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ASP5878, a selective FGFR inhibitor, to treat FGFR3-dependent urothelial cancer with or without chemoresistance

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Key words

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FGF/FGFR **gene aberrations such as amplification, mutation and fusion are associated with many types of human cancers including urothelial cancer. FGFR kinase inhibitors are expected to be a targeted therapy for urothelial cancer harboring *FGFR3* gene alternations. ASP5878, a selective inhibitor of FGFR1, 2, 3 and 4 under clinical investigation, selectively inhibited cell proliferation of urothelial cancer cell lines harboring *FGFR3* point mutation or fusion (UM-UC-14, RT-112, RT4 and SW 780) among 23 urothelial cancer cell lines. Furthermore, ASP5878 inhibited cell proliferation of adriamycin-resistant UM-UC-14 cell line harboring MDR1 overexpression and gemcitabine-resistant RT-112 cell line. The protein expression of c-MYC, an oncoprotein, in gemcitabine-resistant RT-112 cell line was higher than that in RT-112 parental cell line and ASP5878 decreased the c-MYC expression in both RT-112 parental and gemcitabine-resistant RT-112 cell lines. Once-daily oral administration of ASP5878 exerted potent antitumor activities in UM-UC-14, RT-112 and gemcitabine-resistant RT-112 xenograft models without affecting body weight. These findings suggest that ASP5878 has the potential to be an oral targeted therapy against urothelial cancer harboring *FGFR3* fusion or *FGFR3* point mutation after the acquisition of gemcitabine- or adriamycin-resistance.**

Urothelial cancer can arise anywhere along the epithelial lining of urinary tract, including the bladder, renal pelvis and ureter. Although urothelial cancers arising in these various locations have similar morphology and gene expression profile,[1] urothelial cancer occurs most frequently in the bladder. Bladder cancer is the most common malignancy involving the urinary system and the ninth most common malignancy worldwide.[2] Bladder cancer is mainly divided into two groups by stage. The stage classification differentiates between non-muscle invasive (Tis, Ta, and T1) and muscle-invasive tumors (T2, T3, and T4) according to the depth of invasion. The standard therapy of muscle-invasive bladder cancer is the combination of chemotherapeutic agents (GC and MVAC). However, despite reasonable response rates to chemotherapy in patients with locally advanced or metastatic bladder cancer, long-term progression-free survival rates remain insufficient,[3] which is thought to be caused by the induction of MDR1 overexpression or the alterations in the apoptotic machinery including overexpression of c-MYC, an oncoprotein.[4,5] Therefore, effective drugs against chemotherapy-resistant bladder cancer are eagerly needed.

The mammalian FGF/FGFR family comprises 18 ligands and four main receptors (FGFR1–4). FGFs induce FGFR dimerization, followed by FGFR autophosphorylation and activation of downstream signaling pathways. In a variety of human cancers, aberrant activation of FGF/FGFR signaling promotes cellular proliferation, migration/invasion and angiogenesis.[6] Five different *FGFR3* point mutations such as R248C, S249C, G372C, Y375C, and K652E account for more than 90% of the point mutations of *FGFR3*, and S249C is the most common (48%) in bladder cancer.[7] The frequency of *FGFR3* point mutation in muscle-invasive bladder cancer is lower than that in non-muscle invasive bladder cancer [15% (7/47): invasive, 58% (58/100): non-invasive].[7] Another report shows that the frequencies of *FGFR3* point mutations in primary muscle invasive urothelial tumors and metastases are 2% (2/161) and 9% (3/33), respectively.[8] Recently, it has been also reported that *FGFR3-TACC3* and *FGFR3-BAIAP2L1*, fusion genes were identified in some urothelial cancer cell lines and cancer tissue samples.[9,10] *FGFR3* fusion genes are observed in 3% (3/114) of muscle-invasive urothelial cancer.[11] Therefore, clinical trials of FGFR inhibitors in urothelial cancer harboring *FGFR3* fusion genes or point mutations are ongoing.[12] The clinical relevance of *FGFR3-TACC3* has been suggested by the clinical report of JNJ-42756493, a pan-FGFR inhibitor, which exerts three out of four partial responses among patients with tumors harboring *FGFR3-TACC3* fusion genes.[13] In a subset of urothelial cancer patients harboring *FGFR3* gene alternation (*FGFR3* fusion gene and point mutation) treated with BGJ398, the overall response rate in 25 evaluable patients was 36% and included one unconfirmed complete response and eight partial

responses.[14] In light of these reports, FGFR3 has been considered as an attractive target for novel therapy in urothelial bladder cancer.

