Piperazine-based Alpha-1 AR Blocker, Naftopidil, Selectively Suppresses Malignant Human Bladder Cells *via* Induction of Apoptosis

YUSUKE U. NAKAGAWA[1,2*], HISAO NAGAYA[1*], TAKEAKI MIYATA[1], YOSHITAKA WADA[1], TSUNEHIRO OYAMA[1] and AKINOBU GOTOH[1]

[1]*Laboratory of Cell and Gene Therapy, Institute for Advanced Medical Sciences, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan;*
[2]*Department of Bioscience, Graduate School of Science and Technology, Kwansei Gakuin University, Sanda, Hyogo, Japan*

Abstract. *A retrospective observational cohort study has shown that exposure to alpha-1 adrenergic receptor (AR) blocker reduces the risk of bladder cancer (BCa). We investigated the antitumor activity of alpha-1 blockers, that are administered long-term therapeutically, in BCa. The antitumor activity of alpha-1 blockers was evaluated in terms of cell viability, cell cycle, competition, and apoptotic signaling in BCa cells. Our cell viability studies showed that naftopidil was one of the strongest alpha-1 AR blockers, regarding its antitumor action in BCa cells, independent of the grade of malignancy, but with no similar action on normal human bladder cells. Oral administration of naftopidil reduced tumor volume in a xenograft model. Our own competitive analysis using an alpha-1 AR agonist and other alpha-1 AR blockers showed that naftopidil activated cell death signaling without inhibitory action on alpha-1 ARs. We conclude that naftopidil has potential as an antitumor drug against BCa in vitro and in vivo. This finding provides a rationale for developing naftopidil in grade-independent treatment of BCa.*

Bladder cancer (BCa) is the sixth most common cancer and the eighth most common cause of death. In 2014, there were approximately 16,600 or 74,690 new cases of BCa and 5,400 or 15,580 deaths attributed to BCa in Japan or the United States (1, 2). About 80% of BCas are non-muscle invasive bladder cancer (NMIBCa) (3). The most common therapeutic regimen comprises of surgery, transurethral resection of bladder tumor (TURBT) at the site of NMIBCa, and treatment with Bacillus Calmette-Guerin (BCG), which stimulates the immune response and prevents recurrence. However, toxicity is a major concern when using BCG. Side-effects include severe cystitis, hematuria, reduced bladder capacity, and systemic toxicity such as fever, toxic hepatitis, and BCG sepsis (4). BCG therapy is, therefore, limited to 1 year, even in high-risk BCa (5). Many trials have aimed to reduce the side-effects of BCG therapy while maintaining its efficacy and have found that the standard dose may be the most effective (4). Some agents, including epirubicin, are effective against NMIBCa in intravesical chemotherapy, but the therapeutic effect is weaker than that of BCG (6, 7).

Drug repositioning allows for development of new therapeutic strategies with drugs that are already in clinical use and have established side-effect profiles. A recent retrospective observational cohort study showed that quinazoline-based alpha-1 adrenergic receptor (AR) blockers, such as doxazosin and terazosin, can reduce the risk of BCa and prostate cancer (PCa) (8, 9). Another study showed that doxazosin suppressed the growth and induced apoptosis in BCa cells *in vitro* (10, 11). The alpha-1D AR blocker and piperazine-based drug naftopidil was shown to induce cell death of PCa cells (12, 13) and to reduce the risk of PCa in a retrospective cohort study (14). Naftopidil is used in the treatment of benign prostatic hyperplasia (BPH) and works primarily by inhibiting alpha-1D ARs (15). We previously reported that naftopidil suppressed BCa, PCa, and renal cancer cells in serum-free *in vitro* cultures (16). Based on these findings, we tested the antitumor effect of a number of alpha-1AR blockers on BCa cells.

*These Authors contributed equally to this work.

Correspondence to: Akinobu Gotoh, MD, Ph.D., Laboratory of Cell and Gene Therapy, Institute for Advanced Medical Sciences, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya 663-8501, Japan. Tel: +81 798456807, Fax: +81 798456806, e-mail: cellgene@hyo-med.ac.jp

Key Words: Bladder cancer, drug repositioning, alpha-1 AR blocker, toxicity, naftopidil.

Because of their alpha-1 AR-blocking activity, some alpha-1 AR blockers have been used for the treatment of hypertension (doxazosin, prazosin, and terazosin) and BPH (naftopidil, prazosin, tamsulosin, and terazosin). Alpha-1 AR blockers are classified in three types based on the target receptor, alpha-1A AR (tamsulosin), alpha-1D AR (naftopidil and prazosin), and non-selective blockers (doxazosin and terazosin). In phenylephrine-induced pupil dilatation, doxazosin, prazosin, tamsulosin, and terazosin were 10.1-, 22.4-, 332-, and 6.4-fold more active, respectively, than with naftopidil (17). Furthermore, the affinity of naftopidil for alpha-1D AR was lower than that of other alpha-1 drugs (18). These reports led us to hypothesize that naftopidil has little or no systemic toxicity.

In the present study, we investigated the effect of alpha-1 blockers on cell growth in BCa cells of different malignancy grades and normal human cells.

