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Medicinal Herb Extract and a Single-Compound Drug Confer Similar Complex Pharmacogenomic Activities in MCF-7 Cells

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Key Words
Anoectochilus formosanus · MCF-7 cancer cells · Pharmacogenomic activities · Metabolite profiling

Abstract
Metabolite profiling and DNA microarray analysis of global gene expression profiles were employed to characterize the bioactivities of the herbal extract of *Anoectochilus formosanus* (AF), a popular folk medicine with anticancer activity, in MCF-7 cancer cells. The pharmacogenomic activities of this plant extract as a crude phytocompound mixture were compared to those conferred by the single-compound drug, plumbagin. A similar level of complexity in transcriptional regulation at the genomic level was observed for both AF extract- and plumbagin-treated MCF-7 cells, as revealed by the number of up- or downregulated genes as well as by the specific but distinct patterns found in the gene-clustering analysis. This finding offers evidence to support the search for fractionated medicinal herb extracts or phytocompound mixtures, in addition to single-compound drugs, as defined therapeutic agents.

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Introduction

Koop [3] recently advocated in *Science* that natural products should be considered important resources for future medicines. However, a conceptual challenge for the development of botanical drugs is the lack of scientific acceptance of using a mixture of multiple phytocompounds or plant extracts as opposed to single-compound drugs. In this study we compared a plant extract fraction from *Anoectochilus formosanus* (AF) with defined bioactivity and a single phytocompound, plumbagin (isolated from another medicinally used plant which is being tested as a chemotherapy drug) for the treatment of human breast adenocarcinoma MCF-7 cells and characterized the functional genomic response of tested cancer cells. Our results demonstrate a similar level of complexity in the global gene expression pattern for both AF extract- and plumbagin-treated MCF-7 cells.

Materials and Methods

Cell Culture

MCF-7 cells obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA) were grown in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All experiments were carried out on confluent cells in the medium at 37 °C in 5% CO_2.

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Plant Materials

In the following stage, fresh AF test plants (September 1999) were obtained from the reputable *Anoectochilus* Cultural Station in Nantou County, central Taiwan, as officially recommended by Dr. Hsin-Sheng Tsay of the Taiwan Agricultural Research Institute, via the National Science and Technology Program in Agriculture Biotechnology, National Science Council, Taiwan. In this study, we used the same plant cultivar grown by the same farmer on the same lot in Puli, Taiwan, under 'tissue culture' and green/net-house conditions, maintaining a normalized/standardized plant growth protocol under good agricultural practice (GAP) conditions.

mRNA Preparation and cDNA Probe Generation

Total cellular RNA samples of MCF-7 cells treated with AF plant extracts, plumbagin, or vehicle control (0.4% DMSO) for 3, 6, 9, 12, 18, and 24 h were prepared using Trizol® reagent and further purified using the RNeasy® kit (Qiagen, Hilden, Germany). Biotin-labeled cDNA probes were generated using an RT-PCR method as described by Lau et al. [4].

Generation, Hybridization, and Colorimetric Signal Detection of cDNA Microarrays

Candidate cDNAs for generating cDNA microarrays were prepared from Image consortium gene pools or self-constructed cDNA clones using PCR. The PCR mixtures (100 μl) consisted of 10 U Taq polymerase (Merck, Whitehouse station, N.J., USA), 0.2 μM of the desired primers, 200 μM dNTPs, and 10 ng of template DNA. PCR reactions were performed with a thermocycling program of 3 min at 95°C, and 40 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C on a PrimusHT thermocycler (MWG-Biotech, High Point, N.C., USA). The PCR products were then precipitated by 70% EtOH (Merck), vacuum-dried, and dissolved in sterilized water to a final concentration of 1.1 μg/μl DNA (~10^8 molecules of DNA/spot), before being spotted onto a nylon membrane. Conversion of coordinate and database constructions was established using GeneTrend™ software (U-Vision Biotech, Taipei, Taiwan). Probe hybridization and color development were adapted from a colorimetric system published elsewhere [4]. A 3,000-dpi light scanner (Umax, Taipei, Taiwan) integrated with Imagene® (BioDiscovery Inc., El Segundo, Calif., USA), QuantArray® software, and statistical programs developed at U-Vision Biotech Inc. (available at http://www.u-vision-biotech.com/) was employed to perform image and microarray data analyses. Information on the above-described and related DNA microarray design followed standard guidelines and fulfilled the requirements for submission of our protocol to the MIAME database system, now established at the Institute of BioAgricultural Sciences and the Institute of Statistical Science, Academia Sinica, Taipei, Taiwan.

Chemical Fingerprint Analysis of the Plant Extract of AF

Analytical HPLC was performed to analyze the chemical fingerprint of the plant extract from AF. UV spectra of candidate index compounds were recorded from a Jasco (Tokyo, Japan) V-550 spectrometer and IR spectra were obtained using a Bio-Rad (San Jose, Calif., USA) FTS-40 spectrophotometer. Electron-impact mass spectrometry (EIMS) and high-resolution electron-impact mass spectrometry (HREIMS) data were collected with a Finnigan (Mass., USA) MAT-958 mass spectrometer; NMR spectra were recorded with Bruker Avance (Rheinstetten, Germany) 500- and 300-MHz FT-NMR spectrometers, at 500 MHz (^1H) and 75 MHz (^{13}C).