In this report, we describe the preclinical profile of ASP5878, which is a selective FGFR inhibitor under clinical investigation (NCT 02038673), targeting *FGFR3*-fusion or -mutation positive urothelial bladder cancer. Interestingly, ASP5878 suppressed the growth of *FGFR3*-fusion or -mutation positive urothelial cancer cell lines even after the acquisition of chemoresistance. Our data indicate that ASP5878 is a potentially effective therapeutic agent for urothelial cancer patients whose tumors express *FGFR3* mutation or -fusion after the acquisition of gemcitabine- or adriamycin- resistance.

Materials and Methods

Reagents. 2-[4-({5-[(2,6-difluoro-3,5-dimethoxyphenyl)methoxy]pyrimidin-2-yl}amino)-1H-pyrazol-1-yl]ethan-1-ol [ASP5878, Fig. 1[15]] was synthesized at Astellas Pharma Inc. (Tokyo, Japan). ASP5878 was dissolved in DMSO or suspended in 0.5% methyl cellulose for *in vitro* and *in vivo* experiments, respectively. Gemcitabine was purchased from Eli Lilly Inc. (Indianapolis, IN, USA), and was dissolved in water or saline for *in vitro* and *in vivo* experiments, respectively. Adriamycin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and was dissolved in water.

Cell lines. HT-1197, HT-1376, J82, RT4, SW 780, TCCSUP, and UM-UC-3 were purchased from ATCC (Manassas, VA, USA). 647-V, BC-3C, BFTC-905, CAL-29, KU-19-19, RT-112, SW-1710 and VM-CUB1 were purchased from DSMZ (Braunschweig, Germany). EJ138, U-BLC1, UM-UC-9 and UM-UC-14 were purchased from ECACC (Salisbury, UK). KMBC-2 and T24 were purchased from JCRB Cell Bank (Osaka, Japan). BOY-12E, and JMSU-1 were provided by the RIKEN BRC (Tsukuba, Japan). These cell lines were cultured according to the guidelines from the suppliers.

To generate chemotherapy-resistant cell lines, UM-UC-14 and RT-112 cell lines were exposed to adriamycin and gemcitabine, respectively, whose concentrations were gradually increased up to 100 and 1000 ng/mL, respectively. Adriamycin-resistant UM-UC-14 and gemcitabine-resistant RT-112 cell lines were maintained in the culture medium containing 50 ng/mL adriamycin and 1000 ng/mL gemcitabine, respectively.

***In vitro* cell growth assay.** The cells were seeded in 96-well plates at 2000 cells per well and incubated overnight. On the following day, the cells were exposed to ASP5878 for 4 days (JMSU-1) or 5 days (other cell lines). The cell viability was measured with CellTiter-Glo (Promega, Madison, WI, USA). Data are presented as means from a single experiment performed in duplicate.

MDR1 expression. Immunoblotting was performed using mouse anti-MDR1 (D-11) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-β-actin (13E5) monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA).

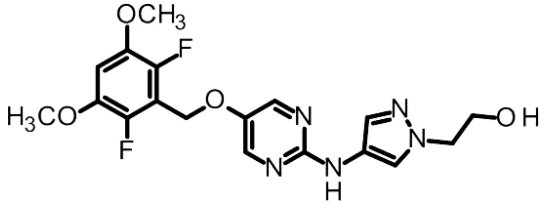

Fig. 1. Chemical structure of ASP5878.

Inhibition of *in vitro* FGFR3 phosphorylation. Cells were seeded in 100 mm dishes at 2×10^6 cells/10 mL/dish and cultured overnight. Media were replaced with ASP5878 containing media at the final concentrations of 0, 1, 10, 100 and 1000 nmol/L, respectively. The final concentration of DMSO in each dish was 0.1%. Following 2-h incubation with ASP5878, cells were rinsed with PBS and collected. Cell pellet was obtained and lysed with cell lysis buffer containing phosphatase inhibitor (Thermo Fisher Scientific, Rockford, IL, USA) and protease inhibitor (Roche, Basel, Switzerland). Cell lysate was centrifuged and then supernatant was obtained as the sample for ELISA assay. Phosphorylated and total FGFR3 were measured by sandwich ELISA assay (DYC2719 and DYC766; R&D Systems, Minneapolis, MN, USA). The ratio of phosphorylated FGFR3 to total FGFR3 is calculated according to the formula: (phospho FGFR3 concentration [pg/mL])/(total FGFR3 concentration [pg/mL]). FGFR3 phosphorylation rate to the DMSO-treated sample was calculated according to the formula: (phosphorylation ratio of ASP5878-treated sample)/(phosphorylation ratio of DMSO-treated sample) × 100 (%).

