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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2846–2849

Rediscovery of natural products using genomic tools

Akira Kawamura,* Angelika Brekman, Yevgeniy Grigoryev, Tal H. Hasson, Anna Takaoka, Stephanie Wolfe and Clifford E. Soll

CUNY—Hunter College, Department of Chemistry, 695 Park Avenue, New York, NY 10021, USA

Received 1 February 2006; revised 7 March 2006; accepted 8 March 2006
Available online 31 March 2006

Abstract—A screening methodology called 'genomic screening' was established to identify natural products that can regulate cellular gene expression. Application of genomic screening to Keishi-bukuryo-gan (KBG), a Japanese herbal medicine formulation, identified a previously unnoticed transcriptional effect by linoleic acid, a known KBG component. The approach opens up a possibility to develop cell-permeable molecular tools for functional genomics research and sets a stage to evaluate the potential of natural products for transcription therapies.

Transcription therapy, or regulation of cellular mRNA expression for therapeutic purposes, is becoming a viable approach for the treatment of various pathological conditions, including cancer.[1] Common approaches for transcription therapy include oligonucleotide therapeutics, such as short interfering RNA (siRNA).[2] However, they have to overcome some technical challenges, such as serum stability and cellular uptake. It is, therefore, important to explore alternative approaches for safe and effective regulation of cellular transcription.

In an effort to discover new classes of compounds for gene regulation, we recently initiated a research program focusing on the identification of natural products that can regulate cellular transcription. Some natural products, such as retinoic acid, have been used for transcription therapy of leukemia.[3] It is likely that many other natural products are potentially useful molecular tools for gene regulation in biomedical research as well as for transcription therapy. Although natural products may not specifically regulate a single gene, they can still serve as useful tools as long as their transcriptional effects are well characterized; speaking of selectivity, however, even oligonucleotide therapeutics are also known to cause non-specific cellular responses.[4] Currently, our knowledge of transcriptional activities by natural products is limited and fragmental because of the lack of appropriate screening methods.

Here, we present a screening methodology based on DNA microarray, which we call 'genomic screening.' Genomic screening identifies compounds that can regulate cellular mRNA transcription. Identification of such compounds is important because they can serve as cell-permeable molecular tools for functional genomics research and transcription therapy. In this proof-of-principle study, we used Keishi-bukuryo-gan (KBG), a Japanese herbal medicine formulation,[5] as the first trial sample to establish the experimental protocol for genomic screening. This report will describe our established scheme for genomic screening together with an unexpected discovery of gene regulatory effects by linoleic acid, one of the KBG components. Although DNA microarray has been employed for biological characterizations of natural products, what sets this work apart from others is that it employs DNA microarray for the purpose of purification of biologically active compounds from complex mixture.

Genomic screening consists of two parts: (1) DNA microarray profiling of cells treated with the compound mixture of interest and (2) identification of active compounds guided by quantitative real-time polymerase chain reaction (qRT-PCR) and LC/MS. The first part, DNA microarray profiling, is important because, at the onset of each study, we do not know which genes are modulated by the compound mixture of interest.

Keywords: DNA microarray; Real-time PCR; Linoleic acid; Heme oxygenase 1; Keishi-bukuryo-gan; Kampo medicine; Transcription therapy.
* Corresponding author. Tel.: +1 212 650 3095; fax: +1 212 772 5332; e-mail: akira.kawamura@hunter.cuny.edu

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doi:10.1016/j.bmcl.2006.03.022

DNA microarray generates mRNA expression profiles of cells treated by the compound mixture of interest, and by the vehicle control, from which differentially expressed genes are identified.[6] Once we know which genes are affected by the compound mixture of interest, the subsequent screening can be guided by qRT-PCR, which is more focused and less expensive tool for mRNA quantitation than DNA microarray.