Materials and Methods

Materials and reagents. Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Dojindo Laboratories (Kumamoto, Japan). Pan caspase inhibitor, z-VAD-FMK, was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Formalin neutral buffer was purchased from Kenei-Pharm (Osaka, Japan). RPMI-1640 medium, penicillin-streptomycin mix solution, and Lipofectamine LTX with Plus Reagent kit for transfection were purchased from Life Technologies Japan (Tokyo, Japan). Phentolamine was purchased from LKT Laboratories (St. Paul, MN, USA). *N,N*-Dimethylformamide (DMF) and sodium dodecyl sulfate (SDS) were purchased from Nacalai Tesque (Kyoto, Japan). Phosphate-buffered saline (PBS) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Forskolin, GF109203X, H89, and methoxamine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). *In situ* apoptosis detection kit was purchased from Takara Bio (Otsu, Japan). Doxazosin, phenylephrine, tamsulosin, and terazosin were purchased from Tokyo Chemical Industry (Tokyo, Japan). Hydrochloric acid, Triton X-100, and yohimbine were purchased from Wako (Osaka, Japan). Naftopidil was provided by Asahi Kasei Pharma (Tokyo, Japan).

Cell lines and cell culture. Three BCa cell lines, KK-47 (grade: I), 5637 (grade: II), and T-24 (grade: III), were used in this study. KK-47 cells were generously provided by Dr. Seiji Naito at the Department of Urology, Kyushu University (Fukuoka, Japan) (19). T-24 and 5637 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Human renal proximal tubular epithelial cells (HRPTEC) were purchased from Kurabo Industries (Osaka, Japan). All cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS and 1.0% penicillin-streptomycin (final concentration: 100 U/ml-100 μg/ml) and were maintained with a humidified atmosphere of 5% CO_2 and 95% air at 37˚C in a CO_2 incubator (Sanyo, Osaka, Japan).

Measurement of cell viability and proliferation using the MTT assay. Cells were seeded in 96-well plates and cultured in RPMI-1640 medium with 10% FBS. The following day, the medium was replaced with RPMI-1640 medium with 5% FBS with alpha-1 AR blockers, and plates were returned to the CO_2 incubator. Cell viability was assayed with the MTT assay, as described previously (20). Cell viability percentage was calculated as (Ax − An)/(Ac − An) ×100 using the following absorbance measurements: An: negative control (no cells: 0%); Ac: positive control (drug non-treated cells: 100% viability) cells; and Ax: drug-treated cells.

Evaluation of antitumor effect of orally administered naftopidil using a xenograft model. Animal experiments were approved by the Hyogo College of Medicine Animal Experiment Committee (#12-028) and performed according to the Regulations for Animal Experimentation at Hyogo College of Medicine. Nude BALB/cAJcl-nu/nu mice (male, 5-6 weeks old) were obtained from Clea Japan (Shizuoka, Japan). KK-47 (1×10^7 cells/mouse) were suspended in 100 μl of RPMI-1640 medium and subcutaneously inoculated into the right flank of mice. The longest diameter (L) and the shortest length (S) of the inoculated tumors were measured using calipers and tumor volume (V) was calculated as: $V=L\times S^2\times0.5$. Seven days after inoculation, mice with tumors were divided into three groups with no significant difference in tumor size; 0, 10, and 100 mg/kg naftopidil. Naftopidil was prepared in 100 μl of saline and was orally administered to xenograft mice with a feeding tube once daily for 36 days.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining. TUNEL staining was performed to detect *in situ* DNA fragmentation, a marker of apoptosis, using an *in situ* apoptosis detection kit. Briefly, cells treated with naftopidil were fixed with 10% formalin diluted with PBS and permeabilized by 0.2% Triton X-100 in PBS. After washing with PBS, DNA nick-ends in cells were labeled with fluorescein isothiocyanate (FITC)-dUTP for 90 min at 37˚C. FITC images were visualized at emission and excitation wavelengths of 488 and 458 nm, respectively, using a confocal scanning laser microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany).

Measurement of caspase-3 activity in living cells treated with naftopidil by fluorescence resonance energy transfer (FRET) probe. pSCAT3.1, an overexpression plasmid for SCAT3.1 protein (a FRET-based indicator of caspase-3 activation), was a kind gifted from Dr. A. Miyawaki (Riken, Wako, Japan) (21). One day after cells were seeded on a 6-well plate, they were transfected with pSCAT3.1 using Lipofectamine LTX with Plus Reagent and cultured for 24 hours. Transfected cells were seeded on 35-mm non-coated glass-bottom dishes (Matsunami Glass, Kishiwada, Japan) and cultured in RPMI-1640 medium with 10% FBS overnight. Medium was replaced with RPMI-1640 medium containing 5% FBS with or without 40 μM naftopidil. SCAT3.1 images were visualized at emission and excitation wavelengths of 477 or 530 nm and 458 nm, respectively, using a confocal scanning laser microscope (LSM 780; Carl Zeiss, Oberkochen, Germany). The fluorescence intensity of SCAT3.1 images at 477 nm (FI, 477 nm) or 530 nm (FI, 530 nm) was measured using ImageJ (Rasband W.S., the National Institutes of Health, Bethesda, MD, USA) and the emission ratio was calculated as: FRET ratio=(FI, 530 nm)/(FI, 477 nm).

Statistical analysis. All data are expressed as the mean±standard error of the mean (SEM). Statistical comparisons between groups were performed using Student's *t*-test of Excel (indicated in each figure legend), where $p<0.05$ was considered statistically significant.