Immunoblotting for the downstream signaling of FGFR3 and c-MYC. Cells were seeded in 100 mm dishes at 2×10^6 cells/10 mL per dish and cultured overnight. Media were replaced with ASP5878 containing media at the final concentrations of 0, 1, 10, 100 and 1000 nmol/L respectively. The final concentration of DMSO in each dish was 0.01%. Following 2-h (for ERK and phospho-ERK) or 48-h (for c-MYC) incubation with ASP5878, cells were rinsed with PBS and collected. The cells were lysed with cell lysis buffer (Cell Signaling Technology) containing phosphatase inhibitor (Thermo Scientific) and protease inhibitor (Roche), and protein levels of ERK, c-MYC and actin, and phosphorylation levels of ERK were determined by immunoblotting. Antibodies were obtained from following sources: ERK (#9102; Cell Signaling Technology) and phospho-ERK (Thr202/Tyr204) (#9101; Cell Signaling Technology), actin (A5441; Sigma-Aldrich, St Louis, MO, USA), c-MYC (#5605; Cell Signaling Technology).

***In vivo* tumor studies.** Five-week-old male nude mice (BALB/c nu/nu) were purchased from Charles River Japan, Inc (Kanagawa, Japan). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Furthermore, Astellas Pharma Inc., Tsukuba Research Center was accredited by AAALAC International. UM-UC-14, RT-112 and gemcitabine-resistant RT-112 cell lines were subcutaneously inoculated into the flank of mice at 3×10^6, 1×10^6 and 1×10^6 cells/0.1 mL (matrigel: PBS = 1:1)/mouse, respectively and allowed to grow. The mice with tumor were divided into 4 or 5 groups (n = 5 or 10) so that the mean tumor volume of the groups were similar on Day 0. ASP5878 (0.3–10 mg/kg) was administered orally once daily to these xenografted mice. Intravenous gemcitabine (100 mg/kg) was given to them twice a week. Tumor volume was determined by length × width^2 × 0.5. Matrigel were purchased from Corning Life Sciences (Tewksbury, MA, USA).

***In vivo* FGFR3 phosphorylation.** Tumor samples were collected from UM-UC-14 tumor-bearing mice at 0.5, 1, 2, 4, 6, 12, 18 and 24 h after single dose of ASP5878 and vehicle. Frozen tumor samples were lysed with cell lysis buffer containing phosphatase inhibitor (Thermo Fisher Scientific) and

protease inhibitor (Roche). Phosphorylated and total FGFR3 were measured by sandwich ELISA assay.

Statistical analysis. Values are expressed as the mean ± SE. Differences between groups were analyzed using Dunnett's multiple comparison test. All data analysis was performed using the SAS statistical software (SAS Institute, Cary, NC, USA), with *P*-values <0.05 considered significant.

Results

Kinase inhibition profile of ASP5878. Materials and Methods of kinase assay were described in the Supporting Information (Appendix S1). ASP5878 potently inhibited the tyrosine kinase activities of recombinant FGFR 1, 2, 3 and 4 with IC_{50} values of, 0.47, 0.60 0.74 and 3.5 nmol/L, respectively (Table S1). The selectivity of ASP5878 was profiled against a kinase panel of 128 human kinases. FGFRs, VEGFR2 and FMS were inhibited by more than 50% by ASP5878 (200 nmol/L) (Tables S1, S2).

Anti-proliferative profile of ASP5878 in urothelial cancer and other FGFR-dependent cell lines. ASP5878 inhibited cell growth of UM-UC-14 [FGFR3_S249C;[16]], RT-112 [FGFR3-TACC3;[9]], RT4 [FGFR3-TACC3;[9]], SW 780 [FGFR3-BAIAP2L1;[10]] and JMSU-1 [FGFR1 overexpression;[17]] with IC_{50} values of <100 nmol/L (Fig. 2). ASP5878, however, was inactive (IC_{50} values ≥ 300 nmol/L) against other urotherial cancer cell lines without *FGFR* genetic alterations (Fig. 2). Additionally, ASP5878 also inhibited cell proliferation of NCI-H1581 [*FGFR1* amplification, lung;[18]], HSC-39 [*FGFR2* amplification, stomach;[19]], and Hep3B2.1-7 [*FGF19* amplification, liver;[20]] which is known as a FGF19/FGFR4-dependent cell line (Table S3, Appendix S1). Thus, ASP5878 has potent anti-proliferative effects in human cancer cell lines harboring gene alterations in *FGF* or *FGFR*.

Inhibitory effect of ASP5878 on FGFR3 and ERK phosphorylation in UM-UC-14 and RT-112 cell lines. ASP5878 (1, 10, 100 and 1000 nmol/L) decreased phosphorylated FGFR3 in UM-UC-14 and RT-112 cell lines after 2 h of treatment (Fig. 3a). ERK phosphorylation, a downstream signaling molecule in the cell lines, was inhibited by ASP5878 in a concentration-dependent manner (Fig. 3b). Thus, ASP5878 inhibits FGFR3 phosphorylation and ERK phosphorylation in urothelial cancer cell lines harboring *FGFR3* gene alternations.