As the first part of genomic screening, the DNA microarray profiling of KBG was carried out using human umbilical vein endothelial cells (HUVEC). Endothelial cells were used in this study because many of the beneficial effects of KBG can be linked to its actions on blood vessels.[5] HUVEC was treated with 100 μg/ml of KBG, which roughly corresponds to the daily dosage of this formulation for adults (~0.15 g/kg). It is noted that KBG does not affect the proliferation of HUVEC at this concentration (see Supplementary data). The expression profiling was carried out using Genisphere 3DNA 900 MPX kit and MWG Human 40 k A array, which detects the expression of approximately 20,000 different transcripts. Hybridized arrays were scanned with Axon GenePix 4000B microarray scanner. The raw data were processed using the TIGR TM4 package.[7] In order to remove false positives and dye bias, two independent experiments with dye-flip procedure were carried out.[8] Table 1 summarizes the list of HUVEC genes differentially regulated by KBG.

Once we found which genes to focus on, we moved onto the second part of genomic screening: identification of compounds responsible for the observed changes in gene expression. To this end, we first prepared a panel of 21 KBG extract fractions as described in Supplementary data. We then screened these fractions with qRT-PCR analysis of heme oxygenase 1 (HMOX1). HMOX1 was selected for this study because its basal expression in HUVEC was the highest among the differentially regulated genes, which allowed the most reproducible analysis of this gene by qRT-PCR. The qRT-PCR experiments were carried out using Applied Biosystems TaqMan® Gene Expression Assays on 7500 Real-Time PCR System.

Table 1. HUVEC genes differentially expressed by KBG (100 μg/ml, 4 h)

GenBank ID	Gene	Average ratio
1040871	Aromatic hydrocarbon receptor	3.4
2833652	Gonadotropin-releasing hormone precursor second form	2.3
4504436	Heme oxygenase 1	2.3
12620187	Unknown (c1orf17)	2.0
2921682	Olfactory receptor; or 19–18	0.5
12653010	NADH dehydrogenase 1 α subcomplex, 1	0.5
4176442	Unknown (dj1042k10.2)	0.5
14042185	cDNA clone moderately similar to zinc finger protein 184	0.4
3152822	p120 catenin isoform 2ab; ctnnd1	0.4

Fold change cutoff: 2-fold. The full list of differentially regulated genes and experimental procedure are described in Supplementary data.

Figure 1 summarizes the results of the qRT-PCR screening of the 21 fractions. The qRT-PCR analysis identified 8 fractions that significantly ($p < 0.01$) affected the expression of HMOX1 when compared to the DMSO control. Fractions **3N**, **3A**, **3B**, **4N**, **4A**, **4B**, and **7N** up-regulated HMOX1, whereas **1N** down-regulated it. At the concentration used for the qRT-PCR screening, none of the fractions significantly affected the growth of HUVEC (data not shown).

In order to identify the KBG component(s) responsible for the differential expression of HMOX1, we then examined the LC/MS profiles of individual fractions. This analysis allowed us to identify molecular ions uniquely correlated to the observed HMOX1 regulatory activity. It turned out that compounds with the molecular weights of 280 and 528 consistently appeared in the LC/MS chromatograms of the active fractions that regulated HMOX1. Figure 2 shows the positive and negative ion profiles of an LC/MS peak (retention time ~27 min), which indicated the presence of a compound with MW 280. Likewise, the compound with MW 528 was indicated by an analogous LC/MS analysis.

Analysis of known KBG components suggested that the molecular weights of 280 and 528 could be those of linoleic acid and pachymic acid, respectively (Fig. 3a). The qRT-PCR analyses of these compounds (commercial samples, both at 1 μg/ml) revealed that linoleic acid indeed induced HMOX1 in HUVEC, whereas pachymic acid did not affect its expression (Fig. 3b). The LC/MS retention time of commercial linoleic acid also matched that of corresponding ions previously observed in KBG fraction analysis (data not shown).

