Award Number: W81XWH-07-1-0158

TITLE: Mechanisms of Chinese Red Yeast Rice Inhibition of Prostate Cancer Growth

PRINCIPAL INVESTIGATOR: Mee Young Hong, Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, CA 90095

REPORT DATE: April 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

□ Distribution limited to U.S. Government agencies only;
report contains proprietary information

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
Prostate cancer is the second leading cause of cancer deaths in men in the United States. Early prostate cancer is androgen-dependent, but in later stages of the disease androgen-independent tumors arise with an eventual fatal outcome. Red Yeast Rice (RYR) is a traditional food spice consumed throughout Asia and contains a family of monacolins, one of which (monacolin K) is identical to lovastatin, with the ability to inhibit cholesterol synthesis. The objective of the study was to determine whether RYR can inhibit the growth of androgen-dependent and -independent prostate tumors in xenograft and to determine the underlying mechanisms. The study showed that RYR inhibited both androgen-dependent and androgen-independent xenograft prostate tumor volume by downregulation of gene expression involved in androgen synthesis (3β-hydroxysteroid dehydrogenase type 2, aldo-keto reductase family 1 member C3 and steroid 5α reductase type 1) and de novo cholesterol synthesizing enzyme (3-hydroxy-3-methyl-glutaryl CoA reductase) and its response element (sterol response element binding protein-2). RYR also reduced androgen receptor gene expression in androgen-independent xenograft. Currently, gene profile as a function of RYR diet is examined in microarray analysis. This study would establish a proof of principle that would strengthen the biological basis for human trials of RYR extract.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>9</td>
</tr>
<tr>
<td>Conclusion</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>Appendices</td>
<td>10</td>
</tr>
</tbody>
</table>
Introduction
Prostate cancer is the second leading cause of cancer deaths in men in the United States (1). Chinese Red Yeast Rice (RYR) is a traditional food spice consumed throughout Asia (2, 3), and RYR contains a family of monacolins, one of which (monacolin K) is identical in structure to lovastatin, with the ability to inhibit cholesterol synthesis and lower plasma cholesterol levels in humans (4, 5). Since de novo cholesterogenesis is required for tumor growth, RYR may inhibit cancer cell growth. Statins are known to have anti-inflammatory properties (7, 8) and inflammation has been proposed as a critical step in prostate carcinogenesis. We hypothesized that RYR prevent against prostate cancer via cholesterol synthesis inhibition, inflammation or both. The primary specific aim of this proposal was to determine whether RYR can inhibit the growth of the androgen-dependent and –independent prostate tumors in vivo. A secondary specific aim was to determine the mechanisms by which RYR suppresses the growth of androgen dependent and androgen receptor-overexpressing androgen-independent LNCaP tumor xenografts.

Body
Task 1
Androgen-dependence of LNCaP cells and androgen–independence of LNCaP-AR cells were established and the effects of RYR on human prostate cancer cell proliferation in vitro model data has been published in Journal of Medicinal Food 11:657-666, 2008 (Title: Chinese red yeast rice vs lovastatin effects on prostate cancer cells with and without androgen receptor overexpression) (This paper is attached in appendix). Based on the in vitro study, androgen-dependent and –independent prostate cancer xenograft model was set up and the in vivo xenograft study as a function of RYR was carried out. RYR inhibited both androgen dependent LNCaP and androgen-independent LNCaP-AR xenograft tumor volume (Figure 1) (P<0.05).

Figure 1. RYR inhibited androgen-dependent LNCaP and androgen-independent LNCaP-AR xenograft tumor volume (P<0.05).
Serum prostate specific antigen (PSA) levels were lower in RYR group compared to control group in LNCaP-AR xenografted animals (Figure 2) (P<0.05). The levels of monacolin K metabolite (lovastatin hydroxyl acid) were measured in serum by high performance liquid chromatograph (HPLC) and they were the highest in RYR groups compared to lovastatin and control groups (Table 1).

![Figure 2](image)  Serum prostate specific antigen (PSA) levels were lower in RYR group compared to control group in LNCaP-AR xenografted animals (P<0.05).