Anti-proliferative effects of ASP5878 in urothelial cancer cell lines with acquired resistance to adriamycin or gemcitabine. It has been reported that *MDR1* mRNA levels in bladder cancer tissues are correlated with the resistance to adriamycin in bladder cancer patients.[4] We therefore established adriamycin-resistant UM-UC-14 cell line as described in "Materials and Methods" and compared MDR1 expression levels in the parental and the adriamycin-resistant UM-UC-14 cell lines. The expression levels of MDR1 protein (Fig. 4a) and mRNA (Fig. S1, Appendix S1) in the adriamycin-resistant UM-UC-14 cell line were much higher than those in the parental cell line. Adriamycin exhibited 8.7-folds weaker anti-proliferative effect in the adriamycin-resistant UM-UC-14 cell line (IC_{50} = 58 ng/mL, 95% CI: 17–203, n = 3) than that in the parental cell line (IC_{50} = 6.7 ng/mL, 95% CI: 2.3–20, n = 3) (Fig. 4b). While ASP5878 exerted anti-proliferative effects in both the parental and adriamycin-resistant UM-UC-14 cell lines with similar IC_{50} values of 8.7 (95% CI: 2.3–32, n = 3) and 11 nmol/L (95% CI: 3.9–34, n = 3), respectively (Fig. 4c).

Gemcitabine is also one of the chemotherapeutic agents for invasive/metastatic bladder cancer. However, despite reasonable response rates to initial chemotherapy including gemcitabine in patients with locally advanced or metastatic bladder cancer, long-term progression-free survival rates remain insufficient.[4] Therefore, we established gemcitabine-resistant RT-112 cell line as described in "Materials and Methods" and examined effects of ASP5878 on the proliferation and downstream signaling of FGFR3 in the gemcitabine-resistant RT-112 cell line. Gemcitabine inhibited the proliferation of the parental RT-112 cell line with IC_{50} value of 0.95 ng/mL (95% CI: 0.18–5.0, n = 3) but not inhibited that of the gemcitabine-resistant RT-112 cell line up to 1000 ng/mL (Fig. 5a). ASP5878 inhibited the proliferation of the parental and the gemcitabine-resistant RT-112 cell lines with similar IC_{50} values of 8.7 (95% CI: 3.9–20, n = 3) and 10 nmol/L (95% CI: 3.7–27, n = 3), respectively (Fig. 5b) and decreased the level of ERK phosphorylation in the gemcitabine-resistant RT-112 cell line as with that in the parental cell line (Figs 3b,5c). It has been reported that gemcitabine-resistant cell growth in urothelial cancer cells is related to upregulation of c-MYC expression which is involved in cell proliferation.[5] Upregulation of c-MYC protein in the gemcitabine-resistant RT-112 cell line was also observed (Fig. 5d). Interestingly, ASP5878 decreased the expression of c-MYC in both the gemcitabine-resistant RT-112 and the parental cell lines (Fig. 5d). From these findings, it is possible that ASP5878 can inhibit cell proliferation and c-MYC expression independent on gemcitabine-resistant status of urothelial cancer cell line harboring *FGFR3* gene alternation. Thus, ASP5878 has growth inhibitory activities against urothelial cancer harboring FGFR3-TACC3 fusion or FGFR3_S249C even after the acquisition of adriamycin or gemcitabine resistance.

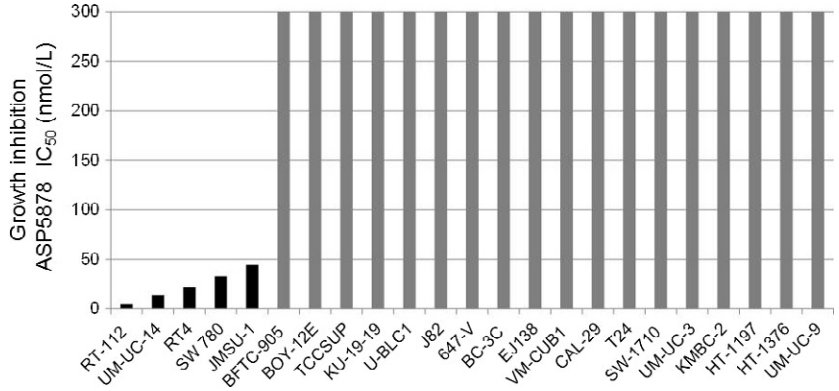

Fig. 2. Cell panel assay for the identification of ASP5878-sensitive bladder cancer cell lines. The 23 bladder cancer cell lines were treated with ASP5878 or 0.1% DMSO (control) for 4 (JMSU-1) or 5 days (other cell lines). The cell viability on day 4 or day 5 was measured by quantitating the amount of ATP in cell lysate. The IC_{50} value of ASP5878 on the cell proliferation of each cell line was indicated with each bar graph. Data are presented as means from a single experiment performed in duplicate.

