbol) and 2, 4, 8 and 16 mg/kg TAC-101 (open triangle, closed triangle, open circle and closed circle, respectively)-treated groups is shown. The numbers of animals were seven in both vehicle and treated groups. * *P*<0.05, ** *P*<0.01, *** *P*<0.001 (two-sided Welch's *t*-test). Bar, SD.
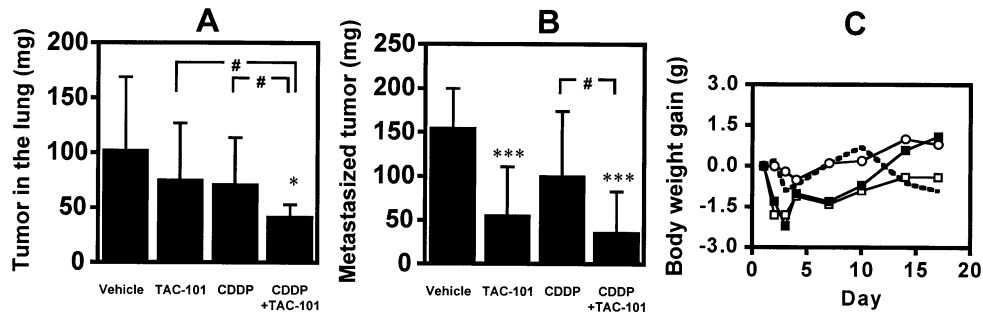
Fig. 2. Combination treatment with TAC-101 and CDDP. LLC cell suspension admixed with "MATRIGEL" was orthotopically implanted into the left lung of mice. CDDP was given i.v. on day 1, and TAC-101 was administered p.o. for 14 days, starting on day 1. The weight of tumor in the lung for orthotopic tumor (A) and at the mediastinal lymph node for metastasized tumor (B) was measured on day 17 after the implantation. Body weight of each group was measured twice a week during the experiment (C). Mean body weight gain of untreated (dotted, no symbol), 8 mg/kg TAC-101-treated (open circle), CDDP-treated (open square) and combined treatment (closed square) groups is shown. The numbers of animals in the control and each treated group were 12 and 10, respectively. ∗ $P<0.05$, ∗∗∗ $P<0.001$ (two-sided Welch's t-test); # $P<0.05$ (one-sided Welch's t-test, all rejecting rule against *a-priori* ordered hypothesis, comparing TAC-101 alone and CDDP alone with the combined treatment). Bar, SD.
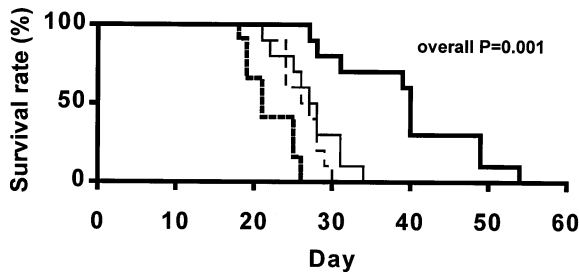

Fig. 3. Life-prolonging effect of TAC-101, CDDP and the combination treatment. Life-prolonging effect was determined from the survival time (day) of LLC-tumor-bearing mice. Survival curves of untreated (dotted, bold), 8 mg/kg TAC-101-treated (line), CDDP-treated (dash) and combined treatment (line, bold) groups are shown. The numbers of animals in the control and each treated group were 12 and 10, respectively. Overall P value was determined by use of the one-sided Wilcoxon's exact test according to the all rejecting rule against *a-priori* ordered hypothesis, comparing TAC-101 alone and CDDP alone with the combined treatment.

