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Genomic and *in vivo* evidence of synergy of a herbal extract compared to its most active ingredient: *Rabdosia rubescens* vs. oridonin

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Abstract. *Rabdosia rubescens* is a herbal root extract of traditional Chinese medicine (TCM) used to treat inflammatory diseases and oral cancers. A key principle of TCM is that multiple ingredients in a plant extract are more effective and less toxic than a single purified active ingredient or a purified drug derived from a plant product. *Rabdosia rubescens* extract (RRE) contains terpenoids and flavonoids, but the most active ingredient within the extract attributed to the inhibition of cancer is the kaurene diterpene, oridonin. In order to research synergy with a complete plant extract, the effects of RRE on the inhibition of prostate cancer cell proliferation were compared to the effects of pure oridonin alone *in vitro*. Three groups of 8 SCID mice bearing human prostate cancer xenografts (LAPC-4) were administered either RRE containing 0.02 mg/g oridonin, pure oridonin at a dose of 0.02 mg/g, or pure oridonin at a dose of 0.1 mg/g, by gavage for 5 days/week for 4 weeks. RRE and pure oridonin at 0.1 mg/g inhibited tumor growth to a similar extent, while oridonin at a dose of 0.02 mg/g did not. Therefore, in comparison to RRE, five times more pure oridonin was required to obtain equivalent prostate xenograft growth inhibition. Since the nuclear factor-κB signaling pathway and inflammation are implicated in prostate carcinogenesis, gene microarray analysis was conducted and demonstrated activation of genes by RRE that was not evident with oridonin treatment alone. This study demonstrated that genomic methods and xenograft studies are capable of demonstrating the benefits of the synergy of whole plant extracts rather than active ingredients isolated and purified as drugs.

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Introduction

Botanical extracts are used for the prevention and treatment of common conditions by 80% of the world's population according to estimates made by the World Health Organization (1). Drugs are produced from botanical sources by isolation and purification of the most active ingredients, while other substances in the raw fraction are separated. Synergistic interactions of mixtures of bioactive constituents and related substances or analogs in plant extracts have been proposed to occur with the most active ingredient. These interactions help explain the improved effectiveness of extracts containing multiple ingredients compared to drugs developed from single constituents.

We previously studied the action and metabolism of Chinese red yeast rice (*Monascus purpureus Went*) (CRYR), a dietary supplement containing monacolins, one of which (Monacolin K) is identical in structure to the statin drug lovastatin, and to unsaturated fatty acids and phytosterols capable of lowering low-density lipoprotein (LDL) cholesterol in humans (2-5). The apparent bioactivity of CRYR containing 6 mg of Monacolin K was equivalent to 20 mg of purified lovastatin. Moreover, CRYR is well tolerated in patients who are intolerant to statin drugs (6), suggesting that the lower amounts of active substances, enhanced action and more complete metabolism in comparison to a drug made from a single constituent may prevent adverse muscle side effects. Our group previously demonstrated similar interactions for phytochemicals in cranberry, pomegranate and green tea (7-9). In studies demonstrating synergistic interactions of a mixture of herbs against prostate cancer by isobolographic analysis, *Rabdosia rubescens* extract (RRE) was found to be particularly active among five herbs in a PC-SPES mixture (10). Purified oridonin was shown to be active against a number of different types of cancer (11), which motivated the present study, designed to demonstrate the interaction of multiple components in RRE in comparison to purified oridonin. In order to understand the mechanism of the observed synergistic interactions, we conducted gene microarray analysis of the nuclear factor-κB (NF-κB) pathway, which is implicated in prostate carcinogenesis, in order to identify differential gene expression as a result of treatment with the plant extract compared to the most active component of the plant extract.

Materials and methods

Preparation of Rabdosia rubescens extract. An oridonin-enriched extract of *Rabdosia rubescens* (Henan, China) from the aerial part of the plant was standardized to 4% oridonin using methods established at the UCLA Center for Human Nutrition. RRE was administered to animals at doses based on the average amount of oridonin contained in a single dosage of Donglingcao, a tablet currently used in China for human consumption. The equivalent dose to that administered to a 70-kg human was calculated to be 0.5 mg of oridonin for a mouse with a body weight of 25 g. For RRE, which contains 4% oridonin, the dose to be administered was calculated to deliver the same amount of oridonin as above and was determined to be 10.4 mg per 25-g mouse. Both oridonin and RRE were suspended in 200 μl of water with 1% carboxymethylcellulose.

