Pharmacological Studies on Causal Factors and Pharmacological Therapies for Urological Cancer

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Abstract

Globally, the number of older adults is increasing at a rapid pace. The number of people aged 65 and older is projected to grow from an estimated 524 million in 2010 to nearly 1.5 billion in 2050 [1, 2]. Consequently, urological cancers such as prostate cancer (PCa) and urothelial cancer have been increasing, and these cancers are one of the most common cancers in the world. Moreover, the approach to urological cancer treatment has huge impact on patient's health-related quality of life (HRQOL), however, the approach to urological cancer treatment is by no means satisfactory, and new progress has been required. In this study, I performed pharmacological studies on causal factors and pharmacological therapies for urological cancer in order to satisfy this unmet medical needs (UMN).

In the first chapter, I described the biochemical and cell biological analysis of aldo-keto reductase family 1 member C3 (AKR1C3) inhibitor ASP9521 that is a therapeutic agent for PCa. ASP9521 inhibited conversion of androstenedione (AD) into testosterone (T) potently by recombinant human or cynomolgus monkey AKR1C3 in a concentration-dependent manner. ASP9521 showed >100-fold selectivity for AKR1C3 over the isoform AKR1C2. In LNCaP-AKR1C3 cells stably expressing human AKR1C3, ASP9521 suppressed AD-dependent prostate specific antigen (PSA) production and cell proliferation. In CWR22R xenografts, single oral administration of ASP9521 (3 mg/kg) inhibited AD-induced intratumoral T production and this inhibitory effect was maintained for 24 h. Based on these findings I discussed the physiological role of AKR1C3 in PCa androgen metabolism.

In the second chapter, I described the inhibitory mechanism of a selective fibroblast

growth factor receptor (FGFR) inhibitor ASP5878, targeting *FGFR3*-fusion or -mutation positive urothelial cancer. ASP5878 showed potent anti-proliferative and antitumor activity in urothelial cancer cell line harboring *FGFR3-transforming acid coiled coil 3 (TACC3)*, *FGFR3-BAI1-associated protein 2-like 1 (BAIAP2L1)* or *FGFR3* point mutation and their tumor xenografted models. This anti-proliferative activity is based on inhibitory effects of FGFR3 phosphorylation and extracellular signal-regulated kinase (ERK) phosphorylation, a downstream signaling molecule in these urothelial cancer cell lines harboring *FGFR3* gene alternations. Additionally, I established chemotherapy-resistant cell lines: adriamycin-resistant UM-UC-14 and gemcitabine-resistant RT-112. ASP5878 also inhibited the proliferation of these cell lines. These findings suggest that ASP5878 has therapeutic potential against urothelial cancer harboring *FGFR3-TACC3*, *FGFR3-BAIAP2L1* or *FGFR3* point mutation even after the acquisition of gemcitabine- or adriamycin- resistance.

On the basis of two studies, I propose that AKR1C3 inhibitor against PCa and FGFR3 inhibitor against urothelial cancer harboring *FGFR3-TACC3*, *FGFR3-BAIAP2L1* or *FGFR3* point mutation can lead to a novel therapeutic approach for these urological cancers. I believe these studies can contribute to developing new therapeutic agent of urological cancer.

Abbreviations

17βHSD5	17β-hydroxysteroid dehydrogenase type 5
95% CI	95% confidential interval
AA	abiraterone acetate
AD	androstenedione
ADT	androgen deprivation therapy
AKT	protein kinase B
AKR1C3	aldo-keto reductase family 1 member C3
AR	androgen receptor
BAIAP2L1	BAI1-associated protein 2-like 1
BG	background
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GC	gemcitabine/cisplatin
HRQOL	health-related quality of life
IC_{50}	50% inhibitory concentration
LH-RH	luteinizing hormone releasing hormone
MAPK	mitogen-activated protein kinase
mCRPC	metastatic castration-resistant prostate cancer
MDR1	multidrug-resistant transporter 1
MIBC	muscle-invasive bladder cancer
MVAC	methotrexate/vinblastine/adriamycin/cisplatin
NADP	nicotinamide adenine dinucleotide phosphate
NMIBC	non- muscle-invasive bladder cancer
PBS	phosphate-buffered saline
PAGE	polyacrylamide gel electrophoresis
PCa	prostate cancer
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PSA	prostate specific antigen

Ras	rat sarcoma
SDS	sodium dodecyl sulfate
STAT	signal transducer and activator of transcription
T	testosterone
TACC3	transforming acid coiled coil 3
UMN	unmet medical needs
WHO	World Health Organization

General Introduction

According to World Health Organization (WHO), cancer is one of the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases in 2012 [3]. The number of new cases is expected to rise by about 70% over the next 2 decades. Cancer is the second leading cause of death in the world, and was responsible for 8.8 million deaths in 2015. Globally, nearly 1 in 6 deaths is due to cancer.

Urological cancers, which consist of PCa, urothelial cancer, and renal cell cancer are common malignancies with increasing incidence and mortality worldwide. According to the most recent cancer statistics, urological cancers are major causes of morbidity and mortality: The American Cancer Society estimates that 318,980 new instances of urological cancer will be diagnosed and that 59,690 deaths will occur from urological cancers in 2017 in the USA [4]. I particularly focused on PCa and urothelial cancer because of these high UMN.

PCa is the most common cancer in males, with a projected 161,360 new cases and 26,730 deaths estimated in the USA in 2017 [4]. PCa cells usually require androgen hormones, such as T, to grow, therefore, a common treatment option for PCa is to lower the levels of androgen hormones in male body. Androgen signaling plays a pivotal role in the proliferation of PCa, and androgen deprivation therapy (ADT) is the standard treatment. ADT can be achieved either by surgical castration through bilateral orchiectomy or medical castration through the use of luteinizing hormone-releasing hormone (LH-RH) agonists or antagonists, which is the mainstay for treatment in PCa [5-7]. ADT elicits a response in majority of patients with PCa. However, some patients will have recurrence of their disease after treatment and will progress to the castration-resistant prostate cancer (CRPC) or metastatic CRPC (mCRPC) over time. CRPC can be

defined as either progressively rising levels of serum tumor marker prostate-specific antigen (PSA) or detection of new or progressive metastatic tumors by radiographic scans, despite castrate T levels (below 50 ng/dL and recently updated to less than 20 ng/dL [8-10]). Progression of disease in patients with mCRPC can lead to development of worsening symptoms and patients may experience decline in their HRQOL with ensuing increased pain [11-13]. mCRPC remains driven by the androgen axis, and despite the use of ADT, most patients with metastatic PCa will progress to CRPC, which still depends on androgen synthesis and androgen receptor (AR) signaling for proliferation. In addition, increased intratumoral production of androgens is also thought to result from upregulation of androgen biosynthesis enzymes. Two hormonal therapy agents, abiraterone acetate (AA) which inhibits androgen biosynthesis and enzalutamide which interferes with androgen-receptor signaling, have been approved in many countries, and have proven to be effective in the treatment of mCRPC (Figure 1) [14-18]. Unfortunately, many patients treated with these two agents will fail to respond to initial treatment with these drugs [14-17]. Furthermore, within 24 months of initiating treatment, even those who initially respond to these drugs will develop resistance, therefore new methods by which treatment resistance develops in PCa are constantly identified.

One of the key enzymes involved in adrenal and *de novo* intratumoral steroidal biosynthesis is AKR1C3, also called 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5). This enzyme plays a crucial role in the synthesis of dihydrotestosterone (DHT), by catalysing the conversion of the adrenal androgens dehydroepiandrosterone (DHEA), AD and 5α-androstanedione into DHT [19]. AKR1C3 is highly expressed in PCa cells [20-23], and its expression increases with increasing tumor aggressiveness [24], as demonstrated by upregulated AKR1C3 levels in patients with CRPC compared with

benign prostate and early stage PCa [25-31]. This is one of the reasons why AKR1C3 has been implicated in CRPC progression.

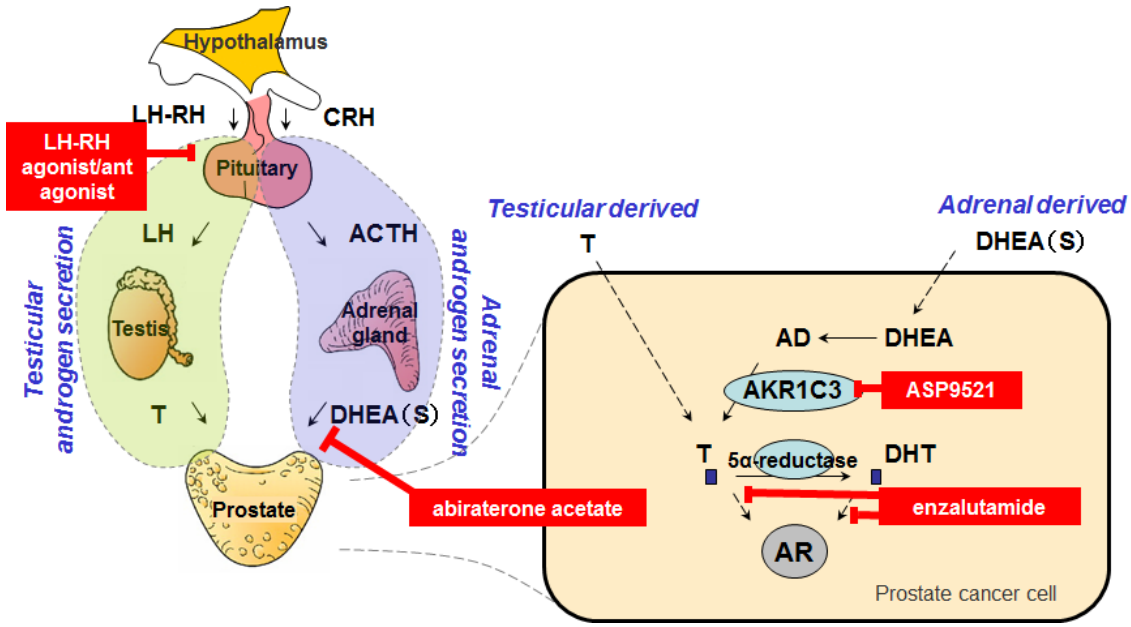

Figure.1 hormonal therapy agents

Urothelial carcinoma of the bladder cancer is the fourth most common cancer in males and the 11th most common in females, with a projected 79,030 new cases and 16,870 deaths estimated in the USA in 2017. The incidence and death rate is approximately four times higher in males than females [4]. 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 [32], urothelial cancer occurs most frequently in the bladder. Bladder cancer is mainly divided into two groups by stage. The stage classification differentiates between non-muscle-invasive and muscle-invasive tumors according to the depth of invasion. Non- muscle-invasive bladder cancers (NMIBC) highly recur, and at least 50% of patients will have recurring tumors. More than 60% of patients with the disease recur

within 4 months after surgery. For patients with unresectable or muscle-invasive bladder cancer (MIBC), first-line treatment with platinum-containing chemotherapy is considered the best available treatment option. Most efficacious are cisplatin-containing chemotherapy regimens, specifically cisplatin-gemcitabine (GC) and the combination of methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC). While these treatments have demonstrated anti-tumor activity among this population, cisplatin-containing chemotherapies are associated with significant toxicities, and for this reason, nearly half of patients are ineligible to receive them [33]. Moreover, despite reasonable response rates to chemotherapy in patients with locally advanced or metastatic bladder cancer, long-term progression-free survival rates remain insufficient, [34] which is thought to be caused by the induction of multidrug-resistant transporter 1 (MDR1) overexpression or the alterations in the apoptotic machinery including overexpression of c-MYC, an oncoprotein [35, 36].

