

Research Article

Analyzing Gene Expression Profile in K562 Cells Exposed to Sodium Valproate Using Microarray Combined with the Connectivity Map Database

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To explore the mechanism underlying antileukaemia effect of sodium valproate, the growth and survival of the K562 cell line were investigated. Global profiles of gene expression in K562 cells exposed to sodium valproate were assessed and validated. The differentially expressed genes identified were further used to query the connectivity map database to retrieve a ranked list of compounds that act on the same intracellular targets as sodium valproate. A significant increase in cell apoptosis and a change in gene expression profile were observed in valproate-exposed K562 cells. The significant enrichment analysis of gene ontology terms for the differentially expressed genes showed that these genes were involved in many important biological processes. Eight differentially expressed genes involved in apoptosis were verified by quantitative real-time PCR. The connectivity map analysis showed gene expression profile in K562 cells exposed to sodium valproate was most similar to that of HDACi and PI3K inhibitors, suggesting that sodium valproate might exert antileukaemic action by inhibiting HDAC as well as inhibiting PI3K pathway. In conclusion, our data might provide clues to elucidate the molecular and therapeutic potential of VPA in leukaemia treatment, and the connectivity map is a useful tool for exploring the molecular mechanism of drug action.

1. Introduction

Sodium valproate (VPA) is a well-known antiepileptic drug, also used to treat bipolar disorder, migraine, and neuropathic pain. Recently, VPA has been described as an HDAC inhibitor, resulting in an increased interest for its use in cancer therapy. Studies show that VPA, alone or in combination with other antileukaemic compounds, possesses significant antileukaemic actions on acute myeloid leukemia (AML) cells [1], chronic myeloid leukaemia (CML) cells [2–4], acute lymphoblastic leukemia (ALL) cells [5], and chronic lymphocytic leukemia (CLL) cells [6, 7]. Clinical trials

show that VPA therapy is of clinical benefit in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [8–11]. However, the exact molecular mechanisms of VPA action on leukemia treatment remain poorly understood.

Microarray is a high-throughput tool which allows for the analysis of global gene expression profile in a single experiment and has been widely used for exploring molecular mechanisms of pathogenesis and drug treatment. Based on this high-throughput technology, the molecular mechanisms underlying the observed antileukaemic activity of VPA in CLL cells [12] and AML cells [13, 14]

have been described. So far, however, there has been no study exploring genomewide gene expression changes in CML.

The Connectivity Map (CMAP) is a collection of genomewide transcriptional expression data from cultured human cells treated with bioactive small molecules. It connects drugs, genes, and diseases together through the transitory feature of common gene-expression changes. By comparing gene-expression signatures, this tool can be used to find connections among small molecules drugs affecting common molecular pathways and putative mechanisms of action of unknown drugs. CMAP has previously been used to discover the mechanisms of drug action [15–18] and disease pathogenesis [19]. In the current study, we first investigated effects of VPA on apoptosis and gene expression profiles of K562 cells, a model for CML, and then mined the CMAP database to explore the molecular mechanism underlying the observed anti-CML effect of VPA.

2. Methods and Materials

2.1. Culture of K562 Cell Line. K562 cells, human chronic myelogenous leukemic cell line, were procured from Sun Yat-sen University Cancer Center. The cells were grown in RPMI (Life Technology Corporation, Camarillo, CA, USA), supplemented with 15% fetal bovine serum, 100 U/mL penicillin, and 1 mg/mL streptomycin. Cultures were incubated at 37°C in 5% CO₂.

2.2. Assessment of Apoptosis by Annexin V/PI Dual Staining Method. In order to determine the effect of VPA treatment on apoptosis rate of K562 cells, FACS analysis was carried out as previously described [20].