Results and Discussion

AF is a folk medicine traditionally used in Taiwan for treating cancer and liver disease. AF extracts were subjected to metabolite profiling using reverse-phase HPLC to define the reproducibility of the constituents in lot-to-lot prepared extracts (fig. 1). Eight major compounds, including compounds **1** (nicotinic amide), **2** (adenosine), **3** (cytosine), **4** (isohamnetin 3,4′-O-β-*D*-diglucopyranoside), **5** (isorhamnetin 3-O-β-*D*-glucopyranoside), **6** (caffeic acid), **7** [(6*R*, 9*S*)-hydroxy-megastima-4,7-diene-3-one-9-O-β-*D*-glucopyranoside], and **8** (kinsenone) [6], were structurally identified using IR, MS, and NMR spectrometry. The AF extract, thus defined, was observed to strongly and specifically inhibit the proliferation of human breast adenocarcinoma MCF-7 cells, as judged by MTT and ^3H-thymidine incorporation assays; more than 50% of AF extract-treated MCF-7 cells were observed to exhibit apoptotic activities according to flow cytometric analysis (data not shown and submitted for publication elsewhere).

A series of DNA microarray experiments [1] was then conducted to characterize the functional genomic expression profiles of a spectrum of genes (~10,000 genes) in MCF-7 cells, treated either with the defined AF extract (as a phytocompound mixture) or with plumbagin. Plumbagin, a single-compound drug isolated from *Plumbago rosea*, is a known plant naphthoquinone with demonstrated antitumor and antibacterial properties [2]. As shown in figure 2a, b, the global mRNA expression profiles in MCF-7 cells were found to exhibit similar complexities using stringent statistical analysis when cells were treated with either plumbagin or the AF phytocompound mixture (fig. 2b). Analysis of microarray data showed that 39 known genes were upregulated and 20 known genes downregulated (a >3-fold difference in expression levels) in AF-treated MCF-7 cells, while 50 known genes were upregulated and 30 known genes downregulated in plumbagin-treated cells (a >3-fold difference). A second microarray experiment confirmed a similar level of genomic complexity for both treatments.

From the two microarray experiments, upregulation of genes encoding caspase 8 and cytochrome c was observed in AF-treated cells (as confirmed by Western blot analysis and data submitted for publication elsewhere). Cyclin-dependent kinase inhibitor (p21Waf1/Cip1) and DNA topoisomerase II (TOP II) genes were upregulated in plumbagin-treated cells. Plumbagin was previously reported to induce apoptosis of mouse embryonic fibroblast cells [2] and human KB3-1 carcinoma cells [5] via upregu-