<table>
<thead>
<tr>
<th>Table 1. Lovastatin hydroxyl acid level in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP Control</td>
</tr>
<tr>
<td>LNCaP Drug</td>
</tr>
<tr>
<td>LNCaP RYR</td>
</tr>
<tr>
<td>LNCaP-AR Control</td>
</tr>
<tr>
<td>LNCaP-AR Drug</td>
</tr>
<tr>
<td>LNCaP-AR RYR</td>
</tr>
</tbody>
</table>

ND: not detected

Task 2
RNAs of xenograft tumors were extracted and the qualities were verified. Then microarray analysis using the tumor RNA was performed using illumine microarray. The data is being analyzed.

Task 3
The gene expressions of androgen-independent tumor related genes (androgen receptor, 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), aldo-keto reductase family 1 member C3 (AKR1C3), steroid 5α reductase type 1 (SRD5A1) were determined as a function of RYR diet. RYR downregulated HSD3B2, AKR1C3 and SRD5A1 genes for androgen synthesis more than two fold in both tumor xenografts (p<0.05) (Figure 3-5). RYR downregulated androgen receptor gene expression only in androgen-independent LNCaP-AR xenografts (P<0.05) (Figure 6).
Figure 3. RYR downregulated 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) gene expression in both LNCaP and LNCaP-AR xenografts (P<0.05).

Figure 4. RYR downregulated aldo-keto reductase family 1 member C3 (AKR1C3) gene expression in both LNCaP and LNCaP-AR xenografts (P<0.05).

Figure 5. RYR downregulated steroid 5α reductase type 1 (SRD5A1) gene expression in both LNCaP and LNCaP-AR xenografts (P<0.05).
Figure 6. RYR downregulated androgen receptor (AR) gene expression in LNCaP-AR xenografts (P<0.05).

Task 4
It was investigated if the effect RYR inhibits the gene expression involved in de novo cholesterogenesis. RYR downregulated HMGCR and SREBP-2 genes compared to control (P<0.05) (Figure 7 and 8).

Figure 7. RYR downregulated 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) gene expression in both LNCaP and LNCaP-AR xenografts (P<0.05).
Figure 8. RYR downregulated sterol response element binding protein-2 (SREBP-2) gene expression in both LNCaP and LNCaP-AR xenografts (P<0.05).

Task 5
RYR effects on gene expression involved in inflammation and oxidative stress were also determined. The oxidative stress and inflammation related genes (NFκB, iNOS2, COX-2, 8-oxodeoxyguanosine, 8-oxoguanine-DNA glycosylase 1, glutathione S-transferase, superoxide dismutase and catalase) were not significantly different among groups (data not shown).

Task 6
One paper has been published in Journal of Medicinal Food and four abstracts have been submitted to scientific conferences. Research data have been presentation in professional conferences. Another manuscript is being prepared for journal submission.

Key research accomplishments
1. For the first time, this study showed RYR inhibited both androgen-dependent LNCaP and androgen-independent LNCaP-AR xenograft tumor volume.

2. This study showed RYR decreased serum prostate specific antigen (PSA) levels in LNCaP-AR xenografted animals.

3. Lovastatin (monacolin K) amount was measured in serum

4. This study demonstrated that RYR downregulated the gene expression of enzymes involved in androgen synthesis in both LNCaP and LNCaP-AR xenografts: androgen 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), aldo-keto reductase family 1 member C3 (AKR1C3) and steroid 5α reductase type 1 (SRD5A1).
5. This study illustrated that RYR downregulated androgen receptor (AR) gene expression in LNCaP-AR xenografts.

6. This study also showed RYR downregulated the gene expression of rate limiting enzyme of cholesterol synthesis and the response element in both LNCaP and LNCaP-AR xenografts: 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) and sterol response element binding protein-2 (SREBP-2) gene expression in both LNCaP and LNCaP-AR xenografts (P<0.05).

7. Part of data of this study has been published in a scientific peer reviewed journal.

8. Some data of this study has been presented in professional national meetings.

9. The data have been used as preliminary data for NIH RO3 grant (PAR-06-313) application.

**Reportable outcomes**

1) **Paper publication**

2) **Abstract submission and research presentation**

3) **Abstract submission and research presentation**

4) **Abstract submission and research presentation**
Conclusion
We were able to demonstrate that Chinese Red Yeast Rice inhibits androgen-dependent and androgen-independent human prostate tumor growth by downregulation of genes involved in in vitro cholesterogenesis and androgen receptor. We will provide more gene expression data based on microarray gene array profile.

