Title: Oral naftopidil suppresses human renal cell carcinoma by inducing G1 cell cycle arrest in tumor and vascular endothelial cells
Title: Oral naftopidil suppresses human renal cell carcinoma by inducing G1 cell cycle arrest in tumor and vascular endothelial cells.

Short running title: Phenylpiperazine derivative naftopidil impairs human renal cell cancer

Yoichi Iwamoto (1), Kenichiro Ishii (2), Takeshi Sasaki (1), Manabu Kato (1), Hideki Kanda (1), Yasushi Yamada (1), Kiminobu Arima (1), Taizo Shiraishi (2), Yoshiki Sugimura (1)*

(1) Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, Mie, Japan

(2) Department of Oncologic Pathology, Mie University Graduate School of Medicine
* Corresponding author

Requests for reprints: Yoshiki Sugimura, Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan.

Tel: +81-59-231-5026, Fax: +81-59-231-5203

E-mail: sugimura@clin.medic.mie-u.ac.jp

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Abstract

Renal-cell cancer (RCC) is an angiogenesis-dependent and hypoxia-driven malignancy. As a result, several targeting-agents are being investigated. However, the efficacy of current regimens is generally insufficient for their toxicity and poor overall response rates. We have recently reported that naftopidil exerts growth-inhibitory effects on human prostate cancer cells. In this study, we investigated the biochemical mechanisms by which naftopidil produces growth-inhibitory and anti-angiogenic effects on RCC. We first tested the effects of naftopidil on the proliferation of ACHN and Caki-2 RCC cells. Next, we set up a model simulating the tumor microenvironment, in which ACHN cells were grafted onto the renal capsule of mice. We then tested the effects of naftopidil on human vascular endothelial cells (HUVEC) cell proliferation and Matrigel plug vascularization. Finally, to establish the antitumor activity of naftopidil on RCC, we tested the anti-tumor effects of naftopidil on excised tumor specimens from 20 RCC patients that were grafted beneath the renal capsule of mice. Naftopidil showed similar in vitro growth-inhibitory effects on all cell lines. FACS analysis revealed an increase in G1 cell cycle arrest in all naftopidil-treated cell lines. In vivo tumorigenic studies showed a significant reduction of ACHN tumor weight,
Ki-67 index, and microvessel density (MVD) in naftopidil-treated mice. Naftopidil attenuated neovascularization in an in vivo Matrigel plug assay. Studies in xenograft models also showed a significant MVD reduction in naftopidil-treated excised human RCC. The growth-inhibitory effects of naftopidil suggest it may a novel anti-cancer agent and a potential preventive option for RCC.
Introduction

Each year, ~270 000 patients are diagnosed with renal cell carcinoma (RCC), resulting in ~110 000 deaths, making it the sixth leading cause of cancer deaths in Western countries (1, 2). RCC accounts for 80% to 95% of kidney tumors and has poor prognosis when diagnosed at advanced stages; currently, about 30% of patients with RCC have metastatic disease at diagnosis (3).

Since RCC is angiogenesis-dependent and hypoxia-driven (4), several angiogenesis-targeting agents, including inhibitors of the mTOR and VEGF pathways, are being actively investigated in preclinical and clinical studies (5, 6). Targeted agents that have recently received approval for clinical use include receptor tyrosine kinase inhibitors (TKIs), such as sorafenib and sunitinib. Although these inhibitors have shown clinical benefit in RCC, they cause a number of side effects. In addition, patients eventually fail these targeted therapies, developing refractory disease. Therefore, development of novel treatment approaches that are well tolerated and improve clinical outcome remains a high priority.