FGFR3 is a member of a structurally related family of tyrosine kinase receptors (FGFR1–4) that regulate a variety of cellular activities, including proliferation, differentiation, and survival. Ligand binding promotes receptor dimerization, transphosphorylation of key tyrosine residues, and recruitment of adaptor proteins, ultimately leading to the activation of multiple downstream signaling cascades, including PI3K/AKT, RAS/MAPK, STATs, and phospholipase Cγ [37]. Point mutations of the *FGFR3* gene such as S249C, Y375C, and K652E are one of the most frequent genetic alterations seen in bladder cancer, occurring in around 75% of all cases of NMIBC [38, 39]. 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 [40, 41] Both *FGFR3-TACC3* and *FGFR3-BAI1AP2L1* translocations generate

constitutively activated and oncogenic FGFR3 kinase protein products, and cellular dependence on these drivers confers sensitivity to selective FGFR inhibition [40, 42]. In light of these considerations, FGFR3 has long been considered an attractive actionable target for novel therapeutic approaches in urothelial bladder cancer [43].

Based on these two studies, I propose that AKR1C3 inhibitor and FGFR3 inhibitor can lead to a novel and feasible therapeutic approach for PCa or urothelial cancer harboring *FGFR3* fusion or *FGFR3* point mutation.

Chapter I: *In vitro* and *in vivo* characterisation of ASP9521: a novel, selective, orally bioavailable inhibitor of 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5; AKR1C3)

1. Introduction

Ever since the pioneer work by Huggins and Hodges more than 70 years ago [44, 45], androgens are known to play a pivotal role in the growth and perpetuation of prostate cancer (PCa) cells [46]. Consequently, androgen deprivation therapy (ADT) has become a standard component of therapy for patients with advanced PCa [47]. It often consists of medical castration using LH-RH agonists or antagonists, which target testosterone (T) production by the testes. Although most prostate tumors initially respond well to castration, the majority eventually (i.e. after a median time of 2–3 years) progress despite castrate serum T levels. This is defined as castration-resistant PCa (CRPC) [47-49].

Several mechanisms have been implicated in the development of CRPC, few of which may act independently of androgen receptor (AR) signaling, e.g. through upregulation of anti-apoptotic molecules, activation of c-myc, alterations in the PI3K/Akt/mTOR pathway or aberrant activation of other growth and survival pathways [47-49]. However, the major mechanisms involved in CRPC development rely on either ligand-dependent or ligand-independent continuous activation of the AR [47-49]. Ligand-independent activation may arise from adaptive changes in the AR –including amplifications, mutations, splice variants and/or post-translational modifications, allowing AR activation by alternate ligands or cross-talk with other signalling pathways. However, most abundantly, the AR is activated by its proper ligands, i.e. by androgens. Although surgical

or medical castration with LH-RH agonists or antagonists reduce serum T levels by 90–97%, the total androgen pool in the circulation and intraprostatic levels of dihydrotestosterone (DHT) are only reduced by approximately 60% [49, 51]. These 'androgens that are untouched by castrative efforts' are formulated either via conversion of so-called 'weak' androgens, previously synthesized by the adrenal glands, into DHT in peripheral tissues, or via increased intratumoral (intracrine) *de novo* synthesis of androgens, often due to upregulation of enzymes involved in steroidal biosynthesis [47-49].

One of the key enzymes involved in adrenal and *de novo* intratumoural steroidal biosynthesis is aldo-keto reductase 1C3 (AKR1C3), also called 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5). This enzyme plays a crucial role in the synthesis of DHT, by catalysing the conversion of the adrenal androgens dehydroepiandrosterone (DHEA), androstenedione (AD) and 5α-androstanedione into DHT (Fig. 1-1) [19]. AKR1C3 is highly expressed in PCa cells [20-23], and its expression increases with increasing tumor aggressiveness [24], as demonstrated by upregulated AKR1C3 levels in patients with CRPC compared with benign prostate and early stage PCa [25-31]. This is one of the reasons why AKR1C3 has been implicated in CRPC progression, and is the target for ASP9521, a novel AKR1C3 inhibitor.

Based on the above-mentioned concepts, the overall objective of the current study was to ascertain if inhibition of AKR1C3 by ASP9521 would lead to reduced adrenal androgen biosynthesis and utilization in PCa tissue. Three fundamental questions were formulated: 1) Does AKR1C3 mediate conversion of adrenal androgens into T in preclinical models of CRPC?; 2) Does ASP9521 selectively inhibit AKR1C3?; 3) Where does ASP9521 accumulate, in PCa tissue or in plasma? The results of the study described

in this manuscript indicate that ASP9521 selectively inhibits AKR1C3, thereby reduces prostatic intratumoral androgens and PSA production. These observations led to a phase I/II study to investigate the safety, tolerability and anti-tumor activity of ASP9521 in patients with metastatic CRPC [52].

2. Materials and Methods

2.1. Synthesis of ASP9521 and crystal structure

ASP9521 is a 1-{1-[(5-methoxy-1H-indol-2-yl)carbonyl] piperidin-4-yl}-2-methylpropan-2-ol synthesized at Astellas Pharmaceutical Co. Ltd. (Tsukuba, Japan). It was discovered through a high-throughput screening approach to identify compounds inhibiting AKR1C3-mediated conversion of AD into T and subsequent *in vitro* and *in vivo* optimization of a lead compound with a non-steroidal scaffold. The synthesis of ASP9521 and the crystal structure of AKR1C3 in complex with ASP9521 have been reported elsewhere [53].

2.2. Cell lines and stable transfection

HEK293 cells, a cell line derived from human embryonic kidney cells, and LNCaP cells (American Type Culture Collection, Manassas, VA, USA), a cell line commonly used as a model for castration-sensitive human PCa, were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) and RPMI-1640 (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), respectively. As endogenous expression levels of AKR1C3 are very low in both cell lines [20, 54], I established HEK293 and LNCaP cells stably expressing AKR1C3, i.e. HEK293-AKR1C3 and LNCaP AKR1C3. The cDNAs encoding human or cynomolgus monkey AKR1C3 with a FLAG-tag in the N-terminus were cloned into a pcDNA3.1 vector (Life Technologies, Carlsbad, CA, USA). Cells were transfected with pcDNA3.1-AKR1C3 (human or monkey; 10 μg DNA) using FuGENE® 6 Transfection Reagent (30 μL) (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions. Stable transfectants were selected in media containing 0.5–1.0 mg/mL geneticin (Invitrogen, Minato-ward,

Tokyo, Japan). AKR1C3 protein expression levels in parental cells and stable transfectants were examined by Western blot analysis. 10 μg protein from cell lysates was loaded and separated using SDS-PAGE, transferred to a PVDF membrane and probed with 3 μg/mL mouse monoclonal anti-AKR1C3 antibody (Sigma-Aldrich; clone NP6. G6. A6). CWR22R cells, a cell line derived from a human PCa xenograft, which endogenously expresses human AKR1C3, were kindly provided by Dr. Gregory CW (University of North Carolina, Raleigh, NC, USA) and maintained by *in vivo* passage in male Balb/c athymic nude mice (Charles River Japan Inc., Atsugi-City, Kanagawa-Pref, Japan).

2.3. *In vitro* enzyme assay

ASP9521 and AD (Sigma-Aldrich) were dissolved separately in dimethyl sulfoxide (DMSO), and then diluted with 100 mmol/L potassium phosphate buffer (pH 6) to final concentration of 30–10,000 nmol/L and 10 μmol/L, respectively. To obtain human AKR1C3 enzyme or AKR1C3 homologues from other species, *E. coli* BL21 strain (GE Healthcare Life Sciences, Hino-City, Tokyo, Japan) were transformed with pGEX-2T vector (GE Healthcare Life Sciences) containing human AKR1C3 (NM_003739), cynomolgus monkey AKR1C3 (17βHSD5; DQ266251) , rat AKR1C1 (NM_001033697) or mouse AKR1C6 (NM_030611) (see also: **http://www.ncbi.nlm.nih.gov/gene/?term=hsd17b5**) [55], followed by purification of the respective enzymes from these strains. Each purified enzyme was diluted with 20 mmol/L NADPH, 2% CHAPS buffer and 100 mmol/L potassium phosphate buffer (pH 6). About 5 μg/mL enzyme was added to the reaction mixture containing ASP9521 and AD. Reaction mixtures containing human or cynomolgus monkey AKR1C3 were

incubated at room temperature (RT; 20°C) for 1.5 h, while mixtures containing rat or mouse homologues were incubated at RT for 0.5 h. T levels in the reaction mixture were determined using Wallac DELFIA Testosterone reagents® (PerkinElmer Life Sciences Inc., MA, USA), based on the time-resolved fluorescence assay methods (i.e. competition between fluorescently labeled T and sample T for binding sites on polyclonal T antibodies). Fluorescence intensity was measured using a multi-well plate reader ARVO (PerkinElmer Life Sciences Inc.).

2.4. AKR1C enzyme assay by monitoring NADPH oxidation

Recombinant human enzymes AKR1C3 and AKR1C2 were purified from *E. coli* BL21 strain (GE Healthcare Life Sciences). All assays were performed on SAFIRE spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland). The potency of ASP9521 was determined by measuring its ability to inhibit NADPH-dependent reduction of 9,10-phenanthrenequinone (9,10-PQ) catalysed by human AKR1C as previously described [56]. Purified AKR1C3 or AKR1C2 (10 μg/mL), 9,10-PQ (4 μmol/L) (Wako chemicals, Tokyo, Japan), NADPH (200 μmol/L) and ASP9521 were mixed in 100 mM potassium phosphate buffer (pH 6.0) to give a total volume of 80 μL, and incubated at RT for 20 min. The change in absorbance of NADPH at 340 nm at RT was measured. The amount of oxidation of NADPH in the presence of the compound was obtained as a relative value, with the amount of NADPH in the absence of the enzyme set at 0%, and the amount of NADPH in the absence of ASP9521 set at 100%. The IC_{50} values were calculated using Sigmoid-Emax model non-linear regression.

