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A Novel Flavonoid Composition Targets Androgen Receptor Signaling and Inhibits Prostate Cancer Growth in Preclinical Models[1,2]

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Abstract

The high prevalence and long latency period of prostate cancer (PCa) provide a unique opportunity to control disease progression with dietary and nutraceutical approaches. We developed ProFine, a standardized composition of luteolin, quercetin, and kaempferol, and investigated its potential as a nutraceutical for PCa in preclinical models. The three ingredients of ProFine demonstrated synergistic *in vitro* cytotoxicity and effectively induced apoptosis in PCa cells. ProFine markedly affected the transcriptome of PCa cells, suppressed the expression of androgen receptor, and inhibited androgen-regulated genes. Oral administration of ProFine did not exhibit obvious toxicities in mice, and the three ingredients retained their individual pharmacokinetic and bioavailability profiles. Importantly, ProFine significantly retarded the growth of PCa xenografts in athymic nude mice and extended the survival of animals. This study provides preclinical evidence supporting the promise of ProFine as a safe, efficacious, and affordable intervention to control PCa progression and improve clinical outcomes.

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Introduction

Prostate cancer (PCa) is the most common nonskin cancer in American men, with a lifetime risk for diagnosis of approximately 15.9%. It is estimated that 164,690 new cases are diagnosed and 29,430 patients die in 2018 [1]. Most cases of PCa are low risk and have a good prognosis, even without any treatment. Nonetheless, about 30% of PCa patients harbor a higher-grade cancer and eventually progress to metastatic and castration-resistant status, which has no cure [2]. Currently available therapies can only extend the median survival by approximately 3 months. These expensive treatments (usually ranging from $21,500 to $93,000 for a typical course of treatment) pose a huge burden on patients, their families, and the healthcare system.

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The high prevalence and long latency period of PCa provide a unique opportunity to control disease progression, reduce mortality, and improve the quality of life of patients using dietary or nutraceutical approaches [3]. Numerous epidemiologic studies have indicated an important role of diet in PCa progression and therapeutic response, and dietary management of PCa is being actively pursued due to low dose-limiting toxicities and negligible side effects [4]. Promising efficacy has been reported in several trials. For example, in a double-blind, placebo-controlled randomized study, an oral capsule (Pome-T) containing a blend of pomegranate, green tea, broccoli, and turmeric demonstrated a significant short-term and favorable effect on the median prostate-specific antigen (PSA) levels in PCa patients [5]. Despite these encouraging clinical results, however, most studies using dietary supplements still suffer from low patient number, short treatment duration, and absence of proper placebo control. Importantly, the lack of standardized formulations and nonspecific effects of dietary supplements make it difficult to validate and compare their clinical efficacy among various trials. Therefore, a nutraceutical with defined composition and potent anticancer activity is highly desired to provide a safe, efficacious, and affordable therapy for early-stage and low-risk PCa.

Luteolin, quercetin, and kaempferol are among the most common flavonoids found in plants, including some vegetables and fruits that have been thought to have anticancer benefits, such as onions, olives, grapes, tea, pomegranate, broccoli, and cauliflower [6]. Epidemiological evidence has associated the dietary consumption of these flavonoids with reduced risk of developing various diseases, including cancer [7]. Molecular studies demonstrated that luteolin, quercetin, and kaempferol have diverse pharmacological activities, including antioxidant, anti-inflammatory, and anticancer effects [8]. Despite a large body of epidemiological and preclinical evidence suggesting potential preventative and therapeutic benefits of flavonoids in human cancers, very few clinical trials have been or are being conducted using pure flavonoid compounds or defined compositions. To provide a standardized formulation of luteolin, quercetin, and kaempferol for clinical evaluation of their therapeutic efficacy in PCa patients, we developed ProFine, a unique combination of the three flavonoids at a specific ratio. In this study, we determined the *in vitro* and *in vivo* activities of ProFine in preclinical models of PCa and investigated the mechanism of action of ProFine against PCa progression.