Fig. 4. Inhibition of LLC cell invasion and cytotoxicity by TAC-101. *In vitro* invasion assay was performed with LLC cells (1×10^5), treated with various concentrations of TAC-101 for 24 h. Invaded cells were detected by the crystal violet staining method. The results represent the mean±SD of quadruplicate cultures. ∗ $P<0.05$, ∗∗ $P<0.01$, ∗∗∗ $P<0.001$ (two-sided Dunnett's t-test). Bar, SD. Cytotoxic concentration of TAC-101 was determined by 24 h incubation of LLC cells with various concentrations of TAC-101, using the crystal violet staining method (triplicate cultures). Absorbances for invasion assay and cytotoxicity assay are indicated on the left and right vertical bar, respectively.

and CDDP showed an increased therapeutic effect on tumor metastasis and survival rate.

***In vitro* effect of TAC-101 on tumor invasion and invasion-related molecules** To examine the anti-metastatic properties of TAC-101, invasion assays were performed using "MATRIGEL." TAC-101 at non-cytotoxic concentrations of less than 10 μM showed dose-dependent inhibition of the invasion of LLC cells (Fig. 4). Because blocking of the u-PA/plasminogen system is able to inhibit LLC cell invasion,[28–30] we next examined the effect of TAC-101 on the production of u-PA. As shown in Fig. 5A, two fibri-
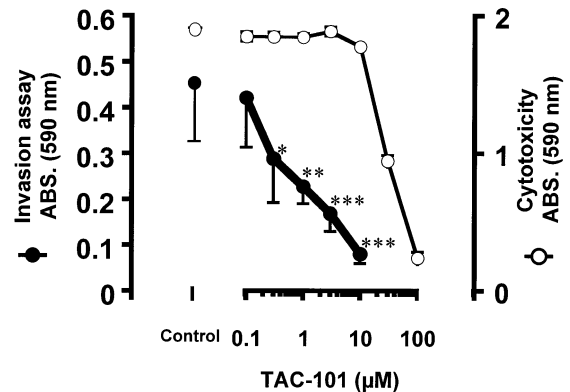
nolytic bands were detected at approximately 100 kDa and at 55 kDa in the zymogram. Since it has been reported that there are four bands i.e., u-PA/PAI-1 complex, tissue-type plasminogen activator, high-molecular-weight u-PA (free form of full-size mature u-PA) and low-molecular-weight u-PA of approximately 100, 70, 55 and 33 kDa, respec-

tively, in fibrinolytic zymograms,[31] the fibrinolytic bands of 100 kDa and 55 kDa were considered to be the PAI-1-bound and free forms of u-PA, respectively. Both fibrinolytic bands disappeared after treatment with TAC-101 in a concentration-dependent manner (Fig. 5, A and B). RT-PCR showed that LLC expressed mRNA for u-PA, u-PAR and PAI-1, but not for PAI-2 (Fig. 6). Treatment with TAC-101 for 3, 6, 12 or 24 h resulted in a significant decrease of the mRNA for u-PA and u-PAR (Fig. 7). Thus, TAC-101 inhibited the production of u-PA through reducing the mRNA for u-PA. Moreover, reduction of the mRNA for u-PAR by TAC-101 may be involved in the inhibition of anti-invasive ability by TAC-101.

Anti-AP-1 activity of TAC-101 The gene expression of u-PA is considered to be transactivated by AP-1, because AP-1 binding motifs are located in the promoter regions of

u-PA, as well as u-PAR.[14, 32] Since we previously reported that TAC-101 inhibited the binding of AP-1 to its consensus DNA,[11] inhibition of AP-1 activity by TAC-101 was also examined using the dual luciferase assay system. Treatment with TAC-101 for transfected LLC cells (6 h) inhibited AP-1 transcriptional activity in a concentration-dependent manner (Fig. 8A). Ten micromolar TAC-101 significantly decreased AP-1 activity at both 6 and 24 h (Fig. 8B), which suggests that inhibition of u-PA and u-PAR expression by TAC-101 is mediated by reduction of AP-1 activity.