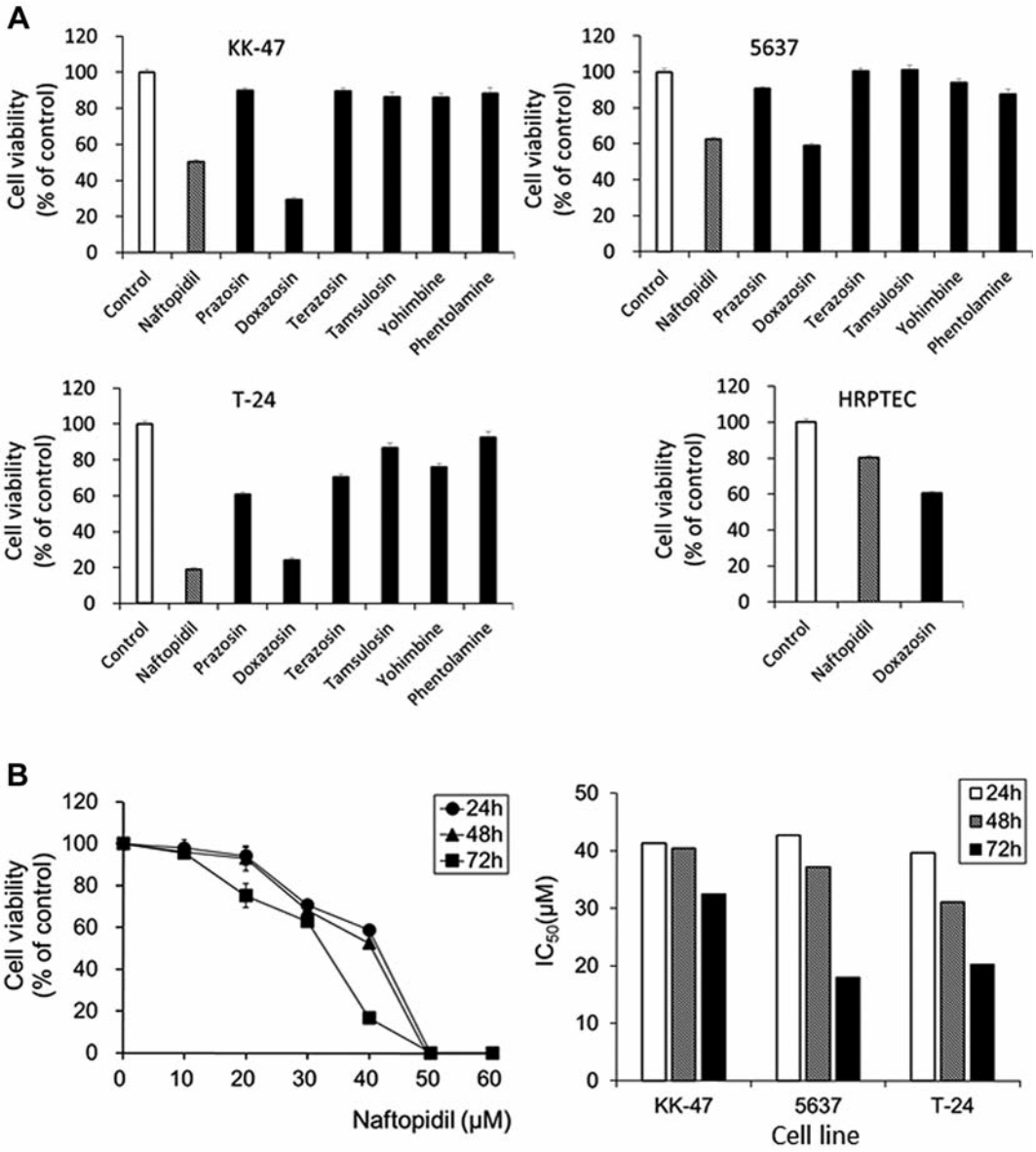

Figure 1. *Effects of naftopidil on bladder cancer cell viability. A: Effects of naftopidil, other alpha-1 adrenergic receptor (AR) blockers (prazosin, doxazosin, terazosin, and tamsulosin), and non-selective alpha AR blockers (yohimbine and phentolamine) on bladder cancer cell viability. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out in KK47, 5637, and T-24 cells untreated and treated with naftopidil or other blockers at 30 μM for 48 h. Each point represents the mean±SEM (MTT intensities for cells untreated; n=5 independent experiments). B: MTT assay was carried out in bladder cancer cells treated with naftopidil at the concentrations indicated for 24, 48, and 72 h. Each point represents the mean±SEM (MTT intensities for cells not treated with naftopidil; n=4 independent experiments).*

Results

Naftopidil reduces the viability of BCa cells independently of malignancy grade but not that of normal human urothelial cells. We previously reported that naftopidil reduced the viability of BCa cells in serum-free conditions (16). In order to investigate the antitumor effect of naftopidil, we used other alpha-1 AR blockers (doxazosin, prazosin, terazosin, and tamsulosin) and non-selective alpha AR blockers (yohimbine and phentolamine), and three BCa cell strains (KK-47, 5637, and T-24 cells) were treated with each drug at 30 μM for 48 h. As shown in Figure 1A, naftopidil and doxazosin strongly inhibited cell viability of all BCa cell lines. Viability of T-24 cells was weakly suppressed by prazosin, terazosin and yohimbine. To investigate the toxicity of naftopidil and doxazosin, normal human cells, HRPTEC, were treated with

