Table 2 Risk of PC diagnosis

Variable		3 Months or longer				12 Months or longer				36 Months or longer			
		Cancer/total (%)	Univariate analysis† *P*-value	Multivariate analysis‡ Hazard ratio	95% CI	Cancer/Total (%)	Univariate analysis† *P*-value	Multivariate analysis‡ Hazard ratio	95% CI *P*-value	Cancer/Total (%)	Univariate analysis† *P*-value	Multivariate analysis‡ 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	16/298 (5.3)	<0.0001	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	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.

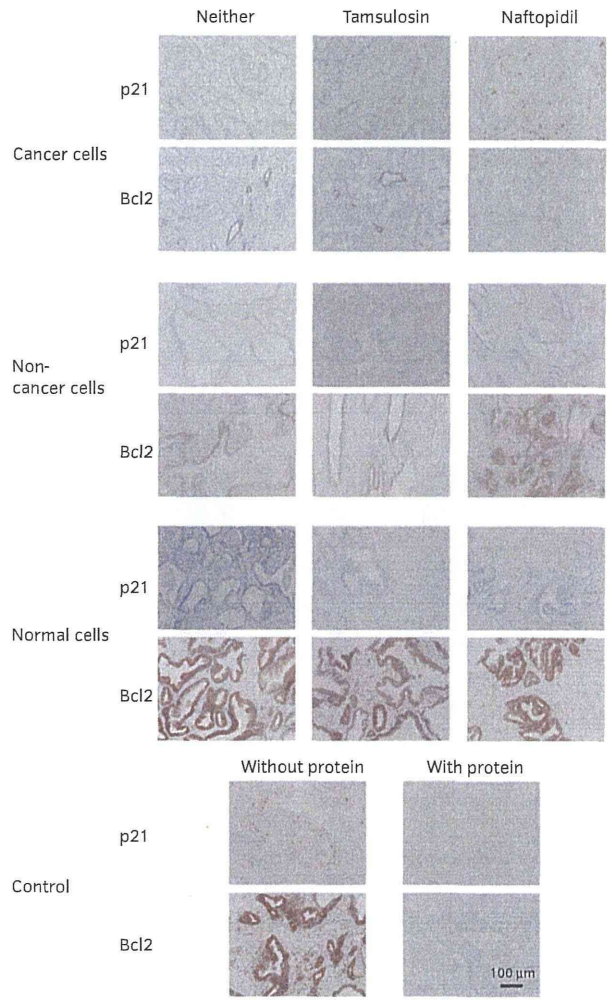

Fig. 2 Immunohistochemical expression of p21 and Bcl2 in prostatic cells. Cancer cells from men exposed to naftopidil showed higher p21 expression and lower Bcl2 expression compared with men exposed to tamsulosin or neither. In contrast, non-cancer cells from men exposed to naftopidil showed higher Bcl2 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).

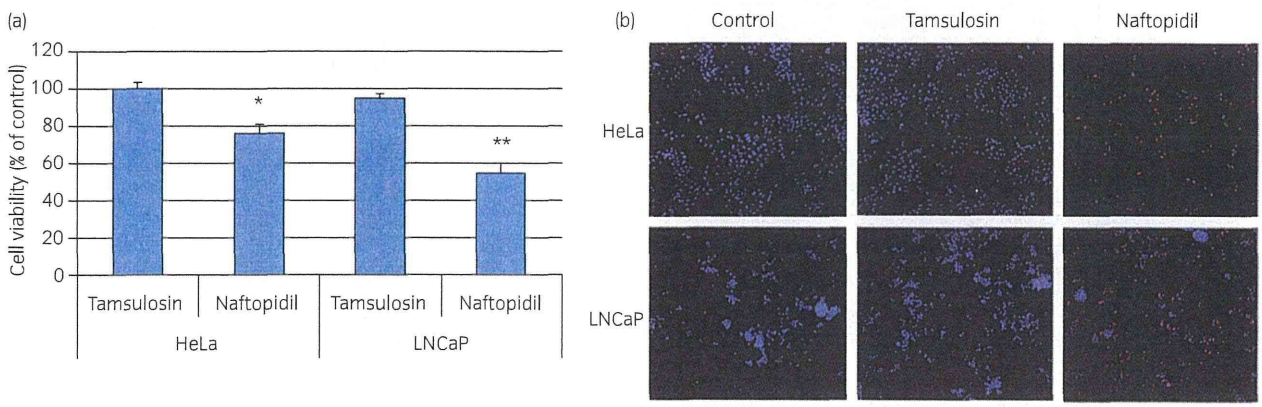

Fig. 3 Cell viability and apoptosis assay. (a) Cell viability (% of control). Cells treated with 10 μmol/L of naftopidil, showed significantly reduced viability compared with tamsulosin or vehicle (control). *$P < 0.05$, **$P < 0.01$. (b) TUNEL assay. Double labeling of cells with DAPI (blue) and TUNEL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