2.5. Cell-based AKR1C3 enzyme assay

HEK293-AKR1C3 cells or CWR22R cells, obtained by excising xenografted PCa tumors from mice and digesting them with 0.1% protease from *Streptomyces griseus* (Sigma-Aldrich), were seeded in 96-well plates at 2×10^4 cells/100 μL/well in medium (DMEM for HEK293-AKR1C3 cells; RPMI-1640 for CWR22R cells) supplemented with 10% heat-inactivated charcoal-dextran-stripped FBS (Gemini Bio-Products, CA, USA). After overnight incubation at 37 °C in 5% CO_2, AD (final concentration 300 nmol/L) was added to each well, with or without ASP9521. Four hours after incubation, the cell supernatants were collected to measure T concentration using Wallac DELFIA Testosterone reagents® according to the manufacturer's instructions. IC_{50} values, defined as 50% inhibition of the conversion from AD into T, were calculated using Sigmoid-Emax model nonlinear regression.

2.6. *In vitro* cell proliferation/PSA expression assay

LNCaP-AKR1C3 cells stably expressing human AKR1C3 were seeded in 96-well plates at 1×10^4 cells/100 μL/well in RPMI-1640 medium supplemented with heat-inactivated charcoal-dextran-stripped FBS (1% for the PSA expression assay and T measurement and 5% for the cell proliferation assay). After 24 h incubation, AD was added to each well with or without ASP9521 (0.3-100 nmol/L). The cell culture media were collected 24 h after administration of AD to measure T concentration and 6 days after administration of AD to measure either PSA levels using Human Kallikrein 3/PSA Quantikine® ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA) or cell proliferation using Cell-Titer Glo (Promega). For comparison of cell morphology between LNCaP cells and LNCaP-AKR1C3 cells, cells were supplemented with T, AD or DHEA (all reagents purchased from Sigma-Aldrich) (final concentration for each

reagent: 10 nmol/L in RPMI-1640 medium supplemented with 10% heat-inactivated charcoal-dextran-stripped FBS) for 7 days and morphology was assessed using a Nikon ECLIPSE Ti microscope (magnification 100×).

2.7. *In vivo* model for intracrine T synthesis in CWR22R xenografts

All experiments were performed in accordance with the regulation of the Animal Ethics Committee of Astellas Pharma Inc. Male Balb/c athymic nude mice (Charles River Japan Inc.) (4–6 weeks old) were used for the CWR22R xenograft model and were castrated before tumor implantation. Mice were maintained on a standard diet throughout the experiments under specific-pathogen-free conditions. Established CWR22R tumors from 3 host mice were minced into small fragments and digested with protease (0.1%) from *Streptomyces griseus* (Sigma-Aldrich). The digested cells were suspended in RPMI-1640 medium with 20% FBS and then mixed with Matrigel® (Becton Dickinson Co., NJ, USA) (1:1 v/v) solution to a concentration of 1×10^7 cells/mL. Approximately 100 μL of the cell suspension was subcutaneously injected into the flank of each castrated mouse. Approximately 3 weeks after implantation, mice carrying CWR22R tumors with similar sizes were divided into different groups (n=6 for each group). A control group for background (BG) intratumoral T concentration (no AD, no ASP9521) and a placebo group (AD, no ASP9521) were treated with vehicle (0.5% methyl cellulose), while the other groups were treated with ASP9521 (single oral administration directly into the mouth of the mice; 1, 3 or 10 mg/kg). AD (1 ng/100 mm^3) was injected directly into the xenografted tumors 1 h before mice were sacrificed. This lag time of 1 h was chosen based on pilot experiments in the absence of ASP9521, showing that intratumoral T production reached a maximum 1 h after AD administration.

Blood samples were obtained from the central vein just before the sacrifice. Tumor tissues were removed, weighed and homogenated with 200 mmol/L phosphate buffer (pH 7.4). T was extracted by *tert*-butyl methyl ether and T concentrations of the reconstituted extracts were determined using Wallac DELFIA Testosterone reagents®. ASP9521 concentrations in tumor tissue or plasma were determined using the high-performance liquid chromatography (Waters Alliance 2690 HPLC separation module, Waters Corporation, Milford, MA, USA)-mass spectrometry/mass spectrometry (API4000, AB SCIEX, Framingham, MA, USA) (HPLC-MS/MS) method.

2.8. Accumulation of ASP9521 in HEK293 xenografts

To investigate the role of AKR1C3 in the accumulation of ASP9521 in tumor tissue, HEK293 cells with or without AKR1C3 expression were xenografted into male Balb/c athymic nude mice (Charles River Japan Inc.) (5–6 weeks old). HEK293(−AKR1C3) cells were cultured *in vitro* in DMEM supplemented with 10% FBS and were dispersed in PBS (Sigma-Aldrich). The dispersed cells were mixed with Matrigel® (1:1 v/v; final concentration 3×10^7 cells/mL), and about 100 μL of the mixture was subcutaneously injected into the flank of each mice. Approximately 2–3 weeks after implantation, mice carrying HEK293 or HEK293-AKR1C3 tumors with similar sizes were selected and randomly divided into 5 groups (n=3 for each group). All groups were treated with ASP9521 (single oral administration; 3 mg/kg). Plasma (from the central vein) and tumor tissues were collected at 0.25, 0.5, 1, 2 and 4 h after administration of ASP9521, and ASP9521 concentrations were determined using the HPLC-MS/MS method.

3. Results

3.1. AKR1C3 is required for adrenal hormone conversion to T and PSA production

LNCaP cells and LNCaP cells stably expressing AKR1C3 (LNCaP-AKR1C3) (Fig. 1-2) were supplemented with T, AD or DHEA. After supplementation with T, cell morphology was similar between both cell lines. However, supplementation with the adrenal androgens AD or DHEA induced a neuroendocrine-like phenotype in LNCaP cells, but not in LNCaP-AKR1C3 cells (Fig. 1-3). Similarly, LNCaP cells secreted lower levels of T and PSA in the culture medium than LNCaP-AKR1C3 cells (Fig. 1-4). Moreover, in LNCaP-AKR1C3 cells, ASP9521 inhibited both AD (10 nmol/L)-induced PSA production and cell proliferation in a concentration-dependent manner, with IC_{50} values of 11 nmol/L and 6.6 nmol/L, respectively (Fig. 1-5).

3.2. ASP9521 selectively inhibits AKR1C3 activity in CRPC

In vitro, ASP9521 inhibited the conversion of AD into T by recombinant human and cynomolgus monkey AKR1C3 in a concentration-dependent manner, with IC_{50} values of 11 and 49 nmol/L, respectively. In contrast, ASP9521 did not inhibit the conversion by rat and mouse homologues (AKR1C1 and AKR1C6, respectively) up to a concentration of 10 μmol/L. In an *in vitro* assay to evaluate the potency of ASP9521, inhibition of AKR1C-dependent oxidation of NADPH was monitored. ASP9521 showed moderately high selectivity (>100-fold) for human AKR1C3 (IC_{50}: 120 nmol/L) over the human isoform AKR1C2 (IC_{50}: >20,000 nmol/L).

In HEK293 cells stably expressing human or cynomolgus monkey AKR1C3 (HEK293-AKR1C3), ASP9521 inhibited conversion from AD into T in a concentration-dependent manner, with IC_{50} values of 1.9 and 6.2 nmol/L, respectively. Similarly, in CWR22R cells,

ASP9521 inhibited AD-induced T production in a concentration-dependent manner, with an IC_{50} of 0.88 nmol/L. Consistent with that, in murine models harboring CWR22R xenograft tumors, single oral administration of ASP9521 suppressed AD-induced intratumoral T production in a dose-dependent manner (2 h after administration of ASP9521: 52% inhibition for 3 mg/kg dose and 103% inhibition for 10 mg/kg dose) (Fig. 1-6). Moreover, this inhibitory effect was maintained for 24 h after single oral administration of ASP9521 (Fig. 1-7).

3.3. AKR1C3-dependent accumulation of ASP9521 in prostate tumors

To investigate ASP9521 accumulation, the concentration of ASP9521 in plasma and tumor tissue was measured over time in nude mice bearing HEK293 tumors with or without AKR1C3 expression. After single oral administration of ASP9521, plasma concentrations of ASP9521 reached maximum values within 0.25 h (mean: 767.3 ng/mL and 648.2 ng/mL for HEK293 and HEK293-AKR1C3 cells, respectively), but decreased rapidly thereafter (Fig. 1-8). Similarly, the intratumoral concentration of ASP9521 in HEK293 tumors lacking AKR1C3 expression rapidly decreased from 845.8 ng/g after 0.25 h to undetectable levels after 4 h. In contrast, in HEK293 tumors expressing AKR1C3, the maximum intratumoral ASP9521 concentration was considerably higher (mean: 1,905.0 ng/g after 0.25 h), and elevated ASP9521 levels were maintained for at least 4 h. These results suggest that accumulation of ASP9521 in tumor tissue depends on AKR1C3 expression.

4. Discussion

The principle findings of this study relate to the inhibitory activity of ASP9521. The observations suggest that inhibition of AKR1C3 by ASP9521 in preclinical models of CRPC: 1) reduces androgen biosynthesis and PSA production, 2) is selective and 3) is maintained in the prostatic cancer tissue and not in plasma. In light of these data, ASP9521 was tested in a pilot phase I/II study in men with metastatic CRPC progressing after chemotherapy [52]. I hypothesized that administration of ASP9521 to patients with castrate levels of T would lead to complete suppression of androgen biosynthesis, by targeting mechanisms of hormonal drug resistance in CRPC [48-50], i.e. *de novo* intratumoral steroidogenesis through upregulation of AKR1C3 [57, 58] and ligand independent activation of AR signaling [59]. However, the planned phase I/II study was terminated prematurely due to lack of observed change to pharmacodynamic markers of activity [52].

Selectivity of ASP9521 for AKR1C3 was investigated using various AKR1C homologues and isoforms. ASP9521 was shown to selectively inhibit conversion of AD into T by human and cynomolgus monkey AKR1C3, but not by rat (AKR1C1) and mouse (AKR1C6) homologues [60]. Furthermore, ASP9521 showed high selectivity for AKR1C3 over its closely related isoform AKR1C2. Selectivity for AKR1C3 in PCa tissue is an absolute requirement for AKR1C inhibitors, as the isoforms AKR1C1 and AKR1C2 are both involved in catabolism and deactivation of DHT within the prostate at steady state (Fig. 1-1) [61, 62]. Thus, inhibition of these isoforms would potentially lead to increased DHT tissue levels, increased AR signaling and PCa progression.

