Discussion

In this study, we found that overexpression of AM, Ang-1, and VEGF-A by adenoviral infection significantly restored erectile function in terms of ICP, histology of the penis, and expression of several proteins in the penis. Among several combinations, infection with AdAM plus AdAng-1 mostly mimicked the erectile function in the age-matched positive control. AdAM plus AdAng-1 infection restored ICP and AUC of the ICP traces to a similar level observed in the positive control. The pattern of the increase and decrease of ICP after electrical stimulation was similar between the AdAM plus AdAng-1-infected group and the positive control group. Expression of SMA and VE-cadherin was also similar between the AdAM plus AdAng-1-infected group and the positive control group. In contrast, although ICP rose sufficiently after electrical stimulation in the AdVEGF-A- and AdVEGF-A plus AdAng-1-infected groups, the rise and fall of ICP occurred more rapidly, as assessed by Tmax and $T_{1/2}$, resulting in a smaller value of AUC compared with the AdAM plus AdAng-1-infected group and the positive control. Infection with AdVEGF-A and AdVEGF-A plus AdAng-1 induced "hypertrophy" of the trabeculae of the cavernous body and overproduction of collagen fibers in the trabeculae. Infection with AdVEGF-A induced aberrant angiogenesis in the trabeculae. We propose the following scenario about the effect of AdVEGF-A- and AdVEGF-A plus AdAng-1-infection. Because the trabeculae of the cavernous body were "hypertrophied," the area of the cavernous sinus was relatively small in these rats. Therefore, once erection started, blood filled in the cavernous sinus rapidly, but once erection ceased, blood outflow from the cavernous sinus also occurred rapidly. That is why the level of ICP rose and fell rapidly in the AdVEGF-A- and AdVEGF-A plus AdAng-1-infected groups. Another possibility is that AdVEGF-A infection did not sufficiently improve corporal veno-occlusive dysfunction (CVOD). It is well known that CVOD is the most prevalent cause of ED. This disorder results from an inadequate relaxation of the corporal smooth muscle, resulting in insufficient compression of the subtunical veins against the tunica albuginea [20]. Decreases in the amount of smooth muscle cells (SMC) and/or increases in the amount of collagen in the cavernous body appear to result in dysfunction of the corporal smooth muscle and CVOD [21,22]. Infection with AdAM plus AdAng-1 restored the amount of SMC in the cavernous body without increases in the amount of collagen fibers, whereas infection with AdVEGF-A and AdVEGF-A plus AdAng-1 increased the amount of collagen as well as SMC. This may be the reason why the blood flowed out of the cavernous body rapidly after cessation of electrical stimulation in AdVEGF-A- and AdVEGF-A plus AdAng-1-infected groups compared with the AdAM plus AdAng-1-infected group.

Although VEGF are essential for vasculogenesis and have probably the most potent proangiogenic activity, overproduction of VEGF potentially causes a harmful effect on organ physiology [12,13]. In fact, it was reported that the VEGF-A gene transfer using the adeno-associated virus into rabbit ischemic hind limb skeletal muscles caused aberrant angiogenesis and fibrosis in skeletal muscles [23]. It was also reported that the blockade of the type 2 receptor for the VEGF family resulted in attenuation of fibrosis in the kidney in a unilateral ureteric obstruction model [24]. Therefore, it appears that an appropriate amount of VEGF-A is necessary for physiological angiogenesis and that overproduction of VEGF-A potentially causes aberrant angiogenesis, inflammation, and fibrosis.

Ang-1 reportedly restored erectile function in a diabetes model and a hyperlipidemia model [11,25]. In addition to proangiogenic activity, Ang-1 appears to have an inhibitory effect on fibrosis. It was shown that a variant of Ang-1 (cartilage oligomeric matrix protein-Ang-1) that is stable and has a potent activity, inhibited fibrosis in a unilateral ureteric obstruction model of the kidney [26]. Furthermore, Ang-1 reportedly inhibits tissue inflammation via suppression of macrophage infiltration and activation [27,28]. Thus, it seems to be reasonable to use Ang-1 for the treatment of ED.

AM was originally isolated from human pheochromocytoma tissue and has potent natriuretic, vasorelaxant, and proangiogenic activities [5–8]. We have shown that overproduction of AM in the penis restores erectile function via stimulation of the regeneration of the cavernous tissue and via restoration of endothelial function [4]. Furthermore, it was reported that AM inhibited tissue fibrosis in the heart and the kidney [29–31]. Thus, AM also has proangiogenic and antifibrotic activities like Ang-1. In this study, we found that infection with AdAM plus AdAng-1 in the penis restored erectile function more potently than infection with AdAM alone or AdAng-1 alone.