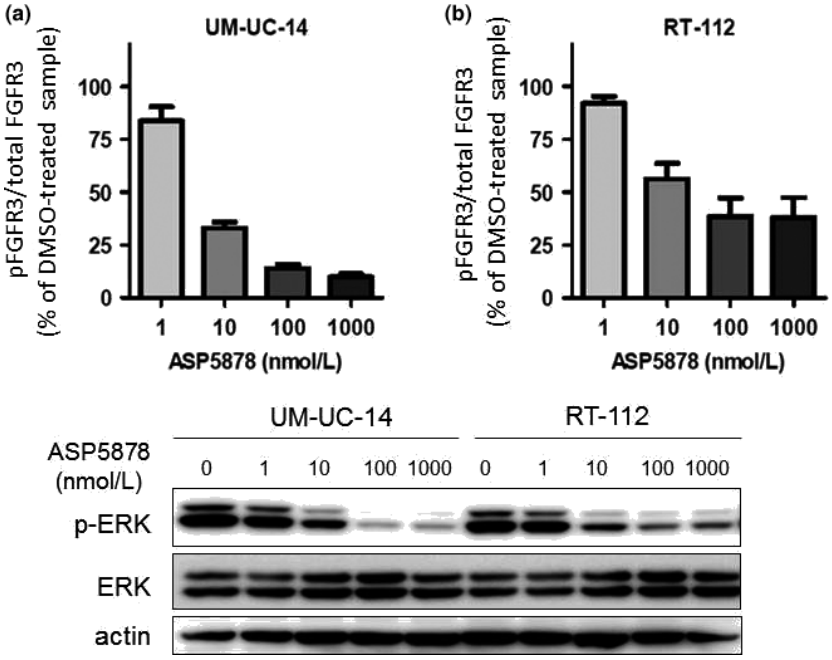

Fig. 3. Inhibitory effects of ASP5878 on FGFR3 and ERK phosphorylation in UM-UC-14 and RT-112 cell lines. (a) UM-UC-14 and RT-112 cell lines are incubated for 2 h with each concentration of ASP5878 or 0.1% DMSO. Cells are then lysed and assessed FGFR3 phosphorylation rate by sandwich ELISA assay. (b) UM-UC-14 and RT-112 cell lines are incubated for 2 h with each concentration of ASP5878 or 0.01% DMSO. Phosphorylated ERK (p-ERK), ERK and actin were detected by immunoblotting.

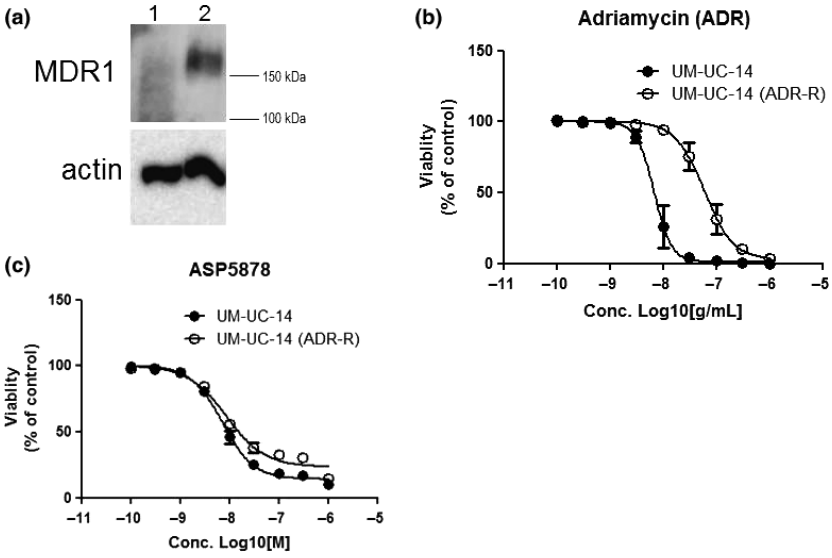

Fig. 4. Anti-proliferative effect of ASP5878 in adriamycin-resistant UM-UC-14 cell line. (a) MDR1 protein expression in adriamycin-resistant UM-UC-14 (lane 2) and parental UM-UC-14 (lane 1) cell lines was detected by immunoblotting. (b, c) Anti-proliferative effect of ASP5878 (b) and adriamycin (c) in adriamycin-resistant UM-UC-14 (ADR-R) and parental UM-UC-14 cell lines. These cell lines were treated with ASP5878 or adriamycin for 5 days [control: 0.1% DMSO (ASP5878), water (adriamycin)]. Values are expressed as the mean ± SE from three separate experiments.