DISCUSSION

The present study demonstrated the anti-tumor effect of TAC-101 on the orthotopic tumor growth and mediastinal lymph node metastasis of LLC caused by intrapulmonary implantation and also examined anti-AP-1 activity of
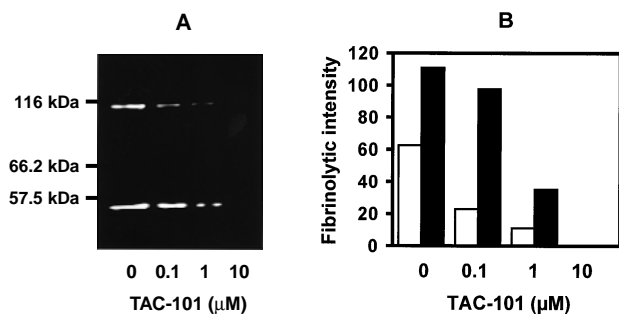

Fig. 5. Effect of TAC-101 on u-PA activity of LLC. Fibrinogen-zymography was performed using conditioned medium of LLC in the presence or absence of TAC-101 (A). Fibrinolytic intensity was determined with a Master Scan Gel Analysis System (B). u-PA/PAI-1 complex (open column), free-u-PA (closed column).
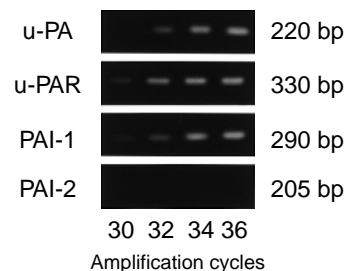

Fig. 6. Detection of mRNAs for u-PA, u-PAR, PAI-1 and PAI-2. RT-PCR was performed using reverse-transcribed cDNA of LLC. PCR products were electrophoresed on 1.5% agarose gel. The numbers under the picture indicate the number of amplification cycles.
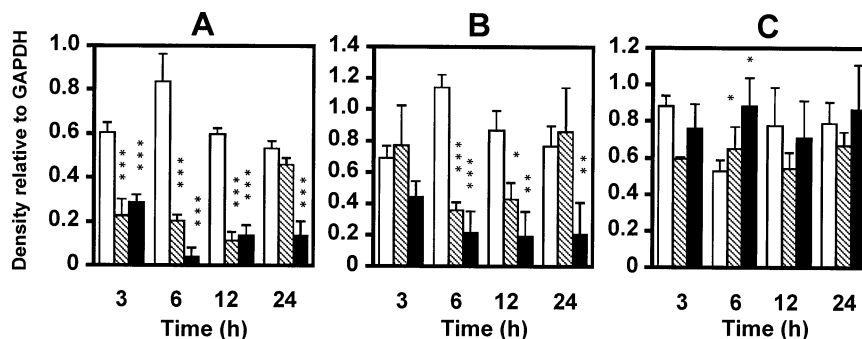

Fig. 7. Effect of TAC-101 on the expression of mRNAs for u-PA (A), u-PAR (B) and PAI-1 (C). LLC cells were incubated with TAC-101 (untreated, open column; 1 μM, shadow column; 10 μM, closed column) for 3, 6, 12 and 24 h. RT-PCR was performed and PCR products were electrophoresed on 1.5% agarose gel. Density ratio to GAPDH was determined with a Master Scan Gel Analysis System. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (two-sided Dunnett's t-test). Bar, SD.
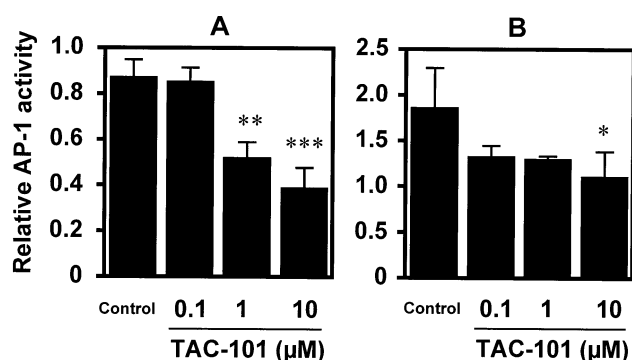
Fig. 8. Effect of TAC-101 on AP-1 transcriptional activity. LLC cells, cotransfected with AP-1 and TK reporter plasmid, were incubated with TAC-101 for 6 (A) and 24 (B) h. Relative AP-1 activity was determined by normalization with respect to pTK-luciferase activity.