Expression of SMA and VE-cadherin in the penis increased more significantly in the AdAM plus AdAng-1-infected group than in the AdAM-infected and AdAng-1-infected group without overproduction of type I collagen. The expressions of SMA and VE-cadherin in the AdAM plus AdAng-1-infected group increased to a level similar to that observed in the positive control group. Thus, vascular networks in the cavernous sinus appear to be restored more significantly by AdAM plus AdAng-1-infection than by AdAM infection alone or AdAng-1 infection alone. This can be a mechanism whereby AM and Ang-1 additively restore erectile function. Another possible mechanism is that AM and Ang-1 additively restored vascular endothelial function. In fact, we have reported that AM stimulates phosphorylation and activation of endothelial nitric oxide synthase (eNOS) in the penis [4]. Another study reported that Ang-1 stimulates phosphorylation of eNOS in the penis [11]. Furthermore, AM and Ang-1 reportedly inhibit the production of reactive oxygen species (ROS) [32,33]. As it has been shown that ROS are implicated in ED in diabetes [34,35], AM and Ang-1 may restore erectile function via suppression of ROS production. Therefore, it is possible that AM and Ang-1 additively restores erectile function via improvement of vascular endothelial function by activating eNOS and/or suppressing ROS production in an additive manner. Future studies are required to elucidate these possibilities.

Limitations of This Study

Adenovirus was injected into the penis. Thus, some portions of the injected adenovirus may have entered the systemic circulation and got trapped in the tissues of organs such as the liver, which may then have produced AM, Ang-1, or VEGF-A. These cytokines produced outside the penis may have entered the systemic circulation and affected the results of this study. However, we believe that this effect was minimal, if present at all, because no decline in blood pressure was observed after injection of AdAM into the penis (data not shown). Because AM has a potent vasodilator activity, blood pressure should have decreased if systemically delivered adenovirus played a major role in erectile function.

AM, Ang-1, and/or VEGF-A may have also improved erectile function through regenerative effects on the autonomic nervous system. This possibility should be addressed in a future study.

Conclusions

Combination therapy with AM and Ang-1 additively restored erectile function to a level very similar to that observed in the age-matched Wistar rats. This combination therapy effectively regenerated vascular structure in the cavernous body without inducing aberrant angiogenesis, overproduction of collagens and "hypertrophy" of the trabeculae of the cavernous body. This combination therapy will be useful to treat ED resulting from old age and diabetes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 Blood glucose levels before and after adenoviral infection. Casual blood glucose levels were measured in each group 4 weeks after STZ injection (before adenoviral injection: white boxes) and 10 weeks after STZ injection (4 weeks after adenoviral injection: black boxes). Casual blood glucose levels were also measured in age-matched positive control rats (PC: dotted box). †: $P < 0.05$ vs. positive control rats. N = 6 in each group.

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Original Article: Laboratory Investigation

Reduction of prostate cancer incidence by naftopidil, an α_1-adrenoceptor antagonist and transforming growth factor-β signaling inhibitor

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Abbreviations & Acronyms
BPH = benign prostatic hyperplasia
BSA = bovine serum albumin
DAPI = diamidino-2-phenylindole
DMSO = dimethylsulfoxide
IC_{50} = half maximal inhibitory concentration
PC = prostate cancer
PSA = prostate-specific antigen
R1 = receptor 1
RT = room temperature
TGF-β = transforming growth factor-β
TUNEL = terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling
TUR-P = transurethral resection of the prostate

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Objectives: Quinazoline-based α_1-adrenoceptor antagonists are known to inhibit prostate tumor growth through induction of apoptosis. We investigated the effect of a naphthalene-based α_1-adrenoceptor antagonist, naftopidil, on prostate cancer incidence, apoptosis of prostatic cell and transforming growth factor-β signaling.

Methods: Prescription records were linked to pathological data for men who continued naftopidil ($n = 766$) or tamsulosin ($n = 1015$) for 3 months or longer between 2003 and 2010. Prostate cancer incidence was analyzed by log–rank test and the Cox proportional hazards model. Apoptosis and cell cycle arrest in human tissues were assessed by immunohistochemical detection of Bcl2 and p21, respectively. Growth inhibition and apoptosis treatment with naftopidil and tamsulosin were assessed in cancer cell lines. Interference with transforming growth factor-β signaling was examined by western blot analysis.

Results: Prostate cancer incidence was significantly lower in men who received naftopidil for 3 months or longer compared with tamsulosin ($P = 0.035$). Multivariate analysis confirmed a decreased hazard ratio, 0.46, for naftopidil use ($P = 0.013$), which was more evident with longer treatment. Immunohistochemical positivity for Bcl2, a marker for resistance to apoptosis, was less frequently detected in prostate cancer cells of men who received naftopidil compared with tamsulosin ($P < 0.05$). Naftopidil inhibited cancer cell growth, induced apoptosis and blocked Smad2 phosphorylation activated by transforming growth factor-β in cell lines, with a half maximal inhibitory concentration of 1.1 μmol/L.