Future study
1) Task 1: Measure cell proliferation and apoptosis in tumors using immuno-histochemistry
2) Task 2: Real time PCR to validate the microarray data using quantitative real time PCR analysis
3) Task 6: Paper submission

References

Appendices
1) Paper publication
2) Abstract submission and research presentation

Statin use has been associated with a reduced risk of prostate cancer. Chinese Red Yeast Rice (RYR) is a traditional food spice containing a family of eight monacolins one of which (monacolin K) is identical to lovastatin. The effects of 5% RYR in the diet on the growth of androgen-dependent (LNCaP) and androgen-independent (LNCaP-AR) prostate cancer xenografts in SCID mice over 8 wks was examined. The expression of genes regulating androgen biosynthesis and cholesterogenesis were determined by quantitative real time PCR. RYR inhibited both androgen-dependent and -independent prostate tumor xenograft growth by 54% and 40 %, respectively (P<0.05). RYR downregulated HSD3B2, AKR1C3 and SRD5A1 genes for androgen synthesis more than two fold in both tumor xenografts (p<0.05). RYR also downregulated HMGCR and SREBP-2 genes involved in de novo cholesterogenesis (P<0.05). RYR downregulated androgen receptor gene expression only in androgen-independent xenografts (P<0.05). Androgens are known to increase the growth of prostate cancer xenografts but this is the first study to demonstrate that RYR inhibits tumor growth and gene expression for both cholesterol biosynthesis and androgen biosynthesis. These studies demonstrate one of several possible pathways of RYR action in prostate cancer and suggest potential biomarkers for human studies. Supported by the UCLA CNRU CA 42710 and DOD W81XWH-07-1-0158.

3) Abstract submission and research presentation

Large cohort studies demonstrate that users of cholesterol-lowering drugs have a reduced risk of prostate cancer. Xenograft studies demonstrate that statins can inhibit PCa xenograft growth by depleting lipid raft cholesterol. Chinese Red Yeast Rice (RYR) is a food spice containing a family of monacolins one of which is identical to lovastatin (LV). We have previously demonstrated inhibition of cell proliferation with RYR treatment in human prostate cancer cells in vitro. The present study examined the effects of RYR on growth of androgen-dependent and androgen-independent human prostate cancer xenografts in SCID mice. LNCaP, and androgen-independent LNCaP-AR cells were inoculated subcutaneously in mice receiving LV or 5% RYR diets over 8 wks. RYR inhibited both androgen-dependent and –independent prostate tumor volume by more than 60 % in SCID mice (P<0.01).
RYR showed a more potent effect in reducing tumor size than LV. RYR diet produced lower serum cholesterol and prostate specific antigen levels compared to control and LV in LNCaP-AR xenograft mice (P<0.05). RYR food spice and nutritional strategies for lowering cholesterol are planned to explore the anti-cancer activity of RYR and statins in humans. Supported by UCLA CNRU CA 42710 and DOD W81XWH-07-1-0158.

4) Abstract submission and research presentation

Early prostate cancer is androgen-dependent (AD), but in later stages of the disease androgen-independent (AI) tumors arise with an eventual fatal outcome. RYR contains monacolin K (MK) which is identical to lovastatin, with the ability to inhibit cholesterol synthesis. Since increased cholesterol level in prostate tissues is correlated with its malignancy, we hypothesized that RYR may protect against prostate cancer by inhibiting cancer cell growth via downregulation of de novo cholesterogenesis. Two human prostate cancer cell lines, either AD (LNCaP) or AI (LNCaP-AR), were treated with RYR or MK-free RYR. Cell proliferation and apoptosis were determined using the Cell Titer-Glo Luminescent viability assay and Cell Death Detection ELISA assay, respectively. Transcription levels of 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) and sterol response element binding protein-2 (SREBP-2) were determined by real time PCR. RYR inhibited cell proliferation in both prostate cancer cell lines (p<0.001) and stimulated apoptosis only in LNCaP cells (p<0.01). MK-free RYR showed similar results as the RYR treatment. Mevalonate (end product of HMGCR) treatment reversed the RYR’s anti-proliferative in LNCaP-AR cells but not in LNCaP cells. RYR increased mRNA expression of HMGCR and SREBP-2 (p<0.01) but MK-free RYR decreased expression in both cell lines (p<0.01). This study demonstrated that RYR inhibits cancer cell growth due to its MK component in AI prostate cancer cells, while via its other constituents and MK in AD cells. Funds: UCLA CNRU CA 42710 and DOD CDMRP PC060044.
Chinese Red Yeast Rice Versus Lovastatin Effects on Prostate Cancer Cells With and Without Androgen Receptor Overexpression