Cell culture. DU-145, CWR22Rv1, LNCaP and PC-3 prostate cancer cells were purchased from the American Type Tissue Culture Collection (Rockville, MD, USA) and maintained in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37˚C. Confluent cells (70-80%) were treated with oridonin at 10-100 μg/ml for 48 h, dissolved in DMSO and mixed with complete cell medium. The final concentration of DMSO used was 0.1% (vol/vol).

Cell viability (MTT) assay. The effect of oridonin on the viability of cells was determined based on the uptake of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by measuring the absorbance at 540 nm in a UV spectrophotometer. Cells were plated at a density of 10,000 cells/well in 200 μl of complete culture medium containing 10-100 μg/ml concentrations of oridonin in 96-well microtiter plates for 48 h. After incubation for specified times at 37˚C in a humidified incubator, MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for 2 h, after which the plate was centrifuged at 1,800 x g for 5 min at 4˚C. The absorbance at 540 nm was measured with a microplate reader. The effect of oridonin on growth inhibition was assessed as the percentage of cell viability, where 0.1% DMSO-treated cells were deemed 100% viable. DMSO at the concentrations used did not affect cell viability.

In vivo tumor xenograft animal model. The UCLA Animal Research Committee approved all animal experimental procedures. Male SCID mice (Taconic Farm, Germantown, NY, USA) were bred in a pathogen-free colony and housed in groups of 4 per cage under pathogen-free conditions with a 12-h light-dark cycle. The animals were fed an autoclaved diet *ad libitum* of sterilized food pellets and water. A total of 24 SCID mice (Taconic Farms) were injected subcutaneously at 5 weeks of age with $2x10^5$ androgen-dependent LAPC4 prostate cancer cells (a gift from Charles Sawyers). Mice were divided into four groups consisting of 6 animals each and were administered the two doses of oridonin, RRE or water alone (control) by gavage 5 days/week for 4 weeks. Tumor size was measured with calipers three times a week starting on day 7. After 4 weeks, the mice were sacrificed, and both serum samples and tumor tissues were harvested. Tumor volume was calculated by the formula 0.5238 x L1 x L2 x H, where L1 is the long diameter, L2 is the short diameter and H is the height of the tumor.

Transfection and NF-κB luciferase assay. Cells were transfected using Effectene transfection reagent (Qiagen). SBE luciferase reporter gene plasmid was obtained from Panomics Inc. (Fremont, CA, USA). SBE (SBE_{3X}-Lux) luciferase reporter constructs were used to monitor NF-κB transactivation with a vector containing multiple repeat-specific consensus binding sites. The Renilla luciferase vector pRL-CMV (Promega) was co-transfected with the NF-κB reporter vector as a control to assess transfection efficiency. Twelve hours after transfection, cells were subjected to treatment with either oridonin or RRE at various doses. Cells were then harvested, and luminescence was measured in a Turner $20/20^n$ single-tube luminometer (Turner Biosystems, Sunnyvale, CA, USA).

RNA isolation and PCR arrays. Total RNA was isolated from 100 mg of tumor tissue using TRI Reagent according to the manufacturer's instructions. RNA concentration and purity were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). RNA quality was required to have absorbance ratios at 260 nM compared to 280 nM ratios (nucleic acid/protein ratio) >2.0 and 260/230 nM ratios (estimate of organic compound contamination) >2.0. RNA (5 μg) was treated with the RT^2Nano PreAmp cDNA Synthesis kit (SA Biosciences, Frederick, MD, USA) according to the manufacturer's instructions in order to generate cDNA and pre-amplify the cDNA template. Briefly, after genomic DNA elimination, the reverse transcription reaction was performed at 42˚C for 15 min and then heated at 95˚C for 5 min to inactivate the enzyme. The cDNA was then pre-amplified and mixed with RT^2 SYBR Green/ROX qPCR master mix (SA Biosciences). Aliquots (25 μl) were loaded into each well of a Human NF-κB Signaling PCR-array (catalog #PAHS-025; SA Biosciences) according to the manufacturer's instructions on a One Step Plus real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Conditions for amplification were as follows: 1 cycle of 10 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. A dissociation curve from 65 to 95˚C was performed on each plate immediately after the PCR run to determine the quality of the specific products amplified in each well. Dissociation curves with multiple peaks were not included in the analysis.