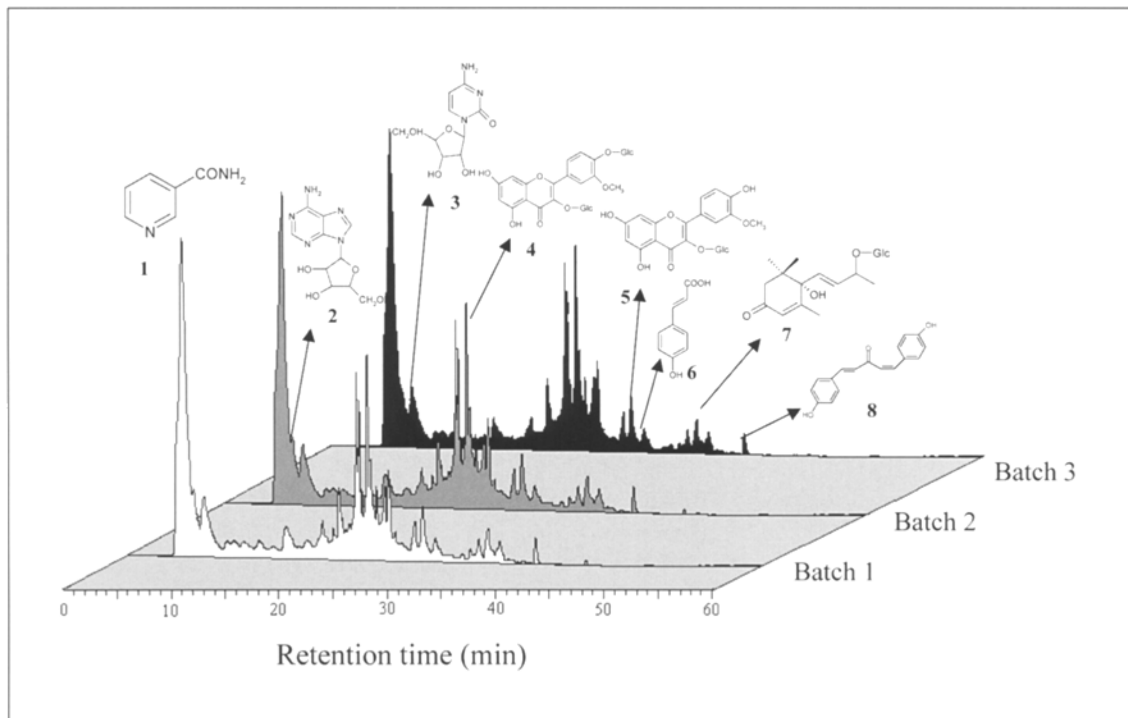

Fig. 1. Metabolite profiling of different lot preparations of plant extract from AF. The three chromatograms were obtained using a C-18 reverse-phase HPLC system. The structures and identities of the 8 index compounds were identified using IR, mass, and NMR spectrometric analyses. **1** = Nicotinic amide; **2** = adenosine; **3** = cytosine; **4** = isohamnetin 3,4′-*O*-β-*D*-diglucopyranoside; **5** = isorhamnetin 3-*O*-β-*D*-glucopyranoside; **6** = caffeic acid; **7** = (6*R*, 9*S*)-hydroxy-megastima-4,7-diene-3-one-9-*O*-β-*D*-glucopyranoside; **8** = kinsenone.

Fig. 2. Non-distinguishable complexity of functional genomic reactivities in human MCF-7 mammary carcinoma cells in response to chemotherapy using a singular compound (plumbagin) or a crude plant extract preparation derived from the medicinal herb, AF. **a** Comparison of the MCF-7 mRNA expression patterns displayed on duplicate-spotted DNA microarrays. MCF-7 cells were treated with a single compound (plumbagin) or AF plant extracts. **b** Scatterplot showing differential gene expression patterns in response to drug/herbal treatments providing statistical significance in multiples (threshold) of change. In the present study, 9,600 (~10,000) genes were studied in the original experiment, and total numbers of up- and downregulated genes with known functions for each pair of test sets were scored (e.g., ~80–90 known genes observed for each test pair with ≥3-fold change in expression), as described in the text. In later experiments, due to the large number of expression sequence tag (EST) but unknown (potentially novel) genes included, whose biological significance was uncertain in this study, we then selected 2,000 known genes of interest for further study. These 2,000 functional genes (each with 2 replicate dots) were then rearranged onto a single DNA chip, and used for a second run of hybridization with test cell RNA, and for scatterplot analysis. Cases (~500 genes) were excluded from our data analyses if (1) the reproducibility of the 2 replicate dots for the same gene showed ≥50% difference in activity or (2) the signal-staining intensity was too low (< threshold), too high (over saturation/linear range), or had background interference problems. Based on the above exclusion criteria, only about 1,500 genes/chip were analyzed by the scatterplot analysis program developed by U-Vision Biotech Inc. (Taipei, Taiwan). All treatment (control vs. test) pair combinations were again subjected to scatterplot analysis, and typical examples are represented. **c** Time-course movement in functional clustering implying gene/pathway crosstalk and pharmacogenomic mechanisms. Data represent examples of the results of specific gene clustering on indicated treatment time points, based on normalized (~1,500) gene expression profiles obtained in **b**, using statistical software as described in 'Materials and Methods'. Here, apoptosis-related genes (e.g., caspase 8, cytochrome c, p21, and TOP II) were suggested as being responsive to plumbagin or AF for antitumor cell activity. All analytical software was generated and provided by U-Vision Biotech Inc. (available at http://www.u-vision-biotech.com).

lation of p21Waf1/Cip1 protein and TOP II-mediated DNA cleavage activities, respectively. In this study, plumbagin induced both genes in apoptotic MCF-7 cells, and this is the first evidence to support the hypothesis that these two proteins are regulated at the transcription level in tumor cells. Figure 2c demonstrates that specific but distinct gene clustering patterns were also obtained for AF- and plumbagin-treated MCF-7 cells, implying that specific but distinct gene expression patterns are definable for these two treatments. Further preliminary evidence also revealed that global gene expression complexity was observed in taxol-treated MCF-7 cells (data not shown).

In conclusion, in terms of genomic expression complexity, we could not distinguish a difference between the effect of a single-compound anti-cancer drug (plumbagin) and a relatively crude medicinal herb extract (AF) in MCF-7 cells. A major reservation about herbal medicines is that they produce 'complex, undefinable' effects in biological systems, whereas single-compound drugs have

been classically portrayed as eliciting specific (definable) effects. However, our study has shown that this is simply not true, and that, at the very least, this widely held argument will have to be reassessed so that serious scientific consideration is given to the enormous resources offered by complex botanical preparations. Therefore, we suggest here that a combination of functional genomics, metabolomics, defined signal transduction pathways (e.g., specific apoptotic mechanisms) and correlatable bioactivity assays be used together as an effective approach to build a research platform for the evaluation and application of botanical drug products in the form of compound mixtures, just as is done for single compound drugs.

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