2.3. Analysis of Gene Expression Profile. To analyze gene expression profile, K562 cells were first cultured for 12 hours with 2 mM VPA or without VPA as a control. Then, the K562 cells were harvested and total RNA was extracted using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. Total RNA quality was evaluated using formaldehyde agarose gel electrophoresis and was quantified via spectrophotometry (Nanodrop, Wilmington, DE). RNA was amplified and labelled according to a previous protocol [21]. Briefly, 100 ng of total RNA was used to synthesize the double-strand cDNA. RNA was amplified by in vitro transcription using Ambion's MessageAmp II aRNA Amplification Kits (Life Technologies, Austin, TX, USA). Then, aRNA was reverse-transcribed into cDNA and further labelled with Klenow enzyme. cDNA from VPA-pretreated K562 cells was labelled with Cy3-deoxycytidine triphosphate and cDNA from control K562 cells was labelled with Cy5-deoxycytidine triphosphate. Fluorescent dye-labelled cDNA was hybridised to an Agilent SurePrint G3 Human GE 8 × 60 K Microarray. Hybridisation, scanning, and washing were carried out on Agilent's Microarray Platform according to Agilent's standard protocols. The array data were extracted with Agilent Feature Extraction software. After global mean

normalization, probes with an intensity <400 were filtered out for further analysis. Differentially expressed genes were further analysed based on a significant enrichment of GO terms using hypergeometric distribution in the R language package software.

2.4. Validation of Differentially Expressed Genes. To explore the mechanism of the observed VPA-inducing apoptosis, eight differentially expressed genes involved in apoptosis were selected for validation of the microarray results by quantitative real-time PCR (qRT-PCR). Total RNAs were first separated from K562 cell line treated with or without VPA. The isolated total RNAs were treated with DNase and exclusively used for validation of the microarray results. Following DNase treatment, 1 µg of DNase-treated RNA was reverse-transcribed cDNA with oligo (dT)15 using M-MLV reverse transcriptase (Life Technologies) in a total volume of 20 µL reaction volume. After reverse transcription reaction, 1 µL of the reaction mixture was used for a qPCR program of 45 cycles consisting of melting (30 s at 94°C), annealing (30 s at 58°C), and extension (30 s at 72°C). The 20 µL reaction mixture contained 1 × PCR Buffer (Mg²⁺ Plus), 0.5 µM of forward primer, 0.5 µM of reverse primer (Table 1), 200 µM of each dNTP, and Eva Green Master Mix in a LightCycler 480 Real-Time PCR System (Roche Applied Science). Data were analysed by the 2^{-ΔΔCt} method [22]. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a reference gene. All other results were shown as fold change relative to GADPH control.

2.5. Statistics. The microarray data have been deposited in NCBI's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE32189. The significance between groups was analyzed by the Student's *t*-test (Microsoft Excel, Microsoft Corporation, Seattle, WA). A *P* value of less than 0.05 (*P* < 0.05) was considered statistically significant.

2.6. Comparison of Gene Expression Profiles with the CMAP Database. To further explore the mechanisms underlying the observed effects of VPA, the gene expression profile of VPA treatments was used to query the CMAP database (build 02), which contains more than 7,000 expression profiles representing 1,309 compounds [23]. The similarity between the gene expression profiles of the query signature and a CMAP instance is measured by the connectivity score, ranging from -1 to 1. A high positive connectivity score indicates inducement of the expression of the query signature by the corresponding drug, a high negative, inhibition. Prior to the query in the CMAP database via the Internet (<http://www.broad.mit.edu/cmap/>), the probe ID of Agilent SurePrint G3 Human GE 8 × 60 K Microarray was transformed into the probe ID defined by the Affymetrix GeneChip Human Genome U133A array according to the probe corresponding gene.

TABLE 1: Primer pairs for qRT-PCR validation of differentially expressed genes involved in apoptosis.