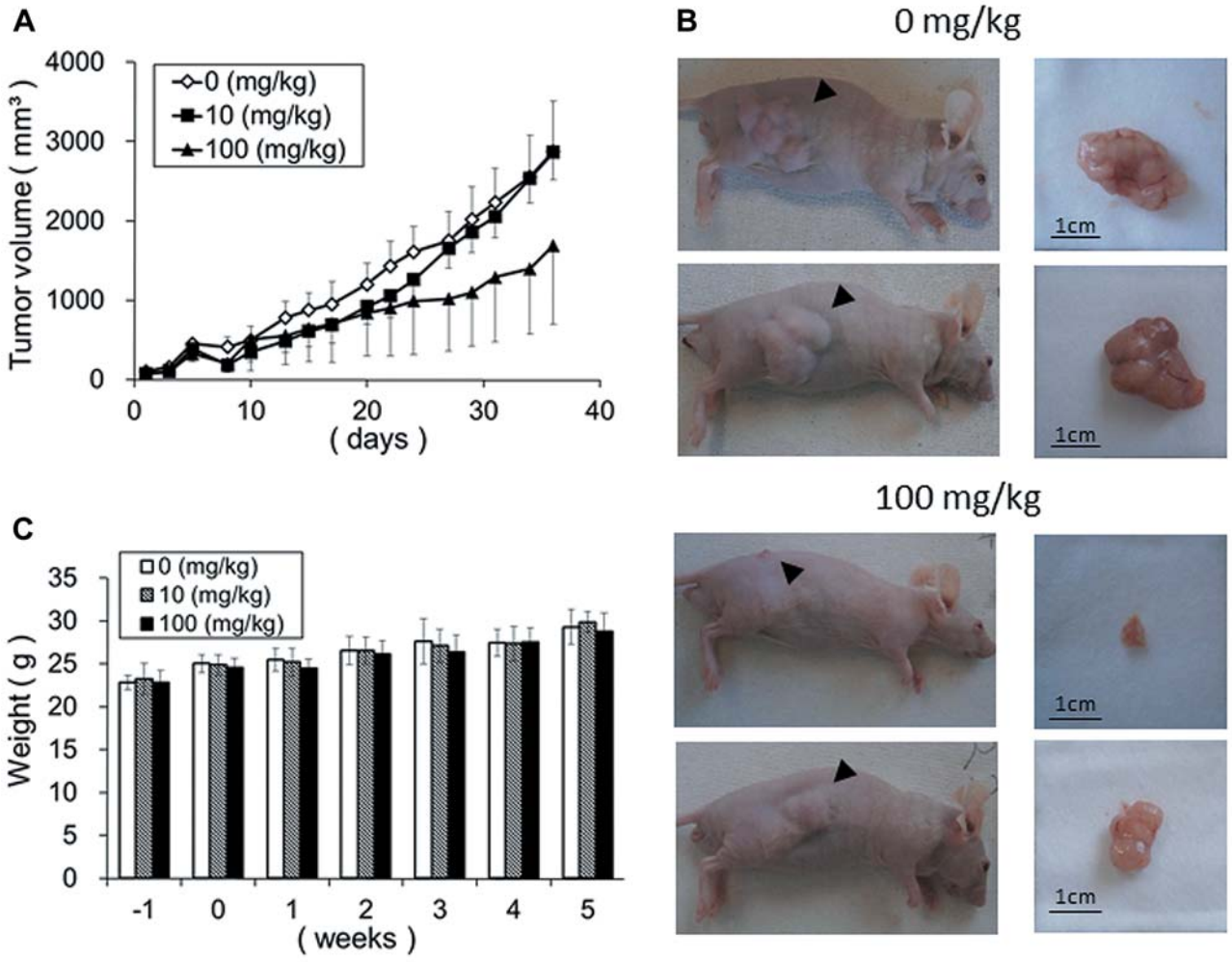

Figure 2. *In vivo study of antitumor action against bladder cancer cells. Nude mice were subcutaneously injected with KK47 cells (1×10^7 cells/mouse). The mice were divided into three groups (n=6, 5, and 5), and naftopidil (0, 10, and 100 mg/kg/day) was administered orally daily. Tumor volume (A) was measured every 2 to 3 days and body weight (C) was measured once weekly. B: Nude mice at 36 days after starting treatment. Note the tumor on the right flank of each mouse (left) and the tumor removed from each mouse (right).*

naftopidil and doxazosin. Cell viability of HPRTECs decreased to 80.3% and 60.5% following treatment with naftopidil and doxazosin, respectively. The dose–response curve for viability of KK-47 cells treated with naftopidil is shown in Figure 1B. The 50% inhibitory concentrations (IC_{50}), that were calculated from the dose response of cell viability in KK-47, 5637, and T-24 cells, were 41.3, 42.7 and 39.6 μM at 24 h; 40.4, 37.2 and 31.1 μM at 48 h; and 32.5, 18.0 and 20.3 μM at 72 h (Figure 1B, right). IC_{50} values showed that naftopidil inhibited cell viability of all BCa cells in a time-dependent manner. Our findings indicated that naftopidil has a high antitumor activity against BCa cells and low cytotoxicity against normal cells.

Antitumor effect of naftopidil in vivo. The antitumor effects of naftopidil were examined in a xenograft model of BCa. KK-47 cells were subcutaneously injected into the abdominal region of nude mice. Antitumor therapy with naftopidil was initiated 7 days later, and mice received 0, 10, or 100 mg/kg naftopidil daily for 36 days. On day 36, the mean tumor size (±SEM) was 2,875.6±628.5, 2862.3±341.3, and 1,690.0±989.0 mm^3 in the 0, 10 and 100 mg/kg naftopidil-treated groups, respectively (Figure 2A). The 100 mg/kg naftopidil dose caused a suppression of tumor growth without significantly affecting body weight (Figure 2B), suggesting that naftopidil has antitumor effects in KK-47 tumors without severe side-effects.

