Determination of Rottlerin in Pancreatic Cancer Cells and Mouse Xenografts by RP-HPLC Method

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Abstract

Rottlerin is a natural polyphenolic ketone isolated from the pericarps of Mallotus philippinensis. In previous studies we showed that parenteral administration of rottlerin reduced tumor growth in murine xenograft models of pancreatic cancer. The aim of this study was to develop a simple and validated method for the quantitative determination of rottlerin in plasma and tumor tissues of mice fed a rottlerin diet. A xenograft model of pancreatic cancer was prepared by injection of 2×10⁶ HPAF-II cells subcutaneously into nude mice. One week before tumor implantation, mice were randomly allocated to standard diet (AIN76A) and standard diet supplement with 0.012% rottlerin (n=6 per group). Mice were sacrificed after 6 weeks on diets. Rottlerin was extracted from the plasma and tissues using protein precipitation-extraction and analyzed by reverse-phase HPLC-DAD method. The same HPLC method was also applied to determine rottlerin levels in conditioned culture media and in cell lysates from HPAF-II cells exposed to 25 μM concentration of rottlerin. A substantial amount of rottlerin was detected in tumor (2.11 ± 0.25 nmol/g tissue) and plasma (2.88 ± 0.41 μM) in mice fed rottlerin diet. In addition, significant levels of rottlerin (57.4 ± 5.4 nmol/mg protein) were detected in cell lysates from rottlerin-treated HPAF-II cells. These data indicate that rottlerin is efficiently absorbed in cells and tissues both in vivo and in vitro and suggest a strong potential for rottlerin as a preventive or adjuvant supplement for pancreatic cancer.

Keywords: Rottlerin; Tissue distribution; HPLC; Pancreatic cancer; In vivo; Cell uptake

Introduction

Rottlerin (1-[6-[[3-acetyl-2,4,6-trihydroxy-5-methylphenyl]methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzo[γpyran-8-yl]-3-phenyl-2-propen-1-one, also known as mallotoxin) is a natural polyphenolic ketone (Figure 1) isolated from the pericarps of Mallotus philippinensis (common names Monkey-puzzle, Monkey Face Tree, Kamala Tree). It is a traditional Indian medicine that is used against tapeworm, scabies, and herpetic ringworm. Recent scientific research has demonstrated that rottlerin has a range of molecular targets and anti-tumor activities, such as cell growth suppression [1], apoptosis [2], anti-angiogenesis [3] and inhibition of reactive oxygen species formation [4]. Rottlerin is most well-known as an inhibitor of protein kinase C (PKC) with selectivity for PKC δ [5]. It is also a mitochondrial uncoupler that depolarizes the mitochondria membrane potential, reduces cellular ATP levels and activates 5'-AMP activated protein kinase (AMPK) and affects the mitochondrial production of reactive oxygen species [6,7]. Moreover, rottlerin can target many key regulatory kinases including p38 regulated / activated kinase, cAMP-dependent protein kinase, casein kinase II, glycogen synthase, kinase 3-beta, AKT/PKB, and calmodulin-dependent kinases [8].

Our research team demonstrated recently that rottlerin at concentration range of 2.5-10 μM has potent proapoptotic and antitumor activities in pancreatic cancer in vitro, which is mediated by disrupting the interaction between prosurvival Bcl-2 proteins and proapoptotic BH3-only proteins [2]. Importantly, we showed rottlerin significantly reduced tumor growth in both pancreatic cancer subcutaneous and orthotopic xenograft murine models [2]. Thus rottlerin may represent a promising novel agent for the treatment of pancreatic and other cancers. However, the bioavailability of this natural compound in experimental animals has not been reported. The aims of this study were to develop a simple and validated HPLC-UV method for the quantitative determination of rottlerin in plasma, liver, pancreas and tumor tissues of mice fed with a rottlerin diet, and for the examination of rottlerin uptake by cultured pancreatic cancer cells after cells were exposed to rottlerin.

