Ethnopharmacological communication

Nephrotoxicity study of total rhubarb anthraquinones on Sprague Dawley rats using DNA microarrays

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Abstract
Total rhubarb anthraquinones (TRAs) are the active therapeutic components from the rhizomes of \textit{Rheum palmatum} \textit{L.} (Polygonaceae), which are widely used in traditional Chinese medicines (TCMs) and have been reported to have cell toxicity recently. This study focuses on the toxicity of TRAs on Sprague Dawley (S.D.) rats. TRAs administrated per os for 13 weeks induced nephrotoxicity on S.D. rats as renal tubule epithelial cells swelled and denatured in tissue slice examination. After high-density oligonucleotide microarrays scanning, we have identified mitogen-activated protein kinase (MAPK) kinase 6 to be the target gene which causes cell cycle arrest and proliferation inhibition and contributes to nephrotoxicity on S.D. rats.

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Keywords: Total rhubarb anthraquinones; Oligonucleotide microarrays; Toxicogenomics

1. Introduction
Hydroxyanthraquinones are the active components from the rhizomes of \textit{Rheum palmatum}, which is widely used in TCMs for a long time for cathartic, febrifugal, and antidotal purpose. Recent studies demonstrated that TRAs could effectively retard the progression of chronic renal failure, thus developed the new usage. 3-Methyl-1,6,8-trihydroxyanthraquinone (emodin) is an anthraquinone derivative possessed a number of biological activities such as vasorelaxative (Huang \textit{et al.}, 1991), immunosuppressive (Kuo \textit{et al.}, 2001), hepatoprotective (Lin \textit{et al.}, 1996), and anti-tumor activity (Shi \textit{et al.}, 2001). Recent studies suggested that emodin could also induce apoptosis in several kinds of cancer cells (Srinivas \textit{et al.}, 2003). However, so far there is little study concerning the toxicology of TRAs and its effect on kidney. Our findings showed that TRAs cause nephrotoxicity in high dose and long time administration in S.D. rats. Further studies found that mitogen-activated protein kinase (MAPK) kinase 6 may account for the toxicity of TRAs that caused the swelled and denatured renal tubule epithelial cell in S.D. rats.

2. Material and methods

2.1. Chemicals
TRAs, the active therapeutic components from the rhizomes of \textit{Rheum palmatum} \textit{L.} (Polygonaceae), were produced by Nanjing ZhongShan Pharmaceutical factory with Lot No. 020801. The plant was authenticated by Prof. Ping Li, Department of Pharmacognosy, China Pharmaceutical University. A reference sample (NJ 0401022) was retained at the department. It consisted mainly of 3-methylchrysazin, emodin and the purity was above 50%.

2.2. Animals
S.D. rats (Shanghai Sipper-BK Lab Animal Co. Ltd., China) were used for the study. All animals had free access to clean water and pellet food (Jiangsu JiangPu Lab animal forage factory, China). Experiments were performed according to regulation for the administration of affairs concerning experimental

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animals (Ministry of Science and Technology, China, 1988) and approved by the Ethics Committee of China Pharmaceutical University. On the day of dosing the rats were in the weight range 200–225 g and were housed 5 to a cage in suspended polypropylene cages with wire grid floors. The room temperature and relative humidity controls were set at 21 °C and 50%, respectively.

2.3. Treatments

To study the toxicity of TRAs, 20 S.D. rats were randomly allocated into four groups with 5 ones each. TRAs were dissolved in 0.5% sodium carboxymethylcellulose (CMC-Na) solution and each group was administrated per os once daily for 13 weeks at a dose of 0, 140, 794, 4500 mg/kg body weight.

2.4. In vivo treatments and tissue collection

All rats were housed in accredited animal facilities with free access to pelleted food and tap water. After that, rats were sacrificed under halothane anesthesia and all main organs and glands were taken for pathohistology studies, including heart, liver, spleen, lung, kidney, adrenal gland, thyroid gland, bladder, prostate, jejunum, ileum, duodenum, stomach, optic nerve, brain, testis or uterus and ovary. The tissues were fixed in 10% phosphate-buffered formalin solution, and embedded in paraffin. Sections of 4 μm thickness were cut and stained with hematoxylin and eosin (HE).

Additional tissues samples were quickly frozen into liquid nitrogen or stored at −80 °C for RNA isolation usage.

2.5. RNA isolation

Total RNA was isolated from frozen kidney tissues of individual rats using RNeasy Midi kits (Qiagen, German) basically as described by the manufacturer. Equivalent amounts of RNA from individual samples within each group were pooled to provide a representative sample for microarray analysis (n = 4). Aliquots of RNA from individual animals were saved for subsequent confirmatory evaluations of gene expression.