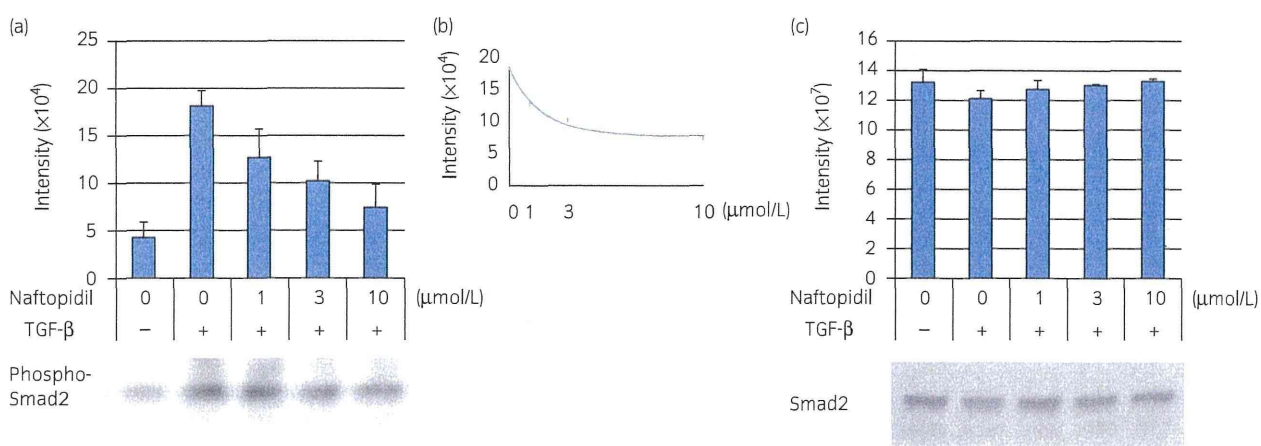

Fig. 4 Western blot analysis and model of inhibition. (a) Intensity of three times western blot analyses and representative data of phospho-Smad2. Phosphorylation of Samd2 induced by TGF-β was suppressed by naftopidil dose-dependently. (b) Model of phosphorylate inhibition of Smad2 by naftopidil. $Y = a \exp(-bX) + c$; $a = 1.0 \times 10^5$ $b = 0.58$ $c = 7.6 \times 10^4$ 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