Gene symbol	GenBank Accession		Primer (5' → 3')
GSN	NM_198252	F	CGAAGAGGTTTCCTGGTGAGC
		R	GCTGGGTCCGTCTCGATGTA
TP53INP1	NM_033285	F	CTTCTCATTGAACATCCCAGCA
		R	TGGGTTGTTCCAGAAAAGTTGTA
TNFSF9	NM_003811	F	GAGCTTTCGCCCGACGAT
		R	GCTCCTTCGTGTCCTCTTTGTAG
DDIT4	NM_019058	F	CCGAACCTCCCACCCAGCA
		R	GCAACGACACCCCATCCA
POLR2G	NM_002696	F	AAGATTGTGGGGACCCGTGT
		R	GGATGAGTTGCCTTCCTTGC
LGALS1	NM_033101	F	CAACCCTCGCTCAACGC
		R	GGCGGTTGGGGAACCTTGA
BBC3	NM_014417	F	CGGACGACCTCAACGCAC
		R	GCACCTAATTGGGCTCCATCT
FOXO3	NM_001455	F	GGGGAACCTCACTGGTGCTAA
		R	AACGGTATCACTGTCCACTTGC
PUF60	NM_014281	F	CTGTTTCCCAGTCAGAGCG
		R	CTCCTCTGTACCTCCCCTTC
RRAGA	NM_006570	F	TGGTCATGTCAGATCCGTCG
		R	AAGGCAATTCAGCATTTTCAGA

3. Results

3.1. Analysis of Apoptosis Using the Annexin V/PI Dual Staining Method. Using the Annexin V/PI dual staining method just as we previously reported, we measured apoptosis rate of K562 cells. In this study the similar increase in apoptosis was also observed in K562 cells exposed to 2 mM VPA for 48 hours (11.47 ± 0.25 ; $P < 0.05$, $n = 3$), compared to the K562 control (4.77 ± 0.40) [20].

3.2. Differentially Expressed Genes in K562 Cells Pretreated with VPA. Considering that the detection of differentially expressed genes is more easily in cells during the primary response phase of a drug exposure at certain doses that result in a 10–20% decrease in cell viability, we pretreated K562 cells with 2 mM VPA for 12 hours. This treatment resulted in an approximately 12% decrease in cell viability. The transcription profiles of K562 cells pretreated with or without VPA were analyzed using Agilent gene expression microarray. A total of 706 transcripts were identified as being significantly differentially expressed. The significant enrichment analysis of GO terms for the differentially expressed genes using the R language software package showed that apoptosis was the mostly enrichment function genes, and 34 genes involved in apoptosis were listed in Table 2.

3.3. Validation of the Differentially Expressed Genes Using qRT-PCR. Based on the microarray results, eight differentially expressed genes involved in apoptosis were selected for qRT-PCR confirmation. Consistent with the results obtained

from microarray analysis, PERP, GSN, TP53INP1, NXA4, FI6, ERPINB9, and CDKN2D were upregulated and NFKB1 was downregulated (Figure 1).

3.4. Comparison of Gene Expression Profiles with the CMAP Database. To gain a better understanding of the anticancer and proapoptotic mechanisms of VPA exposure in a CML model, the gene expression profile of differentially expressed genes related to VPA treatment was used to compare with those of 1,309 compounds in the CMAP database [23, 24]. The similarity of the gene expression profile between the query signature and a CMAP instance were measured by the connectivity score, which ranges from -1 to 1 . Only drugs with positive connectivity scores, which induce the expression of the query signature, are listed (Table 3). The results showed that the top ten drug hits belong to HDACi PI3K inhibitor, selective estrogen receptor modulator, antipsychotic or antimycobacterial agent, consistent with VPA's pharmaceutical actions including recently reported action of inhibiting HDAC. Gene expression profile in K562 cells exposed to VPA observed in this study was most similar to that of HDACi and PI3K inhibitor in CMAP database.

4. Discussion

VPA is a short-chain, branched fatty acid that has recently been described as a potent HDACi at therapeutic concentrations. Unlike other HDAC inhibitors, which are associated with various toxic side effects, VPA is clinically available. It can be taken orally, can cross the blood-brain barrier, and can be used for extended periods. HDAC inhibition is

TABLE 2: Differentially expressed genes involved in apoptosis detected by significant enrichment analysis of GO terms using the R language software package.