Conclusions: Naftopidil seems to reduce prostate cancer incidence, possibly by inducing apoptosis, preferentially in cancer cells, and blocking transforming growth factor-β signaling.

Key words: adrenoceptor, incidence, naftopidil, prostate cancer, transforming growth factor.

Introduction

Some α_1-adrenoceptor antagonists, such as doxazosin and terazosin, can reduce PC incidence, whereas other α_1-adrenoceptor antagonists; that is, tamsulosin, cannot.[1–5] This inhibitory effect is reportedly mediated by apoptosis induction, and independent from the property of α_1-adrenoceptor antagonist; overexpression of α_1 adrenoceptor did not influence apoptosis and cell viability;[1] exposure to phenoxybenzamine, an irreversible inhibitor of α_1-adrenoceptors, failed to prevent the apoptotic effect of doxazosin.[3] G1 cell cycle arrest has been implicated in the growth inhibitory property of naftopidil, another α_1-adrenoceptor, in human PC cell lines.[6]

In the present study, we investigated the effects of naftopidil on PC incidence in an observational cohort and the possible role of TGF-β signaling in its inhibition mechanism.

Table 1 Backgrounds in men treated with naftopidil or tamsulosin for 3 months or longer

	3 Months or longer			12 Months or longer			36 Months or longer		
	Naftopidil (*n* = 766)	Tamsulosin (*n* = 1015)	*P*-value	Naftopidil (*n* = 433)	Tamsulosin (*n* = 506)	*P*-value	Naftopidil (*n* = 203)	Tamsulosin (*n* = 208)	*P*-value
Baseline age (years)	71 (64–76)†	71 (65–78)	0.09	71 (64–76)	72 (66–78)	0.10	71 (64–76)	72 (66–79)	0.12
Baseline PSA level (ng/mL)	2.5 (1.1–4.9)†	2.3 (1.0–4.6)	0.14	2.5 (1.2–4.9)	2.3 (1.0–4.4)	0.09	2.3 (1.1–4.5)	2.1 (1.1–3.9)	0.27
Methods for examination of the prostate									
Biopsy	197 (25)‡	250 (24)	0.60	124 (29)	134 (26)	0.46	64 (32)	56 (27)	0.30
TUR-P	93 (12)‡	120 (11)	0.83	49 (11)	67 (13)	0.37	12 (6)	21 (10)	0.11
Either	239 (31)‡	313 (30)	0.86	147 (34)	169 (33)	0.85	68 (33)	69 (33)	0.94

†Median (quartile range). ‡Cases (%).

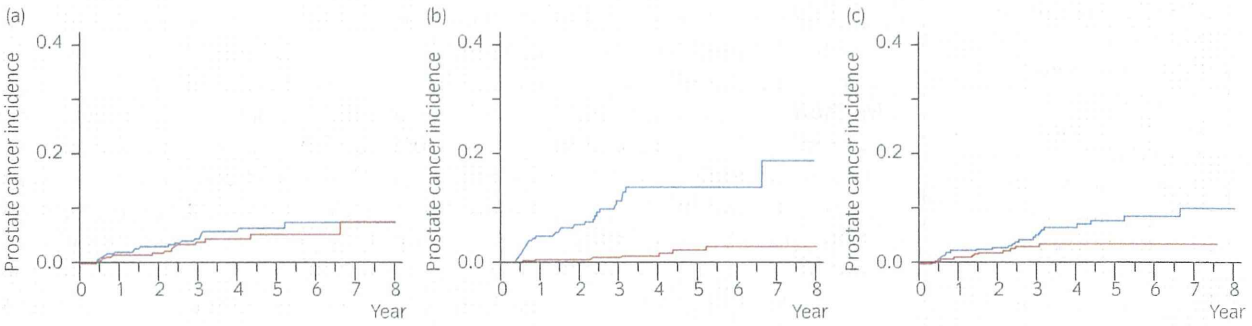

Fig. 1 Cumulative PC incidence in men during treatment with α_1-adrenoceptor antagonists. (a) There was no significant difference between men aged 71 years or older (——) and men aged less than 71 years (——). (b) The incidence was higher in men with PSA ≥4.0 ng/mL (——) than in men with PSA <4.0 ng/mL (——; $P < 0.001$). (c) It was lower in men receiving naftopidil (——) than in men receiving tamsulosin (——; $P = 0.035$).

Methods

A retrospective observational cohort study was carried out on men who received naftopidil or tamsulosin at the Fraternity Memorial Hospital in Tokyo, Japan, between 2003 and 2010. Medications, including α_1-adrenoceptor antagonists and hormonal agents, were listed from prescription records of the pharmacy. Patient data, including pathological results, were linked by the identification number. Men receiving hormonal therapy or diagnosed with PC before treatment with α_1-adrenoceptors were excluded. All the men had PSA checked before starting medication. The time zero of the present study was the time treatment with α_1-adrenoceptor antagonists was started. The end-points of follow up were stopping of the α_1-adrenoceptor antagonist or starting hormonal therapy (censored), or diagnosis of PC (evented). The follow up ended in December 2010 in patients who continued the same α_1-adrenoceptor antagonists without diagnosis of PC.