We have previously reported that naftopidil, a selective alpha1-adrenoceptor (α1-AR) antagonist, inhibits tumor growth in PC-3 human prostate cancer (PCa)
cells, induces G1-phase cell-cycle arrest, and decreases microvessel density (MVD) (7) without the α1-ARs and the α1-AR signals (8). However, the mechanisms of naftopidil on reducing MVD are still unclear. Clinically, α1-AR antagonists have been widely used to treat benign prostatic hyperplasia (BPH), a common prostatic disease in elderly men. Recent research has demonstrated exciting anticancer effects of α1-AR antagonists that are independent of their effects on α1-AR. Garrison et al. reported that an α1-AR antagonist, doxazosin, reduced endothelial cell viability, thus suppressing tumor vascularity in PCa xenografts (9). Epidemiologically, Harris et al. reported that quinazoline-based α1-AR antagonists, such as doxazosin and terazosin, significantly decreased PCa incidence (10). In addition, Sakamoto et al. found that the α1-AR antagonists doxazosin and DZ-50 significantly reduced RCC metastatic potential (11). These data led us to hypothesize that naftopidil has anticancer activity against RCC, in addition to its effects against PCa.

The principle aim of our study was to determine whether naftopidil has unique growth-inhibitory and anti-angiogenic effects on RCC cells. To conduct our study, we present here results of studies using RCC cell lines, in vitro and in vivo, in two different RCC model systems.
Materials and Methods

Materials

Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) kindly provided naftopidil and tamsulosin. Monoclonal anti-human Ki-67 antibody was purchased from Dako (Glostrup, Denmark), rabbit anti-CD31 (ab28364) was from Abcam (Cambridge, UK), rabbit anti-p21 (2947) and rabbit anti-CDK2 (2546) were from Cell Signaling Technology (Danvers, MA, USA), and monoclonal anti-actin (AC-15) was from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Cell culture and treatment conditions

The human RCC cell lines, ACHN and Caki-2, were obtained from the European Collection of Cell Cultures (ECACC), a Public Health England Culture Collection via DS Pharma Biomedical Co., Ltd. (Osaka, Japan). And the vendor authenticated both cells by the STR-PCR method. Human umbilical vein endothelial cells (HUVEC) were from Lonza Walkersville Inc. (Walkersville, MD, USA). ACHN cells were cultured in Minimum Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA), and Caki-2 cells were cultured in McCoy’s 5A Medium (MP Biomedicals, in Santa Ana, Ca, USA), both with 10% fetal
bovine serum (FBS; Thermo Scientific HyClone, Logan, UT, USA). HUVEC cells were cultured in endothelial cell growth medium-2 (EGM-2) (EGM-2 Bullet Kit; Lonza Walkersville Inc.) with 5% FBS. For treatment with naftopidil and tamsulosin, ACHN and Caki-2 cells (2.0 × 10⁴ cells/well) were cultured in 12-well plates for 5 days. Test samples (0-15 µM) or vehicle (0.1% DMSO) was added on day 2. Cytotoxicity was determined as previously described (7). To investigate whether naftopidil affected endothelial cells, HUVEC cells (2.0 × 10³ cells/well) were cultured in 12-well plates for 5 days, and naftopidil (0-40 µM) or vehicle (0.1% DMSO) was added on day 2. The cells were cultured for an additional 3 days, and cell growth was determined as previously described (12).

Cell cycle analysis

ACHN, Caki-2, and HUVEC cells were cultured in 90-mm dishes (6.0 × 10⁵ cells/dish) for 3 days. Naftopidil, tamsulosin, or vehicle (0.1% DMSO) was added on day 2, and cell cycle analysis was performed 24 hours later by flow cytometry. Flow cytometry was performed as described previously (7).

Preparation of cell lysates and western blot analyses
ACHN, Caki-2, and HUVEC cells were cultured for 3 days in 90-mm dishes (6.0 × 10^5 cells/dish). Naftopidil, tamsulosin, or vehicle (0.1% DMSO) was added on day 2; cell lysates were prepared 24 hours later and analyzed by western blotting (WB), as previously described (7). Western blots were probed with anti-p21 (1:1,000), anti-CDK2 (1:1,000), or anti-β-actin (1:5,000) antibodies and visualized on LAS-4000 Mini (Fuji Photo Film, Tokyo, Japan).

Animal studies

All animals were maintained in a pathogen-free environment, under experimental protocol guidelines approved by Mie University’s Committee for Animal Investigations. Female athymic nude mice (BALB/c, nu/nu, 6–8 weeks old) were from CLEA Japan, Inc. (Tokyo, Japan).