Genes	GenBank accession	Fold change	Gene description
PDCD7	NM_005707	-2.20	Programmed cell death 7
RRAGA	NM_006570	2.03	Ras-related GTP binding A
DAPK3	NM_001348	2.04	Death-associated protein kinase 3
PUF60	NM_014281	-2.00	Poly-U binding splicing factor 60kda isoform c
FOXO3	NM_001455	2.16	Forkhead box O3A
ARHGEF18	NM_015318	2.20	Rho/Rac guanine nucleotide exchange factor 18
BBC3	NM_014417	-2.18	BCL2 binding component 3 isoform 1
GSN	NM_198252	5.02	Gelsolin isoform c
LGALS1	NM_033101	2.58	Galectin-1
NFKB1	NM_003998	-3.33	Nuclear factor kappa-B, subunit 1 isoform 1
SH3GLB1	NM_016009	2.82	SH3-containing protein SH3GLB1
POLR2G	NM_002696	2.02	DNA directed RNA polymerase II polypeptide G
DDIT4	NM_019058	-2.23	Homo sapiens DNA-damage-inducible transcript 4
STEAP3	NM_182915	-2.52	Dudulin 2 isoform a
DRAM1	NM_018370	2.29	DNA-damage regulated autophagy modulator 1
RNF130	NM_018434	2.07	Ring finger protein 130 precursor
AVEN	NM_020371	-2.62	Cell death regulator Aven
TRIB3	NM_021158	-2.19	Tribbles 3
BAX	NM_138764	2.06	BCL2-associated X protein isoform beta
BID	NM_197966	-2.76	BH3 interacting domain death agonist isoform 1
PERP	NM_022121	9.42	PERP, TP53 apoptosis effector
SPTAN1	NM_003127	2.63	Spectrin, alpha, nonerythrocytic 1
BUB1B	NM_001211	-2.20	Budding uninhibited by benzimidazoles 1 beta
VDAC1	NM_003374	-2.37	Voltage-dependent anion channel 1
BCL2L12	NM_138639	2.19	BCL2-like 12 isoform 1
CUL1	NM_003592	-2.08	Cullin 1
YARS	NM_001040436	-2.30	Tyrosyl-TRNA synthetase
TNFSF9	NM_003811	2.38	Tumor necrosis factor (ligand) superfamily
IER3	NM_003897	2.17	Immediate early response 3
ARHGEF2	NM_004723	-2.10	Rho/Rac guanine nucleotide exchange factor 2
TP53INP1	NM_033285	3.03	Tumor protein p53 inducible nuclear protein 1
ITM2B	NM_021999	2.32	Integral membrane protein 2B
LITAF	NM_004862	2.25	Lipopolysaccharide-induced TNF-alpha factor
ESPL1	NM_012291	-2.11	Separase

-: indicate downregulation.

responsible for the acetylation of histones and nonhistone proteins such as hsp90 [25] and p53 [26]. This event has been associated with the induction of apoptosis in many models of leukaemia [27, 28]. Thus, the use of VPA prior to or concurrently with anticancer drugs may prove to be a beneficial treatment of leukaemia [8, 29]. The K562 cell line, originating from a patient diagnosed with CML in terminal blast crisis, is highly undifferentiated and of the granulocytic series. In this current study, we investigated the apoptosis rate of K562 cells exposed to VPA. The observed apoptosis result was in agreement with our previous reports [20].

Although the molecular mechanisms underlying antileukaemic activity of VPA in CLL [12] and AML [13, 14] models

have been reported, genomewide gene expression changes in CML, however, have not been described. CML is a type of myeloproliferative disease associated with a characteristic chromosomal translocation called the Philadelphia chromosome. Conversely, CLL affects B-cell lymphocytes, and AML is characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. The observed clinical features and pathology of CML patients, as well as the therapy options, are quite different from those of CLL and AML patients. Consequently, the effect of VPA treatment on CML patients and the underlying molecular mechanisms may be different from effects observed in CLL and AML

TABLE 3: Top CMAP hits correlated with gene expression signature induced by VPA.