Naftopidil inhibits cell viability through a non-AR mechanism. We found that naftopidil strongly inhibited cell viability of all three BCa cell lines. It is known that

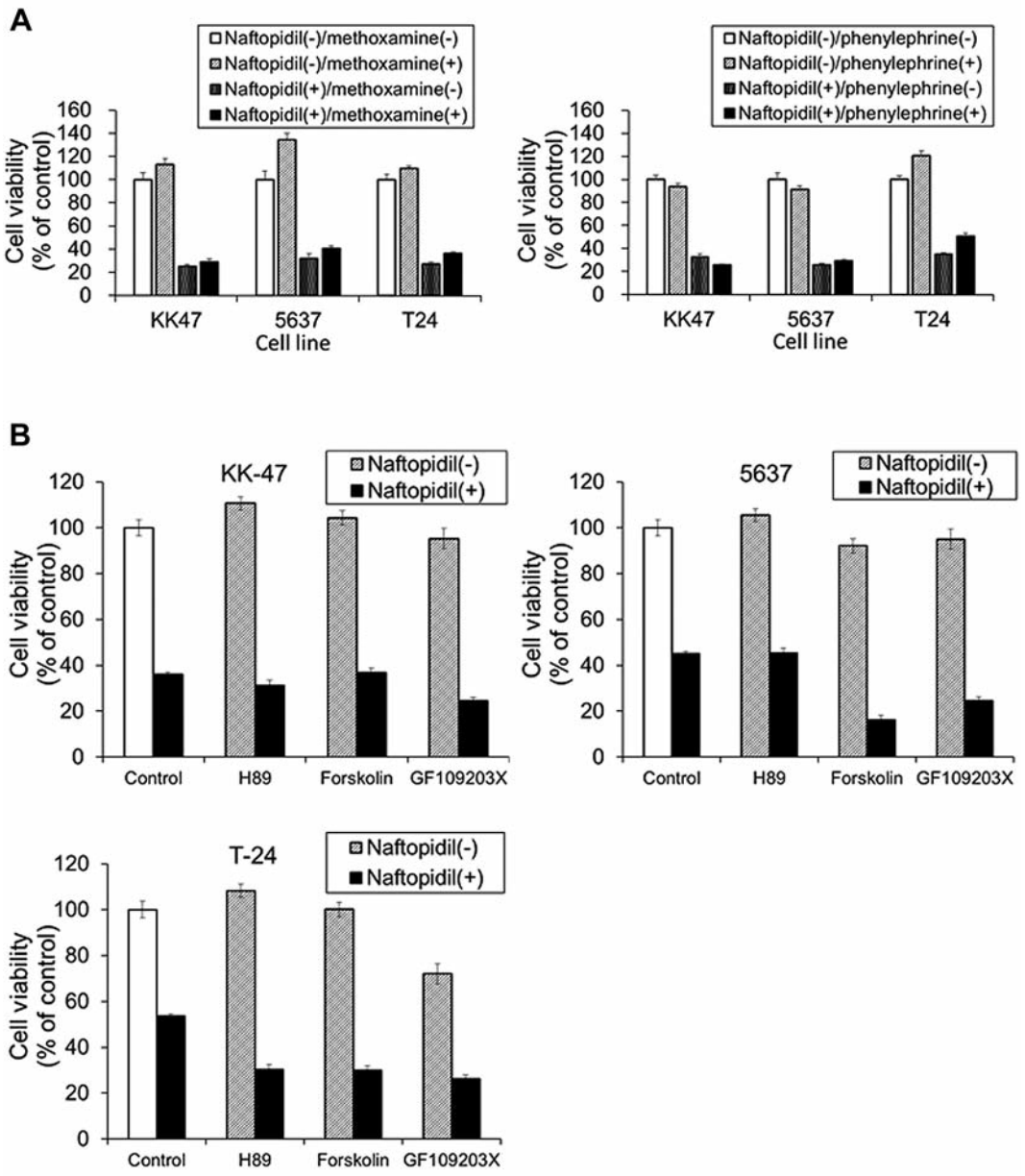

Figure 3. *Naftopidil inhibits cell viability via a non-adrenergic receptor (AR) pathway. A: Effects of alpha-1 AR agonists (phenylephrine and methoxamine) on bladder cancer cell viability. Bladder cancer cells were treated with both naftopidil (40 μM) and the alpha-1 agonist, methoxamine (30 μM) or phenylephrine (40 μM) for 48 h. Each point represents the mean±SEM (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) intensities for cells untreated with naftopidil or other blocker; n=5 independent experiments). B: Effects of drugs acting on the alpha-1 AR pathway (H89, forskolin, and GF109203X) on viability of bladder cancer cells treated with naftopidil. BCa cells were treated with H89, forskolin, and GF109203X in the absence or presence of naftopidil (40 μM) for 48 h. Each point represents the mean±SEM (MTT intensities for cells not treated with naftopidil or other inhibitors; n=5 independent experiments).*

naftopidil has a high affinity for alpha-1D AR but very low inhibitory activity (17, 18). To investigate the role of alpha-1 AR in naftopidil-induced inhibition of cell viability, three BCa cell lines were treated with both naftopidil and the alpha-1 agonist, methoxamine or phenylephrine. Alpha-1 agonist treatment had no effect on naftopidil-induced inhibition of cell viability (Figure 3A). Since adrenaline agonists induce protein kinase A (PKA) and protein kinase C (PKC) activation *via* alpha-1D AR (22), we tested whether PKA and PKC were involved in naftopidil-induced inhibition of cell viability using the PKA inhibitor H89, the PKA activator forskolin, and the PKC inhibitor GF109203X.