Subconfluent cultures of ACHN cells were trypsinized, and 5 x 10^5 cells were counted and suspended. ACHN tumors cells were grafted into female athymic nude mice (6 to 8 weeks old) divided into groups of 5. Animals were weighed twice weekly, and treatments started 7 days after transplantation. Naftopidil (10 mg/kg/d) suspension in 0.5% carboxymethylcellulose or vehicle control was administered orally through a 22-gauge gavage needle for 28 days, as
previously described (8).

Twenty patients diagnosed with clear cell RCC underwent radical nephrectomy or partial nephrectomy between December 2011 and December 2012 in Mie University Hospital. The institutional review board at the institution approved the study, and informed consent was obtained from all patients. Specimens of 2 mm³ were prepared from 20 fresh RCC patient tissues obtained at the time of surgery. Of these, 12-16 specimens were grafted into the bilateral renal capsule. Animals were weighed twice weekly, and treatments started 7 days after transplantation. Naftopidil (10 mg/kg/d) suspension in 0.5% carboxymethylcellulose or vehicle control was administered orally through a 22-gauge gavage needle for 28 days.

**Histology and immunohistochemistry**

Tumors were harvested from animals on day 35 after grafting, fixed in buffered formalin, and embedded in paraffin. Tissue slices (3 μm) were stained with hematoxylin and eosin (H&E), and tissue morphology was visualized. Tissue samples from ACHN tumors were analyzed for altered patterns of tumor cell proliferation (percent Ki-67 positivity), MVD (CD31-positive microvessels)
and cell cycle (percent p21cip1 or CDK2 positivity) between control and naftopidil-treated animals by immunohistochemical staining as previously described (7).

**Matrigel plug assay**

Matrigel (BD Biosciences) was injected subcutaneously into the flanks of nude mice (BALB/c, nu/nu, 6–8 weeks old), divided into groups of 5 each. Treatment began 1 day after transplantation with naftopidil or control vehicle, administered orally (10 mg/kg/d), for 55 days. After 56 days, the mice were perfused with PBS containing 2 mM ethylenediaminetetraacetic acid for 40 minutes after intravenous injection with 1% Evans blue dye, and the amount of Evans blue eluted with formamide from the Matrigel plugs was measured with a spectrophotometer (620 nm), as previously described (13).

**Statistical analysis**

Unless otherwise stated, a minimum of three independent experiments were performed for all quantitative studies, and data are expressed as the mean ± SD. Statistically significant differences between treatment and control groups were
determined using one-way ANOVA and Tukey’s Multiple Comparison Test, and the correlation between RCC preoperative tumor characteristics and MVD ratio was determined using \( t \) test and Pearson’s correlation coefficient (PCC) test, followed by data analysis with JMP version 8.01. \( P \)-values less than 0.05 were considered significant.
Results

**Growth inhibition of ACHN and Caki-2 cells by naftopidil or tamsulosin**

To investigate the effects of naftopidil and tamsulosin on RCC cells *in vitro*, ACHN and Caki-2 cells were treated as described in the Materials and Methods. Naftopidil exerted more cytotoxic effect than tamsulosin, which had a slight dose-dependent, cytotoxic effect on ACHN and Caki-2 cells (Fig. 1A).

To determine the mechanism by which naftopidil inhibits proliferation in RCC cells, we examined PI-stained cells by flow-cytometry. Compared with vehicle, naftopidil increased the population of cells in G1 phase and decreased the cell populations in S and G2 (Fig. 1B); no appreciable change was observed between tamsulosin treatment and vehicle.

To determine which key cell-cycle regulators are involved in naftopidil-induced G1 arrest, we investigated the expression of positive and negative G1/S checkpoint modulators in ACHN and Caki-2 cells, by western blot analysis (Fig. 1C). In ACHN and Caki-2, naftopidil increased p21cip1 and decreased CDK2, whereas tamsulosin caused no appreciable change in the levels of p21cip1 and CDK2.
Tumor growth delay of ACHN xenograft by oral administration of naftopidil

We analyzed the effects of naftopidil in vivo using subcutaneous (s.c.) ACHN xenograft tumors in BALB/c nude mice (Fig. 2A). Naftopidil induced a significant delay in tumor growth (P < 0.05).