TAC-101. TAC-101 potently inhibited the metastatic growth of LLC at mediastinal lymph nodes rather than the growth of the tumor at the implantation site (Fig. 1). TAC-101 also inhibited the invasion of LLC cells *in vitro* (Fig. 4). Downregulation of u-PA and u-PAR expressions due to inhibition of AP-1 activity is considered to be associated with anti-invasive and anti-metastatic effects of TAC-101 (Figs. 5, 7 and 8).

The u-PA/plasminogen system is thought to be involved in the development of metastatic disease through fibrinolytic degradation of extracellular matrix, activation of growth factors and mitogen for tumor cells and vascular endothelial cells.[33] Increased expression of u-PA is observed in various types of cancers and is related to the metastatic potential.[34] In some cases of lung cancer, the prognosis of patients with high expression of u-PA is reported to be significantly worse than that of patients with lower expression of u-PA.[35] Lymphatic metastasis of lung cancer is correlated with enhanced expression of u-PA.[36, 37] It has been reported that the invasion and metastasis of LLC cells were inhibited by blocking the u-PA/plasminogen system.[28–30]

In addition, the c-Jun proto-oncogene product, which is a component of transcription factor AP-1, is also overexpressed in lung cancer patients with metastasis.[38, 39] AP-1 is a major transcriptional enhancer for the transactivation of u-PA and u-PAR genes, expression of which is depen-

dent on the activation of AP-1, although another factor, PEA3, binds to their promoter region.[12–16, 32] AP-1 also regulates other matrix-degrading enzymes,[40] though our preliminary experiment suggested that LLC cells did not express a major metastasis-associated gene, MMP-9 (data not shown), which contains an AP-1-binding site on the promoter region. Moreover, AP-1 antagonism has been reported to cause a decrease of *in vivo* tumorigenesis.[41] These findings suggest that the anti-AP-1 activity of TAC-101 resulted in growth inhibition of the metastatic lymph node tumor due to repression of AP-1-related genes such as u-PA and u-PAR.

The prognosis of lung cancer patients who have recurrent metastatic disease is generally poor.[1, 42] Among the potent combined modalities, platinum-based chemotherapy is used as a standard modality for treatment of lung cancer patients.[17, 18] Thus, we investigated the combined therapeutic effects of TAC-101 and CDDP in the LLC model. As shown in Fig. 2, TAC-101 alone caused a significant inhibition of mediastinal lymph node metastasis, while CDDP alone did not inhibit metastasis. Combination treatment with TAC-101 and CDDP caused an increased antitumor effect against both proliferation of the tumor at the implantation site and lymph node metastasis. Although the mechanism of the marked combined effect against tumor growth in the lung remains to be elucidated in detail, u-PA reduction by TAC-101 may be partly associated with the suppression of tumor neovascularization, because interaction between u-PA and its receptor appears to be mandatory for the angiogenic effect of u-PA.[43] Furthermore, this combined treatment significantly prolonged the survival time of mice as compared with treatment with each agent alone (Fig. 3). These findings suggest that this combined treatment with TAC-101 and CDDP delays the progression of lymphatic metastasis of lung cancer, even though the mechanisms of action of TAC-101 and CDDP are independent of each other.

In conclusion, TAC-101 inhibited spontaneous lymphatic metastasis of murine lung cancer. The combination of TAC-101 and CDDP caused marked anti-tumor and life-prolonging effects. TAC-101, which has anti-metastatic properties based on repression of AP-1 activity, may offer improved therapeutic efficacy for patients with lymphatic metastasis of lung cancer.

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