Antitumor activities of ASP5878 in urothelial cancer xenograft models. Once-daily oral administration of ASP5878 dose-dependently inhibited tumor growth and induced tumor regression at more than 1 mg/kg in UM-UC-14 subcutaneous xenograft mouse model (Fig. 6a). Single administration of ASP5878 (1, 3, and 10 mg/kg) inhibited FGFR3 phosphorylation in UM-UC-14 subcutaneous tumor and the duration of inhibition was dose-dependent (Fig. 6b), which indicates a reasonable antitumor activity of ASP5878 in UM-UC-14 subcutaneous xenograft mouse model. ASP5878 also dose-dependently inhibited tumor growth in RT-112 (Fig. 6c) and gemcitabine-resistant RT-112 (Fig. 6d) subcutaneous xenograft mouse models. Body weight was not affected at any dose of ASP5878 examined in these experiments (data not shown). Thus, ASP5878 has the antitumor activities in urothelial cancer models harboring FGFR3_S249C or FGFR3-TACC3 fusion after the acquisition of gemcitabine resistance.

Discussion

FGFR tyrosine kinases are frequently activated by diverse genetic alterations in cancer, and therefore, FGFR inhibitors may be effective in patients with *FGFR* genetic alterations. In this study, we examined the therapeutic potential of ASP5878 in urothelial cancer cell lines and xenografts harboring *FGFR3* gene alternations. ASP5878, an FGFR tyrosine kinase inhibitor with a high selectivity against a number of other kinases (Tables S1,S2), has potent anti-proliferative effects on FGFR1, 2, 3 and 4-depencent cell lines (Table S3). In 23 urothelial cancer cell lines, ASP5878 inhibited the proliferation of

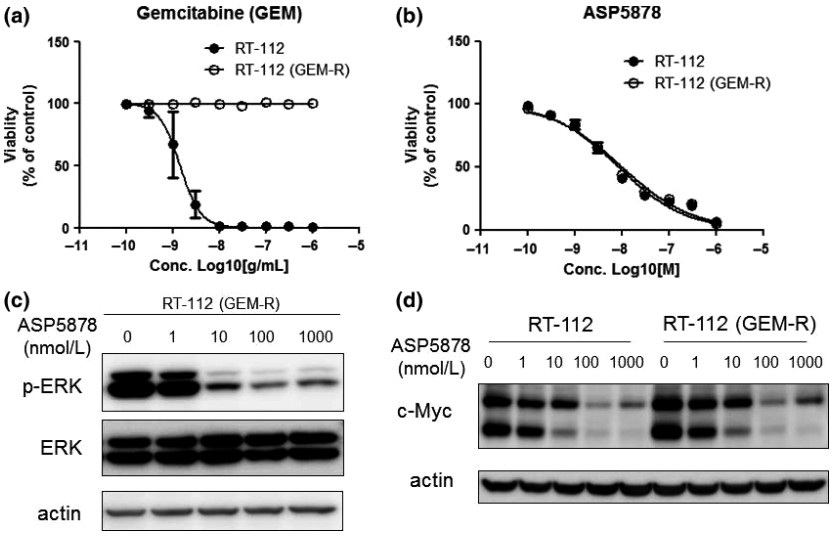

Fig. 5. Anti-proliferative effect of ASP5878 in gemcitabine-resistant RT-112 cell line. (a, b) Anti-proliferative effect of gemcitabine (a) and ASP5878 (b) in gemcitabine-resistant RT-112 (GEM-R) and parental RT-112 cell lines. These cell lines were treated with gemcitabine and ASP5878 for 5 days [control: 0.1% DMSO (ASP5878), water (gemcitabine)]. Values are expressed as the mean ± SE from three separate experiments. (c) Gemcitabine-resistant RT-112 cell line was incubated for 2 h with each concentration of ASP5878 or 0.01% DMSO. Phosphorylated ERK (p-ERK), ERK and actin were detected by immunoblotting. (d) RT-112 and gemcitabine-resistant RT-112 cell lines were incubated for 48 h with each concentration of ASP5878 or 0.01% DMSO. c-MYC and actin were detected by immunoblotting.