Taken together our data demonstrate the utility and potential of genomic screening. The current study

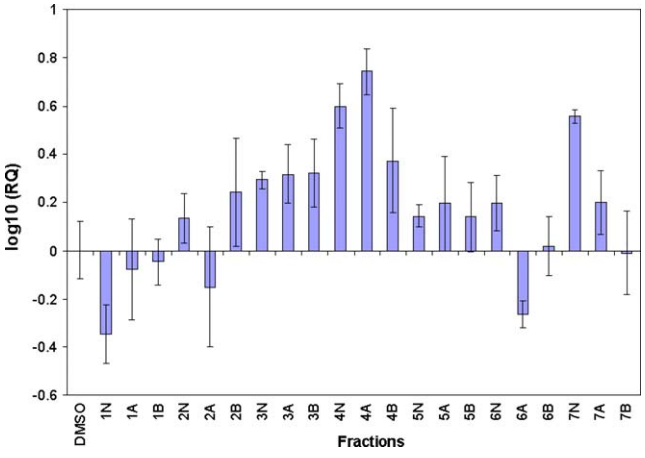

Figure 1. Screening of KBG fractions with HMOX1 qRT-PCR. Minimum triplicate experiments were carried out for each condition. The RQ (Relative quantitation) values, expressed in the log scale here, correspond to the fold change ratios between drug-treated and control (DMSO) samples. The qRT-PCR data were normalized by GAPDH (endogenous control), as well as by the DMSO vehicle control. Detailed experimental procedure for qRT-PCR is described in Supplementary data.

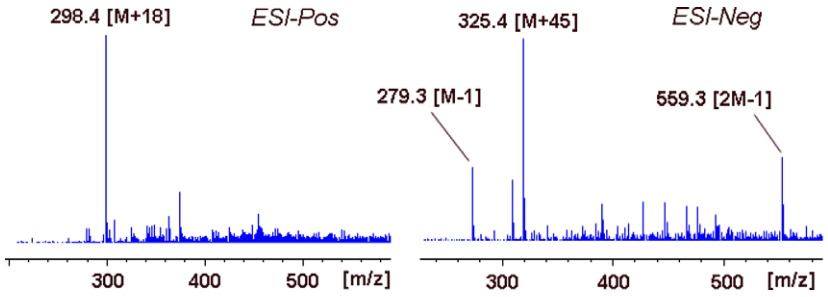

Figure 2. Positive and negative ion profiles of an LC/MS peak at ~27 min, which were commonly seen in the active fractions. The shown data were obtained from **7N**. LC/MS conditions are described in Supplementary data.

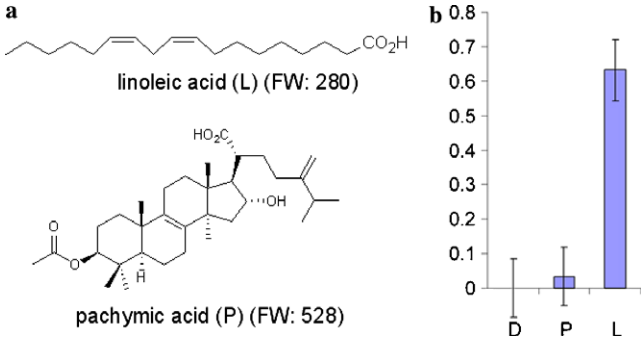

Figure 3. (a) Structures of linoleic acid (MW 280) and pachymic acid (MW 528). (b) Quantitative RT-PCR (HMOX1) of each compound (1 μg/ml). D (DMSO vehicle control), P (pachymic acid), and L (linoleic acid).

identified linoleic acid as an inducer of the HMOX1 gene. It is known that linoleic acid can modulate cellular transcription through PPAR, SREBP, etc.[9] HMOX1, however, is not one of the genes known to be regulated by linoleic acid. HMOX1 is generally considered as a stress-responsive protein induced by various oxidative agents,[10] although recent studies indicate that it is also regulated by interleukin-10,[11] a potent anti-inflammatory cytokine. Linoleic acid can serve as a useful molecular probe to gain novel insights into the signaling cascades leading to HMOX1 induction.