The decision to carry out prostate biopsy or TUR-P was made clinically by the primary physicians. Men with follow up less than 3 months were excluded because of minimal exposure to the agents.

Immunohistochemistry

We used fresh prostatic tissues obtained from men with or without PC who received naftopidil, tamsulosin or neither ($n = 10$, each). The specimens were fixed in formalin, dehydrated, embedded in paraffin and cut into 3-μm sections using a Yamato large-scale microtome (TU213; Yamatokohki, Saitama, Japan). After deparaffinization, the sections were hydrated and incubated for 10 min in 3% hydrogen peroxide in methanol. Sections were heated in a steel pressure oven in 10 mmol/L citrate buffer, pH 6.0, to 120°C for 20 min. Non-specific binding was blocked using 2% normal goat blocking serum (X0907; Dako Cytomation, Glostrup, Denmark). Primary and secondary antibodies were incubated for 1 h and 0.5 h at RT, respectively. After washing, sections were incubated with EnVision (K4063; Dako Cytomation) at RT for 1 h, visualized with 3,3′-diaminobenzidine and stained with hematoxylin. The primary antibodies used were anti-p21 (#2947; Cell Signaling Technology, Danvers, MA, USA) and anti-Bcl2 (M0887; Dako Cytomation), both diluted 1:50. To confirm specificity of staining, primary antibodies were mixed with p21 (#1055; Cell Signaling Technology) and Bcl2

Table 2 Risk of PC diagnosis

Variable		3 Months or longer					12 Months or longer					36 Months or longer				
		Cancer/total (%)	Univariate analysis†	Multivariate analysis‡			Cancer/total (%)	Univariate analysis†	Multivariate analysis‡			Cancer/total (%)	Univariate analysis†	Multivariate analysis‡		
			P-value	Hazard ratio	95% CI	*P*-value		*P*-value	Hazard ratio	95% CI	*P*-value		*P*-value	Hazard ratio	95% CI	*P*-value
Baseline age (years)	Less than 71	19/852 (2.2)	0.44				9/434 (2.0)	0.58				3/188 (1.5)	0.58			
	71 or higher	27/929 (2.9)					13/505 (2.5)					5/223 (2.2)				
Baseline PSA level (ng/mL)	Less than 4.0	10/1206 (0.8)	<0.0001	1.00			6/641 (0.93)	<0.0001	1.00			4/298 (1.3)	0.08	1.00		
	4.0 or higher	36/575 (6.2)		9.00	4.63–19.2	<0.0001	16/298 (5.3)		7.51	3.07–21.0	<0.0001	4/113 (3.5)		3.56	0.83–15.1	0.083
Medicine	Tamsulosin	32/1015 (3.1)	0.035	1.00			15/506 (2.9)	0.15	1.00			7/208 (3.3)	0.07	1.00		
	Naftopidil	14/766 (1.8)		0.46	0.23–0.84	0.013	7/433 (1.6)		0.46	0.17–1.09	0.081	1/203 (0.4)		0.16	0.008–0.92	0.039

†Log-rank test. ‡Cox proportional hazards model.