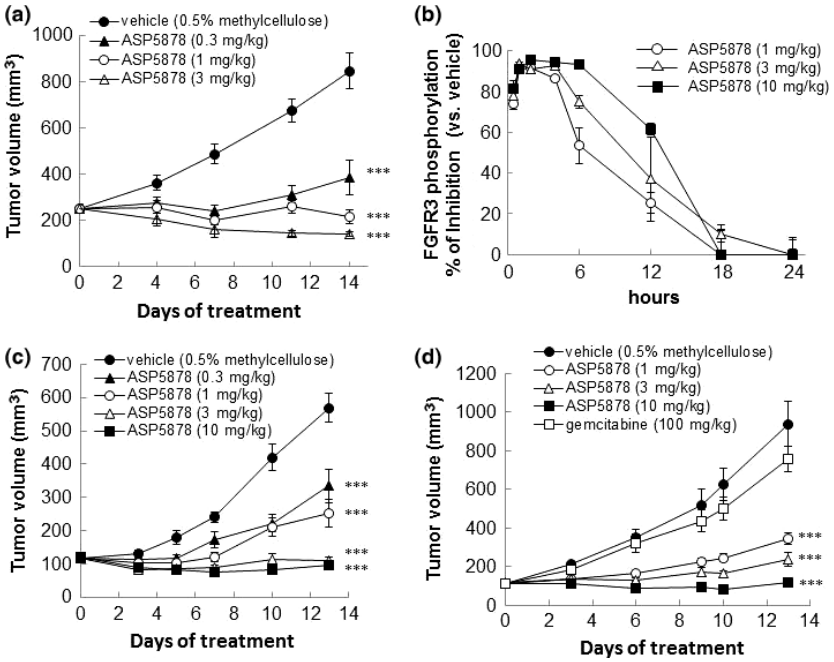

Fig. 6. ASP5878 treatment leads to tumor regression in UM-UC-14, RT-112 and gemcitabine-resistant RT-112 subcutaneous xenograft models. (a) ASP5878 was administered by oral gavage once daily to nude mice bearing UM-UC-14 tumors (n = 10). (b) Tumor samples were collected from UM-UC-14 tumor-bearing mice at various time points (0.5, 1, 2, 4, 6, 12, 18 and 24 h) after single dose of ASP5878 and vehicle. Phosphorylated FGFR3 and total FGFR3 were measured by sandwich ELISA assay (n = 3). (c) ASP5878 was administered by oral gavage once daily to nude mice bearing RT-112 tumors (n = 5). (d) ASP5878 or gemcitabine was administered by oral gavage once-daily or intravenous injection twice-weekly to nude mice bearing gemcitabine-resistant RT-112 tumors (n = 5). Each point represents the mean ± SE. Statistical analysis for antitumor tests was performed the values on the final day of each experiment (***$P < 0.001$, Dunnett's multiple comparison test).

RT-112 and RT4 harboring FGFR3-TACC3, SW 780 harboring FGFR3-BAIAP2L1, UM-UC-14 harboring FGFR3_S249C and JMSU-1 harboring FGFR1 overexpression (Fig. 2). FGFR3-TACC3 displayed ligand-independent constitutive activation of FGFR3 kinase activity and dimerization through a coiled-coil domain in TACC3.[9,15] BAIAP2L1 has Bin-Amphiphysin-Rvs (BAR) domain which contributes to dimerization and constitutive activity in FGFR3-BAIAP2L1 fusion protein.[10] FGFR3_S249C mutation induces disulfide bond formation by introducing an additional cysteine in the extracellular domain of FGFR3, thereby causing constitutive dimerization and activation of the receptor.[21] Aside from *FGFR3* gene alternations, JMSU-1 cell line, an urothelial cancer cell line harboring FGFR1 overexpression, has been demonstrated to have FGFR1-dependent cell growth activity by using *FGFR1* siRNA.[17] These findings suggest that FGFR3-TACC3, FGFR3-BAIAP2L1, FGFR3_S249C mutations and FGFR1 overexpression may be predictors of the sensitivity to ASP5878 in urothelial cancer.

Currently, combination chemotherapy such as MVAC and GC are the first-line therapy for metastatic bladder cancer patients. Unfortunately, the treatment success of bladder cancer is limited resulting in a median survival of 12–16 months.[4] Treatment failure can be commonly caused by development of resistance to chemotherapy.[3,22]

MDR1 is a cell membrane efflux pump involved in drug resistance. Expression of MDR1 was detected in both pre- and post-chemotherapy tumor tissue samples from patients with bladder cancer and a higher expression in post-chemotherapy patients was reported.[23,24] We also obtained adriamycin-resistant UM-UC-14 cell line harboring MDR1 overexpression by stepwise increasing concentrations of adriamycin (Fig. 4). In addition, some studies have highlighted the important role of c-MYC in the development of drug-resistant phenotypes in cancer.[25,26] It

has been reported that KU19-19/GEM, gemcitabine-resistant urothelial cancer cells, upregulated c-MYC expression in the presence of gemcitabine and the growth of KU19-19/GEM cells was suppressed by KSI-3716, a c-MYC inhibitor.[5] As is the case of KU19-19/GEM cells, we also successfully established gemcitabine-resistant RT-112 cell line harboring c-MYC upregulation by stepwise exposure to gemcitabine (Fig. 5). Furthermore, c-MYC overexpression has been observed in urothelial cancer tissues.[27–29] Thus, c-MYC is thought to be relevant to drug-resistance in cancer. On the other hand, it has been reported that c-MYC expression was decreased by PD173074, an FGFR inhibitor, in lung cancer cell lines harboring FGFR1 overexpression.[30] Activated ERK, a downstream molecule of FGFR, stabilizes c-MYC in melanoma cells.[31] In the present study, ASP5878 also inhibited ERK phosphorylation and induced c-MYC down-regulation in urothelial cancer cell line harboring *FGFR3* gene alternation independent on gemcitabine resistant status (Fig. 5d). These findings suggest that c-MYC expression may be regulated by the FGFR/ERK signaling pathway.