The Ki-67 proliferation index was determined, using fixed tumor sections in order to better understand the mechanism mediating the tumor growth delay. As shown in Fig. 2B, naftopidil caused an approximate 50% reduction in proliferating cells, compared with vehicle control (p < 0.01). The MVD was significantly decreased in naftopidil-treated tumors, as shown in Fig. 2C, with an approximate 75% reduction in proliferating cells, compared with vehicle-treated tumors (p < 0.01). Naftopidil treatments also significantly increased the abundance of p21cip1 (Fig. 2D) and decreased the abundance of CDK2 (Fig. 2E) in ACHN tumors.

Analyses of anti-angiogenic effects of naftopidil on the Matrigel plug assay and on HUVEC cells

In order to further address the functional role of naftopidil in mediating angiogenesis, we used an in vivo Matrigel plug assay. Naftopidil treatment
markedly attenuated neovascularization (Fig. 3A). Evans blue was used to quantify the extent of neoangiogenesis. The naftopidil group (10 mg/kg/day) demonstrated a reduction of about 35% in Evans blue content (Fig. 3A).

Naftopidil, at 2.5 µM dose or higher, suppressed cell proliferation of HUVEC cells in a dose-dependent manner (Fig. 3B). The number of naftopidil-treated HUVEC cells in G1 phase was higher than for untreated HUVEC cells (Fig. 3C). Naftopidil treatment increased p21cip1 in dose-dependent manner, while tamsulosin treatment showed the least change (Fig. 3D).

Establishment and analysis of RCC xenografts in nude mice

To develop an adoptive transfer murine model to study the effects of naftopidil on RCC tumor progression, RCCs from a total of 20 patients were grafted into nude mice. MVD was significantly decreased in naftopidil-treated mice (Table 1 and Fig. 4B). Pearson’s correlation coefficient test showed strong positive correlations between the naftopidil/control MVD ratio and preoperative greatest tumor dimension which were measured by a pathologist after formalin-fixed (Table 2), whereas no appreciable association was observed between naftopidil/control MVD ratio and age, sex, Fuhrman grade, or initial
MVD of RCC.
Discussion

Cell cycle inhibition is a potentially important target for cancer management, since there is an established association between deregulated cell cycle progression and cancer (14, 15). This antiproliferative effect suggests the potential value of naftopidil in RCC therapy. Cyclin-dependent kinases (e.g. CDK2) are critical, positive regulators of progression through the cell cycle G1/S checkpoint (16). CDK inhibitors of the Kip1/Cip1 and INK4 families prevent activation of CDKs and their entry into S phase in mammalian cells (17). p21 (cip1/waf1) is a CKI that directly inhibits the activity of cyclin E/CDK2 complexes, and p21 functions as a regulator of cell cycle progression at S phase (18). Our study showed that expression of p21cip1 was significantly increased in ACHN and Caki-2 cells by naftopidil treatment but was not affected by tamsulosin treatment (Fig. 1C); expression of CDK2 was significantly decreased by naftopidil treatment. These results indicate that naftopidil-induced G1 cell cycle arrest is mainly associated with p21cip1 levels in ACHN and Caki-2 cells. Furthermore, it was the same results with in vivo ACHN xenograft model (Fig. 2D and Fig. 2E).