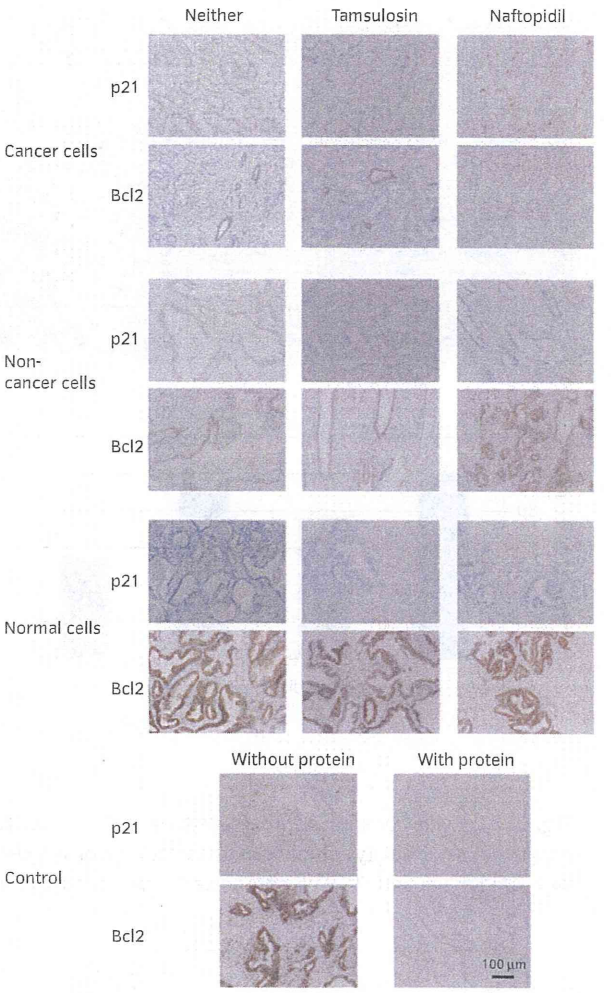

Fig. 2 Immunohistochemical expression of p21 and Bcl2 in prostatic cells. Cancer cells from men exposed to naftopidil showed higher p21 expression and lower Bcl2 expression compared with men exposed to tamsulosin or neither. In contrast, non-cancer cells from men exposed to naftopidil showed higher Bcl2 expression compared with men exposed to tamsulosin or neither. Normal cells showed no significant difference in expression among medication groups. Staining with p21 and Bcl2 (without protein) was specifically diminished of pre-incubation with the corresponding protein (with protein).

(B25-30G-20; Signalchem, Richmond, BC, Canada) before staining in control specimens. Expression levels were determined by counting positive epithelial cells in 10 separate microscopic fields at ×100 magnification. Cancer cells and non-cancer cells were collected from men with PC, and normal cells were from men without PC. The results were independently reviewed by two blinded investigators.

Cell viability assay

HeLa cell and LNCaP cell were seeded on 12-well dishes (1×10^4 cells/well) in DMEM/F12 (#11320; Life Tech.

Table 3 Proportion of epithelial cells positive for p21 and Bcl2 (%)

			Neither ($n = 10$)	Tamsulosin ($n = 10$)	Naftopidil ($n = 10$)
Men with prostate cancer	Cancer cells	p21	1 (0–5.5)	4 (0.7–9.5)	7 (3.5–10)*
		Bcl2	11 (4.5–23)	11 (5–13)	4 (2.5–5)**
	Non-cancer cells	p21	0 (0–1)	0 (0–0)	0 (0–2.2)
		Bcl2	61 (27–78)	60 (44–69)	82 (66–94)**
Men without prostate cancer	Normal cells	p21	1 (0–1)	0 (0–1.2)	0.5 (0–1.2)
		Bcl2	71 (50–82)	71 (68–76)	74 (67–88)

*$P < 0.05$: versus neither. **$P < 0.05$: versus neither and versus tamsulosin. Median (quartile range).

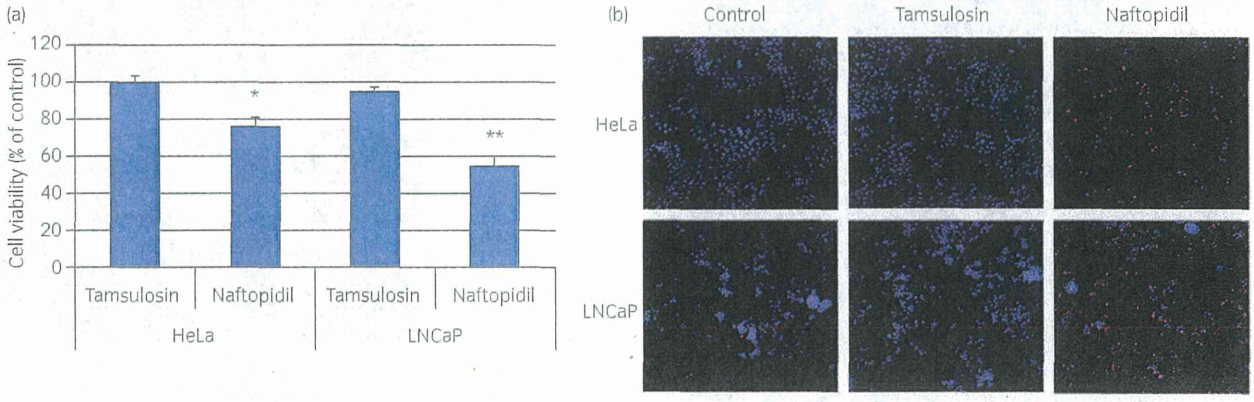

Fig. 3 Cell viability and apoptosis assay. (a) Cell viability (% of control). Cells treated with 10 μmol/L of naftopidil, showed significantly reduced viability compared with tamsulosin or vehicle (control). *$P < 0.05$, **$P < 0.01$. (b) TUNEL assay. Double labeling of cells with DAPI (blue) and TUNEL (red) showed significant increments of apoptotic cells treated with 10 μmol/L naftopidil.