Mee Young Hong, Navindra P. Seeram, Yanjun Zhang, and David Heber

Center for Human Nutrition, David Geffen School of Medicine, University of California, Los Angeles, California

ABSTRACT Chinese red yeast rice (RYR), a food herb made by fermenting Monascus purpureus Went yeast on white rice, contains a mixture of eight different monacolins that inhibit cholesterogenesis and also red pigments with antioxidant properties. Monacolin K (MK) is identical to lovastatin (LV). Both LV and RYR contain statins, which could inhibit de novo cholesterogenesis, which is critical to the growth of tumor cells. Dysregulation of the cholesterol biosynthetic pathway has been demonstrated during progression to androgen independence in xenograft models, and it has been proposed that cholesterogenesis and androgen receptor (AR) up-regulation are essential to androgen-independent cell survival. This study was designed to examine the differences between the effects of RYR and LV on androgen-dependent LNCaP cells and androgen-independent cells overexpressing AR (LNCaP-AR). RYR showed more potent inhibition effect on prostate cancer cell growth compared to LV. Both the pigment and monacolin-enriched fractions purified from RYR inhibited proliferation (P < .001) to a lesser extent than intact RYR. While mevalonate, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), restored proliferation in LV-treated cells, it failed to do so in RYR-treated cells. Expression of the HMGCR gene was up-regulated by LV (P < .001) but not RYR in both LNCaP and LNCaP-AR cells. These results suggest that the RYR matrix beyond MK alone may be bioactive in inhibiting androgen-dependent and -independent prostate cancer growth. In vivo studies are needed to further establish the potential advantages of RYR over LV in prostate cancer chemoprevention and in the prevention of the emergence of androgen independence.

KEY WORDS: • Chinese red yeast rice • cholesterogenesis • 3-hydroxy-3-methylglutaryl coenzyme A reductase • lovastatin • monacolins • pigment • prostate cancer

INTRODUCTION

Prostate cancer (PCa) is currently the most common malignancy in men in the United States, comprising 32% of all cancers and remains the second most common cause of cancer death in men in the United States, accounting for 11% of all cancer deaths.1 The early stage of PCa is androgen-dependent and treatable.2-7 However, after successful treatment, the emergence of androgen-independent PCa is common as the result of dysregulated gene expression leading to an adaptive up-regulation of cell survival genes, including the androgen receptor (AR).3,4 These tumors are more difficult to treat, and they lead progressively to metastasis and death.2-7 Therefore, novel approaches are needed to treat advanced androgen-independent PCa in order to reduce overall PCa mortality.

Chinese red yeast rice (RYR) is produced through solid-state fermentation of the yeast Monascus purpureus Went. on white rice.8-13 RYR contains predominantly rice starches and sugars, yeast polyketides (called monacolins), fatty acids, pigments, and condensed tannins.12,13 The major monacolin found in RYR is monacolin K (MK), which is identical in structure to lovastatin (LV). Other polyketides in RYR are structural analogs of MK.13 Monascus pigments comprise more than 10 compounds, six of which are well known: monascin, ankaflavin, monascorubin, rubropunctatin, monascorubramine, and rubropunctamine.14-17 Recently, it has been reported that the pigments have antimicrobial18,19 and anticancer20-22 activities.

LV has been used throughout the world as a prescription cholesterol-lowering drug that can inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR),23-26 which forms mevalonate (MV), a key intermediate in the synthesis of cholesterol.