The PCR array data were analyzed by the ΔΔCt method. Genes with Ct values >35 cycles were considered as non-detectable and assigned a value of 35. The average of two housekeeping genes [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB)] was used to obtain the ΔCt value for each gene of interest. The ΔΔCt value for each gene was calculated by the difference between the ΔCt of the treated and the control groups. The fold-change for each gene was calculated by $2^{\Delta\Delta Ct}$, and statistical analysis to determine differences between treatments was performed using the RT^2 Profiler PCR Array Data Analysis web-based software from the SA Bioscience website (http://www.sabiosciences.com/pcrarraydataanalysis.php).

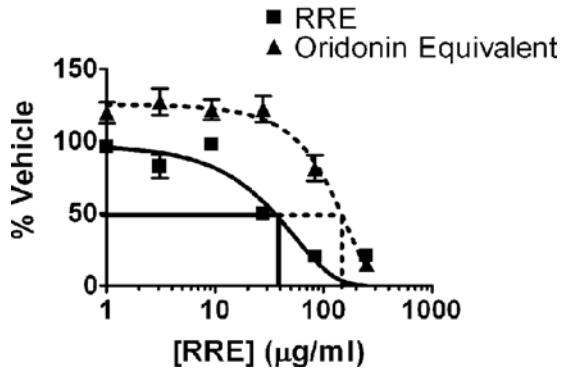

Figure 1. *Rabdosia rubescens* extract (0.02 mg oridonin equivalent) inhibits the growth of prostate cancer cell lines more potently than oridonin (0.02 mg/g). Data with the 22Rv1 cell line are shown as an example of this inhibition. Each point represents the average of three triplicate wells ± SD.

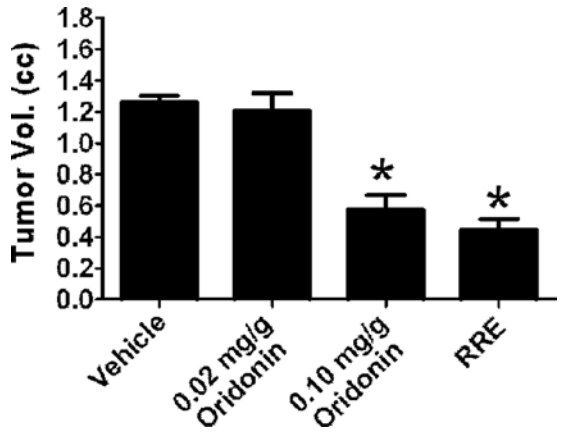

Figure 2. Oridonin at 0.1 mg/g must have the same activity as RRE (0.02 mg/g of oridonin equivalent) to inhibit the growth of LAPC-4 xenograft prostate tumors in SCID mice. Each bar represents the average tumor volume of 8 animals ± SD (*p<0.0001 compared to the vehicle).

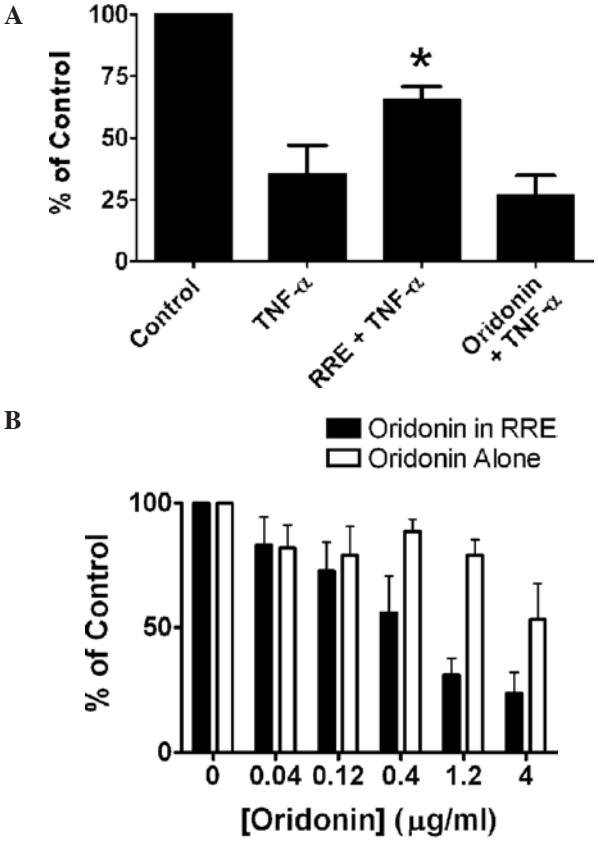

Figure 3. (A) RRE (0.02 mg/g oridonin equivalent) stabilizes IκB-α protein from TNF-α-induced IκB-α degradation in DU-145 cells. Each bar represents the average quantification (relative to the untreated control) of three separate Western blot experiments ± SD (*p<0.05 compared to the TNF-α-treated control). (B) RRE inhibits TNF-α-induced activation of an NF-κB luciferase reporter vector. Each bar represents the average of three triplicate wells ± SD.