The selectivity of ASP9521 spans more than inhibition of the AKR1C3 enzyme, but also relates to its lack of interference of precursor biosynthesis pathway components,

unlike that of other novel androgen biosynthesis inhibitors, such as abiraterone acetate (AA) (Fig. 1-1). AA targets CYP17A1 hydroxylase/lyase, an enzyme that converts pregnenolone and progesterone into DHEA and androstenedione via 2 subsequent reactions, catalyzed by CYP17α-hydroxylase and CYP17,20-lyase. However, inhibition of CYP17α-hydroxylase also decreases serum cortisol levels, leading to a subsequent rise in adrenocorticotropic hormone and mineralocortoids upstream of CYP17α-hydroxylase [63]. To suppress potential side effects related to this mineralocorticoid excess, AA requires coadministration with steroids such as prednisone. In contrast, ASP9521 acts further downstream in the steroid biosynthesis pathway and does not interfere with glucocorticoid metabolism (Fig. 1-1). So, ASP9521 may not require coadministration with prednisone [19]. In addition, ASP9521 was shown not to have any appreciable affinity for testosterone, oestrogen, glucocorticoid, mineralocorticoid or progesterone receptors (ASP9521 (10 μmol/L) had no appreciable affinity.). As such, ASP9521 itself is thought to be devoid of androgenic activity.

AKR1C3 catalyzes both the conversion of DHEA and AD into androstenediol and T via the ‘classical’ pathway, and the conversion of 5α-androstanedione into DHT via the ‘alternative’ pathway (Fig. 1-1) [19], thereby increasing intratumoral steroidogenesis. Upregulation of AKR1C3 has been suggested to be an adaptive response to androgen deprivation or to inhibition of AR activity [29, 31]. So, it is not surprising that prior treatment with AA was recently reported to be associated with increased levels of AKR1C3 expression [57, 58]. Taken together, administration of ASP9521 following progression on AA is a scientifically plausible path for further investigation. A limitation to the current study is the fact that we did not assess ASP9521 inhibitory activity in combination with AA or post AA treatment.

Although the current study appropriately demonstrates the inhibition of AKR1C3 by ASP9521, further limitations to the results were noted. The selectivity of ASP9521 for AKR1C3 over AKR1C1 was not specifically investigated. However, ASP9521 showed >100-fold selectivity for AKR1C3 over AKR1C2, and AKR1C1 shares 98% sequence identity to AKR1C2, with both isoforms differing only in 1 amino acid at respective active sites [19, 64, 65]. Therefore, it is very likely that ASP9521 is also highly selective for AKR1C3 over AKR1C1. I did not assess the inhibitory potential of ASP9521 on the 'backdoor' pathway of androgen biosynthesis, but mainly on the 'classical' and 'alternative' pathways. The 'backdoor' pathway requires 5α-reductase activity as a first step in the conversion of pregnenolone and progesterone into androstanediol (3α-diol), which is then back-converted to DHT (Fig. 1-1) [58, 66, 67]. This pathway has been implicated in CRPC as contributing to hormonal drug resistance. I also did not evaluate the effect of ASP9521 on the levels of DHT and/or precursor steroidal hormones in the biosynthesis pathway. Knowledge of the impact of ASP9521 on these endocrine levels would allow for a more well-rounded understanding of the activity and full mechanistic profile of ASP9521.

In summary, the current study, even with its limitations, demonstrated that ASP9521 is a potent, selective, orally bioavailable inhibitor of AKR1C3-mediated conversion of AD into T, both *in vitro* and *in vivo* in CWR22R xenografted castrate mice. Furthermore, expression of AKR1C3 in tumor tissue was found to be a prerequisite for intratumoral accumulation of ASP9521. These results provided the rationale for testing ASP9521 in patients with mCRPC. In patients with mCRPC, ASP9521 demonstrated dose-proportional increase in exposure over the doses evaluated, with an acceptable safety and tolerability profile. However, the novel androgen biosynthesis inhibitor showed no

relevant evidence of clinical activity [52]. The discrepancy between the preclinical and clinical results illustrates the complexity of T synthesis pathway in CRPC. Further research is needed to find out why the promising preclinical results could not be reproduced in the clinical setting.

5. Figures

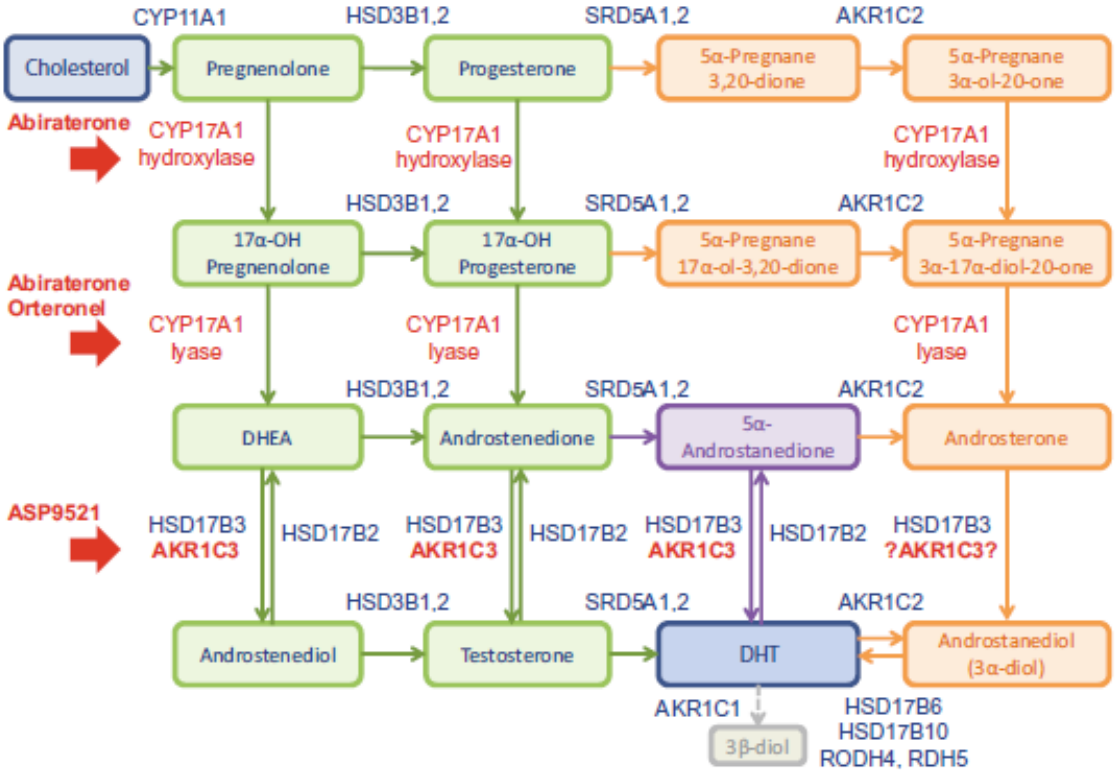

Figure 1-1. Androgen biosynthesis pathways.

The 'classical', 'alternative' and 'backdoor' pathways of androgen biosynthesis are indicated in green, purple and orange, respectively. The target enzymes of abiraterone/orteronel and ASP9521 are indicated in red. AKR1C1: aldo-keto reductase family 1, member C1; AKR1C2: aldo-keto reductase family 1, member C2 (3α-hydroxysteroid dehydrogenase type III); AKR1C3: aldo-keto reductase family 1, member C3 (17β-hydroxysteroid dehydrogenase V); CYP11A1: cytochrome P450, family 11, subfamily A, polypeptide 1; CYP17A1: cytochrome P450, family 17, subfamily A, polypeptide 1; DHEA: dihydroepiandrosterone; DHT: dihydrotestosterone; HSD17B10: 17β-hydroxysteroid dehydrogenase X; HSD17B2: 17β-hydroxysteroid dehydrogenase II; HSD17B3: 17β-hydroxysteroid dehydrogenase III; HSD17B6: 17β-hydroxysteroid dehydrogenase VI; HSD3B1,2: 3β-hydroxysteroid dehydrogenase 1,2; RDH5: retinol dehydrogenase 5; RODH4: 17β-hydroxysteroid dehydrogenase VI; SRD5A1,2: steroid 5α-reductase 1,2

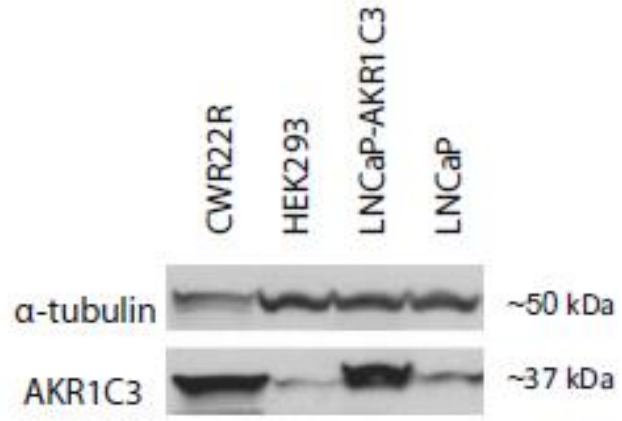

Figure 1-2. Comparison of AKR1C3 expression levels in CWR22R, HEK293, LNCaP-AKR1C3 and LNCaP cell lines by Western blot analysis, using mouse monoclonal anti-AKR1C3 antibody.

10 μg protein was loaded per lane and α-tubulin was used as a loading control.

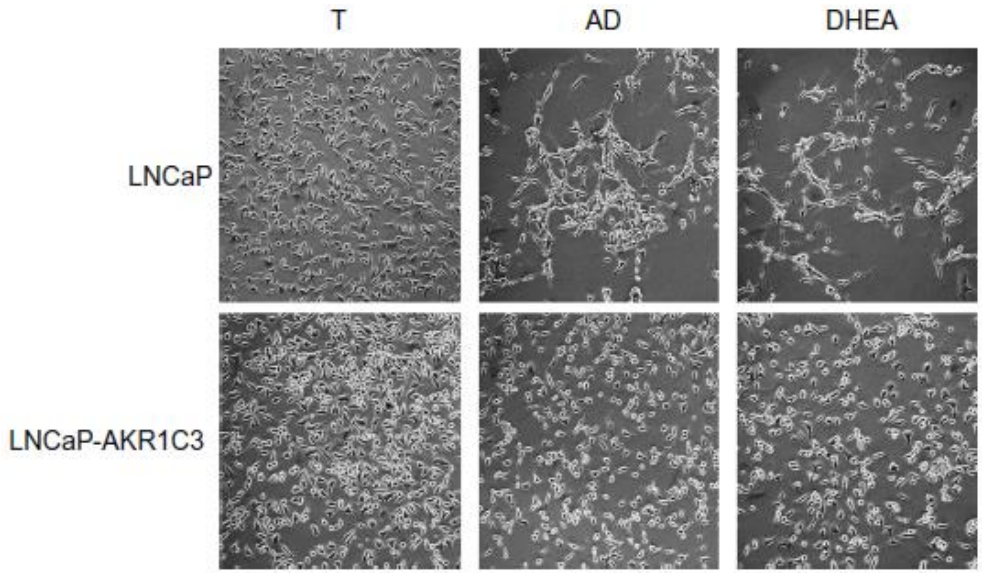

Figure 1-3. Comparison of cell morphology between LNCaP cells and LNCaP-AKR1C3.

