Toxicology 393 (2018) 160–170

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Full length article

Integrative omics data analyses of repeated dose toxicity of valproic acid *in vitro* reveal new mechanisms of steatosis induction

Simone G.J. van Breda*, Sandra M.H. Claessen, Marcel van Herwijnen, Daniël H.J. Theunissen, Danyel G.J. Jennen, Theo M.C.M. de Kok, Jos C.S. Kleinjans

Department of Toxicogenomics, Grow School for Oncology and Developmental Biology, Maastricht University, P.o. Box 616, 6200 Md, Maastricht, The Netherlands

ARTICLE INFO

Keywords:
Valproic acid
Integrative omics data analyses
Human liver
Adverse outcome pathway
Primary human hepatocytes
Genomics

ABSTRACT

Valproic acid (VPA) is a very potent anti-cancer and neuro-protective drug probably by its HDAC inhibiting properties, which may cause steatosis in the liver. The present study investigates the effect of repetitive VPA treatment of primary human hepatocytes (PHH) on whole genome gene expression-, DNA methylation-, and miRNA changes, using microarrays and integrated data analyses. PHH were exposed to a non-cytotoxic dose of VPA for 5 days daily which induced lipid accumulation. Part of the PHH was left untreated for 3 days for studying the persistence of 'omics' changes. VPA treatment appeared to inhibit the expression of the transcription factors *HNF1A* and *ONECUT1*. *HNF1A* interacted with 41 differentially expressed genes of which 12 were also differentially methylated. None of the genes present in this network were regulated by a DE-miR. The subnetwork of *ONECUT1* consisted of 44 differentially expressed genes of which 15 were differentially methylated, and 3 were regulated by a DE-miR. A number of genes in the networks are involved in fatty acid metabolism, and may contribute to the development of steatosis by increasing oxidative stress thereby causing mitochondrial dysfunction, and by shifting metabolism of VPA towards β-oxidation due to reduced glucuronidation. Part of the changes remained persistent after washing out of VPA, like *PMAIP1* which is associated with cellular stress in liver of patients with NASH. The *MMP2* gene showed the highest number of interactions with other persistently expressed genes, among which *LCN2* which is a key modulator of lipid homeostasis. Furthermore, VPA modulated the expression and DNA methylation level of nuclear receptors and their target genes involved in the adverse outcome pathway of steatosis, thereby expanding our current knowledge of the pathway. In particular, VPA modulated *PPARγ,* and *PPARα, AHR* and *CD36* on both the gene expression and the DNA methylation level, thereby inhibiting β-oxidation and increasing uptake of fatty acid into the hepatocytes, respectively. Overall, our integrative data analyses identified novel genes modulated by VPA, which provide more insight into the mechanisms of repeated dose toxicity of VPA, leading to steatosis.

1. Introduction

Valproic acid (VPA) is a branched short-chain fatty acid (Burton, 1882), which at the moment, is one of the most prescribed drugs to treat epilepsy and −bipolar disorders, and to prevent migraine headaches (Perucca, 2002). In spite of its wide range of therapeutic applications, VPA shows severe hepatotoxic side effects, with steatosis as the main phenotypic endpoint (Powell-Jackson et al., 1984). It is, however, unclear how chronic VPA treatment causes accumulation of triglycerides in the liver.

As a fatty acid analogue, VPA is a competitive inhibitor of fatty acid metabolism, in particular for the mitochondrial fatty acid β-oxidation pathway. It is furthermore hypothesized that reactive intermediates which are generated during various metabolic routes of VPA, cause mitochondrial dysfunction, which has also been associated with the induction of steatosis (reviewed in Silva et al. (2008). It has been suggested that VPA influences homeostasis of fatty acid metabolism via interaction with lipid-sensing nuclear receptors such as *PPARα, PPARγ*, *LXRα*; *LXRβ, PXR,* and *AHR*, leading to downstream activation of target genes involved in cholesterol and lipid metabolism (Balmer et al., 2014;