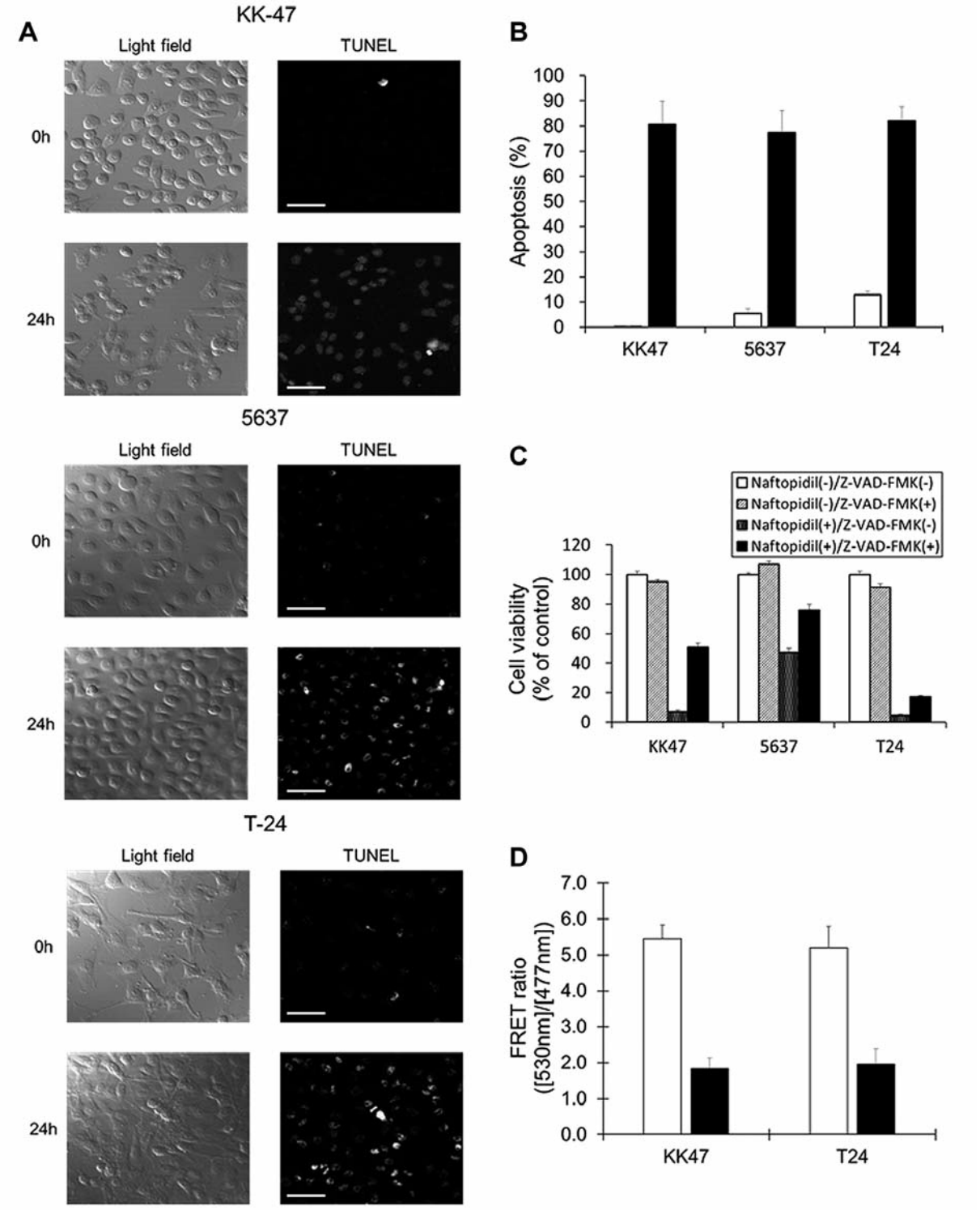

Figure 4. *Apoptosis analysis. A: Bladder cancer cells were treated with naftopidil (con, naftopidil: 0, 40 μM) for 24 h and then terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed. Bars=50 μm. B: TUNEL-positive cells, shown as percentage of the control from A. C: Naftopidil induced caspase activation in all bladder cancer cells. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was performed in KK47, 5637, and T-24 cells not treated and treated with pan-caspase inhibitor, z-VAD-FMK, and naftopidil (40 μM) for 48 h. Each point represents the mean±SEM (MTT intensities for cells not treated with naftopidil and z-VAD-FMK; n=5 independent experiments). D: Fluorescence resonance energy transfer probe for caspase-3 activity, SCAT3.1, was overexpressed in bladder cancer cells. These cells were treated with naftopidil (con, naftopidil: 0, 40 μM) for 24 h and observed.*

These drugs did not affect naftopidil-induced cell death (Figure 3B) suggesting that cell death by naftopidil was regulated through a pathway not involving alpha-1D AR.

Naftopidil-induces apoptosis of BCa cells via caspase-3 activation. A previous study that examined the mechanism of action of naftopidil in PCa cell growth using the TURBT method *in vivo* found that naftopidil-induced cell death does not involve apoptosis (12). Therefore, we examined whether the grade-independent inhibition of cell growth was attributable to increased apoptosis. As shown in Figure 4A and B, naftopidil treatment dramatically increased the number of TUNEL-positive cells in all BCa cell lines. These results clearly demonstrate that naftopidil induces apoptosis of BCa cells. The pan-caspase inhibitor z-VAD-FMK markedly reduced naftopidil-induced cell death (Figure 4C). Moreover, in the analysis of FRET ratio reduction by caspase-3 activation using a SCAT3.1 indicator, naftopidil significantly reduced the FRET ratio of SCAT3.1 in KK-47 cells and T-24 cells (Figure 4D). Taken together, these results indicate that naftopidil induces cell death *via* apoptosis.