Cells were supplemented with T, AD or DHEA for 7 days and morphology was assessed. Final concentration for each reagent: 10 nmol/L in RPMI-1640 medium supplemented with 10 % heat-inactivated charcoal-dextran-stripped FBS

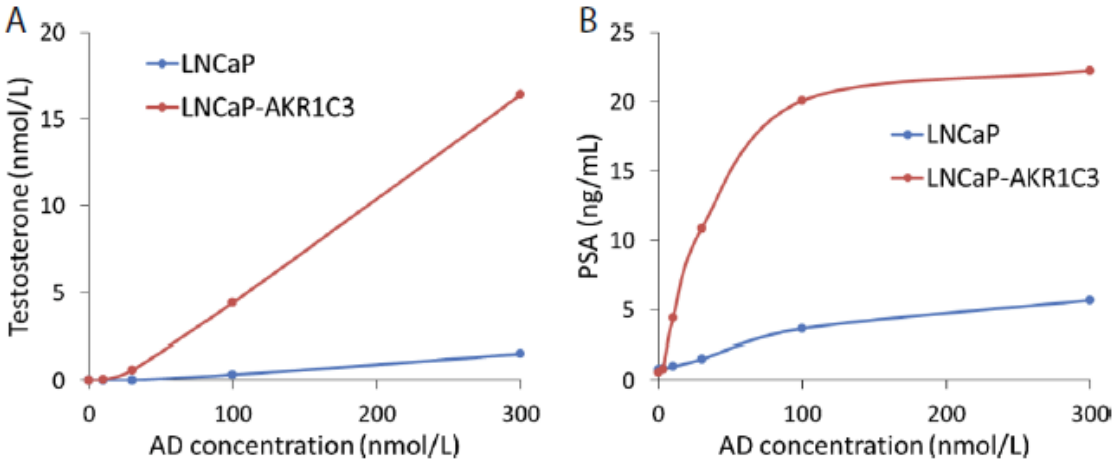

Fig. 1-4. Comparison of (A) testosterone and (B) PSA formation between LNCaP cells and LNCaP cells stably expressing AKR1C3 (LNCaP-AKR1C3).
Cells were treated with AD at various concentrations (range: 10–300 nmol/L). Testosterone and PSA concentration in the medium were measured 24 h and 6 days after administration of AD, respectively.

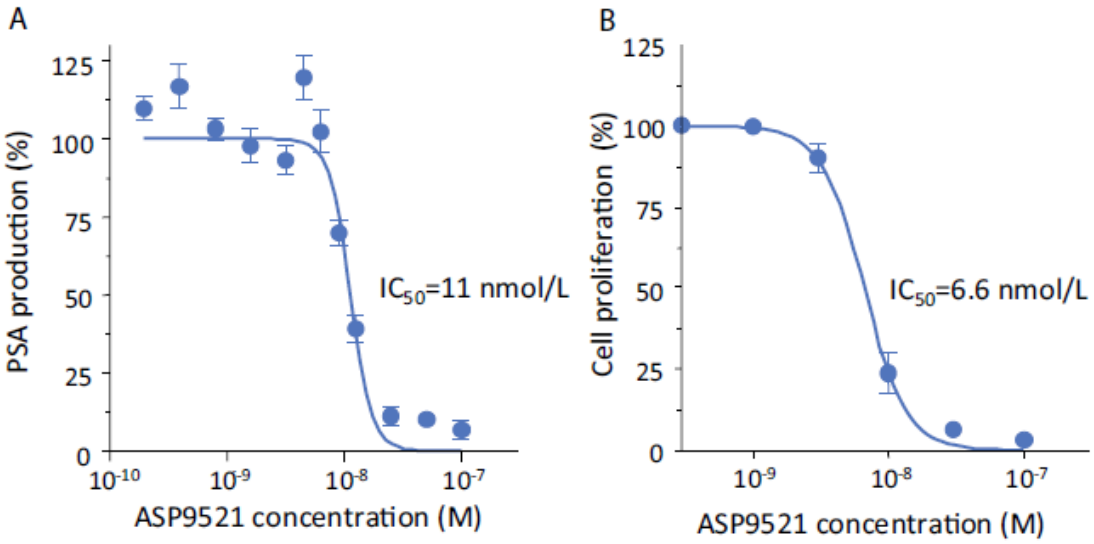

Fig. 1-5. Inhibitory effect of ASP9521 on AD-dependent (A) PSA production and (B) cell proliferation in LNCaP prostate cancer cells stably transfected with AKR1C3.
PSA production and cell proliferation were measured 6 days after administration of AD (final concentration 10 nmol/L) and various concentrations of ASP9521. Results are expressed as percentage inhibition compared with PSA production/cell proliferation in the absence of ASP9521

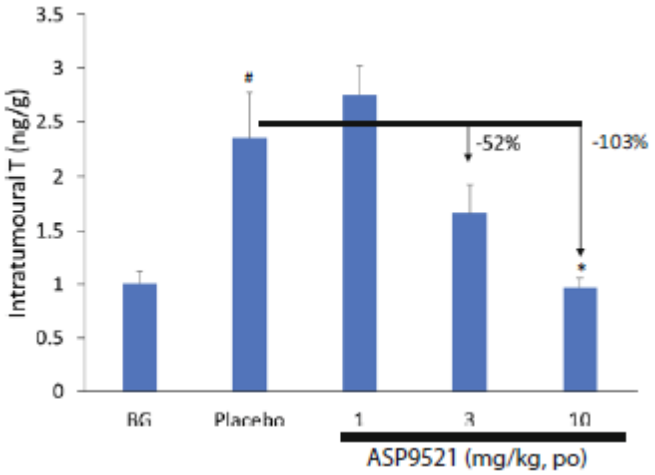

Fig. 1-6. Dose dependency of inhibitory effect of ASP9521 on AD-induced intratumoral T production in mice bearing CWR22R xenograft.

Mice were sacrificed 2 h after administration of a single oral dose of ASP9521. For each group, mean ± standard error is depicted (n=6 per group). Percentage inhibition of intratumoral T was calculated taking the mean value for the placebo group (AD only, no ASP9521) as 0% inhibition and that for the BG group (no AD, no ASP9521) as 100% inhibition. #P<0.05 vs BG group (Student's t-test); *P<0.05 vs placebo group (Dunnett's test)

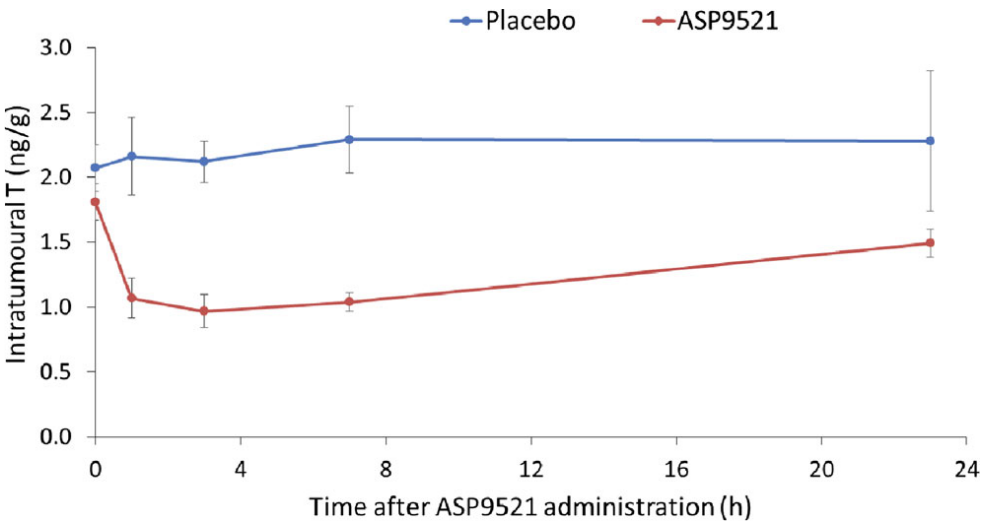

Fig. 1-7. Duration of inhibitory effect of ASP9521 on AD-induced intratumoral T production in mice bearing CWR22R xenograft.

Mice were treated with ASP9521 (3 mg/kg, po) or placebo and sacrificed 0.25, 0.5, 1, 2, 4, 8 and 24 h afterwards (n=6 per group). AD was injected into the xenografts 1 h before the sacrifice. For each group, mean ± standard error is depicted. The dashed line indicates the background level of intratumoral T production (no AD, no ASP9521)

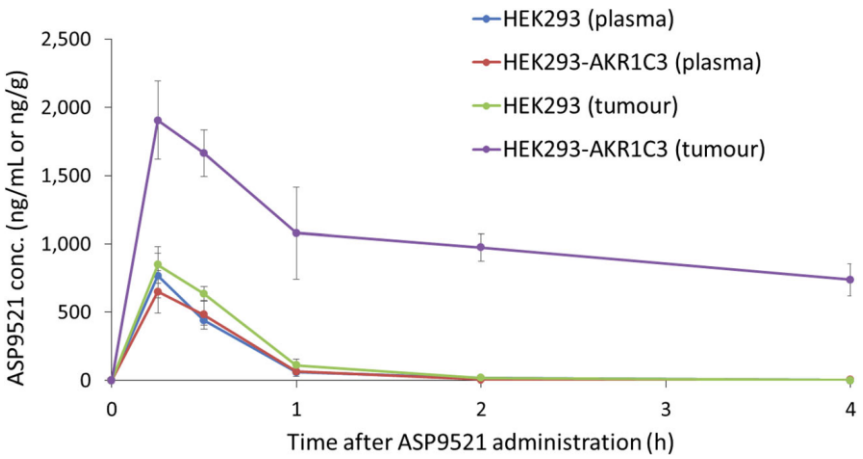

Fig. 1-8. Concentration of ASP9521 over time in plasma (ng/mL) and tumor tissue (ng/g) of mice bearing HEK293 xenograft with or without AKR1C3 expression.
Mice were treated with ASP9521 (3 mg/kg, po) and sacrificed 0.25, 0.5, 1, 2 and 4 h afterwards (n=3 per group). For each group, mean ± standard deviation is depicted

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

1. Introduction

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 [32], urothelial cancer occurs most frequently in the bladder. Bladder cancer is the most common malignancy involving the urinary system. 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 [34], 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 [35, 36]. Therefore, effective drugs against chemotherapy-resistant bladder cancer are eagerly needed.