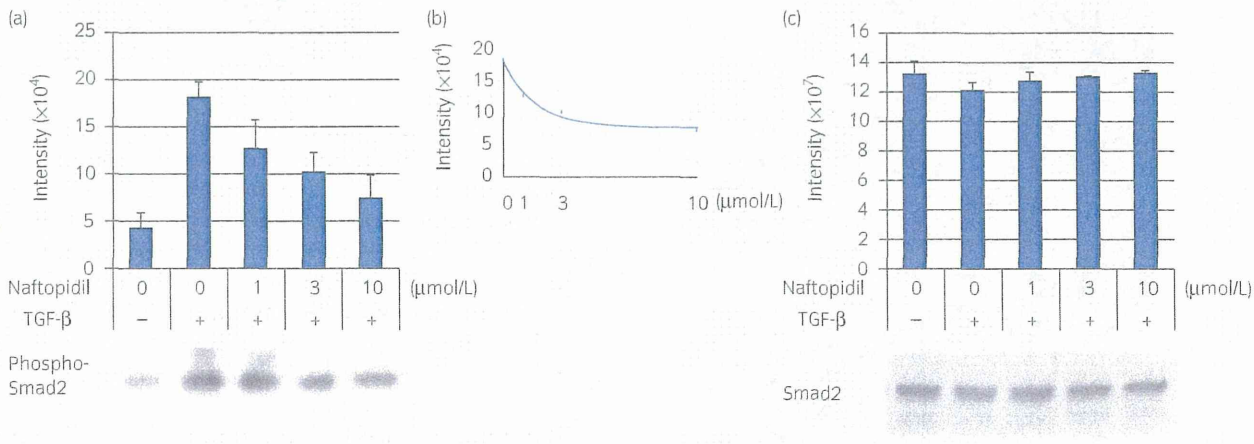

Fig. 4 Western blot analysis and model of inhibition. (a) Intensity of three times western blot analyses and representative data of phospho-Smad2. Phosphorylation of Samd2 induced by TGF-β was suppressed by naftopidil dose-dependently. (b) Model of phosphorylate inhibition of Smad2 by naftopidil. $Y = a \exp(-bX) + c$; $a = 1.0 \times 10^5$ $b = 0.58$ $c = 7.6 \times 10^4$ IC_{50} was 1.1 μmol/L. (c) Intensity of three times western blot analyses and representative data of Smad2. Expressions of Smad2 were not changed by TGF-β or naftopidil treatment.

Carlsbad, CA, USA) and RPMI (#22400; Life Tech) containing 10% fetal bovine serum. Naftopidil, which was kindly provided by Asahi Kasei (Tokyo, Japan), and tamsulosin (T1330; Sigma-Aldrich, St. Louis, MD, USA) were dissolved in DMSO and then diluted 1000 times for use with cells (final 0.1% DMSO). Cells were treated with 10 μmol/L of naftopidil, tamsulosin or control (vehicle) for 2 days. Then cells were detached by trypsinization (#12605; Life Tech) and counted using Coulter counter (Z1; Beckman Coulter, Brea, CA, USA). Cell viability was assessed by

trypan blue exclusion assay (#15250; Life Tech) and expressed as a percentage of the control.

Apoptosis detection

HeLa cell and LNCaP cell were seeded on 96-well dishes (1×10^3 cells/well) and on collagen-coated 96 well dishes (3×10^3 cells/well), respectively. The next day, cells were treated with 10 μmol/L of naftopidil, tamsulosin or control (0.1% DMSO) for 1 day, and apoptotic cells were detected by TUNEL assay using an *in situ* detection kit (#2156792; Roche, Basel, Switzerland). DAPI (D1306; Life Tech) was used to counter staining. Cells were visualized using a fluorescence microscope (BZ9000; KEYENCE, Chicago, IL, USA).

TGF-β induction and western blotting

HeLa cells were seeded on 6-cm dishes. Recombinant human TGF-β1 (100-21; PeproTech, Rocky Hills, NJ, USA) was dissolved in 10 mmol/L citric acid, pH 3.0, in 0.1% BSA, and then diluted 1000 times for use with the HeLa cells (final 10 μmol/L citric acid). Controls contained the same concentrations of citric acid, BSA and DMSO. TGF-β1 1 ng/mL was added the next day, in the presence or absence of naftopidil. After a 30-min incubation, cells were solubilized with radio-immunoprecipitation assay buffer (1.5% Triton X-100, 20 mmol/L Tris, pH 7.5, 150 mmol/L $MgCl_2$ and 1 mmol/L ethylenediaminetetraacetic acid) containing protease inhibitors (dithiothreitol, leupeptin, aprotinin and phenylmethanesulfonylfluoride) and phosphatase inhibitor cocktail3 (P0044; Sigma-Aldrich). Lysates were subjected to western blot analysis. Membranes were detected by anti-phospho-Smad2 (Ser465/467, #3101; Cell Signaling Technology) and anti-Smad2 (#5339; Cell Signaling Technology) with Can Get Signal (NKB-101T; Toyobo, Osaka, Japan). We drew Smad2 and phospho-Smad2 western blotting figures based on three experiments by densitometry scan (Science lab 2005 Multi Gauge ver. 3.0; FUJIFILM, Tokyo, Japan). The average intensity of phospho-Smad2 was analyzed and IC_{50} was calculated using Bio Data Fit (Chang Bioscience, Castor Valley, CA, USA).