Statistical methods. For the cell proliferation assays, data were expressed as a percentage of untreated cells (i.e., treatment value-blank/vehicle value-blank) with the mean ± SE of at least three separate experiments. Statistical methods included ANOVA and Fisher *post-hoc* analysis. For PCR arrays, gene expression was calculated as fold-change relative to the average expression in the vehicle control. Differences were evaluated by ANOVA followed by a pairwise t-test. The level of statistical significance was set at $p<0.05$.

Results

Oridonin in Rabdosia rubescens inhibits prostate cancer cells more potently than oridonin alone. Both RRE and pure oridonin inhibited the proliferation of DU-145, PC3, LNCaP and 22Rv1 prostate cancer cells *in vitro*. As noted with the 22Rv1 cell line (Fig. 1), treatment with RRE (10-100 μg/ml for 24 h) or oridonin (50-200 μg/ml or 20-80 μmol for 24 h) resulted in a dose-dependent inhibition of cell growth in all four prostate cancer cell lines. We characterized our RRE and determined that it contained 4% oridonin. Therefore, oridonin plus other compounds in RRE were more potent than oridonin alone in inhibiting prostate cancer cell growth *in vitro*. The less aggressive cell lines, 22Rv1 and LNCaP, were much more sensitive to the inhibitory effects of oridonin than the more aggressive DU-145 and PC3 cell lines (data not shown).

In the absence of RRE, five times the oridonin concentration is required to inhibit prostate xenograft tumor growth. Treatment with RRE significantly inhibited tumor growth in SCID mice implanted with LAPC-4 prostate cancer cells. However, 0.02 mg/g oridonin failed to inhibit tumor xenograft growth. When animals were administered oridonin at 0.1 mg/g, tumor growth was inhibited to a similar degree as with administration of RRE (0.02 mg/g oridonin equivalent). Tumor volume in mice treated with 0.1 mg/g of oridonin was 0.600±0.295 mm^3 compared to 1.261±0.104 mm^3 in control animals and 0.440±0.182 mm^3 in animals treated with RRE (Fig. 2). The tumor latency period was prolonged to 14 days in animals receiving 0.1 mg/g oridonin and to 16 days in animals receiving RRE.

Oridonin in RRE is more effective at inhibiting the NF-κB pathway than oridonin alone. Using IκB-α protein disappearance as an indication of activation of the NF-κB pathway, the effects of RRE or an equivalent oridonin dose alone on the

Table I. Fold change in LAPC-4 xenograft tumors between control (PBS)- vs. oridonin- or RRE-treated animals.

Gene symbol	Gene name	Oridonin	RRE
ATF1	Activating transcription factor 1		2.9
BIRC2	Baculoviral IAP repeat-containing 2	2.1	
NOD1	Nucleotide-binding oligomerization domain containing 1	2.3	2.3
CFLAR	CASP8 and FADD-like apoptosis regulator	1.6	1.8
EDARADD	EDAR-associated death domain	1.3	1.6
ELK1	ELK1, member of ETS oncogene family		1.7
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog		2.8
GJA1	Gap junction protein, α1, 43 kDa	1.6	1.4
HMOX1	Heme oxygenase (decycling) 1		1.8
HTR2B	5-Hydroxytryptamine (serotonin) receptor 2B	2.6	2.7
IFNG	Interferon-γ	-2.6	
IKBKG	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase γ		1.6
IL1A	Interleukin 1α	2.2	
IL1R1	Interleukin 1 receptor, type I	1.7	1.8
IL8	Interleukin 8		2.8
LTA	Lymphotoxin α (TNF superfamily, member 1)		1.9
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	2.8	3.4
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	2.7	2.6
PPM1A	Protein phosphatase 1A (formerly 2C), magnesium-dependent, α isoform	1.7	1.6
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	2.7	2.6
TRIM13	Tripartite motif-containing 13	1.3	1.4
TICAM2	Toll-like receptor adaptor molecule 2	2.0	2.1
TLR3	Toll-like receptor 3	2.7	3.4

stabilization of IκB-α protein in TNF-α-treated DU-145 cells was examined. A 15-min incubation with 5 ng/ml of TNF-α was sufficient to degrade ~70% of IκB-α protein in DU-145 cells. RRE, but not pure oridonin in the amounts found in RRE, abrogated the effect of TNF-α to cause a decrease in IκB-α protein at 4 h (Fig. 3A).