Abbreviations: VPA, valproic acid; PHH, primary human hepatocytes; HDAC, histone deacetylase; AOP, adverse outcome pathway; MeDIP, Methylated DNA Immunoprecipitation; PSW-ANOVA, Probe Sliding Window-ANOVA; DMR, differential methylated regions; DMG, differentially methylated genes; MoA, modes of action; MIE, molecular initiating event; MMP, matrix metalloproteinase; ECM, extracellular matrix

* Corresponding author at: Department of Toxicogenomics, Faculty of Health, Medicine and Life Sciences, Maastricht University, Universiteitssingel 40, 6229 ER, Maastricht, The Netherlands.

E-mail address: s.vanbreda@maastrichtuniversity.nl (S.G.J. van Breda).

https://doi.org/10.1016/j.tox.2017.11.013
Received 6 October 2017; Received in revised form 31 October 2017; Accepted 9 November 2017
Available online 14 November 2017
0300-483X/

Krug et al., 2013; Landesmann et al., 2012; Waldmann et al., 2014). However, only a limited number of studies have been carried out which investigate the effect of VPA on these nuclear receptors in human liver systems. It remains unclear whether modulation of these nuclear receptors actually contributes to VPA induced steatosis.

Furthermore, epigenetic effects of VPA have been indicated. VPA has been defined to represent a novel class of histone deacetylase (HDACs) inhibitors, which is related to its potential anti-tumor and neuro-protective effects (Cincarova et al., 2013). The HDAC-inhibiting activity of VPA has been associated with increased expression of hundreds of genes involved in multiple cancer-related processes leading to differentiation, inhibition of cell growth and increased apoptosis, demonstrated in a variety of *in vitro* and *in vivo* systems (reviewed by Duenas-Gonzalez et al. (2008). In addition, a number of studies describe a role for VPA in active DNA demethylation of genes (Detich et al., 2003; Milutinovic et al., 2007). These effects of VPA become also manifest on the level of microRNA expressions which cause the suppression of genes involved in the development of neural tubes and forebrain neurons (Meganathan et al., 2015), as well as in regulating the expression of genes which stabilize mood-disorders such as schizophrenia (Hunsberger et al., 2013).

However, the contribution of epigenetic changes and microRNA expression modulations of genes which play an adverse role in steatosis development has not yet been investigated in depth. In addition, epigenetic changes in genes encoding for nuclear receptors involved in lipid metabolism have not been studied. Investigation hereof will undoubtedly provide more insight into the molecular mechanisms of VPA induced steatosis.

Interestingly, several studies have indicated that certain chemical exposures are associated with persistent epigenetic changes which may have life-long phenotypic consequences (Heijmans et al., 2008). Therefore, persistence of epigenetic changes upon terminating the repeated dosing of VPA is highly relevant, as these changes might contribute to steatosis development on longer term. We already have shown the relevance of persistent effects on the epigenetic level in two earlier studies in which primary human hepatocytes (PHH) were exposed to aflatoxin B1 (Rieswijk et al., 2016), and to cyclosporine A (Wolters et al., 2016). Furthermore, an in-depth sequencing study on DNA methylation changes of nuclear and mitochondrial DNA of PHH exposed to VPA, showed persistent changes on nuclear DNA methylation level, but not on mitochondrial level (Wolters et al., 2017). However, persistence of cross-omics responses to VPA has not been evaluated previously.