Targeting angiogenesis in patients with advanced and/or metastatic RCC
has become the standard of care. The use of targeted therapies, including angiogenesis inhibitors, TKIs, and mTOR inhibitors, has contributed to increased progression-free survival and overall survival in RCC patients (19-21). However, these therapies are often not curative and produce a range of adverse effects (AEs), and the majority of patients develop recurrent disease. Therefore, it is important to continue investigating novel treatment approaches that may potentially improve treatment outcome in RCC. In the present study, naftopidil inhibited the proliferation of HUVEC cells by inducing G1 cell cycle arrest \textit{in vitro} and neovascularization \textit{in vivo}. Here, we have shown that naftopidil plays a critical role in endothelial cell growth \textit{in vitro} and angiogenesis \textit{in vivo}. Moreover, our immunohistochemistry in RCC xenograft model results provide supportive evidence that naftopidil suppressed neovascularization. The steps leading to creation of new blood vessels include activation of endothelial cells that form the blood vessel wall, synthesis of matrix metalloproteinases (MMP) that break down the extracellular matrix (ECM), invasion through the matrix, and endothelial cell proliferation. Eventually, new endothelial cells organize into hollow tubes, creating new networks of blood vessels that supply tissues (22). These processes are dynamically controlled by many proangiogenic and
antiangiogenic factors. Normally, the vasculature is quiescent outside of select physiological processes, such as wound repair and the female menstrual cycle (22). The turnover time of these cells may be several years (23). In contrast, endothelial cells during neovascularization can proliferate with a turnover time of several days (24). The understanding that the growth of tumors depends on angiogenesis has led to the development of novel strategies for treatment directed at the tumor vasculature. Antiangiogenic compounds have had striking success in preclinical models, and new agents are rapidly entering clinical trials (22). In addition, targeted therapies, the only treatments currently available for RCC or metastatic RCC beside surgical resection, cause a number of AEs. Most AEs associated with TKIs and mTOR inhibitors may be managed effectively with medical or supportive measures (25). However, some toxicities can affect the patient’s quality of life or present medical challenges in treating patients with comorbidities (25). For example, the TKIs sunitinib and sorafenib are commonly associated with hypertension, diarrhea, fatigue, hand-foot syndrome, thyroid dysfunction, elevated lipase, and myelosuppression (25).

The present study used two selective \( \alpha_1 \)-AR antagonists: naftopidil, which has higher selectivity for \( \alpha_{1D} \)-AR than tamsulosin and is marketed only in Japan
(26), and tamsulosin, which is selective for α\textsubscript{1A}-AR- and α\textsubscript{1D}-AR- and is used worldwide (27). Clinically, α\textsubscript{1}-AR antagonists such as naftopidil and tamsulosin have been widely used to treat benign prostatic hyperplasia (BPH), a common prostatic disease in elderly men; naftopidil has high tolerability with few AEs (28). As the incidence of BPH increases with age (29), this would suggest that administration of α\textsubscript{1}-AR antagonists for BPH might often precede diagnosis of RCC. Thus, there may be some prospective clinical benefits from long-term use of naftopidil for BPH. Naftopidil may also be considered for long-term use to prevent RCC. Moreover, since naftopidil has been available clinically, it may be used in treating RCC without the costs associated with development of a new drug.

In conclusion, our present study suggests that naftopidil can suppress tumor growth and angiogenesis by inhibiting the growth of human RCC and vascular endothelial cells, through G1 cell cycle arrest. Naftopidil may be used, not only to better resolve urinary morbidities associated with BPH without compromising safety in elderly male patients (28), but also as an off-label drug to suppress development of RCC. However, additional studies are needed to verify the observed effects, using clinically appropriate doses, before naftopidil can be
used to treat RCC in patients.
Acknowledgements:

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References


Figure and Table legends

Figure 1. Effects of α₁-AR antagonists on ACHN and Caki-2 cell cycle.

(A) ACHN and Caki-2 cells exposed to various concentrations (0, 5, 10 and 15 µM) of each α₁-AR antagonist for 5 days. (B) Distribution of cell-cycle phases is shown for ACHN (upper) and Caki-2 (lower) cells treated with 50 µM of each α₁-AR antagonist for 24 hours. (C) Cell-cycle regulatory protein expression, determined by Western blot. Total cell lysates (30 µg) were western blotted and probed with antibodies to p21 and CDK2. Protein levels were compared with β-actin loading controls.

Values represent the means ± SD. *P < 0.05. **P < 0.01 versus untreated controls

Figure 2. Effects of naftopidil on tumorigenesis in vivo in ACHN RCC cells.