Our group conducted the first clinical trial of RYR in the United States.27 A dose of 2,400 mg/day RYR, containing 0.4% by weight monacolins, resulted in an 18% decrease in
total cholesterol, a 23% decrease in low-density lipoprotein cholesterol, and a 15% decrease in triglycerol concentrations. De novo cholesterogenesis is required for tumor cell growth and for androgen synthesis. In fact, a recent case-control study reported that hypercholesterolemia was associated with a 50% increase in the risk of PCa, and a growing body of evidence supports the notion that statins, including LV, may inhibit PCa cell growth in animal models and in humans.

The present study was carried out to examine the differences between RYR and LV treatment on PCa cell growth in both the LNCaP cell and in the androgen-independent LNCaP-AR cell lines. The mechanism of action was studied by examining expression of the HMGCR gene and its transcription factor, sterol response element binding protein-2 (SREBP-2), following treatment with LV and RYR.

MATERIALS AND METHODS

Extract and standard preparation

Chinese RYR powder purchased from Botanica Bio-Science (Ojai, CA) was extracted with methylene chloride and evaporated under vacuum at 40°C. The MK concentration of the RYR extract was determined by high-performance liquid chromatography/mass spectrometry analysis (LCQ Classic Finnigan LC-MS/MS Systems, ThermoFinnigan, San Jose, CA) using an authentic standard (AG Scientific, San Diego, CA) as previously reported. For MK-free RYR, endogenous MK in RYR was removed by injecting a sample of the RYR extract onto a Prep-LC 4000 system coupled with a model 490E Programmable Multiwavelength UV detector (Waters Corp., Milford, MA) with conditions as follows: column, Phenomenex (Torrance, CA) Spheri-clone (250 × 21.2 mm × 10 mm), isocratic solvent system of methanol/water (8:2, vol/vol), flow 5 mL/minute, detection λ = 237 nm. To obtain the monacolin-rich fraction (MF-RYR) and pigment-rich fraction (PF-RYR) of RYR, the powdered RYR was extracted with a mixture of dichloromethane and acetone (1:1, vol/vol) solution and purified by silica-gel flash column chromatography, eluting with hexane and acetone (8:2, vol/vol), followed by pure acetone. The purified fractions—PF-RYR and MF-RYR—were 10% and 90% of RYR by weight, respectively.

Cell culture

The LNCaP human PCa cell lines was obtained from American Type Culture Collection (Rockville, MD), and LNCaP-AR cells were a generous gift from Dr. C. Sawyers (University of California Los Angeles, Los Angeles, CA). LNCaP and LNCaP-AR PCa cells were grown in RPMI 1640 medium, and the medium contained 10% fetal bovine serum (Life Technologies, Grand Island, NY) in the presence of 100 U/mL penicillin and 0.1 g/L streptomycin (Life Technologies). Cells were incubated at 37°C with 95% air and 5% CO2. All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

Cell proliferation assay

Proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). When added to cells, the assay reagent produces luminescence in the presence of ATP from viable cells. Cells (5 × 10^3 per well) were seeded in 0.1 mL of the medium in sterile 96-well plates. After 24 hours, the medium was removed and replaced with treatment media. For the LV dose curve, cells were treated with LV (5, 9.3, 20, 40, or 80 μM) for 48 hours. The 5.93 μM LV is equivalent to the MK amount in 50 μg/mL RYR. For the RYR dose experiment, cells were treated with RYR (0–150 μg/mL) for 48 or 72 hours. To test the function of MK in RYR on PCa cell growth, cells were treated with MK-free RYR (0–100 μg/mL) for 48 hours. To compare the effect of whole RYR, MF-RYR, and PF-RYR on cell growth, cells were treated with RYR, MF-RYR (90% of RYR concentration), or PF-RYR (10% of RYR concentration) for 48 hours. MV (Sigma-Aldrich, St. Louis, MO) at 25 μM was used to test if the effect of RYR and its fraction is by de novo cholesterogenesis. All stock solutions of LV, RYR, MF-free RYR, MF-RYR, PF-RYR, and MV were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in medium was <0.2%. At the end of treatment, plates were equilibrated, and then assay reagent was added to each well to induce cell lysis. The luminescence signal and results were read on an Orion Microplate Luminometer (Bertholds Detection Systems, Pforzheim, Germany). All plates had control wells containing medium without cells to obtain a value for background luminescence. Data are expressed as ratio to control (0.2% DMSO), and at least three independent experiments were replicated.