The importance of the current study, however, goes beyond the finding of linoleic acid as a regulator of HMOX1 expression. The current study tells us that there is still a lot to learn from natural products, even from those compounds that are generally considered 'well-known.' Genomic screening can now be applied to a wide variety of natural resources to re-evaluate their biological properties. The methodology can be used to examine many natural resources that did not yield 'active compounds' with traditional screening methods, such as cytotoxicity assays. For example, genomic screening is particularly useful for the study of herbal formulations that do not exhibit toxicity at their therapeutic concentrations. It is our expectation that genomic screening will uncover previously unnoticed transcriptional effects by many natural products. Once active compounds are identified, the exact nature of transcriptional effects, that is, whether compounds affect mRNA synthesis or stability, can be further defined by the examination of nuclear run-on RNA.[12] The resulting compounds can serve as powerful molecular tools for functional genomics research and will set a stage for the examination of their potential for transcription therapies.

Acknowledgments

This study was supported by the NIGMS-MBRS/SCORE Program under Grant S06 60654 to Hunter College. RR-03037 from NCRR/NIH, which partially supports the infrastructure of Hunter core facilities, is also acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.022.

References and notes

1. Pandolfi, P. P. *Oncogene* **2001**, *20*, 3116.
2. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494; Robinson, R. *PLoS Biol.* **2004**, *2*, E28.
3. Melnick, A. *Leukemia* **2005**, *19*, 1109.
4. Marques, J. T.; Williams, B. R. *Nat. Biotechnol.* **2005**, *23*, 1399.
5. Noguchi, M.; Ikarashi, Y.; Yuzurihara, M.; Kase, Y.; Chen, J. T.; Takeda, S.; Aburada, M.; Ishige, A. *J. Endocrinol.* **2003**, *176*, 359; Sekiya, N.; Kainuma, M.; Hikiami, H.; Nakagawa, T.; Kouta, K.; Shibahara, N.; Shimada, Y.; Terasawa, K. *Biol. Pharm. Bull.* **2005**, *28*, 294; Shimada, Y.; Yokoyama, K.; Goto, H.; Sekiya, N.; Mantani, N.; Tahara, E.; Hikiami, H.; Terasawa, K. *Phytomedicine* **2004**, *11*, 404; Goto, H.; Shimada, Y.; Sekiya, N.; Yang, Q.; Kogure, T.; Mantani, N.; Hikiami, H.; Shibahara, N.; Terasawa, K. *Phytomedicine* **2004**, *11*, 188; Nakagawa, T.; Yokozawa, T.; Terasawa, K.; Nakanishi, K. *J. Pharm. Pharmacol.* **2003**, *55*, 219; Chen, J. T.; Shiraki, M. *Maturitas* **2003**, *45*, 199; Sekiya, N.; Goto, H.; Tazawa, K.; Oida, S.; Shimada, Y.; Terasawa, K. *Phytother. Res.* **2002**, *16*, 524.
6. Brown, P. O.; Botstein, D. *Nat. Genet.* **1999**, *21*, 33; Lockhart, D. J.; Winzeler, E. A. *Nature* **2000**, *405*, 827.
7. Dudoit, S.; Gentleman, R. C.; Quackenbush, J. *Biotechniques* **2003**, Suppl 45; Saeed, A. I.; Sharov, V.; White, J.; Li, J.; Liang, W.; Bhagabati, N.; Braisted, J.; Klapa, M.; Currier, T.; Thiagarajan, M.; Sturn, A.; Snuffin, M.;

Rezantsev, A.; Popov, D.; Ryltsov, A.; Kostukovich, E.; Borisovsky, I.; Liu, Z.; Vinsavich, A.; Trush, V.; Quackenbush, J. *Biotechniques* **2003**, *34*, 374.
8. Quackenbush, J. *Nat. Genet.* **2002**, *32*, 496.
9. Jump, D. B.; Clarke, S. D. *Annu. Rev. Nutr.* **1999**, *19*, 63.
10. Poss, K. D.; Tonegawa, S. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10925.
11. Lee, T. S.; Chau, L. Y. *Nat. Med.* **2002**, *8*, 240.
12. Cheadle, C.; Fan, J.; Cho-Chung, Y. S.; Werner, T.; Ray, J.; Do, L.; Gorospe, M.; Becker, K. G. *Ann. N.Y. Acad. Sci.* **2005**, *1058*, 196.