In the present study, we hypothesized that VPA is able to modulate the expression of genes which are relevant for steatosis development, by changing the methylation status of these genes and/or by modulation of particular microRNAs which target the transcripts of these genes. To investigate the persistence of induced molecular changes relevant for steatosis upon terminating treatment, a subset of PHH was subjected to a washout period of three days. Identification of differentially expressed genes, microRNAs, and methylated genes was performed using whole genome microarrays and subsequent data analyses using R included in the open source software framework BioConductor. In order to identify the main affected biological processes and key modulated gene networks, we analyzed our data using the molecular functional interaction database ConsensusPathDB (Kamburov et al., 2011; Pentchev et al., 2010) in combination with the network visualization program Cytoscape (Shannon et al., 2003). We the aim to identify genes with the highest level of interactions, as these could play a central role in VPA induced changes associated with development of steatosis (Georgiadis et al., 2016). Furthermore, we projected our data onto the previously published adverse outcome pathway (AOP) of steatosis (Landesmann et al., 2012; Vinken, 2015) in order to visualize the cross-omics changes related to the nuclear receptors and their target genes in this established framework, for the purpose of gaining more insight into the molecular mechanisms of VPA-induced steatosis.

2. Material and methods

2.1. Cell culture and valproic acid treatment

Cryopreserved PHH and culture media were purchased from Life Technologies (Bleiswijk, The Netherlands). In order to reduce the influence of inter-donor variability, PHH from 3 donors (lots Hu4197, Hu8084 and Hu4227) were pooled. Characteristics of the donors are displayed in Supplementary data Table 1. PHH were cultured in precoated multi-well plates in a 2-layer collagen sandwich, according to the supplier's protocol. For RNA and DNA, 0.7 *10^E6 cells and 2 *10^E6 cells were seeded, respectively. Before treatment, cells were allowed to acclimatize for 3 days in order to restore an *in vivo*-like cellular configuration and expression of enzyme levels.

The optimal incubation concentration of VPA was chosen after a screening experiment examining a range from 0 to 30 mM of VPA. For this, cytotoxicity and fat deposits were assessed using MTT and Bodipy staining, respectively. PHH showed no signs of cytotoxicity in the MTT assay at any incubation concentration considered, but fat accumulation was observed in a dose-dependent manner after 24 and 48 h of exposure (data not shown). An incubation concentration of 15 mM VPA was selected for the main experiment. This relatively high incubation concentration is apparently required for creating a diffusion gradient across the collagen layer to generate an intracellular effective dose sufficient for inducing a clearly visible fat accumulation, but still without a complete displacement of the cytoplasm, and thus without causing cytotoxicity. PHH were exposed to VPA in a 24 h repeated dose testing regime. Medium was changed daily thereby providing a new incubation concentration of VPA to the PHH each day. A bi-phasic treatment regime was applied, combining a 5-day VPA exposure with a subsequent 3-days washout period during which the PHH were exposed to medium only. In view of its rapid metabolism (half-life 9–16 h (20)) this period is sufficient to cause VPA depletion. 1% Ethanol was used as solvent control. Cell lysates were collected on days 5 and 8, and prepared for transcriptomic, miRNA and DNA methylation analyses. All experiments, as well as all 'omics' analyses were carried out in triplicate.

2.2. Methylated DNA analyses

2.2.1. DNA isolation

DNA was isolated from PHH as described previously (Rieswijk et al., 2016). The total amount was at least 10 µg DNA, the 260/280 ratio ranged between 1.7-1.9, and the 260/230 ratio appeared higher than 1.6. A total of 12 DNA samples were prepared. DNA was used for MeDIP-Chip analyses.

2.2.2. Methylated DNA immunoprecipitation (MeDIP), whole genome amplification and methylation enrichment assessment

Genomic DNA was sonicated to obtain fragments ranging from 200 bp to 600 bp, cleaned up using silica columns (Zymo Research) and eluted in TE buffer. MeDIP was performed using the MagMeDIP kit (Diagenode, Liège, Belgium) according to the manufacturer's protocol as described previously (van Breda et al., 2014). Methylation enrichment in the paired samples MeDIP/Input was derived from qPCR data by calculating the ratio positive control/negative control, applying the ΔΔCq method using the primers included in the kit.

2.2.3. MeDIP-Chip

For analysis of DNA methylation levels, the Human DNA Methylation 2.1 M Deluxe Promoter Array (Roche NimbleGen) was used. Labeling and hybridization of arrays was performed according to the manufacturer' protocol as described previously (van Breda et al., 2014).