Apoptosis assay

Cells (10^5 per dish) were plated in 60-mm-diameter dishes for 24 hours, and then cells were treated with control (0.2% DMSO), LV (5.93 μM), RYR (50 μg/mL), or MK-free RYR (50 μg/mL) for 48 hours. Following treatments, apoptosis was assessed by measuring DNA fragmentation using the Cell Death Detection enzyme-linked immunosorbent assay ELISAPLUS Assay (Roche, Indianapolis, IN) as previously described. Two replicates per condition were assayed, and data averaged from three or four separate experiments are presented.

RNA extraction and reverse transcription (RT)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified by measuring the absorbance at 260 nm with a Gene Quant Spectrophotometer (Amersham-Pharmacia Biotech, Piscataway, NJ). RT was performed on 3 μg of RNA by using oligo(dT)12-18 primers (Invitrogen, Carlsbad, CA) with SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction (PCR)

Expression of the genes for HMGCR and SREBP-2 was determined using Taqman Universal PCR master mix and
primers (Applied Biosystems, Foster City, CA) by quantitative real-time PCR using the ABI 7900 HT Sequence Detector (Applied Biosystems). The transcription levels of target genes were normalized to r18S expression. Some RT reaction repeated on a separate occasion, followed by PCR and quantitation to confirm the reproducibility of the assay. In addition, every set of RT reactions contains a without-RT negative control to confirm that no contamination or anomaly has occurred.

Statistics

Data for the proliferation, apoptosis, and gene expression were analyzed by Student’s t test or one-way analysis of variance followed by Student-Newman-Keuls test with GraphPad PRISM version 3.0 (GraphPad Software, San Diego, CA).

RESULTS

Cell proliferation

Growth of the human PCa cell line LNCaP (P < .01) and LNCaP-AR (P < .01) cells was inhibited by LV in a dose-dependent manner at 48 hours (Fig. 1A). At a concentration of 5.93 μM, LV decreased prostate tumor cell growth by 20% and 15% in LNCaP and LNCaP-AR, respectively (P < .01) (Fig. 1A). Based on the chemical composition of RYR,
50 μg/mL RYR provides a concentration of 5.93 μM MK, and this led to 47% and 77% inhibition of LNCaP and LNCaP-AR cell growth in 48 and 72 hours of treatments, respectively (P < .001) (Fig. 1B). MK-free RYR treatment still decreased cell proliferation in LNCaP cells and LNCaP-AR cells (P < .05) (Fig. 1C). Addition of 25 μM MV to the medium of cells treated with 5.93 μM LV partially but significantly restored proliferation of LNCaP cells (P < .05) and fully in LNCaP-AR cells (P < .05) (Fig. 2). However, the same concentration of MV had no effect on the antiproliferative activity of RYR in LNCaP cells or LNCaP-AR cells (Fig. 2).

In order to determine which fraction of RYR exhibited the greatest antiproliferative potential, the effects of MF-RYR, PF-RYR, and RYR were compared on tumor cell growth. Both MF-RYR and PF-RYR inhibited cell growth in a dose-dependent manner in both LNCaP and LNCaP-AR PCa cells (P < .001) (Fig. 3). The two purified fractions obtained from RYR each only partially inhibited cell growth by comparison to intact RYR, which was more potent. In-
incubation with 25 μM MV partially abolished the antiproliferative effect of MF-RYR in both cells (P < .05) (Fig. 4A). In contrast, PF-RYR inhibited tumor cell growth regardless the MV treatment (P < .05) (Fig. 4B).

**Apoptosis**

The relative amount of induction of apoptosis was determined using an ELISA-based apoptosis assay, which quantitatively detects fragmented DNA. LV (5.93 μM) enhanced apoptosis in both LNCaP and LNCaP-AR cells by 1.7- and 2.1-fold, respectively (P < .01), and incubation with MV decreased the pro-apoptotic action of LV (Fig. 5A). Apoptosis was increased with RYR treatment at the level of 50 μg/mL in LNCaP (P < .05) (Fig. 5B). Incubation with MV did not decrease apoptosis in LNCaP cells treated with RYR (Fig. 5B). MK-free RYR also induced apoptosis in the LNCaP cells by more than 50% compared to controls (P < .05) but had no effect on the LNCaP-AR cells (Fig. 5C).