(A) Upper: Comparison of tumor weights from ACHN cells treated with vehicle control or with naftopidil (10 mg/kg) after grafting into animals. ACHN tumors cells were grafted into female athymic nude mice (6 to 8 weeks old) divided into groups of 5. The tumor weights were measured 35 days after transplanting.

Lower: Gross appearance of tumors grafted into the renal capsule. (B) Ki-67
index in ACHN cells after treatment with naftopidil. Ki-67 staining was used to assess cell proliferation at 400x magnification. (C) MVD in ACHN tumors. CD31-positive vessels with lumens were counted in 8-10 different areas at 200x magnification. (D) p21cip1 index in ACHN cells after treatment with naftopidil. p21cip1 staining was used to assess cell cycle at 400x magnification. (E) CDK2 index in ACHN cells after treatment with naftopidil. CDK2 staining was used to assess cell cycle at 400x magnification.

Values represent the mean ± SD. Arrow: CD31-positive vessels with lumens. *P < 0.05. **P < 0.01 versus vehicle-treated control.

**Figure 3.** Anti-angiogenic effects of naftopidil on the Matrigel plug assay and on HUVEC cells.

(A) The functional role of naftopidil in mediating angiogenesis as assessed using an in vivo Matrigel plug assay. The amount of Evans blue dye was quantified by A_{620} and normalized to Matrigel weight. (B) Survival of HUVEC cells treated with naftopidil (0, 2.5, 5, 10, 20, 30 and 40 µM) for 5 days. (C) Distribution of cell-cycle phases of HUVEC cells treated with 50 µM of each α_{1}-AR antagonist for 24 hours. (D) Western blot of p21 in 30 µg of total lysates, from naftopidil and
tamsulosin-treated HUVEC cells. p21 levels were shown with β-actin loading control.

Values represent the mean ± SD. *p < 0.05. **p < 0.01 compared with untreated controls

**Table 1.** Patient characteristics and analysis of RCC tumors grafted into nude mice.

Demographics and MVD ratios of patients whose tumors were used in RCC xenografts. Twenty patients diagnosed with RCC underwent radical nephrectomy or partial nephrectomy. All RCC patient tissues were obtained at the time of surgery and grafted into the bilateral renal capsule. All different RCC specimens remained engrafted successfully 35 days after transplantation.

Values represent the mean ± SD.

**Table 2.** Association between RCC preoperative tumor characteristics and MVD ratio, using Pearson’s correlation coefficient test.

The calculations showed strong positive correlation between the naftopidil/control MVD ratio and preoperative tumor greatest dimension (PCC:
0.52, 95% CI: 0.11, 0.78, p=0.01).

**Figure 4.** Gross appearances of RCC xenografts and relationships of MVD of RCC xenografts between control and naftopidil groups.

(A) Gross appearance of tumors grafted into the renal capsule. Arrow: engrafted yellow RCC tumors grafted after 35 days.

(B) Relationships of MVD of RCC xenografts between control and naftopidil groups.
<table>
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<th>Patient number</th>
<th>Age</th>
<th>Sex</th>
<th>Fuhrman grade</th>
<th>Tumor greatest dimension (mm)</th>
<th>MVD of RCC (number/field)</th>
<th>Naftopidil / Control CD31 positive MVD ratio</th>
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Fig. 3

A. Weight of Evans blue (g/g) measured in the Control and Naftopidil groups.

B. HUVEC Cell survival fraction (%) plotted against the Concentration of naftopidil (μM).

C. Cell cycle analysis showing percentages of G0/G1, S, and G2/M phases for Control, Naftopidil, and Tamsulosin groups.

D. Western blot analysis of p21 and Actin expression levels across different treatments with Naftopidil and Tamsulosin concentrations.
Fig. 4

A. Gross appearance

Control  | Naftopidil

B. Patients number

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<th>Patients number</th>
<th>Microvessel density (number/field)</th>
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Control  | Naftopidil
Cancer Prevention Research

Oral naftopidil suppresses human renal cell carcinoma by inducing G1 cell cycle arrest in tumor and vascular endothelial cells.

Yoichi Iwamoto, Kenichiro Ishii, Takeshi Sasaki, et al.


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