Materials and Methods

Reagents and chemicals

Rottlerin (96% purity), butylhydroxytoluene (BHT), ascorbic acid, butylhydroxyanisole (BHA), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic acid, butylhydroxytoluene (BHT), ascorbic aci

Figure 1: Chemical structure of rottlerin.
from Invitrogen, Carlsbad, CA, USA) Cultures were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin mix solution and 11.0 μg/mL sodium pyruvate (all from Fisher Scientific, Pittsburg, PA, USA). All solvents used were HPLC grade medium. After incubation, cells and conditioned media were collected and analyzed as indicated in the following sections.

**Sample preparation**

For the analysis of rottlerin in plasma, 10 μL of 3, 3', 4'-trihydroxyflavone (IS, 100 μM), and 200 μL acetone containing 1% of BHT were added to 100 μL of plasma. The resulting mixture was thoroughly vortex-mixed for 2 min and then centrifuged at 1,300×g for 5 min. The precipitate was extracted one more time with acetone. Supernatants were combined and subjected to HPLC analysis. The precipitate was dried, weighed and dissolved in protein lysis buffer for protein concentration measurement using 2D Quant kit (Amersham, Piscataway, NJ, USA).

Aliquots of 100 μL cell culture medium were taken at 0 (control), 0.5, 1, 3, 6 and 24 h during cell incubation with rottlerin. Aliquots were mixed with the same volume of acetone, centrifuged and an aliquot of each supernatant was injected into the HPLC.

**Stability of rottlerin in mouse plasma and liver homogenates**

An aliquot of rottlerin (20 μL, 80 μg/mL) and IS were added to 500 μL pooled blank plasma. The resulting plasma was equally divided into 5 samples. Mouse liver (0.374 g) was ground in buffer containing 1% ascorbic acid and the homogenate was equally divided into 4 samples. Rottlerin (20 μL, 16 μg/mL) was added to each sample. Samples were incubated at 37°C for 2 h and aliquots were taken at 0, 40, 80, 120 min. Rottlerin was extracted from plasma as described above. Acetone was added to liver homogenates, followed by precipitation-extraction as described above.

**Method validation**

The specificity was evaluated by analyzing the chromatograms of blank plasma samples from six mice for possible interferences at the retention time of rottlerin and the IS. The analytical response was expressed as rottlerin to IS peak area ratios and calibration curves were generated by using the peak area ratios versus concentrations. The limit of detection (LOD) in plasma and tissues was defined as the lowest concentration resulting in a signal-to-noise ratio of 3:1. The intra-day and inter-day precision and accuracy were determined by replicative analysis of three QC samples at concentrations of 200, 2000 and 8000 ng/mL for rottlerin on the same day and on three consecutive validation days.
days, respectively. The intra-day and inter-day precisions were expressed by the relative standard deviation (% RSD), while the relative error was used to evaluate the accuracy. The extraction recovery was determined by comparing the ratio of the analyte peak areas of the extracted QC samples with the standard solutions of the same concentration.

Statistical analysis

Descriptive statistics, such as mean and SD, were used to summarize the results. Data were analyzed by paired student t-test. Statistical significance was defined by p-value of 0.05.

Results and Discussion

Understanding the absorption, distribution, metabolism of a bioactive compound is important for its application as a potential chemopreventive or therapeutic agent. Extensive experimental evidence has demonstrated the correlations between tumor size and levels of the bioactive compounds found in tumor in various animal models [10,11]. To the best of our knowledge, pharmacokinetics and tissue bioavailability studies that relate efficacy and toxicity have not been carried out for rottlerin. In this paper, we describe the development of an analytical methodology which would allow the quantitative analysis of rottlerin in the mouse plasma, tissues and in pancreatic cancer cells.

Plant phenolic compounds are often found in the plasma and tissues of animals as the conjugates of glucuronide and sulfate of the parent compound. Particularly in plasma, the conjugates may be the predominant form [9,12,13]. Therefore, we first treated plasma and tissue samples by adding β-glucuronidase/sulfatase to hydrolyze glucuronide and sulfate conjugates. After the incubation and liquid-liquid extraction, we found the samples with added β-glucuronidase/sulfatase did not produce a higher peak area by HPLC analysis in comparison to the samples without enzyme treatment, suggesting that rottlerin may not form conjugates as other phenolic compounds.