2.6. Microarray protocol and expression analysis

Oligonucleotide microarrays Rat Genome 230 2.0A (Affymetrix, USA) were used, and all the operating procedures were performed exactly as described by the manufacturer.

2.7. Data processing

Expression profiles were analyzed using GenMAPP (Gene MicroArray Pathway Profiler) 2.0Beta software developed by Gladstone Institution of California University at San Francisco.

2.8. Real-time PCR confirmation

The differential expression rates of some key genes related to toxicity were confirmed using real-time PCR methodology. SYBR GreenI (Invitrogen, USA) was used to detect double-stranded DNA product. Primers were designed with Oligo 6.0 and mRNA was copied to cDNA by reverse transcriptase (Promega, USA) with oligo dT primers. The cDNAs were denatured at 94 °C, annealed at 50 to ∼60 °C due to different cases and extended at 72 °C for 40–50 cycles using icycler (Bio-rad, USA). By monitoring the signals of SYBR GreenI, the relative concentration of initial template can be concluded (Rajeevan et al., 2001).

3. Results

3.1. Pathohistology analysis

In TRAs treatment group (4500 mg/kg/day), nephrotoxicity were discernable at 13 weeks. There was no clear morphologic change in the kidney of the control group, while in TRAs treated group, swelled and denatured renal tubule epithelial cells were observed (Fig. 1).

3.2. Gene with differential expression

Data fluctuation above 2.0-fold is considered to be differential expression. Among the 102 up-regulated genes, 28 are functionally related to metabolism, 12 to immunology, 14 to oxidative

Fig. 1. Comparison of control and TRAs treated kidney of S.D. rats.
catalysis, 23 to signal transduction, while 25 are unknown ESTs. In addition, 12 of the 100 down-regulated genes are functionally related to metabolism, 10 to immunology, 19 to signal transduction, 23 to renal filtration and endocrine, 38 are unknown ESTs.

3.3. Data processing result

In TRAs treated groups, expression of hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) of cholesterol biosynthesis pathway increased by 34-fold, while isopentenyl-diphosphate delta isomerase down changed by 3-fold. In electron transport chain, the expression of gene encoding mitochondrial brown fat uncoupling protein increased by 6.5-fold. In pentose phosphate pathway, the expression of gene encoding transketolase increased at most by 4.3-fold, indicating the acceleration of glucose metabolism. The expression of gene encoding caspase3 and p53 decreased by 2.6-fold, respectively in TRAs treated group. Caspase3 and p53 up-regulation are known to trigger the process of cell apoptosis. Thus we conclude, in TRAs treated group, caspase3 and p53 pathways obviously did not account for the apoptosis of cells. Expression of metallocinein, heme oxygenase 1 and NADPH dehydrogenase increased by 4.3-, 4.3- and 3.0-fold, respectively, indicating that the oxidative stress activation led to the up-regulation of the three anti-oxidative genes. Concerning p38 MAPK pathway, expression of MAPK kinase6 was found decreased by 2.3-fold, which may account for the cell injury to some extent. In cell cycle regulation pathway, expression of cyclin D1 decreased by 2-fold, which was essential for the control of the cell cycle at the G1/S (start) transition. Cyclin-dependent kinase 1 (CDK-1), whose expression level decreased by 2.1-fold in TRAs treated group, played a key role in the control of the eukaryotic cell cycle, which was required in higher cells for entry into S-phase and mitosis.