To further verify that the NF-κB pathway was inhibited by RRE, we examined the ability of RRE or an equivalent dose of oridonin alone to inhibit TNF-α-induced NF-κB transactivation in DU-145 PCa cells. The cells were first transfected with either a null vector or a luciferase reporter gene containing NF-κB binding elements (NBE_{3X}-Lux), and the luciferase signal was used as an index of NF-κB transactivation. Cells were treated with TNF-α alone, or pre-treated with RRE or an equivalent concentration of oridonin alone for 2 h before exposure to TNF-α, and the luciferase signal was monitored and normalized to Renilla luciferase. Use of the null vector did not result in spontaneous activation (data not shown). Conversely, TNF-α was capable of inducing a very robust increase in luciferase reporter activity at all concentrations, ranging from 0.5 to 50 ng/ml at 2 h. When cells were pre-treated with RRE before exposure to TNF-α, NF-κB transactivation activity was significantly reduced (Fig. 3B). Equivalent concentrations of oridonin alone had little or no effect on NF-κB activity (Fig. 3B). MTT assays were used to confirm that the inhibitory effects were not due to toxicity of the treatments (data not shown).

Gene microarray analysis of the NF-κB pathway shows up-regulation of inflammatory and oxidative stress genes unique to RRE. Gene microarrays were used to determine differences between the regulation of NF-κB pathway genes by oridonin vs. RRE in the LAPC-4 xenograft tumors. Oridonin treatment alone (0.02 and 0.1 mg/g) induced the transcription of 16 genes (Table I); three of these were unique to oridonin. The three genes expressed solely after oridonin treatment included BIRC2, interleukin 1α and LTBR. The majority of the 16 genes expressed in response to oridonin are known to play a role in inflammation and cell death. Also unique to oridonin was the down-regulation of interferon-γ (IFNG) gene expression. By comparison, RRE treatment increased the expression of 23 different genes, six of which were unique to RRE (Table I). These included ATF1, ELK1, v-FOS, HMOX1, LTA and IKBKG, which are genes controlling inflammation and oxidative stress.

Discussion

Rabdosia rubescens (aka Donglingcao) is a traditional Chinese medicine used to treat oral cancer. Oridonin, the most biologically active molecule purified from RRE, has been studied extensively for its effects on breast cancer (12,13), leukemia (14), cervical cancer (15), melanoma (16-18) and prostate cancer (19). Oridonin has previously been shown to inhibit the proliferation of a wide variety of human cancer cells,

including prostate (LNCaP, DU-145 and PC3), breast (MCF-7 and MDA-MB-231) and non-small cell lung (NCI-H520, NCI-H460 and NCI-H1299) cancers, acute promyelocytic leukemia (NB4) and glioblastoma multiforme (U118 and U138) (11). In the aggressive HT1080 fibrosarcoma cell line, oridonin has been shown to induce apoptosis through a p53-mediated mechanism that is regulated by NF-κB (20). Another study with the human melanoma A375-S2 cell line also demonstrated that oridonin acts via a p53-mediated mechanism inhibited by blocking PI3-K pathway activation (21).

Our microarray results suggest that the enhanced inhibition of RRE to reduce prostate xenograft tumor growth may occur via the up-regulation of genes in the inflammatory pathway (Table I). This suggests that RRE may have affected the immune response to the xenografts by recruiting immune cells to the tumor site. Supporting a possible involvement of oridonin in regulating immune cells and immune system, a previous study showed that oridonin enhanced the phagocytosis of apoptotic U937 cells by macrophage-like U937 cells through the release of TNF-α and IL-1β (22).

Synergy research has, as its main aim, the establishment of a scientific basis for the therapeutic superiority of plant extracts from traditional medicines, fruits, vegetables and grains, as compared to single constituents isolated and purified as drugs to maximize potency. Synergistic effects of the mixtures of bioactive constituents and their byproducts contained in plant extracts may account for the apparent enhanced potency of plant extracts compared to individual constituents (23,24). However, the mechanisms underlying this synergy remain to be established. We used gene expression arrays to uncover targets of RRE that were not activated by oridonin. Future research on synergy utilizing genomics, proteomics and systems biology may provide the impetus for the development of new botanical dietary supplements that fulfill the necessary quality, safety and efficacy standards while maximizing efficacy and minimizing potential toxicity.

Acknowledgements

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