**HMGCR and SREBP-2 gene expression**

LV treatment up-regulated the expression of the *HMGCR* gene by more than fivefold in both LNCaP and LNCaP-AR prostate cancer cells (P < .001), but RYR did not (Fig. 6A). Cells treated with LV increased the expression of the *SREBP-2* gene by more than twofold in both LNCaP and LNCaP-AR cells (P < .05) (Fig. 6B). RYR treatment did not increase expression of the *SREBP-2* gene in LNCaP cells (Fig. 6B). In LNCaP-AR cells, RYR enhanced *SREBP-2* expression, but the increased amount was lower than that of LV (P < .05) (Fig. 6B).

**DISCUSSION**

Recent epidemiologic studies suggest a potential protective effect of statins in the patient against the risk of cancer at multiple sites, including the prostate. While some studies show no effect of statins on PCa, a recent, large cohort study showed a substantially reduced risk of metastatic or fatal PCa among statin users, with evidence of decreased risk with increasing duration of use.

Cholesterol is a required intermediate in sex steroid synthesis, and reduction of testosterone precursors may influence the risk of progression and biology of PCa by suppressing steroid hormone production within the PCA cell. While several groups have shown that in men treated with

---

**FIG. 4.** MV effect on MF-RYR- or PF-RYR-treated PCA cell growth. (A) Incubation with 25 μM MV partially abolished the antiproliferative effect of MF-RYR in both cells. (B) In contrast, PF-RYR inhibited tumor cell growth regardless the MV treatment. Control contained 0.2% DMSO. Data are mean ± SEM values (n = 3–6). *Significantly different from control at P < .05. †Significantly different from MF-RYR at P < .05.
agents that modulate serum testosterone, tissue androgen levels are relatively unchanged. There may be effects of statins on intracellular androgen synthesis in the PCa cell. The mechanisms by which prostatic tissue maintains tissue androgens may include metabolism of adrenal androgens or de novo synthesis from cholesterol. Statin may decrease androgen synthesis by reducing the precursor (i.e., cholesterol) of androgen via inhibiting de novo cholesterologenesis in prostate tissue. In patients undergoing androgen deprivation therapy to treat PCa, statins could influence disease progression via effects on residual androgen production, which might help explain the association between PCa progression and statin use. Other mechanisms through which statins may influence PCa severity have also been proposed.
as decreasing prostate-specific antigen in small studies\textsuperscript{41} and increasing prostate epithelial cell sensitivity to apoptosis.\textsuperscript{42}

The AR has been implicated in the development and progression of recurrent PCa, and its expression is frequently up-regulated in androgen-independent PCa.\textsuperscript{43–45} Although variation of expression of AR protein has been correlated with response to androgen deprivation therapy,\textsuperscript{46,47} AR expression appears similar in androgen-dependent and recurrent PCa, but the receptor can be mutated.\textsuperscript{48} When characterized functionally, most of the mutant ARs retain transcriptional activity in response to androgens, and some have altered steroid-binding specificity that changes the spectrum of ligands capable of activating androgen receptor.\textsuperscript{49–51} Therefore, it is likely that up-regulation of the AR contributes to the emergence of androgen-independent PCa by enhancing the response to androgens in the circulation and those synthesized in the PCa cell.

\textit{De novo} cholesterogenesis may be a key target for the prevention of the emergence of androgen-independent PCa. Much convincing evidence indicates that cells manifest a higher flux through the MV pathway when proliferating than when they are in the cell cycle arrest condition; furthermore, tumors undergo deregulated cholesterogenesis mainly at the critical rate-controlling juncture (\textit{i.e.}, the reaction catalyzed by HMGCR). The MV component of the cholesterol biosynthesis plays a key role in controlling cell proliferation by generating prenyl intermediates, particularly farnesyl and geranyl-geranyl moieties.\textsuperscript{52} These isoprenoids covalently modify and thus modulate the biological activity of signal transducing proteins. Therefore, depletion of MV may affect the processing and the transforming activities of growth signals in the prostate cell, androgen biosynthesis, and membrane cholesterol composition.