Therefore, we used protein precipitation-extraction with acetone and determined the levels of rottlerin in plasma and tissues. Of note, mice fed rottlerin diet grew smaller tumors than those fed control diet (unpublished data). No rottlerin was detected in samples collected from mice fed control diet.

To examine the stability of rottlerin during the extraction procedure, we spiked rottlerin to liver tissue homogenates in pH 7.0 buffer and to plasma of control mice. The resulting samples were then incubated at 37°C for 2 h under dark condition and aliquots of each sample were taken at 40, 80, and 120 min. Table 2 lists the rottlerin concentration in plasma and tissues shows a peak with same retention time at 21.7 min as standard reference rottlerin. On-line UV-VIS maxima of the diode-array detector responses facilitated the confirmation of the compound. Under the selected chromatographic condition, separation of rottlerin was achieved without the interference of endogenous peaks within the time frame of a single analysis. For the calibration curves, there were linear relationship between peak area and concentration in the range. The equation for rottlerin was y=59.61x−0.064 with R²=0.9996. The lower limit of quantitation for rottlerin was 135 ng/mL.

To determine the in vivo effect of rottlerin on pancreatic tumor growth we used a xenograft mouse model as described in the Material and Methods section. Mice were fed standard diets supplemented with rottlerin (0.012% or 120 mg/kg diet) for 6 weeks. Based in daily food consumption, we estimated that the mice received an average of 0.5 mg/mouse/day rottlerin. Rottlerin in the diets was stable at room temperature for 3 days as determined by HPLC analysis.

The sample preparation method described above was applied to the measurement of rottlerin in plasma, selected tissues, and tumors obtained from mice fed control and rottlerin diet for 6 weeks. While we determined the distribution of rottlerin in pancreas and tumor as target tissues, we also investigated liver uptake as liver is a site of metastases for pancreatic cancer. Table 1 shows the average concentration of rottlerin in plasma, tumor, pancreas and liver. Levels of rottlerin were highest in liver (6.56 ± 0.13 nmol/g), followed by pancreas (2.46 ± 0.29 nmol/g) and tumor (2.11 ± 0.25 nmol/g) tissues. Average plasma level is 2.88 ± 0.41 µM. The extraction efficiency is 87.2% for plasma and 85.2-104.5% for tissues and tumors. Of note, mice fed rottlerin diet grew smaller tumors than those fed control diet (unpublished data). No rottlerin was detected in samples collected from mice fed control diet.

Table 1: Rottlerin concentration in plasma and tissues of mice fed 0.012% rottlerin diet for 6 weeks (n=6).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Plasma (µM)</th>
<th>Liver (nmol/g)</th>
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<tbody>
<tr>
<td>0</td>
<td>6.19 ± 0.06</td>
<td>6.01 ± 0.38</td>
</tr>
<tr>
<td>40</td>
<td>6.29 ± 0.29</td>
<td>5.48 ± 0.01</td>
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<tr>
<td>80</td>
<td>6.62 ± 0.15</td>
<td>5.86 ± 0.56</td>
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<tr>
<td>120</td>
<td>6.29 ± 0.21</td>
<td>5.56 ± 0.64</td>
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Table 2: Stability of rottlerin in plasma and liver tissue homogenates with time (n=3).
To summarize, we described here a simple and a validated HPLC-UV method for the quantitative determination of rottlerin in plasma, selected tissues and tumors of the mice fed standard diet containing low content of rottlerin. Rottlerin was found in plasma, liver, pancreas and s.c. tumor tissues. Rottlerin was also found to be accumulated in pancreatic cancer HPAF-II cells and degraded gradually in culture medium with time. Our data can be used to provide important information on evaluating the potential chemo-preventive or therapeutic efficacy of rottlerin.

Acknowledgments

This work was supported by the National Institutes of Health (P01AT003960) and the Hirshberg Foundation for Pancreatic Cancer Research.

References