Materials and Methods

Composition of ProFine

For *in vitro* studies, ProFine was prepared as a stock solution of 100 mg/ml, containing 24.68 mg/ml luteolin, 26.06 mg/ml quercetin, and 49.35 mg/ml kaempferol in 100% dimethyl sulfoxide (DMSO). The composition of ProFine formulation for oral gavage administration includes inactive ingredients hydroxypropyl methylcellulose (50%, w/v), corn oil (35%, v/v), Tween 80 (5%, v/v), and ethanol (10%, v/v). Ultrasonication was used to form a yellow-colored, well-dispersed colloid.

Microarray and Gene Set Enrichment Analysis (GSEA)

Total RNAs from triplicate preparations of ProFine- and DMSO-treated C4-2 cells as well as reference total RNA samples were amplified and hybridized to Agilent 44 K whole human genome expression oligonucleotide microarray slides. Spots of poor quality or average intensity levels <300 were removed from further analysis. Analysis of Microarrays (SAM) program was used to analyze expression differences between groups using unpaired, two-sample *t* tests and controlled for multiple testing by estimation of *q*-values using the false discovery rate method. The genes were ranked according to their *t* test scores and used to conduct GSEA to estimate pathway enrichment. We utilized the Hallmark pathways from within the MSigDBv6.0.

In Vivo Efficacy of Oral ProFine in PCa Xenograft Models

All animal procedures were approved by Augusta University Institutional Animal Care and Use Committee. For the subcutaneous (s.c.) model, male athymic nude mice (5 weeks; Harlan Laboratories) were randomly divided into three groups ($n = 5$ in control group, $n = 5$ in 100 mg/kg ProFine group, $n = 7$ in 200 mg/kg ProFine group). A total of 2×10^6 C4-2-luc cells were mixed with Matrigel and inoculated subcutaneously into two flanks of each mouse. Twenty-two days following tumor inoculation, mice were treated with ProFine (100 mg/kg or 200 mg/kg) or vehicle control, three times per week, *via* oral gavage. Tumors were measured three times per week using a caliper, and tumor volume was calculated using the formula: $(\text{width})^2 \times \text{length}/2$. Bioluminescence imaging of s.c. C4-2-Luc tumors was also performed.

For the intratibial model, male athymic nude mice (5 weeks) were randomized and evenly divided into two groups ($n = 8$ in control group, $n = 9$ in ProFine group). For each mouse, a total of 2.0×10^6 C4-2 cells were inoculated into bilateral tibia. Tumor-bearing mice were treated with ProFine (100 mg/kg) or vehicle control, three times per week, *via* oral gavage. Mice were weighed twice per week, and tumor growth in bilateral tibia was followed by serum PSA once a week. At the end point, X-ray radiography was performed using MX-20 System (Faxitron, Tucson, AZ).

Statistics

Two-way analysis of variance was performed to test the overall difference across the control and treatment groups during the entire study period. The effects on animal survival were determined by log-rank survival test. Errors are SE values of averaged results, and values of $P < .05$ were taken as a significant difference between means. All *in vitro* data represent three or more experiments. GraphPad Prism 7.0 program (GraphPad Software Inc., La Jolla, CA) was used to perform the statistical analyses.

Results

In Vitro Cytotoxicity of ProFine in PCa Cells

ProFine is a defined composition consisting of luteolin, quercetin, and kaempferol at the molar ratio of 1:1:2. *In vitro* cytotoxicity assays found that, as single compounds, luteolin, quercetin, and kaempferol have weak to modest activities in PCa cells. For example, the half minimal inhibitory concentration (IC_{50}) of luteolin, quercetin, and kaempferol in androgen receptor (AR)-positive C4-2 cells is 114.02, 55.25, and 157.81 μM, respectively. In comparison, ProFine exhibited enhanced cytotoxicity compared to any of the three individual components, with the IC_{50} of 16.56 μg/ml in C4-2 cells (equivalent to 14.28, 14.28, and 28.60 μM of luteolin, quercetin, and kaempferol, respectively) (Figure 1*A*). Indeed, isobologram analysis showed that the combination index (CI) achieved as low as 0.11 when the three ingredients were used at low concentrations, indicating a strong synergy among them (Figure 1*B*; Table S1). Fluorescence-activated cell sorting analysis demonstrated that ProFine