The mammalian FGF/FGFR family comprises 18 ligands and 4 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 [37]. 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 [39]. 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] [39]. 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 [68]. 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 [40, 41]. *FGFR3* fusion genes are observed in 3% (3/114) of muscle-invasive urothelial cancer [69]. Therefore, clinical trials of FGFR inhibitors in urothelial cancer harboring *FGFR3* fusion genes or point mutations are ongoing [70]. The clinical relevance of *FGFR3-TACC3* has been suggested by the clinical report of JNJ-42756493, a pan-FGFR inhibitor, which exerts 3 out of 4 partial responses among patients with tumors harboring *FGFR3-TACC3* fusion genes [71]. 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 1 unconfirmed complete response and 8 partial responses [72]. In light of these reports, FGFR3 has been considered as an attractive target for novel therapy in urothelial bladder cancer.

In this thesis, I 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. My 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.

2. Materials and Methods

2.1. Reagents

2-[4-({5-[(2,6-difluoro-3,5-dimethoxyphenyl)methoxy]pyrimidin-2-yl}amino)-1H-pyrazol-1-yl]ethan-1-ol [ASP5878 [73]] 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.

2.2. Cell lines

HT-1197, HT-1376, J82, RT4, SW 780, TCCSUP, UM-UC-3, NCI-H1581 and Hep3B2.1-7 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). HSC-39 was purchased from IBL (Takasaki, 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.

2.3. *In vitro* cell growth assay

HSC-39 was seeded into 96-well clear flat plates at 500 cells/well, Hep3B2.1-7 was at 1000 cells/well, and other cells were 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-GloTM (Promega, Madison, WI, USA). Data are presented as means from a single experiment performed in duplicate.

2.4. 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).

MDR1 mRNA expression in adriamycin-resistant UM-UC-14 cell line was quantified by qPCR method using the following *MDR1* and GAPDH primer sets; forward and reverse primer sequences for *MDR1* were 5'-CCCATCATTGCAATAGCAGG-3' and 5'-GTTCAAACTTCTGCTCCTGA-3', and forward and reverse primer sequences for GAPDH were 5'-CCTGACCTGCCGTCTAGAAAA-3' and 5'-CGCCTGCTTCACCACCTT-3, respectively.

2.5. 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-hour 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 (%).

2.6. Immunoblotting for the downstream signaling of FGFR3 and c-MYC

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 0.01%. Following 2-hour (for ERK and phospho-ERK) or 48-hour (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), c-MYC (#5605; Cell

Signaling Technology).

2.7. *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/0.1mL (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).

2.8. *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 hours 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.

2.9. 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), with P values less than 0.05 considered significant.

3. Results

3.1. Kinase inhibition profile of ASP5878

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. 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) [74].

3.2. Anti-proliferative profile of ASP5878 in urothelial cancer and other FGFR-dependent cell lines

ASP5878 inhibited cell growth of UM-UC-14 [FGFR3_S249C; [75]], RT-112 [FGFR3-TACC3; [40]], RT4 [FGFR3-TACC3; [40]], SW 780 [FGFR3-BAIAP2L1; [41]] and JMSU-1 [FGFR1 overexpression; [76]] with IC_{50} values of less than 100 nmol/L (Fig. 2-1). ASP5878, however, was inactive (IC_{50} values ≥300 nmol/L) against other urothelial cancer cell lines without FGFR genetic alterations (Fig. 2-1). Additionally, ASP5878 also inhibited cell proliferation of NCI-H1581 [FGFR1 amplification, lung; [77]], HSC-39 [FGFR2 amplification, stomach; [78]], and Hep3B2.1-7 [FGF19 amplification, liver; [79]] which is known as a FGF19/FGFR4-dependent cell line (Table 2-1). Thus, ASP5878 has potent anti-proliferative effects in human cancer cell lines harboring gene alterations in FGF or FGFR.

3.3. 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 hours of treatment (Fig. 2-2a). ERK phosphorylation, a downstream signaling molecule in the cell lines, was inhibited by ASP5878 in a

concentration-dependent manner (Fig. 2-2b). Thus, ASP5878 inhibits FGFR3 phosphorylation and ERK phosphorylation in urothelial cancer cell lines harboring *FGFR3* gene alternations.

3.4. 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 [35]. I 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. 2-3a) and mRNA (Fig. 2-3b) 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. 2-3c). 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. 2-3d).

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 [35]. Therefore, I 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 lineup to 1000ng/mL (Fig.2-4a). ASP5878 inhibited the proliferation of the parental and the gemcitabine-resistant RT-112cell lines with similar IC_{50} values of 8.7 (95% CI: 3.9-20, n=3) and 10nmol/L (95% CI: 3.7-27, n=3), respectively (Fig.2-4b) and decreased the level of ERK phosphorylation in the gemcitabine-resistant RT-112 cell line as with that in the parental cell line (Fig. 2-2b, 2-4c).It has been reported that gemcitabine-resistant cell growth in urothelial cancer cells is related to up-regulation of c-MYC expression which is involved in cell proliferation [36]. Up-regulation of c-MYC protein in the gemcitabine-resistant RT-112 cell line was also observed (Fig. 2-4d). Interestingly, ASP5878 decreased the expression of c-MYC in both the gemcitabine-resistant RT-112 and the parental cell lines (Fig. 2-4d). 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.

3.5. 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. 2-5a). 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. 2-5b), 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. 2-5c) and gemcitabine-resistant RT-112 (Fig. 2-5d) subcutaneous xenograft mouse models. Body weight was not affected at any dose of ASP5878 examined in these experiments (Fig. 2-5e). Thus, ASP5878 has the antitumor activities in urothelial cancer models harboring FGFR3_S249C or FGFR3-TACC3 fusion after the acquisition of gemcitabine resistance.

4. 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, I 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 [74], has potent anti-proliferative effects on FGFR1, 2, 3 and 4-depencent cell lines (Table 2-1). In 23 urothelial cancer cell lines, ASP5878 inhibited the proliferation of 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. 2-1). FGFR3-TACC3 displayed ligand-independent constitutive activation of FGFR3 kinase activity and dimerization through a coiled-coil domain in TACC3 [40, 73]. BAIAP2L1 has Bin-Amphiphysin-Rvs (BAR) domain which contributes to dimerization and constitutive activity in FGFR3-BAIAP2L1 fusion protein [41]. 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 [80]. 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 [75]. 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 to 16 months [35]. Treatment failure

can be commonly caused by development of resistance to chemotherapy [34, 81].

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 [82, 83]. I also obtained adriamycin-resistant UM-UC-14 cell line harboring MDR1 overexpression by stepwise increasing concentrations of adriamycin (Fig. 2-3). In addition, some studies have highlighted the important role of c-MYC in the development of drug-resistant phenotypes in cancer [84, 85]. It has been reported that KU19-19/GEM, gemcitabine-resistant urothelial cancer cells, up-regulated 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 [36].

As is the case of KU19-19/GEM cells, I also successfully established gemcitabine-resistant RT-112 cell line harboring c-MYC up-regulation by stepwise exposure to gemcitabine (Fig. 2-4). Furthermore, c-MYC overexpression has been observed in urothelial cancer tissues [86-88]. 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 [89]. Activated ERK, a downstream molecule of FGFR, stabilizes c-MYC in melanoma cells [90]. 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. 2-4d). 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 [91, 92]. 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 urothelial cancer in the preclinical models. Therefore, I 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. 2-3a) and ASP5878 inhibited the proliferation in common with the parent UM-UC-14 cell line (Fig. 2-3c). Furthermore, ASP5878 inhibited the cell growth in gemcitabine-resistant RT-112 cells *in vitro* and *in vivo* studies. (Fig. 2-4b, 2-5d). In addition to gemcitabine and adriamycin, cisplatin is also a key chemotherapeutic agent. However, I 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, I demonstrated antitumor activities of ASP5878 using mouse models xenografted with urothelial cancer cell lines (Fig. 2-5). 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 gemcitabine-resistant urothelial cancer harboring *FGFR3* gene alternations.

Hyperphosphatemia has been commonly observed with other FGFR inhibitors (e.g.

JNJ-42756493 and BGJ398) [71, 72]. In line with the findings, ASP5878 also induced serum phosphate increase in rodents. 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 [71, 93]. In the present study, I showed the duration of FGFR3 inhibitory activity after single administration of ASP5878 was relatively short in mice (Fig. 2-5b), 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.

5. Table

Table 2-1. Anti-proliferative effect of ASP5878 in FGFR-dependent cell lines.

Cell line	Origin	FGF/FGFR alternation	Cell growth IC_{50} (nmol/L)	95% confidence interval
UM-UC-14	Urothelial cancer	FGFR3_S249C	**7.8**	7.3-8.3
RT-112	Urothelial cancer	FGFR3-TACC3	**3.7**	3.2-4.4
NCI-H1581	NSCLC	FGFR1 overexpression	**4.6**	2.3-9.2
HSC-39	Stomach cancer	FGFR2 gene amplification	**2.8**	1.8-4.3
Hep3B2.1-7	Hepatocellular carcinoma	FGF19 overexpresison	**8.5**	6.7-11

Data represent the mean of IC_{50} and 95% CI of 3 individual experiments.

6. Figures

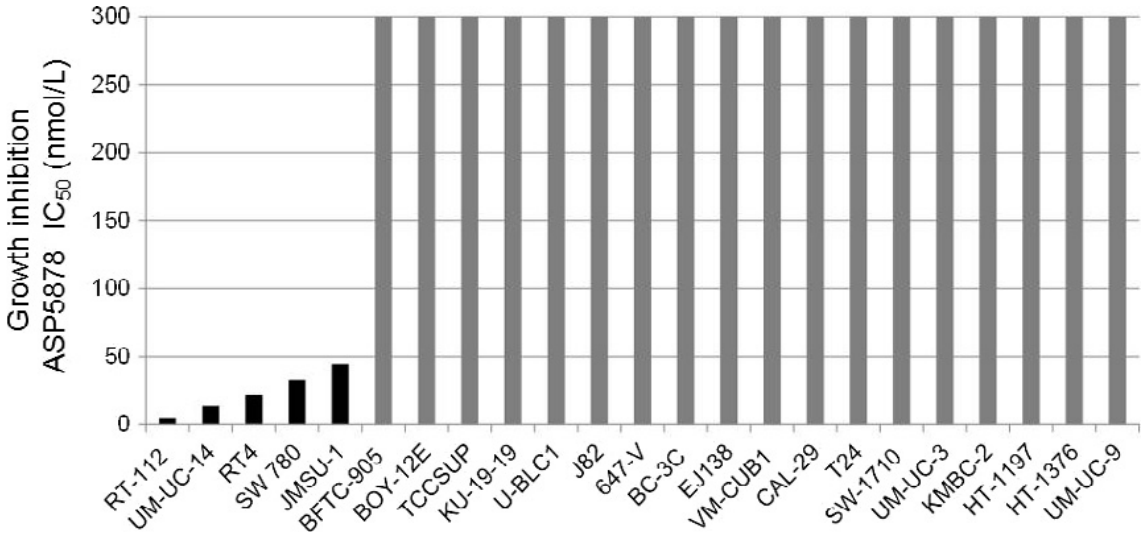

Fig. 2-1. Cell panel assay for the identification of ASP5878-sensitive bladder cancer cell lines.