Statistical analysis

Data were analyzed using JMP 9.0.2 (SAS Institute, Cary, NC, USA). PC incidence was analyzed by log–rank test. PC risk was calculated as the hazard ratio using the Cox proportional hazards model. Variables were baseline PSA level and medicine. Other comparisons between groups were analyzed by Wilcoxon rank–sum test.

Results

Prostate cancer incidence

We identified 1121 men treated with naftopidil and 1654 men with tamsulosin by tracking 17 497 and 20 870 prescriptions for naftopidil and tamsulosin, respectively, between 2003 and 2010. A total of 355 men on naftopidil and 639 men on tamsulosin were excluded because of treatment was shorter than 3 months (226 and 382, respectively), combination with hormonal therapy (32 and 49, respectively), interrupted prescriptions (29 and 36, respectively), PC before prescription (50 and 150, respectively) and PC diagnosed during the first 3 months of treatment (18 and 22, respectively). The remaining men received continuous treatment with either medicine for 3 months or longer; naftopidil group ($n = 766$) and tamsulosin group ($n = 1015$). No significant differences were detected for baseline age, serum PSA level, and the methods for examination of the prostate histology between the naftopidil and tamsulosin group (Table 1).

During the observational period ranging 3–96 months (median 13 months), 46 men (2.5%) were diagnosed with PC. The incidence of PC was significantly higher in men with the baseline PSA levels greater than 4 ng/mL ($P < 0.001$, 6.2% *vs* 0.8%, Fig. 1b and Table 2), and significantly lower in men receiving naftopidil ($P = 0.035$, 1.8% *vs* 3.1%, Fig. 1c and Table 2). Multivariate analysis showed that the odds ratio of developing PC was 9.00 for men with high PSA ($P < 0.0001$) and 0.46 for men on naftopidil ($P = 0.013$, Table 2). Along with the extension of observation, the increased risk of PC by high PSA became less evident; the ratio was 9.00, 7.51 ($P < 0.0001$) and 3.56 ($P = 0.083$) for men treated for 3 months or longer, 12 months or longer and 36 months or longer, respectively. By contrast, a reduced odds ratio by naftopidil treatment was more pronounced: 0.46, 0.46 ($P = 0.081$) and 0.16 ($P = 0.039$) for men treated for 3 months or longer, 12 months or longer and 36 months or longer, respectively. The Gleason scores in men diagnosed with PC did not differ between the two groups ($P = 0.86$, median 6 and 6, respectively).

Immunohistochemical analysis

Typical immunohistochemical staining for p21 and Bcl2 in prostatic epithelial cells are shown in Figure 2. The specificity of staining was ensured by completely negative staining in specimens pre-incubated with the corresponding proteins. Expression of p21 was more enhanced in cancer cells than normal cells, and it was significantly more enhanced in men treated with naftopidil than in men treated with neither α_1-adrenoceptor antagonists ($P < 0.05$, Table 3). By contrast, expression of Bcl2 was more suppressed in cancer cells than normal cells. In men receiving naftopidil, as compared with men treated with tamsulosin or neither, Bcl2 expression was significantly suppressed in cancer cells ($P < 0.05$) and significantly increased in non-cancer cells ($P < 0.05$, Table 3).

Cell viability and apoptosis

Cell viability was significantly suppressed by naftopidil in HeLa and LNCaP cell lines (Fig. 3a). TUNEL assay showed apoptosis by naftopidil for both cell lines (Fig. 3b).

Naftopidil significantly suppressed cell variability and induced apoptosis in both cell lines, whereas tamsulosin did not.

Western blotting

TGF-β induced Smad2 phosphorylation, which was inhibited by naftopidil in a dose-dependent manner (Fig. 4a). The IC_{50} against Smad2 phosphorylation was 1.1 μmol/L (Fig. 4b). Smad2 expressions were not changed by TGF-β or naftopidil (Fig. 4c).

Discussion

The growth inhibitory and apoptotic effects of adrenoceptor antagonists were initially reported in 1994.[7] Subsequently, PC incidence was found to be significantly lower in men exposed to terazosin or vasopressin than in unexposed men.[2] However, another α_1-adrenoceptor antagonist, tamsulosin, failed to induce apoptosis or inhibit tumor cell growth in PC cell lines.[1] In the present study, we found a significant reduction of PC in men receiving naftopidil (1.8%) compared with men receiving tamsulosin (3.1%). The odds ratio of PC was 0.46 for men on naftopidil by multivariate analysis, and the ratio was further lowered along with longevity of naftopidil administration.