Discussion

Based on the evidence from previous studies, we hypothesized that alpha-1 blockers may be an effective and low-toxic drug option for BCa. Of all the alpha-1 AR blockers studied, naftopidil and doxazosin exhibited the highest antitumor activity. Moreover, naftopidil had lower toxicity than doxazosin in normal human cells. The antitumor activity of naftopidil was independent of malignancy grade of BCa cells. Naftopidil induced cell death in BCa cells not by inhibition of alpha-1 AR but by activating the caspase-3-dependent apoptotic pathway. Taken together, these results demonstrate that, compared to other alpha-1 AR blockers, naftopidil had higher antitumor activity against BCa cells and lower cytotoxicity towards normal cells and that this action was independent of its alpha-1 AR blocker activity.

Cell death by naftopidil is not associated with the apoptotic pathway *in vivo* (14). Numerous reports have shown that apoptosis is related to activation of proteases, such as caspases, condensation of nuclear chromatin, fragmentation of genomic DNA, and redistribution of phosphatidylserine to the extracellular surface (21-23). Our data show that naftopidil induced genomic DNA fragmentation by TUNEL and caspase-3 activation by FRET. Naftopidil-induced cell growth suppression was inhibited by a pan-caspase inhibitor. These findings indicate that naftopidil induces apoptosis *via* a caspase-3-dependent cascade.

Thus far, BCG is the only prophylactic measure available clinically for NMIBCa. Cancer prophylaxis without serious side-effects is the main objective of cancer chemoprevention (4, 6, 7). We show that naftopidil selectively induced cell death in BCa cells without altering body weight of mice. Naftopidil for BHP treatment is orally administered at a higher dose than other alpha-1 blockers (the medicinal dosage for BHP; naftopidil, doxazosin, prazosin, terazosin, and tamsulosin: 50 mg/day, 4 mg/day, 1 mg/day, 2 mg/day, and 0.2 mg/day, respectively) because the affinity of naftopidil for alpha-1 AR (K_i for 1A, 1B, and 1D: 23, 7.8, and 4.4 nM, respectively) is lower than that of other alpha-1 AR blockers (prazosin Ki for 1A, 1B, and 1D: 0.12, 0.028, and 0.078 nM, respectively; tamsulosin Ki for 1A, 1B, and 1D: 0.012, 0.12, and 0.03 nM, respectively) (18). The molecular weight of naftopidil (392.5 g/mol) is similar to that of other alpha-1AR blockers (doxazosin, prazosin, tamsulosin: 547.6 g/mol, 383.4 g/mol, and 408.5 g/mol). Our results indicate that of all alpha-1AR blockers, naftopidil had the lowest ratio of (antitumor effect)/(alpha-1AR block) at an effective concentration. Taken together, these data indicate that the toxicity of orally administered naftopidil in humans may be low.

In conclusion, our study suggests that piperazine-based alpha-1 AR blockers (doxazosin and naftopidil) but not the sulfonamide- (tamsulosin) or the quinazoline-based alpha-1 AR blockers (prazosin and terazosin) induce cell death in BCa cells *via* a mechanism independent of alpha-1 AR blockade. Future studies will explore the apoptotic signaling triggered by naftopidil *via* the caspase pathway. Perhaps naftopidil may be a viable therapeutic option in recurrence prevention after TURBT for patients with BCa.

Conflicts of Interest

No potential conflicts of interest were disclosed by any Authors.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 23592354 and 15K10613 (to A.G.), by Cooperative Research Grant of the Institute for Enzyme Research, Joint Usage/Research Center, the University of Tokushima (to A.G. and H.N.), by Grant-in-Aid for Researchers, Hyogo College of Medicine, 2014 (to H.N.), by Hyogo Prefecture Health Promotion Association (to H.N.), and by Asahi Kasei Pharma Corp. (Tokyo, Japan, to A.G.).

The Authors wish to thank Satoko Kodama for her help in the completion of the manuscript.

References

1 Foundation for Promotion of Cancer Research: CANCER STATISTICS IN JAPAN, 2014-2015. http://ganjoho.jp/en/professional/statistics/brochure/2014_en.html

2 Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin *64(1)*: 9-29, 2014.

3 Miñana B, Cózar JM, Palou J, Unda Urzaiz M, Medina-Lopez RA, Subirá Ríos J, de la Rosa-Kehrmann F, Chantada-Abal V, Lozano F, Ribal MJ, Rodríguez Fernández E, Castiñeiras

Fernández J, Concepción Masip T, Requena-Tapia MJ, Moreno-Sierra J, Hevia M, Gómez Rodríguez A, Martínez-Ballesteros C, Ramos M, Amón Sesmero JH, Pizá Reus P, Bohorquez Barrientos A, Rioja Sanz C, Gomez-Pascual JA, Hidalgo Zabala E, Parra Escobar JL and Serrano O: Bladder cancer in Spain 2011: Population-based study. J Urol *191(2)*: 323-328, 2014.

4 Ali-El-Dein B, Barakat TS, Nabeeh A and Ibrahiem E-HI: Weekly intravesical bacillus Calmette-Guerin (BCG) alternating with epirubicin in Ta and T1 urothelial bladder cancer: An approach to decrease BCG toxicity. Urol Ann *5(2)*: 103-108, 2013.

5 Sylvester RJ, Van Der Meijden APM, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, Newling DW and Kurth K: Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: A combined analysis of 2596 patients from seven EORTC trials. Eur Urol *49(3)*: 466-477, 2006.

6 Melekos MD, Chionis HS, Paranychianakis GS and Dauaher HH: Intravesical 4'-epi-doxorubicin (epirubicin) versus bacillus Calmette-Guérin. A controlled prospective study on the prophylaxis of superficial bladder cancer. Cancer *72(5)*: 1749-1755, 1993.