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.

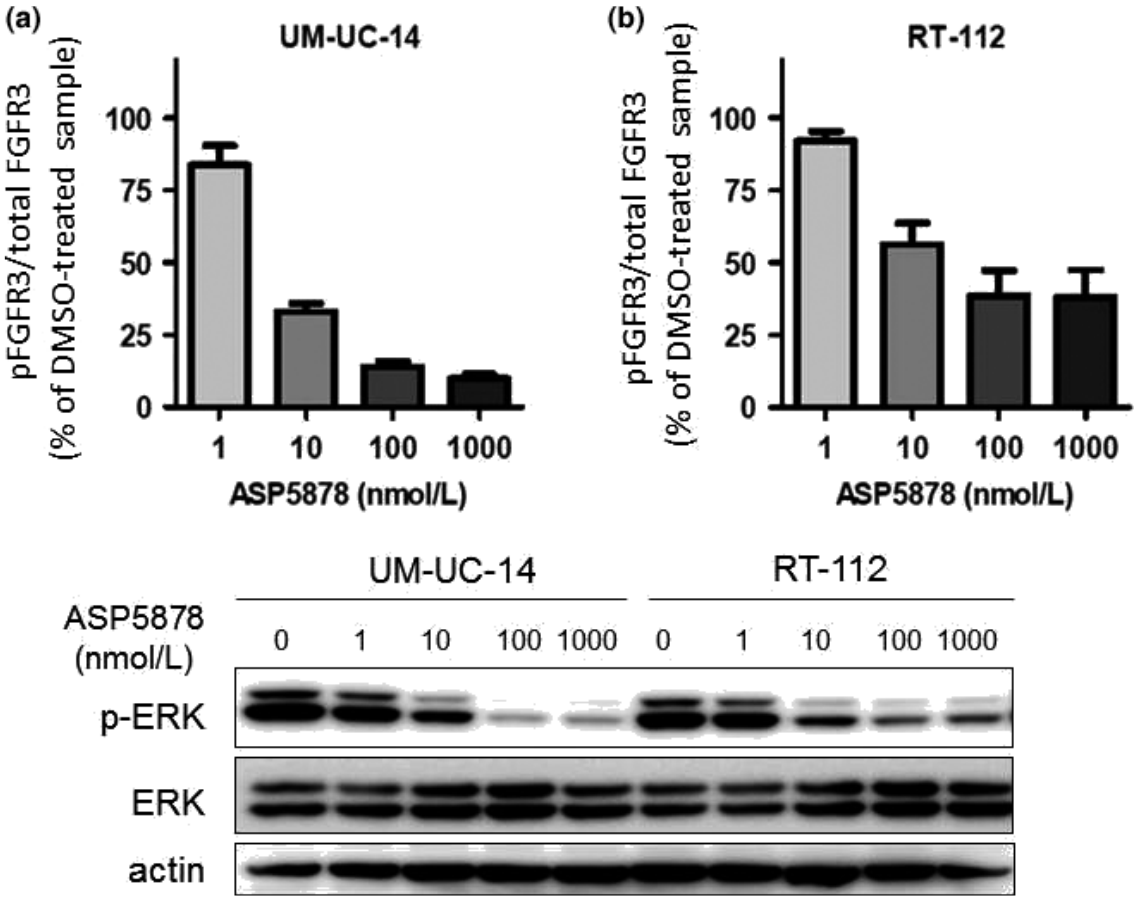

Fig. 2-2. Inhibitory effects of ASP5878 on FGFR3 and ERK phosphorylation in UM-UC-14 and RT-112 cell lines.

(upper) UM-UC-14 (a) and RT-112 (b) 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. (bottom) 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.

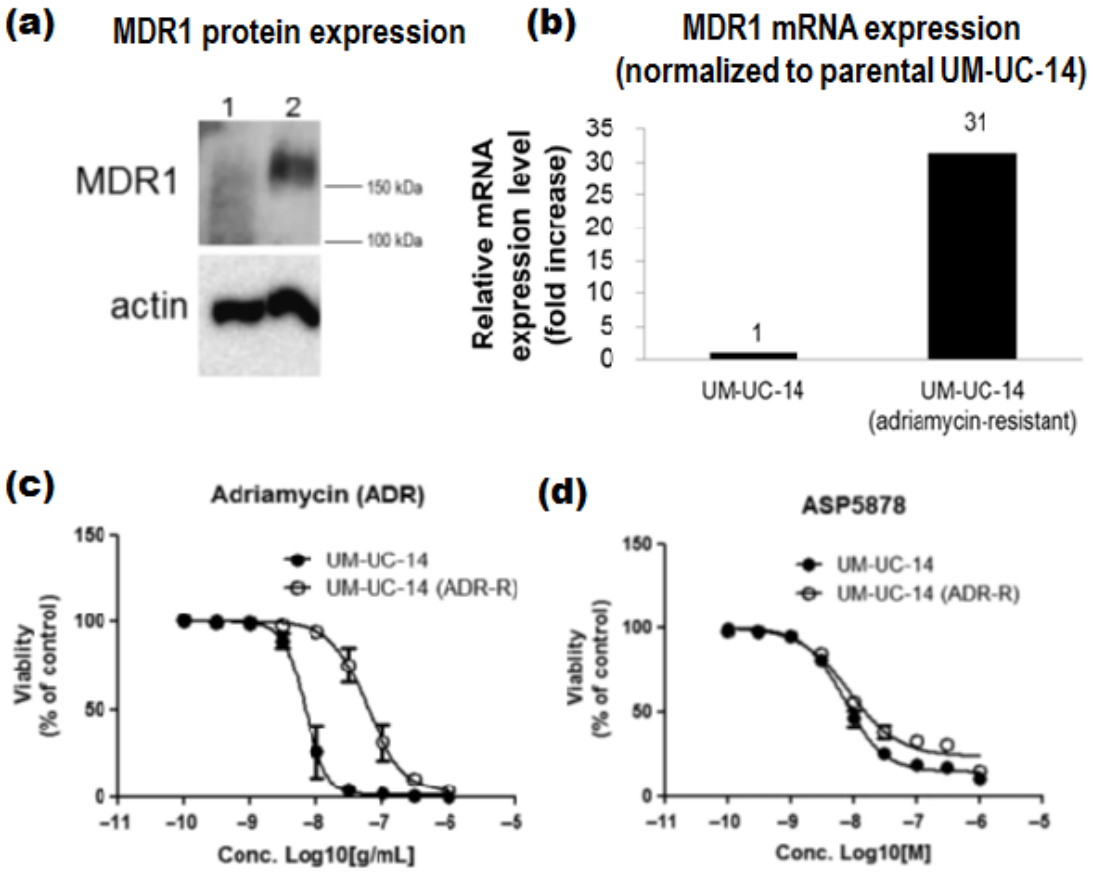

Fig. 2-3. Anti-proliferative effect of ASP5878 in adriamycin-resistant UM-UC-14 cell line.

(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) MDR1 mRNA expression in adriamycin-resistant UM-UC-14 cell line was detected by qPCR (normalized to parental UM-UC-14 cell line). (c, d) Anti-proliferative effect of adriamycin (c) and ASP5878 (d) 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.

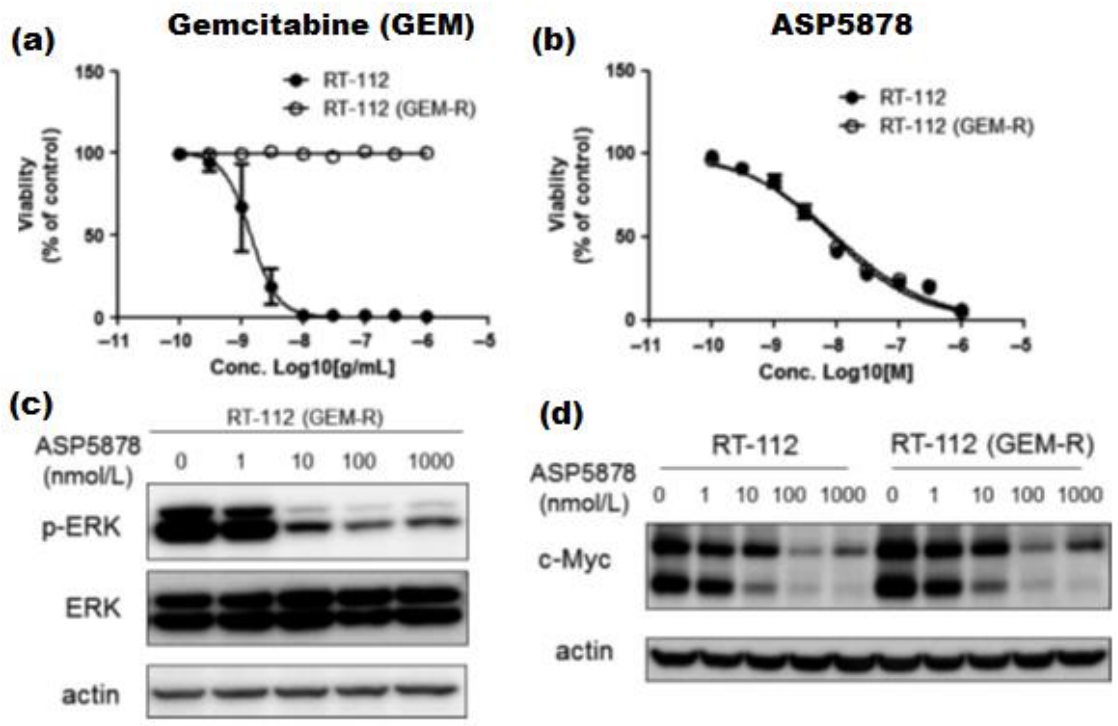

Fig. 2-4. Anti-proliferative effect of ASP5878 in gemcitabine-resistant RT-112 cell line.

(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.