The histological analysis showed higher cell cycle arrest and higher apoptosis in human PC cells compared with non-cancer cells, and this reciprocal relationship was more pronounced in men treated with naftopidil. Compounds with inhibitory effects on PC cell growth all contain a piperazine group, which is absent from tamsulosin (Fig. 5). Naftopidil shares structural similarly with terazosin and doxazosin in terms of containing piperazine and naphthalene groups, which might be important for PC inhibition. Some signaling pathways that induce apoptosis through α_1-adrenoceptors include the death receptor,[8] vascular endothelial growth factor,[9] Smad4 and TGF-β pathways.[10] Cancer cells are known to be more susceptible to apoptosis because of their cellular deviation and it exerts protective effect against neoplasms.[11–13] Actually, PC with less apoptosis index tends to show biochemical failure after total prostatectomy.[14] In this context, it is notable that TGF-β induces apoptosis in normal cells, but promotes the proliferation of cancer cells.[15–17] There are five types of TGF-β inhibitors: oligonucleotides, antibodies, small-molecule inhibitors, interacting peptides and vaccines.[18] Naftopidil is made up of small molecules and might therefore inhibit TGF-β R1 kinase-like pyrazole inhibitors (Fig. 5), which attach to the adenosine triphosphate-binding site of the TGF-β R1 kinase domain.[19,20] Smad2, which is rapidly phosphorylated by TGF-β R1 bound to TGF-β, is known to function as a signal transducer for TGF-β signaling. The observed rapid inhibition (less than 30 min) after TGF-β stimulation in the present study supports the idea that naftopidil might interact directly with TGF-β R1 and block TGF-β signaling. Based on the pharmacokinetics of naftopidil,[21,22] it is estimated that oral administration of 320 mg is required to attain serum concentration of

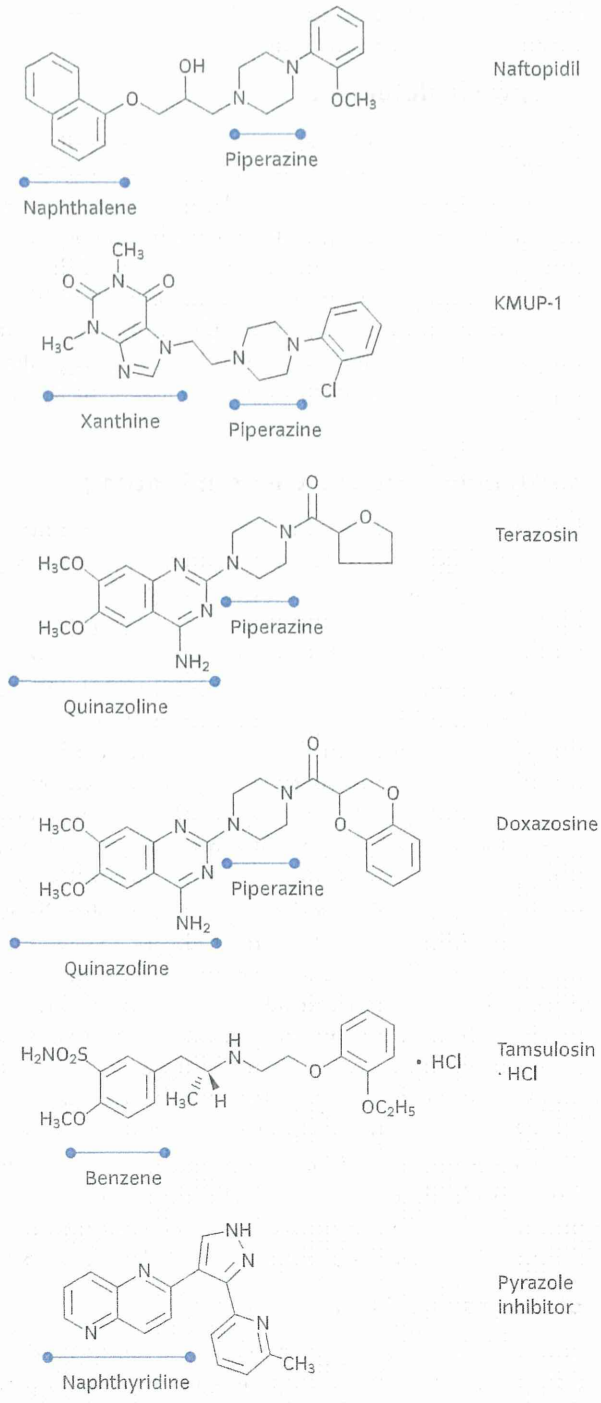

Fig. 5 Structural formula of medicines. Naftopidil, KMUP-1, terazosin and doxazosin all contain piperazine, but tamsulosin does not. Naftopidil also contains naphthalene, which is similar to xanthine, quinazoline and naphthyridine.