Rank	CMAP drug	<i>P</i>	Mean score	Enrichment	Specificity	Percent nonnull	Function
1	Vorinostat	0.00000	0.918	0.974	0.0201	100	HDACi
2	Trichostatin A	0.00000	0.817	0.957	0.0000	96	HDACi
3	Wortmannin	0.00000	0.335	0.695	0.0065	77	PI3K inhibitor
4	Ly-294002	0.00000	0.325	0.564	0.0201	65	PI3K inhibitor
5	VPA	0.00000	0.370	0.516	0.0000	61	Anti-epileptic; HDACi; Mood stabilizer
6	Raloxifene	0.00002	0.302	0.789	0.0000	85	SERM
7	Trifluoperazine	0.00002	0.303	0.594	0.1154	68	Antipsychotic
8	Scriptaid	0.00004	0.728	0.968	0.0185	100	HDACi
9	Rifabutin	0.00004	0.802	0.967	0.0052	100	Antimycobacterial agent
10	Thioridazine	0.00008	0.260	0.486	0.2831	65	Antipsychotic

The connectivity score, similarity measurement of the gene expression profile between the query signature and that of a CMAP instance, ranges from -1 to 1 . Only drugs with positive connectivity scores, which induce the expression of the query signature, are listed. HDACi: histone deacetylase inhibitor. SERM: selective estrogen receptor modulator. VPA: valproic acid.

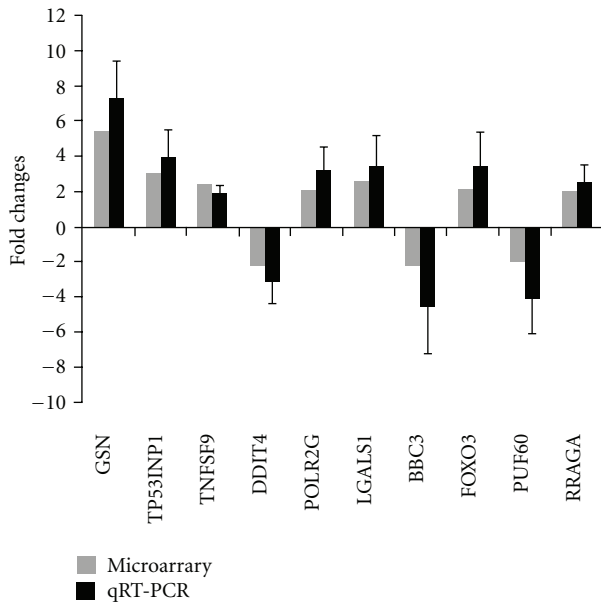


FIGURE 1: Alterations in the expression levels identified by microarray were confirmed using qRT-PCR. Fold changes between K562 cell line treated with and without valproate detected by microarray were compared with those measured by qRT-PCR. In qRT-PCR assay, RNA was isolated from K562 cell line treated with or without valproate exclusively for validation of differentially expressed genes. mRNA levels were normalized with GAPDH and fold changes were calculated by dividing mRNA level of each K563 cell line treated with valproate by mean mRNA level from control samples in triplicate. Data are mean values \pm standard deviations of the means from three independent experiments performed in triplicate.

patients. Based on this information, we investigated the global gene expression profile of K562 cells exposed to VPA using gene expression microarray. Our microarray data demonstrated that VPA exposure resulted in widespread gene expression changes in this cell line. The significant

enrichment analysis of GO terms revealed that genes involved in apoptosis were significantly altered by VPA treatment. This alteration was in accordance with the increase in cell apoptosis observed in VPA-pretreated K562 cells using flow cytometry.