Table 1 Validation of array-base gene expression profile by real-time PCR

<table>
<thead>
<tr>
<th>Gene name (accession no.)</th>
<th>Relative expression</th>
<th>Validation (yes, no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear factor I/X (AB012235)</td>
<td>−4.0</td>
<td>−2.2</td>
</tr>
<tr>
<td>Cypl1 (NM_012540)</td>
<td>3.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Heme oxygenase 1 (NM_012580)</td>
<td>4.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Caspase3 (NM_012922)</td>
<td>−2.6</td>
<td>−2.6</td>
</tr>
<tr>
<td>NADPH dehydrogenase (NM_017000)</td>
<td>3.0</td>
<td>−5.0</td>
</tr>
<tr>
<td>Cell division cycle 2 homolog A (NM_019296)</td>
<td>−2.1</td>
<td>−4.4</td>
</tr>
<tr>
<td>Tumor protein p53 (NM_030989)</td>
<td>−2.1</td>
<td>−1.0</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2D2 (NM_031001)</td>
<td>3.0</td>
<td>−3.5</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase 6 (NM_053703)</td>
<td>−2.3</td>
<td>−3.0</td>
</tr>
<tr>
<td>Metallothionein (NM_138826)</td>
<td>4.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Cyclin D1 (NM_171992)</td>
<td>−2.0</td>
<td>−2.1</td>
</tr>
<tr>
<td>DNA replication licensing factor MCM6 (U17565)</td>
<td>−2.5</td>
<td>−6.8</td>
</tr>
<tr>
<td>Mitochondrial HMGCoA synthase (M33648)</td>
<td>34.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 1 (NM_013144)</td>
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<td>5.1</td>
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<tr>
<td>Acetyl-coenzyme A acyltransferase 1 (NM_012489)</td>
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<td>5.3</td>
</tr>
<tr>
<td>G protein-binding protein CRFG (NM_053689)</td>
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<tr>
<td>CYP450 BC (NM_019184)</td>
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<td>−6.7</td>
</tr>
<tr>
<td>Renal specific organic anion transporter (NM_030837)</td>
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<td>−1.4</td>
</tr>
<tr>
<td>Unknown ESTs (B1255575)</td>
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<td>−1.3</td>
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<tr>
<td>Unknown ESTs (BE107450)</td>
<td>−4.9</td>
<td>−2.9</td>
</tr>
</tbody>
</table>

Note: relative expression is calculated as ratio of expression levels compared with housekeeping gene such as GAPDH. “+” “−” are used to indicate genes up- or down-regulation, respectively. A gene is considered differentially expressed in this article if its relative expression is two-fold or greater.

3.4. Real-time quantitative PCR result

As the result shows (Table 1), 17 assays came in consistency with microarray results and 3 assays in wrong direction of differential expression between two detection systems. RNA quality was the single most important variable in two assay systems and may partially account for the inconsistency of results by two detection systems.

4. Discussion and conclusion

TRAs are reported to have cell toxicity especially inducing apoptosis and genotoxicity (Mueller and Stopper, 1999). Naturally occurring anthracene derivatives, 1,8-dihydroxyanthraquinones are pharmacologically active laxatives. A carcinogenic potential was reported for 1,8-dihydroxyanthraquinone, danthron in rodents (Mori et al., 1985, 1986). However, for other more abundantly occurring anthraquinone derivates even antimitogenic properties were found (Su et al., 1995). In our experiment, TRAs treatments at fairly high dosage caused nephrotoxicity in S.D. rats, and the results of gene differential expression study indicated the TRAs affect mostly at oxidative stress pathway, cell cycles, nutrients metabolism, thus caused renal tubule epithelial cells swelled and denatured in histopathology study.

CYP1A1 was involved in an NADPH-dependent electron transport pathway in oxidative stress and the gene increases by 3-fold in S.D. rats by TRAs treatment. It oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics. Thus, CYP1A1 is regarded as a carcinogen-metabolizing enzyme (Michael et al., 2001) and its dramatic up-regulation in TRAs treatments may account for genotoxicity.

MAPK kinase 6, functioned as a mitogen-activated protein kinase kinase, was decreased by 3-fold in real-time PCR analysis at mRNA level after TRAs treatment. MAPK, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals because they are
activated by a wide variety of extracellular signals, and are rapidly phosphorylated on threonine and tyrosine residues. This protein phosphorylates and activates p38 MAPK in response to inflammatory cytokines or environmental stress. As an essential component of p38 MAPK mediated signal transduction pathway, this protein is involved in many cellular processes such as stress induced cell cycle arrest, transcription activation and apoptosis. Although previous reports showed that the toxicity of emodin, the principle active ingredient of TRAs, is due to its effect on caspase3 cascade (Chen et al., 2002) and p53 pathway (Shieh et al., 2004), the expression of these two genes were down-regulated after TRAs treatment in our experiment. After analysis of the other apoptosis related pathways, p38 MAPK was identified to be the main cause of cell cycle arrest and apoptosis in this experiment.

It may be concerned that the discernable nephrotoxicity was only observed at the high dose group of S.D. rats. Clinical therapeutic dosage of TRAs is 420 mg/day, and if one person weighs 60 kg, then the dosage used in our experiment is more than 600 times ordinary clinical dose. In TRAs treatment group, the β2-microglobulin and urinary albumin increased dramatically at 13-week, which revealed the injury of kidney function (Zhang et al., 2004). By gene differential expression study, we found MAPK kinase 6 and CYP1A1 may contribute to the renal tubule epithelial cells injury and genotoxicity, respectively.

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References