Despite a lot of studies related to the mechanisms of chemoresistance, an effective therapy for chemoresistant urothelial cancer is still unestablished. Recently, FGFR inhibitors such as BGJ398 and CH5183284/Debio1347 have been reported to have an antitumor effect in FGFR3-dependent urothelial cancer models.[32,33] And also several FGFR inhibitors including BGJ398, CH5183284/Debio1347, JNJ-42756493 and AZD4547 are being developed for treatment of urothelial cancer. In a subset of urothelial cancer patients harboring *FGFR3* gene alternation, JNJ-42756493 and BGJ398 exerted partial responses. However, these FGFR inhibitors haven't been shown to have therapeutic potential against chemoresistant urotherial cancer in the preclinical models. Therefore, we evaluated ASP5878 for the treatment of chemoresistant urothelial cancer by using chemoresistant urothelial cancer cell lines. In adriamycin-resistant UM-UC-14 cell lines, MDR1 expression was increased (Fig. 4a) and ASP5878 inhibited the proliferation in common with the parent UM-UC-14 cell line (Fig. 4b). Furthermore, ASP5878 inhibited the cell growth in gemcitabine-resistant RT-112 cells *in vitro* and *in vivo* studies (Figs 5b,6d). In addition to gemcitabine and adriamycin, cisplatin is also a key chemotherapeutic agent. However, we have not obtained the data that ASP5878 inhibits cell proliferation in cisplatin-resistant cells, because we currently do not succeed in the establishment of cisplatin-resistant cells. In the present study, we demonstrated antitumor activities of ASP5878 using mouse models xenografted with urothelial cancer cell lines (Fig. 6). Patient-derived xenograft models are thought to be useful to make sure the efficacy of ASP5878 in urothelial cancer harboring *FGFR3* gene alternation. These are future tasks to be confirmed. From these findings, ASP5878 may exert antitumor activity against adriamycin-resistant and gemicitabne-resistant urotherial cancer harboring *FGFR3* gene alternations.

Hyperphosphatemia has been commonly observed with other FGFR inhibitors (e.g. JNJ-42756493 and BGJ398).[13,14] In line with the findings, ASP5878 also induced serum phosphate increase in rodents (unpublished data). The safety, pharmacokinetics, and pharmacodynamics of ASP5878 are currently evaluated in clinical phase I study. In addition to the safety information, it has been reported that $T_{1/2}$ values of JNJ-42756493 and BGJ398 are quite large according to clinical information of these compounds.[13,34] In the present study, we showed the duration of FGFR3 inhibitory activity after single administration of ASP5878 was relatively short in mice (Fig. 6b), which might be a benefit for the management of plasma phosphate levels.

In conclusion, ASP5878, a selective FGFR inhibitor, showed potent anti-proliferative and antitumor activity in urothelial cancer cell line harboring *FGFR3-TACC3*, *FGFR3-BAIAP2L1* or *FGFR3* point mutation and their tumor xenografted models. ASP5878 also inhibited the proliferation of adriamycin-resistant UM-UC-14 and gemcitabine-resistant RT-112 cell lines. These findings suggest that ASP5878, which is currently being evaluated in phase I clinical trials, has therapeutic potential against urothelial bladder cancers harboring *FGFR3-TACC3*, *FGFR3-BAIAP2L1* or *FGFR3* point mutation after the acquisition of gemcitabine- or adriamycin- resistance.

Disclosure Statement

All authors are employees of Astellas Pharma Inc.

Abbreviations

95% CI	95% confidential interval
BAIAP2L1	BAI1-associated protein 2-like 1
DMSO	dimethyl sulfoxide
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GC	gemcitabine/cisplatin
IC_{50}	50% inhibitory concentration
MDR	multidrug-resistant transporter
MVAC	methotrexate/vinblastine/adriamycin/cisplatin;
PBS	phosphate-buffered saline
TACC3	transforming acid coiled coil 3

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Supporting Information

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

Appendix S1. Supporting Materials and Methods.

Figure S1. MDR1 mRNA expression in adriamycin-resistant UM-UC-14 cell line.

Table S1. Kinase inhibitory profile of ASP5878 against 128 kinases.

Table S2. Inhibitory activity against 128 kinases.

Table S3. Anti-proliferative effect of ASP5878 in FGFR-dependent cell lines.