In the current study, RYR decreased HMGCR expression in both androgen-dependent and -independent PCa cells. However, there was a different effect of RYR on SREBP-2 expression in the two PCa cell lines: RYR increased SREBP-2 in LNCaP-AR but not in LNCaP cells. Since LNCaP-AR cells are androgen independent with overexpression of AR, this may be related to the differential expression of SREBP-2 in LNCaP-AR compared to LNCaP cells. However, we need further research to answer why RYR increased SREBP-2 expression in LNCaP-AR but not in LNCaP cells. SREBP-2 is one of the factors known to affect the transcription of \textit{HMGCR}. Expression of the \textit{HMGCR} gene was not induced with RYR treatment regardless of an-
HONG ET AL.

cholesterogenesis metabolic pathway. On the other hand, the RYR effect on cell proliferation and apoptosis was not affected by addition of MV, even though RYR contained the same range of MK concentrations as the medium containing MK alone. Furthermore, MK-free RYR still inhibited cell proliferation. These data suggest that RYR has an effect on proliferation that is independent of the MK in RYR. A matrix with other structural analogs and other substances including pigments was able to inhibit PCa cell proliferation and stimulate apoptosis. While our studies clearly demonstrate that there are other factors beyond MK mediating some of the effects of RYR, further studies are needed to determine the effects of other active components in RYR, including sterols, isoflavones, and tannins, on PCa cell growth and apoptosis.

In the present study, RYR showed decreased cancer cell proliferation and induced apoptosis in LNCaP cells. It has been reported that LV reduced DNA synthesis by a significant induction of p21WAF1/Cip1 protein expression in vascular smooth muscle cells, which may, in part, explain the potential mechanism of RYR on inhibition of cancer cell growth. Simvastatin potentiates tumor necrosis factor α-induced apoptosis through the down-regulation of nuclear factor κB signaling pathway in squamous cell carcinoma SCC4 cells. This study also showed that statin administration induces apoptosis by increase of caspase-3 and poly(ADP-ribose) polymerase cleavage in cancer cells. In another study, LV decreased AKT protein expression in SCC6 cells, which suggests the involvement of phosphatidylinositol 3-kinase signaling on apoptosis induction. Therefore, RYR, which naturally contains LV, may enhance apoptosis via down-regulation of nuclear factor κB and phosphatidylinositol 3-kinase/AKT signaling as well as via induction of caspase-3 and poly(ADP-ribose) polymerase.

Interestingly, there was a difference in the apoptotic sensitivity in LNCaP cells compared to LNCaP-AR. RYR had no effect on apoptosis in the LNCaP-AR cells. We speculate that LNCaP-AR cells are an advanced type of PCa cells so that they may be resistant to the apoptotic process. RYR decreased advanced androgen-independent prostate tumor cell growth mainly by inhibition of cell proliferation rather than induction of apoptosis.

We have recently showed that food components can alter the expression of AR gene and genes involved in androgen synthesis. Therefore, RYR may down-regulate gene expression of AR- and androgen-synthesizing enzymes, which contribute to attenuation of the risk of advanced PCa. The present study obviously supports the inhibition effect of RYR on growth of LNCaP-AR PCa cells, which indicates the potential use of RYR as an anticancer agent against advanced-stage PCa.

RYR, a traditional Chinese food herb and a modern dietary supplement, has demonstrated in vitro effects including stronger inhibition of tumor cell growth compared to LV treatment in human androgen-dependent and -independent prostate cancer cells. Furthermore, LV increased expression of the gene for HMGCR, while RYR did not. The advantage of using RYR over LV, which is a drug, is that RYR decreases the cholesterol level without elevation of expression of the gene for HMGCR. The multiple effects of RYR in vitro suggest that further investigations in animal models and ultimately in humans to confirm the anticancer activity of RYR are warranted.

ACKNOWLEDGMENTS

We thank Dr. Simin Liu for allowing us to use his real-time PCR equipment. This study was funded by UCLA/NCI Clinical Nutrition Research Unit grant CA 42710. This work was also funded by grant W81XWH-07-1-0158 from the Department of Defense (Principal Investigator: M.Y.H.).

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES