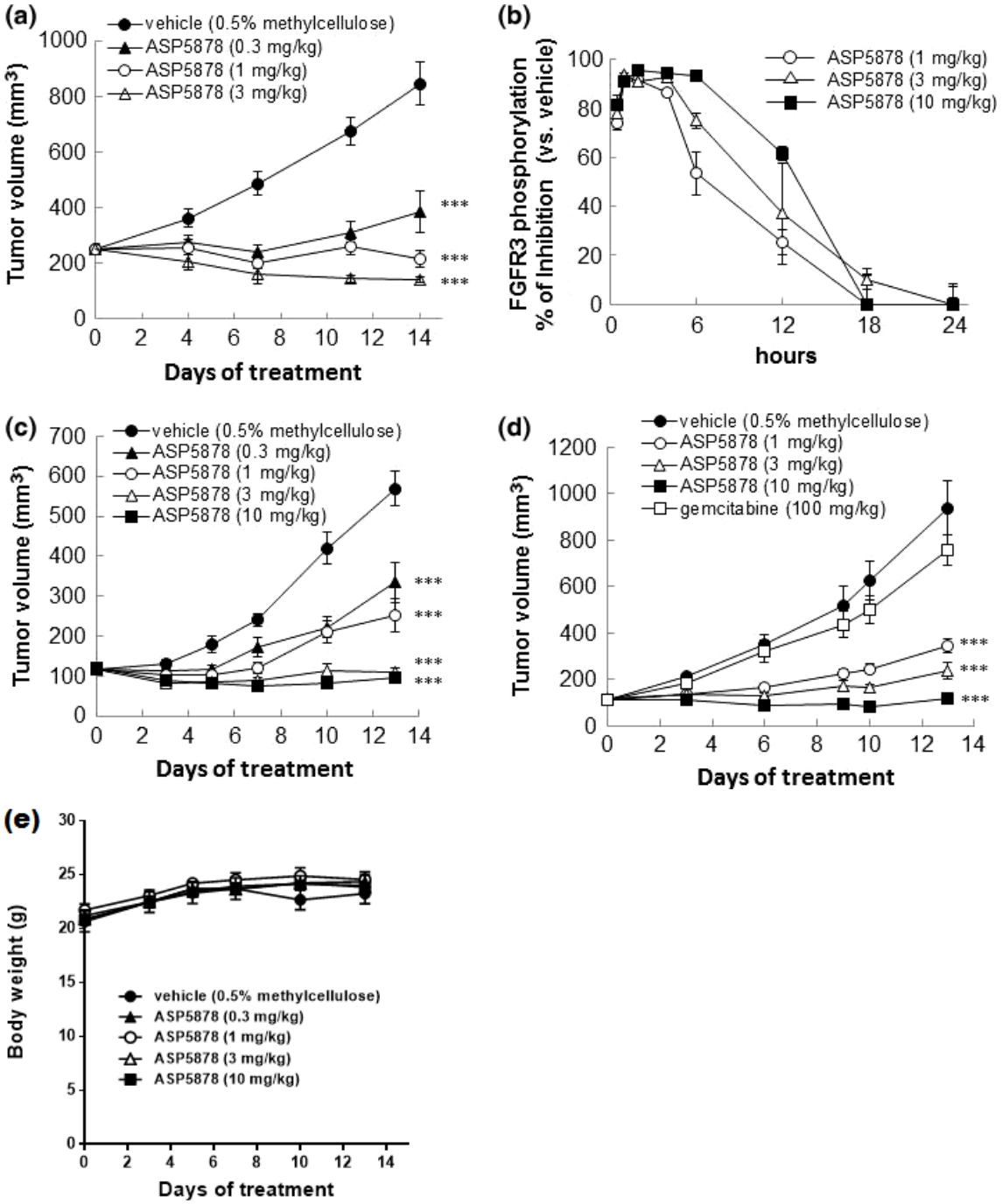

Fig. 2-5. ASP5878 treatment leads to tumor regression in UM-UC-14, RT-112 and gemcitabine-resistant RT-112 subcutaneous xenograft models.

(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). (e) Body weight of once-daily oral administration of ASP5878 for 14 days in nude mice bearing RT-112 cells (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).

General Discussion

The medical treatment techniques for cancers are largely divided in the following three categories: surgery, chemotherapy (anticancer agent), and radiotherapy. The checkpoint inhibitor has recently demonstrated effectiveness against some cancers, however the use has been limited. Surgery and radiotherapy are treatment only for local tumors, and patient's HRQOL is markedly decreased such as depression of sexual function, urinary disorder and urinary tract diversion by surgery approach. On the other hand, anticancer agent may treat widespread deposits of tumor throughout the body, therefore, anticancer agents are remarkably helpful treatment for metastatic cancer. In this study, I focused attention on the anti-urological cancer agents by inhibition of tumor cell growth and proliferation in resistant status against existing treatments.

In the first chapter, I described about the importance of the AKR1C3 function in androgen metabolism pathway produced in PCa. The production of androgen occurs not only in the testes, involving the conversion of AD to T by AKR1C3 [94], but also in the prostate by adrenal-derived DHEA [95]. Indeed, although surgical or medical castration with LH-RH agonists or antagonists reduce serum T levels by 90–97 %, the total androgen pool in the circulation and intraprostatic levels of DHT are only reduced by approximately 60 % [40, 43]. AKR1C3 is one of the important enzymes in adrenal and *de novo* intratumoral steroidal biosynthesis, that is, AKR1C3 is involved in the conversion steps of DHEA to androstenediol and androstanedione to DHT, in addition to conversion of AD to T in PCa [96]. AA also inhibits androgen synthesis by the adrenal gland. AA targets CYP17α-hydroxylase/CYP17, 20-lyase, an enzyme that converts pregnenolone and progesterone into DHEA and AD via 2 subsequent reactions [97]. Both DHEA and AD are eventually transformed into T and DHT, the most potent androgen. However,

inhibition of CYP17α-hydroxylase also decreases serum cortisol levels, leading to a subsequent rise in adrenocorticotropic hormone and mineralocorticoids upstream of CYP17α-hydroxylase. To suppress potential side effects related to this mineralocorticoid rise, AA requires concomitant use of steroids such as prednisone. Furthermore, although it is initially effective, most patients receiving AA will progress in their disease by 15 months of treatment [14]. Determining the mechanisms of resistance to first therapies has been being investigated and several adaptive pathways have been uncovered. One of the resistance mechanisms may be partially due to the presence of AR splice variants and mutation of the AR. Clinically, detection of AR-V7 in prostate cancer patients may indicate AA resistance [98], and the progesterone-activated T878A AR mutant is associated with resistance to AA [99]. In addition to such AR modification, increased expression of steroidogenic enzymes is another likely contributor to both PCa progression and AA resistance by increasing androgen levels and inducing AR activation [100, 101]. In AA or enzalutamide-resistant PCa cells, AKR1C3 is overexpressed and the levels of intracrine androgens are elevated. In addition, AKR1C3 activation increases intracrine androgen synthesis and enhances AR signaling via activating AR transcriptional activity. Treatment of AA or enzalutamide-resistant cells with AKR1C3 inhibitor, overcomes resistance and enhances AA therapy both *in vitro* and *in vivo* by reducing the levels of intracrine androgens and diminishing AR transcriptional activity [102, 103]. Then, AKR1C3 activation contributes to CRPC drug resistance in patients treated with both AA and enzalutamide, and it has been suggested as a biomarker for assessing prostate cancer progression [102, 104]. Increased expression of AKR1C3 is associated with PCa progression and aggressiveness. Moreover, it is not detectable in normal prostatic epithelium, however it is highly elevated in metastasized PCa. AKR1C3 may also act as

an AR selective coactivator that promotes tumor growth, and this coactivator function could be blocked with small molecule enzyme competitive inhibitors [59]. In the first chapter, I demonstrated that ASP9521 is a potent, orally bioavailable inhibitor of AKR1C3-mediated conversion of AD into T, both *in vitro* and *in vivo* in CWR22R xenografted castrate mice. ASP9521 also inhibited AD-dependent PSA production and proliferation in LNCaP-AKR1C3 cells. AKR1C2, closely related isoforms of AKR1C3, is involved in DHT metabolism, and inhibition of this isoform may lead to the accumulation of DHT in the prostate and thereby induce the development or progression of PCa. Therefore, AKR1C3 inhibitors without any inhibitory activity against AKR1C2 is desirable for the treatment of PCa, and ASP9521 showed high selectivity to AKC1C3 against AKR1C2. Furthermore, expression of AKR1C3 in tumor tissue was found to be a prerequisite for intratumoral accumulation of ASP9521. These results provided the rationale for ASP9521 in the treatment of mCRPC by inhibition of androgen synthesis necessary for PCa growth and proliferation. In addition, for cancer resistance, combination of ASP9521 with AA or enzalutamide is expected to be more effective. In ASP9521 phase I/II study, since none of the patients had received prior treatment with AA, it is hypothesized that AKR1C3 expression was insufficient to exert significant effects of ASP9521 in these patients. It is suggested that the importance of patient selection with the expression level of AKR1C3 and the concomitant use of a 5α-reductase inhibitor to completely block DHT production should be considered to have significant effect of ASP9521 in clinical study.

In the second chapter, I described the FGFR3 inhibition mechanism in *FGFR3*-mutant cells by demonstrating the preclinical profile of ASP5878, which is a selective FGFR inhibitor targeting *FGFR3*-fusion or -mutation positive urothelial bladder cancer. Among

several large sequencing studies in urothelial bladder cancer which include NMIBC/MIBC and metastatic/nonmetastatic disease, 35–55 % of tumors had a mutation, translocation, or amplification of *PIK3CA*, *EGFR*, *ERBB2*, *ERBB3*, or *FGFR3* [69, 105-107]. Genomic alterations of *FGFR3* have been identified in heterogeneous subsets of such urothelial bladder cancer patients. In 23 urothelial cancer cell lines, ASP5878 inhibited the proliferation of 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. 2-1). 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 [76]. These findings suggest that FGFR3-TACC3, FGFR3-BAIAP2L1, FGFR3_S249C mutations and FGFR1 overexpression may be predictors of the sensitivity to ASP5878 in urothelial bladder cancer. Over the past 20 years, the standard therapy of MIBC or metastatic bladder cancer has been the combination of chemotherapeutic agents (GC and MVAC), however, their therapeutic effects have been generally unsatisfactory. One of the reasons is resistance to these chemotherapeutic agents, and induction of MDR1 overexpression or the alterations in the apoptotic machinery including overexpression of c-MYC, the resistance factors. I established adriamycin-resistant UM-UC-14 cell line harboring MDR1 overexpression and gemcitabine-resistant RT-112 cell line harboring c-MYC up-regulation by stepwise increasing concentrations of adriamycin, or gemcitabine. ASP5878 inhibited the proliferation of these resistant cell lines with similar potency to their parent cell lines. 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. It is suggested that c-MYC expression may be regulated by the

FGFR/ERK signaling pathway in this study. ASP5878 also dose-dependently inhibited the tumor growth in gemcitabine-resistant RT-112 subcutaneous xenograft mouse model. In the second chapter, ASP5878, a selective FGFR inhibitor, showed potent anti-proliferative and antitumor activity in urothelial bladder cancer cell lines 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 has therapeutic potential against urothelial bladder cancers harboring *FGFR3-TACC3*, *FGFR3-BAIAP2L1* or *FGFR3* point mutation even after the acquisition of gemcitabine- or adriamycin-resistance.

While great strides have been recently made in anticancer drug treatment, there remain considerable UMN from cancers that still cannot be effectively treated even today. My studies showed that the discovery and the development of anti-cancer drug for resistant status against existing treatments are possible based on their diverse physiological responses. AKR1C3 inhibitor for PCa and FGFR3 inhibitor for urothelial cancer harboring *FGFR3* fusion or *FGFR3* point mutation could lead to a novel and effective therapeutic approach for such urological cancer.

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