To gain a better understanding of the antileukaemic mechanisms of VPA exposure in a CML model, the gene expression profile of differentially expressed genes related to VPA treatment was utilised to compare with those of 1,309 compounds in the CMAP database. CMAP analysis showed that the top ten drug hits included HDACi, PI3K inhibitor and VPA itself. These results were consistent with VPA's pharmaceutical actions including recently reported action of inhibiting HDAC, indicating the reliability of our result of gene expression profile. In this study, VPA ranked in the top five drug hits. This result not only reflects the reliability of our result of gene expression profile, but also indicates that the connectivity map is a reliable tool for exploring molecular mechanism of drug action. The top four drug hits were vorinostat (suberoylanilide hydroxamic acid, ZolinzaTM), trichostatin A, Wortmannin, and LY-294002. Vorinostat and trichostatin A belong to HDACi. HDACi has a multitude of effects on cancer cells. It can induce cell-cycle arrest, promote differentiation, and stimulate tumor cell death [30]. It activates the apoptosis of cancer cells by the death-receptor (extrinsic) pathway and/or the mitochondrial (intrinsic) pathway [31]. The latter is induced by stress stimuli that disrupt the mitochondrial membrane. Recent studies have showed that HDACi treatment generates reactive oxygen species (ROS) and causes DNA damage in AML cell line, AML patient-derived blasts cultured ex vivo [32], and CML cell line [33], suggesting that ROS inducing intrinsic apoptosis might be one of important mechanisms underlying pro-apoptotic effect of HDACi. The intrinsic apoptosis may be dependent or independent on p53 pathway. We found that expressions of many genes involved in p53-dependent apoptotic pathway were up-regulated by VPA, for example, TP53INP1, PERP, and BAX (Table 2). TP53INP1

promotes p53/TP53 phosphorylation in response to double-strand DNA breaks. PERP plays a role as an effector in the TP53-dependent apoptotic pathway. BAX is regulated by p53 gene. It forms the mitochondrial pore leading to mitochondrial depolarization by oligomerization with BAK [34] and confers an essential gateway for the activation of caspases in the intrinsic apoptosis. Moreover, GO analysis showed that the genes involved in release of cytochrome c from mitochondria (GO: 0001836) were significantly enriched ($P = 0.00066$) (data not shown). It has been reported that VPA produces DNA oxidative damage in liver cells [35, 36], increases ROS formation, and induces apoptosis in postimplantation embryos [37]. Taken together, VPA might induce ROS and activate p53-dependent apoptosis of k562.

The expression of the BCR/ABL oncogene is the major pathogenic event in CML and the abnormal BCR/ABL tyrosine kinase promotes leukaemogenesis by inducing the phosphorylation of multiple downstream protein targets that mediate growth promoting and antiapoptotic signals, including MAPK/ERK and PI3K pathways [38–40]. PI3K is required only for the proliferation of BCR/ABL-dependent leukemic cells but not of normal hematopoietic cells [41, 42]. Wortmannin and LY294002 are the earliest and still widely employed inhibitors of PI3K. Studies have shown they are able to block proliferation of Ph1-positive cells [38, 43, 44] and enhance the antileukemia effect of Bcr-Abl tyrosine-kinase inhibitor [45–47], the initial choice of treatment for most CML patients. Based on CMAP analysis, we speculate that VPA might inhibit PI3K pathway too, and such inhibition might be one important mechanism by which VPA exerts antileukaemic action. We found that one member of the forkhead transcription factor O (FOXO) family, FOXO3, was upregulated by VPA (Table 2). FOXO family plays an important role in stress defence and it is negatively regulated by AKT-dependent phosphorylation [48].

In conclusion, we observed that VPA exposure altered mRNA expression in the K562 cell line. The CMAP analysis suggests that these alterations might be associated with inhibiting effect of VPA on HDAC as well as PI3K pathway. The data obtained in this study might provide clues to elucidate the molecular and therapeutic potential of VPA in leukaemia treatment, and the CMAP, by which we confirmed GO analysis and analyze the possible mechanisms of VPA antileukaemia, is a useful tool for exploring molecular mechanisms of drug actions.

Abbreviations

CML: Chronic myelogenous leukaemia
 VPA: Sodium valproate
 GO: Gene ontology
 CMAP: Connectivity Map.

Conflict of Interests

The authors declare no potential conflict of interests.

Authors' Contribution

These authors contributed equally to this work X. Z. Zhang and A. H. Yin.

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