7 Duchek M, Johansson R, Jahnson S, Mestad O, Hellström P, Hellsten S and Malmström PU; Members of the Urothelial Cancer Group of the Nordic Association of Urology: Bacillus Calmette-Guérin is superior to a combination of epirubicin and interferon-α2b in the intravesical treatment of patients with stage T1 urinary bladder cancer. A prospective, randomized, Nordic study. Eur Urol *57(1)*: 25-31, 2010.

8 Martin FM, Harris AM, Rowland RG, Conner W, Lane M, Durbin E, Baron AT and Kyprianou N: Decreased risk of bladder cancer in men treated with quinazoline-based alpha 1-adrenoceptor antagonists. Gene Ther Mol Biol *12(2)*: 253-258, 2008

9 Harris AM, Warner BW, Wilson JM, Becker A, Rowland RG, Conner W, Lane M, Kimbler K, Durbin EB, Baron AT and Kyprianou N: Effect of alpha1-adrenoceptor antagonist exposure on prostate cancer incidence: an observational cohort study. J Urol *178(5)*: 2176-2180, 2007.

10 Benning CM and Kyprianou N: Quinazoline-derived alpha1-adrenoceptor antagonists induce prostate cancer cell apoptosis *via* an alpha1-adrenoceptor-independent action. Cancer Res *62(2)*: 597-602, 2002

11 Kyprianou N and Benning CM: Suppression of human prostate cancer cell growth by alpha1-adrenoceptor antagonists doxazosin and terazosin *via* induction of apoptosis. Cancer Res *60(16)*: 4550-4555, 2000.

12 Kanda H, Ishii K, Ogura Y, Imamura T, Kanai M, Arima K and Sugimura Y: Naftopidil, a selective alpha-1 adrenoceptor antagonist, inhibits growth of human prostate cancer cells by G1 cell-cycle arrest. Int J Cancer *122(2)*: 444-451, 2008.

13 Hori Y, Ishii K, Kanda H, Iwamoto Y, Nishikawa K, Soga N, Kise H, Arima K and Sugimura Y: Naftopidil, a selective alpha1-adrenoceptor antagonist, suppresses human prostate tumor growth by altering interactions between tumor cells and stroma. Cancer Prev Res *4(1)*: 87-96, 2011.

14 Yamada D, Nishimatsu H, Kumano S, Hirano Y, Suzuki M, Fujimura T, Fukuhara H, Enomoto Y, Kume H and Homma Y: Reduction of prostate cancer incidence by naftopidil, an α1-adrenoceptor antagonist and transforming growth factor-β signaling inhibitor. Int J Urol *20(12)*: 1220-1227, 2013.

15 Castiglione F, Benigni F, Briganti A, Salonia A, Villa L, Nini A, Di Trapani E, Capitanio U, Hedlund P and Montorsi F: Naftopidil for the treatment of benign prostate hyperplasia: a systematic review. Curr Med Res Opin *30(4)*: 719-732, 2014.

16 Gotoh A, Nagaya H, Kanno T and Nishizaki T: Antitumor action of alpha1-adrenoceptor blockers on human bladder, prostate and renal cancer cells. Pharmacology *90(5-6)*: 242-246, 2012.

17 Michel MC, Okutsu H, Noguchi Y, Suzuki M, Ohtake A, Yuyama H, Yanai-Inamura H, Ukai M, Watanabe M, Someya A and Sasamata M: In vivo studies on the effects of alpha1-adrenoceptor antagonists on pupil diameter and urethral tone in rabbits. Naunyn Schmiedebergs Arch Pharmacol *372(5)*: 346-353, 2006.

18 Tatemichi S, Kobayashi K, Maezawa A, Kobayashi M, Yamazaki Y and Shibata N: Alpha1-adrenoceptor subtype selectivity and organ specificity of silodosin (KMD-3213). Yakugaku zasshi *126*: 209-216, 2006.

19 Hisazumi H, Kanokogi M, Nakajima K, Kobayashi T, Tsukahara K, Naito K, Kuroda K and Matsubara F: Established cell line of urinary bladder carcinoma (KK-47): growth, heterotransplantation, microscopic structure and chromosome pattern. Nihon Hinyōkika Gakkai zasshi *70(5)*: 485-494, 1979 (in Japanese).

20 Nagaya H, Gotoh A, Kanno T and Nishizaki T: A3 adenosine receptor mediates apoptosis in *in vitro* RCC4-VHL human renal cancer cells by up-regulating AMID expression. J Urol *189(1)*: 321-328, 2013.

21 Nagai T and Miyawaki A: A high-throughput method for development of FRET-based indicators for proteolysis. Biochem Biophys Res Commun *319(1)*: 72-77, 2004.

22 Lin RZ, Chen J, Hu ZW and Hoffman BB: Phosphorylation of the cAMP response element-binding protein and activation of transcription by alpha1 adrenergic receptors. J Biol Chem *273(45)*: 30033-30038, 1998.

23 Zhou JR, Mukherjee P, Gugger ET, Tanaka T, Blackburn GL and Clinton SK: Inhibition of murine bladder tumorigenesis by soy isofiavones *via* alterations in the cell cycle, apoptosis, and angiogenesis. Cancer Res *58(22)*: 5231-5238, 1998.

24 Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA and Henson PM: A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature *405(6782)*: 85-90, 2000.

25 Segawa K, Kurata S, Yanagihashi Y, Brummelkamp TR, Matsuda F and Nagata S: Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. Science *344(6188)*: 116-1168, 2014.

Received November 17, 2015
Revised February 16, 2016
Accepted February 17, 2016