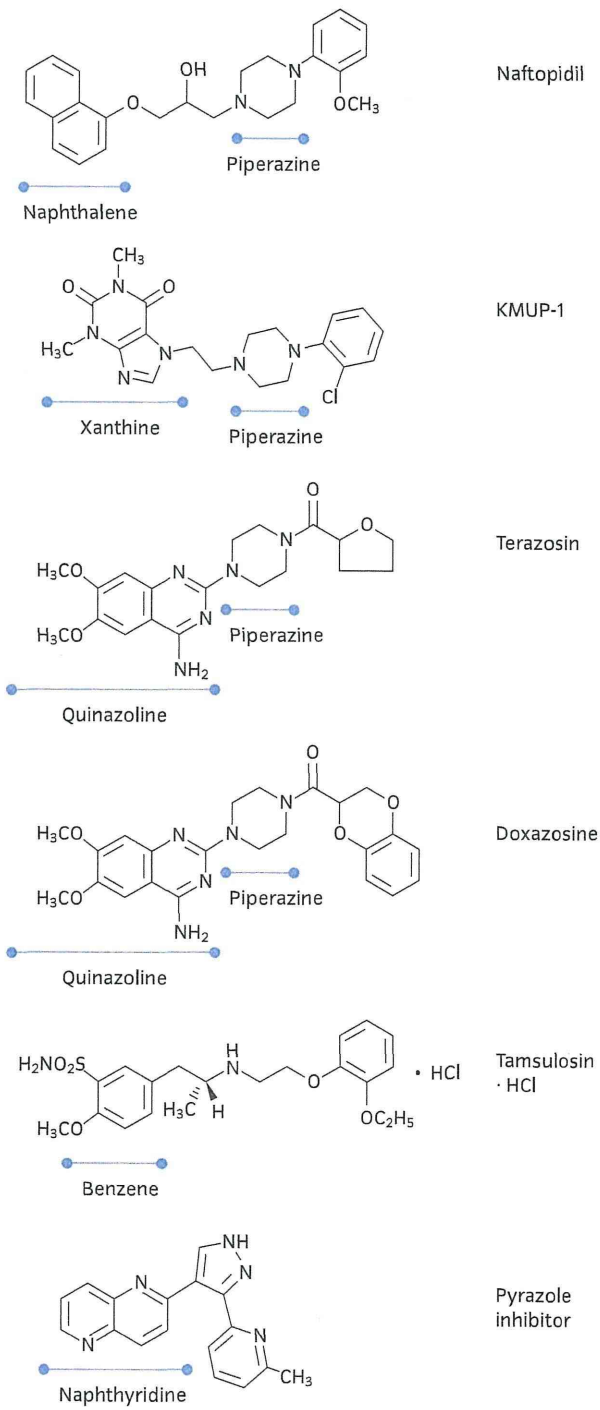

Fig. 5 Structural formula of medicines. Naftopidil, KMUP-1, terazosin and doxazosin all contain piperazine, but tamsulosin does not. Naftopidil also contains naphthalene, which is similar to xanthine, quinazoline and naphthyridine.

1.1 μmol/L (IC_{50} for Smad2 phosphorylation). The normal dose of naftopidil for BPH (25–75 mg in Japan) is unlikely to achieve a therapeutic effect on PC, but might prevent prostate carcinogenesis.

The limitations of the present study included its retrospective nature of a cohort study on BPH men and restricted exploration of TGF-β signaling pathways. The relative risk between users and non-users of α_1-adrenoceptor antagonists was not evaluated in the present study. Further investigations are warranted to clarify the cancer inhibitory property of naftopidil and its mechanisms involved.

The results of the present study show for the first time that naftopidil use might reduce the PC incidence by possibly inducing apoptosis preferentially in cancer cells and blocking TGF-β signaling.

Acknowledgments

We thank Dr Shinichi Teshima and Masakazu Takahira for assisting with the immunohistochemical analyses. This study was supported in part by a Grant-in-Aid #21592066 (to HN) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest

Professor Y Homma received grants from Asahikasei (Tokyo, Japan) and Astellas (Tokyo, Japan).

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Supporting information

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

Fig. S1 Demographic chart of the male population in Sumida-ku and Japan. The ubiety if the Fraternity Memorial Hospital is this Sumida-ku, whereabouts of the Fraternity Memorial Hospital, represent well the average Japanese population.

Editorial Comment

Editorial Comment from Dr Chen to Reduction of prostate cancer incidence by naftopidil, an α1-adrenoceptor antagonist and transforming growth factor-β signaling inhibitor

To date, the following considerations are our concern for the treatment on prostate cancer (PC) cells and tissues with small molecule drugs: (i) α1A-adrenoceptor antagonist activity; (ii) apoptotic activity on PC cells; (iii) inhibition activity on PC cell growth; (iv) chemical structure with piperazine moiety; (v) low incidence of fibrosis; and (vi) low incidence of PC.

In the present article,[1] we can see that naftopidil fulfils the requirements as mentioned above. Tamsulosin fails to induce apoptosis or inhibits tumor cell growth in PC cell lines, indicating that it is not suitable for the treatment of PC; but it has limited use for the treatment of benign prostatic hypertrophy-induced urinary obstruction. Although adrenoceptor antagonists, doxazosin and terazosin, both with piperazine moiety in chemistry, could not reduce PC incidence, but less potent than naftopidil, which is characterized with naphzoline base. In contrast, tamsulosin, although selectively binding with α1A receptor, but without significant apoptotic activity on cancer cells.

In the present study, the follow up of study ended in December 2010 in patients who continued the same α1-adrenoceptor antagonists without diagnosis of PC have arisen some uncertainty problem. I suggest that patients receive monitoring of serum PSA levels during treatment according to a time schedule provided by the hospital.

The relative risk between users and "non-users" of α1-adrenoceptor antagonists was not evaluated, perhaps because of a lack of patients in this study who where "non-users". However, classification of users who used different α1-adrenoceptor antagonists can be used to compare the risk, as described in the present study.

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DOI: 10.1111/iju.12174

Conflict of interest

None declared.

Reference

1 Yamada D, Nishimatsu H, Kumano S *et al.* Reduction of prostate cancer incidence by naftopidil, an α1-adrenoceptor antagonist and transforming growth factor-β signaling inhibitor. *Int. J. Urol.* 2013; **20**: 1220–7.

Editorial Comment

Editorial Comment from Dr Murtola to Reduction of prostate cancer incidence by naftopidil, an α1-adrenoceptor antagonist and transforming growth factor-β signaling inhibitor

This interesting study used a two-staged approach to discover whether naftopidil, an alpha-blocker, could affect prostate cancer.[1] First, the incidence of prostate cancer was compared between naftopidil and tamsulosin users in a cohort of Japanese men. The incidence was found to be lower among naftopidil users. Second, naftopidil was studied *in vitro*, and found to reduce cell growth and to affect markers of apoptosis in prostate cancer cells. Such an approach is powerful, as it provides biological plausibility for the epidemiological findings.