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

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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 oncprotein.\(^4,5\) 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
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-[[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).

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 lysis 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 rate 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 × 10^5 cells/0.1 mL (matrigel: PBS = 1:1) per mouse 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 / 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

Fig. 1. Chemical structure of ASP5878.
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 IC₅₀ 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 IC₅₀ values of <100 nmol/L (Fig. 2). ASP5878, however, was inactive (IC₅₀ values ≥ 300 nmol/L) against other urothelial 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 Hep3B.2.1-7 [FGF19 amplification, liver;(20) ] which is known as a FGF19/FGFR4-dependent cell line (Table S3, Appendix S1). Thus, ASP5878 has potenti anti-proliferative effects in human cancer cell lines harboring genetic alterations in FGF or FGFR. 

**Inhibitory effect of ASP5878 on FGFR3 and ERK phosphorylation in UM-UC-14 and RT-112 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 IC₅₀ values of <100 nmol/L (Fig. 2). ASP5878, however, was inactive (IC₅₀ values ≥ 300 nmol/L) against other urothelial cancer cell lines without FGFR genetic alterations (Fig. 2).

**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 IC₅₀ 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.
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 models. 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.

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-dependent cell lines (Table S3). In 23 urothelial cancer cell lines, ASP5878 inhibited the proliferation of models harboring FGFR3_S249C or FGFR3-TACC3 fusion after the acquisition of gemcitabine resistance.
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 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. 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.

In conclusion, ASP5878, a selective FGFR inhibitor, showed potent anti-proliferative and antitumor activity in urothelial cancer cell line harboring FGFR3 gene alternation and its relationship to mutation status and prognostic variables in bladder cancer. J Pathol 2007; 213(1): 91–8.

Disclosure Statement

All authors are employees of Astellas Pharma Inc.

Abbreviations

95% CI 95% confidential interval
BAIAP2L1 BA11-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
IC50 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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Table S3. Anti-proliferative effect of ASP5878 in FGFR-dependent cell lines.

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.